Excerpts from

PROCESSES IN BIOLOGICAL VISION:

including,

ELECTROCHEMISTRY OF THE NEURON

This material is excerpted from the full β-version of the text. The final printed version will be more concise due to further editing and economical constraints. A Table of Contents and an index are located at the end of this paper.

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Vision Concepts

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5 The Photochemistry of Animal Vision [1]

"The concept in psychophysics that the visual spectral sensitivities are unknowable and irrelevant is unthinkable." (This Author)

"Solid state events involving conduction are evident in animate aggregations and may well be an essential characteristic of life, which may be an electromagnetic phenomenon."
Gutmann, Keyzer & Lyons (1983)[2]

5.1 Introduction

Saari said in 1994; "Nature has exploited the relatively simple retinoid structure to full advantage. The molecule mediates a bewilderingly complex set of biologic functions with only a single functional group and a set of conjugated double bonds."[3] This chapter will show that when Nature added a second functional group, resulting in two distinctly different sets of conjugated double bonds, She expanded this exploitation considerably.

The vision community within the field of biology has sought to determine the detailed nature of the chromophores of animal, and particularly human, vision for a very long time. Even after the industrial and technological revolutions, these chromophores are not known, except in conceptual form, in the vision literature. This is unfortunate since the necessary scientific knowledge has been available since the 1930-50's in other non biological disciplines. In the absence of the transfer of this knowledge into the vision community, the community has suffered from a lack of knowledge about exactly what they are seeking and the conditions required in the laboratory to isolate it. On the other hand, **the specific conditions under which the chromophores of vision can be isolated and identified are extremely demanding** compared to conventional chemistry. Many conventional chemical tests lack the specificity required to isolate the chromophores completely. This Chapter will provide the background and detailed conditions required to isolate the chromophores of vision, and the exact formula and state of matter of the chromophores of vision.

Example: The four chromophores of animal vision are resonant conjugate retinoids that only exhibit their unique spectral performance when in the liquid crystalline state. They must not be attacked by strong detergents or either oxidizing or reducing agents. Their spectra can only be measured in the transient mode unless they are in quantum contact with another material that can continually de-excite the excitons created by the incident photons.

CHAPTER SYNOPSIS

[xxx some duplication with above insert]
This Chapter will present the actual chromophores of vision, labeled the Rhodonines and derivable from a number of feedstocks, including the retinol family, consist of relatively small molecules with a molecular weight of either 285 (R5 & R9) or 299 (R7 & R11). They are retinoids of the resonant conjugate type. They are also carboxylic-ion systems and exhibit a negative charge in their fundamental form. The molecules are relatively easily generated in the laboratory in pure form. However, they exhibit a number of unique properties that have made their isolation difficult. They only exhibit the properties of a visual chromophore when in the liquid crystalline state. Their absorption characteristic is a transient one unless a means of de-exciting the molecules of the liquid crystal is present. Finally, they are extremely sensitive to destruction by oxidants and alkali metal ions.

The Rhodonine chromophores consist of a family of twelve molecules; four related to Retinol (Vitamin A1 and found in saline-based animals), four related to 3,4 dehydroretinol (Vitamin A2 and associated with the freshwater-based animals) and four related to 3-hydroxyretinol (Vitamin A3 and found primarily in the two-winged flies, the Diptera Order of *Arthropoda*). The functional absorption spectra of these three sets, when in the liquid crystalline state, are virtually identical. This is due to the primary quantum-mechanical mechanism involved and the identical form of their chromophoric structure. The members of each set are spaced at 95 ± 2 nm.

Each Rhodonine molecule exhibits a large number of functional groups. It requires careful laboratory technique to differentiate them from simpler structures which may not have all of their characteristics. They satisfy all of the

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[1] Released: April 30, 2017
[2] Gutmann, F. Keyzer, H. & Lyons, L. (1981) Organic semiconductors: Part B Malabar, FL: Robert E. Krieger Publishing Co. Pg. 319
[3] Saari, J. *in* Sporn, M. Roberts, A. & Goodman, D. (1984) The retinoids. NY: Academic Press pg. 351

historical tests for a retinene but are not retinenes. Their carboxylic-ion structure exhibits the characteristics of both an alcohol and an aldehyde simultaneously. This fact has caused confusion in the literature for years.

When utilized in the vision process, the Rhodonine chromophores are formed into a liquid crystalline state on the surface of a substrate, known generically as the protein opsin. It appears that the chromophores are held to the opsin substrate by very weak bonds of the hydrogen bond type. This linkage does not disturb the unique electronic configuration of the chromophoric material.

The unique spectral absorption of the Rhodonines contains two visual band components, an isotropic absorption associated with the conjugated dipole molecular structure of the molecule, and a anisotropic absorption associated with an additional resonant slow-wave structure intimately associated with the triplet electrons of the oxygen atoms of the molecule. The unusual relaxation properties of these molecules are also associated with these triplet state electrons. The Rhodonines do not fluoresce or phosphoresce significantly while in a dilute liquid solution.

The chromophores are aggregated into a liquid crystalline structure wherein they are able to conduct the excited electrons resulting from photoexcitation at a given location to a second location where they are de-excited in the process (developed in **Chapter 11**) of generating a signal within the dendrites of the photoreceptor cells. The chromophores of vision are produced in the RPE cells of the retina and not in the photoreceptor cells as conventionally assumed. This complex procedure is developed in **Chapter 7**.

This Chapter begins with a comprehensive review of the quantum-mechanical properties of organic molecules and how this affects their photon excitation. A series of detailed definitions and concepts are presented that are not normally found in biological treatises. These concepts are vital to an understanding of the mechanisms involved in the photochemistry of vision.

5.1.1 The conventional wisdom of the vision community

The vision community has had great difficulty in describing the chromophores of vision. The nomenclature has varied from being based on the color of light absorbed, the color of light not absorbed (their appearance by reflection or transmission), the chemical chromogen they include, whether they are sensitive to a given short, medium or long wavelength region, the numerical value of the absorption peak, the animal or cell from which they were obtained, or some combination of the above[4]. The problem has been compounded by the different perspectives adopted by the biological, electrophysiological and psychophysical communities.

Since the time of Wald's demonstration that rhodopsin, the conceptual chromophore of vision relied upon retinol as a chromogen and Hubbard's contemporary proposal that photodetection involved an isomerism of the chromophore, the conventional wisdom has adopted that position. To support these proposals, Collins proposed that a Shiff-base was the mechanism connecting the protein opsin to the retinoid retinol in forming rhodopsin. This structure is defined as N-retinylidene-opsin. Since this proposal was found to have serious problems on energy grounds, an additional conceptual proposal was made by Bownds suggesting protonation of the Schiff-Base, to form N-retinyl-opsin. Hubbard proceeded to promulgate a complex series of chemical reactions leading to the transition of the initial 11-*cis*-retinol ligand to all-*trans*-retinol. However, the community has not been able to demonstrate the accuracy of those proposals or to confirm any of these reactions under biological conditions. An extremely large volume of literature has used their unconfirmed proposals as their foundation.

As reviewed more fully in **Section 5.5.2.1**, no independent laboratory confirmation of the Wald, Hubbard, Collins, or Bownds proposals have appeared from outside of their institution in over fifty years. Goldsmith concurred in this position[5].

5.1.1.1 Putative isomerism in the chromophore

The experimental work based on the assumption that rhodopsin consists of a conjugated protein consisting of opsin and retinol joined via a Schiff base has been studied for over 60 years. The work has failed to discover how the chromophores of vision achieve their high spectral absorption, broad spectral line widths and their specific central

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[4]Goldsmith, T. (1994) Ultraviolet receptors and color vision: Evolutionary implications and a dissonance of paradigms. *Vision Res*. vol. 34, no. 11, pp 1479-1487

[5]Goldsmith, T. (1990) Optimization, constraint, and history in the evolution of eyes. *Quart. Rev. Biol*. vol. 65, no. 3, pg 297

spectral wavelengths. In 1979, Boynton[6] stated unequivocally that "No primate pigments have ever been extracted." Birge provided a wide ranging review of this conventional wisdom in 1981 where Rhodopsin was described as the generic name for all vertebrate and invertebrate visual pigments[7]. It included a large number of other reviews and recent references to the work of Honig and his team.

> While the Birge review was in preparation, Honig, et. al. published a paper with an extremely significant note added in proof[8]. In this note, they recognize that "it was possible. though unlikely, that there was no counter-ion and that the protonated nitrogen is 'solvated' by dipolar groups in the opsin which provide a negative environment in the vicinity of the Schiff base." e. g., the putative protonated Schiff base may not be an acceptable model.
>
> The note continued; "our discussion would be applicable if the counter-ion would be replaced by formal charges on electrically neutral dipolar groups such as carbonyls."

Bridges, writing in Sporn, et. al[9]. in 1984 said: "Since the key to understanding visual pigment regeneration lies in obtaining an insight into how 11-*cis*-retinoid is formed from all-*trans*-retinoid, this section is devoted to this difficult as yet unsolved problem." He continued on the following page: "While there has been repeated failure to form 11-*cis*-retinoids from all-trans-retinoids in *in-vitro* situations, it is clear that this conversion can be effected by the living organism." He offered no evidence of the latter claim and was not able to resolve the primary issue within the following five pages. Note the generalized term retinoid used by Bridges.

Writing ten years later in the 2nd edition of Sporn, et. al[10]. Saari discussed the conventional wisdom concerning biological photosensing in considerable detail, including the assertion that it was the aldehyde form that was used in vision while the alcohol form was used in reproduction. He closed by saying: "The term 'Visual Cycle' is used to describe the set of dark (thermal) reactions used to produce the critical 11-*cis*-retinoid needed for vision from all-*trans*-precursors." His figure 3 defined multiple independent processes to perform these reactions. He did not provide further evidence to confirm the reality of the set(s) of Hubbard equations.

In 2000, Gartner published an extensive list of putative invertebrate visual pigments[11]. These are in fact the structural proteins that form the disks in mammals and the equivalent supporting structures in invertebrates. Gartner tabulated the length of each molecule, and gave the nucleic sequence for many based on a specific software package. However, he did not document the presence of retinol in them. The molecules exhibit long sequences common to many of these proteins as expected. While they probably do contain a ligand of retinol, they are not photosensitive. Gartner offered no spectrographic data showing the molecules listed were photo-reactive in any way. Neither did he offer a proposed peak spectral wavelength for the chemicals in his tables.

5.1.1.2 Gates in the putative Outer Segment membrane

During the 1980s and 1990s, a number of investigators have defined a wide variety of chemical process sequences that explain the generation of an electrical signal as the result of photon excitation of the Outer Segment of the photoreceptor cells[12]. These have been largely conceptual. At best they have been based on chemical kinetics. None have resulted in a mathematical description of the luminous efficiency function or the dark adaptation function of the human visual process.

Also during the 1980s and 1990s, many investigators explored concepts wherein gates were introduced into a putative plasma membrane surrounding the Outer Segment. These gates were controlled following the activation of rhodopsin

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[6]Boynton, R. (1979) Human Color Vision Holt, Rinehart & Winston. pg 156, comment to footnote 9.
[7]Birge, R. (1981) Photophysics of light transduction in rhodopsin and bacteriorhodopsin. Ann. Rev. Biophys. Bioeng. Vol. 10, pp 315-354
[8]Honig, B. Ebrey, T. Callender, R. Dinur, U. & Ottolenghi, M. (1979) Photoisomerization, energy storage, and charge separation: A model for light energy transduction in visual pigments and bacteriorhodopsin. Proc. Natl. Acad. Sci. USA, vol. 76, no. 6, pp 2503-2507 (note f on pg 2503)
[9]Bridges, C. (1984) in Sport, M. Roberts, A. & Goodman, D. The Retinoids, Vol. 2. NY: Academic Press, pp 157-162
[10]Saari, J. (1994) in Sporn, et. al. The Retinoids, 2nd ed. NY: Raven Press, Chapter 9
[11]Gartner, W. (2000) Invertebrate visual pigments *In* Stavanga, D. DeGrip, W. & Pugh, E. *eds*. Handbook of Biological Physics, Vol. 3. NY: Elsevier Chap. 7
[12]Hargrave, P. & McDowell, J. (1992) Rhodopsin and phototransduction. *International Rev. Cytol*. Vol 137B, pp 49-97

by light. They have all been highly conceptual and have involved complex multi-step processes portrayed using a variety of cyclic schematics. The proposal by Hargrave & McDowell is typical of these[13]. These representations are far more complex than that presented in this work, yet they do not lead to closed form equations of any part of the visual process.

5.1.1.3 Moving toward a carboxyl ion system

In the above note, Honig, et. al. came very close to defining the actual chromophores of vision as defined in this work. And, in the last paragraph of the first page, they continued to discuss the possibility of a carboxylate group of the protein being located approximately 3 Angstrom from the nitrogen of the Schiff base. There remains a problem and an alternative. The nitrogen of a Schiff base does not possess an unpaired electron that can be contributed to a chemical resonance spanning the suggested 3 Angstrom. In addition, the two oxygen atoms of the carbonyl group are not separated by sufficient distance to result in absorption within the visual spectral band. The solution is to employ an oxidized form of retinol where the second oxygen atom is removed from the oxygen of the alcohol by a similar distance, thereby forming a carboxyl-ion system instead of a simple carbonyl prosthesis. The resulting material is no longer a retinene. This chromophore is not joined to the substrate opsin except through a hydrogen bond. The following material will develop the details regarding the actual generic chromophores of biological vision, the Rhodonines.

5.1.2 The definition of photochemistry

The global field of photochemistry has been defined in Coxon & Halton as "the study of chemical reactions initiated by light."[14] Although succinct, it may be too restrictive for the purposes of vision. The field might be better described by replacing the expression chemical reactions by chemical processes or chemical interactions. The revised definition would allow mechanisms, such as sensitization of one species by another without any change in species to occur. This is the context used by Maron & Lando[15]. It would also allow extension of the field to include a critical process in vision, the entirely quantum-mechanical sensitization of the neural system by the simultaneous de-excitation of an excited species. An alternate wording for the above mechanism is the electronic excitation of the neural system by the simultaneous de-excitation of the chromophores of vision employing quantum-mechanical techniques. These latter interpretations will be employed in this work. **Photochemistry will be defined as the photosensitization of a chemical species by light followed by a desensitization of the resulting species coupled with a second process.** Coxon & Halton come close to this interpretation in their discussion of the transfer of excitation energy between species in what they define as an intersystem crossing[16]. In vision, this intersystem crossing eventually results in a free electron within the neural system. This mechanism, involving the generation of a free electron by an excited state in an associated molecular (liquid crystalline) structure, has been defined as translation in this work. No chemical reaction has occurred during this sequence of events.

Using the above definition, the process of a photo-induced isomerization involves an excitation followed by a de-excitation involving a rearrangement. Rearrangement and dissociation were two of the few possibilities for de-excitation known during the 1920-30's.

5.1.3 Tools for identifying ligands in large molecules

Identifying the specific ligand in a complex molecule is extremely difficult. While magnificent progress has occurred recently in identifying specific ligands in the DNA complex (and their sequence of occurrance), this progress has not been equaled in more general chemistry. Harding & Chowdhry have recently reviewed the bulk techniques used in the broader field of protein chemistry[17].

5.1.4 Redefining the baseline of photodetection in vision

Building on the experimental base of the 1920-50's, the Wald school attempted to explain the photodetection process in vision based on the technology base available at that time. The most advanced concept available initially involved a stereomeric change in a molecular structure. The effort was not successful although the community followed and expanded on the defined baseline for 50 years. The technology knowledge base was not adequate. No investigator has

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[13]Hargrave, P. & McDowell, J. (1992) Rhodopsin and phototransduction. Int. Rev. Cytol. vol. 137B, pp 49-97
[14]Coxon, J. & Halton, B. (1987) Organic photochemistry. Cambridge: Cambridge University Press. pg. 1
[15]Maron, S. & Lando, J. (1974) /fundamentals of Physical Chemistry. NY: Macmillan pp 724-725
[16]Coxon, J. & Halton, B. (1987) Op. Cit. pg. 11-13
[17]Harding, S. & Chowdhry, B. *eds.* (2001) Protein-Ligand Interactions: hydodynamics and calorimetry. Oxford: Oxford Press pp 4-10

been able to confirm in-vitro the spectral absorption characteristic of even one of the putative chromophores according to the Wald school and its descendants.

There are a large number of reasons to revert to an alternate baseline. The most important is the fact that the chromophores of vision are not formed within the photoreceptor cell. There is an extensive database on how they are formed and stored within the RPE cells and subsequently transported to the Outer Segments for coating of the protein substrate known as opsin (**Section 4.6**).

There are a large number of reasons to discount the simple photon-stereomeric approach, that is endothermic, in favor of considering available modern choices based on quantum-mechanics. The field of photographic dyes provides a well-understood alternative based on resonant conjugated carbon chemistry. This approach is energy neutral (marginally exothermic). When combined with the solid state physics derived from the transistor, it offers an alternate hypothesis that is confirmable in the laboratory.

The quantum-mechanical approach coupled with a newly discovered electrolytic semiconductor device, the Activa, provide a traceable solution to the photodetection problem and forms the basis of this Chapter as well as the overall work.

5.1.4.1 Redefining the role of rhodopsin in vision

Rhodopsin is an interesting and complex molecule. It was defined conceptually in the 1930's. It has been defined technically since the mid 1950's. In recent times, it has been elucidated in detail via the genetic code and x-ray crystallography. Hargrave et al[18]. first presented the caricature in **Figure 5.1.1-1** in 1983. As developed below, the caricature is archaic. Here, the left side of the caricature has been modified to show the extent of the bilayer lemma of a neuron beyond the coordinates defined by Hargrave et al. The extent is usually taken as 80 Angstrom in more recent work. The unmodified caricature has been reproduced repeatedly over subsequent decades but incorporating a variety of simplifications that tend to obfuscate several important features.

Crouch & Ma [19] have provided a description of the analogous molecule in salamander red rod rhodopsin using the same caricature at the gross level. Their version omits several of the features and all dimensions provided by Hargrave et al. and Dratz & Hargrave. The material analyzed by Crouch and Ma appears to be rhodopsin from a disk supporting the M–channel spectral channel of vision as discussed in **Section 3.1.5**. This conclusion is based on their morphological description of the material *in-vivo*. Connell et al[20]. have presented a composite figure showing the specific peptide differences between bovine, mouse and rat rhodopsin.

In this work, rhodopsin is shown to perform in its conventional role as a structural protein.. Rhodopsin is used to form the substrates (disks) that are coated by a liquid crystalline monolayer of one of four retinine chromophores. **This is its exclusive, and passive, role**. This assertion is supported by the fact that no investigator has ever demonstrated the spectral absorption of rhodopsin, in any configuration, that matches that of a spectral channel of vision.

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[18]Hargrave, P. McDowell, J. Curtis, D. et al. (1983) The structure of bovine rhodopsin *Biophys Sr Mech* vol 9(4), pp 235-244

[19]Crouch, R. & Ma, X-J. (2000) The role of Vitamin A in visual transduction *In* Livrea, M. *ed*. Vitamin A and Retinoids: An Update. . . Berlin: Birkhauser Verlag pp 59-72, pg 61

[20]Connell,G. Molday, L. Reid, D. & Molday, R. (1991) Chapter 43 *in* Hollyfield, J. et. al. *eds*. Retinal Degenerations Boca Raton: CRC Press

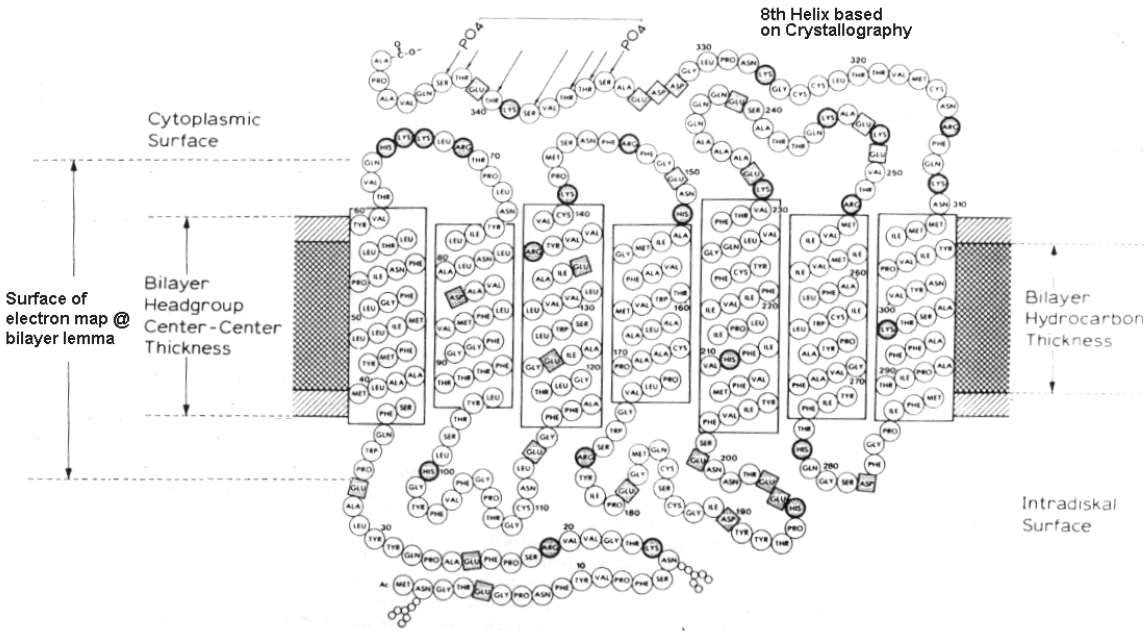

Figure 5.1.1-1 An early caricature of rhodopsin prior to crystallographic investigation. Widely used pedagogically into the 2000's. THE CONCEPT IS ARCHAIC. As demonstrated by more recent crystallography, the individual a-helices do not span the molecules forming the bilayer lemma of the sensory receptor neurons. The location of the chromophore is usually given as a ligand of either Lys296 or Glu113. In both cases, the ligand is physically isolated from the exterior of the molecule. Note the addition of the label for an 8th α-helix at the upper right, based on more recent crystallography. See text. Modified from Hargrave et al., 1983.

A single molecule of retinoid is attached to the lysine amino acid at position 296 in this unrolled representation. The retinoid is a retinene and not a chromophore of the retinine family. The seven individual helices shown (of the eight actual helices known from crystallography) completely surround the retinoid and isolate it from the exterior environment. This is more clearly represented in **Figure 5.1.1-2** from Dratz & Hargrave[21]. It shows the retinol ligand completely enclosed by the individual helices of the opsin. They provide many volumetric numbers related to the opsin layer that are useful. However, this work does not support their caricature of the large scale disk or their concept of a chromophore embedded in the lemma of the outer segment. (see **Section 4.3.5**).

The arrangement shown is not compatible with the requirements of quantum mechanics for two distinct reasons. First, the retinol ligand connected to the opsin protein by a Schiff Base does not form a chromophore. To be an effective chromophore, the retinol must be converted to a diol, such as a Rhodonine. Second, to be an effective absorber of light, the retinol ligand (after conversion to a diol) must have its long axis parallel to the direction of the light beam attempting to excite the chromophore. It is also important that the chromophore be in fluid contact with the inter photoreceptor matrix (IPM) as discussed in **Section 4.3.5**. A third consideration is the low absorption cross section for the retinene within the rhodopsin molecule relative to the area cross section of rhodopsin. At about 6%, the absorption cross section ratio is very low for an optimally performing biological sensory system.

Various investigators have described rhodopsin as either pink or purple. Neither of these labels are used to describe a "spectral" sources. The peak absorption of the isolated retinenes, either retinol or retinaldehyde occurs at about 500 nm. In transmitted light, these retinenes appear to be the compliment of 500 nm (frequently described as 500C). This compliment is not a spectral color. It is best described as a purplish-red or a purplish-pink depending on the degree of color saturation. See **Section 2.1.1.3** or **Appendix S** . In the language of subtractive color, retinene is represented by a *magenta*.

[21]Dratz, E. & Hargrave, P. (1983) The structure of rhodopsin and the rod outer segment disk membrane *TIBS*, pp 128-131

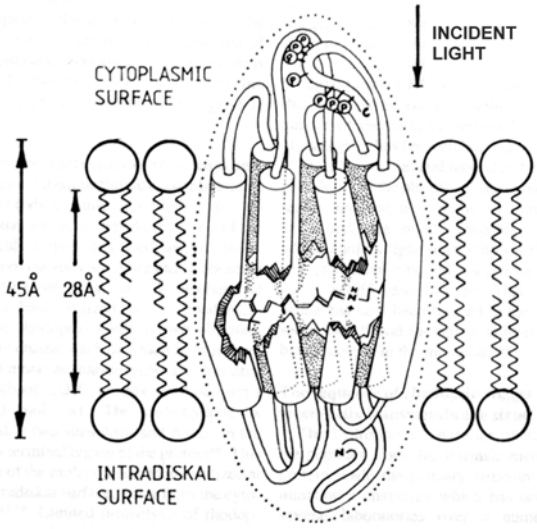

Figure 5.1.1-2 Putative diagram of a cross-section of rhodopsin forming a disk. THE CONCEPT IS ARCHAIC and QUANTUM-MECHANICALLY UNSOUND for two reasons. First, the retinol ligand connected to the protein by a Schiff Base does not constitute a chromophore, it remains a virtually transparent structure. Second, an optimal chromophore must have it long axis parallel to the direction of the applied light. See text.

5.1.4.2 Critical niche chemistry in vision

The rapid expansion of the chemical knowledge base during the last fifty years offers an alternate description of the photodetection process in vison. The mechanisms involved in vision are not normally discussed even in upper level college textbooks because of their limited general utility. To completely understand the very sophisticated design of the visual system, relative to man's current knowledge, the serious investigator must look to the specialty literature. Key niches to explore are,

+ the unique status of oxygen. It exhibits a triplet ground state that provides a unique property in quantum-mechanics related to its excitation and its lifetime in the excited state.

+ the unique nature of a dicarbonyl polymethine and particularly a conjugated dicarbonyl polymethine, also known as a carboxylic ion system. The Rhodonine family, as a constituent of this system, exhibits a unique confluence of three different definitions of the term resonance (See Glossary, **Section 5.2.3**). As a result, they exhibit a set of spectral characteristics identical with those found in vision.

+ the unique transfer of energy between one chemical species of material and a second semiconducting structure of other species through a more broadly defined mechanism than that described by Coxon & Halton as an intersystem crossing (ISC). This mechanism generates a free electron in the neural system for each photon absorbed by the chromophores (with a second order exception in the red region).

The roles of these three niches are explored in this chapter and **Chapters 4 & 8**. With their contribution to the knowledge base, the operation of the photodetection process of vision can be explained without employing any crutches.

5.1.4.3 Resolving the nomenclature issues between niche fields of chemistry

In explaining the photodetection process in vision, there remains the ever present problem of clashes in nomenclature as two previously separate fields of chemistry become more entwined. In this case, they are the field of photosensitization chemistry as used in photography and the field of dye chemistry as used in the textile industry. The reader is cautioned to review **Section 5.4.2** before proceeding beyond that section. Nassau has presented a very comprehensive review of the phenomenon of color as it relates to the physics and chemistry of materials[22]. His chapter 6 reviews color phenomena in organic molecules. However, it stops short of developing the theory applicable to the chromophores of vision, the conjugated carbon systems named polyenes enhanced by the addition of two oxygen atoms. The critical form can be described as a carboxylate where the two oxygen atoms are separated by a polyene chain. This configuration forms a dipole resonant carboxylate. The same materials are known as oxynols in photographic chemistry.

5.1.4.4 Crystallography of rhodopsin

As noted elsewhere in this work, rhodopsin, the primary structural protein forming the disks of the outer segment of the visual sensory neurons, is not an active element in the visual transduction process. On the other hand, it does contain a ligand of a retinene coupled through a Shiff Base to the protein portion of the opsin.

Because of the conventional wisdom that rhodopsin plays a major part in transduction, it has been studied extensively, most recently by x-ray crystallography (**Section 7.1.1.4**). The Zhang laboratory at the University of Michigan is a major investigator and repository of data related to rhodopsin[23]. The database cites 31 articles related to rhodopsin under a

[22]Nassau, K. (1983) The Physics and Chemistry of Color: The Fifteen Causes of Color. NY: John Wiley & Sons.
[23]Zhang Laboratory http://zhanglab.ccmb.med.umich.edu/GPCR-EXP/

variety of conditions. Their putative chromophore, rhodopsin is described as a G-protein-coupled receptor (GPCR), also known as seven-transmembrane domain receptors, 7TM receptors, heptahelical receptors, serpentine receptor, and G protein–linked receptors (GPLR). The letter G represents the ubiquitous guanine group. Guanine is a nucleoside (as distinguished from a nucleotide) found as one of the four nucleosides creating DNA and RNA. The list of putative GPCR is currently endless[24]. The interpretation of the labels on the cited page can be found at HUGO[25]. Only the Class A GPCR's relate to the spectral performance of the visual modality.

> The term transmembrane helix is largely archaic and not descriptive of a GPCR. It was used to caricaturize the proteins believed to be present in the walls of bilayer lemma before the day of precision crystallography. The labels 7TM and and heptahelical receptors are also misleading as shown via crystallography. The protein + retinoid complexes of interest in vision actually contain 8 α-helices. The label, serpentine receptor remains viable, but only when the helices are interpreted as all within the envelope of the rhodopsin molecule.
>
> As shown in the following figures, the individual helices are generally not long enough to extend through the full thickness of the nominal bilayer lemma. They are also too small in diameter to transport a molecule through the lemma. The current concept in the literature is a vague one; it is proposed that the overall molecule changes conformation as a result of a photon striking a retinene molecule perpendicular to its long axis. Based on the dimension above, the absorption cross section of the retinene is only about 6% of the ellipsoidal cross section of the protein moiety. Such a low percentage is not compatible with an optimal biological design.
>
> The conceptual model of the transduction process under the chemical theory of the neuron remains so crude, it can not be interpreted here in sufficient detail to evaluate vis-a-vis the Electrolytic Theory of the Neuron.

A particularly valuable data source from the Zhang laboratory database is Teller et al[26]. This paper was assembled by a multi-disciplinary team from several departments of the University of Washington (Seattle). The work employed bovine rhodopsin that has been shown to be very similar structurally to human rhodopsin. Figure 1 does not label the retinal within the molecule but is appears to be shown in red. In figure, 2, "Lys^{296} forms this protonated Schiff base with retinal and is shown as a black filled circle. Glu^{113} is a counterion of the Schiff base and is shown as a red filled circle." The difficulty of representing the retinal in x-ray crystallography is indicated by the following quotation from the caption of their figure 5(c); when comparing bovine rhodopsin and bacterirhodopsin, "Note the irregular and kinked helix in rhodopsin as well as the differences in the location of the Schiff base attachment and orientation of the retinal chromophore." The differences in the orientation of the retinal are sufficient to cause a major difference in sensitivity to the applied illumination. These differences may be required to accommodate the difference in application of the two molecules. The paper is accompanied by very detailed supplemental material.

Teller et al. describe the spectral performance of their rhodopsin as follows; "Rhodopsin is a red-colored protein due to a prosthetic group chromophore, 11-cis-retinal (Figure 1, chromophore in red). The chromophore is bound in the hydrophobic core of the molecule, causing its absorption maximum at approximately 380 nm to be shifted bathochromically to that characteristic of intact rhodopsin at 500 nm." Citations are provided to support this assertion but they are quite old. Two date from the early work of Wald in 1968 and appear in popular scientific journals. One is by Kowoser from 1988[27]. Kowoser appears to rely upon a very old interpretation of the chemical theory of the neuron and a very complex concept of chromophore excitation. Kowoser appears to assert incorrectly, "The bilayer thickness is taken as 36 A (24 amino acids in an α-helix)." This appears to be a miscalculation. [xxx sort through these calculations] As noted in the next paragraph, The length of 24 amino acids arranged in an a-helix only advances, has a length of, 10 Angstrom. Furthermore, the thickness of the bilayer forming the outer lemma of a sensory neuron is known to be 80 Angstrom. The Kowoser paper has only been cited by 38 people in 27 years. Note, the fact that a peak absorption at 500 nm does not correspond to the spectral peak of any resonant conjugated chromophore (Section xxx). This peak is associated with the non-resonant but conjugated retinal. Figure 2 of Kowoser may contribute information concerning the location of retinal within the rhodopsin molecule, but it is probably dated. Kowoser goes on, "The arrangement in Fig. 2 is only approximate, arbitrarily counterclockwise in helix order and schematic, since the overall structure of rhodopsin is not known. Bacteriorhodopsin, for which both fine and overall structural details are established, and rhodopsin may be similar, but the similarity hypothesis cannot be applied without further information in view of the

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[24]IUPHAR/BPS Guide to Pharmacology (2016) http://www.guidetopharmacology.org/GRAC/ReceptorFamiliesForward?type=GPCR

[25]HUGO Gene Nomenclature Committee (2016) http://www.genenames.org/genefamilies/GPCR

[26]Teller, D. Okada, T. Behnke, C. et al. (2001) Advances in Determination of a High-Resolution Three-Dimensional Structure of Rhodopsin, a Model of G-Protein-Coupled Receptors (GPCRs) http://zhanglab.ccmb.med.umich.edu/GPCR-EXP/pdf/gpcr2.pdf

[27]Kosower, E. (1988) Assignment of groups responsible for the "opsin shift" and light absorptions of rhodopsin and red, green, and blue iodopsins (cone pigments) *PNAS USA* vol 85, pp 1076-1080 http://www.pnas.org/content/85/4/1076.full.pdf

rather different functions of the two proteins."

Teller et al. provide valuable information concerning the dimensions of the overall molecule. "Examination of the figures in the 'standard view' (figure 1) shows an ellipsoidal shape. The dimensions of the ellipsoid are ~75 Angstrom perpendicular to the membrane, ~48 Angstrom wide in the standard view, and ~35 Angstrom thick in this view." In their supplemental material Teller et al. describe the tilt angles of their individual helices relative to the axis of the molecule. It is important to note this molecule is not large enough to be a transmembrane protein in the manner it is typically depicted. The external lemma of a typical neuron is nominally 80 Angstrom thick (**Section 2.2.2.7**). Bovine rhodopsin is certainly not large enough for seven of it's a helices to cross the external lemma of a sensory neuron seven times. The helices of bovine rhodopsin are in the range of 102 to 183 Angstrom according to Teller et al. (page 4) based on a1.5 Angstrom advance per residue of an α–helix and 3.6 residues per turn (**Section 7.1.1.4.4** from Glusker et al.) "Bovine rhodopsin contains 348 amino acids, has a molecular mass of ~40 kDa, and folds into seven transmembrane helices varying in length from 19 to 34 residues (102 to 183 Angstrom) and one cytoplasmic helix (Figure 1, blue rods encapsulating α-helices; Figure 2)."

Figure 5.1.1-3 reproduces Figure 1 from Teller et al. with a realistic lemma added from Dratz & Hargrave[28]. Both protein and lemma are shown to the same approximate scale. Because dimensions are poorly defined at atomic and molecular levels, several dimensions are shown for the lemma; The 28 Angstrom value indicates the nominal distance between the ends of the aliphatic tails of the typical phospholipid bilayer lemma. The 45 Angstrom value is the distance between the centroids of the phospholipid head groups of the lemma. The 80 Angstrom value is suggestive of the distance between the distant edges of the electron density maps associated with each phospholipid of a bilayer lemma. Such phospholipids may also have a Helmholtz layer of water molecules associated with both the inner and outer surfaces of the lemma (**Section xxx**). It is necessary to define the actual phospholipid molecules in greater detail (particularly their head groups) in order to specify their actual length more precisely. In addition, when employed as chemical receptor cites, each molecule of the phospholipids of the outer lemma bilayer may also include an additional ester of an amino acid.

The size of the putative GPCR of vision is clearly disruptive of the phospholipid bilayer lemma as illustrated by Dratz & Hargrave. A 48 Angstrom diameter GPCR is roughly five times the diameter of a single phospholipid molecule (9.76 Angstrom when present in a liquid crystalline lattice). The individual α-helices are not sufficiently widely spaced to intertwine their way through the lemma between phospholipid molecules as frequently suggested by caricatures in the literature (Dratz & Hargrave, fig 3).

To date, no electron micrograph or other physical evidence of a GPCR interconnecting the extracellular side and the cytoplasmic side of a sensory neuron lemma has appeared in the literature. Similarly, no evidence has appeared showing the reconfiguration of the GCPR actually causing excitation of any of a broad range of proteins (a typical putative case is phospholipase C).

[28]Dratz, E. & Hargrave, P. (1983) The structure of rhodopsin and the rod outer segment disk membrane *TIBS*, pp 128-131

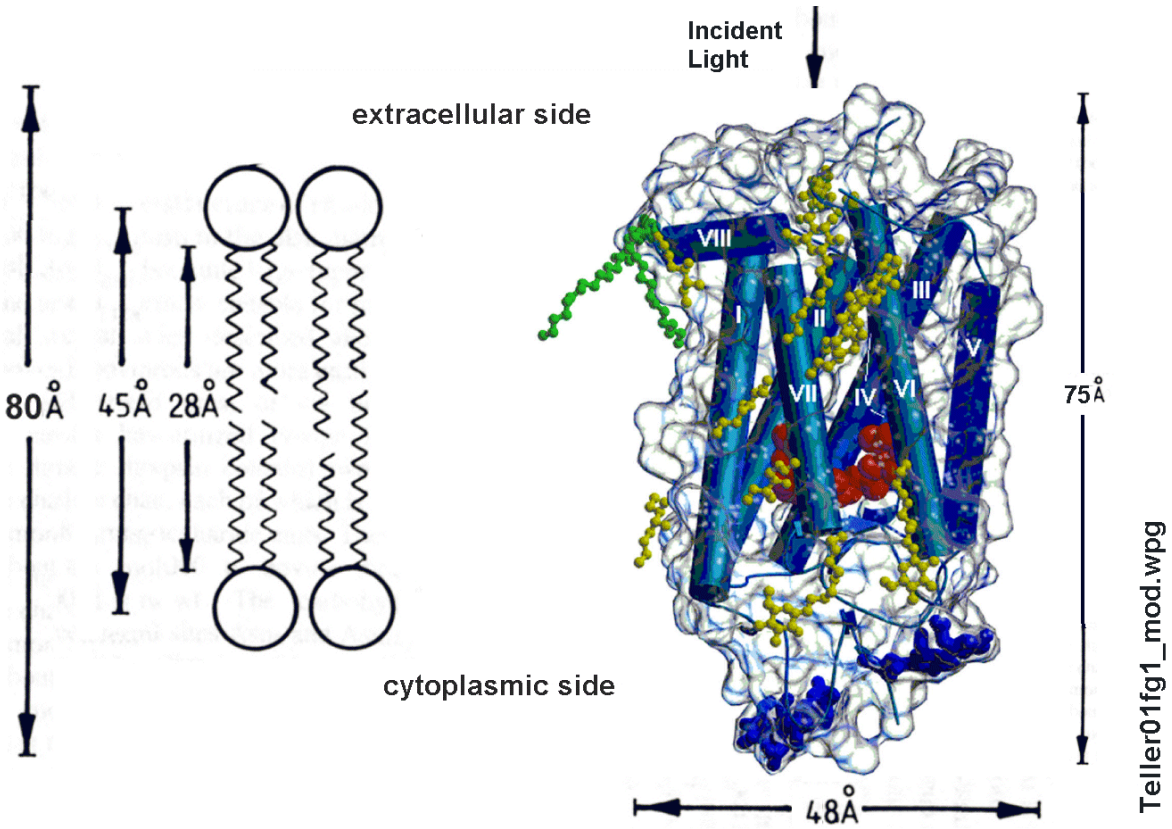

Figure 5.1.1-3 Bovine rhodopsin from x-ray crystallography and a sensory neuron lemma shown to the same scale. The retinene ligand within rhodopsin is shown by the red balls in the center of the molecule. The dimensions of a lemma are difficult to show with precision. 45 Angstrom indicates the distance between the centroids of the head groups of the phospholipids of the bilayer lemma. The distance between the distant edges of thetwo electron maps of the bilayer lemma is nominally 80 Angstrom without considering any Helmholtz layer associated with the lemma. See text. Lemma from Dratz & Hargrave, 1983. Protein from Teller et al., 2001.

Kosower introduced a different method of solving/avoiding the transmembrane helices problem. He assumed a much thinner bilayer membrane of only 36 Angstrom. He did this by merely defining the bilayer lemma as having a nominal thickness equal to that of 24 peptide residues of an α-helix (without concern for the angular tilt of the individual helices relative to the axis of the rhodopsin complex or the orientation of the lemma. He accompanied his schematic with the assertion that also applied to his figure 1, "The arrangement in Fig. 2 is only approximate, arbitrarily counterclockwise in helix order and schematic, since the overall structure of rhodopsin is not known." Kosower was a physical organic chemist and approached the problem by examining the potential movement of charge between atoms and groups within the peptides in a single prototypical opsin +retinene complex adequate to justify the large "bathochromic spectral shifts suggested by the literature for the other chromophores that were derived from the rhodopsin base line. Using this approach, he noted; "Two serious difficulties stand in the way of an exact calculation of electrostatic and other effects. First, the amino acid side chains can exist in a number of different local conformations that may interconvert by "single group rotations." The distances between charged groups then cannot be exactly specified. Second, the dielectric constant of the regions between the charges may vary in a complex way from 2 to 78 (35-39). The electrostatic interaction difference between the ground state and the excited state can thus be estimated only roughly." The Kosower paper cannot be relied upon.

Teller et al. note, "Another interesting conformational issue concerns the orientation of the ß-ionone ring with respect to the rest of the chromophore. The diffraction data at 2.8 Å resolution are sufficient to identify and orient the ring, although there is a possibility of partial occupancy of rings rotated by 180˚ about the C6–C7 bond. Solid-state NMR measurements have shown the orientation of various C–C vectors in the ring with respect to the membrane normal. The NMR results are consistent with a ring rotated 180˚ from the ring found in the crystal structure; see Figure 3B. However, other studies indicate that the retinal chromophore is in the twisted 6-s-*cis* conformation in rhodopsin, in contrast to the planar 6-s-*trans* conformation found in bacteriorhodopsin. The inconsistency between the X-ray crystallographic and solid-state NMR studies requires further study, but it should be pointed out that the binding site for the ß-ionone ring was demonstrated to accommodate a variety of substituents. Thus, it can be oriented as either 6s-*cis* or -*trans* in the

ground state (K. Palczewski, unpublished)."

5.1.5 Chemical Analogs to Vision Chemistry

Research in vision has followed a relatively isolated path; that is so common in science. Very few references are found in the vision literature to concepts brought over from other fields except in the popular field where the eye is frequently compared to a camera. When inter-disciplinarians have tried to contribute to the vision field, they have encountered massive resistance. (Rose 1976).

The resistance to cross-disciplinary ideas has now reached the stage where the Emperor of conventional thinking in vision research "has no clothes." This is particularly true in the area of photodetection where the field has been dominated for a very long time by the concept of photo-isomerization of a molecule that has never been characterized in terms of its chromophoric properties. More recently, this photo-isomerization has been linked to the movement of Calcium and/or Sodium ions through an enveloping membrane (surrounding the Outer Segment of the photoreceptor cell) that has never been observed, intact or after fracture, even under the electron-microscope. Furthermore, there has never been an assay of Outer Segments that showed a significant concentration of calcium or sodium bearing molecules to be present.

[xxx repititious]
Based on some early work in the 1930's, the assumption has been that the chromophores of vision are based on retinol or retinal coupled to a protein, opsin, in such a way as to form a chromophore. The variation in the location and possibly the style of coupling has been assumed to account for the actual spectral absorption of the material. After massive efforts, this premise has still not been demonstrated. However, it has caused a great deal of concentration on retinol and retinal as the fundamental structure of the chromophores. To this day, no laboratory experiments have shown either of these two retinoids exhibit a spectral absorption characteristic matching those of vision, human or otherwise. **The retinols are neither chromophores nor analogs of the chromophores of vision.** The retinols are chromogens of the chromophores of vision.

The study of structural analogs of retinol has been extensive. However, there are no reports in the literature of any of the retinals or their analogs exhibiting the isotropic spectral peak at 500 nm or the anisotropic peaks at 437, 532, or 625 nms that characterize the chromophores of vision. A true analog of the chromophores of vision must exhibit both an isotropic and anisotropic spectra. This requires that the material be analogous to Rhodonine in more than just the structural sense. The material must exhibit quantum mechanical conjugated molecular resonance. This requires the material contain two heavy atoms separated by a conjugated chain. It must also be present in the liquid crystalline state to achieve the necessary spectral line broadening and enhanced absorption cross section. Only one material reported in the literature has exhibited the appropriate spectra of a quantum mechanically resonant conjugated retinoid. It will be discussed below. **While this material is not a chromophore, it is an analog of the chromophores of vision, the Rhodonines.**

[xxx repititious]
This work takes a completely different path based on the application of scientific tools unknown before the 1960's, and still essentially unknown in the biological literature. It also incorporates a set of retinoid molecules into the theory of vision that can be shown to have the required chromophoric properties that Vitamin A aldehyde clearly does not possess. Neither retinol modified by a Schiff base nor further protonation of that Schiff base offers the required absorption spectrum[29].

The photoreceptors of the eye utilize "dyes" to convert the incoming light into at least the precursor to a nervous system signal. The field of dye chemistry is extremely well developed and broad. It is also extremely complex. For many years, dye chemistry delineated itself between natural dyes and man-made dyes. This was done for a number of reasons; the natural dyes were generally inferior in absorption, stability and applicability to all but the first few man-made counterparts and the natural dyes involved grossly different fabrication techniques. However, the chemical rules derived during the development of man-made dyes are also applicable to the natural dyes. In recent years, the separation between natural dye chemistry and man-made dye chemistry has begun to disappear. Many natural dyes can now be classified in the same tables as the man-made dyes. This will be demonstrated below.

The term "dye" is frequently used to describe two major chemical classes; colorants that affect the perceived color of other materials, and transducers that accept photons of a specific wavelength and generate a technical response that can take on a number of forms. Dyes as colorants are not total analogs to visual photo-reception, but the chemistry of photography is. This field has nearly all of the same constraints and requirements as does vision chemistry. It can be very instructive to review dye chemistry as it applies to photography and transition that analog to the natural vision

[29]Shichi, H. (1983) Biochemistry of vision NY: Academic Press pg. 137

chemistry arena.

It was only shortly after the dawn of silver halide photography that natural organic dyes were found capable of extending the spectral range of the basic emulsions[30]. The use of these dyes proceeded to expand rapidly on an empirical foundation until the 1950-60's. At that time, the work in colorant chemistry and photographic chemistry began to merge and even more effective progress was made, including the detailed description of the why's and how's of the underlying chemistry.

Areas of particular interest to the vision area were in defining the rules which determined the absorption effectiveness of a chromophore, the elucidation of the "liquid crystalline" state of matter, the electronic characteristics of useful chromophore and the mechanisms of interfacing between chromophores and their associated "signal" receivers.

It was quickly found that any truly effective chromophore had to contain a conjugated chain of (usually) carbon atoms terminated by a polar atom on each end. Everything else was secondary, but still important for other reasons such as stability. It was also found that these effective materials exhibited very strange physical characteristics; they aggregated into crystal like structures in high molarity solutions and on surfaces. These structures exhibited unusual electronic properties, including the transmission of energy through the breadth of the structure. Although developed toward different purposes, the man-made liquid crystal is now one of the most pervasive materials in our everyday life. In addition, the liquid crystal is now being recognized as the predominant structure in most biological connective tissue. Mees & James provide a variety of figures showing overall concept of, and the specific details for, individual photographic dyes[31]. Shichi has presented a series of analogs of retinal that unfortunately do not meet the basic requirement of two polar atoms separated by a conjugated carbon chain[32].

One of the basic problems in dye chemistry as applied to photography was; how does a low energy photon get absorbed by a dye and cause the excitation of a silver halide crystal that requires a higher energy? This "intersystem" problem was solved when it was found that the energy could be accumulated in the liquid crystal and transferred to the silver halide in multiples of the incoming energy. This process has been exploited widely in the photographic field and also in the dye laser field where "frequency doublers" are now widely used. A frequency doubler is an organic dye that accepts long wavelength photon energy, accumulates the energy and causes the emission of a photon at a wavelength corresponding to approximately twice the energy of the incoming photons. Frequency tripling, and higher orders, are also achieved but usually at much poorer efficiencies. This capability is exploited by the long wave chromophore of vision.

In photography, the result of energy accumulation in the liquid crystals led to a 2-photon process which resulted in an essentially nonlinear effect. The transfer characteristic between input photons and the number of atoms developed in the output negative was linear for the halide portion of the spectrum but *quadratic* in the dye portion of the spectrum. This caused the overall spectral response of the emulsion to vary depending on the light level of the exposure. This became, and remains, a significant problem in color photography where the higher the exposure, the redder the hue of the output photograph. That is why indoor and outdoor color film types are sold commercially to this day, they are "balanced" for different color temperature light sources. This effect also arises in the mesopic range of vision where it is associated with the Purkinje Effect.

Modern color photography utilizes highly tailored dye molecules containing multiple auxochromes (photographically effective functional groups) to provide the widest possible spectral absorption without using multiple dyes in individual dye layers. The auxochromes invariably lie in a plane for this is a condition for forming effective liquid crystals capable of sharing energy within the crystal. This energy is then transferred to the silver sulphide "site" on the silver halide crystal where development occurs. This involves the conversion of a silver ion to free silver.

Chromophores containing more than two auxochromes can exhibit multiple peaks in their visual region absorption spectrum. However, effective chromophores consisting of only two auxochromes in resonance exhibit a single peak that is usually much higher than that of their non-resonant molecular and dipole based spectral absorption peaks. These resonant peaks can be mathematically described based on first principles. Examples of these peaks are shown in [**Figure 5.5.10-1**] below. Note the resonant peak(s) is far from the molecular peak and at least seven times higher in the case of the man-made dyes. Indigo is one of the best natural plant-based dyes. Its absorption coefficient is only one tenth of that of the man-made dyes.

One feature, only now appearing in photographic emulsions, has been effectively utilized in vision; the stacking of individual layers of liquid crystalline material to achieve higher absorption coefficients in the desired region--and

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[30]Hamer, F. (1964) The cyanine dyes and related compounds. NY: Interscience publishers, John Wiley & Sons.
[31]Mees, C. & James, T. (1966) The Theory of the Photographic Process, 3rd ed. NY: Macmillan. Chap. 2
[32]Shichi, H. (1983) Op. Cit. pp. 78-81

effectively suppressing any undesired minor absorption peaks.

5.2 Glossary

Because of their family relationships, many terms in this section are grouped into subsections below. Only individual terms are defined in this section.

Fibrillar colloid–A liquid crystalline structure that appears to consist of individual fibers.

Hydrogen bond–A bond between a hydrogen nuclei and two other entities. One bond is a strong ionic bond (with the donor atom) while the second is a weak covalent bond (frequently shown by a dashed line) with a second structure. The only elements concerned in hydrogen bonding are nitrogen, oxygen, fluorine and sometimes chlorine.

x,y,z axes–used to define the spectral absorption axes of a complex molecule. x describes the length or major axis of the molecule. y describes the width of the molecule and z describes the thickness of the molecule perpendicular to the x,y plane.

5.2.1 Terms specific to the liquid crystalline state of matter

Gray has provided an excellent book on the liquid crystalline state as known in 1962[33]. It includes many detailed definitions. Although he notes the wide occurrence of liquid crystals in biological tissue, he limited his discussion of such crystals to the introduction. Materials in the liquid crystalline state can be divided into two major groups, those that are thermotropic and lyotropic.

Lyotropic– A material initially in solution that changes state as a function of the concentration of the solution.

Thermotropic– A material that changes state primarily as a function of temperature.

Amphiphilic–A compound with a hydrophilic head and a hydrophobic, organic, tail.

Amphoteric–Capable of acting as either an acid or a base.

Each of the above groups can be divided into a series of phases describing the orderliness of the molecular associations. The definition of these phases is still in evolution and a reference is useful[34, 35].

Nematic– The axes of the molecules are parallel or nearly (locally) parallel. The molecules are generally not side-by-side. Compare to Smectic.

Smectic– The axes of the molecules are parallel. The molecules are arranged side-by-side in a two-dimensional film. The films may be stacked in layers. There are second order parameters describing the molecular array more specifically. The basic smectic arrangement can be found in both thermotropic and lyotropic liquid crystals.

5.2.2 Terms specific to Spectral absorption

Rotational absorption–A low energy-level change in the rotational state of a molecule characterized by absorption in the far infrared.

Vibrational absorption–A low energy-level change in the vibrational state of a molecule characterized by absorption in the near infrared.

Molecular absorption–A higher energy-level change involving a change in the energy levels of a molecule and characterized by isotropic absorption in the visible or ultraviolet range.

Dipole molecular absorption– A medium energy-level change involving a change in the energy levels of a molecule characterized by anisotropic absorption in the visible or ultraviolet range.

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[33]Gray, G. (1962) Molecular structure and the properties of liquid crystals. NY: Academic Press
[34]Brown, G. Doane, J. & Neff, V. (1971) A Review of the Structure and Physical Properties of Liquid Crystals. Boca Raton, FL: CRC Press.
[35]Brown, G. (1977) Op. Cit.

Conjugated Dipole absorption–A specific type of dipole absorption represented by a structural resonance related to the conjugated backbone of a molecule and characterized by isotropic absorption in the visual spectrum.

Resonant absorption–A specific type of conjugate dipole absorption associated with two auxochromes separated by a conjugated chain of carbon atoms characterized by absorption in the visual spectrum. The resonance is due to the low velocity of charge transport along the molecule. As a result, the effective wavelength of the molecule is equal to that of the incident photons. The absorption is generally anisotropic.

The shifts in the peak absorption that may result from changes in solvation are defined in Nassau (1983, pg 111) as:

Hyperchromic–displaying a higher extinction coefficient, e.g., higher absorption coefficient.
Hypochromic–displaying a lower extinction coefficient.
Hypsochromic–displaying a peak shifted toward shorter wavelength.
Bathochromic–displaying a peak shifted toward longer wavelength.

Since the chromophores of vision are in the liquid crystalline state and not solvated, these terms are usually not required when discussing *in-vivo* absorption.

5.2.3 Terms specific to pharmacology and retinoid transport

The chemistry of the chromophores of vision are closely tied to the chemistry of natural dyes and the field of pharmacology where many of these materials were first isolated. The following classes describe the relevant terms.

Active-A term originally used to indicate whether a given retinoid showed biological activity as a vitamin in humans. More properly used to describe such activity in any species, order or phylum.

Carotene-A hydrocarbon consisting of a conjugated carbon chain attached to an ionone ring. Most frequently, a conjugated carbon chain terminated at each end with an ionone ring.

Protovitamin-A material, usually a dimer, that can be cleaved to produce one or more materials exhibiting biological activity associated with vitamins.

Retinoid-A term undergoing nearly continual modification in order to accurately describe the characteristics of a large family derived from the carotenes and xanthophylls. The family exhibits specific properties required in vision, metabolism and reproduction. Many of these properties are dependent on the stereo-chemistry of the materials. Only some of the stereo-chemical features may be required in a given application. The definition of Sporn, et. al. in 1994 is the most widely used. However, it does not stress the visual properties required to form a chromophore in vision.

> The retinoids of interest in vision all contain an ionone ring attached to a conjugated carbon side chain and two oxygen atoms with conjugation maintained between the two oxygens.

Xanthophyll-A carbohydrate consisting of a conjugated carbon chain attached to a ring structure containing oxygen. Most frequently, a conjugated carbon chain terminated at each end with a ring structure containing oxygen.

The definiton of a vitamin and a hormone are given in **Section 5.2.3**. However, this work has led to the definition of a new vitamin which is active in Order *Diptera* of *Arthropoda*. As will be discussed below, Wolf & Johnson say this type of Vitamin A, their structure III, does show biological activity in rats.

Vitamin A3-Specifically 3-hydroxyretinol. The aldehyde and acid forms may also be effective as vitamins but it is the alcohol in the all-trans form that is used in the vision of some *Arthropoda*. Generally derived from the cleavage of the protovitamin, zeaxanthin.

The literature related to the carotenes and xanthophylls is very large because of its importance in human nutrition. However, the scope of the literature devoted to Vitamin A_2 is minuscule by comparison. The literature devoted to Vitamin A_3 is even smaller.

5.3 Terminology

The photochemistry of vision depends on a quantum-mechanical foundation and involves states of matter that may not be familiar to the average investigator. These must be carefully defined. In the absence of careful definition, it is impossible to account for the spectral characteristics of vision. Recently, science has defined a variety of states of matter beyond the conventional gas, liquid and solid. These states may each be observed in several forms

5.3.1 States of Matter

More and more forms of matter are being found that deserve specific identification because of their unique properties that do not fit the descriptions of the historical states of gas, liquid, and solid. These forms are recognizable at the macro molecular cluster level and above. The most important of these in animal vision is a mesophase becoming more widely known as the liquid crystalline state. It has an orderly arrangement of molecules and atoms and can be described using the terminology of crystallography. Whereas conventional crystals exhibit compression strength, liquid crystals, normally exhibit little or no compression strength.

Technology has spawned the development of many man-made liquid crystals. However, it should be noted that most organic hydrocarbons can exist in a liquid crystalline configuration. When they exist within a very limited temperature range, they may exhibit unusual properties. This range is frequently defined as the biological temperature range.

As the complexity of molecules increases, it is found that they can exist in a variety of liquid crystalline states. Additional categories, called phases, must be defined within the liquid crystalline state. Brown & Wolken[36] have studied these phases of matter as they relate to biological situations. In a more specific analysis, Brown describes many of the features of these states but concentrates on the thermotropic sub-state[37]. Small has addressed the liquid crystalline state from a narrower biological perspective[38]. He also discusses the lyotropic state from this perspective although he focuses on the formation of the liquid crystal by the penetration of a crystal by a solvent instead of as a result of raising the concentration of a solution. In more detaileded descriptions of the lyotropic sub-state than found in Brown, the labels nematic, smectic and cholosteric also appear. **Figure 5.3.1-1** presents the best available description of the two liquid crystalline materials of significance in vision. The most important fact is that the Rhodonines are smectic liquid crystals formed lyotropically in polar solutions. This figure is discussed in greater detail in **Section 4.1.1**.

[36]Brown, G. & Wolken, J. (1979) Liquid crystals and biological structures. NY: Academic Press.
[37]Brown, G. (1977) Structures and properties of the liquid crystalline state of matter. *J. Colloid Interface Sci.* Vol. 58, pg. 534
[38]Small, D. (1977) Liquid crystals in living and dying systems. *J. Col. Interface. sci.* vol. 58, no. 3, pp. 581-602

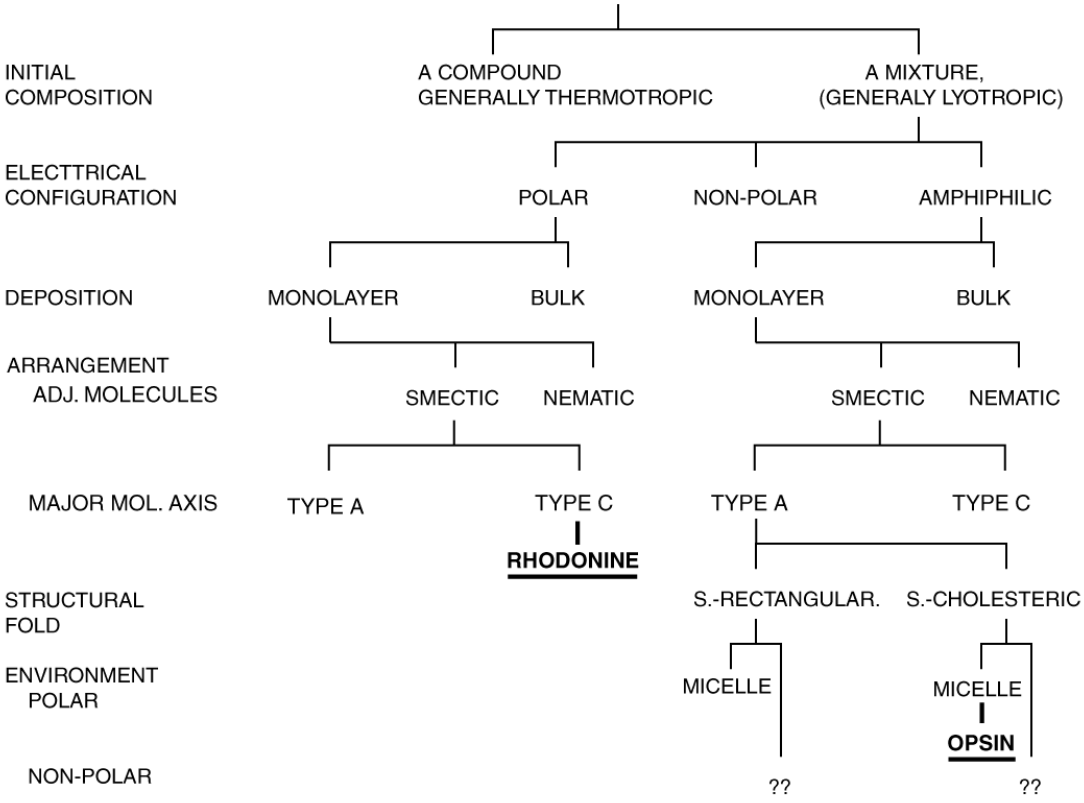

Figure 5.3.1-1 Characteristics of the liquid crystalline materials critical to vision. Both Rhodonine and opsin are formed lyotropically in the smectic phase in a polar environment . See text in Section 4.1.1.1.3

A material in the liquid crystalline state can be in a pure form such as hydronium, the liquid crystalline form of water. In this case, hydronium is described as a macromolecule consisting of a large number of individual water molecules exhibiting a precise crystalline structure but exhibiting negligible compression strength. This is not the form of water found in ice.

Alternately, a liquid crystal can exist as a special situation within the general category of solutions. In this case, the material may exist in an extended molecular structure in conjunction with the solvent. The result is generally described as a gel or colloid, materials exhibiting some structural rigidity but very little compression strength. The crystal structure of these materials is difficult to describe by normal crystallographic techniques due to its size and its minimal rigidity.

5.3.1.1 The Liquid Crystalline State

The liquid crystalline state of matter is found in several different situations, particularly related to large organic molecules but also occurring in the case of water.

Most large organic molecules will assume a fixed configuration if caused to precipitate from a solution or otherwise arrange themselves. This configuration will exhibit the properties of a liquid crystal. These properties include unusual electronic properties generally associated with a semiconductor. In most, if not all biological cases, these materials can be described as of the p-type. Their hole conductivity is higher than their electron conductivity. With great care, these materials can be described using crystallographic techniques.

Hydronium is a liquid crystalline state of water found under a variety of special conditions. It is usually described as a macromolecule. It is crucial to the operation of the animal nervous system due to its specific electronic properties. When used as the base material in the Activas of the neural system, the hydronium liquid crystal has a thickness of less than 100 Angstrom. Hydronium is a n-type semiconductor material with a significant hole velocity in its valence band relative to the electron velocity in its conduction band.

A sandwich-like liquid crystalline structure consisting of two large organic molecules with a liquid crystal of hydronium between them is the key to the electronic mechanisms of the nervous system. This structure is known as an Activa. Its characteristics and mode of operation is the subject of **Chapter 8**.

5.3.1.2 Forms within the Liquid Crystalline State

The large organic molecules that can be described using crystallographic techniques are found to occur in several structures not found in normal crystals. These additional structures are known as the nematic and smectic phases of the liquid crystalline state. They are inadequately described in the vision literature. One of the few descriptions of limited applicability is in Wolken[39]. These materials have assumed great importance recently in the graphic displays associated with computer terminals. The applicable foundation in this area is presented in the terminology section of Chapter four.

Most of the molecules of interest in vision and biology are prepared lyotropically. There are three main classes, the nematic, the smectic, and the cholesteric-nematic (frequently simplified to just cholesteric) When deposited on a substrate, the chromophores of vision assume a structure of smectic class. This class is further subdivided into types A through H. Type A liquid crystals have their major molecular axis perpendicular to the surface of the film. Type C crystals have their major molecular axis at some other angle. Assuming an analogy of similar chromophores in photography, it is most likely that the chromophores of vision are of type C.

5.3.1.3 Energy coupling between chemical structures

A variety of energy (Jablonski) diagrams have appeared to define the transfer of quantum energy within the electronic structure of various chemical and physical structures when in a crystalline state. The figures have generally been drawn for pedagogical purposes. **Figure 5.3.1-2** provides an extended energy diagram that expands on several important concepts. The terminology is basically that of Coxon & Halton[40] and similar to a slightly more complex diagram in Zollinger[41]. The conventional diagram shows absorption, A, on the left coupled with a transition involving the singlet state. The presence of oxygen introduces an alternate possibility, absorption causing the direct transition of an electron to the excited triplet state. The figure shows two distinct types of radiative de-excitation, fluorescence, F, and phosphorescence, P. The difference between these two phenomena is clear. Fluorescence involves a radiative de-excitation without a change in singlet state and is very fast, typically occurring in less than 10^{-9} seconds. Phosphorescence involves a transition from an excited triplet state to a ground singlet state and involves a much longer time interval, typically 10^{-3}– 10^{-4} seconds. V symbolizes a vibrational mode of de-excitation that is partial in extent and thermal in character. Coxon & Halton speak of two general mechanisms for non-radiative de-excitation of an electron within a crystalline structure of one species, internal conversion, IC, and intersystem crossing, ISC.

A more complex arrangement is shown by the couple on the right. This involves a third mechanism, the transfer of energy to a separate structure. This mechanism can be considered a broader concept of intersystem crossing. Coxon & Halton discuss it as involving the sensitization of the second structure and the quenching of the first. They define two types of excitation energy transfer, *resonance-excitation* transfer and *exchange-energy* transfer, within the context of simple molecular structures. Both involve "collisional transfers" over distances of nominally 5 nm. A broader definition is needed to describe energy transfer between more complex (crystalline or semi-crystalline) structures. This transfer may involve different concepts than discussed by those authors because the distances involved may be more than 50 nm. Whether the coupling of energy between these structures is defined in terms of exciton transfer or phonon transfer is inconsequential to vision. Similarly, whether this process is specifically defined in terms of an intersystem crossing is also inconsequential. This is the process defined as translation in this work. All of the transition times shown in Coxon & Halton are much shorter than that required to accommodate the bandwidth of the visual system. Transition times associated with translation reported in other papers are also quite short, typically 10^{-4} – 10^{-5} seconds. This interval is generally less than the time of phosphorescence.

Coxon & Halton also present specific details of the stereometric and energy state properties of oxygen. In addition, they present details of the stereometric and energy state properties of the n-π^* transition in the carbonyl group.

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[39]Wolken, J. (1986) Light and life processes. NY: Van Nostrand Reinhold pg. 46
[40]Coxon, J. & Halton, B. (1987) Op. Cit. pp. 9-17
[41]Zollinger, H. (1991) Color Chemistry NY: VCH pg. 25

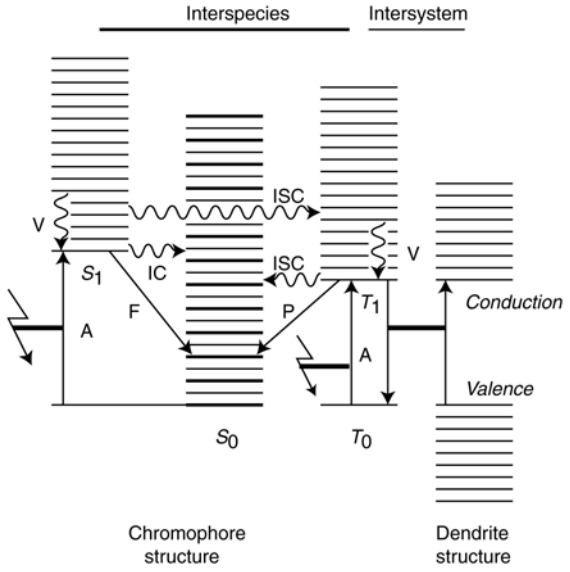

Figure 5.3.1-2 Expanded Jablonski diagram illustrating energy transfer into and between different chemical structures. The notation follows Coxon & Halton, 1987. See text.

5.3.2 The concepts of resonance important to vision

The term resonance is used in a myriad of different and important ways in vision chemistry. Many of them appear at various places in Venkataraman[42]. One of the most important, the slow-wave structure associated with structural resonance, is difficult to find in the literature and is discussed in Platt[43]. Virtually all of the forms of resonance discussed below are found in the Rhodonines, the chromophores of vision. Thus, care is required to be precise in using this term when discussing vision.

5.3.2.1 Chemical resonance

At the atomic level, resonance is used to describe the unique properties associated with a pair of covalent electrons exhibiting opposite spin vectors.

At the molecular level, resonance is generally used to describe the presence of a chemical duality that contributes to a lower energy state than found in otherwise similar situations.

Also at the molecular level, resonance is used to describe the ability of conjugated chains of atoms, primarily carbon but also including oxygen and nitrogen chains, to exhibit two arrangements of the conjugated bonds.

There is a special case of great significance to vision. That is the duality exhibited by a chain of carbon atoms terminated by a polar atom of either oxygen or nitrogen at each end. This structure can exhibit conjugation over its entire length.

5.3.2.2 Quantum-mechanical resonance

There are a variety of interaction modes between photons and chemicals. They can generally be grouped as involving destruction, ionization and excitation. They can also be sorted with respect to whether they impact a single atom, a ligand or the entire molecule. The mechanism of interest in vision involves the quantum-mechanical excitation of the entire molecule by photons with a wavelength within the visual spectrum.

The absorption of a photon can result in the cleaving, ionization or excitation of a molecule. Cleavage and ionization usually involve ultraviolet wavelength photons and are not of primary interest in vision. Isomerization is a process in the same energy regime as cleavage and ionization. Isomerization normally requires photons with higher energy than visual (particularly long wavelengths) photons. It will be shown that no destruction, ionization or even physical rearrangement (isomerization) is involved. It is the excitation of molecules by visible wavelength photons that is the key to vision.

The nondestructive photo-excitation of a molecule by light is controlled by several mechanisms related to the bonding and mass of individual elements of the molecule. These include rotation about a bond, vibrational between two ligands separated by a bond, and the vibration of the entire molecule.

Molecular excitation by photons can be subdivided into several cases depending on the mass distribution within the molecule. The first involves the entire molecule acting as a unit of roughly uniform mass density. Alternately, the molecule can be considered either a monopole (with one area of significantly higher mass density) or a dipole. A molecular dipole generally has more clearly definable modes of oscillation than the other cases. These modes are even more clearly defined if the molecular structure between the two mass concentrations is rigidized by double-bonds, triple-bonds or other rigidizing structures. The use of a conjugated carbon chain is a particularly well recognized technique for rigidizing a molecule. Photons interacting with any of the above structures generally do so isotropically. The

[42]Venkataraman, K. (1952) The Chemistry of Synthetic Dyes, 2 volumes. NY: Academic Press
[43]Platt, J. (1964) Systematics of the electronic spectra of conjugated molecules. NY: John Wiley & Sons, paper #20

efficiency of interaction can be described in terms of an absorption cross section for the molecule. Upon absorption of a visual band photon, the molecule is raised to an excited quantum-mechanical state that generally involves singlet electrons transferring into the sigma energy band of the molecule.

The absorption cross section associated with the above quantum-mechanical resonance is strongest for a straight molecular structure. This leads to a maximum in the spectral absorption characteristic for a dipole molecule when in the all-trans form. For other configurations, the structure exhibits several lesser structural resonances along different axes that lead to shorter wavelength spectral absorption peaks. This effect was studied extensively by Scheibe[44] and an excellent figure in Venkataraman describes it.

In the electronic regime, the excited states of the individual atoms of the structure are found to be shared energy-wise. Any excited electrons may travel along the molecular structure (in a quantum-statistical sense). Excitation may occur at one point and de-excitation may occur at another.

A critical feature in the conjugated molecule is the effective rate of propagation of energy along the length of the molecule. This rate can be considerably slower than the speed of light. The result is what Platt called a slow-wave structure. Because of this feature, the structure exhibits a resonant *frequency,* determined by the structural length of the molecule divided by the velocity of energy propagation, that is equal to the *frequency* of photons in the visual spectrum. As a result of this phenomenon, visual wavelength photons can interact efficiently with these structures. This slow-wave structure is particularly apparent in dipole molecules containing two atoms of oxygen, each with at least one unpaired electron. These electrons exist in the triplet quantum state. When excited, they remain in the triplet state. Such electrons are recognized as contributing to the slow-wave structure of the molecule that is aligned with the length of the conjugated chain of the molecule extending between the two oxygen atoms. As a result of the above structural and quantum-electronic mechanisms, an anisotropic enhancement of the absorption cross section of these molecules is observed. This unique combination can be called resonance dipole molecular absorption or just resonance absorption for short. It is the key to the biological vision process and is a cornerstone of the photographic process. Its interpretation can be obscured by the more familiar but less capable conjugated dipole absorption.

In complex molecules, such as the Rhodonines, it is important to recognize that both the dipole molecular and resonance molecular absorption can coexist. It is only resonance absorption is functionally important in vision. The spectral absorption associated with both may be observed in the laboratory. However, the dipole absorption is normally only observed in the liquid crystalline state. The mechanics of the resonant molecular process will be detailed in **Section 5.4.3.**

5.3.3 Nomenclature in photon-chemical interactions and the retinoids

The process of photodetection in vision involves processes studied in three distinct fields of chemistry. The field of photodetection associated with photography and the field of photon absorption and reflection at the base of the dye chemistry field of the textile industry differ over the words chromophore, chromogen and auxochrome[45]. Gurr has attempted to avoid this problem by defining a colligator as a specific type of auxochrome[46]. The fields of biology and dye chemistry differ over the foundation of the term chromogen. In biology, the suffix *gen* is usually associated with a precursor to something, such as a chromophore, whereas in dye chemistry, chromogen is used as the overall substance incorporating a chromophore.

The above differences stem from different starting points. The dye chemists have generally started by considering the absorption spectra of chemicals in the ultraviolet region. In this region, a single simple vinylene group can be considered a fundamental chromophore. However, to an investigator interested in vision or photography, such a group is completely colorless and of no significance. For him, a chromophore must absorb in the visual region of the spectrum. In the case of an auxochrome, a dye chemist is concerned with the broad solubility of the dye while the biologist and photographic scientist are only concerned with solubility in a much narrower range. The dye chemist speaks of an auxochrome as "weakly salt forming" even though exceptions are known, consisting of large families of auxochromes, that are not salt forming. Venkataraman provides the best discussion in this area.

This work will use these three terms in a specific context restricted to the visual spectrum. A chromogen is a chemical precursor to a complete chromophore. There may be a series of precursors based on different criteria. A chromophore is a material exhibiting significant spectral absorption in the visual spectrum. The actual chromophore may only exist in a specific state of matter. In that case the same chemical species may be a chromogen when in a different state of matter. One, but generally two or more, auxochromes are found within a chromophore and are the critical element in the absorption of light. The auxochrome contains unshared covalent electrons when in the unexcited state. The

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[44]Scheibe, St. Harwig & Mueller (1943) *Z. Electrochem*. vol. 49, pg. 372 (pg. 364 in Venkataraman)
[45]Venkataraman, K. (1952) Op. Cit.
[46]Gurr, E. (1971) Synthetic Dyes. NY: Academic Press.

solubility of the chromophores is only important in the deposition of the liquid crystalline form of the materials. When in the highly structured liquid crystalline state, chromophores may exhibit significantly anisotropic absorption at different wavelengths. This performance is related to the orientation of the chromophores within the molecules within the structure. While the molecules may exhibit two distinct axes, the complete crystal may exhibit three distinct axes.

Since the 1970's, the scope of the retinoid family of chemicals has evolved greatly, spurred by the focus on pharmacology. This progress has left the description of the structure of the retinoids in disarray. The initial system was defined by Karrer. Later, a set of structural rules was evolved by the IUPAC before the explosion in man-made retinoids. Later, Frickel introduced a set of systematic rules. None of these sets of rules contained any elements related to the electronic structure of the chemicals involved. This has left a void that requires attention. **Section 5.5.12** will present a modification of the Frickel proposal that focuses on the relationship between the electronic and structural features of the retinoids of vision without detracting significantly from his systematic notation.

Goodwin has provided a useful summary of the structural chemistry applicable to the retinoids of vision[47]. However, the book is not as comprehensive as needed to address all of the types of Vitamin A. He also refers to a separate essay on the structural notation he uses[48]. Taylor & Ikawa have provided additional nomenclature, more semisystematic names and excellent information on laboratory evaluation techniques[49].

5.3.3.1 Chromophore is preferable to photopigment

The term photopigment has a long history in visual research. However, its utilization in vision has become more precarious as the scientific era has progressed. A pigment, at least in additive color, has come to mean a substance with a high reflectivity in a specific wavelength region. The perceptual color response to energy at this wavelength is frequently used to name the pigment. However, the photo-sensitive material of the neural sensors exhibit a negligible reflectance at the wavelength of their peak sensitivity. In fact, their absorption is so high when dark adapted, both their reflectance and their transmittance are negligible in this region. Outside of this region, the material is transparent. Thus the photo-sensitive material is more like a filter than a pigment. This material will be described as a chromophore in this work based on that usage in general biology; a chemical group capable of selective light absorption resulting in the coloration of certain organic compounds.

The chromophores of vision are like those of photography. Since they act like filters, they can be stacked within an optical beam and each will remove a different spectral range from the incident light. This is a feature of subtractive color, and is quite different from the use of pigments in additive color.

5.3.3.2 The stereo-chemistry of the retinoids of vision

Stereo-chemistry has played a large role in attempting to understand the visual process. In the case of the retinoids forming the chromophores of vision, their stereo-chemistry is uniquely important during their transport from th liver to the site of utilization. At the site of utilization, their stereo-chemistry assures that the chromophores of a specific wavelength sensitivity are assembled into a disk (to the exclusion of all others). Many different systems of notation have been used over the years to describe stereo-geometry. Goodwin provides a broad review of this terminology[50].

5.3.3.3 The pharmacology of the retinoids of vision

The trivial names of the chromogens and the chromophores used in vision appear in complete disarray because they were assigned by researchers pursuing other avenues of study. A great number of authors have named various chromogens independently. This section will provide a framework for proceeding based on the use of systemic names with some of the trivial names shown in parentheses. A finding of this work that there are actually three types of Vitamin A active in the visual process further complicates the matter. The general occurrence of these types of Vitamin A were presented in **Section 1.2.1.** Most marine based animals (including most terrestrial mammals) rely upon Vitamin A_1 form all their retinoid needs. Freshwater fish generally rely upon Vitamin A_2 for their needs and certain orders within Arthropoda rely upon Vitamin A_3.

The term active has historically been used to define protovitamins (dimers) that resulted in material biologically active with respect to humans when cleaved (in this case Vitamin A_1). It is only infrequently that an author will introduce a

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[47]Goodwin, T. (1984) The Biochemistry of the Carotenoids Volume II, Animals. 2nd ed. NY: Chapman & Hall Chapter 1.
[48]Goodwin, T. (1973) xxx *Essay Biochem*. vol. 9, pg 103
[49]Taylor, R. & Ikawa, M. (1980) Gas chromatography, gas chromatography-mass spectrometry, etc. *Methods Enzymol*. Vol. 67, pp 233-261 *attached to Groenendijk in personal files*
[50]Goodwin, T. (1974) Prochirality in biochemistry, *Essays Biochem*. Vol. 9, pp 103-160

caveat that some protovitamin A material can be considered active because it results in Vitamin A_2. There has been virtually no previous literature regarding Vitamin A_3. In this work, a protovitamin can be cleaved to form any one of the three types of Vitamin A. Many are dimers that can be cleaved to form two monomers of Vitamin A. The resulting monomers need not be the same type of Vitamin A. When discussing the term active in this work, it is more closely aligned to the phylum and order of the animal than to the chemical formula of the compound.

5.3.3.3.1 Hormones, Vitamins and Chromophores

Our understanding of the role of Vitamins has evolved rapidly in the last fifty years. However, as pointed out in Sporn, et. al. in 1984[51] and again in the 2nd edition of 1994, the descriptor, Vitamin A is a global designation for a large group of compounds affecting the growth, maturation and healing of groups of animals in a specific manner. To accommodate the fact that certain animal groups do not respond to these compounds equally, the subcategories of Vitamins A_1, A_2 and now A_3 have arisen.

Morton[52] writing in 1974, reviewed the situation up to that point. He relied upon the 1969 definition of Folkers to describe the "vitamin concept."

> Folkers defines a vitamin as "An organic substance of NUTRITIONAL NATURE present in LOW CONCENTRATION as a NATURAL COMPONENT OF ENZYME SYSTEMS and CATALYZES REQUIRED REACTIONS and may be derived EXTERNALLY TO THE TISSUES or by INTRINSIC BIOSYNTHESIS."

Writing contemporaneously, Wolf & De Luca said[53]: "There is now, we believe, general agreement that vitamin A is to be regarded as a hormone rather than a vitamin in the classical sense of a conenzyme. By 'hormone' we mean a substance secreted into the blood stream which influences tissues and organs so as to differentiate and elaborate new cell types and new enzymes." While the retinoids of vision are secreted into the blood stream in support of the above roles, those destined for use in the visual process are transported in a more sophisticated manner that isolates them from the blood stream.

These definitions illustrates the fact that the roles of retinol as a vitamin and/or hormone have little connection to its role as a vitamin. A more current description of the role of the retinoids considered part of the Vitamin A group is to

+ perform as a hormone in the role defined by Wolf & De Luca,

+ to also perform in a reproductive role in the testes, and

+ to perform in a third, and independent, role as the chromogens leading to the chromophores of vision.

The first two of these roles appear to focus on the conformal chemistry associated with the external profile of the molecules. The latter role focuses on the internal, quantum-mechanical structure of the molecules. While retinol, in its metabolic role as a vitamin participates in the manufacture of components of the disks of the Outer Segment of the Photoreceptor cells, it primary role is the last one. It acts as the critical chromogen, independent of any vitamin or hormonal role, leading to the production of chromophores in the retinal pigment epithelium (RPE) cells of the eye. Morton noted this role specifically: "In the retina, retinol is indubitably a precursor."

5.3.3.3.2 The source of the monomer retinoids

There are three biological forms of Vitamin A. Vitamin A_1 is based on the all trans form of retinol, a conjugated alcohol of 15 carbons and including a β-ionone ring containing one double bond. Vitamin A_2 is structurally the same except the ionone ring has been dehydrogenated, leading to two double bonds within the ring. A new, variant defined as Vitamin A_3 is defined in this work. Vitamin A_3 is structurally the same as Vitamin A_1 except a hydroxyl ligand has been attached to position 3 of the ring. These three vitamins can be related to their role in vision in terms of the host animal by the following table. It also provides a consistent nomenclature for the chromophores of vision, the Rhodonines. As will be shown below, the chromophore families are not identical to the chemicals on the left. Each of these chromophore families consist of four members to be defined within the parentheses.

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[51]Sporn, M. Roberts, A. & Goodman, D. (1984) The Retinoids, Volume 1, NY: Academic Press, pg 2-5

[52]Morton, R. (1974) The Vitamin Concept. *In* Harris, R. et. al. Vitamins and Hormones, NY: Academic Press Pp 155-166

[53]Wolf, G. & De Luca, L. (1970) *in* De Luca, L. & Suttie, J. *eds*, The Fat-Soluble Vitamins, Madison, WI: Univ. of Wisconsin Press, pg 257

Trivial name	systemic name	principal occurrence in	Chromophore families
Vitamin A_1	retinol (β-ionone ring)	marine based and marine derived animals	$Rhodonine_1( )$
Vitamin A_2	3,4 dehydroretinol	freshwater based animals	$Rhodonine_2( )$
Vitamin A3	3-hydroxyretinol	2-winged flies feeding on carrion	$Rhodonine_3( )$

Vogt presents the three compounds listed by their systemic names in their aldehyde form as the structural formulas of the known visual pigment chromophores. He names the visual pigments rhodopsin, porphyropsin and xanthopsin respectively. Based on his work, he proclaims "there are now three visual pigment chromophores known to occur in nature." Vogt originally named the 3-hydroxyretinol form xanthopsin. This work has extended his statement to label the 3-hydroxyretinol as Vitamin A_3 for continuity (A recently found 1993 paper by Stavenga, et. al. has used this designation also[54]). In this work, the materials become chromophores when deposited on an appropriate substrate in the liquid crystalline state. The opsin involved in the substrate is irrelevant.

The form of Vitamin A has historically been associated with the original environment of the species during its evolution. However, the above table suggests the form is chosen based on the diet, the actual form of the carotinoid or retinoid absorbed by the digestive system. This clarification provides a reasonable rationale for the change of vitamin (and chromophore) type with type in migrating aquatic animals. It also explains why the carrion eating flies exhibit a special type of vitamin.

Vogt suggested in 1989 that the family of forms of Vitamin A based on modifications to the ionone ring was now complete. However, two papers by one team describing a fourth potential form of Vitamin A have appeared[55,56]. These authors suggested a 4-dehydroxyretinol form. Stavenga, et. al. noted this form briefly in 1993. The form was labeled Vitamin A4.

As will be discussed below and in **Section 7.1**, whether the ingested monomer chemical is in fact the alcohol or the aldehyde is largely irrelevant to the visual process. However, the acid form is generally not usable for vision purposes. It appears to be the alcohol form that is initially released by the liver for participation in the visual system. When operating as a chromophore, the actual material contains both an alcohol and an aldehyde group in a resonant state.

In the biological environment, there are a large number of dimers, either carotenes or xanthophylls, that are easily cleaved to provide the above monomers. Some authors suggest oxygen must be present, but this is only relevant if the aldehyde form is desired. Simple cleavage in the omnipresence of water will result in the alcohol form[57]. In earlier works concentrating on human nutrition, the focus was on the symmetrical dimer known as carotene. Carotene is a hydrocarbon whereas many of the more complex retinoids are carbohydrates since they also contain oxygen prior to cleavage. These retinoids are defined as xanthophylls. The number of retinoids is known to exceed 1000[58]. Many of these are either carotenes or xanthophylls. The symmetrical form of the dimer carotene was originally described as β-carotene to denote the nature of the *b*-ionone rings it contained. In the following discussion, β-carotene will be re-labeled β,β-carotene to provide continuity with other precursors of the above Vitamins. By employing two scripts to describe the individual rings, it becomes clear that there can be a large number of protovitamins depending on the number of cardinal values for each script. At the current time, it is known that each subscript can have three values, those describing the ring structures in the above table, that produce significant vitamin (hormonal) activity.

As simple examples, β,β-carotene will provide two monomers of Vitamin A_1 when cleaved. The retinoid and the xanthophyll known as β−cryptoxanthin will provide one monomer of Vitamin A_1 and one monomer of Vitamin A_3 when cleaved. Zeaxanthin when cleaved will provide two monomers of Vitamin A_3. Goodwin (vol. 2, pg 148) references Barua & Goswami[59] concerning a xanthophyll that cleaves into one monomer of Vitamin A_2 and one of Vitamin A_3 (his structure 8.30) in the freshwater fish, *Saccabranchus fossillis*. Goodwin proposes that these cleavages occur as the

[54]Stavenga, D. Smits, R. & Hoenders, B. (1993) Simple exponential functions describing the absorbance bands of visual pigment spectra *Vision Res* vol. 33, no. 8, pp 1011-1017

[55]Masui, S. Seidow, M Uchiyama, I. et. al. (1988) 4-Hydroxyretinal, a new visual pigment chromophore found in the bioluminescent squid, *Watesina scintillans*. *Biochimica et Biophysica Acta* vol 966, pp 370-374

[56]Kito, Y. Partridge, J. Seidou, M. et. al. (1992) The absorbance spectrum and photosensitivity of a new synthetic visual pigment based on 4-hydroxyretinal *Vision Res* vol. 32, pp 3-10

[57]Goodwin, T. (1984) The Biochemistry of the carotenoids, Volume 2, Animals, 2nd ed. NY: Chapman & Hall, pg 181

[58]Sporn, M. Roberts, A. & Goodwin, D. (1994) The Retinoids, Vol. 2. Xxx

[59]Barua, A. & Goswami, U. (1977) Biochem. J. vol. 166, pg 133

protovitamin crosses the intestinal wall. Wolf & Johnson, quoting Balasundraram, et. al. indicate that Vitamin A_3 does have biological activity when fed to rats[60].

5.3.3.4 The structure of the retinoids important to vision

While most animal species use about a dozen of the known xanthophylls and carotenes for various purposes[61], they typically only cleave one into the monomer used in the formation of the chromophores of vision. When complete, these chromophores all exhibit the same structure relative to vision, an all-*trans* conjugated carbon backbone separating two oxygen atoms. Vogt confirmed the all-*trans* form in extracts from the fly[62]. The precise structure of the ring is not relevant. At the most, the presence of the ring provides some thermal stability to the chromophore. However, it has a negligible impact on the spectral sensitivity of the chromophore. It appears that the Vitamin used by an animal to create the chromophores of vision is not uniquely specified. As in the euryhaline fish, it may change routinely based on the environment. Besides the above structural requirement, there may be a second requirement relative to its compatibility with the transport proteins specific to the formation and delivery of the chromophores to the disks.

5.3.4 Quantum mechanics & spectral plots

The mechanism of photon energy absorption by complex organic molecules is based on Fermi-Dirac statistics, not Gaussian statistics. The only role for Gaussian statistics in spectral absorption is due to the effect of the Central Limit Theorem. Careful experimental design can eliminate this effect completely.

As in individual atoms, the energy states (both empty and occupied) of electrons present in a complex organic molecule are described by probability functions. Where multiple energy states exist in close proximity to each other, they overlap and blend. The resulting energy profile of the resulting energy band is also expressed by Fermi-Dirac statistics. Even more important, if it is possible for an electron to transfer from one such energy band to a second band, the probability of this transfer occurring is also represented in Fermi-Dirac statistics. This probability of transfer is what is actually described when an absorption spectrum is plotted as a function of wavelength or wave-number.

5.3.4.1 The fundamental characteristic of spectral absorption

Stiles and Dartnall both provided early empirical descriptions of the absorption bands of the visual system. However, their work lacked any theoretical basis. Lewis provided an early theoretical description of the long wave absorption edge of a visual photon absorber based on simple quantum mechanics[63]. While the form of his simplified equation was correct, he did not pursue this partial solution. He did not have faith in his complete solution. Lewis did provide the first explanation for the fold back in the human perception of color in the far red (600-900 nm).

In the case of an energy band of a complex molecule, the profile of each edge of the band is described by a simple exponential function. The value of this function reaches a plateau either above or below a specific energy level and decreases exponentially on the other side of this energy value. By combining the exponential functions describing the two profiles associated with a specific band, an energy band function is obtained that exhibits a flat top and very steep sloping sides. By subtracting two such functions to obtain the transfer probability function, the resulting function also exhibits a flat top and steep sides. This is the characteristic of the typical spectral absorption function for a wide band absorber. It does not exhibit a distinct peak value. If either of the two energy bands is narrow, the resultant spectral absorption function will have a peak value contained by the two steeply sloping sides. However, this peak will not be explicitly defined mathematically. However, it can be determined by evaluating the derivative of the overall function in the normal way. The important point is that there is no explicit absorption peak in the spectra of a complex organic molecule.

5.3.4.2 The variation in wavelength with solvent and concentration

To precisely quantify the spectral characteristics of the chromophores of vision, it is important that the variation in measured spectral peak with solvent and concentration be recognized. In dilute solutions, a variation in peak absorption of ±10 nm is easily obtained by varying these parameters. A variation with temperature is also found. However, this variation is smaller. Because of this variation, the literature contains a variety of peak isotropic absorptions for dilute

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[60]Wolf, G. & Johnson, B. (1960) Metabolic transformations of Vitamin A. *Vitamins and Hormones*, vol 18 pp 403-415

[61]Goodwin, T. (1984) Op. Cit. vol 2, 2nd ed.

[62]Vogt, K. (1989) in Stavenga, D. & Hardie, R. *eds*. Facets of Vision, NY: Springer Verlag, pg 140

[63]Lewis, P. (1955) A theoretical interpretation of spectral Sensitivity curves at long wavelengths *J Physiol* vol 130, pp 45-52

solutions of individual retinoids. The range of these peaks is from 494 to 502 nm for a putative rhodopsin dissolved in various alcohols. Baylor, Nunn & Schnapf summarize the variety of peak wavelengths measured by different investigators in different environments[64]. They give a preferred value of 491 nm but it is based on very coarse spectral measurements (interval of 20 nm) and a sample of only ten cells. More importantly, they describe the long wavelength absorption of this isotropic spectrum. It exhibits a very rapid fall that is precisely described by a Fermi-Dirac mechanism. Their measured short wavelength skirt does not follow a Fermi-Dirac curve. It is because this cumulative spectral measurement is influenced by the β-peak in the absorption spectrum in the region of 350 nm.

In the case of vision, the chromophores of vision are usually explored in the laboratory while in solution. However, *in-vivo*, they are not in solution but in the liquid crystalline state. This state of matter causes the chromophores to exhibit an entirely separate spectral absorption characteristic.

Vogt & Kirschfeld have noted the significance of this situation with respect to the ultraviolet spectrum in Diptera without providing details of their test conditions and test set[65]. In their figure, presented as figure 4 in Vogt[66], they plot the ultraviolet spectrum of the aldehyde and alcohol of the putative chromophore of that species along with the in-vivo absorption spectra of a fly. Unfortunately, the data is all normalized to the same peak value. The peak in the in-vivo absorption is quite close to the theoretical 342 nm predicted for the quantum mechanical resonance of the molecule. They listed the peak value of their spectrum as 350 nm. However, it shows possible bleed-through of the intrinsic absorption with a peak at 332 nm for the alcohol and 369 nm for the aldehyde. The peak in their absorption spectra at 485 nm probably is the result of inadequate spectral filtering to separate the 437 nm and 532 nm components of the overall spectrum. Alternately, it could the the Bezold-Brucke Peak occasionally observed at this wavelength. In figure 9, Vogt provides evidence of the *in-vivo* peak in the region of 437 nm for another member of the order *Diptera, Simulium*, based on high spectral resolution via an electroretinogram. This figure again shows the difference between the intrinsic UV peak near 350 nm and the resonant peak near 342 of vision. The resonant absorption characterisitic does not show the fine structure of the intrinsic characteristic. Menzel & Backhaus, writing in the same volume[67], gave the correct peak spectral responses of another Arthropod (honey bee) as 335, 435 & 540 nm. These numbers are each within seven nm of the theoretical values proposed by this work.

It appears that Vogt took an overly simplistic approach in his figure 6. To account for the two spectral peaks at 350 nm and 485 nm that he measured for the in-vivo fly, he suggested in caricature that two separate chromophores should be associated with the same opsin molecule to form a chromophore with a dual peaked spectrum. The first retinoid was attached to the protein in the conventional manner suggested by Collins, via a protonated Schiff-base. The second was shown outside the protein and connected to the opsin by two hydrogen bonds. In accordance with the conventional wisdom, the axes of both ligands were arranged to be perpendicular to the incident radiation when the opsin was considered a trans-membrane component of the disks. He apparently did not appreciate that each ligand within a molecule can introduce a separate absorption peak as a function of wavelength, whether intrinsically in solution or in the liquid crystalline state. See **Section 5.4**. This situation is true in both visual band spectroscopy and NMR spectroscopy.

5.3.4.3 The frequently observed spectral absorption

The actual spectral absorption generally observed in vision research is not the actual function for several reasons, some of which will be discussed in the next Section. However, a major reason, is related to the design of the experiment. The visual spectra of the individual chromophores of vision are relatively narrow. If an experiment is designed where the absorption spectrum is sampled using a finite width spectral filter, the measured spectrum is defined by the convolution of the actual spectral transmission function and the filter sampling function. The resulting mathematics can assume three forms.

If the function of the filter is much narrower than the absorption features of the material under a test, the resulting convolution will closely approximate that of the original absorption spectra. In vision, this result is obtained if the filter has a width of less than five nanometers.

If the width of the filter is approximately equal to the narrowest feature of the absorption spectra, the resultant mathematical function will be different, it will resemble a sum of one or more Gaussian functions and it will not correctly represent the original absorption function. Individual features will be suppressed relative to the overall function. This

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[64]Baylor, D. Nunn, B. & Schnapf, J. (1984) The photocurrent, noise and spectral sensitivity of rods of the monkey Macaca fascicularis. *J. Physiol*. Vol. 357, pp 575-607

[65]Vogt, K. Kirschfeld, K. (1984) Chemical identity of the chromophores of fly visual pigment Naturwissenschaften vol. 71, pp 211-212 (in English)

[66]Vogt, K. (1989) *in* Stavenga, D & Hardie, R. *eds*. Facets of Vision, NY: Springer Verlag, Chap. 7

[67]Menzel, R. & Backhaus, W. (1989) *in* Stavenga, D & Hardie, R. *eds*. Facets of Vision, Chap. 14

is the situation normally seen in the vision literature where a filter of ten to twenty nanometers has been used.

If an even wider filter is used or a series of individual measurements is made and combined, generally by averaging the values obtained for each of a number of distinct wavelengths, the result will be characteristically different. For the first case, the details present in the absorption spectra will be obliterated and the result will be smoothed to the point where it will resemble a Gaussian Function. In the second case, the result will be well represented by a Gaussian Function due to the Central Limit Theorem. This Theorem insures that the overall result will approach the shape of a Gaussian Function, regardless of the original physics and/or characteristics of the individual waveforms.

This later case is the true explanation for the shape of the various C.I.E. Luminosity Functions of 1924 and 1964. Nearly all of the original data was acquired with filters of 30 nm width. The resulting waveforms were then averaged at arbitrary wavelengths without any statistical analysis to prove the data were collected with adequately calibrated filters. All of the details associated with the actual absorption spectra of the Human Eye have been lost. One of the leading researchers of that time has provided an interesting and contemporaneous insight into the establishment of these functions[68]. More significantly, the Chairman of the Committee essentially disowned the 1931 standard, particularly in the blue region, within only a few years of promulgation. He offered a corrected version that has not been widely referenced.

> **There is an additional difficulty in experimental design where one or both of the C.I.E. Luminosity Functions are used as a reference function. The Central Limit Theorem insures that any results obtained through the addition to or subtraction from these reference functions will contain all of the errors associated with these functions.**

5.3.4.4 The use of spectral templates

Many experimenters and analysts have attempted to create templates describing the absorption spectra associated with the chromophores of vision. These have generally been derived from the limited (although very significant) work of one experimenter and his attempt to rationalize and extrapolate his work with respect to the work of others. These attempts invariably began with the assumption that the true functions were Gaussian in form and the eye operated in a linear mode relative to photodetection. Later optimizations deviated from one or the other of these assumptions in order to agree more closely with laboratory results. However, these templates have always had significant limitations and were only useful in the first order for making estimates.

> **With the actual detailed spectral parameters of the chromophores and the method of combining the resultant signal now available, the use of graphical templates is no longer necessary or desirable.**

Further discussion of this subject will be found in **Section 5.7** of this Chapter.

5.3.5 Spectral and Cumulative Absorption Measurements

Absorption measurements have been a staple of chemistry for a very long time, with spectral absorption measurements becoming important with the development of photoelectric detectors. The basic theory and terminology were developed using solutions of simple inorganic salts in water. The absorption properties of these simple solutions are stable and easy to characterize. They conform to Beer's (or Lambert-Beer's) Law over a wide range of molarities. In addition, the solutions generally display a distinct saturation point above which the solute precipitates in a rapid and simple manner. With the interest in more complex organic chemistry, the simple precepts of absorption measurements are frequently no longer adequate or appropriate.

The fundamental characteristics mentioned above begin to deteriorate when measurements are made on more complex, and particularly more complex organic, molecules. It deteriorates further if there is a third constituent present which is not pH neutral. The familiar pH indicators are typical examples of this complexity in the absorption spectra of organic molecules. A second example is the denaturing of biological molecules at high temperatures. This process is frequently associated with a change of state of the material, from a liquid crystal to a solid, as well as a significant change in color.

The saturation characteristic of complex organic molecules also becomes more complex. As the solution approaches saturation, the solute may undergo changes of molecular configuration which drastically change the absorption characteristic of the solution.

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[68]Wright, W. (1969) *As reproduced in* Boynton, R. (1979) Human Color Vision. NY: Holt, Rinehart & Winston, pp. 393-405

Of even greater importance is the manner in which the solute leaves or modifies the solution. In one easily identified case, the solute does not separate from the solvent but forms a liquid crystalline lattice incorporating the solvent, a state of matter frequently described as a gel. In another case, the solute begins to leave the solution by forming clusters of solute molecules suspended in the solvent. These clusters are frequently liquid crystalline in nature and exhibit individual absorption characteristics that are highly anisotropic. However, the orientation of these clusters within the solvent is random and the externally observed absorption characteristic of the mixture (it is no longer just a solution) appears isotropic. If these clusters continue to aggregate or to precipitate onto a substrate, they form a liquid crystal with distinct spectral absorption characteristics that are significantly different from the parent solution.

The spectral characteristics of complex organic molecules in the liquid crystalline state are subject to an additional parameter which is normally overlooked in the literature. The observed absorption characteristic is strongly affected by the "energy" environment of the liquid crystal. This is true for both the conjugated class and the resonant conjugate class of hydrocarbons, the dominant classes of colored hydrocarbons. The "energy" environment is important because absorption measurements involve a two-step process. The molecule is initially excited and becomes more transparent. It is then de-excited, returns to its earlier opacity and is subject to re-excitation. If the molecules are not de-excited, the target material is said to bleach during observation and only transient measurements can be made. If the molecules of the material are de-excited within a time period which is very short, typically milliseconds or less, the absorption measurements can be made on a steady state basis with little error (no bleaching).

The anisotropic absorption spectral characteristics, and the related reflectance spectra, of a biological liquid crystal on an appropriate "energy" substrate is typified by the appearance of a hummingbird to the eye. The bird typically appears lime green under diffuse illumination, with regions of bright red and yellow that appear only at specific angles of specular illumination and observation. The lime green color is related to a conjugated carbon molecular structure similar to that of the chromophores of animal vision. However, it is not the absorption peak used in the visual process. The bright flashes of color, due to highly anisotropic reflections, are those related directly to the spectral peaks used in animal vision. These peaks are related to the resonant-conjugated-molecular structure of the liquid crystals, specifically the thickness of the liquid crystalline structure measured between the two resonant auxochromes.

To obtain meaningful spectral measurements in vision, it is important that the "energy environment" be controlled. This environment is represented by the pH of the surrounding solution as well as the methods of de-excitation available to the chromophore.

Many liquid crystalline materials containing a conjugated, or a resonant conjugated, carbon chain will bleach during measurement if they are not in a satisfactory "energy" environment. This is a complication that should be avoided by the investigator in the interest of accuracy and communications. This is the specific condition found in the color granules of the RPE. It is also the condition found in attempting to make measurements on the OS of a photoreceptor cell after removal of, or interference with, the dendritic structure emanating from the IS.

The photographic community encountered the problem of the appropriate "energy" environment many years ago. They found that the materials of interest in extending the spectral range of photography were very particular about the environment. Many of the materials exhibited different and transient absorption characteristics depending on the molarity of the solution, the pH of the solvent, the specific solvent of the solution, the electrical characteristics of the solvent, or the substrate involved in the case of a precipitate. Fortunately their substrate of choice, silver halide crystals in the presence of minute quantities of silver sulphide, provided the necessary "energy" environment. It was therefore easy to record the absorption spectra of different dyes in a permanent form.

For many years, it was not recognized by the photographic researchers that the presence of silver sulphide was critical to the efficient exposure and recording of the spectra. It was only known that the best emulsions were made from gelatins derived from certain cows living in certain pastures (primarily in upstate New York at the time). It was the sulphide in the animal-derived gelatin that combined with the silver halide crystals during the preparation process that resulted in the correct "energy" environment.

There are additional requirements on the required "energy" environment related to the spin states of the excited electrons that will be discussed in more detail later in this PART. Suffice to say here that Oxygen as an auxochrome exhibits a rare property related to the triplet spin state. In addition, the symmetrical nature of the resonant conjugated carbon molecule prevents this class of molecules from de-exciting spontaneously through thermal or fluorescent means. Thus, the resonant conjugated carbon organic molecules with Oxygen as the polar atom exhibit unique and highly desirable properties with respect to the visual process.

5.3.5.1 Summary of the Absorption coefficient measuring regime

Figure 5.3.5-1 provides a summary of the various absorption regimes that must be recognized when considering the

properties of the chromophores of vision. The Table is extensive and complex. However, no similar presentation could be located in the literature. It will be discussed in two steps. First, a line by line description of the figure will be provided. Second, a more global discussion of the significance of the chart will be given.

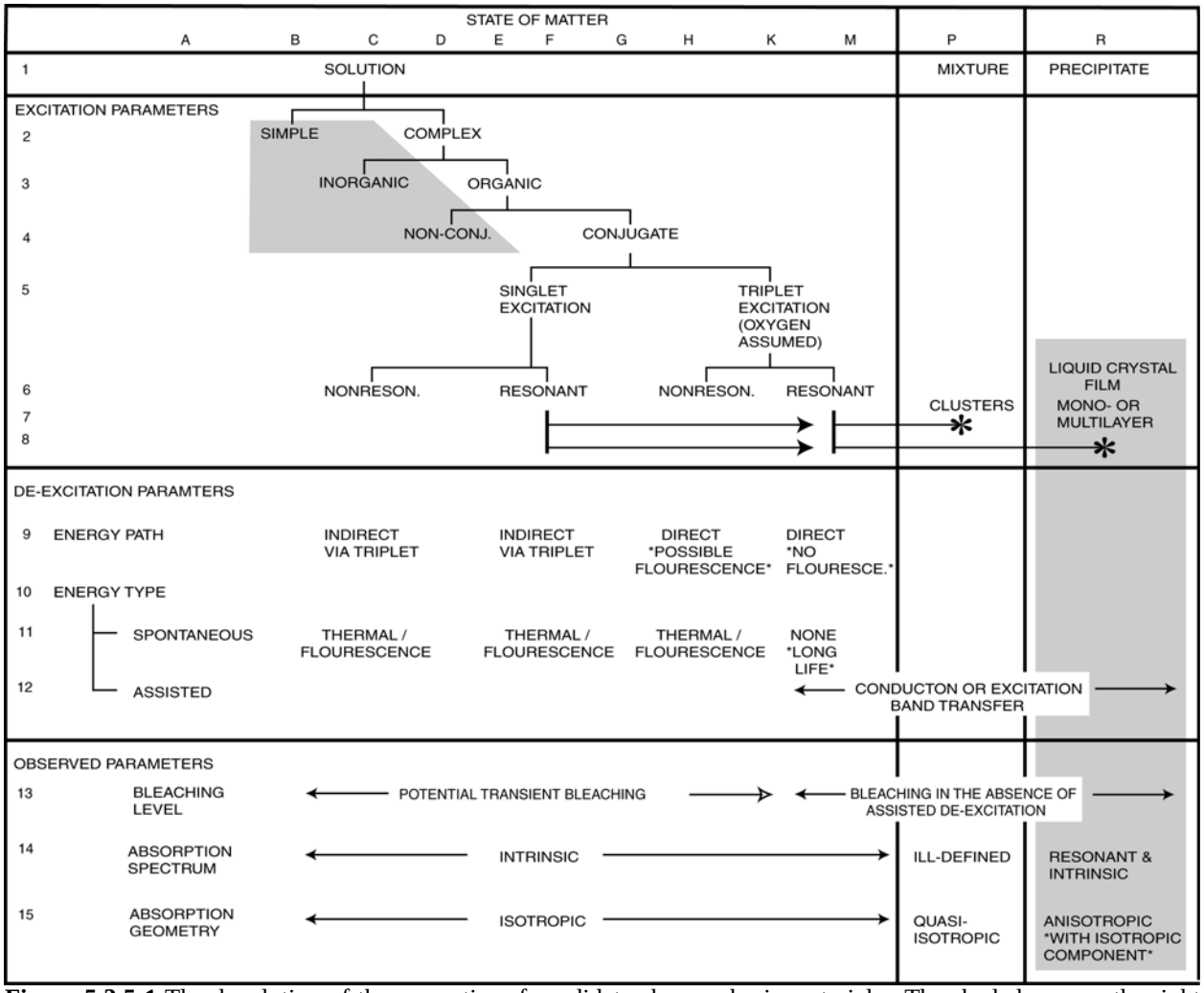

Figure 5.3.5-1 The devolution of the properties of candidate chromophoric materials. The shaded area on the right highlights the unique properties of the chromphores of vision. This is the only state in which the functional properties of the chromophores can be determined in the laboratory. The shaded area in the upper left is only shown for completeness and will not be discussed in detail. See text.

The table illustrates these features:

1 The Table illustrates three distinct chemical conditions, a true solution, a mixture and a precipitate.

2 The Table is divided into solutions containing simple and complex molecules for completeness.

3 The Table is divided into inorganic and organic molecules in solution for completeness.

The activity of simple, inorganic and non-conjugated organic molecules are not of interest in vision research. These areas have been shaded in the figure.

4G All of the retinoids within the Vitamin A Group are conjugated hydrocarbons and exhibit the same intrinsic characteristic absorption spectra (±10 nm depending on the solvent).

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5 There are two principle excitation mechanisms associated with the quantum-mechanical structure of organic molecules, singlet and triplet excitation. Singlet excitation is the dominant type except in the presence of terminal atoms of Oxygen. The Vitamin A Group can be divided into two subgroups, those molecules with heavy atom terminals containing Oxygen and those that do not.

6 The above group can be further subdivided into those members containing two heavy atom terminals and those containing only one. Those with two such atoms exhibit additional spectral characteristics of great interest to vision--but only under specific conditions as illustrated. The mechanical condition is that they be aggregated into clusters of molecules or precipitated as a film. In either case, the molecules are able to assume the liquid crystalline state of matter and thereby achieve the second condition, the sharing of excited electronic states. The result is a unique and accentuated absorption characteristic described as the J-band in the photographic community.

6F & 6M Resonant conjugated organics will only exhibit an intrinsic spectrum when in dilute solution, typically less than 10^{-2} molarity. The spectra will be isotropic. The material will change properties as the molarity rises, becoming a mixture (**7P**) or resulting in a precipitate (**8R).**

7 The nonresonant conjugated molecules are not of interest in vision. The resonant conjugated molecules appear to be lyotropic and are prone to form liquid crystals as the concentration of the solution is increased. In the absence of a suitable substrate, they will form clusters (globules) of highly colored material. The phase of the liquid crystal formed in this way by the chromophores is unknown.

8 In the presence of a suitable substrate, the resonant conjugated molecules are prone to precipitate as liquid crystals with the structure of the smectic phase. They form monolayers with the molecules aligned side-by-side.

9 The de-excitation path available to conjugated organic molecules is controlled by quantum-mechanical rules which are complex. Some molecules will relax spontaneously, other will not (within a reasonable time) without assistance from another material/mechanism. The presence of Oxygen is a special case. Resonant conjugated molecules with two Oxygen atoms will not fluoresce and there only means of de-excitation internally is by means of a direct transition that is not allowed because of the presence of the triplet state. The nonresonant conjugates normally de-excite thermally via a two-step process.

10 The energy released during de-excitation is a key to the quantum-mechanical mechanisms involved. The energy may be released spontaneously without any assistance. Alternately, a more complex transition of the energy to another species may be involved.

11C to H The conjugated organic molecules containing terminal atoms other than Oxygen, or only one Oxygen, normally de-excite spontaneously through thermal or fluorescent mechanisms associated with the terminal atom.

11M The resonant conjugated organics containing two terminal oxygen atoms are unique in the stability of their π^* excited state. The symmetry associated with this molecule prevents it from de-exciting spontaneously. Once excited, the molecule will remain excited indefinitely.

12 It is possible to assist the de-excitation of any excited molecule by providing an alternate mechanism with a higher probability of occurrence than the native mechanism.

12M-R A unique situation occurs in vision. The dendrites of the photoreceptor cell are in direct quantum-mechanical contact with the disks coated with the chromophores. The probability of the energy being transferred to the suitable structure in the dendrites is much higher than the probability of direct de-excitation (probability ~0.0). This biological union of chromophore and dendrite is only available when the chromophore has been precipitated into the liquid crystalline state.

13 As organic molecules considered dyes become more complex, they become more susceptible to photon excitation (absorption) without the necessary spontaneous de-excitation. They are described as bleaching under illumination. The resonant conjugate dyes (chromophores) of vision bleach completely and become transparent in the absence of assisted de-excitation. Many related chromogens bleach and then return to their normal color over a short time interval (normally less than minutes).

14 The absorption spectrum of most molecules is simple, stable and considered intrinsic to the material. The resonant conjugated organics conform to these conditions with regard to their intrinsic absorption spectra.

However, as they achieve the condition described by (**7P)** and (**8R**), they exhibit an additional absorption spectrum. This additional spectrum is poorly defined in mathematical terms under condition (**7P**) because the size of the clusters is highly dependent on the exact molarity of the solution and other parameters determining this parameter. It is more easily defined in (**8R**) because the size of the film is more easily characterized.

14R The chromophores of vision when in the precipitated smectic state exhibit both their normal intrinsic spectral absorption characteristic and an enhanced absorption characteristic related to the resonant condition that generally overshadows the normal characteristic. It is this enhanced characteristic that is employed in vision.

15 The unique absorption geometry of the conjugated molecules as a function of their state of matter is illustrated here. When in dilute solution, the molecules exhibit an isotropic absorption characteristic.

15P The resonant absorption spectrum of the conjugated molecules is difficult to measure when they exist as clusters within a solvent, a mixture. The individual clusters may exhibit an anisotropic resonant spectrum but the clusters are subject to Brownian motion within the solvent. The result is a less pronounced, apparently isotropic, resonant spectrum superimposed on the intrinsic isotropic spectrum of the molecules remaining in solution.

15R The absorption spectrum of the resonant conjugate molecules is quite apparent along specific axes and is clearly anisotropic. These molecules absorb radiation preferentially. They absorb optimally when the Poynting Vector of the radiation is parallel to the mechanical axis of the molecules connecting the two Oxygen atoms. The spectral peak of this absorption is related to the resonant length of the chromophore. The molecules will still absorb radiation isotropically. This absorption characteristic will exhibit a peak of lesser intensity that is related to the length of the conjugated structure of the molecule.

One of the major paradoxes of the current time is that no investigator has isolated and measured the visual spectrums of the chromophores of vision in the laboratory. The above figure illustrates why. The chromophores of vision only exhibit their unique spectral characteristics when in the liquid crystalline state. Furthermore, in the absence of an appropriate de-excitation mechanism, the molecules are excited rapidly (bleach) due to ambient laboratory light and remain in the excited state indefinitely. To achieve a steady-state condition allowing repetitive spectral absorption measurements, a de-excitation mechanism must be provided.

The chromophores of vision are a unique set of polymethines, the Rhodonines, employing two atoms of Oxygen per molecule that are separated by a conjugated carbon chain. The resonance between these two molecules accounts for the specific absorption characteristics of the material. This absorption characteristic is not apparent until the absorption cross-section of the material is raised. This is accomplished by forming the material into the liquid crystalline state. In this state, the molecules are able to share their susceptibility to excitation. When excited, the electrons are transferred into the relatively unusual triplet state because of the unique properties of Oxygen. Once excited, the electrons within this shared molecular structure exhibit a very long lifetime because of their transfer into a triplet state. The transfer of these electrons from the excited triplet state back to a singlet ground state is quantum-mechanically forbidden. These electrons can only be de-excited through an auxiliary mechanism. *In-vivo*, this mechanism involves the transfer of the energy of the chromophore molecules to the hydronium liquid crystal found within the surface structure of the vital (living) neural dendrites.

This transfer of energy from an excited state of one molecule to another medium, although sophisticated, is found in many application. It is commonly used to excite gas lasers. It is also a phenomenon associated with plasma discharge devices.

Special circumstances must exist to measure the spectral absorption characteristics of the Rhodonines *in-vitro* in the laboratory (where *in-vitro* implies the separation of the chromophores from their connection to the neurons) under steady-state conditions. The material must be in the liquid crystalline state and an artificial method of de-excitation must be provided.

5.3.5.3 Details of the absorption coefficient regime

It is important to understand that the absorption regime of the visual system is not based on material present in a continuum. Specifically, the absorption relationship known variously as Beer's Law or the Lambert-Beer Law does not apply to materials that are not in dilute solution. The spaceframe architecture of the Outer Segment of the photoreceptor cells is far from a continuous structure and the chromophores are not present in solution. This section will review the basis of absorption in a dilute solution and then move on to the relevant situation.

5.3.5.3.1 Simple solutions

A common undergraduate laboratory experiment is to measure the change in total absorption of light by a solution as a function of concentration or other parameter. The experiment is good training but misleading in its simplicity. The student is frequently left with the impression that the absorptions of most materials correspond to the simple material investigated. To obtain a meaningful absorption spectrum of a given material, it is important to recognize the parameters involved in the measurements based on theory. Most text books[69] provide a simplified derivation of the absorption of light by a solution to arrive at the Lambert-Beer Law. Pokorny, et. al[70]. give a brief introduction to the subject. Most basic Physics and Physical Chemistry texts give a fuller account. However, these introductions are all limited to low molarity concentrations of simple molecules in true solutions. They do not apply to mixtures or high molarity situations involving complex organic molecules.

This derivation is based on the molar extinction coefficient, ε, which is defined so as to be a positive number. For solutions of simple molecules at low concentration, ε is defined using;

$$Log\left(\frac{I_0}{I}\right) = E = \varepsilon \cdot d \cdot c$$

where I_o is the incident illumination, I is the transmitted illumination, E is the total absorption factor, ε is the molar extinction coefficient, d is the distance through the solution sample, and c is the concentration of the solution. By introducing a negative sign, this equation can be written in an alternate form;

$$I = I_0 \cdot e^{-\varepsilon \cdot c \cdot d}$$

The molar extinction coefficient is not a constant in most applications involving complex organic molecules. It varies both with both wavelength and molarity in such solutions. For precision work involving the complex organic molecules of vision, it is necessary to redefine the total absorption factor, E, as;

$E = d^* \int \int \varepsilon(\lambda, M)\, dM\, d\lambda$

In this formulation, the spectral molar extinction coefficient, ε(λ, M), can change drastically with molarity, as it does in resonant conjugated carbon organics. This behavior is illustrated in the next figure.

In the original formulation, the absorption as a function of wavelength included two assumptions. First, it was assumed that the absorption was uniform with wavelength. Second, it was assumed that the incident illumination was uniform in wavelength (generally with respect to energy per unit wavelength). While the second assumption is acceptable for passive absorbers and photoconductors, the chromophores are neither of these. The chromophores of vision are quantum-mechanical devices, like many other photoemissive devices. For the results of absorption measurements to be interpretable, the absorption coefficient should be measured using a light source exhibiting a constant quantum flux per unit wavelength. Otherwise, additional complexity and further integration must be employed to obtain correct answers. There are many conflicts in the vision literature due to this problem. The peak wavelength measured under a constant energy criterion is not the same as that measured under a constant flux criteria.

In both of the above formulations, it is assumed that the sensor used to record the light exhibits a uniform response as a function of wavelength over the region of interest. Here again, the degree of uniformity and whether it is with respect to energy or quantum flux should be noted and included in the published material.

It should be noted that because of the difficulties associated with calibration, it is usual to see plots of the normalized spectral absorption factor for each relevant material in the vision literature. It would be better to obtain relative measurements compared to a standard than to normalize independent measurements.

5.3.5.3.2 More complex analytical situations

As noted in (**1**) of the above Table, the Table is divided into three distinct chemical conditions. The first condition applies only to true solutions, solutions that can pass through the finest physical filter without separation into two distinct

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[69]Zollinger, H. (1991) Op. Cit. pg. 13

[70]Pokorny, J. Smith, V. Verriest, G. & Pinckers, A. (1979) Congenital and acquired color vision defects. NY: Grune & Stratton pp. 62-63

materials. The second condition, labeled mixtures, is meant to include materials that are still fluid but cannot meet the above test. Many solutions of complex organics transition from the true solution to a mixture as the molarity of the solution/mixture is raised. **Figure 5.3.5-2** illustrates this situation as well as the appearance of an entirely new absorption band, the *j*-band. The photographically active diethyl-2,2'-cyanine chloride is a member of *another family* containing the Rhodonines. It is a resonant, conjugate, hydrocarbon where both terminal atoms are Oxygen. However, the backbone chain is simply carbon atoms and hydrogen. Note first that the absorptivity within the spectral band 420 to 560 nm varies considerably with molarity. The low molarity peak at 520 nm is replaced by a new peak at 480 nm. Even if the integral of the absorptivity coefficient with respect to wavelength had the same value for these two molarities, there would be an obvious color change with molarity related to the absorption.

Of much greater importance is the appearance of the *j*–band resonant peak at 575 nm. Below 10^{-3} M, there is no significant absorption at this wavelength. By the time the molarity reaches 1.4×10^{-2} M, the peak at this wavelength is dominant. At this molarity, the solution is no longer a true solution, it is a mixture of a true solution and clusters of liquid crystalline material. The mixture is often described as having a stringy appearance.

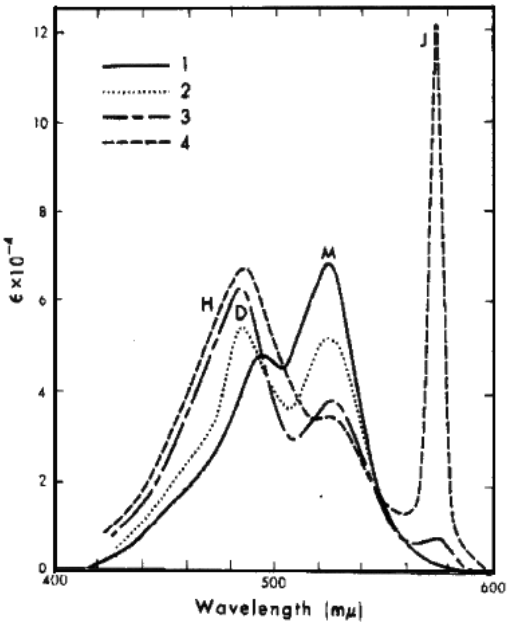

Figure 5.3.5-2 CR Absorption spectrum of aqueous solutions of diethyl-2,2'-cyanine chloride at 25C: (1) 1.3×10^{-5} M; (2) 1.3×10^{-3} M; (3) 7.1×10^{-3} M; (4) 1.4×10^{-2} M. From Mees & James, 1966

The absorptivity of the solution and also the mixture remains isotropic since the clusters are still discrete and randomly oriented within the solution.

This figure provides a clear example of a material having a distinctly different absorptivity when dissolved in a solute and when in the liquid crystalline state. However, this figure does not properly characterize the liquid crystalline state as well as a pure precipitate does. In the pure state, the J-band is much more pronounced, the "clusters" are now arranged and oriented in a crystalline pattern, and the absorptivity will be much higher along a specific axis. Along this axis, it may be very difficult to measure the non-resonant portion of the spectrum. Conversely, perpendicular to this axis, it may be very difficult to measure the resonant portion of the spectrum in the presence of the non-resonant portion.

5.3.5.3.3 More complex psychophysical situations

As noted, the uniformity of the detector used to make absorption measurement and whether it is energy-based or quantum-flux based should be determined. A major problem has occurred in psychophysically determining the apparent absorption profile of the chromatic channels of the eyes. The widely observed assumption has been that the visual system beyond the chromophores is linear and additive across the visual spectrum. Unfortunately, this condition is not even approached. The visual system employs logarithmic summation of signals from multiple independent detection channels. By mathematically manipulating the measured data based on the linearity assumption, a putative but spurious absorption band with a peak absorption near 575 nm has appeared in the psychophysical literature for years.

5.3.5.3.4 Absorption in high absorbance materials

Section 4.3.2.4 has addressed the spaceframe structure used in the Outer Segment of the photoreceptors. This structure is used in part because of the very high absorption coefficient of the chromophores of vision. If the individual liquid crystalline layers of these materials were placed directly in contact with each other, the resulting change in dielectric index at the entrance to the disk stack would cause considerable reflection at this surface. To overcome this problem, the individual layers are separated into a spaceframe of disks. To analyze this structure, the algebra of a continuum used in **Section 5.3.5.3.1** is inappropriate. The net absorption of the structure must be calculated as the product of a large number of discrete absorbing elements where each element includes an active absorber layer and a passive low index of refraction layer. Although the net result will be much the same for the case of a complete Outer Segment of about 2000 discrete disks, a significant difference will result if the absorption coefficient of a single monolayer of chromophoric material is simply multiplied by the length of an Outer Segment.

The net effect of the spaceframe structure used in vision is that the overall absorption spectrum of a multilayer section of chromophore material is considerably broader on a relative basis than that measured for a single monolayer of chromophore.

5.3.5.3 Summary of the absorption regime related to vision

If one accepts the premise that the Rhodonines are the chromophores of vision,

+ they only exhibit the unique spectral properties used in vision when in the liquid crystalline state,

+ they require the presence of a de-excitation agent to exhibit the appropriate stable absorption spectra and

+ their absorptivity is so high that a space frame structure is required to simultaneously overcome the resulting difference in dielectric constant and achieve maximum absorption.

These characteristics are developed in **Chapter 4**, this chapter and the remainder of **PART B**. [**Figure 5.3.3-1** above] provides a number of highly relevant corollaries to the premise with regard to vision. These corollaries explain many of the conundrums found in the vision literature.

5.3.5.3.1 Self screening as a limited concept

The literature occasionally refers to the self-screening of a chromophore. This is a concept based on an approximation of the exponential absorption characteristic applicable to a low molarity liquid solution. The "correction for self screening" is given by the ratio of the actual absorption divided by the absorption predicted by the first order term of a series expansion of the exponential expression for the absorption characteristic. The concept is not directly applicable to a situation involving high molarity solutions or liquid crystalline aggregations of complex organic molecules such as found in the photoreceptors of vision. It also avoids any reflection at the interface between an absorber and the surrounding medium. The latter factor is very important in the design of the disks and disk stack of vision because of the very high absorption coefficient of the in-vivo chromophores. To minimize reflection, the design of the Outer Segment relies on quantum-mechanical and spatial relationships to maintain an effective index of refraction of the Outer Segment near 1.3

5.3.5.3.2 In-vivo conditions

First, the absorption spectra of the Outer Segments of the photoreceptor cells rely on the resonant condition of one of the Rhodonines when it is present under specific conditions (Col. **R** of the Table). These conditions are that it is present as a liquid crystalline surface film on the disks of the segment (**8R**) and in contact with a de-excitation agent associated with the Inner Segment of the same cell (**12R**).

Second, the absorption characteristic of each Rhodonine chromophore is highly directional (**15R**). This anisotropic absorption is only observed for radiation applied perpendicular to the surface of the film, i.e., parallel to the axis of the Outer Segment. The peak absorption wavelength for resonant absorption by these chromophores is nominally either 342, 437, 532 or 625 nm. The chromophore is not polarization sensitive for excitation along this axis. For radiation applied along other axes, such as transverse to the axis of the OS, only the intrinsic absorption characteristic due to conjugate absorption and shared by all retinoids of the Vitamin A Group will be observed. This intrinsic spectrum has a nominal spectral peak at 502 nm at 37C.

Even *in-vivo*, careful experimentation using transverse radiation relative to the OS will always exhibit the intrinsic conjugate absorption spectra with a peak near 502 nm. This conjugate spectrum is polarization sensitive. It is maximum when the E-field of the radiation is parallel to the mechanical axis of the individual molecules, generally parallel to the axis of the OS.

The absorption spectrum of the disk stack is not polarization sensitive to radiation applied perpendicular to the surface of the individual chromophoric films, i.e., along the axis of the OS. There is some data that the individual film layers of some types of liquid crystals are polarization sensitive[71].

Any action that disturbs the de-excitation process integral to the photoreceptor cell will cause the OS to become transparent to continual excitation, i.e., the chromophore molecules will all become excited and the result will be the OS

[71]Chen, S. (1999) xxx Nature, 11 Feb 1999

will bleach to transparency (**13R**).

5.3.5.3.3 In-vitro conditions

The molecules constituting the Rhodonines are extremely sensitive, as are all molecules sensitive to excitation by light of less than 1-2 electron-volts energy, corresponding to the visual spectrum. Furthermore, these molecules will only exhibit the appropriate spectra when in the conditions described above. In the process of transitioning to in-vitro laboratory conditions, experimenters have inevitably destroyed the necessary conditions for observing the resonant visual spectra associated with these chromophores. The scenario is generally as follows;

1. disconnect the OS from the IS, thereby removing the de-excitation mechanism.

2. solubilize the material in the OS, thereby removing the material from the liquid crystalline state.

3. separate and purify the non-protein material using detergents and salts of metallic ions, frequently causing a reaction that modifies the molecules. One common result is the removal of one of the Oxygen atoms.

4. Preparing a dilute solution of the retinoid extract in either water or an alcohol-based solvent.

Each of these activities insures that the resonant absorption spectra of the molecule will not be exhibited. The result is that all of the chromophores so processed will now exhibit only the intrinsic, isotropic spectrum associated with molecules located to the left of the double line in the Table. They will exist in one of the states related to (**6C, 6F, 6H or 6M**) depending on whether one or more of the Oxygen atoms have been removed or replaced within the molecule. If only one Oxygen is related, these states are generally related to the retinoids known as the Vitamin A Group. Only very sophisticated tests will determine whether the resultant molecule is now a retinene or whether it is a pseudo-retinene with the remaining Oxygen atom attached to a carbon located along the conjugated chain instead of at location 15.

To isolate the Rhodonines and then obtain the visual spectrum associated with the chromophore, the material must be returned to the condition illustrated by (Col. **R**), i.e., (**8R**) assisted by (**12R**) resulting in a stable situation typified by (**14R**) and (**15R**). This can be done as long as the chromophores have not been physically modified by excessively aggressive detergents or oxidizing and reducing agents. An additional step is needed since the retinoid extract from any retina will consist of a mixture of the individual chromophores. The extract must be processed further, probably by re-crystallization, to separate the chromophores before they are returned to the liquid crystalline state for measurement. This re-crystallization might be considered a racemization[72].

The transient spectral response can be obtained after depositing the liquid crystal on any substrate. However, to obtain a steady state spectral response a de-excitation agent must be available. This can be in the form of the appropriate substrate or in the form of a third component. As indicated above, by incorporating the individual chromophores into a photographic emulsion, exposing the emulsion in a spectrometer and then developing the emulsion, a permanent graphical recording of the spectral response of the chromophore can be obtained.

To obtain an accurate absorption spectrum, and preserve the true shape of the spectrum, a filter width of less than 5.0 nm is recommended. This filter width is easily obtained with modern spectrophotometers.

As indicated in **Section 5.4.6**, it is not appropriate to consider the observed spectral absorption characteristic of a single chromophore as a single function and speak of the half-amplitude points as describing the waveform. The peak wavelength and the two half-amplitude points can be used for less critical work. However, the correct description of the waveform requires that the waveform on each side of the pseudo-peak be plotted as an exponential function and the wavelength specified at which this function is equal to 1/e of its peak value. These two 1/e values properly describe the measured spectral response.

5.4 The Absorption Characteristics of Materials

There have been periodic references in the vision literature to energy level diagrams in order to explain conceptually the spectra of vision. Up to this time, no reference has appeared connecting the elementary concept of energy levels to the more sophisticated energy bands and then directrly to the calculation of absorption spectra. Taking these additional steps

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[72]Norman, R. & Coxon, J. (1993) Principles of organic synthesis. London: Blackie Academic & Professional. pg. 144

leads to an important and precise characterization of both the requirements for the photochemistry of vision and the straightforward specification of the absorption bands of the visual chromophores, the Rhodonines.

5.4.1 The Energy Level Diagram

The basic energy level diagram was developed at the dawn of quantum physics to explain the absorption and emission characteristics of simple atoms, ions and molecules found in the sun and laboratory flames. Such diagrams have been developed further to explain similar absorption phenomena in more complex inorganic molecules and also into the organic molecule domain. It should be stressed that these energy level diagrams normally only apply to individual atoms, ions and molecules. As normally presented, they do not apply to aggregated groups sharing energy levels and/or actual electrons (examples; metals, semiconductors, crystals, and liquid crystals).

Figure 5.4.1-1 is the energy level diagram for Hydrogen, the simplest atom. Note the transitions between the various levels absorb or emit photons in different spectral regions. Note also that the emission and absorption lines are extremely narrow and well defined. The exact center of each line is given to four and five place accuracies. The width of each observed line is a function of temperature. This width can be computed precisely using Fermi-Dirac Statistics. These widths are typically given in Angstroms for atoms, ions and simple molecules at the normal observation temperatures of 1,000-6,000 centigrade.

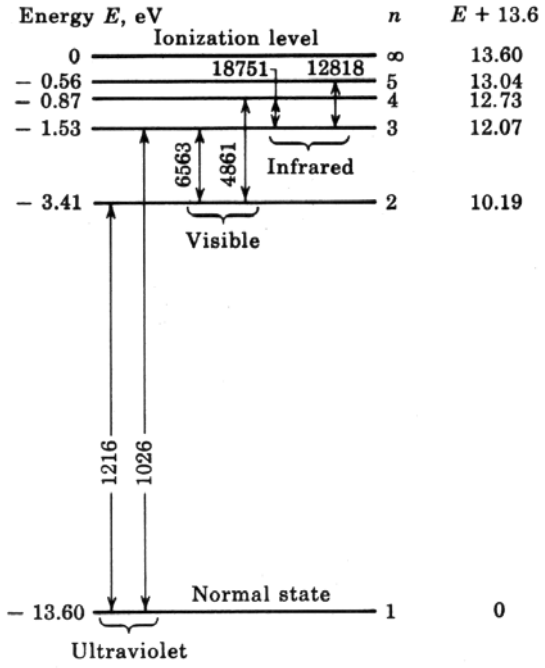

Figure 5.4.1-1 The lowest five energy levels and the ionization level of hydrogen. The spectral lines are in Angstrom. From Millman & Halkias, 1972

Figure 5.4.1-2 from Zollinger[73] extends the simple energy level diagram into the realm of the complex organic molecule where additional levels appear due to the greater physical, and therefore, electronic complexity of the molecules. This type of diagram is very useful at the teaching level. However, it is extremely limited in its ability to explain with precision the nature of the absorption and emission phenomena. There is no definition of the width of any of the energy levels. Note the very simple molecules defined in the caption for this figure and the fact that the great number of energy levels associated with the s-shell are not shown at all. The energy levels shown for the two p-levels were carefully chosen to avoid the most frequent situation-where they are in close proximity and overlap. The fact that the presence of oxygen introduces non-bonding n-electrons in the triplet state (parallel spin) is also a critical change from the condition shown. Coxon & Halton address the situation with regard to oxygen more fully but still only for solutions[74]. They touch on the paramagnetic property of oxygen and its utility in research when using electron paramagnetic resonance spectroscopy.

Coxon & Halton present a caricature of the energy states of the carbonyl ion. However, the situation in the more complex retinoids appears to be more complicated. As suggested in McDowell, the electron paramagnetic resonance spectrum of all-*trans* retinal could not be analyzed because the unpaired electron is distributed over the entire conjugated system[75]. The Rhodonines can be considered to be dicarbonyls with both of the unpaired electrons distributed over the molecule. However, the conjugated character of these materials suggests they are actually carboxyl-ions. As a result, it is more appropriate to call the Rhodonines members of the carboxylic ion system, the practice in photography, than merely dicarbonyls.

The unique and complex properties of the Rhodonines introduced by the paramagnetic properties of the two oxygen atoms place them in a distinct niche area with respect to their quantum-mechanical electronic properties. Only one text could be located that addressed the paramagnetic properties of oxygen reasonably comprehensively[76]. While they discussed measurements involving the liquid and the crystalline state, they did not address the liquid-crystalline state (in 1966). Most other documents only discuss molecular or atomic oxygen as highly reactive radicals capable of quenching most reactions involving the triplet state[77].

On page 24, Zollinger addresses the conventional chemical views of how molecules or ions in the excited state may become de-excited. He limits radiation-less transitions to those "(macroscopically observable by heat formation)." He changes position on page 302 to include "A third and very important type of reaction for newer application of dyes is the energy transfer . . . from the lowest triplet state (T_1) to an acceptor molecule. . . . Such energy transfer processes form the basis of all photosensitized reactions." Exciton-to-exciton transfers across semiconductor boundaries need not release heat.

The importance of the triplet state is addressed in **Section 5.4.5**.

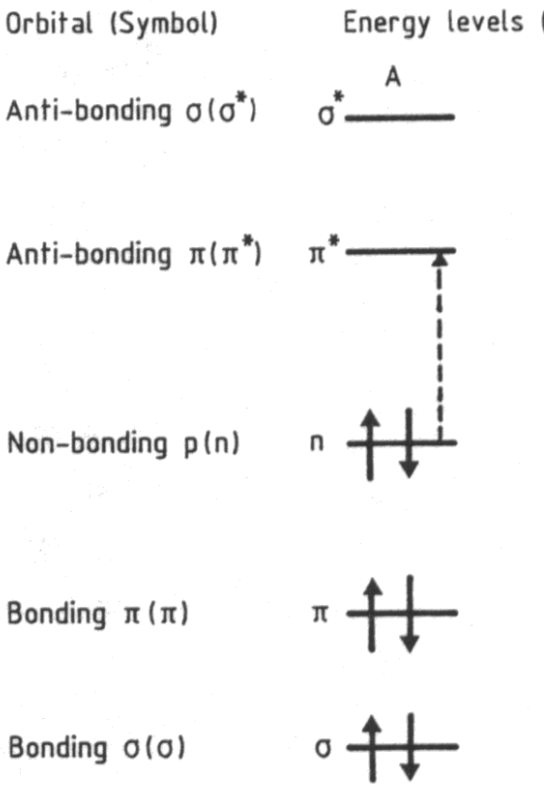

Figure 5.4.1-2 Designation of molecular orbitals & schematic representation of energy levels for a molecule with one σ, one π and one n electron pair. From Zollinger, 1991.

[73]Zollinger, H. (1991) Color Chemistry NY: VCH pg. 20
[74]Coxon, J. & Halton, B, (1987) Op. Cit. pg. 9-10
[75]McDowell, C. (1975) Magnetic resonance. Vol 4 of Physical Chemistry, series 2. London: Butterworth pp 97-98
[76]Bersohn, M. & Baird, J. (1966) An introduction to electron paramagnetic resonance. NY: W. A. Benjamin
[77]Ranby, B. & Rabek, J. (1977) ESR spectroscopy in polymer research. NY: Springer-Verlag

5.4.2 The Energy Band Diagram

When two or more molecules are brought into close proximity, the use of the Energy Level Diagram is no longer adequate to describe their electronic states. **Figure 5.4.2-1** from Millman & Halkias[78] illustrates a more general form of diagram, the Energy Band Diagram and how it derives from the Energy Level Diagram. The transition is required by the Pauli Exclusion Principle. As the individual molecules are brought into close interatomic spacing, their outer (or valence) electronic structures begin to overlap and merge into a single electronic system. Since Pauli has shown that no two energy levels can have exactly the same energy in a molecular system, the individual energy levels are distorted. The result is a broadened energy band made up of many very closely spaced energy levels. The band becomes contiguous for purposes of absorption and emission and the overall energy band width can also be computed from Fermi-Dirac Statistics.

Note that it is impossible to assign a given energy level within each energy band to a specific molecule, the individual levels are shared between the various molecules of the system and thus the levels cannot be described as belonging to the s or p subshells of a given molecule-- electrons at these levels are not orbiting a specific molecule but are now free to travel within the energy band of the system. The electrons belong to the system as a whole. This is a key characteristic of a molecular system, whether of the crystalline or liquid crystalline type. It says that a photon need not interact with a single molecule to elicit excitation; it need only interact with the total system. This increases the absorption cross-section of the material immensely over that of a group of relatively closely but unassociated molecules. This increase is a very key aspect of photo-reception in the vision process--as it is in all high performance, organically based, photon detection systems.

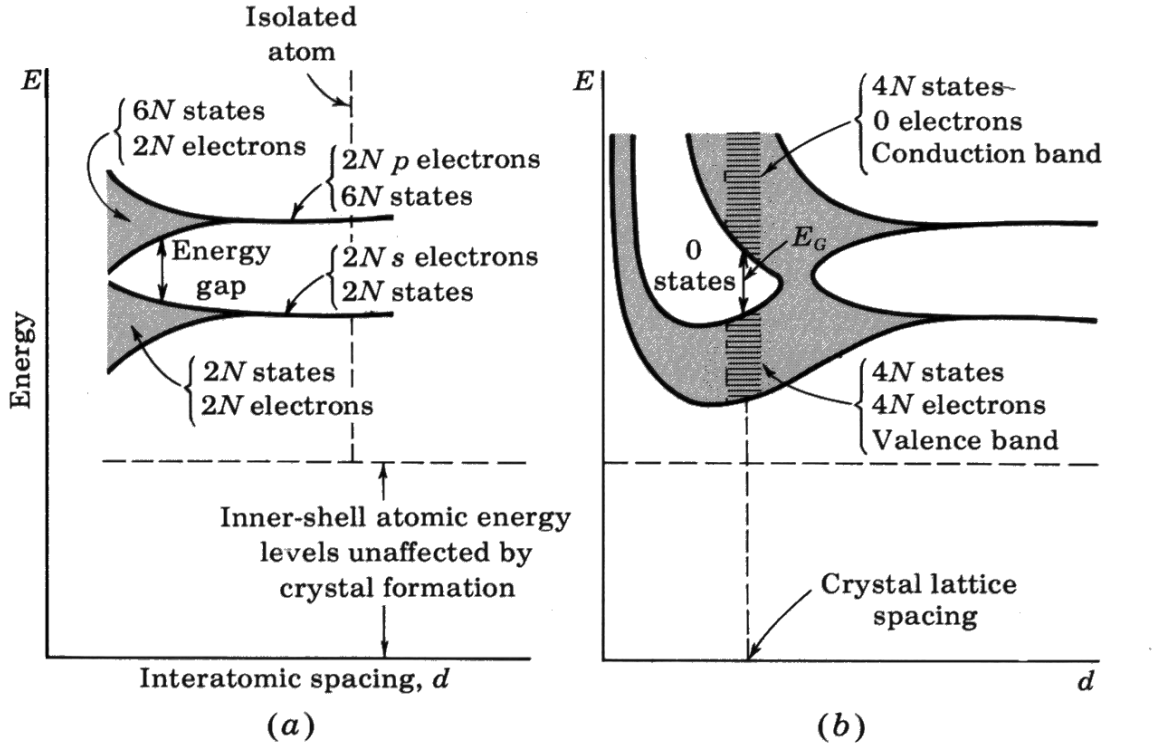

Figure 5.4.2-1 Illustrating how the energy levels of isolated atoms are split into energy bands when these atoms are brought into close proximity to form a crystal. From Millman & Halkias, 1972

[78]Millman, J. & Halkias, C. (1972) Integrated Electronics. NY: McGraw-Hill pg.15

It is important to understand the characteristic differences found in the absorption spectra related to different classes of materials, conductors, insulators and semiconductors (semi-insulators).

+ The conduction band of a conductor extends from zero energy to a specified energy level within the material. Because of this fact, the absorption spectra of a conductor can be considered a low pass function; any photon with greater energy than the bandgap of the material will be absorbed and an electron will appear in the conduction band. Photons with greater energy than the minimum energy of the valence band can actually cause photo emission into a vacuum from many conductors.

+ The excitation levels (there may be several) of an insulator do not extend to the "vacuum level" of zero energy. Any photon absorbed by an insulator will result in an electron from one of the unexcited energy levels corresponding to the valence band transferring to one of the excitation levels. The absorption spectra related to these photons can be considered a band pass function; only photons with an energy bounded by specific values will excite the material. The excited electrons cannot move about within the molecular structure of the insulator. They are normally de-excited by fluorescence or phosphorescence.

+ The excitation levels of semiconductors (semi-insulators) do not extend to the "vacuum level" of zero energy. However, due to the crystal or crystal like structure of the material, electrons excited into one of the excitation bands may travel within the band throughout the crystal. These excited electrons may be de-excited at dislocations in the crystal, at edges of the crystal or by fluorescence and phosphorescence.

+ Organic materials, due to their great variation in structure, can be further divided into three classes; simple organics, conjugate organics and resonant conjugate organics. Simple organics are generally insulators. The conjugate and resonant conjugate organics are generally described as insulators when in dilute solution and semiconductors when in the crystalline or liquid crystalline state. The spectral bands associated with these materials are of the bandpass type. The width of the bandpass is related to the relative stiffness of the molecular structure. The resonant conjugates are generally stiffer than their conjugated cousins.

5.4.3 Absorption Bands of a Molecular System(s)

Absorption of one or more photons by a molecular system is accomplished in much the same way as in a simpler system except that now an individual photon may have any energy within a range of energies. The range of energies is determined from the energy band diagram just as they were for the energy level diagram except there must be more attention paid to the finite width of the bands and the shape of the band edges. Thus, there is now a finite and calculable probability that a given photon will excite an electron found in one energy band, causing it to move up to an empty energy level in a higher energy band. For any reasonable size molecular system, containing a multitude of individual molecules, it is not necessary to speak of an energy level within an energy band; because of thermal line spreading, the levels merge so completely that the energy band can be considered a continuum.

Nassau has provided a tabulation of different "causes of color" related to various mechanisms found in science[79]. If his category "transitions between molecular orbitals" were expanded, it would include the mechanism found in vision. This is the transition between molecular orbitals of conjugated oxygen atoms. He also provided a set of energy band structures. However, these did not include the case of a pure organic semiconductor. Such a semiconductor does not exhibit a conduction band, only an excitation band. Nassau failed to address the critically important Pauli Exclusion Principle that is the only explanation for the broadened absorption spectra of liquid crystalline materials[80] (although he provided an energy band diagram incorporating the concept for pedagogical purposes).

5.4.3.1 The Energy Band Structure

Figure 5.4.3-1 illustrates a series of more complex molecular energy band structures associated with organic molecules.

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[79]Nassau, K. The causes of color, *Scientific American* vol. 243, pp 124-154
[80]Nassau, K. The Physics and Chemistry of Color (1983) Op. Cit. pg 157

Most brief descriptions of the visual transduction process begin and end with a discussion of a simple energy **level** diagram **(a)** as usually presented in a college entry level course for non-physics majors. It is able to describe the line spectra of simple excited molecules. The energy differences between these bands are those usually found in undergraduate chemistry and biochemistry texts. This energy level diagram does not apply to the resonant retinoids of vision.

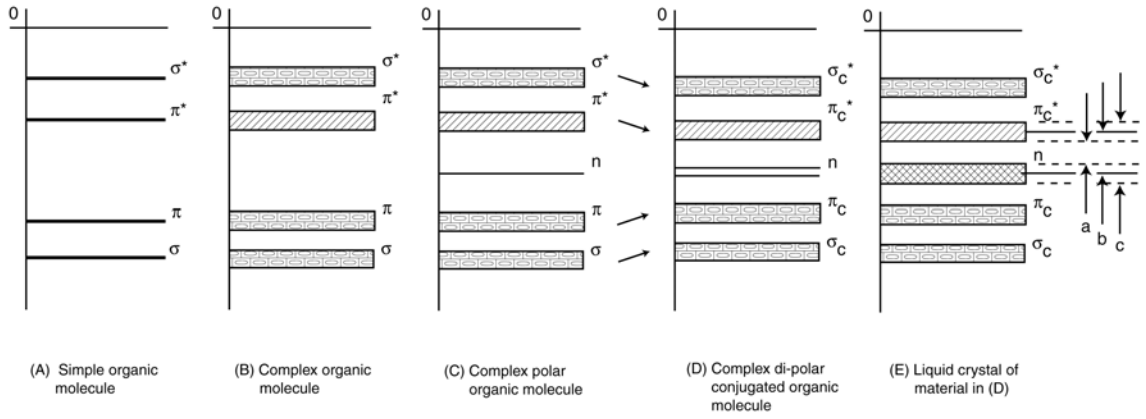

Figure 5.4.3-1 Energy Band structure for organic molecules. See text for details.

If one analyzes a molecule as complex as even the simple retinoid, retinol, one finds a large number of electrons having essentially the same configuration relative to their associated atom. Pauli[81] (Wolfgang Pauli, not Linus Pauling) defined his Exclusion Principle many years ago specifying that no two electrons could occupy the same energy level in a given molecule. This means that in a complex molecule, the energy level diagram **is replaced** by an energy **band** diagram as in **(b)**. In concept, each of these bands is made up of a large group of individual energy levels attributable to individual electrons. However, at temperatures above absolute zero, these individual lines have finite widths and they are found to overlap into a continuous band structure at room and *in-vivo* temperatures. This band structure, that is defined by Fermi-Dirac Statistics, has a *mean value* near the value normally shown in (a).

> Note the upper unexcited levels are broadened just like the lower bands. The quantum-mechanics of this phenomenon are beyond the scope of this work.

5.4.3.1.1 The complex organic molecule with a polar atom

Frame (c) shows the next most complex organic molecule containing a polar atom, an atom with at least one unpaired electron. This unpaired electron is in the ground or neutral state. Its energy level can be precisely defined as in (a) and is shown as the narrow n-band in (c). Excitation of this electron is difficult in dilute solution because of its low absorption cross section relative to the other bands.

5.4.3.1.2 The conjugated carbon bond energy band

If a complex organic molecule contains a conjugation of bonds, the impact on the electronic states of the molecule is significant. The conjugation tends to physically rigidize the molecular structure. It also tends to allow more complete sharing of the electrons associated with individual atoms than found in non-conjugated structures. This mechanism tends to further broaden the bands, again due to the Pauli Exclusion Principle. Frame (d) illustrates this effect. The arrows between (c) and (d) are meant to suggest the broadening causes the lower energy edges of the unexcited bands move up and the higher energy edges of the excited bands move down. Since the amount of broadening is a function of the number of electrons (or potential electrons) present at each energy level in the molecule, increasing the level of conjugation reduces these differences. The significance of the resulting reduction in the minimum energy difference between the bands will be discussed below. The labels related to these modified bands are shown with a "c" subscript to denote conjugation.

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[81]Pauli, W. (1925) --- Hamburg, Germany: Institute of Theoretical Physics

It should also be noted that the sharing caused by conjugated carbon bonds is at its maximum in the all-*trans* configuration. For *cis* configurations, the sharing is reduced and the subsequent absorption cross section is generally lower with a peak at a shorter wavelength.

5.4.3.1.3 The n-electron energy band

If there is more than one polar atom in the molecule, conjugate carbon chemistry and Pauli's rules force the unexcited electrons associated with these atoms into different individual energy bands. These bands remain difficult to detect because of their low absorption cross section relative to the other bands.

In polar atoms attached to organics, there are frequently unpaired electrons present. These electrons are directly associated with the polar atom(s) but indirectly with the rest of the molecule acting *en mass*. When only one unshared electron is present, its energy is shown in (**c**). This is the so-called non-bonding or *n*-electron energy level. For a single molecule having only one unshared electron, the energy level is of finite width. If there are two or more unshared electrons in a single molecule, the n-electron levels are then in a technically shared condition and must again obey Pauli's Exclusion Principle. The result is the two discrete energy levels shown in (**d**).

The presence of two n-electron levels is not important until molecules are encountered with two polar atoms separated by a conjugated carbon chain. They then participate in the same characteristic mentioned above, However, the number of electrons involved is still quite small. If this complex molecule, containing two polar groups, is now aggregated with other similar molecules into a "liquid crystalline state," **(e),** the associated molecules exhibit overlapping electronic configurations which must also conform to the Pauli Principle. Therefore, the *n*-electron level becomes a very broad energy band at an energy level close to the highest energy state associated with the π*-band of the diagram.

5.4.3.1.4 The n-electron band/ π_c* band interaction

A n-electron excited from anywhere in its band can move up into a higher band, with the lowest energy choice being the π_c* band. Thus, the minimum-energy-difference band for derivatives of retinol having two polar groups connected by a conjugated carbon chain and existing in a liquid crystalline state can exhibit a minimum energy difference band (n⇒ π_c*) that occurs at a considerably longer wavelength than its progenitor. This band is also quite intense due to the large number of electrons per "equivalent molecule" available when the material is in the liquid crystalline state. The material exhibits a very large absorption cross-section. This is the absorption band of the aggregated retinoids due to conjugated-dipole-molecular absorption. For retinoids derived from retinol, it typically exhibits an isotropic peak wavelength between 495 and 502 nm depending on the solvent present. If the length of the side chain is extended by one more methine group, the peak wavelength is moved to beyond 540 nm[82].

This is the mechanism proposed in the model for the transduction of visual spectrum photons by the eye. It has led to a very accurate equation for the perceived spectral response of all eyes in the animal world, including the ultraviolet sensitivity of many animals. This is particularly true for the well-documented sensitivity of (many) insects.

When chromophores of the type described here are formed, they have a unique electronic structure due to the delocalized bonds of the conjugated vinylene chain and the non-bonding electrons associated with the auxochromes. The energy difference between the n-electron level and the π_c* level is found to be quite small compared to the difference between the π_c and π_c* levels. Thus, these molecules can be excited by relatively long wavelength radiation and, in the absence of other processes, will re-radiate this energy at a later time. Even more interestingly, when these molecules are aggregated on a surface, they act as an ionic crystal. They exhibit overlapping energy levels that leads to conduction within the crystal. These excited electrons when present in organics do not act quite the same as they do in metallic materials. They are not able to contact a surface as freely as electrons in metallic or metallic materials. For this reason, some authors in the organic semiconductor world speak of these "bound but excited electrons" as excitons. The conduction band is then called an exciton band and the valence band is called the "unbound exciton band." This causes a slight problem here because these same authors call an unexcited exciton a bound exciton (equivalent to an unshared and therefore unbound electron). Other authors in the organic semiconductor world continue to use the labels associated

[82]Zingoni, Y. Crouch, R. Chang, C-H, Govindjee, R. & Ebrey, T. (1986) Effect of variation of retnal polyene side-chain length on formation and function of bacteriorhodopsin analogue pigments. Biochem. vol 25, pp 2022-2027

with metallic semiconductors. Gutmann, et. al. use both nomenclatures in the same book[83].

As this aggregate structure is exposed to radiation in the region of this new spectral peak, the material will gradually become transparent as the n-electrons are excited into the π_c* band. The material will remain transparent until these electrons are de-excited by flourescent, thermal or other means.

> In the literature of organic semiconductors, some authors have chosen to re-label the names of the levels used in solid state semiconductors. The conduction band is known as the exciton band and the valence band is known as the "bound exciton" band. Trap levels are known as bound phonon levels[84]. The "bound exciton" label may lead to problems since this band is actually occupied by non-bonded electrons. Gutmann, et. al. also describe hole transport in their figure 13.3 without using the term. They do use the expression holes, with its normal meaning, in later pages. Their figure 16.1 uses the conventional solid state semiconductor level names when discussing an organic semiconductor. Figure 16.2 presents the hole mobility of the same material in the way consistent with solid state semiconductors.

The principal features of a diagram for an organic chromophore are;

+ The presence of a band populated by non-bonding electrons associated with one or more of the auxochromes.

+ The presence of two bands which are normally empty, the π* band and the σ* band

+ The close proximity of the n-band to the π* band, especially in the case of the resonant organic structure.

It is possible to turn the energy band diagram into an absorption spectrum diagram by replotting it as a probability diagram as a function of energy. However, this is only an intermediate step since it still requires the investigator to calculate the energy differences which will result in excitation. What is desired is to plot a probability diagram as a function of the energy differences (or more conventionally, the wavelength of the required photons) between the various bands. **Figure 5.4.3-2(a)** shows the probability diagram as a function of energy and **Figure 5.4.3-2(b)** shows the probability diagram as a function of energy differences. It should be noted that energy differences exhibit the same probability shapes as due the underlying energy plots because of the exponential form of Fermi-Dirac statistics. **Figure 5.4.3-2(c)** shows the more conventional plot of probability versus the wavelength of the exciting photon. This figure also shows the spectra for a simple molecule versus the spectra for the same chemical material in a molecular system. Note the change in position of peak absorption and the considerable broadening of the absorption band as the molecule is incorporated into a molecular system. These changes are widely observed in the "dye chemistry" associated with the silver halide photography field.

5.4.3.1.5 The n-electron band/ π_c* band interaction for oxygen

The presence of oxygen as the polar atoms of the chromophores introduces another unique property. Atomic Oxygen exhibits a pair of unpaired electrons. The two unpaired electrons should satisfy the Pauli Exclusion Principle in the conventional way, by existing in opposite states of spin. This state is known as the singlet state of spin. However, in the case of oxygen, they do not. Rather, they exist in the same spin state but in slightly different orbital planes. As a result, the unpaired electrons are defined as being in the triplet state and oxygen exhibits paramagnetism.

The triplet state of the unpaired electrons of oxygen play a key role in both the photon excitation and the potential relaxation mode of the excited chromophores of vision. The paramagnetic properties of oxygen provide a definitive method of determining whether oxygen is present in the chromophores of vision, a condition that would eliminate the Shiff-base theory of retinol reaction with opsin to form rhodopsin. The evaluation of the electron paramagnetic resonance of the chromophores of vision is discussed in **Chapter 7**.

5.4.4 Transitions in complex molecules

Normally, when a complex molecule is excited by radiation, a n-electron moves up into a π* or σ* band. This usually involves a n-electron of the singlet type and the Frank-Condon principle requires the transition to proceed into an excited

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[83]Gutmann, F. Keyzer, H. & Lyons, L. (1981) Organic Semiconductors: Part B Malabar FL: Robert E. Krieger Publishing pg. 64 and pg. 197

[84]Gutmann, F. Keyzer, H. & Lyons, L. (1981) Organic semiconductors: Part B Malabar, FL: Robert E. Krieger Publishing Co. Pg. 65

state of the singlet type. The excited state of the singlet type is relatively short lived and is usually associated with fluorescence as a method of decay in molecules. If the n-electron is initially of a triplet type, it can transition directly into an excited state of the triplet type which is a longer life state.

Adamson[85] has pointed out that the oxygen ion is unusual and normally has unexcited n-electrons in the triplet state that can go directly into the excited triplet state. This is why many oxygen containing organics do not exhibit fluorescence that is attributable to the first excitation of oxygen. It is also why it is not necessary to discuss how an excited n-electron from an oxygen ion moves from the singlet to the triplet state.

Witteman has also highlighted an important corollary of quantum theory[86] In a symmetrical molecule containing heavy ions on the ends, it is forbidden for an excited electron to decay through dipole radiation. These molecules have very long excited states, measured in hours, before they decay through other (usually thermal) means such as collision with other molecules or the walls of a container.

Mees & James, and others, have noted that the absolute energy level of the top of the n-electron band (half-amplitude of the electron density profile of the n-band) in most chromophores of photography is located at an absolute energy level near 3.39 electron-volts[87]. This ground level is very near the first excited level of the hydrogen atom shown in [**Figure 5.4.1-1**]. However, this may only be coincidental.

5.4.5 Energy transfers between complex molecular structures

How energy is transferred from the triplet state of the p*–orbitals to the neural system remains to be developed. The dendrolemma in contact with the chromophores is defined as type 2 in Section xxx. In the case of the dendrolemma, this type 2 bilayer is in intimate contact with a second reticular lemma, separated only by a liquid crystalline structure composed of liquid crystalline water (hydronium). In toto, the lemma assembly constitutes an Activa.

The transfer of energy to the Activa formed as above can be considered to occur in either of two ways. The energy could be transferred to the type 2 dendrolemma by the excitation of a conjugated molecule of the lipid forming the outer bilayer. Alternately, the dendrolemma could be considered a “rigid” structure supporting the transfer of an exciton from the chromophore to the hydronium forming the base of the Activa.

The transfer of the energy from the chromophore to the outer bilayer of the dendrolemma would be most likely if the polar portion of the outer lipid of the bilayer contained oxygen exhibiting paired electrons in the triplet ground state. It should be noted that oxygen is the dominant constituent of the polar group of the lipids forming biological bilayers (Lehninger, pg 196).

Like the Rhodonines of the chromophoric layer, the polar portion of the phosophoglyceride lipids forming the bilayers of the dendroplasm are oxygen rich, generally in a conjugated organization about a carbon atom and/or about a phosphorous atom. **Figure 5.4.5-1** shows the structure of a phosphatidic acid, a typical lipid found in lemmas when combined with a serine, glycerol or inositol group. When so combined and with the fatty acid at position 2 unsaturated to the point of conjugation, the molecule exhibits an empty delocalized π^*–orbital band extending nearly the length of the molecule. The precise stereo-specific arrangement between such a molecule when in the liquid crystalline state of the lemma and the nearby liquid crystalline state of the Rhodonine molecules of the chromophores is not known. The charged oxygen at the upper right of the phosphatidic acid appears ideally suited for combining with the chromophoric structure. The molecule appears capable of accepting an excited electron from the delocalized π^*–orbital. of the nearby chromophoric liquid crystal and propagating that excited electron down the length of the fatty acid as described by Fincher et al[88]. (See the work of Heeger discussed in **Section 8.2.4**). When the conjugations remain in the all–trans configuration, the overall size of the molecule is not changed significantly within the structure of the lemma bilayer. The conductivity of this path has been likened to that of copper.

The phosophoglyceride lipids forming the outer layer of the type 2 lemma must be a p-type material according to the electrolytic theory of this work.. There are indications, these external phosphoglycerides may be phophatidyl choline (lecithin) with one of their #2 fatty acid chain fully conjugated. The inner phosphoglycerides may be phosphatidyl ethanolamine (cephalin), again with one of their fatty acids fully conjugated. [xxx **Section 5.2.4.1 of Neuron** discusses

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[85]Adamson, A. (1973) A textbook of physical chemistry. New York: Academic Press pg. 852
[86]Witteman, W. (1987) The CO_2 Laser. Berlin: Springer-Verlag
[87]Mees, C. & James, T. (1966) Op. Cit. pp 269-272
[88]Fincher, C. Park, Y. Heeger, A. & Shirakawa, E. (1977) Electrical Conductivity in Doped Polyacetylene - *Phys Rev Letters* vol 39, pp 1098-1101

the complete lemma of a cell in greater detail.]

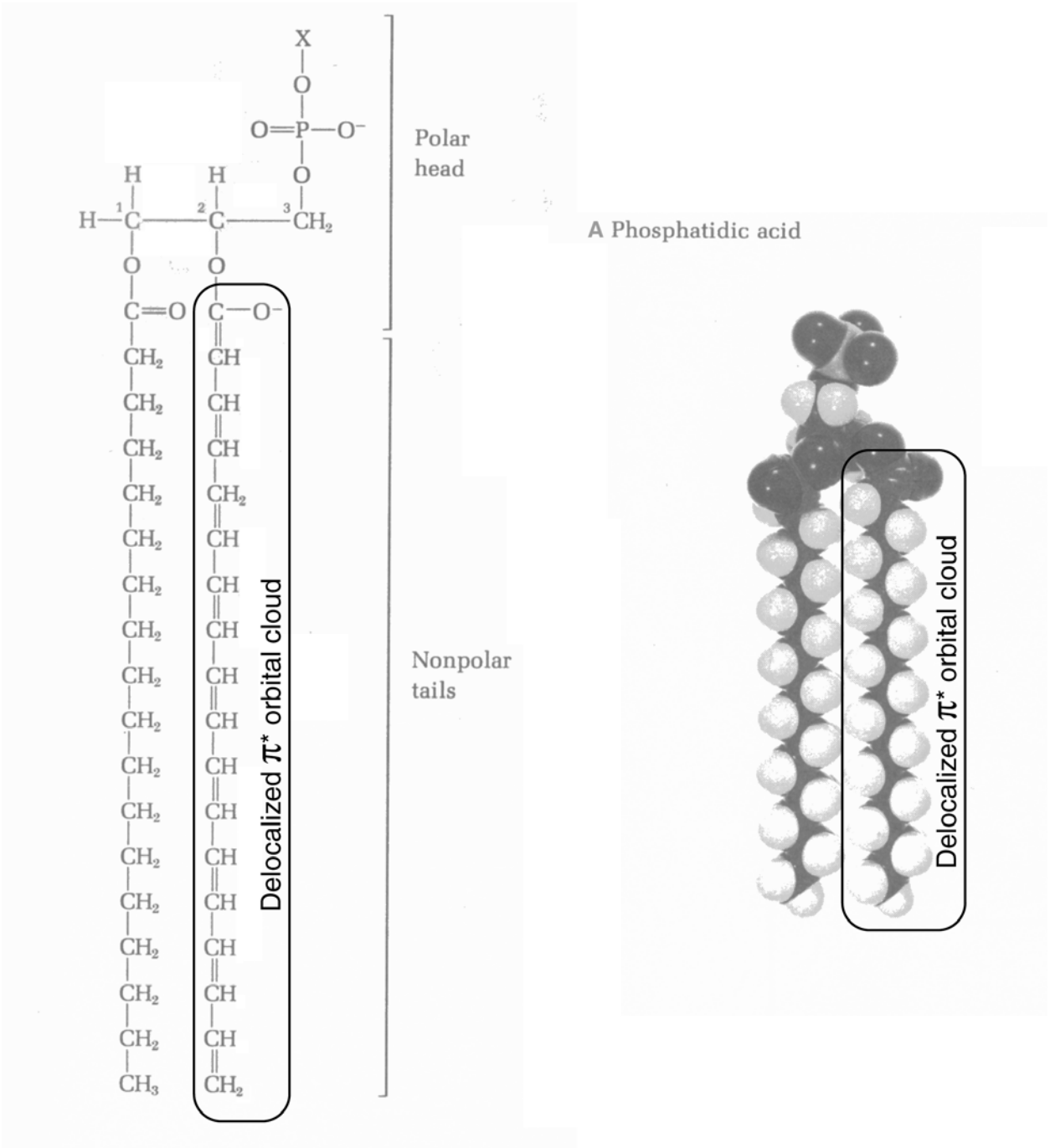

Figure 5.4.5-1 General structure of phosphoglycerides, emphasizing their amphipathic nature. Usually the fatty acid in the 2-position is unsaturated. The left frame shows a fully conjugated fatty acid in the 2–position. X is frequently an oxygen bearing group such as serine, glycerol or especially inositol. The right frame shows a similar space-filled representation. There are hidden hydrogen atoms behind the left-most fatty acid.

When formed into the outer liquid crystalline surface of a type 2 lemma, the conductive fatty acid chains at position 2 are isolated from each other by the surrounding insulating fatty acid chains at positions 1. As a result, the probability of charge flow perpendicular to the axis of the molecules is very low. Using the terminology of light-emitting-diode chemistry (**Sec. 8.2.4.3**), each molecule forms an isolated polaron. Charge transfer in this situation is primarily from one polaron in the outer layer to one polaron of the inner layer. The electrical character of the two polarons constitute an electrical diode relative to their extreme terminals.

5.4.6 Absorption coefficient profiles

One frequently draws the conclusion from the above figure that the energy bands are uniformly populated and have sharp edges. The fact is the bands are not uniformly populated and the edges only describe the half amplitude points associated with an exponential expression developed in the theory of Fermi-Dirac Statistics. **Figure 5.4.6-1** illustrates the actual situation. The lower portion of the figure shows the two most important energy bands of vison. When the Rhodonines move from a solution into a liquid crystalline state, the two isolated energy levels associated with the n-band are now shared throughout the liquid crystal. However, the Pauli Exclusion Principle says that no two electrons can share the identical state within an electronic structure. This applies to both occupied states and potentially occupied states. Therefore, the electronic profiles of the various bands are described by the curves shown where the ordinate is a logarithmic scale. The abscissa is a linear energy scale usually described in terms of electron-volts. The π^*-band is normally unpopulated and the n-band is normally fully populated.

If the probability of an electron making a transition between energy bands is examined, a plot of the probability of transition as a function of the energy of an exciting event can be derived. The energy of excitation can be from any source. In vision, it is usually thought of as a photon. However, for reasons to be presented later, it is important to note that any quanta with adequate energy, whether electromagnetic, chemical or mechanical can excite a material.

If impacted by a photon, the probability of electron excitation is determined by the probability that there is an electron in the n-band that can be excited into an empty site in the π^*-band that is higher in energy by the precise energy of the photon. Otherwise, the photon will not be absorbed by the chromophore. The resulting probability function is shown schematically in the upper part of the figure. The actual mathematics will be addressed in **Chapter 16.** This frame is drawn with two abscissas. The lower scale is linear with respect to the wavelength of the impacting photons. The upper scale is nonlinear and represents the energy of the impacting photons. The curve is drawn to indicate the absorption spectrum of the long wavelength Rhodonine chromophore. Note that this absorption spectrum is achieved by Rhodonine in the liquid crystalline state without its chemical union with any protein such as opsin.

A discussion has surfaced in vision research from time to time concerning whether the spectral absorption coefficients of the photoreceptors (which are actually probability functions related to the absorption of a photon) should be plotted on an abscissa representing equal amounts of energy per interval or one representing equal number of quanta per interval. The important fact is that the relevant calculations are probability functions involving continuous equations related to *energy* and the location of the individual *energy band edges*. They are not calculations based on discrete numbers of particles. **Specifically, the relevant algebra of absorption coefficients, although based on quantum-mechanical theory, is not a quantum-based calculation.**

It is true that in the case of a broadband photoelectric detector, there will be more electrons released per micron interval if the excitation source is tailored so as to provide a constant amount of energy per micron interval. However, this does not have any effect on the coefficient of absorption; the effect is on the number of quanta actually passing through the system. In this respect, **the output signal is a function of the number of quanta absorbed per unit spectral bandwidth**. Most plots of this signal output should employ a stimulus containing equal number of photons per spectral interval.

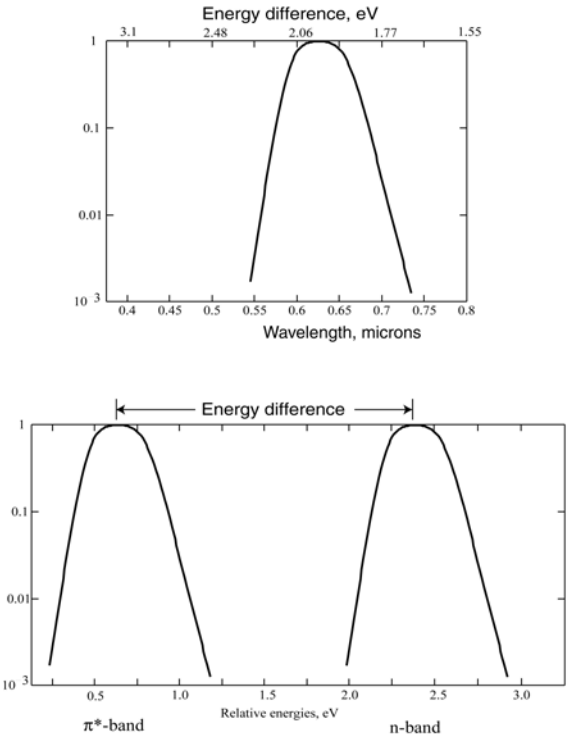

Figure 5.4.6-1 Probability functions as a function of energy--photon wavelength. Lower curves show the probability profiles of two individual energy bands. Normally, the n-band is full and the π*-band is empty. The probability of an electron transferring between these bands when excited by a photon is a function of the energy of the photon. The upper curve illustrates this probability as a function of photon energy and wavelength for the long wavelength photoreceptor of vision. This form is frequently described as the Fermi-Dirac distribution or function.

The photoreceptors of vision are not broadband detectors in the normal sense. They are sensitive to a range of considerably less than 2:1 compared to many metallic semiconductors and many radio antenna systems which are sensitive to ranges of 10:1 to 20:1 or more. Even in these systems, the definition of quality is usually taken as the coefficient of absorption as a function of wavelength (which is usually independent of the number of quanta incident), not how many quanta are absorbed per unit wavelength.

There is a much more important consideration than how to plot the absorption coefficients. That is whether the overall operation of the visual system is even remotely linear. If it is not, the signal related to the quanta out may not be related in any linear way to the quanta in and the above subtlety of how to plot the data becomes trivial. The actual problem is how to define the input-output characteristic of the visual system.

Up until the 1960's, in spite of a great deal of data to the contrary, nearly all of the discussion in the visual field assumed the visual process to be a linear one: this is nearly so today. Frequently, the invocation was made that the system approached linearity over limited operating ranges of interest to a particular investigator. When examined closely, there is practically no region or regime where the visual process approaches linearity. This is especially true in the laboratory where the experimentalist normally and traditionally applies very large stimuli to the system relative to the natural environment.

As this work progresses, it will be shown that the photoreceptor "system" incorporated within the photoreceptor cell includes a sensitivity control mechanism which is a function of the total number of incident particles per unit time (See **Chapter 12** and **Appendix A**). Because of this mechanism, the investigator has the option, mathematically, of considering the absorption coefficient to be constant with regard to the input excitation or to regroup the terms in the equations so as to define a coefficient of absorption which is a function of the total input irradiance over the absorption band of the material. This situation is of little importance except in the case of absorption measurements in the presence of a background irradiance that falls within the overall absorption band of the particular material under test. If this occurs, the effect of the total irradiance must be considered in order to extract the correct coefficient as a function of wavelength. There are many examples in the literature where this was not done; the resulting confusion can only be explained by properly applying the photoexcitation/de-excitation (P/D) equation of the photoreceptor.

Also to be shown in **Chapter 12** is the fact that; in those visual systems employing the L-channel, the transfer function of that channel exhibits a different transfer characteristic than the other channels. Not only are the shorter wavelength channels nonlinear in the presence of large changes in irradiance, the L-channel has another nonlinearity that must be accounted for. It requires care and a correct model to define these different effects independently and to illuminate the subtleties involved.

5.4.6.1 Previously calculated energy band profiles of the visual chromophores

Extensive efforts have been made to calculate the spectral absorption profiles of various forms of the retinenes and those retinenes conjugated with opsin under a variety of conditions. Birge has summarized this work in great detail and with many references[89]. These calculations have not been able to predict the spectral absorption of the visual chromophores. The resulting graphs will not be displayed here. The technical problems have been multifold. The primary problem has been the assumption that retinol played a role in the visual process as a chromophore when its actual role is that of a chromogen. Even making various assumptions concerning delocalization of protons, the results have not been satisfactory. As shown below, the actual chromophores are the Rhodonines acting alone. When the true chromophores are examined, it is not found necessary to invoke a large bathochromic shift or a stereoisomerism to account for the spectral characteristics of vision. The second problem has been not recognizing the liquid crystalline state of the chromophores of vision. The investigators have encountered "severe inhomogeneous broadening (lack of distinct vibronic structure)" of the measured spectral absorption which they look upon as detrimental. In vison, it is actually an attribute brought on by the required line broadening to satisfy the Pauli Principle. Their calculations must be modified to recognize this fact. The third problem is their attempt to treat the absorbing species as a bulk resonator instead of treating it as a slow wave structure ala Platt[90]. This assumption has also led to the general assumption that the axis of the chromophore molecules must be perpendicular to the incident irradiation. In fact, and as shown above, the slow wave structure requires just the opposite condition for maximum absorption efficiency. The axis of the chromophore must be parallel to the incident irradiation.

The concluding remarks of Birge are significant. He *speculates* on why another double-bond isomer of retinal would not work just as well as 11-*cis* retinal (holding to the assumption that a 11-*cis* to 11-*trans* transformation is the critical step in the visual process). Reviewing the earlier definition of photochemistry, it is seen that such a transition is actually one among many possible alternative methods of de-exciting a previously excited species. This work proposes an entirely different method of de-excitation.

5.4.7 Absorption as a function of environment

Beer's Law is widely used in chemistry and it has been generally applied in vision research. This action has significantly hindered the development of the field of vision. Beer's Law applies to general solutions of low molarity containing material that is prone to de-excitation by thermal or re-radiant means. It does not apply to solutions of high molarity or liquid crystals. It also does not apply to materials that depend on other materials for purposes of de-excitation. Under each of these conditions, the material assumes a different mechanical or electronic configuration than envisioned by Beer. In the case of the chromophores of vision, the material only exhibits its absorption spectrum at molarities greater than 10^{-2} and the material usually "bleaches" during *in-vitro* excitation due to a lack of a suitable de-excitation mechanism.

Another problem that will be addressed later is that the chromophores of vision are members of the phthalein family. Their observed color is very sensitive to the pH of the solution.

An additional problem related to the environment is due to the delicacy of the chromophoric molecules. They are easily decomposed in the presence of strong metallic ions and modern detergents. Frequently these agents are applied to the biological material in order to extract the chromophores. The result is partial decomposition of the chromophores.

The remainder of this section will assume that the chromophores of vision are the resonant conjugate retinoids derivable from Retinol and they have no chemical relationship to any protein, such as Opsin, except possibly through hydrogen bonding as a matter of convenience. Any more complex relationship would probably involve an undesired shift in spectra.

5.4.7.1 Chromophores in Solution

Most investigators have attempted to determine the spectral characteristics of the visual chromophores in dilute and frequently organic solvents. Because of the conditions mentioned above, it is unusual for a research worker to observe the actual absorption spectra of the chromophores of vision except *in-vivo*. To successfully record these spectra, it is necessary to insure four conditions:

+ insure the protection of the chromophores to avoid deterioration during purification and concentration.

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[89]Birge, R. (1977) Op. Cit.
[90]Platt, J. (1964) Op. Cit.

+ place the chromophoric materials in a liquid crystalline state to allow absorption of the radiation in the spectral region of interest. This is normally achieved when the molarity of the solution exceeds 10^{-2}.
+ maintain a pH similar to the in-vivo condition.
+ insure the solvent, or a third material that is present, exhibits an excited state that overlaps the energy band of the excited triplet state of the chromophore and is easily de-excited.

The last condition provides a mechanism for measuring the absorption spectra under steady state conditions. In the absence of this condition, it is necessary to make transient measurements and extrapolate back to the true absorption coefficient prior to excitation.

5.4.7.2 Chromophores on a substrate

An alternate procedure for observing the absorption spectra of the chromophores is to precipitate the chromophore on a suitable substrate. If carried out slowly, the precipitate will assume the necessary liquid crystalline form on nearly any substrate. However, in the absence of a substrate, or a third material being present, that has an energy band that overlaps the excited triplet state of the chromophores and is easily de-excited, the material will bleach rapidly and steady state measurements will be inconclusive. Achieving the most accurate measurements requires two conditions, knowing the thickness of the liquid crystal and achieving the necessary electronic contact. The first condition is best achieved by depositing a monolayer. The second condition is most easily accomplished by a careful choice of substrate. It is relatively easy to deposit the chromophore on a metallic semiconductor substrate that provides the required energy band overlap (in the place of the biological material found in the retina -excited). Silver halide crystals, as used in the preparation of a photographic emulsion, are known to be a suitable substrate for most molecules similar to the resonant retinoids. By exposing and developing a silver chloride-based emulsion, a permanent record of the absorption spectra of the visual retinoids is obtained that can be easily separated from that of the substrate. A different substrate should be used to obtain the spectra of the UV sensitive chromophore.

5.4.7.3 Chromophores in the liquid crystalline state

Chromophoric materials in the liquid crystalline state exhibit anisotropic spectral absorption characteristics due to their structure. In general, they have the highest absorption coefficient for irradiation traveling parallel to the long axis of the resonant molecules, i.e., perpendicular to the surface of the crystal in the visual case. For radiation applied perpendicular to the axis of the resonant molecules, the observed spectra will be that of the conjugated structure instead of the resonant structure. This is very important for both *in-vivo* and *in-vitro* testing. The only *in-vivo* testing that exhibits the true spectra of the individual chromophores in their natural condition involves illumination parallel to the axis of the Outer Segment (in the case of *Chordata*). Illumination applied perpendicular to the axis of the OS will exhibit the spectrum related to the conjugation but not the resonance of the molecule. For chromophores derived from Vitamin A, the peak absorption for this conjugate condition always occurs at 502 nm at 37 Celsius, regardless of the resonant chromophore present.

5.4.7.4 A summary of the reported and expected absorptions of the retinoids

The vision literature contains a great variety of different absorption profiles for the chromophores of vision. They have been acquired using a variety of means. The chemist prefers to measure the absorbance of individual chromophores in a standard one centimeter cell as a function of wavelength. The chromophore is usually present in a dilute solution. The psychophysiologist prefers an *in-vivo* test under conditions of differential adaptation. The results from these two methods have shown considerable disparity. This work will show that, for the in-vivo situation (and adequate differential adaptation in the psychophysical situation), the actual L–channel peak is in human vision is at 625 nm. This is the peak wavelength observed in all other biological species.

A primary reason for the above disparities is the critically important structural organization of the chromophores when found *in-vivo*. These relationships make a major (several orders of magnitude) difference in the absorbance of the material and also lead to anisotropic absorption. These relationships have not been maintained by the chemists. A second reason relates particularly to the L–channel. The chromophore of that channel exhibits a more intimate relationship with the electronic portion of the photoreceptor neuron than do the S– and M–channel chromophores. As a result, the L–channel exhibits an effective absorption characteristic very different from that observed by the chemist. This characteristic also accounts for the loss of red response in the mesopic and scotopic regions. These relationships have not been emulated in the environment of the chemist. Failure to emulate these conditions leads to extraneous absorption spectra for the L–channel chromophore. A third reason is due to the frequent chemical reactions occurring in the chemists solutions that he may not be aware of. It has been rare in the past for the chemist to document the pH

of the solutions he has measured. This is a particular problem as mentioned in a later section [**Section 5.5.12**]. The chromophores of vision are members of the "indicator" class of chemicals. Their spectral characteristics are intimately related to the pH of their environment. They are also complex organics. Their spectral characteristics are a function of the organic solvent used. They are also subject to chemical attack. This mechanism has been documented by Wald, et. al. and more recently by Ma, et. al.

Because of these conditions, it is useful to provide a summary of the various chemicals and situations that have been studied because of their potential role as the chromophores of vision. The data in the following table has been collected from later sections of this Chapter, **Chapter 6**, **Section 12.5.2.4** and **Sections 17.2.1** & **17.2.2**. In a table of this type, it is difficult to illustrate all of the absorption lines of complex molecules. Thus, only the major absorption band is listed. The above sections and chapters provide finer data where applicable.

TABLE 5.4.7-1
A SUMMARY OF POTENTIAL CHROMOGENS AND CHROMOPHORES OF VISION

Chemical	Environment	Peak absorp. λ	Max. Absorbance (Optical path)	Comment
IN SOLUTION				
all-trans-retinal$_1$	methanol	325 nm	See Sporn, 1994	
all-trans-retinol$_1$	methanol	350 nm	See Sporn, 1994	
all-trans-retinol$_2$	methanol	375 nm	See Sporn, 1994	
N-Retinyl N^+-Retinyl (w/o hinderance)		~443 nm		See Ma, Znoika(2001)
red rod pigment	hydroxylamine	500 nm	0.06 (1.0 cm)	See Ma, Znoika (2001)
UV-cone (reconst)	PBS*	356 nm		Based on 11-cis-retinal
	HCl acid	443 nm		Based on 11-cis-retinal
UV-cone (reconst)	PBS	360 nm		Based on 11-cis-3,4 dehydroretinal.
	HCl acid	470 nm		"
S-cone	pH 7 water hydroxylamine acid	360-432 (fct of time)	0.06	See Ma, Znoika (2001)
S-cone		**432**-440 nm		See Makino, et. al. (1999) pg 1031
M-cone		502-**521** nm		"
L-cone		562-**620** nm		"
IN SITU (a complete outer segment irradiated axially)				
UV-cone		342 nm	~0.8 (50 microns)	This work & Ma, Znoika
S-cone (Green rod)		437 nm	~0.8 (50 microns)	This work & Ma, Znoika
M-cone (Red rod)		532 nm	~0.8 (50 microns)	This work
L-cone		625 nm (1st order)	~0.8 (50 microns)	This work, photopic level
		610 nm (2nd order)	"	"
IN LIQUID CRYSTALLINE STATE				
Rhodonine( )		498 nm (isotropic)		All four chromophores
Rhodonine(11) (computed)		342 nm (aniso-)	~0.0004 per layer	Bandwidth varies with configuration (# of layers)
Rhodonine(9) (computed)		437 nm "	~0.0004 per layer	"
Rhodonine(7) (computed)		532 nm "	~0.0004 per layer	"
Rhodonine(5) (computed)		625 nm " (1st order)	~0.0004 per layer	"
Rhodonine(5) (Re-computed)		610 nm " (2nd order)	~0.0004 per layer	"

* PBS=phosphate buffered saline PSB=protonated schiff base

While the materials in dilute solution may be chromogens, their spectral peaks and their low absorbances clearly show they are not the chromophores of vision. It appears the absorption spectra of the "reconstituted UV chromophore of Ma,

Kono, et. al. in PBS was, in fact, just the base retinoid[91]. The wavelengths in the presence of HCl are clearly not related to the in-vivo condition. As Ma, Kono , et. al. indicated, the observed wavelengths agree with those of a "free" protonated Schiff base[92]. They speak of the material as being denatured by the HCl, i. e., the material reacted with the HCl. Its relationship to a natural chromophore has become considerably less direct. The same situation appears to apply to the S-cone of Ma, Znoika, et. al. The material appears to be either the all-cis form of either VitaminA1 or Vitamin A2 which is attacked by the hydroxylamine. Although conforming to the conventional wisdom, relying upon nomographs and making many complex "adjustments," particularly to the typically irrelevant ionone ring, Makino et al[93] generally found the S-photoreceptor response to be in the area of 432-442 nm. They provided no spectral responses.

5.4.8 Absorption by the lattice of chromophores associated with an outer segment

The operation of the outer segments is discussed from the operational perspective in Section 12.5.x of this work.

5.5 Candidate Chromophores

Retinol, (a.k.a. Vitamin A) is a well-known chemical with many different properties and uses. These properties fall in many categories, some quite far removed from the obvious pharmacological ones and some which are virtually unknown in the literature. It is important to examine a number of these properties before exploring how retinol and its derivatives participate in the vision process. Because of its peripheral nature, relative to vision, this discussion will be found in Chapter 6.

From here on, our emphasis will not fall on retinol itself but on its derivatives. This is because although retinol derives from a well-known natural dye, carotene; retinol and the retinenes are not dyes themselves. While they exhibit a minor absorption at 500 nm in the visual spectrum when in dilute solution, this absorption feature is not used in the visual system. Normally, it cannot be measured in-vivo because of the much greater absorption coefficients related to the molecules when in the liquid crystalline state.

Similarly, except for **Section 5.5.6.1**, there will be no further discussion of the retinenes combined with nitrogen. Because of the early proposal by Collins, et. al.[94] that retinol participated in a Schiff base to form rhodopsin, this option was originally considered in this work. The overwhelming evidence that this configuration does not, and cannot, provide a visible band absorption spectrum (even when extrapolated to a delocalized proton associated with the nitrogen[95]) is sufficient to eliminate further consideration of this conjecture. Further, there is strong evidence that the chromophores of vision involve two polar atoms of oxygen rather than one or more nitrogen atoms.

While the chromophores of vision are retinoids, derive from retinol in the biological environment, they are characterized by many features not shared with the retinenes.

5.5.1 Organization of the retinoid family

Sporn, Roberts and Goodman[96] have recently provided a broad overview of the field of the retinoids, based on the IUPAC-IUB Joint commission definition that; "Retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner." (The work was updated in 1994) Whereas there is only a handful of naturally occurring retinoids; using this definition, more than a thousand man-made retinoids are known. The IUPAC-IUB bulletin recommended that the term, retinal, not be used as a chemical designation but be reserved as an adjective referring to the retina. They suggested that retinaldehyde be used instead in scientific literature as the name of chemical related to vision.

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[91]Ma, J. Kono, M. et. al. (2001) Salamander UV cone pigment: sequence, expression, and spectral properties *Visual Neurosci* vol. 18, pp 393-399

[92]Ma, J. Znoika, et. al. (2001) A visual pigment expressed in both rod and cone photoreceptors *Neuron* vol. 32, pp 451-461

[93]Makino, C. Groesbeek, M. Lugtenburg, J & Baylor, D. (1999) Spectral tuning in salamander visual pigments studied with dihydroretinal chromophores *Biophys J* vol 77, pp 1024-1035

[94]Collins, F.(1953) *Nature*, vol. 179, pg 469 *and* Morton, R. & Pitt, G. (1955) *Biochem. J.* vol. 59, pg 128

[95]Derguini, F. Caldwell, C. Motto, M. Balogh-Nair, V. & Nakanishi, K. (1983) Bacteriorhodopsins containing cyanine dye chromophores. J. Am. Chem. Soc. vol. 105, pp 646-648

[96]Sporn, M. Roberts, A. & Goodman, D. (1984) The retinoids. NY: Academic Press pg. 3

Sporn, Roberts and Goodman[97] have attempted to catalog all of these retinoids under the following headings:

A. Retinol & Derivatives
B. Retinylamine Derivatives
C. Retinaldehyde & Derivatives
D. Retioic Acid & Derivatives with a modified ring
E. Retinoic Acid Derivatives with a modified side chain and/or polar terminus
F. Etretinate & Derivatives
G. Retinoidal Benzoic Acid Derivatives

Their text and catalog fail to address another group of retinoids that are believed to be crucial to the vision process. These are the "Resonant Retinoids," or retinines which includes the Rhodonines. It is suggested that these Resonant Retinoids be grouped in an additional catalog heading;

H. Resonant Retinoids, or retinines

The principle feature of members of this group are that:

+ they contain a resonant structure that can simultaneously mimic a molecule containing both a aldehyde and a alcohol moiety terminating a conjugated carbon chain, i.e., an isoprenoid chain

+ they contain a β-ionone ring

+ they exhibit the same spectral absorption spectrum in the ultraviolet as their chromogens when in dilute solution

+ They all exhibit the same spectral absorption characteristic when in the dilute (less than 10^{-2} M) liquid state.

+ they each exhibit a distinctly different spectral absorption spectrum only when they are present in the liquid crystalline state.

The unique spectrum when in the liquid crystalline state is due to the resonance phenomenon exhibited by the two terminal groups and the conjugated carbon chain between them, and the ability of electrons to be shared between the individual molecules of the liquid crystal.

5.5.1.1 Concepts of a dye

The use of dyes is so large and the literature of dye chemistry is so correspondingly large that numerous efforts have been made to classify dyes in a systematic manner. The basic problem is of course the immense variety available in the realm of organic chemistry. Most organic molecules of even nominal molecular weight can be analyzed from multiple points of view--and even named based on these points of view. There was a natural division between natural and man-made dyes but it is now only of historical significance.

Dyes are usually divided into water-soluble and water-insoluble groups. The water soluble group is further divided into anionic (acid) and cationic (basic) dyes. Gurr[98] provides a comprehensive chart of the synthetic organic dyes. However, he does not address the resonant amphoteric dyes. The specific placement of the Rhodonines within his chart requires additional analysis and probably laboratory measurements to determine their relative level of amphoterism. The chart does not specifically delineate resonant dyes such as the Rhodonines.

The one nearly universal characteristic of all important (effective) dyes is the presence of a conjugated chain of carbon atoms (and even this structure may be hard to illustrate in more complex multi-ring structures) which are normally in E (*trans*) configuration. Zollinger divides this basic class into two major parts: the polyenes and the polymethines[99]. He defines the polyenes as having chain ends consisting of an alkyl or other group that does not influence the electronic excitation of the molecule. An early study of the absorption spectra of several series of homologous polyenes was by

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[97] Sporn, M. Roberts, A. & Goodman, D. (1984) The retinoids. NY: Academic Press pg. 393

[98] Gurr, E. (1971) Synthetic dyes in biology, medicine and chemistry, NY: Academic Press
[99] Zollinger, H. (1991) Op. Cit. pg. 51

Hausser, Kuhn & Smakula[100]. The most complete series they studied was the diphenyl polyenes: C_6H_5-(CH=CH)$_n$ – C_6H_5, where n=1-7 inclusive. The absorption of this family is in the ultraviolet spectrum. The most important polyene dyes are the carotenoids in which the chain usually ends with an aliphatic or alicyclic group.

Zollinger defines the polymethines as having chain ends consisting of one electron donor D and one electron acceptor A. Zollinger also makes a distinction between the carotenoids and the polymethines based on their source that is not defendable after recognition of the Rhodonines. He defines the carotenoids as natural compounds and the polymethines as synthetic. He also makes a distinction between polymethine dyes and carbonyl dyes. The Rhodonines appear to satisfy both his definitions.

Since the terminal groups do not affect the electronic excitation characteristics of the polyenes, these dyes are invariably very long. b-carotene involves 11 conjugate bond groups to achieve absorption at a wavelength of 450 nm and 478 nm, e=135,000 and 120,000 respectively. Retinol, being much shorter, exhibits a peak absorption at 325 nm, e=52,480 in ethyl alcohol.

The polymethines can be considerably shorter and still achieve significant absorption in the visual spectrum, typically 3 or 4 conjugated double bonds.

Zollinger hastens to point out that the "Carotenoids are natural compounds that are synthesized on a technical scale, using methods which are unique for dyestuff synthesis." He is obviously speaking of only a few carotenoids.

The polymethines rely on the $n \Rightarrow \pi^*$ electronic transition to achieve their long wavelength absorption characteristics, a transition which is uniquely related to the presence of the polar end-groups. The polyenes must rely on the larger energy transitions related to the conjugated chain alone, $\pi \Rightarrow \pi^*$, etc. $\pi \Rightarrow \pi^*$ transitions usually occur in the near ultraviolet and both the $\sigma \Rightarrow \pi^*$ and $\sigma \Rightarrow \sigma^*$ transitions usually occur in the far ultraviolet.

5.5.2 The retinene family as a precursor of the Rhodonines™

This section will discuss the migration from the concept of specific retinenes (embedded in a complex protein named opsin) to a more rational (from a functional perspective) concept of the retinines (with two i's) as stand-alone molecules deposited on the surface of one or more proteins of the opsin family on the disks of *Chordata* OR directly on the surface of the microtubules emanating from te dendrites of the photoreceptors of *Insecta* and *Mollusca* without the participation of any proteins of the opsin family.

5.5.2.1 Background

Although carotene is a well known, and widely used, natural dye; it is a dimer that easily cleaves into two molecules of retinol. Retinol is not an effective visual spectrum dye. Its peak spectral absorption is in the ultraviolet and this characteristic absorption cannot be changed significantly by any reaction involving only its single polar group. It will remain in the polyene class no matter what changes are made at the polar termination. However, if the molecule were modified elsewhere without disturbing its basic conjugated carbon chain, it could become a polymethine and exhibit a significantly different absorption spectrum.

Researchers have been trying to explain how retinal (or retinol) are chromophores in the visual system for more than 40 years with remarkably little success. Dartnall et. al. even claimed that the same retinal-based chromophore accounts for both the broad photopic spectral sensitivity of the eye as well as all three of the color-sensitive photodetectors found in the eye. There may be a problem of semantics related to this claim. In an attempt to synthesize an analog of rhodopsin, they formed a protonated azomethine using retinal and a simple amine, n-butylamine; the resulting peak absorption was at 440 nm. It was hoped the material would have a peak near 500 nm (see further discussion below). Its absorption coefficient was not specified in Zollinger. Although this chromophore exhibits a peak in its absorption near that of the S-channel, it does not explain how the chromophores of the other spectral channels are formed. It is an irrelevant compound.

Saari has made the bold statement that "Three derivatives of 11-cis-retinaldehyde serve as the chromophores of all known visual pigments. They are complexed with a protein component (an opsin), and the resulting protein-retinoid

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[100]Hausser, K. Kuhn, R. & Smakula, A. (1935). Lichtabsorption und Doppelbindung. IV.Diphenylpolyene. *Z Phys Chem B*, vol 29, pp 384-389.

interactions determine the spectral sensitivity of the visual pigment."[101] No reference is given for this statement nor is any explanation of how these complexes exhibit an absorption spectrum in the visual region. However, except for the substitution of all-*trans* for 11-*cis* in the above quotation and a slight modification to the retinoid involved, this work agrees completely with the statement and provides an explanation for how it is applied.

Quoting Berman in 1991, "In current nomenclature, retinol (Vitamin A) belongs to a class of compounds termed retinoids that have three structural features in common: a hydrophobic head (the β-ionone ring), a conjugated isoprenoid side chain, and terminal polar functional groups on carbon 15, which can be chemically or enzymatically modified. . . . Except for the putative 11-cis isomers found in the retina, the retinoids in virtually all body tissues are of all-*trans* configuration." This last sentence should cause any theoretician to pause. Especially if you add the summarizing statement of Tsuda, "The isomeric conformation of retinal varies from one pigment to another; rhodopsin (11-*cis*-retinal), retinochrome (all-*trans*-retinal), light-adapted bacteriorhodopsin (all-*trans*-retinal), dark-adapted bacteriorhodopsin (all-*trans*-retinal/ 13-*cis*-retinal)." But overlooking that implication for the moment, these are interesting properties of retinol but they are not the properties of a chromophore of any known dye family.

Collins[102] made an early attempt to define the chemical structure of the chromophores of vision based on the early work showing retinol was involved and a protein, conceptually labeled opsin, was present in quantity in the Outer Segments of the photoreceptor cells. He appears to have been the source of the proposal that these two materials were combined via a Schiff base, one of the few candidates for low energy binding available at that time. Hubbard, et. al[103] converged on this concept, and attempted to find solutions to the theoretical problems that arose based on kinetic studies. Shriver, et. al.[104] have indicated their quantum-mechanical calculations show there is considerable difficulty with a Schiff base connecting a protein and retinene.

The work of Wald, Collins, Morton and Hubbard in this area was presented in a series of papers in 1953-55[105]. Subsequent authors have not always recognized that there were actually two different Schiff-bases involved. In the early work, Wald introduced the Oxime of Retinal, but only presumed that it was one of the products of his experiments[106]. This was a Schiff-base, an aldimine, involving a double bond between nitrogen and C15. It did not show an absorption peak near 500 nm. Bownds introduced the idea of a protonated Schiff-base in similar attempts to demonstrate a retinoid with an absorption near 500 nm. This also failed. Subsequently, calculations were performed based on a strained proton position in attempts to lower the theoretical energy of excitation and achieve an absorption peak near 500 nm. The results supported a theoretical peak in the area of 440 nm but held little hope for a peak near 500 nm or beyond.

These initial studies assumed rhodopsin was of the form N-retinyl-opsin with the Schiff-base located at the amine terminal of the protein. Bownds, performed experiments that failed to demonstrate this relationship[107]. He then proposed that the Schiff-base must be associated with another amine group within the chain of the protein. It should be noted that lysine is the only common amino acid within a protein with a side chain that can easily form a Schiff-base with an aldehyde or alcohol. Therefore, there are only about six locations within the protein opsin that can support such a Schiff-base. Working in the same laboratory as Wald, Collins, and Hubbard, Bownds then attempted to locate the position of the retinene within the structure of the protein opsin using the protonated Schiff-base assumption and the assumption that the chromophore should lie perpendicular to the axes of the seven elements of the 7-TMS protein. His work was never confirmed. Two quotations from his paper are important. First, "Attempts to find an N-terminal residue in either rhodopsin or opsin have so far been unsuccessful." After considerable discussion of analogs of rhodopsin, he focused on the putative location of the Schiff-base along the length of the rhodopsin molecule and proposed, "This is presumably the e-amino group of a lysyl side chain." He was unable to isolate such a fraction.

Heller noted in 1968 that all of the above work involved "measurements that were all indirect and involved certain assumptions about the molecular weight and molecular absorption of visual pigment". He performed a variety of

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[101]Saari, J. (1994) Retinoids in photosensitive systems. in Sporn, M. Roberts, A. & Goodman, D. The Retinoids 2nd ed. NY: Raven Press pg. 351

[102]Collins, F. (1953) Op. Cit.

[103]Hubbard, R. Bownds, D & Yoshizawa, T. (1966) The chemistry of visual photoreception. *Symposia on Quantitative Biology* NY: Cold Spring Harbor Laboratory, pg 301-315

[104]Shriver, J. Mateescu, G. & Abrahamson, E. (1982) in Methods of Enzymology, vol 81. NY: Academic Press pg 698-703

[105]See the following for references. Sebrell, W. & Harris, R. (1967) The Vitamins, NY: Academic Press, pg 174

[106]Wald, G. & Hubbard, R. (1950) The synthesis of rhodopsin from Vitamin A1. *Proc. Nat. Acad. USA.* vol 36, pp 92-102

[107]Bownds, D. (1967) Site of attachment of retinal in rhodopsin. Nature, vol. 216, pp 1178-1181

exquisite experiments based on the knowledge-base available in 1968-70[108]. He was able to decompose what he described as native visual pigment (Bovine) and subsequent light-exposed material that he labeled an apoprotein. The sequencing was limited to the binning of segments of the proteins. He found that both materials contained a significant amount of sugar. The term light-exposed was introduced to avoid the ambiguity associated with the term "bleached." His work will be discussed in more detail in **Section 4.3.5.4** on the sequencing of the protein, opsin.

Subsequent investigators have not always appreciated the difference between a simple or primary Schiff-base of the aldimine type and the protonated or secondary Schiff-base. Heller stressed this difference. He used the term unsubstituted aldimine to describe a Schiff-base. The term substituted aldimine was used to describe a protonated Schiff-base.

Rubin & Walls provide a conceptual discussion of the anagenesis and the neogenesis of the chromophores of vision as understood up to 1969[109]. It makes interesting reading but includes a large number of conflicts enumerated by the authors. It deviates from the common assumption of a Schiff base connecting the retinoid to the opsin, based on Wald's assumption that a more chemically active sulfhydryl group was probably required[110]. The discussion of the difference between the chromophores based on Vitamin A1 and A2 is superficial. None of the comments concerning the Schiff base or the potential of the sulfhydryl group have been confirmed outside of the Wald laboratory. Most have not been confirmed at all.

Later, Vogt presented a caricature extending Bownds concept. He attempted to show how two chromophores could be attached to the same opsin molecule apparently to obtain one molecule capable of two distinct spectral responses[111]. Since his work found the residue of rhodopsin decomposition was primarily all-trans, specifically 3-hydroxyretinol based on indirect evidence, he proposed this chromophore, in all-trans form, was attached to the outside of opsin by hydrogen bonding between the two oxygens of the chromophore. He also proposed that this chromophore was non-isomerizable. His caricature shows one putative chromophore as N-11-cis-retinyl attached to a lysine at position 296 and the other as all-trans-3, hydroxyretinol.

While 3-hydroxyretinol exhibits two oxygen atoms, they are not in the conjugated form required to achieve the quantum-mechanical resonance required of a chromophore. He suggested that attaching two chromophores to one protein molecule could lead to packaging advantages. However, he also indicated his conceptual arrangement "is by no means conclusive, . . ."

Vogt did not address the question of how multicolored vision was obtained. This work introduced an entirely different aspect of absorption chemistry, characterized by the quantum mechanical resonance of a molecule containing two oxygen atoms separated by a conjugated carbon chain. By changing the spacing between the oxygen atoms, the four spectral peaks associated with the UV-, S-, M- and L-channels of vision can be obtained from the same basic Vitamin A structure. These same four peaks are be obtained whether the chromogen is Vitamin A_1, Vitamin A_2 or Vitamin A_3.

A mathematical model of the putative binding of retinal to a lysine group at location 296 in opsin also appeared in 1989[112]. Such a location would suggest a formulation such as 296, (N-retinyl-lysine)opsin. No laboratory confirmation of either model of a retinene attached to opsin through a Schiff-base has appeared.

Two major conclusions can be drawn from the above discussion. First, no creditable demonstration has been made that rhodopsin is a compound of opsin and a retinene. To date, no residue has been obtained from protein sequencing experiments that contain a retinene. Second, the retinenes cannot be transformed into useful chromophore with visual band absorption spectra without changing their defining structure. Therefore, they are of little interest to vision except as chromogens. As chromogens, they form one of a number of direct paths to the Rhodonines, the actual chromophores of vision. The Rhodonines are retinines. The retinines include two polar groups, both oxygens in the case of visual chromophores, separated by a conjugated carbon chain consisting of methine groups as a minimum. The level of conjugation determines the location of the peak absorption spectrum of the molecule when in the liquid crystalline state..

Because of the above situation, after the following brief section, further discussion of the retinenes will be confined to **Chapter 6**. The sequencing of the protein opsin was addressed in **Section 4.3.5.4**.

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[108]Heller, J. & Lawrence, M. (1970) Structure of the glycopeptide from Bovine Visual Pigment 500, *Biochemistry*, vol. 9, pp 864-869 and vol. 7, pp 2906-2920

[109]Rubin, M. & Walls, G. (1969) Fundamentals of visual science. Springfield IL: Charles C. Thomas. pp 57-105

[110]The sulfhydryl proposal was withdrawn according to Heller, J. (1968) Biochemistry, Vol. 7, pg 2914.

[111]Vogt, K. (1989) In Stavenga, D. & Hardie, R. *ed*. Facets of Vision. NY: Springer-Verlag, pg 140

[112]Krinsky, N. Mathews-Roth, M. & Taylor, R. (1989) Carotenoids. NY: Plenum pg 142

5.5.2.2 Attempts to fabricate rhodopsin from retinal

Wald, et. al[113,114]. performed a set of experiments during the 1940's that purported to demonstrate the formation of rhodopsin from either retinene$_1$ (now known as retinal) or Vitamin A$_1$ and a native protein. While their work involved materials showing a peak absorption at 500 nm, this is the wavelength of peak isotropic absorption of a large number of dipolar retinoids. Such a peak is not exclusive to the chromophores of vision. Neither is it relevant to the anisotropic absorption spectrum of the chromophores of vision.

They did not start from reagent grade chemicals. The experiments involved the in-vitro bleaching of native rhodopsin in solution. They proposed that they caused the disassociation of rhodopsin into a protein and retinaldehyde in accordance with the conventional wisdom of the time. This wisdom conflicted somewhat with the earlier ideas of Kuhne but became the basis of the current conventional wisdom. They claimed to have removed the native retinene$_1$ and then recombined "colorless rhodopsin-protein, free of all native retinene$_1$, with synthetic retinene$_1$ in high concentration. This was accomplished by letting the mixture set in the dark. The pH of these solutions was not described as a function of time. They demonstrated that the material after setting in the dark for 60 minutes exhibited a rise in extinction coefficient of 2:1 over a similar sample without the added retinene$_1$. Both samples exhibited a peak absorption near 500 nm following the experiments. It is not clear why the reference sample showed any absorption at 500 nm if it was truly free of all native retinene$_1$.

With our current knowledge of the photosensitive materials found in the Outer Segments of the photoreceptor cells, it is not at all clear that these experiments demonstrated the recreation of the putative compound rhodopsin from totally chromophore free rhodopsin. Nor is it clear that the recreated material would act as a chromophore when converted into the liquid crystalline state. Several protocols can be described that will produce the same results as obtained by Wald, et. al. They basically involve the excitation of the chromophore present in the native rhodopsin into its excited but highly stable state followed by its gradual degradation back to its initial state over a period of 60 minutes (with or without the presence of additional retinene$_1$). The difference in extinction ratio can easily be accounted for by a small change in pH, see Matthews, et. al[115]. Whether such a change was observed was not discussed.

We now know the entire amino acid sequence of the putative rhodopsin. It is noteworthy that when these sequencing experiments were performed, there was no report of a residue containing a retinoid attached to an amino acid by a Schiff-base. No report could be found that even mentions a retinoid in the residue.

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[113]Wald, G. & Brown, P. (1950) The synthesis of rhodopsin from retinene$_1$. *Proc. Nat. Acad. Sci. USA*, vol. 36, pp 85-92

[114]Wald, G. & Hubbard, R. (1950) The synthesis of rhodopsin from Vitamin A$_1$. *Proc. Nat. Acad. Sci. USA*, vol. 36, pp 92-102

[115]Matthews, R. Hubbard, R. Brown, P. & Wald, G. (1963) Op. Cit.

5.5.2.3 The Rhodonines

The most important characteristics of the Rhodonines are not their hormonal properties, as in the case of the retinenes, but their electronic properties, especially when in the liquid crystalline state. These properties are not directly relatable to their relatives, the retinenes. They are more closely associated with another group of relatives, the oxonols. The oxonols consist of two oxygen atoms connected by a conjugated carbon chain. The oxonols and the phthaleins are members of the carboxylic acid family, a large well-known family of "dyes."

Because of the wide variety of features in complex molecules, naming them is more an art than a science. While both the family names mentioned above have historic roots, it would be useful to consider more rational names for new, but closely related, families based on their structure. To avoid addressing this problem directly, the tradename Rhodonine™ will be used in this work to describe the chromophores of biological vision. The specific systematic names assigned to this family by the Registry Services division of *Chemical Abstracts* are given below and in the appendices.

The conjugated chain of the oxonols may be relatively straight and considered a backbone or it may exist as a wandering path within a more stabilized molecule as it does in erythrosin, a phthalein. In the case of the relatively straight backbone, the material is described as an oxonol, dioxonol, trioxonol, etc. based on its level of conjugation.
When the oxonol backbone is stabilized by the addition of other, usually ring, structures, the new family is given a more descriptive name. In the case of the chromophores of biological vision, one choice would be to describe these molecules as retinienes to reflect their importance in the retina and their conjugated carbon backbone. However, the historical label retinenes has been used to describe a very similar family of monopolar materials with a conjugated carbon backbone that can be considered chromogens but not chromophores. In addition, the dipolar nature of the chromophores is not indicated.

A second name choice might be based on the carboxylic acid system as suggested in Mees. However, no simple name would be suggestive of the most important feature of this family. That feature is the variable length conjugated chain length between the polar atoms (as opposed to the overall conjugated chain length of the carbon atoms alone).

In photographic applications where the dyes are used in emulsions that integrate photons over long time periods (from manufacture until final development), it is extremely important to stabilize the molecules against thermal breakdown and thermal excitation. To aid in this, a sulphur atom is usually incorporated in the above structures. This is not necessary in vision since integration over a long time period is not employed, although thermal breakdown over time might account for why the retinoids in the disks are recycled. It may also be that the β-ionone ring provides a thermal stabilization function in the Rhodonines.

It is interesting to note the statement by Venkataraman that the carboxylated dyes are very effective in dyeing proteins such as cotton and wool[116]. More important, the acid form of the dyes will only **dye** wool and not cotton (wool being an animal protein and cotton being a plant protein). This would suggest the Rhodonines show a particular affinity for animal proteins such as opsin. The particular structure of the Rhodonines is presented in **Section 5.5.8.2.**

5.5.3 Concepts of a liquid crystal

Before achieving its recent commercial importance, the liquid crystalline state of matter has been a laboratory curiosity since at least 1888[117].

In recent times, many if not most biological materials have been recognized as liquid crystals. In the consumer electronics area, nearly every item sold incorporates a liquid crystal display device. The liquid crystalline state of matter exhibits characteristics not found anywhere else in science and engineering. The important and relevant characteristics include:

+ their ability to form monolayers on a substrate which exhibit unique optical properties.
+ their ability to transmit an excited state through the liquid crystal at speeds exceeding the molecular relaxation times of the individual molecules.
+ their great sensitivity to environmental conditions; temperature, pressure, acidity, etc.

The photographic community was one of the first to recognize the importance of the liquid crystal to their business. Considerable work in the area was undertaken before 1940. In photography, it was found that most sensitizing dyes were

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[116]Venkataraman, K. (1977) The Analytical Chemistry of Synthetic Dyes. NY: John Wiley & Sons, pg 6
[117]Blumstein, A. (1978) Liquid crystalline order in polymers. NY: Academic Press, pg. 3

most effective when they deposited themselves in a monolayer covering a major part of the surface if not all of the surface of a photographic grain of silver halide. It was quickly shown that no matter where a photon was absorbed by this monolayer, the resulting energy caused a valence change in an atom of silver found in a silver sulphide complex also located on the grain surface. In addition, it did not need to be a one for one interaction; in the case of long wavelength photons, it was possible to doubly excite the liquid crystal and transmit the sum of the energies to a single molecule of silver sulphide, resulting in the creation of an atom of free silver. This phenomenon allowed the introduction of photographic emulsions with a spectral cutoff wavelength at two or even three times the spectral cutoff wavelength of the underlying silver halide material.

As part of the above investigations, it was found that the organic dye molecules were most effective when they existed in a planar form that could be fitted together like dominoes, although they were usually not rectilinear dominoes.

5.5.3.1 Fundamentals

Brown and Wolken[118] have provided a comprehensive review of liquid crystals as of 1979. This is the appropriate time scale for beginning this discussion. They are able to discuss the fundamentals without becoming lost in the special conditions that have been so useful in consumer electronics applications.

The key feature is that a liquid crystal behaves like a liquid with respect to its mechanical properties and as a crystalline solid in its optical and electronic properties. Beyond that characterization, the individual materials exhibit a very great variety of characteristics. Many of the designations used are adjectives related to prototype members of each group, i.e., the Greek term smectic for soap, slime or grease-like and the term cholesteric for cholesterol-like. Liquid crystals existing in the absence of a solvent are labeled thermotropic. Lyotropic liquid crystals are dispersed in a solvent, frequently water. One of the most important material groups within the lyotropic group are the amphiphiles. The amphiphiles consist of an ionic group that is water soluble and an organic part that is insoluble in water. Lyotropic liquid crystals involving amphiphiles appear to be one of the foundation materials of biology. However, the distinction may become small between lyotropic materials containing water and thermotropic materials on the surface of an interface involving water. As early as 1933, Bernal[119] suggested that the living cell was actually a liquid crystal. The presence of liquid crystals in the animal body extends beyond the cells.

The liquid crystalline state is frequently recognized most simply by heating a thermotropic solid. If the solid transitions to a turbid system before becoming an isotropic liquid, the turbid system is most likely a liquid crystalline system. On cooling, the liquid may revert to the turbid system on the way to the solid phase. However, if heated above a molecular transition point, the material may not return to the turbid state on the way to becoming a solid.

Alternately, a lyotropic material can be dissolved in a solvent. As the solution becomes more concentrated, the isotropic characteristics of the solution may give way to a turbid medium before becoming a solid. It is this turbid state, usually occurring at greater than 10^{-2} M, that is characteristic of a liquid crystal of the lyotropic type. Further concentration may lead to precipitation through crystallization. The material may be quite sensitive to temperature. Many biological materials may be denatured if heated to an excessive temperature. These materials will no longer pass through the liquid crystalline phase as the temperature or concentration is varied. They have been permanently modified by the heat. The biologically active range of liquid crystals is therefore quite limited.

The study of liquid crystals rapidly becomes complex because both the thermotropic and lyotropic types are polymorphic. The lyotropic type exists in at least six phases according to Brown & Johnson. Materials of this type generally exhibit a molecular weight in the range of 250-500. Many of these materials are described as lipids, and frequently as phospholipids. On addition of water to a crystal composed of these materials, the molecular structure initially collapses to form a lamellar structure. Further dilution may result in additional structural changes before an isotropic solution is reached.

The lipids exhibit a unique property of forming mono- or bimolecular layers, depending on the nature of the surrounding material or interfaces:

+ At an air-water interface, they will interject themselves as a monolayer with the hydrophilic ends of the individual molecules closest to the water surface.

Typical: Simple laboratory demonstrations and surface tension experiments involving thin films

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[118]Brown, G. & Wolken, J. (1979) Liquid crystals and biological structures. NY: Academic Press
[119]Bernal, J. (1933) Liquid crystals and anisotropic melts. Trans. Faraday Soc. vol. 29, pg. 1082

+ When formed within water as a solvent, they will form a bilayer with the hydrophobic ends of the individual molecules adjacent to each other.

Typical: Formation of bilayers as found in cell membrane walls

+ When formed on a suitable substrate immersed in water, they will form a monolayer with the hydrophobic ends of the individual molecules adjacent to the substrate.

Typical: The coating of the protein substrate, Opsin, by the chromophore Rhodonine as it is released from the fluid filling the cavity between the RPE and the layer of photoreceptor Inner Segments.

These three cases encompass the situations found in animal vision.

The carotenoids are well known. Many of their properties are reviewed in Brown & Wolken[120]. However, many of their assumptions with regard to rhodopsin are not supported by this work. The monomers of Vitamin A formed by splitting carotene no longer exhibit the properties of carotenoids. Their absorption is limited to the ultraviolet because of their reduced length. Nor do they belong to the class of oxygen-containing derivatives labeled xanthophylls.

5.5.3.2 Spontaneous formation of liquid crystals

When materials susceptible to liquid crystal formation are brought into the proper regime either thermotropically or lyotropically, they will spontaneously form into their characteristic liquid crystalline phase. Blumstein discusses this capability with regard to the formation of microtubules of hemoglobin[121].

5.5.3.3 Change of density constant with excitation

One of the earliest observed properties of liquid crystals, dating from the 1960's, was that they changed density when excited. Local excitation frequently caused a bulge or apparent dislocation in a liquid crystalline film. An interesting ramification of this effect was noted by Baylor, et. al. in 1974[122]. They observed that a probe inserted into an outer segment of a photoreceptor was frequently ejected when the segment was subjected to a high intensity flash of light "as if strong light caused some movement or rapid volume change in the cone."

5.5.4 The (extended) Retinoid family viewed as a liquid crystal

It is generally believed that retinol achieves its pharmacological importance due to its stereo-chemistry. Because of its stereo-chemistry, it and many of its derivatives also exhibit an ability to form a liquid crystal, a physical form found widely in nature but poorly understood by the majority of researchers because of its only recent elucidation. Members of the retinol family are able to align themselves adjacent to each other in a structured form with unique chemical, physical and electrical (electronic) properties. These properties are only exhibited when the material is in the liquid crystalline state, i.e., usually in an aggregated state. Apparently these properties can also be exhibited if the material is in the presence of lyophilic colloids (particularly a gelatin). Under these conditions, a super molecule is formed which also exhibits these light absorption properties[123].

This last point is important since it has prevented many researchers from successfully determining the spectral characteristics of the visual dyes extracted from retinas for many years.

The stereo-chemistry of retinol is well known in the pharmacological literature. However, conversion of retinol into Rhodonine introduces a heavy atom at an asymmetrical location in the structure and the resulting molecules are distinctly non-planar.

5.5.4.1 Crystalline Structure of the Retinol Family using X-RAYS

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[120]Brown, G. & Wolken, J. (1979) Op. Cit. pp 63-65 and part II of Chap. 9
[121]Blumstein, A. (1978) Liqid crystalline order in polymers. NY: Academic Press, pp. 264-265
[122]Baylor, D. Hodgkin, A. & Lamb, T. (1974) The electrical response of turtle cones to flashes and steps of light. *J. Physiol*. vol. 242, pg 706
[123]The Chemistry of Synthetic Dyes and Pigments. (1965) Ed. by H. A. Lubs, NY: Hafner Publishing Co. for the American Chemical Society Monograph Series pg. 675

Kofler & Rubin have provided excellent examples of the x-ray patterns of a variety of retinoids, but not for the chromophores of vision[124]. The crystalline structure of the retinals has been determined by Gilardi, et. al.[125] and by Hamanaka, et. al[126]. This data is presented and reviewed in **Chapter 6**.

5.5.4.2 Typical Stacking Factor for Crystals

Scheibe[127] has presented the parameters related to the stacking arrangement of a cyanine compound when in the liquid crystal or *J-state*. The data is shown in Mees & James[128]. In his caricature of the situation at the cyanine-substrate interface, he suggests that each molecule of the structure has all of its polar atoms on one side of the conjugated backbone. It appears that this is an oversimplification based on what we now know of the stereometric structure of molecules. It appears more likely that the Rhodonine molecules are arranged as shown in **[Figure 5.5.12-3**] and that the various protrusions and indentations contribute to two situations. First, they may control the formation of liquid crystals containing only one species of chromophore. Second, they may play a significant role in the transport of the chromophores through the body.

5.5.4.3 The putative structural arrangement of chromophores

Combining the information available from X-ray studies and Scheibe (See **Chapter 6**), and the studies from electron-microscopy, (See **Section 4.5.1**), a potential arrangement of the chromophores of vision as deposited on the surface of a non-crystalline surface is shown in **Figure 5.5.4-1**. The assumption is that the non-crystalline surface is opsin. The precise shape of the individual molecules of this extruded surface is unknown. The liquid crystalline chromophore is a monolayer. It is shown bonded to the substrate by hydrogen bonds. These are shown as individual dots at the bottom ends of the individual chromophores. The bonding may not be as regular as shown primarily because of the nature of the substrate surface. The dimensions shown are from the Sections referenced above.

Although the physical thickness of the liquid crystal is only about 15 Angstrom, it is quantum-mechanically resonant at the wavelength of the incident photons due to its slow-wave electronic structure. The spatial profile of the anisotropic absorption spectrum is shown without dimensions. It is dependent on the absorption cross section of the liquid crystalline film. The array factor for this array cannot be determined easily using conventional antenna theory because of its sub-wavelength dimensions and other currently unknown parameters.

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[124]Kofler, M. & Rubin, S. (1960) Op. Cit. pp 329-333
[125]Gilardi, R. Sperling, W. Karle, I. & Karle, J. (1971) Crystal structure of the visual chromophores, 11-cis and all-trans retinal. Nature, 232:187-188
[126]Hamanaka, T. Mitsui, T. Ashida, T. & Kakudo, M. (1972) The crystal structure of all-trans-retinal$_1$, *Acta Cryst*. vol. B28, pg 214-222
[127]Scheibe, K. (1948) Kollid-Z. 52: 283
[128]Mees, C. & James, T. (1966) The Theory of the Photographic Process, 3rd ed. NY: Macmillan. pg 245

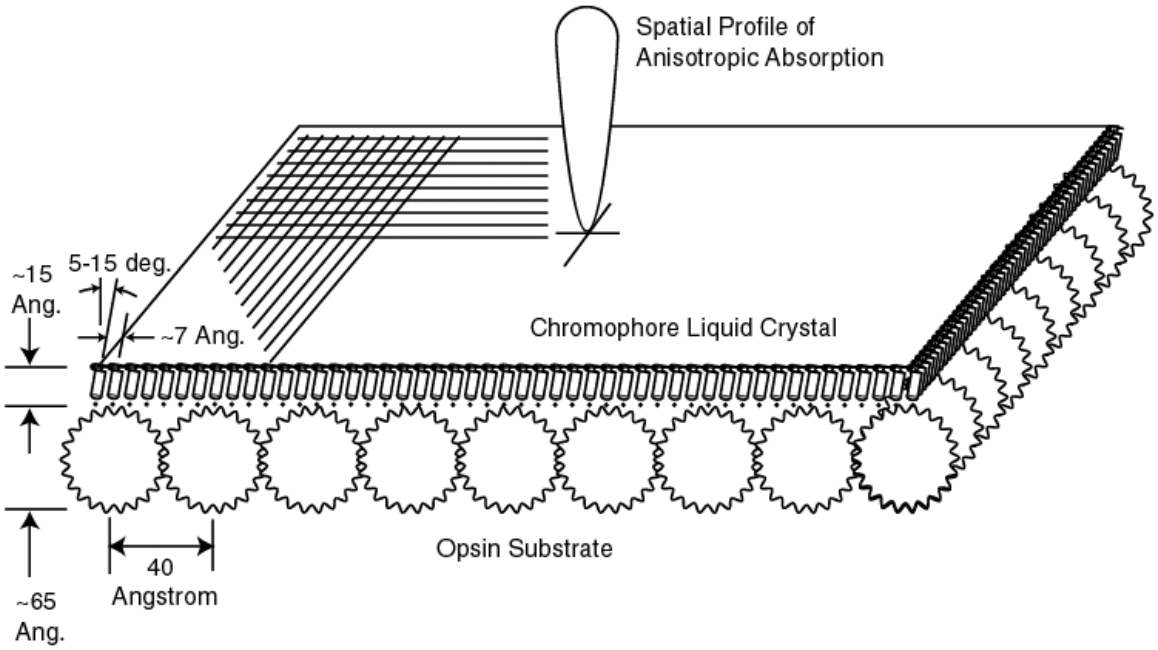

Figure 5.5.4-1 Putative arrangement of a liquid crystalline chromophore on the surface of the opsin substrate circa 2000. The individual molecules are arranged with their long axis nearly perpendicular to the surface of the substrate. The angle of tilt of the array is estimated. It is not documented in both directions and may differ slightly from a straight line drawn between the two auxochromes of the molecules. The pitch and dimensions of the substrate molecules are from Corliss and from Nilsson. The hydrogen bonds between the chromophores and the substrate are shown as dots. The anisotropic absorption profile of the chromophore is illustrative due to the many quantum-mechanical factors in determining it precisely.

5.5.5 Minimal Requirements for an effective Chromophore

The basic requirement for any organic molecule to be an effective absorber of light, i.e., a chromophore, is that it be able to exist in at least two states of energy. One state being the “ground state” of relatively low absolute energy and the second, an excited state--not more than two electron-volts higher than the ground state-- to which it may be raised by the absorption of energy supplied by light of some region of the visible spectrum. Organic dye molecules which satisfy these requirements all contain one or more "chromophoric" systems. Those of greatest importance in a related field, photography, are defined in **Figure 5.5.5-1**. They consist of the Amidinium-ion, the Carboxyl-ion and the Amidic systems. Each of these systems is characterized by the presence of two polar atoms, N or O, elements which are capable of existing in two adjacent states of covalency. These must be joined by a conjugated chain of (usually) carbon atoms with alternating double and single bonds. Under these conditions, it is possible to write each of these structures in two ways which differ only in the way the electrons are disposed, not in the location of any of the atoms--see the (a) and (b) forms of each system in the figure. In all three systems, the vinylene residue (a pair of conjugated methine residues as a minimum) must be taken an integral number of times. Furthermore, in these simple and relatively linear conjugated systems, each vinylene group contributes 0.1 ±0.050 microns to the location in the radiation spectrum of the peak absorption of the ultimate chromophores.

5.5.6 Modification of a retinoid to become a chromophore–a Rhodonine

Based on the preceding paragraphs, it is possible to look at retinol in a different light. **Figure 5.5.6-1** shows retinol and its aldehyde derivative in several equivalent diagrams. Note the ease with which these forms can be changed into a chromophore. Retinol can be converted into an effective chromophore through the substitution of a terminating polar atom at Z in any of the retinol diagrams. Retinal, can be converted into an effective chromophore through the substitution of a connecting polar atom at Z in any of the retinal diagrams. [needs editing to add Z’s or an additional figure.] In either case, the molecule will exhibit a significant absorption spectrum in a region of interest to the study of vision. An explanation is in order for the notation in the bottom row of forms. The conjugate form is maintained across the linear chain to the ionone ring interface if the ring closes onto the leftmost carbon in the leftmost vinylene group. This is only a diagrammatic problem when it is desired to stress the vinylene conjugation in a "tight" form. Of more interest later will be the valuable function performed by the presence of the ring.

Bayliss was the first to describe the spectral properties of the conjugated molecule[129] in significant detail in 1952; considerably after wald’s conceptual pronouncements concerning the non-conjugated retinenes. Bayliss counted the number of conjugated bonds, not the number of conjugated atoms in determining the parameter, n..

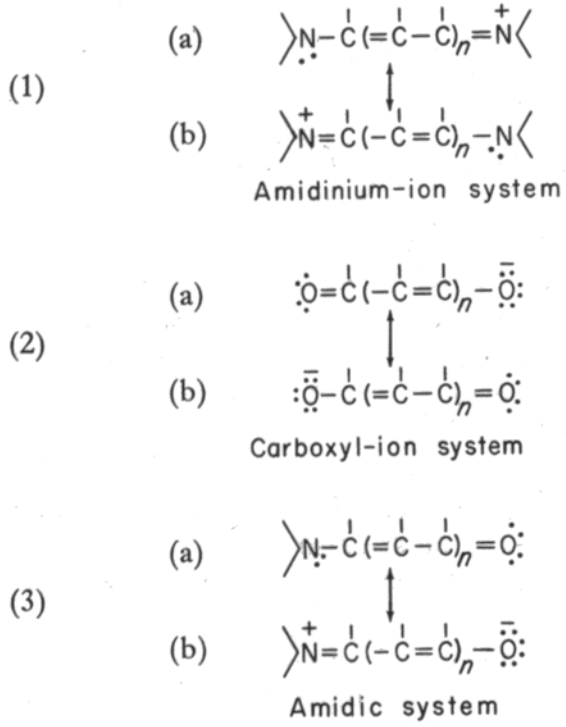

Figure 5.5.5-1 Chromophoric systems used in photography. It will be shown that the carboxylic system is also used in vision.

Before proceeding, it is important to note the additional requirements that must be met by chemicals defined as chromophores on the basis of their structure. To be effective, it is also necessary that they be in the liquid crystalline state of matter. And for maximum effectiveness, they must be associated with a suitable structure that can provide a de-excitation path. This is particularly important in the case of the carboxyl-ion system. Oxygen exhibits a unique atomic structure. Because of this structure, it will not decay thermally after excitation (in a finite

[129]Bayliss, N. (1952) The free-electron approximation for conjugated compounds *Quart Rev Chem Soc* vol 6, pp 319-339

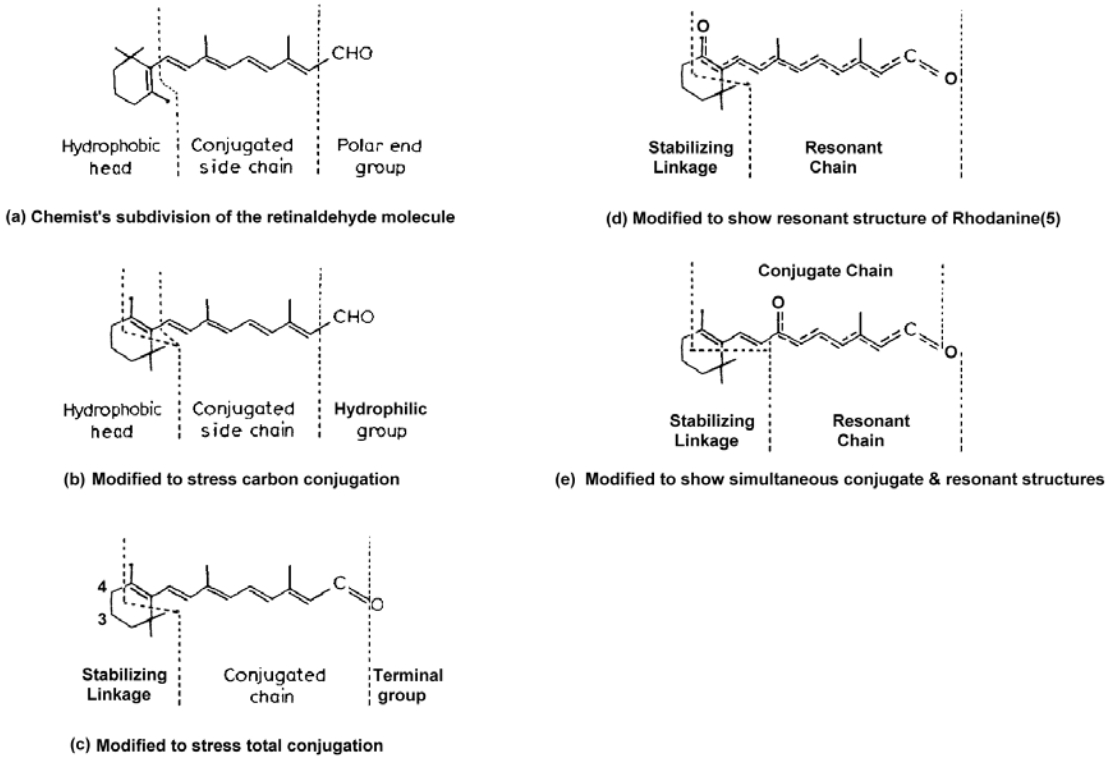

Figure 5.5.6-1 Conversion of the retinenes into chromophores. (a); Retinaldehyde as conventionally displayed. (b); presentation rotated to stress length of conjugated carbon chain. (c); presentation modified to stress total conjugation. (d); presentation modified to illustrate combined conjugation and electronic resonance over the maximum length of the molecule. (e); presentation modified to show the potential separation of the conjugated structure from the electronically resonant structure. See text for details

and useful time interval). Therefore, the material of the correct chemistry and state of matter must also be associated with a special contact or substrate that encourages de-excitation. In the absence of this de-excitation, the material will absorb light efficiently until all of the unpaired electrons have been excited. At that point, the material will be transparent to additional radiation within its normal absorption band.

5.5.6.1 Potential of adding nitrogen to retinol

Nitrogen can be added to retinol through substitution or addition. As noted in **Section 5.5.3.1**, the proposal that retinol is combined with opsin, via a Schiff base (through a substitution), to form rhodopsin has been the baseline of conventional wisdom since the 1950's.

There was no published discussion at that time of the rules of dye chemistry and how the purported material rhodopsin would exhibit compliance with these rules. As indicated above, the most widely used class of dyes incorporates two polar atoms connected to each other by a conjugated carbon chain, and generally named a “chromophoric system.” The presence of a conjugated carbon chain is required as a minimum in nearly all organic material exhibiting a “color” in the visual band. Lack of such a conjugated chain leads to a colorless material. The proteins are such an unconjugated and inherently colorless family of materials. It was proposed that rhodopsin was formed by a condensation between an amide of opsin and the hydroxyl of retinol located at the C-15 position. This Schiff base effectively replaced the one oxygen of retinol with a nitrogen and interconnected the conjugated carbon chain of the retinoid with a non-conjugated carbon chain of the protein. From a dye chemistry point of view, this accomplished very little. The resultant molecule continues to contain only one polar group associated with the conjugated carbon chain and the conjugated chain was not lengthened. Although this material may exhibit a number of absorption peaks in the ultraviolet due to both the protein and retinoid moieties, there is no reason to believe it will exhibit any absorption peak in the visual region and no such

peak has ever been found.

Where visible absorption peaks have been found in natural photoreceptor material, no NMR or X-ray crystallographic data has been put forward to demonstrate the presence of a retinene in the material (See Chapter 7). It is proposed here that any such tests will demonstrate the presence of a Rhodonine instead. Rhodonine contains two polar groups separated by a conjugated carbon chain. It is further proposed that opsin is only present among *Chordata* as a completely passive substrate. Its association with the liquid crystalline Rhodonine is via a weak hydrogen bond that does not change the electronic configuration of the Rhodonines.

> See the new material (ca. 2016) in **Section 7.1.1.1.4** of Chapter 7 relating to the X-ray crysallographic data related to the transport of the retinenes and the recently defined class or retinines (with two i's to describe the diol branch of the retinoid family).

It is possible to add nitrogen to, rather than substitute nitrogen in, a retinene molecule without replacing the oxygen as envisioned in the Schiff base formulation. In this case, the nitrogen could be introduced in place of either a methyl group or possibly a hydrogen. However, to preserve the proposed requirement that conjugation be maintained between the two polar atoms, the nitrogen can only be introduced at certain locations. It can be connected to C-5, C-7, C-9, C-11 or C-13 (and possibly C-3) using Karrer's notation.

No chromophore of biological vision has been found or proposed where an oxygen or nitrogen atom was associated with the C-13 or C-3 positions. The C-11 position will be shown to be the source of ultraviolet vision in many animals.

Nitrogen is not paramagnetic and does not offer the unique triplet state configuration that oxygen does (See **Chapter 6**). All of the data collected in conjunction with the retinoid binding proteins appears to point away from nitrogen as a participant in the chromophores of vision (See **Chapter 7**).

5.5.7 Predicted chromophores derived from natural retinoids

Reviewing the photographic literature, it is possible to estimate the location of peak spectral absorption of the forms shown in **Figure 5.5.7-1** from Mees & James. (Note, the absorption spectrum of the molecules alone is not necessarily the same as the net absorption spectrum of the molecules combined with another quantum-mechanical system.) To a first approximation, the wavelength of peak absorption under appropriate conditions is given as $\lambda_p = n(0.1) + x + y$ microns where the last two terms are determined by the exact nature of the end auxochromes and how tightly they are bound to the chromophoric system. N is the conjugation number of the vinylene series. Platt[130] (also in Radiation Biology, 1956) develops the theoretical foundation for the first term and clearly shows that the quantum-mechanical absorption in this situation is at a wavelength 230 times longer than the length of the individual conjugated chain, L, i.e., $\lambda_p = 500L + x + y$. He also provides us the effective velocity of electrons carrying the excitation within the structure as c/230 where c is the speed of light. In the 1956 paper, Platt[131] develops the x and y parameters in considerable theoretical detail and compares them with a broad range of chromophores of interest here, particularly the cyanines and merocyanines. He provides specific x & y values for end groups similar to those shown in the figure from Mees & James. In some cases, Platt's terminology refers the the change in wavelength with the number of bonds (500 Angstrom/bond) and in the detailed discussions, he refers to the change in wavelength with the number of vinyl groups (1000 Angstrom/group). That of Mees & James always refers to the number of vinylene groups and considers any remaining methine group a portion of a bridge structure.

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[130]Platt, J. (1964) Systematics of the electronic spectra of conjugated molecules. NY: John Wiley pp. 71-123

[131]Platt, J. (1956) Wavelength Formulas and Configuration Interaction in Brooker Dyes and Chain Molecules *J Chem Physics* vol 25, pp 80-105

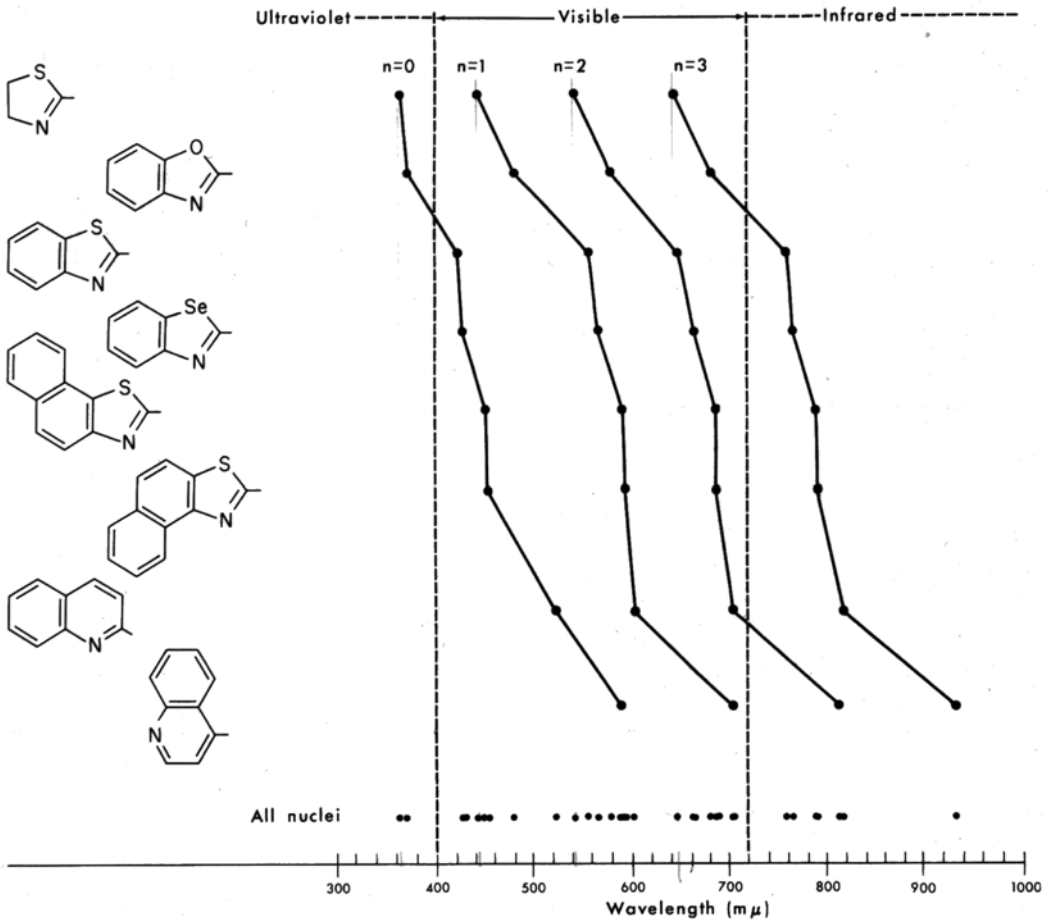

Figure 5.5.7-1 CR Peak spectral absorption of various dyes as a function of the degree of conjugation. For n = 0, there is only a methine bridge but no conjugated structure. For n ≥1, the spacing is proportional to the number of vinylene residues. From Mees, 1966.

It should be noted that the retinol-based and the retinal-based systems represent two different situations; the retinal system requires that it be associated with another atom or molecule (at least a hydrogen atom) through ionic or covalent bonding. However, the retinol system is complete unto itself; by merely substituting a polar auxochrome for Z in the molecule, it will exhibit a peak absorption in the desired spectrum under the appropriate conditions.

The only data in the vision literature assumes that retinol or retinal are themselves the chromophores in the photodetection process. There is extensive literature based on the assumption that retinal is the basic chromophore even though it does not exhibit the necessary properties of a chromophore in the visual region of the spectrum. Its only recorded spectra have peaks in the ultraviolet region which is typical for basic conjugated organics. The assumption in the literature has been that the process of photodetection involves a very circuitous series of steps involving isomerism. This assumption has persisted even after a significant energy deficit was recognized in this process.

Ignoring the isomerism hypothesis for the moment, let us continue to explore the options available if the actual chromophores are based on the discussion above. For the moment, the possible chromophores in the visual process will be defined as Rhodonines; chromophores with a chromogen from the retinol family and exhibiting two auxochromes separated by a conjugated vinylene chain. It has been postulated that the auxochromes consist of a single atom of either oxygen or nitrogen. The presence of two auxochromes of either of two atoms leaves three possible situations, a pair of oxygen auxochromes, a pair of nitrogen auxochromes or a set of one oxygen and one nitrogen auxochrome. Since the conjugate vinylene system is a resonant form, there is no first order difference between putting the oxygen on one end of the chain or the other. There might be a second order effect due to the remainder of the structure of the overall molecule. As indicated, if two oxygen atoms are used, the structure exhibits a net negative electrical charge and the molecule is a member of the Carboxyl-ion system. If two nitrogen atoms are used, the structure exhibits a net positive electrical charge and is a member of the Amidinium-ion system. If a mixed set of auxochromes is used, the molecule is electrically neutral overall (although polarized) and a member of the Amidic system.

It is now appropriate to explore whether an ionic or neutral chromophore is most likely or most desirable. Most discussion in the literature assumes that retinal is chemically bound to a protein opsin.

The only hypothesis in the literature that goes beyond that elementary assumption is the Schiff base hypothesis. This hypothesis dates from the 1950's and proposes that the =O of retinal participates in a reaction with an amino group of opsin to replace the =O with a =N-Opsin[132]. This is a relatively low energy transformation and does result in a stable structure if there is at least one aryl group on either the carbon or the nitrogen (March, pg. 667 in 1968) XXX. Since the presence of an aryl group is very unlikely in a biological environment and replacing the =O with a =N– doesn't accomplish anything in achieving a complete chromophore, it is not likely to be a relevant process.

An alternate hypothesis could be put forward that the second auxochrome is attached to the chromogen at the opposite end of the conjugate chain from the =O or –OH group. This would involve the replacement of a hydrogen or a methyl group by either an =O or =N– in the case of retinol or an –O– or –N= in the case of retinal through substitution. The first case results in a neutral Amidic system. The other three cases are ionic and provide for attachment of a protein or other component. This hypothesis is also compatible with the attachment point varying along the conjugate chain; resulting in a family of chromophores with absorption spectra in the visual region as is shown later.

[132]Hubbard, R. et. al. (1966) Op. Cit.

This hypothesis results in a matrix of possibilities depending on the chromogen, retinol or retinal;

CHROMOGEN	RETINOL	RETINAL
SUBSTITUENT	=O	–O–
	=N–	–N=

Each of these configurations offers the capability of providing an appropriate chromophore under the appropriate conditions.

5.5.8 Stereo Chemistry of effective chromophores–leading to the Rhodonines

Turning again to the related field of photography, a great deal has been learned about the sensitization of silver halide emulsions with dyes similar to what has been defined above; although generally, more complicated structures are used commercially to achieve broadband absorption with only one dye. The important characteristic of all of these dyes is there stereo-chemistry and more specifically their ability to form liquid crystals which are then adsorbed to a surface. These materials begin to exhibit a unique absorption region at long wavelengths when they are dissolved in a solute at relatively high concentrations, typically approaching 0.01 M. When the concentration reaches this level, the viscosity increases significantly until a rigid gel is formed. When the chromophores begin to form a gel, they typically fluoresce (depending on the solvent) at the same or nearly the same wavelength as the new absorption peak.

Under the microscope, the dye is seen to form long filamentary structures in the solute. If the dye is allowed to aggregate on a surface, it will form a "liquid crystalline" structure. This structure will exhibit the unique absorption region at long wavelengths and may or may not fluoresce depending on other conditions related to the liquid crystal and its relation to the substrate. **Appendix F** contains additional information on the extraction & characterization of the Rhodonines.

5.5.8.1 Related physical structures

The detailed geometry of the liquid crystal formed by the Rhodonine family has not been documented to date. However, **Figure 5.5.8-2** illustrates the geometries assumed by related chromophores[133]. **(a)** shows the configuration assumed by 2,2' cyanine when formed as a

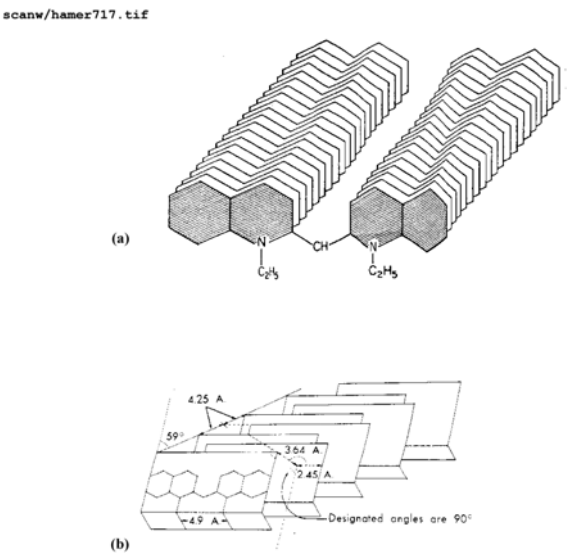

Figure 5.5.8-1 Arrangement of chromophore molecules in the J- or liquid crystalline state of matter on the surface of a substrate. From Scheibe. (a) in Hamer, 1964. (b) in Mees & James, 1966. The rules of perspective used to draw (a) originally appear to be faulty.

[133]Scheibe, K. (1948) Op. Cit.

liquid crystalline film on water[134]. **(b)** shows the same liquid crystalline configuration in more detail[135]. Note the distance between the two Nitrogen atoms which form the dipole of the molecule.

In these and similar chromophores, the physical distance between the auxochromes is much less than the physical distance normally associated with the wavelength of the exciting photon. However, as in many antenna types, it is not the physical distance that is significant; it is the effective distance. If the overall molecular structure is resonant at the same frequency as the incident photon, coupling is achieved and absorption is highly probable. Dewar has defined the "slow wave" structure along the conjugate chains of the liquid crystalline structure[136]. In his analysis, the electrons in the energy bands move along the conjugated carbon chain at a much slower speed than that of the photons in free space (by about 250:1). More recently, Platt has given this ratio as 230:1[137]. This effect, though greater in the magnitude of the ratio, is not unlike the situation in most "loaded" radio antennas where the physical antenna length is shorter than the nominal length required based on the wavelength of the signal to be received. This effect causes the peak absorption wavelength to be some 250 times greater than the length of the chromophore itself.

5.5.8.2 The Rhodonines in stick form

It is proposed here that it is much easier for a chromophore of the Carboxyl type to aggregate than those of the Amidic or Amidinium types, particularly if the Carboxyl ion is completed by the single hydrogen associated with the alcohol form of the chromogen; i.e., the chromophore itself is a compact standalone molecule. Based on this hypothesis, **Figure 5.5.8-2** illustrates the four chromophores of Rhodonine based on Vitamin A1. The stereo-chemistry of these four chromophores is almost identical. They are each able to aggregate into a liquid crystal--and may be able to intermix in a single liquid crystal. For the moment, it will be assumed that they do not normally intermix on a given surface but as in many organic molecules will seek out their own kind in order to aggregate on a surface. The top frame shows a retinene, the standard description of a molecule containing an oxygen atom. The other three frames show retinines, the terminology relating to a molecule containing two oxygen atoms that are in a conjugate configuration. The second “i” is indicative of their diol form. The ending of -ine for these structural forms leads to the designation of the chromophores of vision as rhodonine() where the brackets include the carbon number that the second oxygen atom is associated with. It is this oxygen that is involved in the formation of an ester within the *holo*-SRBP form used to transport the retinine to the RPE of the retina (**Section 7.1**).

[134]Scheibe, K. (1964) Hindered Internal Rotation in Cyanine Dyes *In* Hamer, F. & Weissberger, A. *eds*. The chemistry of heterocyclic compounds. New York: Interscience
[135]Mees, C. & James, T. (1966) The Theory of the Photographic Process, 3rd ed. NY: Macmillan
[136]Dewar, M. (1950) Colour and constitution. I. Basic dyes. *J Chem Soc* pp 2329-2334
[137]Platt, J. (1964) Systematics of the electronic spectra of conjugated molecules. NY: John Wiley & Sons Paper #20

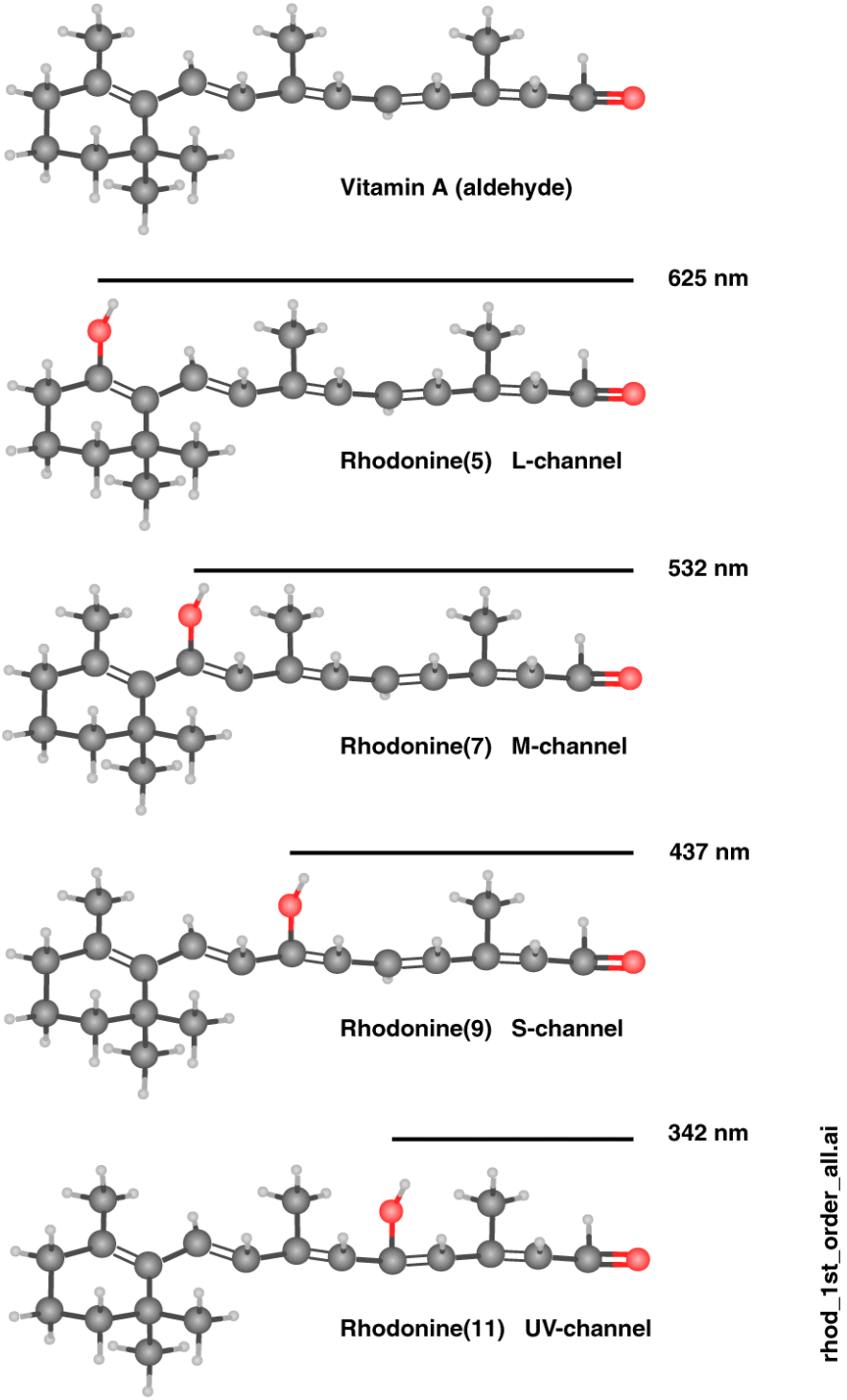

Figure 5.5.8-2 The proposed (**1st order**) chromophores of animal vision, the Rhodonines, based on Vitamin A1. Retinal (Vitamin A1 aldehyde) is shown for reference. Each chromophore is shown as a *planar* molecule in its neutral form. The oxygen atoms are shown in red. The conjugation critical to absorption in the visual region of the spectrum is shown by the alternating single and double bonds. The horizontal line above each molecule shows the relative length of its resonant structure. See text.

5.5.8.3 The Rhodonines in 3D

This paragraph was written based on the 3D forms constructed from the 2D Jmol files of the Royal Society of Chemistry. Unfortunately, these files are generally unreliable with respect to the 3D form of molecules. See the next subsection.

The recent expansion of the chemical libraries of individual compounds based on 3D computerized modeling and visualization has resulted in great progress in understanding. The field remains immature, and the files of various families of chemicals are not necessarily error free.

The development of molecular crystallography, although a demanding technique at present, indicates the actual molecular geometry of the retinines is not necessarily consistent with the computational structure displayed in most 3D databases. See **Section 7.1.3** for more recent representations of the retinine molecules.

In fact, the use of different computer software programs and different visualizer packages can result in different 3D presentations. Several different organizations have begun to maintain repositories of 3D files of different molecular families. The archivists generally accept voluntary contributions of data files, but when asked to defend the accuracy of their archives, deny responsibility for the correctness of these files. The problems in this area have been discussed more completely in this authors work on taste and smell as documented in Chapter 8 of "The Neuron and Neural System." The location of the reference point, 0,0,0 for these programs does not correspond to one of the atoms or to a point along the centerline of the aliphatic portion of the molecules.

Figure 5.5.8-3 illustrates the 3D representation of all-*trans* retinal (CSID 4444397), also known as Vitamin A aldehyde in the above figure, using the DS3.5 visualizer from Accelrys. By highlighting individual atoms, and a variety of structures, in these molecules the precise distance between them is given to at least four digit accuracy. When these measurements are combined with the theory of Platt reported in 1956 (and reproduced in his compendium of 1964) , the peak spectral response of the specific molecule can be determined theoretically.

Care must be observed here. Platt developed the relationship between distance between the chromophores of a molecule and the peak wavelength of absorption, λ, based on the classical absorption frequency of a dipole antenna of length (L) as $\lambda = 460L$ and then rounded the equation to $\lambda = 500L$ for convenience when lecturing.

He notes within his perception of accuracy, "The linear relation between wave length and molecular length is far from exact in the polyenes, but it helps us understand the approximately constant wavelength shifts introduced by adding additional units to a conjugated chain."

A potential correction is to consider the projection of the length L on to the axis of the molecule in order to match the projection to the electronic length of the antenna, a correction commonly employed in antenna work. Another potential correction involves the foreshortening of the molecule due to the puckering of the aromatic ring. Finally, a correction is frequently made for the velocity of the electrons moving along a pair of parallel wires compared to the free space velocity of an electromagnetic wave.

Finally, the speed of light within a liquid crystal does not equal the speed of light in vacuum.

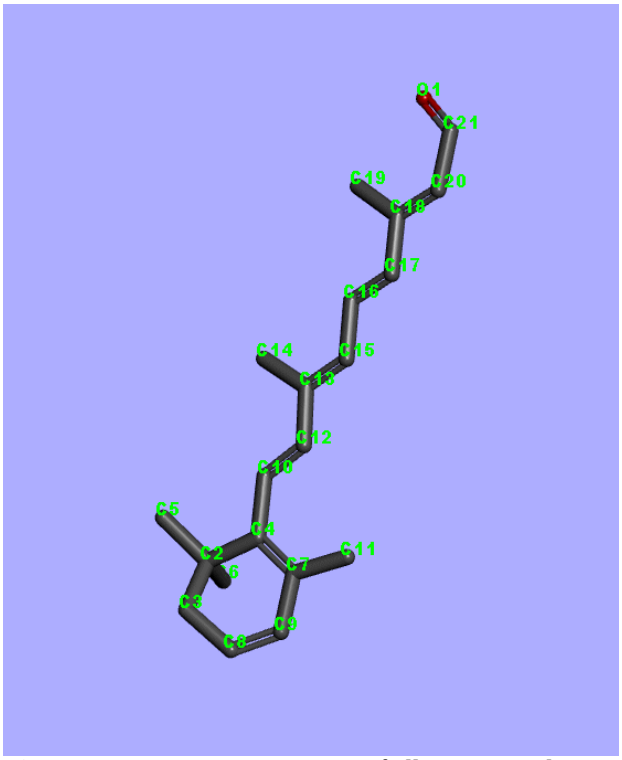

Figure 5.5.8-3 3D representation of all trans retinal using the DS 3.5 visualizer. The visualizer provides the capability of rotating this molecule to any orientation and measuring various distances and angles based on the atom numbering system unique to the 3D modeling technique standardized through the American Chemical Society. Note the numbering associated with the ring structure. The notation rules are documented in their library known as ChemSpider. The molecule has a CSID of 444397.

A caution is important for those attempting to search the various archives and libraries of 3D representations of the polyene molecules. Many of the search engines perform an "or" search rather than an "and" search when you enter multiple words.

In the case of the retinals, the molecule labeled CS 553582 is not all *trans* retinal, it includes a *cis* twist at carbon 9 (using the conventional atom numbering system in these remarks). The molecule labeled CS 4940758 is not all *trans* retinal, it includes two distinct *cis* twists.

Focusing on the retinols for the moment, it is noted that molecule labeled CS ID4940721 is not all *trans* retinol, it is 3,4-didehydroretinol with a twist at C6, or (6cis)-3,4-didehydroretinol and the OH is out of plane.

The molecule labeled CS 8192325 is not all *trans* retinol, it is 3,4-didehydroretinol with a twist at C9 or (9cis)-3,4-didehydroretinol.

The molecule labeled CS 393012 is not all *trans* retinol. It includes at least two twists in its structure.

Different visualizers frequently show minor differences in the distances between the position of the oxygen (or nitrogen) atoms and their carbon neighbors. There are also frequently discrepancies between the locations of the hydrogen atoms between visualizers, although these differences are not important here.

The values of L corresponding to the 2D configurations in the earlier figure, have not been calculated precisely for the equivalent 3D configurations due to the requirement to reload the positions of all of the atoms into the computerized position modeling software. An initial attempt to do this using different modeling software and visualizer packages gave results that could not be interpreted satisfactorily.

5.5.8.3.1 The rhodonines in 3D based on x-ray crystallography

Since the 1990's methods of crystallizing the retinenes have appeared that allow their examination in great detail by x-ray crystallography. This technique establishes the locations of the atoms within even a complex protein molecule to precisions not previously possible. These techniques are discussed in **Section 7.1.1.4**. They establish the fact that the retinenes are not planar molecules when in the liquid crystalline state, the state in which they are crystallized. In that section, the dihedral angle between C5-C6 and C7-C8 is between 54.8 and 62 degrees depending on the investigators. These investigators both described the double bond within the β-ionone ring as C5-C6 and the first double bond along the aliphatic carbon chain external to the ring as C7-C8. By this labeling, the oxygen associated with C5 in the long wavelength sensitive rhodonine(5) is significantly closer to the oxygen associated with C15 than calculating based on the 2D ball and stick model of a planar molecule. The result is a long wavelength chromophore with a peak sensitivity at a shorter wavelength than 625 nm as used in the quartet used in this work previously for the peak sensitivities of the chromophores., 342, 435, 532, 625 nm. The 625 nm value is reduced to be more in line with the 610 nm value proposed by Thornton. (**Section 5.5.10.3**).

> *To avoid rewriting a large portion of this work, the 625 nm value will be considered as the 1st order value as computed using the technique of Platt. The 610 nm value will be considered a 2nd order (more precise) value based on the photometric measurements of Thornton* (**Section 17.2**) *and the crystallographic measurements of both Cowan and Calderone et al.* (**Section 5.5.8.4.1**). The computational technique of Platt still applies but involves a more complicated geometry that must be used in the calculations.

Figure 5.5.8-4 shows the rhodonines in the more appropriate *non-planar* configuration determined by x-ray crystallography. The chromophores in this configuration will be described as the 2nd order chromophores of rhodonine. The aliphatic portions of the molecules remain unchanged. However, the dihedral angle between the aromatic and aliphatic portions of the molecules is now significant, in agreement with the above paragraph.

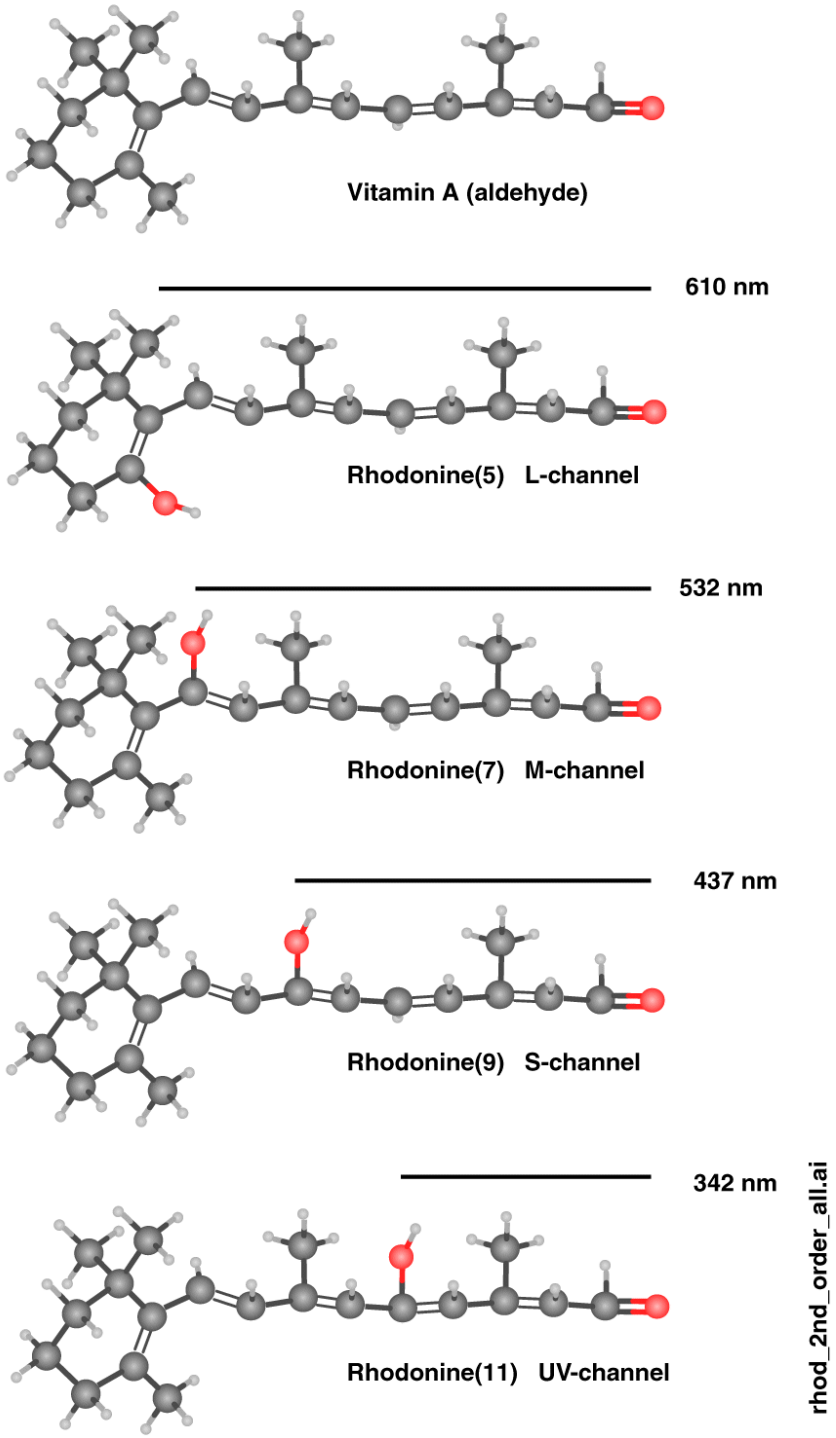

Figure 5.5.8-4 The proposed (**2nd order**) chromophores of animal vision, the Rhodonines, based on Vitamin A1. Retinal (Vitamin A1 aldehyde) is shown for reference. Each chromophore is shown as a *non–planar* molecule in its neutral form. The oxygen atoms are shown in red. The conjugation critical to absorption in the visual region of the spectrum is shown by the alternating single and double bonds. The horizontal line above each molecule shows the effective length of its resonant structure. See text.

5.5.8.4 Visualizing the (other) Rhodonines in 3D

The determination of the precise geometry of the Rhodonines when in the liquid crystalline state is currently a state-of-the-art task. One method, computational chemistry, has proven to be poorly documented and the archives (such as that maintained by the Royal Society of Chemistry, RSC) have been found to be very unreliable and inadequately curated. To date, the RSC has acted as a database collection point without requiring identification of the contributor. The rules employed in computational chemistry remain in a state of development and must be examined in more detail than provided by the RSC.

> It appears the RSC is abandoning their efforts employing their earlier 2D and 3D molecular representations in favor of a new NGL viewer and a curated database. The announcement appears at the following URL;
>
> http://www.rcsb.org/pdb/news.do?year=2016&article=579a47754905dd2004193027&feature=true
>
> Quoting the above site, "These pages now offer summaries for the peptide-like antibiotic and inhibitor molecules described in the wwPDB Biologically Interesting molecule Reference Dictionary (BIRD), including BIRD class, compound details, and description."
>
> Quoting again as of April 2017, "For ongoing technical updates, visit http://status.rcsb.org or subscribe to our Status Update mailing list."
>
> It is now necessary to search the BIRD dictionary for the retinol family and any modifications of that molecule currently entered into that database.

The second method, X-ray crystallography, has been explored by a much smaller group of investigators who have taken great care to describe their protocols.

5.5.8.4.1 X-ray crystallography applied to the Rhodonines INITIAL

Until the papers of Cowan[138] and of Calderone et al[139]., the textbook descriptions of the retinenes, and presumably the retinines, showed the molecules as conjugated carbinols with the methine groups at C5, C7, C9 & C11 all aligned on one side of the carbon chain. This configuration is shown in **Figure 5.5.8-2** above. This is the confirmation portrayed in textbooks since the 1940's. This configuration has been used to calculate the **1st Order** spectral properties of these materials, with peak absorptions near 343, 437, 532 & 625 nm. With the recent ability to grow liquid crystalline masses of these materials, it has been shown the form of these materials is different, and calls for a global change in textbooks portraying the retinenes (and presumably the retinines). **Figure 5.5.8-4** shows the actual conformations determined for these materials. This configuration has been used to calculate the **2st Order** spectral properties of these materials, with peak absorptions near 343, 437, 532 & *610* nm. Both the 1st and 2nd order peaks used the calculation protocol of Platt (1956, **Section xxx**). The experimental literature suggests the long wavelength peak ranges between 600 and 615 nm (**Section 17.2.3.1**).

> *Most of the figures in the following sections were drawn on the 1st Order assumption. They may need to be tailored more carefully once the precise peak of the long wavelength sensory receptor is determined by experiment. For now, the graphics will remain as drawn but the text will reflect a revised peak at 610 nm.*

[xxx discuss effect of pH given in Calderone, pg 848]

5.5.8.4.2 Computational chemistry applied to the Rhodonines EDIT

Section 8.4.1.2.3 describes the poor state of the field of computational chemistry when applied to organic molecules of the complexity of the retinenes and retinines. Because of this situation, this section will not be expanded more than it is.

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[138]Cowan, S. Newcomer, M. & Jones, T. (1990) Crystallographic Refinement of Human Serum Retinol Binding Protein at 2A Resolution *PROTEINS: Struc Func Gen* vol 8, pp 44-61

[139]Calderone, V. Berni, R. & Zanotti, G. (2003) High-resolution Structures of Retinol-binding Protein in Complex with Retinol: pH-induced Protein Structural Changes in the Crystal State *J Mol Biol* vol 329, pp 841–850

Herraez has provided a manual for applying the rules of 3D molecular imaging in the program Jmol to the preparation of 3D visualizations of an arbitrary molecule[140]. The book cited is very helpful. However it is translated from Spanish and is only an introduction. There was a supplement in 2011. Volume 2 had not appeared as of July 2014. Gaining competence in using Jmol requires repetitive implementation of the procedure. A user should search the internet for some calculation templates to simplify the creation of new molecular structures. A new package labeled the Virtual Molecular Model Kit (VMK) has recently appeared along with a monthly application oriented journal[141]. This material is designed for educators. A promising but immature program after five years is Avogadro[142,143]. An initial comparison of the Jmol files for Rhodonine(5)_4444397 gave different d-values of 11.181 Angstrom for the DS 3.5 visualizer and 10.684 Angstrom for Avogadro visualizer (a 5% difference, which is very large). Avogadro did not show the double bonds of retinal, nor did its tabulated bond lengths reflect the presence of double bonds. Avogadro is not a usable program as of July 2014.

The differences in the calculated distances of various visualizer programs leaves the community with the challenge to define which program is correct, or what mathematical formulation underlying the operations of these visualizers is correct.

5.5.8.5 Readdressing the geometry of the liquid crystalline chromophores and substrates REWRITE

[xxx rewrite to include x-ray crystallography]
With the advent of new investigative techniques such as the atomic beam microscope and advanced computer modeling of molecules, it appears to be appropriate to reconfirm many of the dimensions shown in **Figure 5.5.4-1**. New information about the structure and chemistry of the outer segments requires the protocols be carefully written. As an example, separating the disks by using a detergent generally destroys the liquid crystalline array of chromophores and removes the chromophores from the opsin substrates.

The primary goal of the following experiments is to measure the physical separation of the oxygen orbitals of each of four types of chromophores attached to disk substrates. Secondary goals include measuring the pitch of the reticulation of the chromophores in two major (probably orthogonal) directions and the angles between the individual molecules of the chromophores relative to the surface of the underlying substrates.

A potential approach to measuring the dimensions of interest is to use freeze fracturing to expose a fresh surface of a chromophore coated disk. The resulting surface, which will probably be reticulated with a pitch of 5-10 angstrom, can be scanned with an atomic beam microscope or overlaid with a thin coating and scanned with an electron microscope. The goal is to determine precisely the major axes of the reticulation prior to attempts to dice the sample along the major axes in order to allow electron microscope scanning of the cross section of the chromophore layer to determine the spacing between the oxygen orbitals of the chromophores.

An alternate approach is to dissolve the chromophores and reconstitute them in the liquid crystalline state on the surface of a fluid (probably water) as shown earlier. This approach would provide the dimensions of the molecules within the liquid crystalline state independent of their normal growth on the surface of the opsin disk as a substrate.

By measuring the distance between the two oxygen orbitals shown schematically for each of the rhodonine chromophores in **Figure 5.5.8-2**, the distances for these orbital pairs can be measured for these materials in the liquid crystalline state. This would allow calculation of the velocity of light within such liquid crystalline materials (as discussed in **Section 5.5.8.1**) and probably aid in defining the precise lengths calculated by different Jmol models of the chromophores. The experiments involved require a carefully drawn protocol to insure the desired accuracy.

It is important to determine the angles between the plane of the molecules in the liquid crystalline state and the surface of the supporting substrate. These angles determine how the individual molecules of the chromophore coalesce. If possible, it is also important to determine the angles between the axis of each chromophore type and the line drawn between the two oxygen orbitals. These angles could be compared to the equivalent angles suggested by the various Jmol models as presented by various visualizers. It would also allow updating of the cartoon shown in **Figure 5.5.4-1**.

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[140]Herraez, A. (2007) How to use Jmol to study and present molecular structures, Vol 1: . . . Self published http://biomodel.uah.es/Jmol/manual/en/inicio.htm
[141]https://www.youtube.com/watch?v=I4n5un-pizs
[142]Avogadro Program: http://avogadro.cc/wiki/Main_Page
[143]Avogadro Appraisal: http://www.jcheminf.com/content/4/1/17

5.5.9 Electronic structure and absorption in effective chromophores

5.5.9.1 Absorption chemistry background

Section 5.3.5.3 has presented background material relative to the absorption of photons by various configurations of materials. The unique arrangement of the chromophores to utilize the high absorption of the Rhodonines per unit thickness has been dealt with in **Chapter 4**. Placing the Rhodonine family of chromophores in the context of their brethren in the photographic world, it is seen that their spectral absorption characteristics will each exhibit three relative peaks when measured using a thin film of the material on a substrate; the molecular absorption in the region of 0.3 microns, the dimer absorption at 0.50 microns and the J-band absorption related to the excitation described by the $n \Rightarrow \pi^*$ transition of their electronic structure. The absorption coefficient associated with the $n \Rightarrow \pi^*$ transition and generally defined as the resonance peak is usually from two to 20 times higher than for the next higher peak. If multiple thin films of the chromophore are stacked, the overall absorption of the array will be multiplicative resulting in the highest absorption coefficient becoming the dominant coefficient and the resulting overall spectral absorptance exhibiting essentially a single peak at the long wavelength. This peak will exhibit a width determined almost entirely by quantum and temperature considerations.

The complexity of the highly unsaturated Rhodonine family does not lead to the prediction of their spectra by either theoretical calculation or using well established rules[144]. As a result, the performance of the Rhodonines are predicted based on similar unsaturated families of similar complexity and the decomposition of the measured absorption spectra of the eyes of a variety of animals.

Figure 5.5.9-1 from Mees[145] illustrates the location of the peak spectral absorptances for three similar families of photographic chromophores. The exact molecular structure of dyes (9), (10) and (11) are illustrated in Mees.

Notice that the spectral peaks are separated by a relatively fixed spacing of about 0.95 microns. This can be assumed to be the spacing exhibited by the Rhodonine family until more accurate values are determined later in this work. Notice also that the peak spectral wavelength of dye (9) with n=3 is virtually the same as dye(10) with n= 1. Looking at the structure of these two dyes, the distance between the pairs of auxochromes is the same. The generally accepted nomenclature indicates the level of conjugation of the given dye. It does not define the distance between the auxochromes. It only defines the level of conjugation free from involvement in a ring structure. For the purposes of vision, two factors are significant; the level of conjugation is fixed for all of the relevant chromophores. However, the resonant structure length varies. This suggests that a different nomenclature is more useful. The actual resonant distance between the auxochromes is most important. Since the one auxochrome is always attached to carbon(15). The chromophore is completely described by indicating where the second auxochrome attaches to the backbone of the Rhodonine molecule. Thus Rhodonine(9) would have the second auxochrome attached to carbon(9).

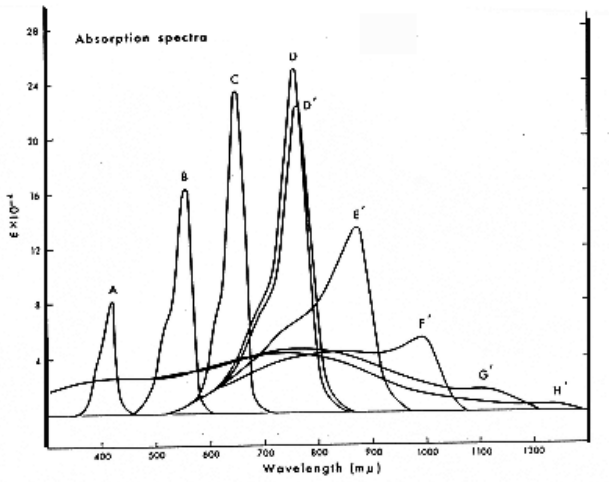

Figure 5.5.9-1 CR A family of absorption spectra in methanol for two families of resonant dyes. A, B, C, D are for dye(9) with n = 0,1,2,3 respectively. D', E' are for dye(10) with n= 1,2 respectively. F', G', H' are for dye (11) with n= 1,2,3 respectively. The molarity was not specified. From Mees & James, 1966.

The various bands described above are discussed briefly in Mees and extensively in other books on physical chemistry. All of the bands are described using relatively simple quantum-mechanical equations. If the bands are plotted as probability densities against wavelength, they are expressed by simple exponential functions. The two half-amplitude parameters of each function provide an accurate description of the probability density function for each of these bands. Being probability density functions, the energy difference function between the various bands is also a probability density function conforming to the algebra of probabilities, i.e., exponentials. These probability density functions are

[144]Hirsch, J. (1974) Concepts in Theoretical Organic Chemistry. Boston, MA: Allyn & Bacon pg 39
[145]Mees, C. & James, T. (1966) The theory of the photographic process, 3rd ed. New York: Macmillan, pg. 203-204

essentially equivalent to the probability that a photon will interact with the material in question; they describe the absorption coefficient of the material and are essentially the foundation for Beer's Law. However, Beer's Law is defined for homogeneous solvents in solution and does not apply directly to nonhomogeneous materials like liquid crystalline materials. The two half-amplitude parameters of each energy difference function provide an accurate description of the probability density function for each absorption band of the chromophore. Each pair of half-amplitude parameters may be used to plot the spectral absorption characteristics of the chromophore.

5.5.9.2 The conjugated-dipole-molecular absorption band of the Rhodonines

The development of the energy band structure of complex organics was discussed in **Section 5.4.3.**
Figure 5.5.9-2 shows the energy band structures applicable to each of the four Rhodonines with additional details related to the absorption spectra of the materials. Both the n-band and the π_c*–band are shown in profile as Fermi-Dirac Distributions. This would be the appropriate description of these bands in the absence of any dopants associated with the lattices of the individual crystalline materials. No hint of any dopant has been found in the data of the literature. The rectangle defining each band is drawn to match the half-amplitude profile of the associated distribution. Dimension **b** indicates the difference in the mean energy level of these two bands. This difference is indicative of the mean absorption spectrum of the chromophore. Dimension **a** indicates the difference in the two nearest half-amplitude energy values. The profile on the right describes the absorption characteristic of the material in terms of a relative probability profile for a photon being absorbed by the material. The equation of this profile is commonly called the Helmholtz-Boltzmann Equation. In this representation, the difference **a** would correspond to the 0.25 amplitude point of the distribution as shown. Dimension **c** indicates the similar difference in the two farthest half energy values.

The probability profile on the right is indicative of the absorption characteristic of each individual chromophore. Note the use of the symbols **c'** and **a'** to indicate the half amplitude points of this profile. Note also that the difference in two exponential profiles remains an exponential profile, albeit with modified parameters. These parameters, and the nominal center energy, represent the conjugated-dipole-molecular absorption band of the Rhodonines. This spectrum is frequently reported in the literature for the chromophores of vision. It is usually attributed to the putative rhodopsin. However, the presence of opsin is not required. Only a conventional concentration of Rhodonine is required to record this isotropic spectrum since it is the only feature in the visual range of the spectrum for these materials.

The best empirical values for this spectral parameter vary somewhat due to experimental technique, particularly due to the solvent used. Although rounded due to the width of the spectral filter used, the values of Wald & Brown are still the most widely quoted[146]. They are a' = 447 nm, b = 502 nm, & c' = 549 nm. It would be easy to interpret the median of this waveform at the mean of the two half-amplitude points, 498 nm. It is common to find the waveform shifted slightly to a center wavelength at 495nm. However, the 502 nm wavelength is frequently reported in an attempt to relate it to the peak of the scotopic spectrum when that spectrum is measured with a broadband spectral filter.

[146]Wald, G. & Brown, P. (1959) Human rhodopsin, Science, vol. 127, pp 222-226

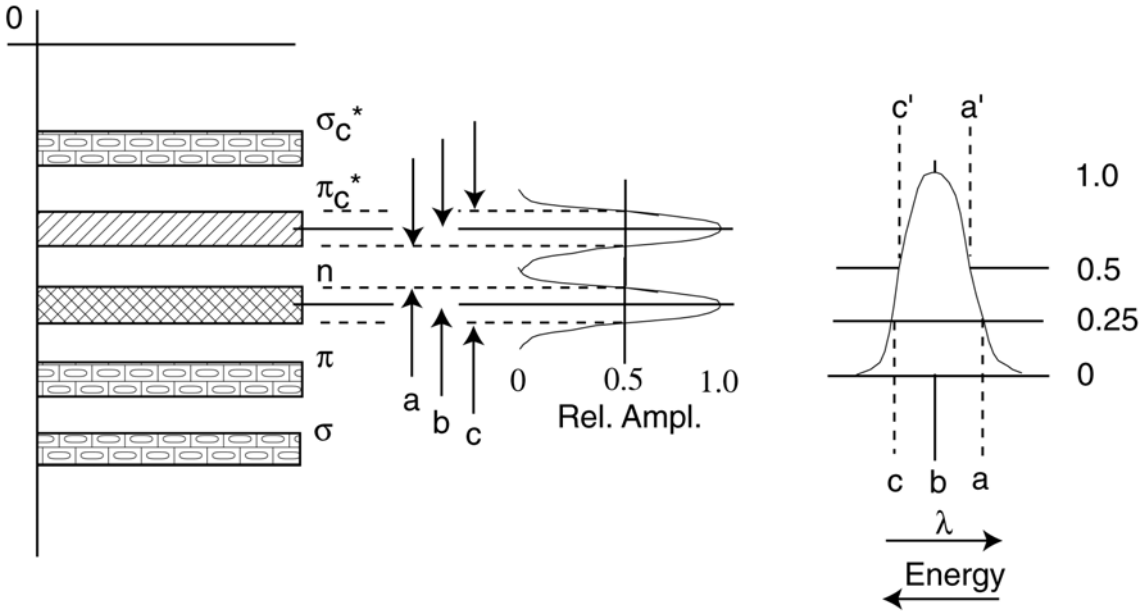

Figure 5.5.9-2 The energy band structures of the Rhodonines in the liquid crystalline state with the relevant profiles and the associated difference in energy profile that describes the molecular absorption spectrum of the molecules when in the liquid crystalline state. See text.

5.5.9.3 Resonant-conjugated-dipole-molecular absorption band of the Rhodonines

Figure 5.5.9-3 shows both the individual functional spectra of long-wavelength trichromatic vision, and the nonfunctional dipole-molecular spectra of the same molecules, in caricature on a linear relative ordinate versus wavelength. This technique allows the information of the figure to be interpreted more easily. However, note that most of the actual data from the laboratory involves a logarithmic ordinate. The ultraviolet spectrum related to R(5) has been omitted for convenience on this graph.

As in the case of the P/D Equation, the absorption spectrum of any retinoid can be described in terms of the absorption cross section of the material.

As discussed above, there are three pertinent situations with respect to the absorption spectrum of the retinoids in the visual range. For most of the retinoids, there is no significant visible spectrum absorption. The dominant mode of molecular absorption is due to bulk excitation and this absorption is most prominent in the ultraviolet. The relevant visual band absorption cross section is zero.

For those retinoids rigidized by conjugation and containing two heavy atoms, the molecules may exhibit a significant absorption spectrum in the visible due to dipole-molecular excitation. This absorption spectrum, peaking at 502 nm, is shown by the solid black line. It can be described in terms of a finite isotropic absorption cross section.

For the case of resonant retinoids (category H in the expanded list of retinoids of **Section 5.4.3**), such as the Rhodonines, there is another aspect of the situation. Because of the slow wave structure of the molecules, the absorption cross section of these materials, when in the liquid crystalline state shows an enhancement that is anisotropic and wavelength dependent. This so-called resonant molecular absorption can be described as a wavelength sensitive enhancement of the dipole molecular absorption cross section. This enhancement was labeled the *J*-band within the photographic dye community beginning in the 1930's. It is directly related to the structural rigidity of the conjugated carbon backbone between the two heavy polar molecules of the molecule and the triplet electronic state of the included oxygen atoms.

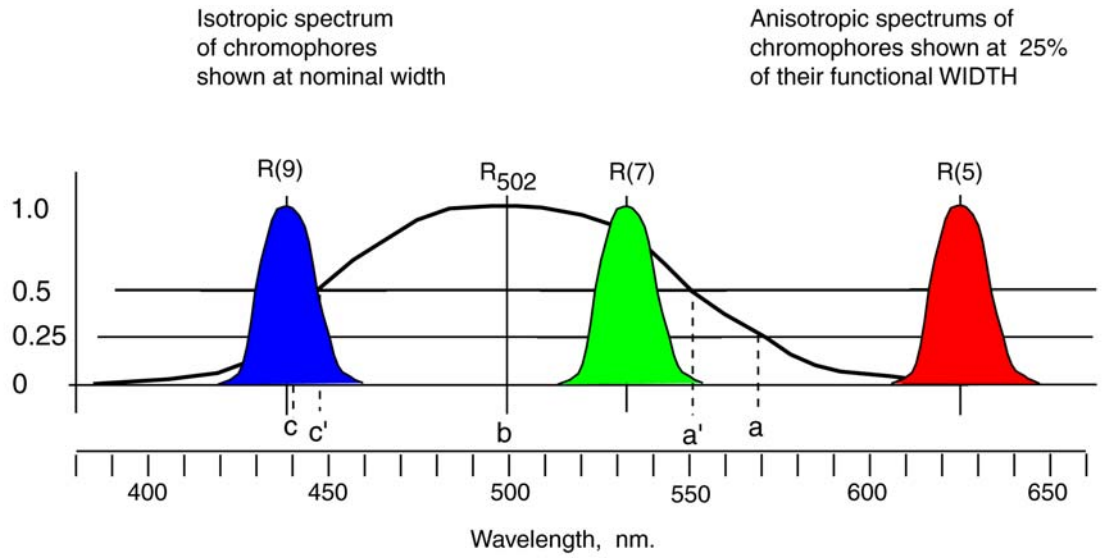

Figure 5.5.9-3 Caricature of the individual S-, M- & L-spectral components of functional importance in long wavelength trichromatic vision combined with the spectrum related to dipole-molecular absorption (solid line). The vertical scale is linear and normalized. The width of the three chromatic spectra are shown at one fourth of their functional width as reported for the human eye. The dipole-molecular spectrum is shown at normal width. See text.

Lacking a detailed theoretical model of how the enhancement of the absorption process should be treated in relation to the energy band diagram, the enhancement will be treated as a multiplier to the underlying dipole-molecular absorption in the spectral region of enhancement. This multiplication factor can be quite large relative to one.

The reason the resonant spectrums are shown at 25% of the width shown in **TABLE 5.5.10-1** will be discussed below.

5.5.9.4 The enhanced overall spectrum of the Rhodonines

This section will discuss the enhanced overall spectrum without regard to the de-excitation environment for the materials under test. It is critically important that this environment be addressed in any in-vitro experiments where the de-excitation mechanism provided by the photoreceptor cells is not present (See **Chapter 12**).

Note as the discussion proceeds, the near total independence of the functional absorption spectra of the Rhodonines, based on their resonant absorption cross section, from the nature of the ionone ring and whether the chromogen was Vitamin A1, A2 or A3. While the dipole-molecular absorption of the Rhodonines in the visual spectrum may reflect a small change relative to the parent chromogen, the enhancement related to resonant-molecular absorption does not.

5.5.9.4.1 Spectra in the in-vitro chemistry laboratory

In the in-vitro environment of the chemistry laboratory, the overall spectrum of each Rhodonine is a composite of the isotropic dipole-molecular and its specific resonance-molecular absorption spectra. In dilute solution, only the dipole molecular spectrum is measurable. As the molarity of the solution is raised, the Rhodonine will begin to aggregate in the solution and an additional feature of the absorption spectrum will begin to appear. Since the aggregations are free to rotate within the solution and exhibit no unique directional orientation, this additional feature will appear to be isotropic. As the solution approaches the molarity of a gel, the overall absorption spectrum will begin to be dominated by the resonance-molecular component. This effect is well documented in the photographic literature. A similar effect has been portrayed by Balogh-Nair & Nakanishi for the same reason in a dipolar retinoid with a modified retinol

backbone, although they attribute it to incubation rather than aggregation[147]. They did not confirm any chemical reaction with the incubation material nor did they report the local pH of the retinoid versus time. Merely a change in pH would be expected to produce a figure like theirs since the material is a member of the indicator group of chemicals. It is proposed that the same result would have been obtained with any other material that could act as a site for aggregation and no chemical reaction should be assumed.

As a result of this enhancement, when the spectral absorption characteristics of the four Rhodonines are plotted on one graph, the resulting family of curves closely resembles [**Figure 5.5.9-1**] from Mees & James.

5.5.9.4.2 Anisotropic spectra involving a tailored substrate

While the above situation resulted in an enhanced but still isotropic absorption cross section, this situation can be changed drastically by the incorporation of a suitably structured physical substrate. If the Rhodonine is deposited as a single layer liquid crystalline material on an inert planar substrate, it will exhibit a highly anisotropic absorption spectrum as a function of its stereometric parameters. However, the spectrum will be transitory. In general, the peak in the anisotropic absorption will be perpendicular to the surface of the substrate, and will be at the peak wavelength for the appropriate member of the Rhodonine family. The relative width of the enhanced absorption compared to the peak wavelength will vary as it does in the figure portrayed by Balogh-Nair & Nakanishi and in the photographic literature.

If the selected planar substrate is able to discharge the excited state of the Rhodonine material, the same anisotropic spectrum will be obtained under steady-state conditions.

5.5.9.4.3 Spectra involving the in-vivo and quasi-in-vivo situation

Both the above experimental conditions involve the Rhodonines in situations of minimal absorption thickness. However, in the in-vivo situation, the individual monolayers of liquid crystalline Rhodonine are about two microns in diameter and are stacked to a depth of typically four thousand layers in humans. This stacking causes a very high degree of absorption at all wavelengths within the absorption spectrum. The result is a much broader absorption spectrum when plotted on a relative basis with respect to the center wavelength. The resulting, highly anisotropic spectrum resembles very closely the spectra recorded for the in-vivo eye in the literature and one of the spectra in [**Figure 5.5.10-1**] below. The parameters of [**TABLE 5.5.10-1**] were gathered based on this situation.

5.5.10 Spectral Characteristics of the Rhodonine family of chromophores

The Rhodonine family of chromophores, when in the liquid crystalline state and with an adequate de-excitation mechanism consists of four distinct chromophores based on retinol$_1$ and four based on retinol$_2$. These are the chromophores of animal vision and their application is pervasive. The presence of all four chromophores in chordate systems is also pervasive. As noted by Neumeyer & Arnold, "In contrast to earlier assumptions, the existence of ultraviolet receptors in vertebrates seems to be the rule rather than the exception."[148] This work defines the ultraviolet chromophore that Neumeyer & Arnold predicted based on their measurement but they say was unknown in 1989.

5.5.10.1 Absorption spectra

As of 2016, this section has been divided into two distinct categories, the analyses performed prior to 2015 and generically labeled the **1st order** spectra of the rhodonines. These analyses were based on a planar retinene/retinine molecules with the C5 carbon considered as part of a linear aliphatic chain where the C5, C7, C9 and C11 carbons were equally spaced along the aliphatic backbone relative to the C15 carbon. This rendition is compatible with a majority of the stick figure representations of the retinenes.

In 2016, the results of single crystal x-ray crystallography experiments were incorporated into the analyses. These evaluations of the structure of a liquid crystalline form of retinal showed that the retinenes were not planar molecules as historically considered, and as incorporated into a vast majority of the 3D chemical databases (such as the non-curated ChemSpider database of the Royal Society of Chemistry). See **Section 5.5.8.4** for changes in the RSC database as of 2017. Instead, the quinone group forming the aromatic portion of these molecules exhibited a significant dihedral angle relative to the aliphatic portion and was rotated to reduce the distance between the C5 and C15 carbon. Spectra based

[147]Dawson, M. & Okamura, W. (1990) Chemistry and Biology of Synthetic Retinoids. Boca Raton, FL: CRC Press. pg 169

[148]Neumeyer, C. & Arnold, K. (1989) Tetrachromatic colour vision in goldfish *Vision Res*. vol. 29, pg. 1719-27

on this configuration have been labeled the 2nd order spectra. The result is a new (2nd order) nominal set of peak spectra at 342, 437, 532 & 610 nm. Only the nominal peak sensitivity of the L–channel chromophore was changed due to this new information. **Section 5.5.10.1.4** will provide data on the new 2nd order set of spectra.

5.5.10.1.1 Absorption by complex photosensitive molecules

As discussed in previous paragraphs, the spectral absorption of the chromophores of vision is much more complicated than that described by Beer's Law for true solutions of low molarity. The absorption of the chromophores of vision is a function of the environment, the chemical state, the spatial relationship, and the orientation of the molecules. They actually exhibit additional, and generally dominant, absorption spectra in the liquid crystalline state that are not found for the same material in low molarity solution. These additional spectra are highly anisotropic. They also exhibit a high absorption coefficient along the preferred axis. Because of these properties, it is extremely difficult to make a comparison of the properties of the chromophores when they are *in-vivo* with their properties when *in-vitro*. Because the chromophores are not in solution, when in-vivo, it is not appropriate to use Lambert-Beer's Law to evaluate them (See **Section 5.3.5.3**). The results of using this law are generally spurious.

The concept of self-screening by a chromophore, as found in the vision literature[149], is based on the fundamental assumptions of Lambert-Beer's Law and a series of simplifications that can be misleading. First, the assumption that the e-vector of the incident light must be parallel to the long axis of the absorbing molecule assumes broadside absorption. This is not the case in vision. The anisotropic absorption of vision is by a separate mechanism. It involves a "slow wave" phenomena as found in end-fire (as opposed to broadside) antennas. A key factor in the absorption of a broadside antenna is the effective length of the antenna relative to the incident photon wavelength. Efficiency calls for this length to be equal to one-half of the wavelength of the incident energy. It behooves proponents of the broadside theory to demonstrate how this effective length is achieved in a molecule less than 0.01 times the length of the photons wavelength.

Second, since each disk of the outer segment is connected to the neural system, the flux absorbed initially is not lost to the overall sensing process. As a result, self-screening is not suggestive of a loss in overall performance. Instead, it contributes to a broadening of the overall absorption spectrum. This is because the wavelengths closest to the absorption peak are absorbed in the first disks of the outer segment. This leaves the photons at wavelengths further from the absorption peak to be absorbed later in the disk stack. They effectively get an increased chance to be absorbed compared to what would be the case for a simple thin slab of absorber. The cumulative effect is to broaden the overall absorption characteristic *in-vivo*.

To obtain more meaningful results, it is necessary to examine the actual physical arrangement of the chromophore, including their index of refraction to account for surface reflectance, and calculate their absorption properties using quantum mechanics (basic physics).

Because of the unique spatial characteristics of the absorption properties of the chromophores of vision, the literature can be misleading. There are frequent references to the orientation of the chromophores with relation to the surface of the disks (or the axis of the disk stack). These references generally proclaim that the long axis of the chromophores is located in the plane of the disks and are, in order to avoid polarization sensitivity, randomly oriented in that plane (See previous reference to Wyszecki & Stiles). Such an assumption is based on the additional assumption that the molecules show maximum sensitivity to radiation applied with a Poynting vector that is perpendicular to the long axis of the molecules and with the E-vector of the field parallel to the long axis. In radio, this corresponds to a crossfire antenna configuration. This analogy is appropriate for *molecular* absorption by an organic molecule. However, it is not appropriate for the *resonant* absorption of the chromophores of vision. These materials, when in-vivo as liquid crystals, operate in an entirely different mode. The mode is described as the end-fire antenna configuration. In this mode, the material is most sensitive to radiation with a Poynting vector parallel to the length of the molecule. In this case, the velocity of the photons traveling along the molecule is the same as the effective velocity of the electrons (excitons) resonating between the two auxochromes of the molecular (liquid crystalline) structure. This is a condition of maximum coupling between the photons and the excitons and leads to simultaneous absorption of the photon and excitation of the electron.

The above explanation is in consonance with the observed situation. The molecules of the chromophore are actually present as a layer on the surface of the disks. The long axis of the molecules is perpendicular to the surface of the disks (as generally found in lipid layers). In this configuration, the molecules exhibit a high and anisotropic sensitivity to

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[149]Wyszecki, G. & Stiles, W. (1982) Op. Cit. pg. 588-591

spectrally unique radiation applied perpendicular to the disk surface (parallel to the axis of the disk stack). This spectrally unique sensitivity is determined by the *resonance* length of the molecules. Simultaneously, these same molecules exhibit a different and intrinsic spectral absorption that is maximal for radiation applied perpendicular to the long axis of the molecules (perpendicular to the axis of the disk stack) and with an E-field that is parallel to the long axis of the disk stack. This latter configuration is that used by Baylor, et. al. up until the 1990's. It always defines the molecular absorption characteristic of the Rhodonines and retinenes as a group. The peak absorption is slightly dependent on environment but is typically near 493 ± 2 nm at mammalian temperatures in-vivo. Regardless of the spectral type of photoreceptor examined, it always exhibits this peak wavelength when tested in this manner.

For experiments where the radiation is applied parallel to the axis of the disk stack, entirely different results are obtained. This is the case of some of the later Baylor experiments and most noninvasive photo-micro-spectrometry studies. These studies, if performed so as to only illuminate one photoreceptor at a time, produce peak spectral absorptions that conform to the actual anisotropic absorption spectra of the chromophores of vision. These peaks are at 437, 532, & 625 nm ± 2 nm at mammalian temperatures.

In the end-fire configuration, also known as the slow-wave configuration in antenna theory, the orientation of the E-field of the Poynting vector is irrelevant. To achieve polarization sensitivity, animals use a different chromophore arrangement and orientation, such as rod-shaped microvilli of the arthropod photoreceptor cells.

5.5.10.1.2 Predicted theoretical spectra

Figure 5.5.10-1 presents the predicted **1st order** spectra of the Rhodonines in the liquid crystalline state and arranged in the spaceframe structure of the Outer Segments of the human photoreceptor cells. The spectra for other animals may be slightly different because of the lower number of disks in individual Outer Segments. Note the absorption spectra are each shown normalized. The relative absorptions of the individual spectrally specific channels are not shown.

The curves are presented in normalized form based on the model developed above and the work of Platt and of Dewar (1950 & 1952). Their procedures give the separation between the peak absorption spectra of adjacent homologs of a polar resonant conjugated chain molecule as 100 ±5 nm. In his 1956 paper, Platt sets the first order separation as 98 nm based on the best values for the physical constants involved ca. 1956. Simultaneously, he notes the ultimate value depends on the state of solvation of the materials. Platt proceeds to examine several **2nd order** effects that may modify these values marginally. His observation was well before the elucidation of the liquid crystalline state so critical to the understanding of the chromophores of vision and photography. By closely examining the available data base, it appears the actual separation of the peaks in the spectra is 95 ±2 nm or ± a marginally smaller value than 2 nm. The half amplitude points of the spectra, when plotted against wavelength, exhibit a slightly different spacing reflecting the variation in the resonance quality, Q, of the individual spectrums. Since the resonance phenomenon employed in these chromophores is based primarily on photon frequency, these curves look somewhat different when plotted as a function of frequency.

Using these values and the resulting parameters from TABLE 5.5.10-1, the **1st order** photopic and scotopic luminosity functions can be computed precisely (See **Chapter 17**).

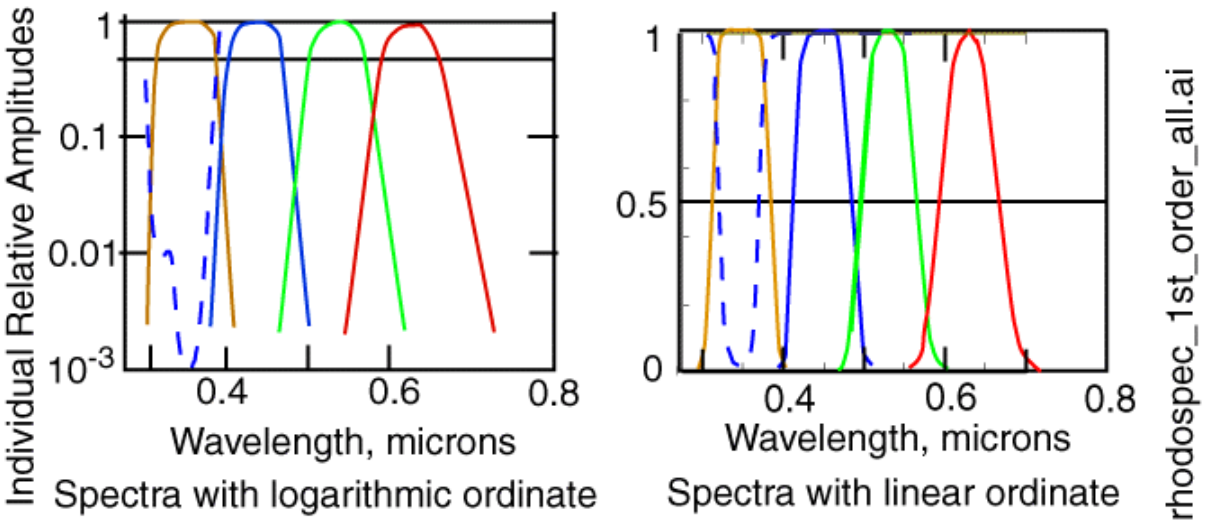

Figure 5.5.10-1 The calculated **1st order** absorption spectra of the Rhodonines, the visual pigments of animal vision, when configured as found in the Outer Segments of the human photoreceptor cells. The parameters of these spectra are those of TABLE 5.5.10-1. The dashed lines are the absorption characteristics of the nominal human lens. These absorption characteristics limit the intrinsically tetrachromatic retina to the observed trichromatic performance of the overall human eye. The lens absorbs nearly all of the ultraviolet light otherwise destined for the retina.

Normally, the distance between the resonant peaks is equally spaced if the parent molecule is a simple conjugated chain. Similarly, the resonant quality, Q, would be expected to be a constant in a structurally uniform molecule. If the molecule contains other groups at various locations along the chain, these may affect the resonant properties of the chain, usually thought of as rigidizing the chain. This may cause the resonant peaks to be nonuniformly spaced or broadened. In the Rhodonines, this would be of primary concern to Rhodonine(5) where the conjugated chain is amalgamated with the cyclogeranyl (ionone) ring.

The above spectra were calculated at a wavelength interval of less than one nm. They exhibit no rounding due to the finite filter width of a spectrometer measuring real spectra. The curves are in good agreement with the data of Neumeyer & Arnold[150]. Their data for goldfish is shown in **Figure 5.5.10-2**. This is a composite figure of data from multiple species, multiple experimenters and collected with a spectrometer of unspecified filter width. It can be assumed from the shape of the curves that a filter width of about 30 nm was used. The transmission characteristic of the eye media of the goldfish is also presented by Neumeyer & Arnold (dashed line). It varies systematically among species and is apparently a genetically controlled variable optimized for the environment. The greater thickness of the lens group of terrestrial animals tends to move the half amplitude of this spectrum toward longer wavelengths. This mechanism significantly limits the ultraviolet capability of the terrestrial eye in any large mammal (**Section 2.4.2**).

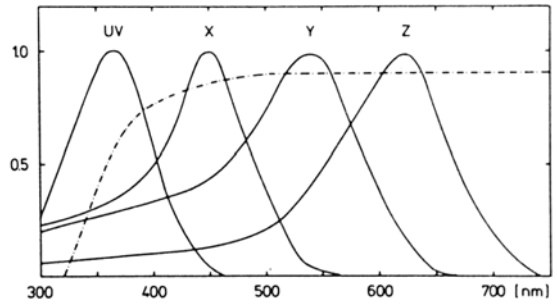

Figure 5.5.10-2 The absorption spectra of fish reported by Neumeyer & Arnold. Note the linear ordinate. The peak wavelengths correspond well to the theoretical values in the previous figure. Neumeyer & Arnold, 1989.

The theoretical half amplitude parameters defining the width of the absorption band of each chromophore are not known analytically. However, it should be pointed out that although the experimentally derived peak amplitudes of the various homologs are recorded in Mees and elsewhere, the equations involved do not define the peak, only the half-amplitude parameters. These half-amplitude parameters are actually equally spaced. Based on an iterative process developed below, the actual values for both the peak

[150]Neumeyer, C. & Arnold, K. (1989) Op. Cit. Pg. 605-625

absorption wavelengths and the half amplitude parameters have been refined to 95 ±2 nm. There has been considerable discussion in the vision literature as to whether the bandwidth, generally loosely defined as the distance between the half amplitude points of an absorption spectrum taken to be Gaussian, is constant for the chromophores in a given animal or whether the bandwidth increases or decreases with wavelength. It has been primarily concerned with how to draw templates. Dartnal used a constant bandwidth approach as a function of frequency. Others have used constant bandwidth approach as a function of wavelength. This work has attempted to use a more fundamental approach by determining the precise frequency of each half amplitude point for each chromophore based on the specific interactions between their spectrums as they are measured in the luminosity and chromaticity functions or as measured by specific experiments measuring the skirts of each absorption function over many orders of magnitude. This approach has provided some very precise values. This method has resulted in the definition of a set of chromophores exhibiting a Q(quality in electronic terms) for the chromophores. The Q is normally defined as the resonance factor =(mean of the frequency of the two half amplitude points)/(frequency difference between the half amplitude points). In this case and for vision generally, the Q has been defined in terms of wavelength instead of frequency. For values of Q greater than about 20, the difference becomes small. However, this choice of definitions does highlight a problem for low Q situations. The peaks of the absorption functions are quite flat. They are based on Fermi-Dirac, not Gaussian statistics. The computed mid frequency is different from the computed mid wavelength using the same half amplitude points.

The equations and their parameters for the spectra predicted by this work for a body temperature of 310 Kelvin are given in the following **Table 5.5.10-1.** The precise values were obtained by comparing a large amount of data in the literature as well as the ability of these values to accurately predict the precise parameters of the human luminosity functions under a range of spectral adaptations. The equations do not exhibit a term describing a "peak wavelength" for each chromophore. The precise peaks can only be determined by setting the second derivative of each equation equal to zero or by making a graphical assessment of each equation. For convenience, the peak absorption wavelength has been taken as the algebraic average of the short (λ_S) and the long (λ_L) half amplitude parameters for each chromophore. The resulting equation for the absorption peaks of each Rhodonine in the series is then given by the equation λ_p=0.095n + 0.147 microns[151,152], where n is the number of conjugated isoprene units incorporated between the polar atoms of the chain. The bands are separated by 0.095 +/-0.005 microns which is a typical spacing for these homologs[153]. These values are believed to be accurate to two digits and can be refined to three digit accuracies by means of this model. The ultraviolet spectrum is only observed in aphakic human subjects.

[151]Fulton, J. (1968) The structure and mechanisms of vision CBS Laboratories, Inc. (Unpublished)
[152] Fulton, J. (1985) The perception of luminance under various states of adaptation, Hughes Aircraft Co. (unpublished)
[153] Mees, C. & James, T. (1966) The theory of the photographic process, 3rd ed. New York: Macmillan, pg 205

TABLE 5.5.10-1
Tables of Parameters based on the **1st order** constant frequency bandwidth assumption for humans *in-vivo* at 310 degrees Kelvin (37 degrees Celsius)

The Helmholtz-Boltzmann Equation (based on Fermi-Dirac Statistics)

$$a_x(\lambda_x) = \frac{1}{\left[1+\exp\left[\frac{1}{KT}\left(\frac{1}{\lambda_x}-\frac{1}{\lambda_{xs}}\right)\right]\right]\cdot\left[1+\exp\left[\frac{1}{KT}\left(\frac{1}{\lambda_{xl}}-\frac{1}{\lambda_x}\right)\right]\right]}$$

where *x* can be replaced with u, s, m, or l to indicate the spectral band of interest. The values of λ_{xs} and λ_{xl} are taken from the appropriate row of the following table to define the short and long wavelength half-amplitude wavelengths for that band.

Receptor	Spectrum Label	Res. chain length	λ_{XS} nm.	Mean Wavelength	λ_{XL} nm.	Resonance Factor Q
Rhodonine(11)	$a_u(\lambda)$	2	300	342	385	4.0
Rhodonine(9)	$a_s(\lambda)$	3	400	437	475	5.82
Rhodonine(7)	$a_m(\lambda)$	4	500	532	565*	8.18
Rhodonine(5)	$a_l(\lambda)$	5	595*	625	655	10.4

***** The difference between the long wavelength half-amplitude point of one chromophore and the short wavelength half-amplitude point of the next longer wavelength chromophore varies between 15 and 30 nm in this table. A difference of 30 nm between λ_{ML} and λ_{LS} is mentioned in Kraft, et. al. (1990) based on Loppnow et. al. (1989), but without further substantiation.

K = Boltzman's constant/(Planck's constant x Speed of light)
K = 0.0000862 electron volts/degree Kelvin, T = 310 temperature in degrees Kelvin, $\therefore$ KT = 0.0267

The resonance factor, Q, can be used as a guide to compare the laboratory results of various experimenters. It is calculated as the quotient of the "peak wavelength" divided by the difference between the wavelengths of the two half-amplitude points for the spectrum of each chromophore. When computing the precise spectral characteristics of the chromophores of vision as found in laboratory experiments (that occurs in **Chapter 16 & 17**) the appropriate Q was determined.

The absolute value of the long-wavelength parameter for Rhodonine(5) is known precisely from the work of Sliney, et. al.[154] in defining the human eye response in the infrared spectra. They showed that the long wavelength half-amplitude point for Rhodonine(5) matches the human visual data over a range of 13 orders of magnitude, a remarkable result of their test accuracy and this theory. There is a slight deviation between the measured and predicted sensitivity in the region beyond 650 nm that will be accounted for in **Section 17.2.2.2.** It has been explored by Brindley. It is explained by the change in chrominance in this wavelength region unrelated to the actual luminance.

The difference in wavelength between the short wave parameter of Rhodonine(5) and the long wavelength parameter of Rhodonine(7) is known precisely because of the sensitivity of the Purkinje Shift to this difference. Similarly, the difference between the short wave parameter of Rhodonine(7) and the long wavelength parameter of Rhodonine(9) is known precisely because of its effect on the visual spectrum in the region of 495 nm, the Bezold-

[154]Sliney, D. Wangemann, R. Franks, J. & Wolbarsht, M. (1976) Visual sensitivity of the eye to infrared laser radiation. J. Opt. Soc. Am. vol. 66, no. 4

Brucke phenomena. These dependencies will be discussed in **Section 15.5**. The short and long wavelength parameters for Rhodonine(9), as well as the very flat top of the spectrum, are known quite precisely from the work of Wald[155]. The short and long wavelength parameters of Rhodonine(11) have been confirmed from the composite spectrum of aphakic humans. See **Section 17.3.3**. The half amplitude full width of these absorptions are in good agreement with the estimates of Wolbarsht when the broader peaks of Fermi-Dirac statistics compared to Gaussian statistics are recognized[156].

The 1964 paper of Wald was accompanied by an appendix provided by Stiles, that essentially questioned the conclusions of the paper (see last paragraph). The obvious concern was how Wald used only "blue" light to isolate the putative "red" receptors of vision. Wald used a Wratten 47B filter and an unspecified color temperature source. As a result, his putative long-wavelength receptor (a result of blue-adaptation) showed a peak response at less than 600 nm (his figures 2 & 3). The paper asserts the peak wavelength of the long-wavelength receptor was either, 575, 580 or 585 millimicrons based on these experiments. His figure 4 shows the long wavelength receptor peak response moving toward the longest wavelength when a partial adaptation was accomplished using a "green" adaptation light (Ilford filter 604, 500-540 millimicron passband peaking at 520 millimicron). Figure 1 of the Stiles appendix clearly shows a variety of spectra with a peak attributed to the long wavelength receptor at 600 millimicrons or longer.

Recently Stockman, et. al.[157] reported on experiments designed to improve on Wald's data for the S–channel receptors. However, it appears their figures incorporated a large number of assumptions and adjustments to the psychophysical measurements. Although they used a very narrow band spectral filter, they appear to have made an unfortunate choice of flicker frequency which introduced a time constant related to the adaptation amplifiers of the photoreceptors. Although titled the spectral sensitivity of the short-wavelength cones, the measurements were actually of the *perceived* sensitivity of the combination of the S–chromophores and the associated adaptation amplifier together in the photoreceptor cell. The paper includes a great amount of material and is well worth reviewing. They adopt the latest template suggested by Lamb to fit their curves. The psychophysically-based Lamb template exhibits a long wavelength skirt similar to a Fermi function but the short wavelength skirt indicates it represents the sum of both the S– and UV–channel spectral responses combined (as developed in this work, **Section 17.2**) and is then truncated. Although they compute two λ_{max} values for these presumed, but truncated, S–channel photoreceptors to five place accuracies, their conclusion is that the most likely photopigment peak is at a different value of 0.44 microns (Table 3). This value agrees well with the theoretical 0.437 microns of this work.

Figures 7, 9 & 11of the Stockman paper shows both complete and partial spectral adaptation when used to isolate the long wavelength skirt of the S–channel response. These responses are in agreement with **Section 17.2** of this work.

It should be noted that the Lamb equation is appropriate for a low frequency filter rather than a resonant phenomenon such as spectral absorption by the chromophores. Its asymptotic character at short wavelengths leads to a half-amplitude value that is quite different from the similar half-amplitude value for a resonant phenomenon of arbitrary resonance factor, Q.

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[155]Wald, G. (1964) The receptors of human color vision. Science, vol. 145, pg. 1009

[156]Wolbarsht, M. (1976) The function of intraocular color filters. Fed. Proc. vol. 35, no. 1, pp 44-50 (caption to figure 3)

[157]Stockman, A. Sharpe, L. & Fach, C. (1999) The spectral sensitivity of the human short-wavelength cones derived from thresholds and color matches. *Vision Res.*, vol. 39, pp. 2901-2927

Recently, a long-wavelength skirt of the absorption spectrum of a putative long wavelength pigment has been presented by Stockman, et. al[158,159]. Their analysis assumes linearity in the visual system. They provided their empirical formula for this response and generated very detailed values based on their analysis of many features of the empirical data base. They have new values for each of the chromophores of vision on their website while awaiting publication. One is cautioned that their values are empirically derived and *do not resemble any theoretically founded absorption spectra.* Their equations are generally based on an arbitrary mathematical power series. The computed peak wavelengths they quote are both arbitrarily defined and more precise (to 6 and 8 decimal point precision) than the underlying data is either precise or accurate. They also appear to have used average values for the absorption of the macula and the lens group across the visual spectrum. While their peak and half amplitude values for the S– and M– channel chromophores agree reasonably well with the above table, their values for the L-channel photoreceptor vary dramatically from those shown in this table and as summarized in **Section 5.5.10**. It appears the psychophysical data they relied upon to compute the L-channel response was not the result of adequate spectral adaptation prior to the measurements. It peaks at 566 nm and resembles curve **C** of [**Figure 5.5.10-5**].

> In 2006, the CIE provided an initial certification of their "physiologically-relevant" LMS functions via one of their Committees, "Fundamental chromaticity diagram with physiological axes – Part 1 Technical Report 170-1." The 2000 papers do not include any physiological or graphical description of the photoreceptors. They are typical investigations involving psychophysics and lack any neurological model of the visual modality.

Although, Rhodonine(11) is not normally associated with human vision, some aphakic patients have been found to respond to irradiation in the 310-360 nm region[160], the very same region to which many UV sensitive animals respond (and far beyond the skirt of the Rhodonine(9) spectra). See **Section 17.3.3.2.2**.

Applying basic antenna theory, it is clear that a single molecule of Rhodonine will be sensitive to the wavelength specified, due to its slow wave structure (a highly anisotropic feature), but its absorption cross-section is extremely low. This is where aggregation into a liquid crystal becomes important. As the size of the aggregated group of molecules increases, the absorption cross-section increases proportionately until it approaches the wavelength of the light in the medium of concern. The shared electrical properties of a liquid crystal approximately the size of the wavelength of light will exhibit a very high absorption cross-section (i. e. absorption coefficient to a chemist).

5.5.10.1.3 Other empirical spectra

As early as 1866, Helmholtz predicted the individual spectra of human vision. He showed them as three equally spaced and overlapping spectrums peaking in the red-orange, green and blue-violet regions[161]. Subsequently, many psychophysicists have presented unequally spaced component spectrums based on a mathematical disassembly of the C.I.E Standard luminous efficiency function using linear algebraic techniques. This has led to the repeated publishing of putative spectrums for the chromophores of vision which are misleading with respect to the location of the spectral peak of the long wavelength chromophore.

Figure 5.5.10-3 compares the typical putative spectrums based on such a linear analysis, β, γ, ρ compared to the actual chromophores, Rhodonines 5, 7 & 9 [with Rhodonine(11) shown for completeness. It is not significant in human vision except for aphakic patients.] Hunt describes the β,γ & ρ spectrums as "probable sensitivity curves of the three types of cones." He did not discuss any rod spectrum in his figure. The "probable sensitivity curves" appear to have been normalized individually. The peak in the ρ spectrum appears to be at a longer wavelength than frequently suggested. However, it is still at too short a wavelength to support the known spectral response of the human eye as illustrated by the Photopic Luminosity Function.

The proposed functions for the Rhodonines are all plotted to the same reference excitation level. The corners of the ultraviolet spectrum are truncated slightly because of the computer graphics program to implement the equation in **TABLE 5.5.10-1**. The longer wavelength chromophores exhibit a slightly lower peak amplitude because of the

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[158]Stockman, A. Sharpe, L. Merbs, S. & Nathans, J. (2000) Spectral sensitivities of human cone visual pigments determined in–vivo and in–vitro *Meth Enzymol* vol 316, pp 626-650

[159]Stockman, A. & Sharpe, L. (2000) The spectral sensitivities of the middle- and long-wavelength-sensitive cones derived from measurements in observers of known genotype *Vis Res* vol 40, pp 1711-1737 data available at http://cvrl.ucl.ac.uk

[160]Gaydon, A. (1938) Colour sensations produced by ultraviolet light. Proc. Phys. Soc. (London) vol. 50, pp. 714-720

[161]Crone, R. (1999) A history of Color. Boston, MA: Kluwer Academic Publishers, pg. 155

variation in resonance factor, Q, associated with these materials. This causes the peak to be lower than the asymptotic value associated with an exponential function fitted to just one side of any of these responses as done by Lamb. The nominal center wavelengths of the Rhodonines differ by very nearly 95 nm. This value is very close to the nominal value (ca. 100 nm) given in and discussed by Backhaus, et. al[162].

The figure also shows the arbitrary wavelengths used to define the 1931 Standard Colorimetric Observer.

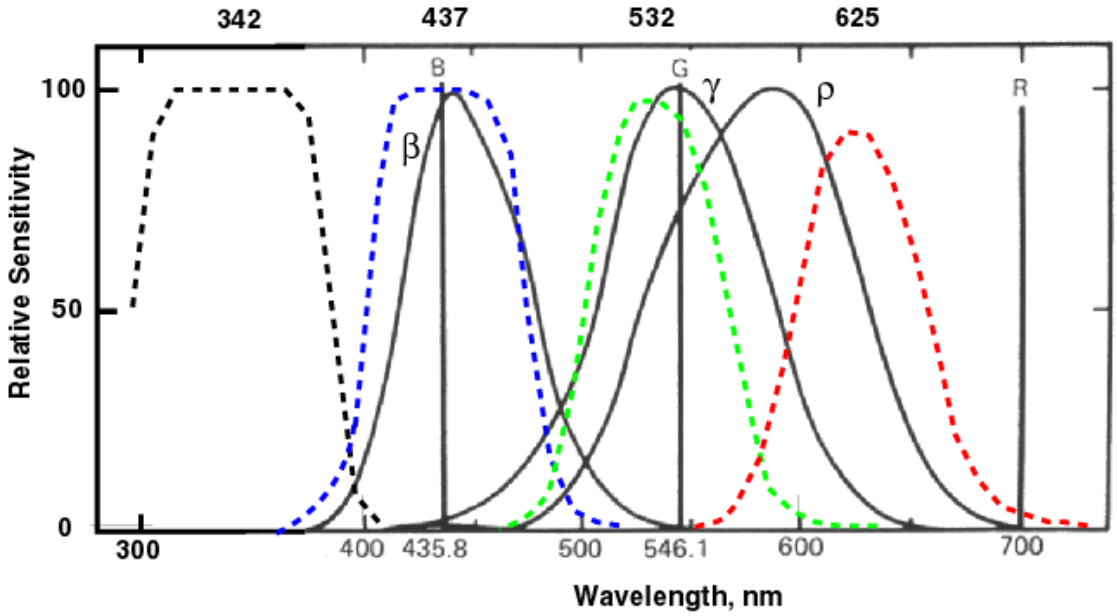

Figure 5.5.10-3 A comparison of various sets of spectrums. Vertical bars, R, G & B are theoretical components used by the CIE 1931 Standard Colorimetric Observer. Solid curves are "probable sensitivity curves of the three types of cones" using the words of Hunt in 1991. Dashed lines are the **1st order** theoretical spectra of the photoreceptors based on this work when excited and viewed axially.

At this time, Stockman, et. al[163]. and Lamb[164] and Baylor et. al.[165] are using empirical expressions for the absorption spectrums of human chromophores. Stockman, et. al. are using a conventional arithmetic series in even powers of the variable. They make no claim to a physical foundation for their series. Lamb says "It needs to be emphasized that the above (his) represents no more than an exercise in curve-fitting, and that neither equation (1) nor equation (2) has any known physical significance. . ." These equations (his equation 2' in particular) are basically attempts to approximate the Helmholtz-Boltzmann equation as it is derived from the Fermi-Dirac equation, by empirical means. In fact, Lamb's equations from curve fitting can be rearranged to the form of the Helmholtz-Boltzmann Equation plus a residue. The residue represents other components on the short wavelength side of the composite absorption spectrum. These components in the empirical data are due to other higher-energy bands of the chromophore. Similar comments apply to the "sixth-order polynomial" in log lambda adopted by Baylor et. al. Baylor, et. al. also note, "The polynomial has no theoretical significance but provides an empirical expression for interpolation."

It is proposed that the closed form equation in **Table 5.5.10-1** does not suffer from the lack of significance noted by

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[162]Backhaus, W. Kliegl, R. & Werner, J. Color Vision: Perspectives from Different Disciplines NY: W de Gruyter pg 177

[163]Stockman, A. & Sharpe, L. T. (2000). Spectral sensitivities of the middle- and long-wavelength sensitive cones derived from measurements in observers of known genotype. *Vision Research, 40,* 1711-1737

[164]Lamb, T. (1995) Photoreceptor spectral sensitivities: common shape in the long wavelength region. Vision Res, vol. 35, pp. 3083-3091

[165]Baylor, D. Nunn, B. & Schnapf, J. (1987) Spectral sensitivity of cones of the monkey *Macaca fascicularis*. J. Physiol. vol. 390, pp 145-160

the above authors with regard to their equations.

As addressed above, the ratio of the peak absorptions of these components is a function of test configuration. See **Section 6.3.4.1**.

In 2017, Taboada et al. provided empirical (behavioral) data on the ultraviolet vision of frogs[166]. They suggest this ultraviolet vision and the corresponding UV reflecting, or fluorescent, polka dot spots on the dermis of frogs is used in remote sexual selection and identification by the frogs.

5.5.10.1.4 Revised retinine spectra ca. 2016 based on x-ray crystallography

The x-ray data of **Section 5.5.8.4.1** has been incorporated into the following **Table 5.5.10-2** to indicate the best available values of the parameters of the chromophores of human vision *in-vivo.*

TABLE 5.5.10-2
Tables of Parameters based on the **2nd order spectral assumption &** constant frequency bandwidth assumption for humans *in-vivo* at 310 degrees Kelvin (37 degrees Celsius)

The Helmholtz-Boltzmann Equation (based on Fermi-Dirac Statistics)

$$a_x(\lambda_x) = \frac{1}{\left[1+\exp\left[\frac{1}{KT}\left(\frac{1}{\lambda_x}-\frac{1}{\lambda_{xs}}\right)\right]\right]\cdot\left[1+\exp\left[\frac{1}{KT}\left(\frac{1}{\lambda_{xl}}-\frac{1}{\lambda_x}\right)\right]\right]}$$

where *x* can be replaced with u, s, m, or l to indicate the spectral band of interest. The values of λ_{xs} and λ_{xl} are taken from the appropriate row of the following table to define the short and long wavelength half-amplitude wavelengths for that band.

Receptor	Spectrum Label	Res. chain length	λ_{XS} nm.	Mean Wavelength	λ_{XL} nm.	Resonance Factor Q
Rhodonine(11)	$a_u(\lambda)$	2	300	342	385	4.0
Rhodonine(9)	$a_s(\lambda)$	3	400	437	475	5.82
Rhodonine(7)	$a_m(\lambda)$	4	500	532	565*	8.18
Rhodonine(5)	$a_l(\lambda)$	5	580*	610	640	10.2

***** The difference between the long wavelength half-amplitude point of one chromophore and the short wavelength half-amplitude point of the next longer wavelength chromophore varies between 15 and 25 nm in this table. A difference of 30 nm between λ_{ML} and λ_{LS} is mentioned in Kraft, et. al. (1990) based on Loppnow et. al. (1989), but without further substantiation.

K = Boltzman's constant/(Planck's constant x Speed of light)
K = 0.0000862 electron volts/degree Kelvin, T = 310 temperature in degrees Kelvin, ∴ KT = 0.0267

The resonance factor, Q, can be used as a guide to compare the laboratory results of various experimenters. It is calculated as the quotient of the "peak wavelength" divided by the difference between the wavelengths of the two half-amplitude points for the spectrum of each chromophore. When computing the precise spectral characteristics of the chromophores of vision as found in laboratory experiments (that occurs in **Chapter 16 & 17**) the appropriate Q

[166] Taboada, C. Brunetti, A. Pedron, F. et al. (2017) Naturally occurring fluorescence in frogs *PNAS* vol 114(14), pp 3672-3677

was determined.

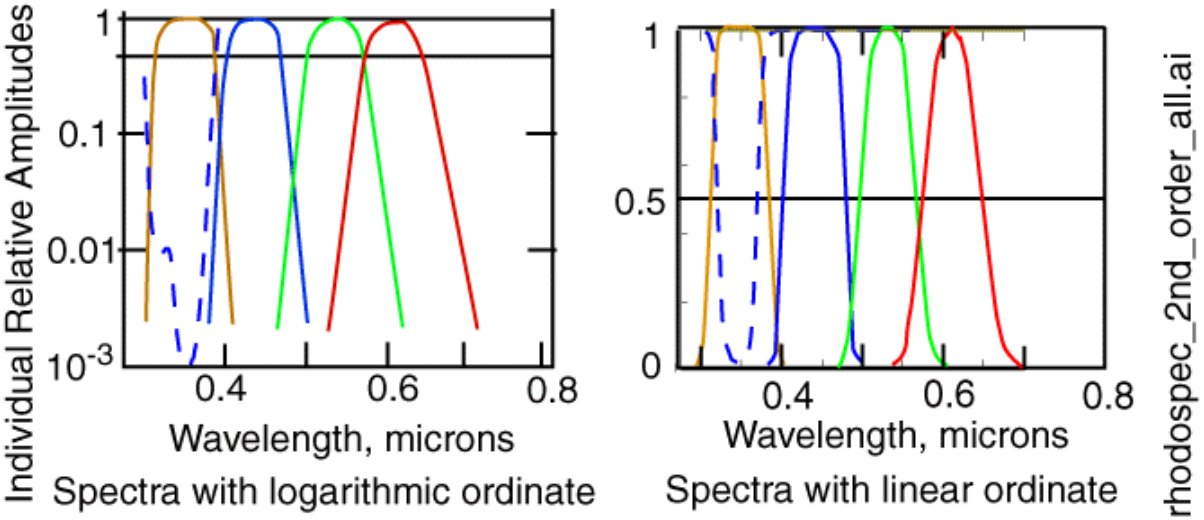

Figure 5.5.10-4 The calculated **2nd order** absorption spectra of the Rhodonines, the visual pigments of animal vision, when configured as found in the Outer Segments of the human photoreceptor cells. The parameters of these spectra are those of TABLE 5.5.10-2. The dashed lines are the absorption characteristics of the nominal human lens. These absorption characteristics limit the intrinsically tetrachromatic retina to the observed trichromatic performance of the overall human eye. The lens absorbs nearly all of the ultraviolet light otherwise destined for the retina.

Figure 5.5.10-4 shows the **2nd order** spectra of the rhodonines based on the above table. The L–channel spectra have been moved marginally, compared to Figure 5.5.10-1, to recognize the shorter distance between the C5 and C15 carbon in the non-planar photoreceptor molecule and the rotation of the aromatic portion compared to earlier calculations.

5.5.10.2 Relaxation modes of the Rhodonine family

To aid in the preparation of the Rhodonines and the understanding of the materials as used *in-situ*, it is important to discuss the relaxation modes of the family.

5.5.10.2.1 In solution

The Rhodonines, like other aromatic members of the carboxylic family are not very soluble in water. They would be expected to be soluble in less polar solvents like ether or alcohol. In dilute solution, their absorption cross section for both their isotropic and anisotropic spectra would be expected to be very small. To the extent they can be excited, their structure would continue to suggest, they will not relax via fluorescence or phosphorescence. Their remaining modes of thermal relaxation and energy transfer to another medium would depend on the electronic characteristics of the solvent. In general, dilute solutions of these materials are found to have relaxation times measured in hours.

As the molarity of the solution is increased, the Rhodonines will begin to coalesce into filaments and globules that show liquid crystalline characteristics. These individual structures will exhibit anisotropic absorption at their resonant wavelengths. However, from a global perspective, the overall absorption of the solution will still appear isotropic but with an added absorption peak. Their relaxation modes will remain the same as above.

If the molarity is further increased, and the material is allowed to precipitate on a substrate, the liquid crystalline structure of the precipitate will exhibit a highly anisotropic absorption spectrum at the resonant wavelength of its chromophore as well as its normal isotropic molecular absorption spectrum. The relaxation characteristic will either remain unchanged or be impacted by the electronic characteristics of the substrate.

The use of a suitably doped silicon substrate could be used to relax the material more quickly.

5.5.10.2.2 In situ

The uncomplexed Rhodonines are found in two locations within the retina. They are found within the pigmented globules of the RPE and they are found deposited on the disks. When in the globules, it is likely that they are in a liquid crystalline state and probably sorted spectrally due to their separate stereo-graphic features. Each individual globule may show significant anisotropic absorption. Their means of relaxation while in this configuration is currently unknown.

When coated onto the disks of the Outer Segment, the materials show both an isotropic absorption spectrum due to their molecular structure as well as the various individual ligands present. However, their primary, and functional mode of absorption is anisotropic. This absorption, due to the resonant properties of the chromophores, is related to the liquid crystalline arrangement of the material on the opsin substrate and is maximized along the axis of the Outer Segment.

The relaxation mode of the chromophores is strictly by energy transfer to the dendrites of the photoreceptor cell when in-situ. The transfer is controlled by the unified Photoexcitation/De-excitation mechanism.

5.5.10.3 Comparisons with the biological literature

The spectral data in the literature agrees quite well with the spectral performance of the Rhodonines predicted by the theoretical part of this work. This is particularly true with regard to the data from the 1970's forward. Of course, it requires that one segregate the isotropic absorption data from the anisotropic data.

Prior to 1960, the conventional wisdom in the textbooks was that humans and only a few other animals could see in color. This changed rapidly beginning at that time as a flurry of laboratory activity showed that nearly all animals had multiple channels of spectral sensitivity[167]. It then became popular to discuss whether the animals with such sensitivity actually processed the collected data in order to perceive color. The laboratory explorations of this subject, primarily psychophysical, were frequently hampered by the indifference of the animal to the investigators desires. Cats in particular were felt to not see in color while in fact they did not want to actively participate in the experiments.

It now appears that nearly, if not all, chordates are tetrachromatic in retinal capability and it would be very strange if this capability was not used to process chrominance as well as luminance information. It is also clear that the human retina is tetrachromatic and that the human processes all of the available information (in spite of a blockage of irradiation at wavelengths shorter than 400 nm by the lens).

During the 1960's, most of the laboratory effort was exploratory in nature. A variety of techniques were employed to differentiate the spectral channels. In animals, electrophysiological techniques were most rewarding. In humans, the techniques were limited to non-invasionary techniques. These were based primarily on differential (chromatic) adaptation and reflection densitometry. The results for humans will be discussed in Section XXX

5.5.10.3.1 Limitations on the applicability of the published database

As discussed in **Sections 17.2.1** & **17.2.2**, the vast majority of the experimental data associated with the luminosity functions has been collected using spectrographic techniques using a 30 nm wide filter and usually 30 nm binning. The data points were then connected to form a smooth curve. The resulting curves always approach a Gaussian form due to the Central Limit Theorem. As a result, the peak wavelength(s) of the underlying function is frequently distorted and the half amplitude points are obscured. Even under these conditions, the resulting curves were frequently interpolated, in some cases down to one nm spacing. Until the recent work of Stockman, et. al. with filters of 4 nm FWHM (full width at half maximum) and a bin spacing of 5 nm, attempts to collect data on chromophore absorption spectra have suffered the same shortcomings. These shortcomings are easily seen by comparing the theoretical curves in [**Figure 5.5.10-2**] with the measured curves in **Figure 5.5.10-3**. The figures of Stockman, et. al. are the first to clearly illustrate the exponential shape of the long wavelength portion of the absorption spectra of the visual chromophores. This shape is identical to that shown in [**Figure 5.5.10-2**].

At the level of spectral resolution and bin spacing used by Stockman, an additional feature is clearly resolved (figures 1, 2, 7 & 9, etc.). The breaks from the main response, generally about 1.5 to 2.0 orders of magnitude down

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[167]Lythgoe, J. & Partridge, J. (1989) Visual pigments and the acquisition of visual information. *J. Exp. Biol.* vol. 146, pp 1-20

from the peak of the subject waveform are usually due to inadequate spectral adaptation. The same distinctive breaks appear in Wald's best earlier data. Figure 11 of Stockman, et. al. appears to show remnant absorption by both the M– and L– channel in the S-channel waveform.

The vast majority of the experimental techniques used to record in-vivo spectral characteristics have relied upon differencing techniques based on an assumption of linearity and without great attention to the presence of other moieties. Merbs & Nathans have provided an index to these methods in their introduction[168]. Figure 3 in Oprian, et. al. provide a sample of the before-and-after in one of these differencing experiments[169]. These methods have consistently failed to provide the correct spectral wavelength for the long wavelength spectral channel. Many of them have also recorded the isotropic spectral characteristic with a peak at 495-502 nm. This characteristic is not a functional response employed by the visual system.

Lewis and Zhaoping have performed a computational analysis of the visual system that indicates the human visual system as described by the spectra proposed by Stockman et al. is far from optimum in the context of the natural luminance and chrominance environment.. They note the need for a long wavelength peak spectral response at or near 610 nm, 50 nm longer than the peak accepted by much of the the psychophysical community, **Figure 5.5.10-6**. Interestingly, a peak at 610 nm is very compatible with the work of Stiles & Burch and of Thornton discussed below (**Section 5.5.10.4.3**), and with this work.

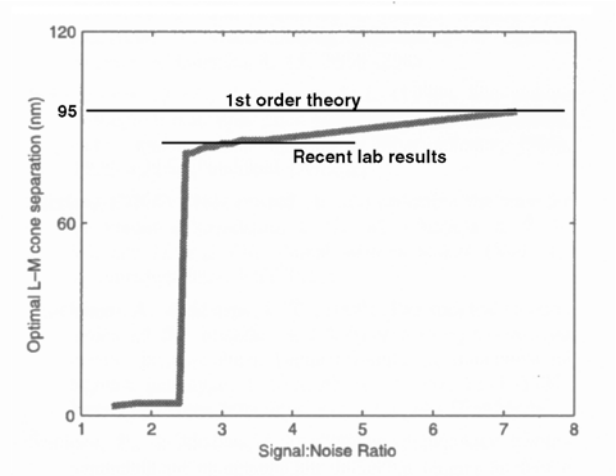

Figure 5.5.10-5 Optimal separation of L– and M–photoreceptors at various levels of noise, for the Vrhel et al. (1994) data, using a Gaussian approximation. The first order theory is from this work. The value of 610 nm proposed by Thornton occurs at the break near 80 nm. From Lewis & Zhaoping, 2006.

A common problem in animal measurements has been the failure to track and report the temperature to a precision of better than 1.0 Celsius during experiments.

5.5.10.3.2 Other mathematical analyses related to spectral absorption EDIT

[combine with 5.5.10.1.3 xxx]

No other closed form solution of the quantum-mechanical absorption characteristic based on Fermi-Dirac Statistics (as presented in **Table 5.5.10-1**) could be found in the literature. A flurry of activity in the 1990's introduced a variety of extensions to simple expressions aimed at curve fitting empirical data.

Based on their supposition that no theoretical description of the spectral absorption of a biological chromophore would become available "for some time to go," Stavenga, et. al. championed staying with the qualitative, heuristic methods as the only ones available[170]. After discussing the so-called log-normal function of Metzler & Harris (which includes a significant extrapolation), they introduced their own variant. These variants all add arbitrary terms (and arbitrary constants) to their putative log-normal function. Lacking a theoretical function, Stavenga, et. al. and others have attempted to fit their empirical forms to what they believed was the most precise available measured data[171]. Stavenga, et. al. focused on calculating the errors between the various empirical equations and the empirical data of Partridge & De Grip.

The log-normal function is unique and does not deserve modification. It occupies a unique position in both botany and biology that is critically related to the processes involved in growth. There are four major classes of statistics of interest in vision. They are the normal, the log-normal, the Stefan-Boltzmann and the Fermi-Dirac statistics. The

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[168]Merbs, S. & Nathans, J. (1992) Absorption spectra of human cone pigments. *Nature*, vol 356, pp 433-435
[169]Oprian, D. Asenjo, A. Lee, N. & Pelletier, S. (1991) Design, chemical synthesis, and expression of genes for the three human color vision pigments. *Biochemistry*, vol 30, pp 11367-372
[170]Stavenga, D. Smits, R. & Hoenders, B. (1993) Simple exponential functions describing the absorbance bands of visual pigment spectra *Vision Res* vol. 33, no. 8, pp 1011-1017
[171]Partridge, J. De Grip, W. (1991) A new template for rhodopsin (vitamin A_1 based) visual pigments *Vision Res* vol. 31, pp 619-630

first is often spoken of as Gaussian Statistics. It relies on a totally random series of outcomes in a linear numerical space. Log-normal statistics rely on a totally random series of outcomes in a logarithmic space. This space is the logarithm of the linear space of Gaussian Statistics. The Stefan-Boltzmann class of statistics apply directly to totally random events constrained in their total energy. They explain the thermal radiation from a physical body. The Fermi-Dirac Statistics are also known as quantum-mechanical statistics. Fermi-Dirac Statistics represent totally random events constrained as to the amplitude of a specific outcome. While Fermi-Dirac Statistics involve exponential functions, their form is not log-normal or Gaussian. Fermi-Dirac Statistics apply directly and specifically to the absorption spectra of chromophores. No arbitrary terms need be added to the Fermi-Dirac Function of well behaved crystalline and liquid crystalline absorbers.

Trying to explain chromophore absorption using the heuristic approach leads to the same problem as encountered in explaining thermal radiation prior to the Stefan-Boltzmann Law. A Eureka Moment must be added to the heuristic process. The tendency among investigators has been to assume absorption is symmetrical about a central value. Fermi-Dirac Statistics describe the probability that an electron will transition across a void defined between two quantized energy states. Their assumption is that the regions beyond the void extend indefinitely. If Fermi-Dirac Statistics are applied to more complex energy state diagrams, the absorption spectrum of any crystalline, liquid crystalline or semi-crystalline material can be described with precision. The resulting calculations do not involve a central moment as that term is used in Gaussian (and indirectly log-normal) mathematics.

Both Stockman, et. al. and Lamb have used general binomial expansions to attempt to fit an equation to the measured data. Stockman, et. al. used a linear series in even powers of x. Lamb used an exponential series. In theory, the Stockman, et. al. expansion can fit both the rising and falling characteristic of an absorption spectrum if they are symmetrical about the mean. The resulting equation is necessarily no more accurate than the underlying data and it gives no insight into the underlying process. The simple exponential series in the Lamb approach[172] is asymmetrical and not simultaneously compatible with both the rising and falling portions of an absorption spectrum. However, it should be noted that only the first term in x is required to fit both the long wavelength portion of the theoretical absorption spectrum (of the equation in **Table 5.5.10-1**) and the measured spectra of Stockman, et. al. in the absence of any perturbations due to poor spectral adaptation or other artifacts. It is a stretch to think either the Stockman or Lamb approach provide any insight into the underlying absorption process.

5.5.10.3.3 Figures from the published database for isotropic Rhodonine

Baylor et. al. have provided recent *in-vitro* isotropic absorption spectrum for Rhodonine based on *Macaca fascicularis*[173]. The data was collected over five and one-half orders of intensity using transverse illumination at "near 37 Celsius." Baylor, et. al. also provide a review of other available measurements and perform a circuitous calculation to compare the isotropic spectrum to the human scotopic luminous efficiency function attributed to Carpenter rather than the C.I.E. Based on ten photoreceptors, the peak sensitivity was estimated to be 491 nm. This is lower than most other investigators. However, data points were only collected at 20 nm intervals using filters of 10 nm nominal half-widths. This sampling plan does not support three digit accuracy in their peak value.

Palacios, et. al[174]. have provided recent data on the peak spectral response of the isotropic spectra of three species assumed to be based on different forms of Vitamin A_1 and Vitamin A_2. A suction pipette approach was used with transverse illumination through the pipette. They did not report the temperature of their in-vitro material during the experiments. Neither did they explicitly state the condition of the animals with respect to their retinol concentration. The retinol state of many amphibians can change over a period of weeks based on their diet. They claimed they only examined "red rods." A mid wavelength chromophore appears reddish because it absorbs maximally at 532 nm. A more precise designation for their samples based on observed color would be magenta instead of red. A more technical description of their results would be the isotropic absorption of mid wavelength photoreceptors examined with transverse illumination of the outer segment. They found the peak to be 503.9 +/– 2.6 nm (n=86) for *Rana pipiens* (frog), 505.8 +/– 1.8 nm (n=24) for *Ambystoma tigrinum* (salamander) and 519.3 nm for *Xenopus* laevis. Their data was difficult to analyze based on these numbers. They therefore performed additional tests on one member of their *Ambystoma* group. Palacios, et. al. say the concentration of $retinal_2$ was 23% and that of $retinal_1$ was 77% in one of their specimens of *Ambystoma*. The range of peak absorption in various subgroups of their

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[172]Lamb, T. (1995) Photoreceptor spectral sensitivities: common shape in the long wavelength spectral region. *Vision Res*. vol. 35, pp 3083-3091

[173]Baylor, D. Nunn, B. & Schnapf, J. (1984) The photocurrent, noise and spectral sensitivity of rods of the monkey, Macaca fascicularis. *J. Physiol*. vol. 357, pp 575-607

[174]Palacios, A. Srivastava, R. & Goldsmith, T. (1998) Spectral and polarization sensitivity of photocurrents of amphibian rods in the visible and ultraviolet *Visual Neurosci* vol. 15, pp 319-331

Ambystoma specimens far exceeded the accuracy range of their peak spectral data reported above. The smaller animals averaged 505.8 nm while the larger averaged 514.6 nm. Clearly, their study did not employ specimen groups that were adequately differentiated based on their retinal concentrations.

The results in their Table 1 show the exploratory nature of their experiments. The peak in the isotropic absorption of the mid wavelength photoreceptors of these species ranged from about 504-505 nm for the largely Vitamin A_1 based animals to a peak near 514-523 (n=11) for the presumed Vitamin A_2 based animals. This is the largest difference based on Vitamin A type found in the literature.

Cameron et al. have recently provided limited visual pigment data on adult teleost fish (in this case green sunfish)[175]. Their protocol included observing the regrowth of retinal tissue after physical excision. Their transverse illuminated optical density measurements were characteristically low, ~0.013 optical density per micron. Their measurements generally showed peak values in two regions, 527 ± 1 nm for the isotropic absorption of Vitamin A_2-based photoreceptors and 620 ± 1 nm for the long wavelength photoreceptors of the native retinas. The mid wavelength spectral response may have been a mixture of the isotropic absorption peaking in the 514-523 nm region (reported above by Palacios et al.) and the operational absorption peaking at 532 nm (predicted by this work). Their regenerated photoreceptors showed slightly longer wavelengths, 526 ± 1 nm and 623 ± 1 nm. Apparently all measurements were made on an equal energy spectral basis. Cameron et al. encountered a lack of correlation between the morphology and the physiology of their photoreceptors as they noted obliquely. "There is a degree of independence between the mechanism that establish cone morphology and pigment content during regeneration, . . ." and "Although anomalous, the long-wavelength single cones may enable regenerated retina to restore the native spectral sampling of the visual scene." They did not observe any photoreceptors with a short wavelength (or UV) response.

5.5.10.3.4 Figures from the published database for long wave trichromats

Figure 5.5.10-6 provides a composite of spectra related to vision taken from Wolken[176] who attributes the left half to Dartnall and the right half to Wald. The labels on the right have been changed in order to provide the comparison in **Table 5.5.10-1**. Note the dotted and dashed lines on the left do not connect with the solid lines on the right. Unfortunately all of the curves are drawn individually to a relative height of 1.00. Because of this, no information is conveyed about the relative heights of one curve to another.

[175]Cameron, D. Cornwall, M. & MacNichol, E. (1997) Visual pigment assignments in regenerated retina *J Neurosci* vol 17(3), pp 917-923

[176]Wolken, J. (1966) Vision, Springfield, IL: Charles C. Thomas, pp. 40-43

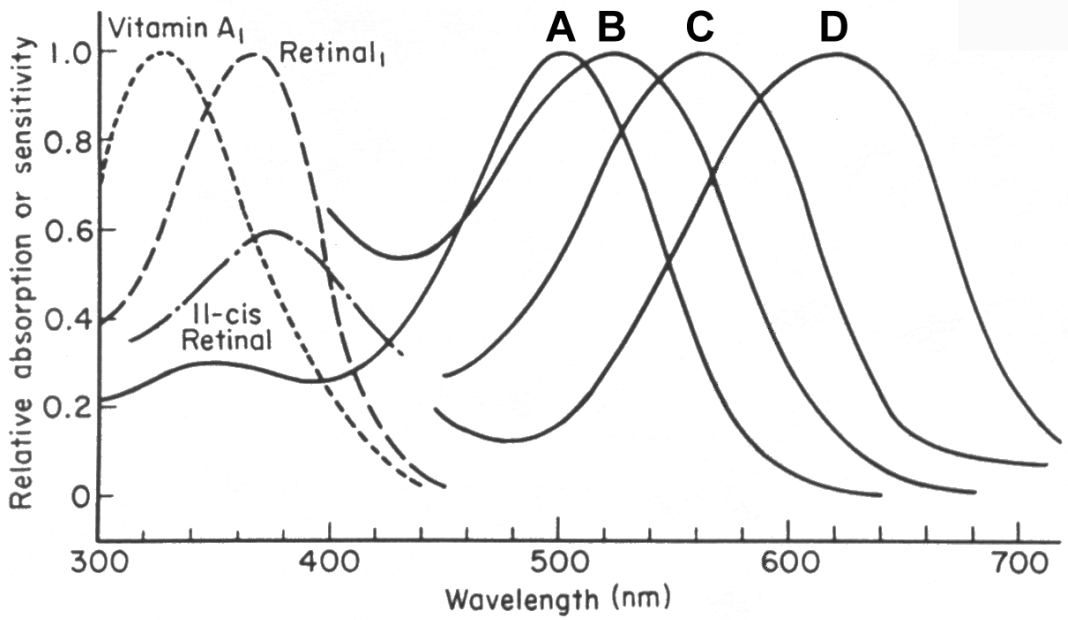

Figure 5.5.10-6 CR Composite of *in-vitro* absorption spectra and psychophysical responses. The discontinuous lines are *in-vitro.* No continuity was claimed between these two data sets although some variants of this figure show 11-*cis*-retinal connected to curve C, the M-channel chromophore. See text. From Wolken, 1966

Note also that the curves on the right are obtained *in-vivo* while those on the left are *in-vitro* and probably in dilute solution.

Note carefully that the short wave peak in the curve for the dilute retinoids (alias rhodopsin), and labeled **A**, does not correspond in wavelength to the peak for any of the *in-vitro* materials. This is to be expected since the *in-vitro* experiments are usually performed with an alcohol solvent. Shifts in wavelength of 15-20 nm as a function of solvent are frequently encountered in *in-vitro* experiments[177].

Menzel & Backhaus have provided a figure showing how the quality of the spectral recording has improved over a period of 22 years[178]. The waveforms were obtained by intracellular recording. Not only does the quality improve significantly, but the recording of the nonfunctional isotropic spectrum with a peak at 500 nm does not appear in the more recent recordings. The recent spectral values correspond to those of this theory within +/- 4 nm.

[177]Sporn, et. al. 2nd ed. pp. 188-189
[178]Menzel, R. & Backhaus, W. (1989) In, Stavenga, D. & Hardie, R. Facets of Vision. NY: Springer-Verlag, pp 282-283

In 1986, Wolken provided a similar set of curves summarizing the data from two other sources. **Figure 5.5.10-8** reproduces this data. The dashed lines are from the worker bee, *Apis mellifera* and are plotted versus the relative

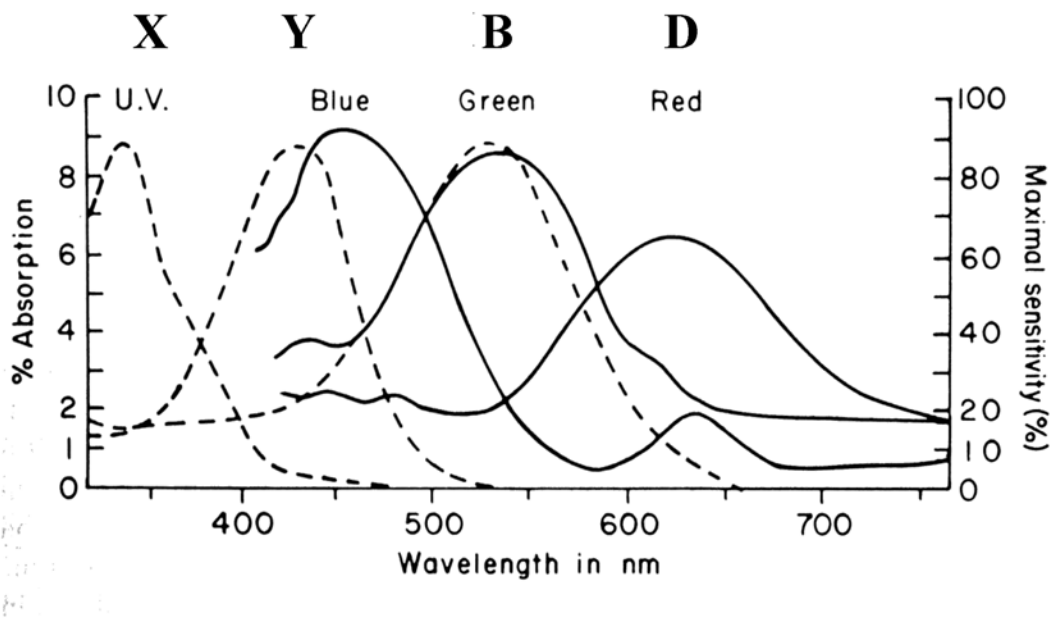

Figure 5.5.10-7 CR Spectral responses of the bee and goldfish as assembled by Wolken in 1986. Solid lines are for goldfish, dashed lines are for the worker bee, *Apis mellifera*.

scale on the left. The solid lines are from the goldfish and are plotted against the scale on the right. Note the likelihood of loss in short wavelength sensitivity of the goldfish near 432 nm due to the absorption in the lens group of the eye. The goldfish curves were obtained by spectral adaptation of a complex eye. Note the likelihood this process was incomplete when measuring the S– and M– channels in the region beyond 600 nm and in the case of the L– and M– channels for the measurements at wavelengths shorter than 500 nm. Here again, the letter labels above the waveforms have been added to support **Table 5.5.10-1**.

TABLE 5.5.10-1
Source of spectra in Figure 5.5.10-5

Curves in Wolken '66	Nominal Peak λ	Visual Channel	This Theory	Names by Wald	Curves in Wolken '86
–	342		Rhodonine (11)		X
–	437	S–	Rhodonine(9)	–	Y
A	495 nm	–	Dilute Retinoid (from Vit. A)	Rhodopsin (see text)	
B	532	M–	Rhodonine(7)	Porphyropsin	B
C	575		Artifact (see text)	Iodopsin	
D	625	L–	Rhodonine(5)	Cyanopsin	D

The peak wavelengths in the table for **X, Y, A, B** and **D** have been assigned on the basis of this theory. The peak wavelength for **C** has been assigned based on the value most commonly found in the literature. Whereas **X, Y, A, B** and **D** can be measured directly by reflection spectrophotometry, **C** is actually obtained by an algebraic calculation based on the assumption that the eye is linear in the psychophysical response following photodetection and signal processing. This assumption is unsupported and unsupportable. A similar peak response has been found by many making psychophysical measurements under conditions of inadequate spectral adaptation and a spectral filter bandwidth of 30 nm or more. Wald has presented a single figure showing both this single broad peak and its equivalent measured with a narrow passband filter. This subject will be developed more fully in **Part D**. Curves **X, Y, B**, & **D** correspond well with the theoretical wavelengths of this work.

There is another complication with regard to the curve with a peak at 502 nm. There are three situations in vision where a spectral curve with a peak near 502 nm is encountered. They can only be distinguished experimentally using spectrophotometers with resolution of 10 nm or better. One is the psychophysical response represented by the CIE 1931 Standard Scotopic Observer (representing smoothed 30 nm data). The second is the isotropic spectral absorption of dilute retinoids structurally related to Vitamin A. Unfortunately, these two curves have been assumed to be related by the vision community for a long time. The result has continued the idea of a single chromophore with a spectral response identical to the smoothed CIE Scotopic Observer, i. e., a rod chromophore. The actual, and mathematical, derivation of the CIE 1931 Standard Scotopic Observer will also be developed fully in **Part D**. The third situation is where a photoreceptor is stimulated by transverse illumination such as in the early suction pipette measurements. In this measurement, the *isotropic* (nonfunctional) retinoid spectral absorption characteristic is recorded regardless of the actual chromophore present. A concurrent measurement using axial illumination would record the actual anisotropic (and functional) absorption characteristics of the chromophore of the cell. This failure to differentiate between these two spectra of the same Outer Segment has led to considerable confusion in the literature[179].

Wolken's figure did not include any spectral response associated with the S–channel (alias blue channel) of vision. This spectral response has been included in the Table for completeness. The S–channel is clearly represented in Wald's papers[180] at a peak wavelength as suggested here.

Wolken has also presented **Figure 5.5.10-8** based on the frog[181]. The measurements were taken *in-vitro* and in an unspecified (three possibilities are mentioned) dilute solution. He also provided similar data for cattle rhodopsin. The data was interpreted as representing a mixture of $retinene_1$ and rhodopsin that changed its spectrum depending on the state of bleaching. However, the fact that the two short wavelength peaks did not agree between the two samples suggests that $retinene_1$ was not present in both samples. There is a simpler explanation. The curves show the typical properties of an indicator dye, such as the Rhodonines, in dilute solution. The difference in the wavelength of the short wavelength peak is due to the fact that the system of fresh water frogs is based on $retinol_2$ and that of cattle is based on $retinol_1$. The long wavelength peaks are the same because these peaks are related to the resonant wavelengths of the chromophores in dilute solution.

[179]Stockman, et. al. (1999) Op. Cit. pg 2921, 2nd paragraph
[180]Wald, G. (1968) The molecular basis of visual excitation. Nature vol. 219, 24 Aug. pg. 806
[181]Wolken, J. (1966) Vision. Springfield, IL: Charles C Thomas. pg. 43

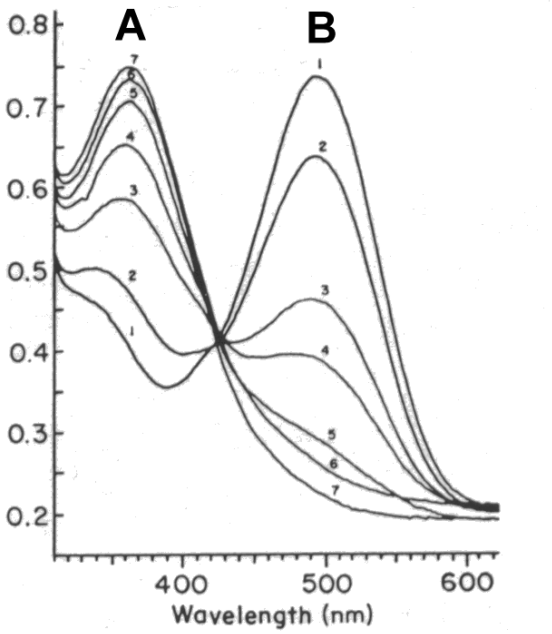

Figure 5.5.10-8 CR Absorption spectrum of putative frog rhodopsin as a function of bleaching level. Curve 1 is unbleached. The material was prepared as a red powder and then placed in dilute solution. From Wolken, 1966. (A) Intrinsic ultraviolet peak of a retinoid. (B) intrinsic isotropic absorption peak of a retinol (al) complexed with opsin. From wolken, 1966.

Based on this theory, the labels can be replaced as follows:

Label	Peak	Wolken's Descriptor		Descriptor based on this Theory
<u>For the frog</u>;				
A	368 nm	$Retinene_1$	<------>	Any $Rhodonine_2$ in dilute solution bleached with long λ light
B	495	Rhodopsin	<------>	Any $Rhodonine_2$ in dilute solution, unbleached
<u>For "cattle"</u>				
A	375 nm	$Retinene_1$	<------>	Any $Rhodonine_1$ in dilute solution bleached with long λ light
B	495	Rhodopsin	<------>	Any $Rhodonine_1$ in dilute solution, unbleached

A similar shift in wavelength between the two peaks could have been obtained by changing the pH of the solution. If this material contained the undamaged chromophores of vision, it would have been interesting to raise the concentration of the solution to the point where the material precipitated. If only one chromophore was present, globules of the concentrated chromophore might have precipitated. This would have surely led to further experiments that would have confirmed the nature of the chromophores of vision.

It should be noted that the bandwidth of the spectrometer used above was not given. It appears that it was about 30 nm. This is too wide to provide definitive data on the shape of the long wavelength feature. Wolken did provide one narrower band (5 nm) spectral scan of the putative rhodopsin from the frog[182]. This scan matches the spectral response of the combination of multiple Rhodonines when logarithmically summed under conditions creating a Brezold-Brucke peak near 487 nm. It also exhibited a secondary peak near 515 nm that cannot be associated with the conventional peak at 500 nm that was also present. This finer resolution scan shows that the long wavelength feature cannot be associated with a single broadband chromophore with a peak at 495-500 nm.

On a subsequent page (69), Wolken shows the spectral scans of a goldfish showing excellent agreement with the

[182]Wolken, J. (1966) Op. Cit. pg. 63

predicted spectral absorption characteristics of the above Rhodonines. Broad peaks are shown in the vicinity of 437 nm, 532 nm and 625 nm and there is an indication of a fourth peak near 342 nm. There is either a labeling problem or the background level is too high to make definitive comments about the Q exhibited by these waveforms. However, they suggest a higher Q (narrower characteristic) at shorter wavelengths. A very similar graph is provided by MacNichol that shows the individual absorption characteristics over a range of 100 to 25 percent of peak absorption[183]. These curves also suggest a higher Q at shorter wavelengths.

Cronin & Goldsmith have provided some uniquely precise information on the photoreceptors of the crayfish, *Orconnectes, procambarus*[184]. They give a peak spectral absorption as 535 nm for what they define as rhodopsin. They quantify this peak in terms of a molecular absorption coefficient multiplied by a quantum efficiency for photoconversion of 0.69. This wavelength does not correspond to the frequently quoted peak of 502 for Rhodopsin. Historically, the 535 nm peak has been associated with porphyropsin. If the expression rhodopsin ⇒metarhodopsin transition is replaced with Rhodonine⇒Rhodonine* transition, the material fits the model of this work precisely. They also present a peak at 510 nm that is based on difference measurements following an adaptation process.

The data collected by Baylor and Hodgkin is shown in **Figure 5.5.10-9**[185]. It leaves little doubt concerning the three spectral peaks found in the absorption spectra of the turtle. It also leaves little doubt as the the broadness of these peaks as described by Fermi-Dirac statistics rather than Gaussian statistics. Notice that there is no absorption with a peak at 502 nm in this figure. They illuminated the photoreceptors end-on and measured the anisotropic absorption expected from this configuration.

On the other hand, Fyhrquist, et. al[186]. have provided data for the toad using the suction pipette approach originated by Baylor, et. al. This technique illuminates the individual photoreceptor from the side. The result is the isotropic absorption spectrum. Although the passband of their spectrometer was not defined, measurements at 10 nm intervals gave a well defined spectrum with a peak at 502 nm at 22C as expected by this theory. They gave the peak value for two different species of toad. However, the difference of 1.5 nm at 502 nm is probably within the error range of their instrumentation. Their statement that the long wavelength tail of the spectrum between 650 and 750 nm "is virtually zero" cannot be supported by this work. Their noise measurements will be useful in describing and evaluating the noise model of the visual process in **Chapter 11**.

MacNichols has provided spectral data[187] similar to that of Baylor & Hodgkins and also summarized the three long wavelength absorbers presented by Marks (See next section). He also included some rare electophysiological recordings from the S-plane of fish that clearly show the long wavelength spectral peaks at 625 nm in fish. His data shows spectral peaks near 455, 530 and 625 nm. He develops the fact that the absorbers operate in a manner consistent with Young-Helmholtz, the neural responses conform more closely to the Hering approach.

Provencio, Loew & Foster have recently provided spectra for the terrestrial lizard, Carolina anoles, *Anolis carolinenisi*[188]. Their results clearly show the peak of the long wavelength photoreceptor at 625 +/– 7 nm based on fifty-five samples. Their appropriate assumption was that the peak resulted from the use of Vitamin A2 in the fabrication of the chromophores of vision.

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[183]MacNichol, E. (1964) xxx Sci. Am. vol. 211, pp. 48-xxx

[184]Cronin, T. & Goldsmith, T. (1982) Quantum efficiency and photosensitivity of the rhodopsin <> metarhodopsin conversion in crayfish photoreceptors. Photochem. Photobiol. vol. 36, pp. 447-454

[185]Baylor, D. & Hodgkin, A. (1973) Detection and resolution of visual stimuli by turtle photoreceptors. *J. Physiol*. vol. 234, pp 163-198

[186]Fyhrquist, N. Govardovskii, V. Leibrock, C. & Reuter, T. (1998) Rod pigment and rod noise in the European toad, *Bufo bufo*. *Vision Res*. vol. 38, pp 483-486

[187]MacNichol, E. (1964) Retinal mechanisms of color vision. *Vision Res*. vol. 4, pp 119-133

[188]Provencio, I. Loew, E. & Foster, R. (1992) Vitamin A2-based visual pigments in fully terrestrial vertebrates *Vision Res* vol 32(12) pp 2201-2208

Figure 5.5.10-9 Absorption spectra of three photoreceptors in turtle. The ordinate is relative quantum sensitivity. Note the long wavelength peak near 625 nm. From Baylor & Hodgkin, 1973.

Hanaoka & Fujimoto published data focusing on five relative peaks in the composite absorption spectra[189]. These were given as 495, 530, 570, 630 & 685 nm. As will be seen in **Section 17.2**, these values, other than the last, are features of the normal composite spectrum of all animals.

Brief mention will be made of the experiments of Stiles & Burch[190]. They chose narrow band laser radiation at wavelengths of 444.4, 526.3 and 645.2 for their experiments. These wavelengths are as closely aligned with the peaks spectral absorptions of the Rhodonines as could be easily obtained with gas laser sources at the time. These wavelengths remain wavelengths of choice today.

5.5.10.3.5 Selected figures from the published database for short wave trichromats

There are an immense number of spectral profiles for the photoreceptors of insect eyes, and the eyes of other short

[189]Hanaoka, T. & Fujimoto, K. (1957) Absorption spectrum of a single cone in the carp retina. *Jap. J. Physiol*. vol. 7, pp 276-285

[190]Stiles, W. & Burch, J. (1959) N.P.L. colour-matching investigations: Final Report. *Optica Acta*, vol. 6, pp 1-26

wavelength trichromats, in the literature.

Schwemer & Paulsen have provided a summary of their spectral findings[191]. However, it is not clear how they normalized their data. They show the three absorption spectra of the moth, *Deilephila elpenor* along with curves showing the net absorption of the eyes under various states of adaptation. Unfortunately, they do not show their actual data points, only smoothed curves probably based on templates. They list the peaks as 345, 440 and 520 nm. These values are very close to the values predicted by this work. They say "The visual pigments found in the moth *Deilephila elpenor* are the physiological bases of trichromatic vision." It is proposed they omitted the critical adjective short-wavelength. They form the physiological bases of short-wavelength trichromatic vision.

They also show three curves peaking in the region of 480 +/-10 nm. This range does not contain 502 nm. The curves were obtained following chemical processing of their extracts.

Backhaus, et. al. have recently provided a similar set of responses for what they label the UV, B & G spectra of honeybee[192]. They label them "best-estimate functions based on intracellular recordings." Although data points are not given, the smooth curves for the UV and B spectra agree very well with this work. The G spectra appears to be following the Dartnall nomographs. It shows a peak slightly beyond 550 nm. Both the B and G responses show significant responses in the region of 350 nm due to molecular absorption.

5.5.10.3.6 Figures from the published database for tetrachromats

The spectral data for various tetrachromats also agrees very well with the above theory. Both Neumeyer & Arnold[193] and Douglas, Bowmaker & Kunz[194] have provided excellent data for the tetrachromats. The Douglas data is truncated at 360 nm but clearly shows the three long wavelength peaks and a peak near 342 nm. The Neumeyer data extends to 300 nm and clearly defines all four peak wavelengths. Both groups used a linear ordinate. Their can be little doubt that these curves support the proposed peak absorptions of the Rhodonines proposed here. It is also clear that these spectra do not conform to a Gaussian shape. The short wavelength tails drawn in these figures are based on templates. As seen in **Figure 5.5.10-10**, the actual data points of Douglas, et. al. do not follow these predicted tails well. The reason is that the templates were originally drawn based on one chromophore . The chromophore showed one anisotropic absorption peak superimposed with the β-peak associated with that chromophore. While investigators "slide" the long wavelength portion of the template horizontally to overlay other anisotropic spectral absorptions, the isotropic absorption near 350 nm is not slidable. Douglas, et. al. also provided data on the absorption by the lenses of the fish.

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[191]Schwemer, J. & Paulsen, R. (1973) Three visual pigments in *Deilephila elpenor (Lepidoptera, Sphingidae*). *J. Comp. Physiol.* Vol. 86, pp 215-229

[192]Backhaus, W. Kliegl, R. & Werner, J. Color Vision: perspectives from different disciplines. Berlin: W. de Gruyter pg 57

[193]Neumeyer, C. & Arnold, K. (1989) Tetrachromatic colour vision in goldfish and turtle. Xxx pp 617-630

[194]Douglas, R. Bowmaker, J. & Kunz, Y. (1987) Ultraviolet vision in fish. *In* Kulikowski, J. Diskinson, C. & Murray, I. *ed*. Seeing Contour and Colour. NY: Pergamon Press, pp 601-616

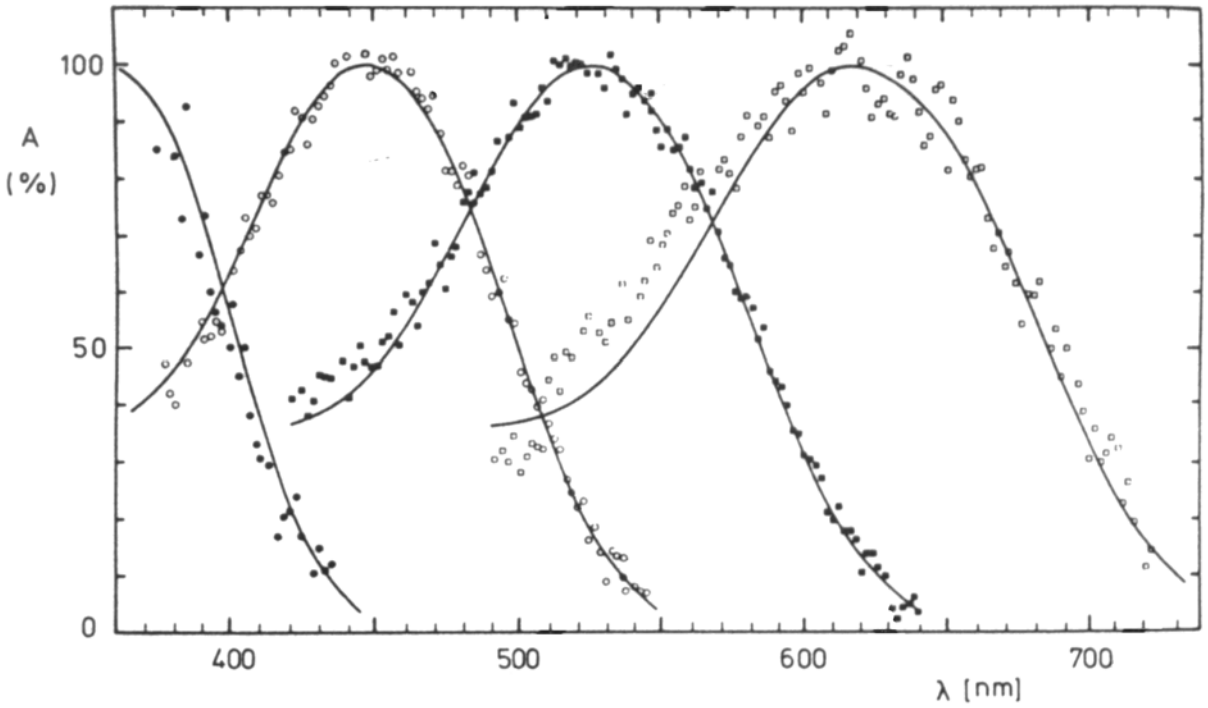

Figure 5.5.10-10 Mean absorption spectra of the four roach photoreceptors. From Douglas, Bowmaker & Kunz, 1987.

It is significant to note the shape of the anisotropic spectrum of the ultraviolet absorber, Rhodonine (11) measured by these workers, does not correspond to the isotropic absorption characteristic of the dilute retinoids. The anisotropic spectrum reflects resonant conjugate absorption in the Rhodonines. Note also the lack of any absorption characteristic with a peak near 502 nm.

Neumeyer and Arnold did not provide the data points to support the shapes of their spectral waveforms. However, they did attempt to create a three dimensional chrominance diagram based on linear algebra. A more satisfactory diagram will be shown in **Chapter 17**. They also provided data on the absorption of the lenses in their fish.

Recently, Hawryshyn & Harosi have presented additional *in-vitro* data for the rainbow trout[195]. Their data appears to suffer from the chemistry used in preparation and unorthodox scaling of their data. The retinas were washed in glutaraldehyde solution and examined under immersion microscopy using glycerine. These materials are known to be antagonistic to reactions involving GABA and glutamic acid reactions. It appears they may have interfered with any electrostenolytic mechanisms associated with these retinas leaving them vulnerable to bleaching without coincident regeneration (See **Chapter 12**). Cronin, et. al. address this chemical sensitivity[196]. As a result, Hawryshyn & Harosi recorded very small absorbances (which they described as optical densities). Maximum values of only 0.015-0.030 were recorded. After recording these small values, they multiplied them by 60:1 as part of their normalization process used in building their figure 5. Their peak long wavelength recorded under these non-functional conditions was reported as 576 nm. They did note that their absorption bands were wider than expected for retinal based chromophores and suggested this was due to a mixture of vitamin A_1 and A_2 based chromophores. While such mixtures are encountered seasonally in eurahaline species, the more consistent explanation is due to the liquid crystalline structure of the chromophores and the Pauli Principle. Their technique did not allow for the normal long wavelength absorption encountered in viable photoreceptors.

An unexpected result of recent research is that the human retina is also tetrachromatic. Although the ultraviolet absorption spectrum has not been isolated, the composite spectral absorption function has been defined in detail. See

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[195]Hawryshyn, C. & Harosi, F. (1994) Spectral characteristics of visual pigments in rainbow trout (Oncorhynchus mykiss), *Vision Res.* vol. 34, no. 11, pp 1385-1392

[196]Cronin, T. Marshall, N. Quinn, C. & King, C. (1994) Ultraviolet photoreception in mantis shrimp. *Vision Res.* vol. 34, no. 11, pp 1443-1452

Section 17.2.

5.5.10.3.7 Reported long wavelength tetrachromats

There have been a series of articles, mostly in the popular press concerning women who may have a fourth chromatic channel in the conventional visual range (exclusive of the UV-sensory channel). There appears to be no scientific basis for this assertion[197]. One quasi-scientific paper describes the additional channel as sensitive to a reddish-orangey-yellow[198]. Yet this is the specific color range of the actual L-channel sensory receptor (610 nm, **Section 17.3.9**) in the normal human eye (as opposed to the putative L-channel of Stockman et al. at 575-585 nm, **Section 5.5.10.1.2**). The factor of 100 in the title to the article is an extrapolation from a poorly founded estimate attributed to Neitz.

The closest to a technical paper on this subject was one by Jameson in 2007[199]. It drew the conclusion that more research was needed to demonstrate any form of long wavelength tetrachromacy (i. e., not involving the UV-channel of the aphakic human eye).

5.5.10.3.8 β-band absorption of retinol vs Rhodonine(11)

During the 1950-60 time period, there was speculation that the ultraviolet sensitivity of animals was due to a secondary absorption band of retinol and retinal designated the β-band. More recent data does not show a good correlation between the peak spectral absorption in the UV band and the peak absorption for these two β-bands. In **Section 17.3.3**, the absorption in the lens due to these two β-bands is seen to occupy a distinctly different spectral region than that of the UV chromophore in humans. If the absorption in the UV was due th the presence of these bands in the chromophores of the S–, M– or L–band photoreceptors, the measured sensitivity in the UV band should correlate with the measured sensitivity in at least one of these other bands. Multiple authors have performed these experiments. They have noted the lack of correlation between the UV sensitivity of their subjects and the state of adaptation of the subjects other spectral bands[200].

5.5.10.4 Comparisons with the human literature

There has been a long standing argument (more than a debate or dichotomy) between the psychology community and the engineering community over the spectral performance of the human eye dating from the 1930's and most intense during the 1970's through the 1990's. Romney & Chiao touched on this difference in 2009[201]. Because of a difference in protocols (both relying on the linearity assumption of Grassman), the psychology community promulgated a tricolor spectral sensitivity for the human eye with the peak sensitivity of the long wavelength channel in the yellow region (peak wavelength ~564 nm). This peak value is inconsistent with much of the psychophysical data acquired by the psychology community itself and is in gross conflict with the engineering community and the inability of industry to build an television or printing system based on a long wavelength photoreceptor with a peak at less than 600 nm. This conflict was addressed indirectly in **Sections 4.6**, resolved chemically in **Section 5.5**, confirmed psychophysically in the following material and demonstrated operationally in Chapter 17 (**Section 17.3**).
The psychology community seriously damaged their position with the development of the CIE L*a*b* and CIE L*u*v* perceptual color spaces in 1976 (**Section 17.3.5**). These empirical representations replaced the linear Grassman assumption with one based on a cube root of the intensity over a limited dynamic range. The cube root is an approximation of the actual natural logarithmic function for the same variable.

While the psychology community has maintained control of the academic press in this area, make no mistake; The

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[197]http://www.sciencealert.com/scientists have found a woman whose eyes have a whole new type of colour receptor (2016)
[198]Ossola, A. (2012) This Woman Sees 100 Times More Colors Than The Average Person *Popular Science* http://www.popsci.com/article/science/woman sees 100 times more colors average person
[199]Jameson, K. (2007) Tetrachromatic color vision. *In* Wilken, P. Bayne, T. & Cleeremans, A. *eds*. Oxford Companion to Consciousness. Oxford: Oxford Univ Press http://www.imbs.uci.edu/~kjameson/jamesonOUP3.pdf
[200]Chapman, R. & Hall, A. (1967) Electroretinogram characteristics and the spectral mechanisms of the median ocellus and the lateral eye in *Limulus polyphemus*. *J. Gen. Physiol*. vol. 50, pp 2284
[201]Romney, A. & Chiao, C-C. (2009) Functional computational model for optimal color coding *PNAS* vol 106(25), pp 10376-10381

spectral performance of the human and mammalian eyes exhibit first order peaks at 435, 532 and 625 nm as confirmed in practice and throughout industry. Second order chemical theory might support the long wavelength photoreceptor peaking at 600-605 nm but not at any shorter wavelength. Laboratory experiments have not shown adequate precision to resolve this potential shortening.

A second flurry of exploratory work in the 1950's and early 1960's focused on determining the spectral channels of the human visual system. Aurebach & Wald summarized the challenge in 1954: "One of the most pressing tasks of visual physiology, still largely unfulfilled, is to identify the various types of cone concerned with human color vision and to learn their properties[202]." The challenge remained largely unfulfilled thirty years later. No chromophore of human vision has been isolated to date. No spectrum of the long wavelength absorber has been quantified by the community to date. The fact that the human visual system exhibited ultraviolet sensitivity was only documented in 1971. See **Section 17.2.2.1**. The fact that this sensitivity was due to a fourth chromophore is presented for the first time in this work.

Unfortunately, the studies of the above years were limited to non-invasionary techniques in live humans. They were all based on the assumption of linearity in the visual system. Based on this assumption, they also made the false assumption that spectral absorptions would add linearly. This was in spite of the realization that artifacts frequently appeared in the spectra that suggested additional mechanisms. Finally, when discussing deutranopia and protanopia, they all focused on the Young-Helmholtz model. Hsia & Graham struggled with the inconsistencies associated with this approach and its extension suggested by Leber and by Fick in the 1870's.

[202]Aurebach, E. & Wald, G. (1954) Identification of a violet receptor in Human color vision, *Science*, vol. 120, pp 401-405

Hsia & Graham presented a paper based on psychophysics[203,204]. Their data shows a consistent hump in the spectral sensitivity function in the region of 625 nm. They also note the tendency of this hump to be suppressed when many spectra are averaged. This is primarily due to a variation in the region of 560 –590 nms of their spectra. **Figure 5.5.10-12**, their figure 2, compares seven normals and five protanopes. It clearly shows the systemic difference between the two types. The normals exhibit significant spectral sensitivity, in the region of 625 nm, not shared with the protanopes. Major features of these spectra are obvious in the region of 437 and 625 nm. Only the mid-wavelength absorption near 532 nm is obscured by the logarithmic summation involved in the overall absorption

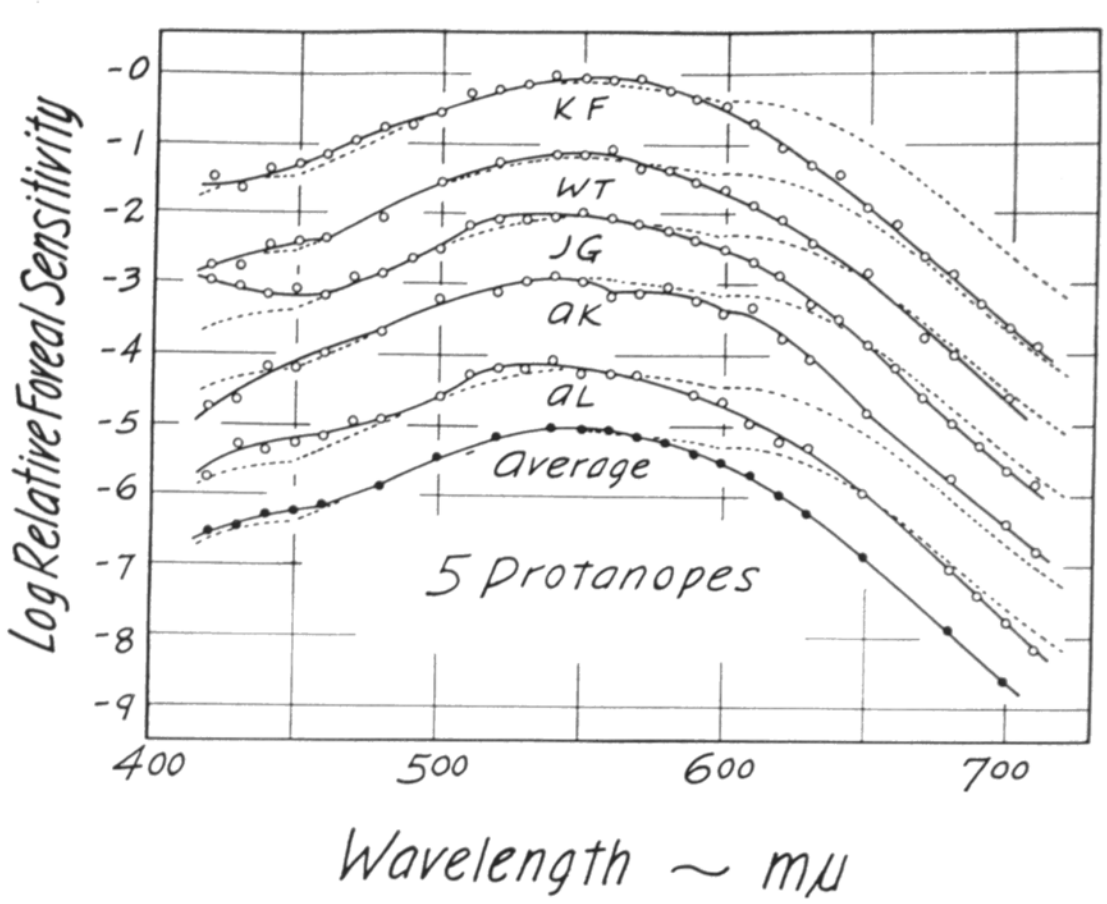

Figure 5.5.10-11 Comparison of 5 protanopes and a normal human. Note the significant rise in sensitivity in the region of 625 nm for the normal subject. Each pair of curves were normalized at 500 nm.

spectra (See **Section 17.2**).

Although a leader in the community of his day, Rushton was frequently equivocal and used very simple floating models. Rushton presented a paper in 1955[205] (based on retinal densitometry), along with a series of talks. This material claimed the long wavelength spectral absorption occurred near 590 nm. In a 1961 paper, he changed his position and ***defined*** the peak as occurring at 575 nm. He was criticized in detail in 1964 for this change in

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[203]Hsia, Y. & Graham, C. (1957) Spectral luminosity curves for protanopic, deutranopic and normal subjects. *Proc. Nat. Acad. Sci*. vol. 43, pp 1011-1019

[204]Graham, C. & Hsia, Y. (1969) Saturation and the foveal achromatic interval *J Opt Soc Am* vol. 59, no. 8, pp 993-997

[205]Rushton, W. (1955) xxx *J. Physiol*. vol. 129, pg 272

position[206]. Simultaneously, he responded to the criticism (by disclaiming the validity of the material) and presenting a new series of papers[207],[208] . The summary of his 1965 paper listed a peak absorption near 635 nm . However, he did not associate it with the term erythrolabe. That paper did include a figure from Hsia & Graham[209] showing a Brezold Peak near 575 nm. It was compared with his own data showing a peak near 570 nm and a clear inflection at a longer wavelength near 625 nm. He claimed chlorolabe had a peak absorption near 540 nm but did not explore the blue region.

Baker & Rushton continued to show that erythrolabe was present in both normals and deutranopes[210]. They also showed the results of differential adaptation but without any calibration of the bleach parameter and a sparse sampling of the spectrum. The figures do not provide unequivocal separation of the spectra.

Ikeda presented two papers which he described as exploratory[211],[212]. They did not contribute significantly to determining the spectral response of the individual spectral channels of human vision. Marks, et. al. used "end-on" microspecrophotometry to measure the spectra of the retinas of both humans and monkeys[213]. The work, admittedly difficult, was performed *in-vitro*. Difference spectra were obtained from "dark adapted" sections of retina. Only two spectra were presented from humans. While orderly, the spectra show a variety of unexplained artifacts. These include rises in absorption at wavelengths longer than 650 nm and a number of unexplained relative peaks in the spectra peaking near 560 & 580 nms.

Boynton, et. al. described psychophysical measurements using an incremental threshold technique[214]. Figures 21 and 22 of their paper show primary spectral channels with peak absorptions near 437, 532 and 610 nms but a caveat concerning the views of individual authors.

Wald continued the effort to quantify the long wavelength chromophore in 1964. This effort will be explored in the following section.

Bowmaker, et. al. reviewed part of the literature in this area in 1978[215]. They also provided data on difference spectra obtained by transverse illumination of *in-vitro* outer segments based on bleaching and red "safe lights." They noted that less than 20 primate cones had been examined up to that time. They also noted a comment by Liebman in 1972 concerning the quality of the database. The frequently reported difference between *in-vivo* and *in-vitro* data was also noted. Without qualification, they chose to use the C.I.E. scotopic luminosity function as an absolute reference in their experiments. Use of a red safe light with outer segments separated from their biological support essentially disqualifies their measurements in the long wavelength region, if not all regions. Separation of the outer segments from the Activa of the photoreceptor cell also eliminates the role of the 2-exciton mechanism in biological vision. It is this mechanism that is used to achieve long wavelength sensitivity in the biological vision system.

They also reported difficulty in isolating any outer segments from "blue cones" although they showed spectral data for two short wavelength absorbers. Apparently they required the cells to recover from bleaching to be considered real.

Bowmaker et al. did identify the isotropic absorption of the Rhodonines with good precision, 502 nm. However, like the work of the Baylor team, their transverse illumination was not appropriate for identifying the axial absorption spectra of the photoreceptors.

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[206]Ripp, H. Weale, R. (1964) On seeing red, *J. Opt. Soc. Am.* vol. 54, pg 272
[207]Rushton, W. (1964) Interpretation of retinal densitometry, *J. Opt. Soc. Am.* vol. 54, pg 273
[208]Rushton, W. (1965) A foveal pigment in the deutranope, *J. Physiol*. vol. 176, pp 24-37
[209]Hsia, Y. & Graham, C. (1957) Spectral luminosity curves for protanopic, deutranopic and normal subjects, *Proc. Nat. Acad. Sci*. vol. 43, pp 1011-1019
[210]Baker, H. & Rushton, W. (1965) The red-sensitive pigment in normal cones. *J. Physiol*. vol. 176, pp 56-72
[211]Ikeda, M. (1963) Study of interrelations between mechanisms at threshold. *J. Opt. Soc. Am.* vol. 53, pp 1305-1313
[212]Ikeda, M. (1964) Further use of the summation index for the study of color vision. *J. Opt. Soc. Am.* vol. 54, pp 89-94
[213]Marks, W. Dobelle, W. & MacNichol, E. (1964) *Science*, vol. 143, pp 1181-1183
[214]Boynton, R. Ikeda, M. & Stiles, W. (1964) Interactions among chromatic mechanisms as inferred from positive and negative increment thresholds. *Vision Res*. vol. 4, pp 87-117
[215]Bowmaker, J. Dartnall, H. Lythgoe, J. & Mollon, J. (1978) The visual pigments of rods and cones in the rhesus monkey, *Macaca mulatta*. *J. Physiol*. vol. 274, pp 329-348

The only figure adopted widely from all of the above studies was Fig 7 of the 1964 paper of Wald. This paper was based on psychophysics and not more intrusive physiology. Because of Dr Wald's charisma and position, no one within the community ever questioned or confirmed his findings. People from the photographic community did raise a flag in 1985 but with little success[216].

Mazzolini et al. repeated some of the experiments of Baylor & Lamb of the 1970's with similar results[217]. Their work lacked an effective model and led to quite confusing terminology. While asserting they were investigating "rods" as opposed to "cones," their terminology was unusual. "The recorded photo-responses was similar for green-sensitive (named also principal "red") and for blue-sensitive ("green") rods ." citing a paper by Röhlich & Szél in 2000. Historically, rods have been conceptualized as broadband receptor cells without any individualizable chromatic sensitivity. The paper did not report any spectrums for their photoreceptors. The abstract to Röhlich & Szél paper dealing with the Xenopus retina is similarly unusual, "The majority of photoreceptors is represented by rods. Altogether 97-98% of the total rod population consists of the principal ("red") rods." In the same year, the Röhlich & Szél team provided another paper of interest here[218].

Mazzolini et al. illuminated their rods with light arriving perpendicular to the axis of the outer segment and without concern for the polarization of the light. As in the majority of the Baylor & Lamb work, this results in acquiring the isotropic spectral sensitivity of the chromophores present in liquid crystalline sheets on the surface of the underlaying material of the disks, dominated by opsin. The expected peak wavelength measured in this way is 502-503 nanometers. Their figure 5 shows how their measurements varied with position relative the position of the illumination relative to the tip of the outer segment. They used "saturating flash illumination." However, their data shows clearly that the photoreceptors under evaluation were not generally saturated (See Section xxx relative to the P/D Equation). The exception is in frame A of figure 6 where the response is totally predictable from below the Hodgkin Condition to full saturation. Their response profiles appear to provide excellent confirmation of the accuracy of the P/D Equation and the electrical properties of the Activa within the sensory receptor cell of this work. Their illumination was centered at 491 nm (slightly shorter wavelength than the peak isotropic sensitivity of rods) and consisted of 20 ms flashes. They achieved "single photon excitation" by merely reducing the intensity of illuminaton until they recorded a minimal response with their instrumentation.

Mazzolini et al. originally assumed the null hypothesis that the outer segments (OS) were filled with a homogeneous material "with marginal inhomogeneities." However, their results demonstrated otherwise, "Therefore, OSs are highly inhomogeneous compartments." This finding is consistent with the stacked coated disks of this work. It is unfortunate that Mazzolini et al. did not stimulate their OS's with light coaxial with the axis of their OS. They would have been able to record the complete anisotropic spectrum of their individual OS and demonstrated that their cells were actually spectrally selective "cones" in the common vernacular.

This work shows that the overall spectra obtained by Hsia & Graham, by Rushton, et. al., by Wald and by Mazzolini et al. are all reproducible using the chromophores defined in this work and the logarithmic summation process at the heart of the perception of luminance (See **Sections 16.3 & 17.2**). Contrary to the widely reproduced proposition by Wald, the three chromophores exhibit peak anisotropic absorptions near 437, 532 and 625 nm. The isotropic absorption (not employed in vision) is centered near 502 nm for all photoreceptors regardless of their operational spectral peak.

5.5.10.4.1 The differential adaptation experiments of Wald

Wald first wrote about differential adaptation in Auerbach & Wald. At that time, they used strong orange-to-red lights (λ> 520 nm) to isolate the short wavelength spectral channel. They then made spectral measurements at one and ten minutes after adaptation and compared the results to Stiles "blue mechanism." They did not measure an isolated short wavelength spectrum. However, they describe the peak absorption near 436 nm as violet since they say most people report this color name when viewing this wavelength. It is suggested that this peak is a major component of their recordings at the end of one minute. They provide a similar figure showing a degree of suppression in the short wavelength region due to chromatic adaptation involving "violet light." Neither the

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[216]Personal communication and response in 1985.

[217]Mazzolini, M. Facchetti, G. Andolfi, L. Zaccaria, R. et al. (2015) The phototransduction machinery in the rod outer segment has a strong efficacy gradient *PNAS* Published online May 4, 2015, pp E2715-E2724

[218]Szél, Á. Lukáts, A. Fekete, T. Szepessy, Z. & Röhlich, P. (2000) Photoreceptor distribution in the retinas of subprimate mammals *JOSA A* vol 17(3), pp 568-579

wavelength or intensity of this light was given.

Figure 5.5.10-12 is presented to illustrate what can be expected based on their observations. Valid measurements of the peak absorption must be made quickly following adequate suppression of the unwanted spectral channels. Auerbach & Wald did not prescribe a maximum time following adaptation within which a meaningful peak in the spectrum could be determined. Neither did they specify a maximum time within which to collect the complete spectral characteristic of the subject. The range of peak absorptions for the L-channel given in the subsequent literature, varying from 565 to 580 nm, is easily understood from the figure.

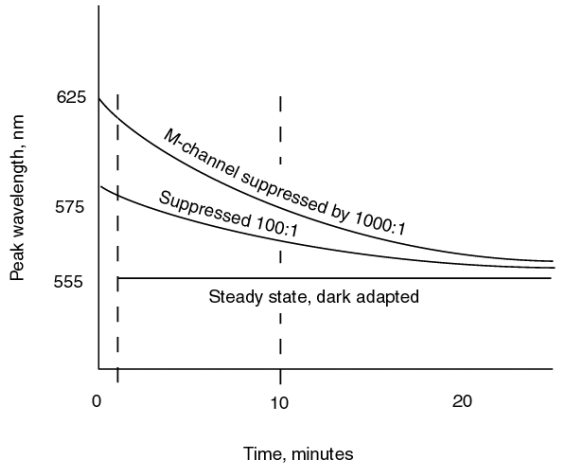

Figure 5.5.10-12 Caricature showing perceived peak spectral sensitivity following suppression of the M-channel by various factors. The true peak in the absorption characteristic is only observed if the suppression due to differential adaptation is adequate and the measurement is made immediately after adaptation.

Ten years later, Wald presented a more comprehensive paper[219]. It involved two separate sets of experiments. One set was designed to isolate each spectral channel of human vision. The second was designed to confirm the data in the first set by suppressing the mid wavelength spectral channel. The work continued to assume linear addition leading to the perceived responses of human vision even though it is widely supposed that the responses are linearly related to the logarithm of the strength of the stimulus[220].

In the first set of experiments, there is a considerable difference between what is said in the introduction and the experiments carried out. Whereas he said he used blue light to isolate the red-receptor and red light to isolate the blue-receptor, he did not. He used light with wavelength greater than 550 nm (distinctly yellow) to isolate the blue. This suppressed both the middle and long wavelength receptors. He used light with wavelength shorter than 500 nm and heavily weighted toward 430 nm in an attempt to isolate the long wavelength receptor. The stated Wratten #47B filter has an optical density of 1.0 at 480 nm and 2.5 at 500 nm compared to its peak near 430 nm. This stimulus only suppressed the short wavelength receptor. In a secondary test, he used a Wratten #47 filter. This lowered the sensitivity of the middle wavelength receptor marginally but did not isolate the long wavelength receptor. As a result, he studies the perceived (logarithmic) sum of the middle and long wavelength receptors. **Figure 5.5.10-13** reproduces his figure 2 with two overlays added. The overlay at the top of the figure is provided only to illustrate the capability of the overall spectral sensitivity equation developed in **Section 16.3.3** and presented graphically in **Section 17.3.3**. This equation is so accurate that the parameters used in it are subject and test set specific. In this case, the Wald curve for subject R.H. is marked dark adapted. However, the test stimulus was undersize (1 degree) and the small shoulder on the right is missing. This shoulder separates the photopic from the scotopic response of the subject. The subject was operating in their scotopic region. The dashed overlay is drawn for the nominal peak absorption amplitudes of k_s:k_m:k_l::100:1000:250 and half-amplitude absorption spectrum widths of 400 to 460, 495 to 550 and 595 to 645 nms respectively. The shoulder in the region of 450 nm was less prominent in Wald's other subject, D.G. Wald did not discuss the precision of his intensity measurements nor provide range bars for his data. The precision of the overall sensitivity equation of this work surfaces variations in the length and/or density of specific spectral types of outer segments in individuals. These variations are treated by adjusting the half-amplitude absorption edges in 1-2 nm increments.

The overlay to the upper of two curves labeled blue adapted (short dashes) is also based on the overall spectral sensitivity equation of this work. The function as drawn includes contributions from all three spectral channels with

[219]Wald, G. (1964) The receptors of human color vision. *Science*, vol. 145, pp 1007-1017
[220]Brindley, G. (1970) Physiology of the retina and visual pathways. Baltimore, MD: Williams & Wilkins, pg 55

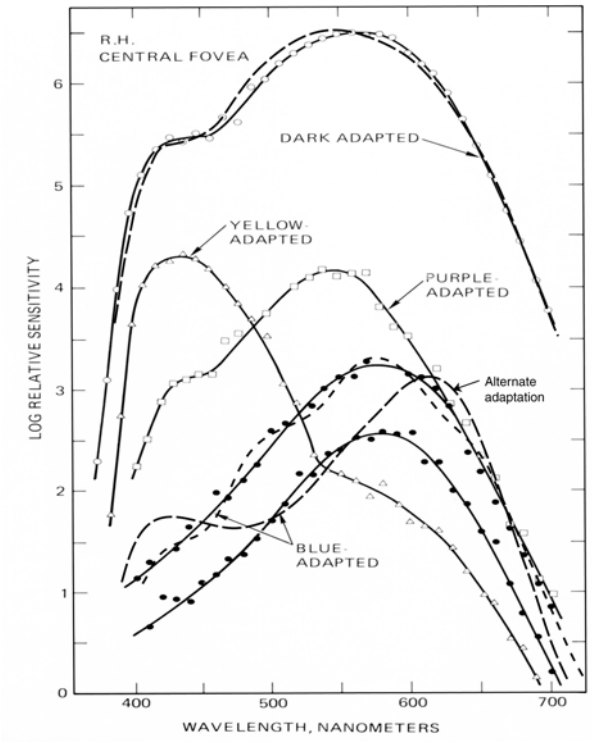

Figure 5.5.10-13 Wald's figure 2 with overlays. The upper overlay (dashed line) shows the overall spectral sensitivity function of this work. The lower overlay(dashed line) shows the overall spectral sensitivity function of this work for a chromatically adapted subject. See text.

peak absorptions at 437, 532 and 625 nm. Their relative amplitudes are k_s:k_m:k_l::50:850:1000 with half amplitude absorption widths of 400 to 460, 495 to 550 and 595 to 645 nms respectively. The data points of Wald can be matched with arbitrary precision by varying the above parameters slightly in the overall spectral sensitivity equation. However, the points to note are:

+ The half amplitude wavelengths for the subject must be known to a precision of <1.0 nm. These vary with the length of the outer segments of the retina of the individual and these lengths vary with location.

+ **Wald was not successful in isolating the L-channel spectral characteristic for this individual**. The measured response included a 3% contribution from the S-channel and a 45% contribution from the M-channel. In addition, a considerable contribution from the Brezold-Brucke Effect has caused the peak absorption to appear at 575 nm.

By more completely suppressing the M-channel, the true spectral peak of the L-channel receptors can be determined. The long-dash overlay (labeled "alternate adaptation") is for such a condition. The relative amplitudes for this overlay are k_s:k_m:k_l::50:50:1000 with the same half-amplitudes as above. Under this more complete adaptation, the peak in the long wavelength receptor channel is seen to occur at 625 nm.

The above discussion combined with that in Section 17.2.2.5.3 have serious ramifications. It is this curve of Wald that has been widely reproduced as authoritative and fundamental. Virtually all of the experiments in psychophysics of the last 50 years have assumed the long wavelength chromophores of vision peaked in the region of 575 nm. They have relied upon a false assumption! The peak spectral absorption of the L-channel in human vision is at 625 nm.

To isolate the long wavelength receptor, he should have used a stimulus extending more uniformly from 540 nm to 430 nm. Among the Wratten filters, #44A would be the preferred choice. A better choice would be a cyan (minus red) filter such as Edmund Scientific #H2536.

In isolating the middle wavelength receptor, he used a Wratten #35 filter. This magenta filter suppressed both the short and long wavelength receptors. He found a significant difference between his subjects in this test. This 60% difference in blue sensitivity more effectively isolated the middle wavelength receptor in subject D.G. Subject R.H. continued to show a distinct shoulder due to the short wavelength receptor at 436 nm. His figures 2 & 3 summarize his isolation experiments. His conclusion was that the receptors exhibited peaks near 430, 540 and 575 nm.

In the second set of experiments, he "green adapted" R.H. in order to isolate both the short and long wavelength receptors. The observed short wavelength peak remained near 430 nm. However, the long wavelength peak moved closer to 580-585 nm. He then "red-adapted" R.H. to isolate the short and middle wavelength receptors. The results are shown in his figure 4, reproduced here as **Figure 5.5.10-14**. It is discussed more fully in **Section 17.2.2.5.3**. The conclusions are the same. Wald did not employ sufficient differential adaptation to isolate the L-channel chromophore of human vision. The peak wavelength of the chromophore is at 625 nm. His figure 6 shows his data corrected for ocular and macular absorptions. The curves for the short wavelength receptor clearly show that isolation was not achieved in the 500-700 nm region. His curves for the putative middle and long wavelength receptors show virtually no roll-off at short wavelengths (less than a factor of 4 to 6 at 400 nm). The curves also show significant absorption beyond 600 nm for the long wavelength receptor. [All of the curves in figures 4 and 6 can be shown to remain composite curves, and can be matched quite closely by theoretical curves developed in **Section 16.3** of this work.]. He then re-plots these curves in normalized form on a linear scale in figure 7. It is this

form that is most often found in the literature to this day. The scatter among the data points is considerable. More critically, **the waveform shown for the L-channel is questionable.**

Summarizing the first set of Wald's experiments;

+ The S–receptors were successfully isolate by suppressing both the L– and M–receptors

+ The M–receptors were successfully isolate by suppressing both the L– and S–receptors

+ **The L–receptors were not isolated** since only the S–receptors were suppressed in the experiment.

The second set of experiments were less than successful because of the minimal amount of suppression of the M–receptors. By suppressing the M–channel by a factor of about 25:1, a logarithmic mixture of nearly equal amounts of M– and L–channel receptors were active. **To successfully isolate the L–channel receptors, the M–channel must be suppressed by a factor of at least 1000:1**.

Wald proceeds to calculate the contribution of each of his putative receptor spectra to the overall spectra using linear algebra. This subject will be addressed in **Section 17.3.3** where it will be compared to the more appropriate (logarithmic) approach.

The problem with Wald's assertion of a peak spectral response for the L-channel near 565-580 nm is in the protocol. This protocol has led others to the same awkward results. This protocol should not be relied upon in the light of the data, including that in the following section.

De Valois, et. al. provided data for the primate based on the macaque monkey in 1963[221]. **Figure 5.5.10-15** shows this data clearly isolates the M- and L-channels based on electrophysiological measurements under more complete adaptation. Using a bleach at 490 nm instead of 510 nm would isolate the L-channel even more effectively.

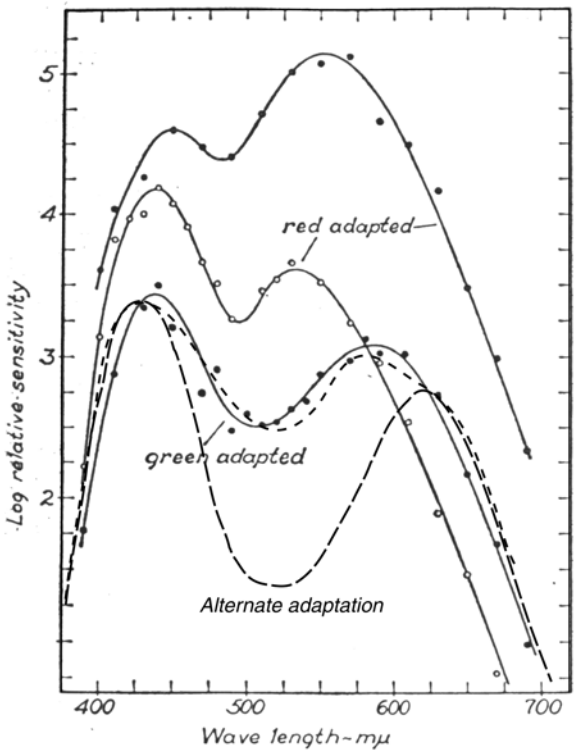

Figure 5.5.10-14 Wald figure 4 with overlay. Wald used the curve labeled green adapted to justify his claim that the long wavelength photoreceptor had a peak absorption at 575 nm. The short dashed curve through the same points contains k_s:k_m;k_l::1000:110:275. The peak near 575-580 nm is actually due to the Bezold Effect. The alternate (long dashes) adaptation curve correctly isolates the peak absorption of the L–channel at 625 nm. See text.

5.5.10.4.2 The mixture data of Wright, Hurvich and others

Both of the above authors have provided differential psychophysical data that differs substantially from the position of Wald. Wright's XXX data clearly shows differential curves showing peak sensitivities in the region of 500-530 nm for the M-channel and less than 450 nm for the S-channel. These values are both consistent with the view based on electrochemistry that the peak wavelengths are near 437 and 532 nm in all animals. The same data would suggest the L-channel chromophore must be at a wavelength longer than 620 nm, even based on the linear assumption. His curves were accepted and adopted by the CIE as a Standard in 1931. His value is consistent with an L-channel peak wavelength at 625 nm as used in this work.

Hurvich & Jameson have provided similar chromatic response functions[222]. However, they show an even better

[221]DeValois, R. Jacobs, G. & Jones, A. (1963) Responses of single cells in primate red-green color vision system *Optik* vol 20, pp87-98

[222]Hurvich, L. & Jameson, D. (1955) Some quantitative aspects of an opponent-colors theory. *J. Opt. Soc. Am.* Vol. 45, pp 602+

agreement on the location of the M-channel chromphore near 532 nm. The show the S-channel peak very near 437 nm. Finally, they show the L-channel peak as near 610 nm +/– 5 nm.

Ingling, et. al. have shown very convincing spectral curves suggesting peaks in the spectral performance spectra associated with 437, 532 and 625 nm[223]. They noted in their title the disparity between various psychophysical methods.

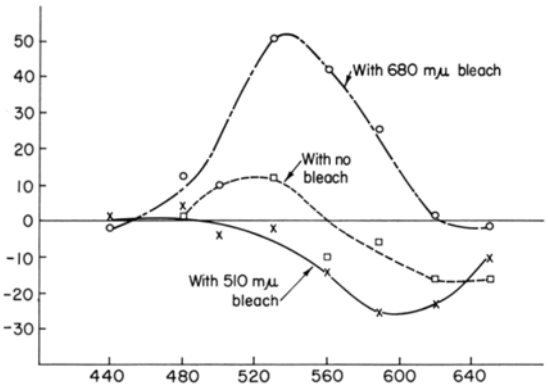

FIGURE 1. Responses of a +G — R cell under three different adaptation conditions. The change in firing rate from the spontaneous rate is plotted at each wavelength. From De Valois, Jacobs, Jones (1963).

Figure 5.5.10-15 Responses of +G–R cell under three different adaptation conditions based on action potential firing rates. The change in firing rate from the spontaneous rate is plotted at each wavelength. From De Valois, et. al. 1963.

5.5.10.4.3 The matching data of Stiles & Burch and Thornton

Stiles & Burch[224] performed a series of color matching experiments in 1958. Their data was plotted as shown in **Figure 5.5.10-16** by Thornton in 1999. Stiles & Burch relied upon the linear assumption of Grassman with the result that they required negative amounts of red to achieve a match in the region of 500 nm (a commonly encountered situation when using the linear assumption). The graph strongly suggests the presence of photoreceptors with peak sensitivity in the regions of 437, 532 and 600 nm. However, the data is quite early and the basic paper leaves the state of adaptation of the subjects and the character of the "bluish-white" light undefined. Thornton has provided many papers analyzing the available data on human spectra. Thornton[225] reviewed the Stiles & Burch data in 1999 and clearly showed that the relative peak amplitudes in color matching experiments depended on the wavelengths of the primary illuminants used in the experiments (his figures19, 20 & 21). His conclusion was that the color matching experiments showed the correct spectral peaks when the wavelengths used in the spectral illuminants matched those peaks. In 1990and using the full set of data used to create the 1964 CIE 10º Standard Observer, he concluded the spectral peaks of the chromophores were most likely at:

L	611 ± 3.1 nm
M	533 ±1.4 nm
S	450 ± 0.9 nm

based on measurements with a spacing of 5 nm and the spectral purity of the sources used by Stiles & Burch. It appears he still employed the linear assumption in his recalculations.

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[223]Ingling, C. Russell, P. Rea, M. & Tsou, B. (1978) Red-green opponent spectral sensitivity: disparity between cnacellation and direct matching methods. *Science*, vol. 201, pp 1221-1223

[224]Stiles, W. & Burch, J. (1959) NPL colour-matching investigation: final report *Optica Acta* vol 6 pp 1-26

[225]Thornton, W. (1999) Spectral sensitivities of the normal human visual system. . . *Color Res Appl* vol 24(2), pp 139-156

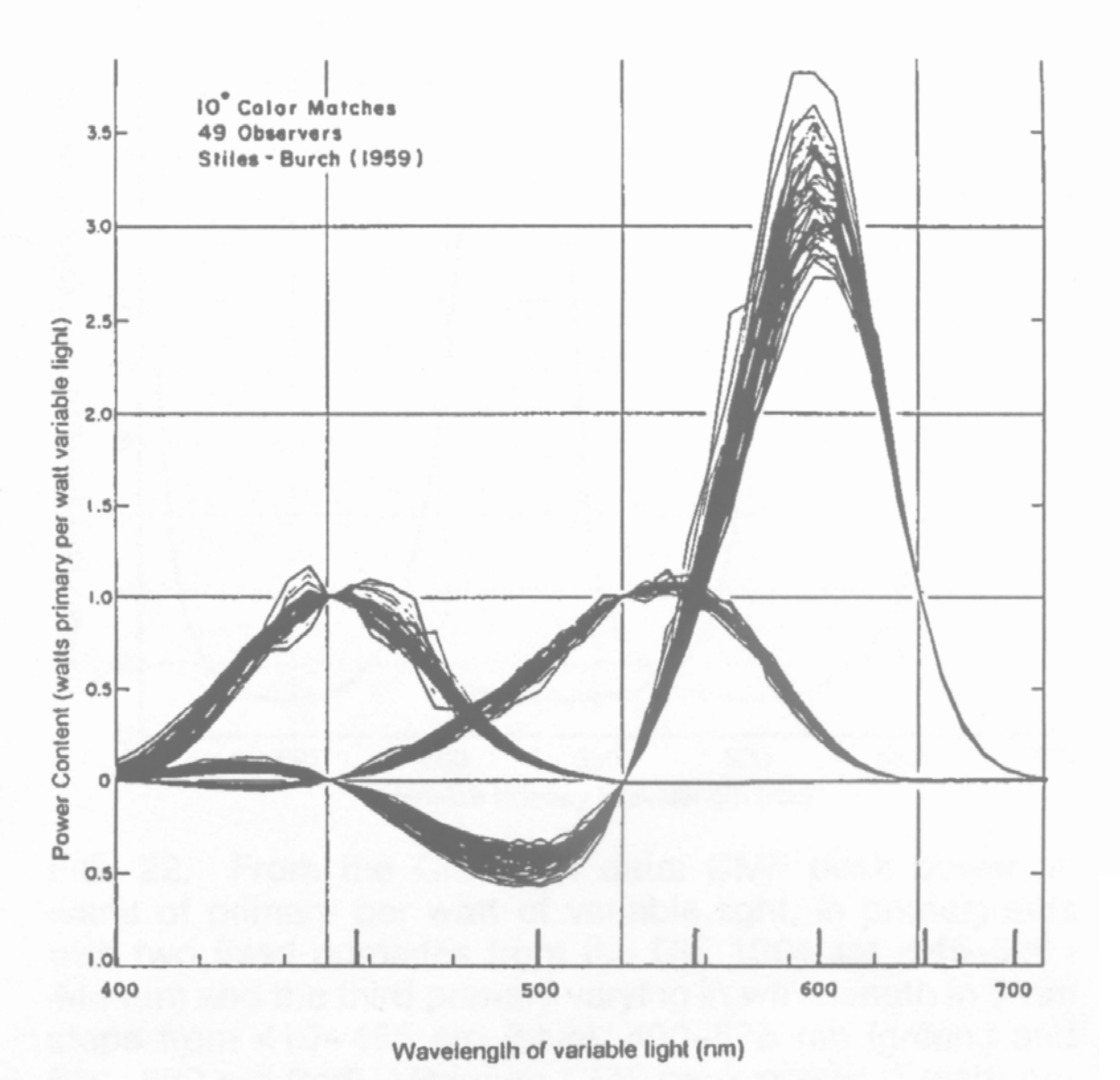

Figure 5.5.10-16 Stiles & Burch color matching functions (49 observers, 10° visual field, real spectral illuminants at 645, 526 & 444 nm). Abscissa converted from wave number to wavelength by authors. From Thornton, 1999.

5.5.10.4.4 The potential second order theoretical spectral peaks in humans

While the peak L–channel response has been documented in a wide range of animals as occurring at or very near 625 nm (see above), the bulk of the data in the literature for humans suggests an L-channel wavelength peaking at somewhat shorter value of 605-615 nm. The question arises as to the source of this difference. While the human chromophores (along with those of most terrestrials and marine animals) are based on Vitamin A_1, and a few of the animal investigated (fresh water fish) have chromophores based on Vitamin A_2 (**Section 5.3.3.3**), the expected difference in wavelengths related to those specimens is believed to be less than a few nanometers (**Section 6.3.4.4.2**).

Besides the empirical values based on a variety of animals, this work has developed a set of theoretical values based

on the Huckel molecular orbital (HMO) method of quantum mechanics and a family of chromophores well studied in the photographic community. The quantum mechanical method develops the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) in order to calculate the visual absorption spectrum of a molecule. The HMO method describes the spacing between the chromophores of biological vision very well (with the possible exception of humans). However, there are other methods of calculation. The Streitweiser method[226] frequently predicts a reduced spacing between the longest wavelength pair, but not generally a reduction compatible with the Smith-Pokorny or Stockman fundamentals.

The difference between Thornton's values and the theoretical values of this work are quite small considering the empirical difficulties involved in gathering psychophysical data. The major difference is between his L–channel value of 611 nm and the proposed first order value of 625 nm.

A particular feature should be noted. If a finite width spectral filter (with finite edge slopes) is used to scan across the spectral response of visual chromophores, the product of the two spectra will generally exhibit a peak shifted toward the direction of approach. Meaningful experiments of this type require the measurements by made by approaching the spectra from both directions and averaging the results.

[226]Streitweiser, A. (1961) Molecular Orbital Theory for Organic Chemists. NY: Wiley

Figure 5.5.10-17 shows Thornton's graphic describing the peak powers per channel required using the modern matching data. The flatness of the functions suggest the peak wavelength is difficult to define precisely.

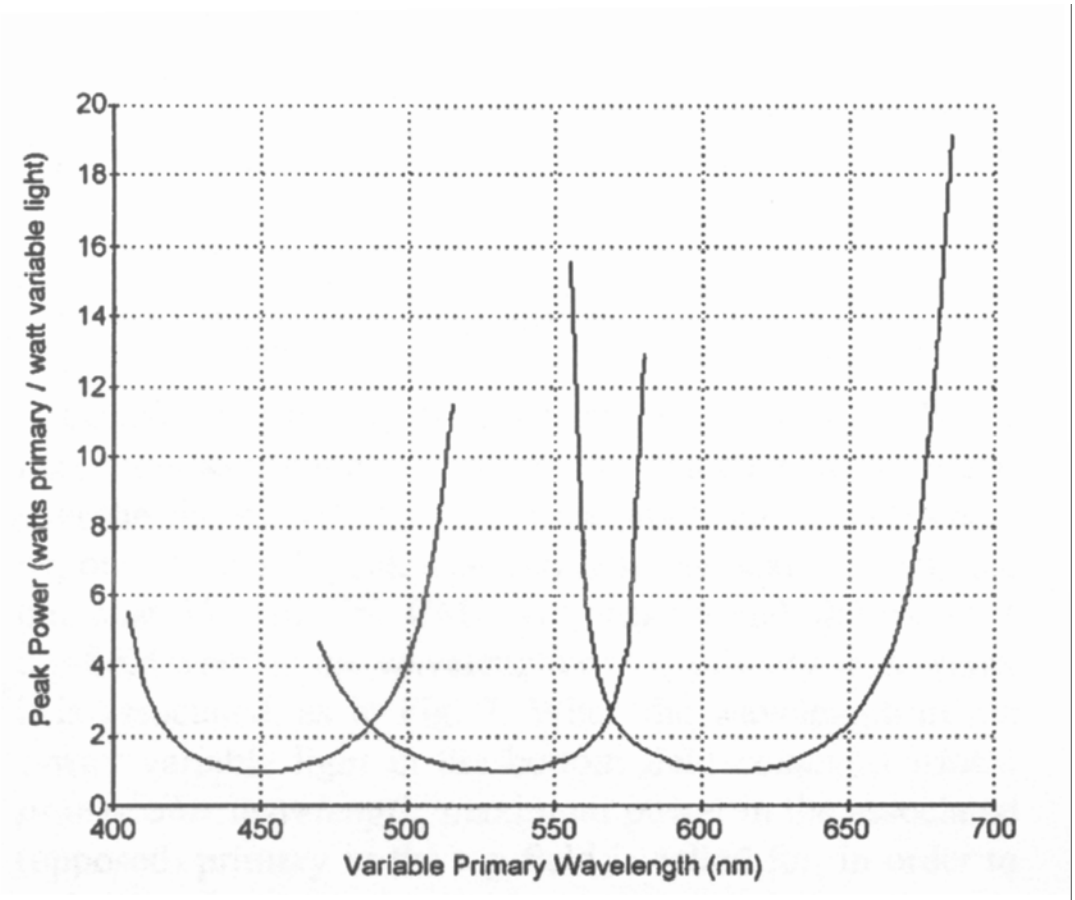

Figure 5.5.10-17 Normalized minimum power color matching functions of Thornton for prime illuminant spectral wavelengths matching the peak wavelengths of the functions, 610, 535, 450 nm to the nearest 5 nm. Note the breadth of the matching functions and their similarity to inverted Fermi Functions. From Thornton, 1999.

As noted, the 1st order estimate of the spectral peaks associated with the rhodonines were obtained using stick and ball models of an aliphatic conjugated chain between two oxygen atoms. These calculations preceded the introduction of both computational chemistry and crystallography chemistry using high speed digital computers to solve more complex configurations for the rhodonines. These computer aided estimates will be defined as 2nd order estimates. The initial 2nd order estimates used computational chemistry based on better but still estimates of the bond lengths between various combinations of atoms within a molecule. These estimates were based on rule-based computations that were based on average bond lengths from a variety of experiments on different molecules containing the bonds of interest. The resulting 3D models tended to show a smaller distance between the mid-wavelength and the long-wavelength chromophores. More recently, the introduction of crystallography techniques offer the capability to measure the distances between the two oxygen atoms within a specific rhodonine with much greater precision. Such *later* 2nd order estimates of peak spectral wavelengths in accordance with Platt's conceptual framework are believed to provide the most precise possible peak spectral wavelengths and be compatible with the values of Thornton given above; 450, 533 & 611 nm. Thornton did not investigate the peak wavelength of the UV photoreceptors. These 2nd order values only differ marginally from the nominal 1st order values defined in this work; 342, 437, 532 & 625 nm. The composite spectra predicted for the retina of *chordata* is not changed significantly by using these *later* 2nd order values of Thornton.

Note the broad peaks in Thornton's empirically derived estimates of the 2nd order spectra. These spectra are

compatible with the Helmholtz-Boltzmann equations of **Section 5.5.10.1.2**. Such broad peaks make the estimated differences between the peaks in nanometers less precise, These 2nd order differences appear to be closer to 80 nm on average compared to the 1st order estimates of 95 nm average.

5.5.10.4.5 Conformity of the predicted spectrum and CMOS imagers

While the xxx school has continued to dominate the academic psychophysical vision literature, the engineering and commercial world of vision applications continues to demonstrate the theoretical spectrum of the individual photoreceptors of this work and the empirical spectrum of Thornton (and more recently of Babucke) is correct. The peak spectral sensitivity of the visual receptors are within one or two nanometers of 437, 532 and 625 nanometers. A recent high performance CMOS imaging sensor demonstrates this fact. **Figure 5.5.10-18** demonstrates the performance of the Piranha4 Color CMOS Camera with its three optimizing color filters in place. Note the high quantum efficiency approaching that of the biological eye (~90% quantum efficiency due to its outer segment organization) and the extended long wavelength response that the biological eye cannot match (cutting off before 700 nm due to the means of transduction employed). Note also, only for completeness, the secondary peaks in the long wavelength region of the spectra due to the color separation filter designs used.

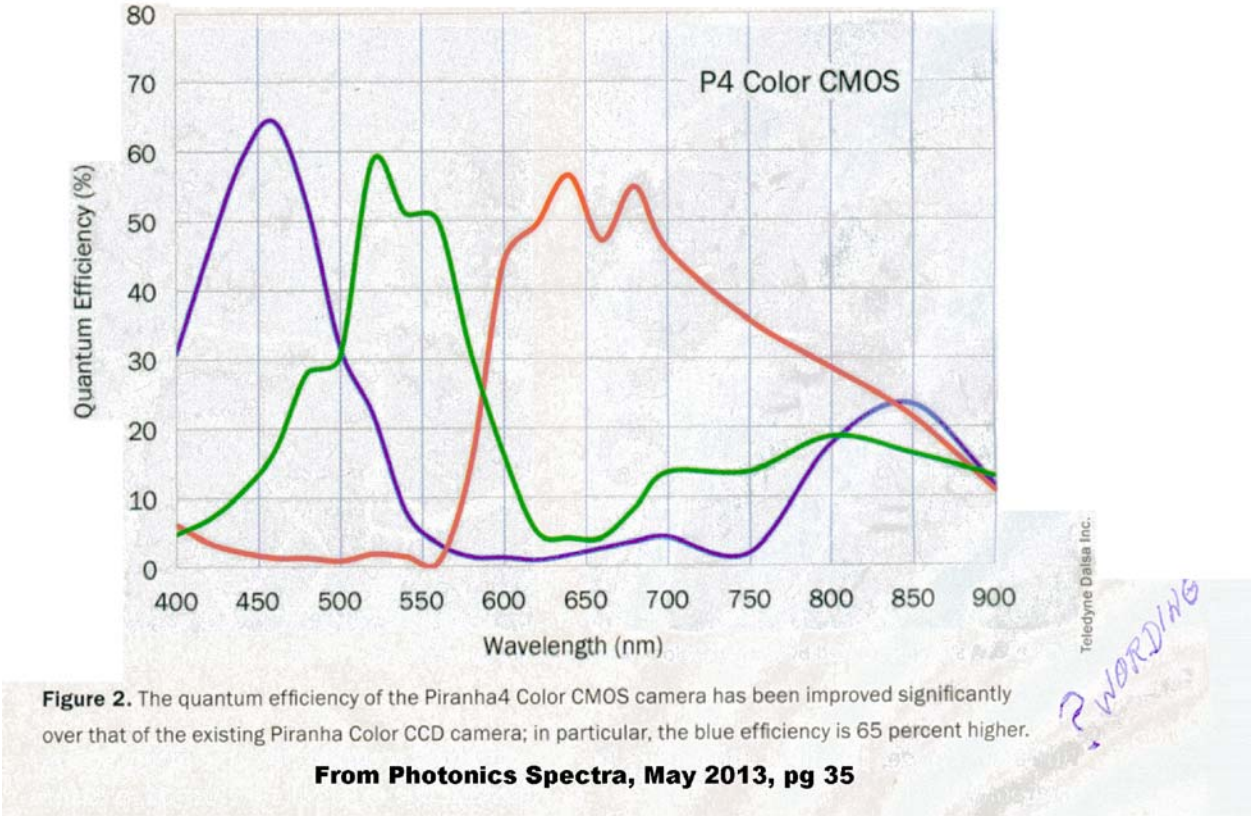

Figure 5.5.10-18 The spectral response of the Piranha4 Color CMOS camera showing its excellent quantum efficiency and its three spectral peaks emulating the performance of the human eye. From, xxx, 2013

5.5.10.5 The next theoretical stage in spectrum description

The above theory fits the available data very well. However, additional experimental investigations could contribute to the refinement of the theory in at least two ways. First, it could confirm the band edge parameters shown in Table 5.5.10-1 and illustrated in Figure 5.5.10-1 for the *in-situ* chromophores. Second, it could further refine the Q factors of the individual chromophores *in-situ*.

5.5.10.5.1 A potential 2nd order absorption theory

The current psychophysical literature is dominated by the papers of Stockman & Sharpe based on the assumption that a deuteranope lacks the M-channel of vision. While not supported here (see **Section 18.1.3**), these writings continue to skew the generally accepted spectrum of the L–channel. The work of Sliney and the nature of the Purkinje Effect clearly support the values quoted in **Table 5.5.10-1**. However, it is conceivable that the long wavelength band edge of the M–channel and the short wavelength band edge of the L-channel are both displaced to shorter wavelengths due to the presence of the ionone ring in the vicinity of the fifth and seventh oxygens of the chromophores. **Figure 5.5.10-19** illustrates the situation using filled models of the molecules involved. **Figure 5.5.8-2** shows the equivalent stick models.

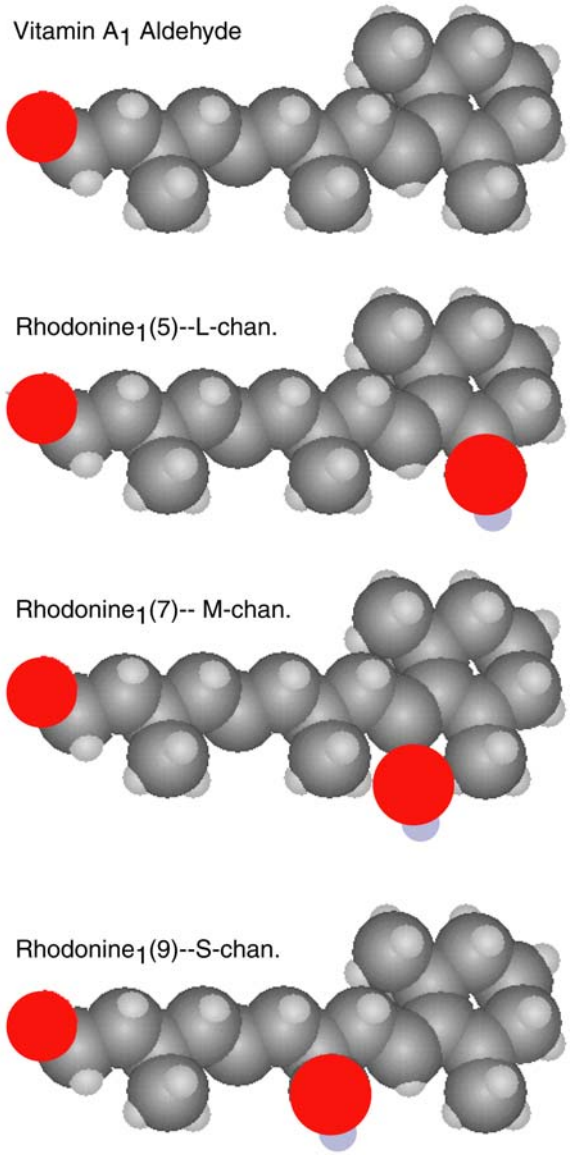

Figure 5.5.10-19 Filled molecular models of the Rhodonines showing the potential impact of the ionone ring on the resonances of the conjugated structures ending at the oxygens located at the 5th and 7th carbons.

No theoretical, or experimental data obtained at the molecular level, could be found that addresses this question. Confirmation of the precise *in-situ* band edges is currently underway (2008) for the L-channel chromophores in the laboratory of Harald Babucke. The work is employing sampling filters with widths in the range of 5-10 nm to ensure minimal smoothing of the expected overall Fermi-Dirac Functions.

An alternate configuration for the vitamin A1 alcohol (retinol) has appeared based on x-ray crystallography experiments (**Sections 7.1.1.4 & 5.5.8.2**). It is shown that retinol is not a planar molecule when in the liquid crystalline form used in their experiments. Their data shows the β-ionone ring with a dihedral angle of the C5-C6 double bond approximately 60 degrees relative to the C7-C8 carbons. of the aliphatic chain. This angle would shift the position of the oxygen associated with the C5 carbon in Rhodonine$_1$(5) significantly closer to the oxygen associated with the C15. More analysis is required. However, if correct, this condition would suggest a peak wavelength for the L–channel chromophore closer to the value promoted by Thornton, about 610 nm (**Section 5.8.3.1**). The peak wavelengths of the other chromophores would not be changed substantially.

5.5.10.5.2 Potential improvements in the calculated quality factor of the Rhodonines

The above figures justify the reliance on the theory of this work related to the chromophores of vision. What is yet to be quantified is the quality factor of the Outer Segments. MacNichol commented on the relative difference in the width of the absorption spectra presented by Marks. MacNichol and Bowmaker, et. al. both noted the long wavelength absorber had a narrower spectrum than that of the middle wavelength absorber. This quality factor is usually described by the equation $\omega = \omega_o (1 +/- 1/2Q)$ where ω is a unit of spectral frequency. Alternately, it can be expressed by $\lambda = \lambda_o(1 +/- 1/2Q)$ where λ is in wavelength. **Figure 5.5.10-20** illustrates the impact of selecting the value of Q when the criteria is based on wavelength. The nominal center wavelengths were 342, 437, 532 and 625 nm. Note the significantly different amount of overlap between the spectra. The curves for Q = 4.8 most closely match the curves of Douglas, Bowmaker and Kunz given above. As the available laboratory data continues to improve in precision, it will be possible to choose a more precise value for Q

and it may be possible to narrow the +/-2 nm tolerance on the nominal peak wavelengths. Once the methodology question is resolved, it may also be possible to assign quality factors to the Outer Segments based on phylum and/or species.

The ancillary peaks related to the Bezold-Brucke and the Purkinje Effects are particularly relevant to determining the precise amount of crossover between the spectral absorption characteristics. A putative peak near 390 nm in the aphakic human eye would also be very useful in evaluating these parameters. See **Section 17.2.4.**

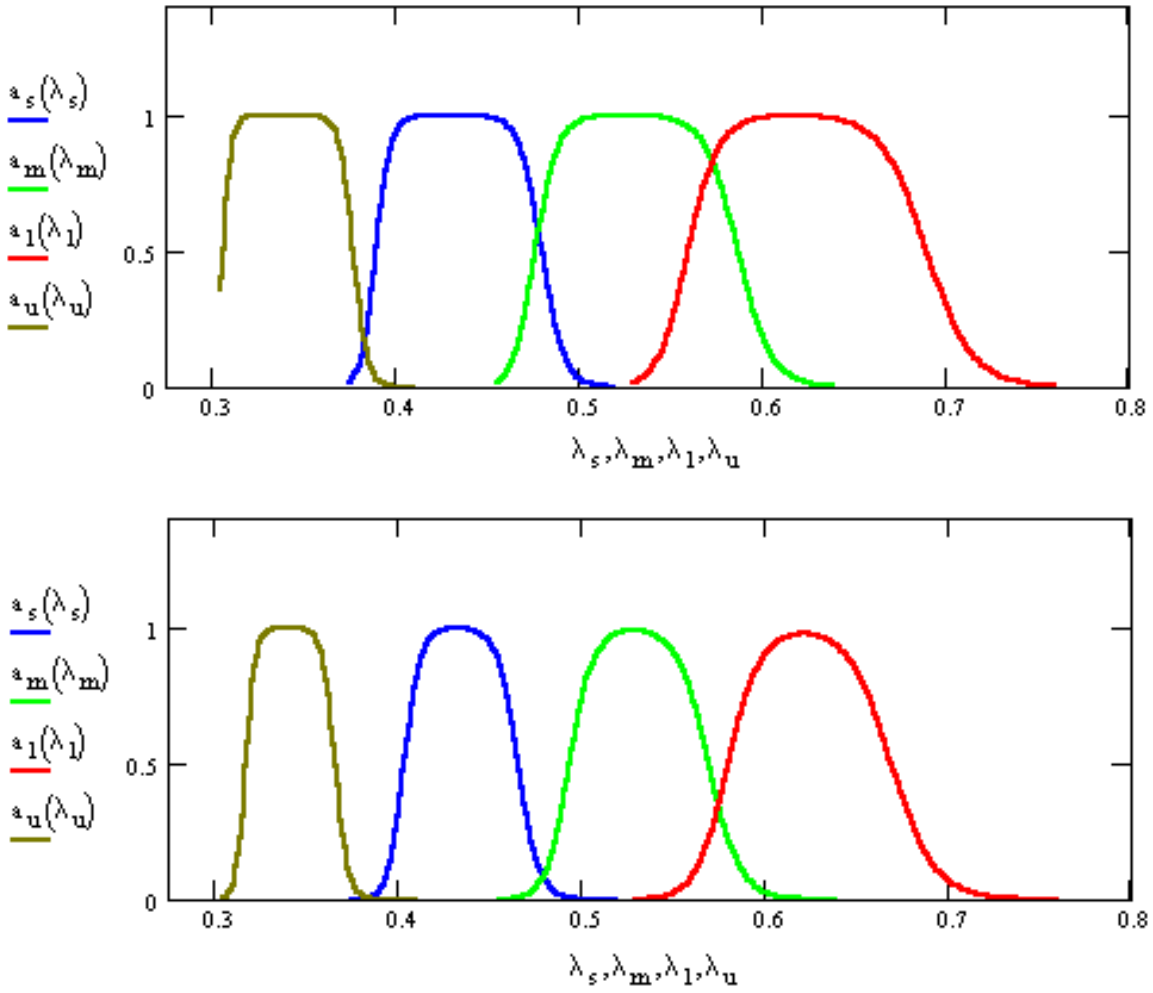

Figure 5.5.10-20 Defining the quality factor associated with the chromophores of vision. Upper curves are for a Q of 4.8 based on wavelength. Lower curves are for a Q of 7.0. Nominal peaks are at 342, 437, 532 and 625 nm.

5.5.10.6 Inferences from Genetics

This section is designed to address the current work in genetics as it applies to the chromophores of vision. **Section 4.3.5.4** addresses the question of the genetic code as it applies to the protein substrate, opsin. There is reason to consider merging these sections in the future. This section will present some fundamental background related to research into the genetic code and an overview of what work has been presented.

Genetics is becoming a powerful force in understanding the nature of the normal and abnormal human. With the transcription, and subsequent interpretation of the genetic code, many questions will be answered about vision.

However, at the current time, matching genetic information with the results of behavioral experiment is the ultimate inferential science. In specific cases, experiments are now being conducted (even on humans) to confirm the inferences between specific readings of the genetic code and specific diseases. These experiments are being approached with the appropriate caution since there may be many undocumented promoters, enhancers and silencers associated with these genes that are not yet recognized in the laboratory. An interesting experiment has been performed. Shichi states, "The opsin synthesized in a wheat germ system containing mRNA for the opsin has the identical amino-terminal sequence to that of rod membrane-associated rhodopsin but the N-terminal methionine is not acetylated. Therefore, acetylation of the N-terminus occurs as a posttranscriptional process."[227] If this is true, a straight forward reading of the genetic code may not describe rhodopsin.

5.5.10.6.1 The genetic code for the casual reader

Watson & Crick first defined the genetic code by its structure, the famous double helix.. They went on to show that each helix consisted of a long string of amino acids. However, the string only contained four types of amino acids (called bases in that community), adenine (A), cytosine (C), guanine (G) and thymine (T). Each helix consists of a very long string of these amino acids along with a sugar and a phosphate group. The name for the complete entity is deoxyribonucleic acid (DNA). Individual sequences (called genes) within each helix defined the code necessary for the ribosomes of a cell to create a specific protein. These codes are copied and transported to the ribosomes by a simpler nucleic acid, ribonucleic acid (RNA). Ribose is the sugar. RNA is slightly different than DNA. In place of the base, thymine (T), it uses one of its close relatives, uracil (U). It typically has a length of 80 to a few thousand bases.

Jones summarized the gross properties of both nuclear DNA (representing the full genome) and the more specialized mitochondrial DNA (representing a specialized region of the genome) in 2001[228]. Using a library analogy, he compares DNA to a small library while RNA can be compared to a pamphlet in that library. "The DNA within the nucleus of the human cell is enormous, made up of 2-3 billion base-pairs distributed among twenty-three pairs of chromosomes, and coding for between 30,000-40,000 genes." The complete human genome is currently under intense attack, with the goal of a complete listing before 2005. The entire human mitochodrial DNA was sequenced in 1981 and contained 16,569 base-pairs. It was first listed, with great fanfare, in *Nature* for April 1981. Printed above portions of the listing describing the specific protein associated with that segment of code (that gene). Other portions were boxed in to represent specific command sequences used to implement specific parts of the gene code (genetic code).

The more focused DNA within a mitochondria contained

When deciphering the genetic code, each base in one of the DNA helixes is uniquely associated with a particular base in the other string. Each G on one string is paired with one C on the other. Each A is paired with a T on the other string. Thus, the second string can be considered the complement of the first, since each of its members is completely defined in terms of the first string. Each pair is labeled an unlike pairing.

The main purpose of the genetic code is to control the manufacture of proteins. The RNA acts as an intermediary between the DNA of the genetic code and the ribosomes that actually manufacture proteins. The ribosomes are made primarily of RNA and other proteins. To distinguish which is which, the RNA carrying the message is labeled "messenger RNA." Note that neither DNA or RNA are proteins.

For those interested in understanding the field of genetic code analysis without becoming immersed in it, Shapiro has presented a very readable primer[229]. He has defined a variety of terms used in the field along with equivalent terms in the vernacular. He uses the analogy of a long string of text, such as produced on a narrow strip of paper by an old teleprinter.

Specific codes
Within a gene as defined below, a specific protein is defined by a sequence of three-letter groups (triplets). GTG indicates valine; CAT, histidine; CTG, leucine; ACT, threonine; CCT, proline; and GAG, glutamic acid. With four possibilities in each location, the potential number of codes is 64. This is more than sufficient to encode the twenty amino acids usually found in animal proteins and allows for a variety of additional codes used for control purposes (much like control codes in a computer program).

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[227]Shicchi, H. (1983) Biochemistry of Vision. NY: Academic Press pg 107
[228]Jones, M. (2001) The Molecule Hunt. NY: Arcade Publishing pp 50-52
[229]Shapiro, R. (1991) The Human Blueprint: The race to unlock the secrets of our genetic script. NY: St. Martin's Press

General concepts
genotype– the observed spelling in the genome related to an observed body type.
phenotype– a body type relating to a specific portion of the genetic code
COS cell– xxx
transfected– xxx
Operational concepts
Cloning– inserting a snippet of DNA into a viral DNA at a controlled location, allowing the virus to reproduce and then removing all of the pieces corresponding to the original DNA snippet.
Codon– A three-letter group (triplet) defining a specific amino acid in a protein.
text cutters– restriction enzymes
text splicers– DNA ligases
text matching– hybridization. The basic process of
shotgun experiment– A nucleic acid experiment where the outcome of the experiment is not well controlled. A mixture of materials may be produced but with a predictable statistical variation in content
unlike pairing– The fact that a specific base is never associated with a base similar to itself
Control codes
Exon– the start of a protein-coding area, defined by the base sequence ATG
intron– an interruption in the code, defined by Shapiro, 1991, as the base sequence TG (Nathans, 1987, says GT).
stop– end of protein definition, defined by the base sequence TAA and two others
Sections of DNA
plasmids– a snippet of DNA, typically a few thousand bases
gene– that length of DNA, the protein code, lying between an exon and a stop code (except for that part defined by intron sequences) describing a particular enzyme (protein).
Second order codes
These are areas of the code that may lie outside of the boundaries of a specific gene (as defined above) but play a major role in controlling the actions promoted by that gene. They may lie upstream or downstream of the gene.
promoters– Sequences such as TATA (the TATA box) and CCAAT (the CAAT box), etc.
enhancers–
silencers–
There is currently a large amount of code that is not understood. It is generally called garbage, junk or nonsense. Some of it may be critically important to defining functions not currently understood. It is very dangerous to discount such code.

Important variations
The genes employ a significant amount of alternate codes to the above codes. These spelling differences come in two forms. Short changes in spelling are called polymorphisms. Longer changes can be called text differences. The formal name for the latter is restriction fragment length polymorphism (frequently abbreviated RFLP and spoken as rifflips) Text differences can be significant enough to deserve special study. These differences can be isolated by specific probes. Some of these differences can be the key to understanding certain genetic diseases. If some of these differences are present in the genetic material from both parents, the resulting condition can range from incidental to lethal.
Analytical tools
chain termination method– dideoxy method. A method of modifying one of the bases so it can participate in the formation of DNA but causes the copying process to terminate at that specific location.
probe– A section of DNA modified by man to exhibit some easily identified property (fluorescence is common). Used to locate its compliment in a single DNA string of a helix.
Interpretation tools
sequencing ladder– a two dimensional table showing the length of fragments obtained when the modified bases of the chain termination method are introduced into a DNA sequence. When completed, the ladder specifies the complete sequence of the DNA sample. Shapiro provides an excellent tutorial on reading these[230].
published transcriptions– The convention is to publish the compliment of the DNA code being discussed. This compliment is the actual code of the probe used to identify the DNA snippet.

As Shapiro points out, it is normal in genetics to first determine a change in the amino acids of a gene and only later (historically much later) decipher the genetic text that accompanies the change. This text may contain many polymorphisms that are not completely understood for an even longer time (such as the differences noted in Section 5.1.4.1). Shapiro has provided an interesting figure, credited to Drayna & White[231], describing the differences in the X chromosomes as it is passed to nine siblings by their mother. They are extensive.

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[230]Shapiro, R. (1991) Op. Cit. pg 89-94
[231]Drayna, D. & White, R. (1985) xxx *Science* vol. 230, pp 753-xxx

5.5.10.6.2 Brief summary of the literature

[XXX consolidate with section 4.3.5.4 or reference. one section applies to the substrate and cell, the other to the chromophores]
A key feature of the above technology is the fact that (as Watson first said) "DNA makes RNA makes protein." This idea was labeled the Central Dogma of biology by Crick in the late 1950's. The important point is that DNA itself does **not** specify fats, sugars, and retinoids. DNA defines proteins. Where necessary, the proteins must take other steps to acquire or manufacture the associated non-proteins, i. e., retinoids.

To date, the genetic literature is unanimously based on the trichromatic theory reflecting the operation of the human eye in object space. It does not reflect the fact that human retina (along with other chordate retinas) is known to be, and is demonstrably, tetrachromatic (see **Section 17.2.2**).

The terminology being used by the geneticists may also be a problem. They appear to be using the term rhodopsin to include three spectrally distinct chromophores (without regard for a fourth UV sensitive chromophore) that only vary in the arrangement of the amino acids in the opsin moiety. Other communities label such materials iodopsins with the term rhodopsin reserved for a fourth (or fifth) achromatic photoreceptor. The geneticists have not addressed the possibility of two types of achromatic photoreceptors, such as the red and green rods of the broader community.

Experimental work in the genetics of vision requires a broader understanding of the functional performance of vision. Any inferential relationship between the human genes and the chromophores of vision must accommodate four spectrally distinct narrowband chromophores of vision (including an ultraviolet, UV, sensitive chromophore). If a truly achromatic rhodopsin is also needed, the total number of genes to be located must equal five.

There has been significant effort expended in trying to relate the genetic code to the simpler conditions of color blindness. However, the papers reviewed below have consistently taken a very elementary view of the operation of the visual system. They have assumed that all failures related to abnormal color vision are based on the chemistry of the chromophores. **Section 18.1** provides a more comprehensive description of the types and potential sources of such failures. **Section 18.1.5.6.4** documents the very important work of Lakowski. Lakowski documents the variety of failures that can occur within the neurological portion of the visual system.

While the papers reflect the highly sophisticated techniques used in DNA and protein analysis, they reflect the use of only the simplest of spectral absorption techniques. Only simple numbers reflecting the absorbance over a one centimeter distance have been reported, rather than absolute absorption coefficients defining the absorption per unit of material present. This matter is extremely important because the chromophores of vision are not present in low concentrations solutions. They are present in highly organized, but extremely thin, layers. While the absorptions reported by the geneticists are typically 0.05-0.20 per cm, the actual absorptions of the chromophores when configured operationally exceed 0.6-0.8 in 50 microns. Thus, the actual absorption is a factor of 500:1 higher in the operational case than in the reports.

The material also appears to use the term solubilization as if it produces a true solution. However, the protein and retinoid materials involved are not water soluble. They do not dissolve in water, even in the presence of detergents and other dispersing agents. The result is the dispersal of the insoluble material into a suspension. Such a suspension can be expected to introduce considerable scatter into any light beam projected through a test cell.

A number of groups have undertaken the task of defining the chromophores of vision based on the genetic code. One of the earliest was Nathans[232]. His papers of the mid 1980's rely upon the conventional trichromatic theory and the putative three forms of rhodopsin. He proposed three specific genes that he inferred were related to three chromophores of vision. No UV gene was identified. The most prolific has been the team consisting of Neitz & Neitz and their associates. More recently, a team led by Oprian has played a major role in the discourse. There are significant differences in the proposed structure and characteristics of the chromophores among these papers. Not all of these can be addressed here. It is indicative of the early stage of the work in this area and the dogged attempt to associate this work with the conventional wisdom of the tricrhomatic theory and the putative rhodopsin molecule.

The 1986 paper of Nathans, Thomas & Hogness include considerable background aiding in identifying their four genes of vision (rhodopsin, red, green & blue) in the larger context of the genome. However, they rely upon the trichromatic theory of the conventional wisdom. They include base sequences (and amino acid equivalents) for their red, green & blue genes. They do not provide such a sequence for rhodopsin or the UV channel of human vision (See Section 17.2.2). Their discussion includes a quandary concerning the number of genes for color vision found in

[232]Nathans, J. (1987) Molecular biology of visual pigments *Ann Rev Neurosci* vol. 10, pp 163-194

their subject, J. N, and the conventional wisdom of the trichromatic theory and their perception of abnormal color vision. The words "must," "surprising" and "presumably" enter the discussion at this point.

Another 1986 paper by Nathans, Piantanida, Eddy et. al. explore the genetics of color blindness they continue to rely upon a very simple trichromatic theory and do not recognize a parallelism between the luminance and chrominance channels of vision[233]. Their analysis of color defects does not distinguish between anomalous trichromats, and the multiple forms of incomplete trichromats (functional trichromats and functional dichromats). This leads them to associate all forms of red-green color blindness with chromophore substitutions, even though complete deutranopes are known to exhibit normal luminance threshold functions at all wavelengths. They do not address the existence of a UV chromophore in humans.

The 1987 Nathans paper is one of the earliest reviewing the relationship of the genetic code to the form of the chromophores of vision and includes many references to other areas of vision. It includes several code sequences associated with three entrons he believes relate to the rhodopsin molecule. Nathan notes that one school of analysis associates color blindness with chromophore deficiencies while another associates the problem with errors in the neural circuitry. However, his paper focuses on deficiencies in the chromophores. He presents a "simplified" explanation of color blindness wherein a total loss of the M–channel chromophore results in deuteranopia while a similar total loss of the L–channel chromophore results in protanopia. These are not the definitions usually found in the vision literature (see **Section 18.1.2.2**) and the absence of the M-channel chromophore has not been reported in the psychophysical or physiological literature. While he associates the human visual process with four light sensitive pigments, he defines these as one achromatic (apparently broadband) pigment and three narrower band chromatic pigments. He does not address the presence of an ultraviolet pigment in human vision (see **Section 17.2.2.1**). The paper does not address the spectral impacts of any errors in the genetic code. Nathans does introduce the proposal that the L–channel chromophore may involve a cyanine like resonant retinoid. However, he does not represent that the retinoid contains two nitrogen atoms. His baseline proposal is that the genetic code controls the expression of the protein (opsin) portion of the putative rhodopsin molecule. He provides the genetic code believed to correspond to a small portion of the complete opsin molecule.

Nathans does introduce the similarity of the chromophores of vision and the cyanines of dye chemistry, although he does not explicitly recognize the requirement for two atoms of either oxygen, nitrogen or phosphorus in a successful chromophore. His conclusion is that only the long wavelength chromophore resembles a cyanine while the others remain polyenes. His figure 4 (from Suzuki, 1967) is interesting in that he shows the nominal peak absorptions of a series of polyenes and cyanines. The uniform progression of wavelength shift with conjugation level of both the polyenes and cyanines are shown. The particular cyanines shown have peak absorptions at 520, 627 and 734 nm. No absorption is shown in the 560 to 575 nm region. The magnitude of the shift for the cyanines is very similar to that of the visual chromophores and about three times larger than for the polyenes.

Nathans also presents a series of genes showing the extensive base differences he predicts between rhodopsin and the distinct narrowband chromophores for the S–, M– & L–channels.

The 1992 Merbs & Nathans paper addresses anomalous color vision based on a dichotomy, the possible complete absence of either the L–channel or M–channel chromophores of vision[234]. Their definition of a complete deutranope as one completely lacking a green, or M–channel, chromophore does not conform to the original definition of the term or as it is used in this work (**Section 18.1**). No report has been found in either the electrophysiological or psychophysical literature of any sighted human, color-blind or not, who totally lacked an operational M–channel in his visual system. At photopic levels of illumination, the most chromatically limited deutranope exhibits a luminous threshold function within the normal statistical variation of color normals.

Merbs & Nathans present the thesis that "Dichromacy and anomalous trichromacy are caused by unequal homologous recombination with the tandem array of genes that encode the red and green visual pigments." This statement suggests that unequal homologous recombination is the only cause of these chromatic shortcomings. Such a position does not recognize the other demonstrable failure modes associated with abnormal color vision.

The 1993 Neitz, Neitz & Jacobs paper is very important and references a large number of papers[235]. It summarizes

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[233]Nathans, J. Piantinida, T. Eddy, R. Shows, T. & Hogness, D. (1986) Molecular genetics of inherited variation in human color vision *Science*, vol. 232, pp 203-210

[234]Merbs, S. & Nathans, J. (1992) Absorption spectra of the hybrid pigments responsible for anomalous color vision *Science* vol. 258, pp 464-465

[235]Neitz, J. Neitz, M. & Jacobs, G. (1993) More than three different cone pigments among people with normal color vision *Vision Res*. vol. 33, no. 1, pp 117-122

the changes in their thinking from that in their earlier papers on page 120. This summary is a caution to those reading their earlier papers. It also defines the conventional wisdom under which they were working at that time. They support the trichromatic theory approach and the rhodopsin hypothesis (the chromophores are formed as a molecule containing a protein opsin and a retinoid). However, they claim a variety of differences in specific chromophores that result in small shifts (3-6 nm) in peak absorption among the chromophores of individuals with otherwise normal vision. They also ascribe these differences to the protein part of the putative rhodopsin molecule. In a 1990 paper, they describe how they have collected psychophysical data on essentially color normals who exhibit small differences in Rayleigh matches. The protocol and instrumentation are not sophisticated by modern standards. This data was used in support of the 1993 paper.

This work suggests the portions of the genetic code ascribed to controlling the expression of the chromophores of vision should be associated with an entirely different mechanism than suggested by Neitz, et. al. Since it is shown in Chapter 7 that opsin and the retinoid portions of the visual process are formed in entirely different location within the body, it is not logical to associate potential changes in opsin with changes in the spectral performance of the chromophores of vision. This work shows it is the retinoids themselves that are changed to exhibit different spectra. But, the retinoids are not proteins. The method of changing the chromophores must be tracked back to a protein intermediary. Two such intermediaries are known. The first is the plasma transport protein, TTR. If this protein exists in four forms, it can cause the delivery of four different chromogens (the rhodonines) to the RPE. These chromogens can then be transported to the disks of the outer segments and be converted to chromophores by their selective conversion to the liquid crystalline form. The second is the IPM transport protein IRBP. This protein transports the chromogens from the RPE to the disks of the outer segment (see Nickerson later in this section).

Under this scenario, the small changes of 3-6 nm between the spectral performance of individuals is accounted for by other (possibly genetically controlled) changes in the signal processing system of stage 2, and possibly changes in the physiological optics of stage 0, rather than in the chromophores themselves.

The literature contains a number of other references to the matching of parts of the genetic code to the putative three variants of rhodopsin. Oprian, et. al[236]. have followed a different path of research. However, it appears to remain wedded to the trichromatic theory and the concept of rhodopsin as a single molecule. They have also discussed the quandary resulting from the assumption that all three pigments of human vision result from only one putative "11-*cis*-retinal chromophore." They indicate their future activity will focus on resolving this dilemma. Many of their matches have involved comparing the genetic material of siblings where one is known to be color blind, generally red-green color blind.

Oprian, et. al. relied upon an early Neitz & Neitz paper of 1986 to define the location of three genes responsible for the chromophores of vision. A "blue gene" was localized to an autosome, chromosome 7. It is not clear what led them to chromosome 7. The inference appears to rely upon the fact that blue color deficiencies are known to be inherited autosomally. Two genes were localized to the X-chromosome. These were identified as green and red because inherited green/red color vision deficiencies are known to be X-linked. They note, "The identification of the color vision genes rests largely on indirect evidence." They did note that the genes they focused on were 350-380 base units long and all ended in a specific sequence including a carboxyl group.

Their paper claims to demonstrate that these genes do in fact code for three proteins that when reconstituted (incubated) with 11-*Cis*-retinal and "purified" have absorption spectra, apparently in dilute solution, that have maxima at 424, 530 and 560 nm. The incubation procedure allows for many uncontrolled chemical reactions. Some of these can convert the retinal into an indicator substance. This makes the pH of the solution of major significance when performing spectroscopic examination. Hubbard showed similar shifts in spectral peaks to those of Oprian, et. al. in the 1950's by changing the pH of the incubated retinal containing solution. The Oprian absorbances were determined using a 1.0 cm cell length at 4° Celsius. The absorbance of the red solution was 3:1 less than the green solution. They did not search for or identify any gene coding for the ultraviolet sensitive chromophore of human vision (see **Section 17.2.2**).

Oprian, et. al. did confirm that each of their genes they described in detail (including their sequence) contained the same terminal sequence. This sequence of eight amino acids is known to be identical to the terminal sequence of opsin (putative portion of rhodopsin) at its carboxyl end. However, they did not discuss how many other proteins exhibited this same terminal sequence.

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[236]Oprian, et. al. (1991) Op. Cit. pg 11371

Zhukovsky, et. al. have provided two papers from the Oprian school[237,238]. These focus on the proposal that Glu^{113} acts as a counterion needed to bring the absorption spectrum of the putative Schiff-base to near 500 nm (contrary to earlier studies that showed a counterion could not accomplish this). They conclude "although these data are also consistent with a model in which Glu^{113} provides a crucial tertiary interaction in the protein that brings a different anion into contact with the Schiff base." The second paper claims the counterion approach was shown to be correct in the first paper without addressing the above caveat. This paper opens with the premise that "Rhodopsin and the visual pigments are a distinct group within the family of G-protein-linked receptors in that they have a covalently bound ligand, the 11-cis-retinal chromophore, whereas all of the other receptors bind their agonists through noncovalent interactions." The paper attempts to show, inconclusively, that the covalent bond between the retinoid and the protein is not required. Both papers appear to involve dilute solutions.

Ma, et. al. presented two papers in 2001 based on the salamander[239,240]. The papers are very broad but contain a number of important proposals. First, they conclude that the morphologically defined green rod and the S–channel cone contain the same chromophore. They did not demonstrate the corollary, that the morphologically defined red rod and the M–channel cone contain the same chromophore. They were not able to isolate the M–channel chromophore. They define four chromophores of vision corresponding to the UV–, S–, red rod and L–channels. Their studies were complicated by the use of larval stage salamander that exhibited a mixture of Vitamin A_1 and Vitamin A_2 based retinoids. The Ma, Kono, et. al. paper suffers from a criticism, apparently raised during the review process, that their proposed UV–chromophore is in fact only retinol in solution. Their spectra for their proposed reconstituted UV cone pigments in a phosphate buffered saline solution are very similar to the narrow spectral peak (peak near 356 nm and FWHM bandwidth = 50 nm) associated with Vitamin A_1 and Vitamin A_2 in methanol as presented by Sporn and shown in [**Figure 6.2.1-1**]. It is also similar to a variety of spectra associated with other of isomers of retinol in hexane[241]. It does not show the broad spectral peak (FWHM bandwidth =100 nm) near 342 nm expected of the *in-vivo* UV chromophore (see **Section 6. xxx**). It appears their spectral sample involved a physical mixture of opsin and a retinoid and not a true compound. They claim that the samples were compounds because when 1μl of concentrated HCl was added to 90 μl of the sample, they exhibited the spectrum of a "protonated Schiff base free in solution." These ostensibly free compounds exhibit a peak absorption at 443 and 470 nm respectively. However, the authors did not demonstrate the presence of a free protonated Schiff base by any other means. Their claim that their material shows the same spectral peak as other attempts to isolate UV chromophores in solution is understandable. However, the in-vivo chromophores show a peak absorption near 342 nm, not 356-360 nm (see **Figure xxx**). This paper did not draw many conclusion but presented a number of options for further study. It did present a listing for the bases in the proposed gene for the UV chromophore of salamander and its amino acid equivalent.

The Ma, Znoiko, et. al. paper begins with a thought provoking Summary. They seek to show that the response of rods and cones cannot be correlated with a topological designation or by their putative associated transducins. The paper tends to use the term opsin, as opposed to rhodopsin. Although discussed indirectly, the conclusion can be drawn that they claim the S–channel cone and the "green rod" are the same entity. They claim that "the spectra of green rods and blue-sensitive cones were nearly identical." They also claim several features supporting the conclusion that these cells are functionally identical. "First, recognition of a 14 amino acid, N-terminal epitope of the pigment is shared by green rods and blue-sensitive cones. Second,380 base pairs of the mRNA's encoding the pigments are also identical. Third, cell spectra, which reflect the opsin-chromophore interactions and are very sensitive to the opsin's tertiary structure, are the same in both cell types." See **Section 3.1.5.3** for a discussion of the origin of red and green rods. This paper follows the Ma, Kono, et. al. paper in defining four spectral channels in the salamander. They are identified as the UV–, S–, red rod & L–channels. They claim that they and others have cloned the opsins relating to each of these channels.

Ma, Znoiko, et. al. also provide data on the effect of adding hydroxylamine to one of their samples at 4° C. The change of the absorption spectrum with time is reminiscent of other tests involving the change in pH of a chromophore. It once again demonstrates that the chromophores of vision involve a retinoid with the complexity

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[237]Zhukovsky, E. & Oprian, D. (1989) Effect of carboxylic acid side chains on the absorption maximum of visual pigments *Science* vol. 246, pp 928-930

[238]Zhukovsky, E. Robinson, P. & Oprian, D. (1991) Transducin activation by rhodopsin without a covalent bond to the 11-cis-retinal chromophore *Science*, vol. 251, pp 558-559

[239]Ma, J. Kono, M. et. al. (2001) Salamander UV cone pigment: sequence, expression and spectral properties *Visual Neurosci* vol. 18, pp 393-399

[240]Ma, J. Znoiko, S. et. al. (2001) A visual pigment expressed in both rod and cone photoreceptors *Neuron* vol. 32, pp 451-461

[241]Dawson, M. & Okamura, W. (1990) Chemistry and Biology of Synthetic Retinoids Boca Raton Fl: CRC Press fig. 2.

and form of an "indicator." Within the context of this work, the chromophore, rhodonine(9) shifted its spectral peak from its functional (anisotropic) peak at 432 nm to its intrinsic (isotropic) peak at 350-360 nm.

The two Ma papers do not discuss the visual appearance of their spectral samples. However, the absorbance levels they report are very low (compared to the peak spectral absorptions of in-vivo photoreceptor cells which are in the 80% range). The measurement of absorbance values in simple spectral cells may not be meaningful due to the fact the sample material does not participate in a true solution.

Hagstrom, Neitz & Neitz have recently presented another paper[242]. Their figure 1 shows how their protocol does not accommodate the presence of a "rod" or of a UV sensitive cone. If they do not allow for the existence of something, they certainly will not isolate it. They do introduce a difference into their analyses compared to earlier papers. They said, "For more than a dozen years it has been known that, contrary to expectation, there is variability in the number of cone pigment genes on the X chromosome. A rich variety of pigment gene arrangements occur in the population, which can include multiple M and multiple L photopigment genes in a genetic array tandemly repeated array." They did not show that any of the variations actually affected the absorption spectrum of any chromophore. Interestingly, they conclude that "the number of L cones outnumbered the M cones in the four subjects that we examined." Their reference to tetrachromatic vision refers to marginal variations in L-channel absorption spectra rather than a UV absorption channel.

Sharpe et al. made an attempt to identify the genes of color vision in 1999[243]. They found multiple copies of genes related to the photoreceptor cells on the q-arm of the X-chromosome at or near Xq28. Other relevant genes were found on the q-arm of the third chromosome near 3q21.3 and 3q24 and the q-arm of the seventh chromosome near 7q31.3 and 7q32.

Neitz & Neitz provided another review in 2000 where they noted, "The molecular genetics of color vision has turned out to be much more compex that originally suspected[244]."

As developed in **Chapter 18**, it is important to differentiate between color blindness due to the absence of a chromophore and color blindness due to a signal processing anomaly. These two different types of disorders may be due to two different genetic problems, or other medical reasons.

Some of these inferential efforts have found it necessary to define four genetic paths, representing proteins associated with three chromatic paths and one achromatic path. Unfortunately, there is no achromatic photoreceptor type and there are actually four variants of the visual chromophores, instead of the commonly recognized three. The fourth is in the ultraviolet and known to be present in the human retina. Further, these chromophores are not proteins. As shown in this work, the protein opsin may come in many varieties but such a variety would likely have little or no effect on the chromophore coating it.

The fact that there are four saline-based chromophores and the experimental evidence that they appear in at least two distinct but overlapping sets of three should be important in inferring the actual relationship between the genes and the chromophores. Comparing the genetic code for the common chromophores between the ultraviolet sensitive members of *Arthropoda* who are believed to lack a L–channel chromophore and the members of *Mollusca* and *Chordata* who do exhibit L–channel sensitivity should prove beneficial. Since most members of *Chordata* are known to be tetrachromatic, focusing on the presence or absence of the ultraviolet chromophore may not be as fruitful. Recent data has confirmed that the system architecture of the human system is tetrachromatic. Our vision in the ultraviolet is limited primarily by the absorption of our lens group.

Very recently, a large group in Japan[245,246] presented their inferential studies that attempted to trace the molecular phylogenetic tree of vertebrate visual pigments. They showed a figure containing four chromatic limbs and one achromatic limb associated with a putative rhodopsin. They used a mixture of amino acid sequencing and deduction

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[242]Hagstrom, S. Neitz, M. & Neitz, J. (2000) Cone pigment gene expression in individual photoreceptors and the chromatic topography of the retina *J Opt Soc Am A* vol 17(3), pp 527-537

[243]Sharpe, L. Stockman, A. Jagle, H. & Nathans, J. (1999) Opsins, genes, cone photopigments, color vision, and color blindness *In* Color Vision: From Genes to Perception. Cambridge, UK: Cambridge Press pp 3-51

[244]Neitz, M. & Neitz, J. (2000) Molecular genetics of color vision and color vision defects Arch Opthalm vol 118, pp 691-700

[245]Hisatomi, O. Satoh, T. & Tokunaga, F. (1997) The primary structure and distribution of Killifish visual pigments. *Vision Res*. vol. 37, no. 22, pp. 3089-3096

[246]Tokunaga, F. et. al. (1999) Evolution of visual pigments and related molecules. In rhodopsins and phototransduction. NY: John Wiley & Sons. pp. 44-53

in the absence of sequencing. They came to an interesting conclusion that the original ancestral chromophore branched into a pair of chromophore groups. One of these they labeled the L-group. In their nomenclature, both the human red and green chromophores were members of their L-group. Subsequent branching produced their S– group (which contained blue, violet, & UV pigments), the MS– group (made up of blue pigments), then the ML– group (containing mostly green pigments), and finally the Rh– group containing unlabeled but apparently achromatic chromophores (based on rhodopsin). Their analysis is not supported by this work.

Another group led by Nickerson[247] has been exploring the retinoid-binding protein, IRBP, found in the IPM from a genetic perspective. They have found that the numeric "four" plays an important role in the genetics of IRBP in humans. They discuss the fourfold "repeat" of a portion of the gene associated with IRBP. They also discuss the fact that the repeats are 80-90% identical cross-species and 30-40% identical intraspecies. They propose three tentative models for the genetic evolution of IRBP. At one point they also note the presence of two separate populations of human IRBP within the IPM based on Edman degradation.

While Oprian, et. al. highlight the fact that each of their gene codes specifies a specific sequence that is the same as that near the carboxyl terminal of opsin, they do not discuss the fact that this terminus is not critically important to the operation of the chromophores in the rhodopsin concept (based on the caricatures of Dratz & Hargrave[248]). Nor do they discuss how many other proteins exhibit this same terminus. In the case of SRBP and IRBP (see **Section 7.1**), the presence of the carboxyl group would provide a ready source of oxygen for converting the retinal (a precursor of the chromophores) into the rhodonines (the chromogens in dilute form and the chromophores of vision when in liquid crystalline form). It is quite possible that opsin, SRBP and IRBP all exhibit a carboxyl terminus with the same sequence of eight amino acids next to it. The fact that the transport protein SRBP is discarded after delivering retinal to the RPE is a significant indication that it has provided an oxygen atom to the retinal, converting it into a rhodonine, during the transport operation.

It remains an early day in correlating the chromophores of vision with the genetic code. However, an alternate explanation of the fourfold repeat would suggest that each fold is related to one of the individual chromophores of vision. This explanation does not include any hierarchal inference and it does not suggest any evolutionary aspect. The same fourfold repeat is found in the earliest animals as suggested by the high cross-species factor mentioned above.

5.5.10.6.3 The limited scope of a recent genetic database from HUGO

The Human Genome Naming Organization (using the acronym, HUGO) has produced **Figure 5.5.10-21** to describe the genetic code database as currently accumulated within the research community[249]. The latest data input related to the Xq28 chromosome is from the early 1990's without subsequent corroboration.

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[247]Nickerson, J. Borst, D. Redmond, T. Si, J-S. toffenetti, J. & Chader, G. (1991) The molecular biology of IRBP: application to problems of uneitis, protein chemistry, and evolution. *In* Molecular Biology of the Retina: basic and clinically relevant studies. NY: Wiley-Liss, Inc. pp. 139-161

[248]Dratz, E. & Hargrave, P. (1983) The structure of rhodopsin and the rod outer segment disk membrane *Trends Biochem Sci* vol. 8, pp 128-131

[249]http://www.genenames.org/genefamilies/GPCRA#OPN

11) Opsin receptors :

Approved Symbol	Approved Name	Previous Symbols	Synonyms	Chromosome
OPN1LW	opsin 1 (cone pigments), long-wave-sensitive	CBBM, RCP, CBP	COD5	Xq28
OPN1MW	opsin 1 (cone pigments), medium-wave-sensitive	GCP, CBBM, CBD	OPN1MW1, COD5	Xq28
OPN1MW2	opsin 1 (cone pigments), medium-wave-sensitive 2			Xq28
OPN1SW	opsin 1 (cone pigments), short-wave-sensitive	BCP	BOP, CBT	7q32.1
OPN3	opsin 3	ECPN	ERO, NMO-1, encephalopsin, PPP1R116	1q43
OPN4	opsin 4		MOP, melanopsin	10q22
OPN5	opsin 5	TMEM13	neuropsin, dJ402H5.1	6p12.3
RGR	retinal G protein coupled receptor		RP44	10q23
RHO	rhodopsin	RP4	OPN2, CSNBAD1	3q22.1
RRH	retinal pigment epithelium-derived rhodopsin homolog		peropsin	4q25

Download gene family data

Figure 5.5.10-21 The identified Opsin receptors of vision curated by the HUGO organization, ca. 2016. After more than 80 years on intensive research, this listing is obviously incomplete and inconsistent. See text. Other than the HUGO organization approving 5 generic names for opsin 1 through opsin 5, the data presented shows no relationship to a rationale description of either the genes or the resultant receptor molecules of the sensory neuron receptors of the visual modality.

To suggest this database is incomplete after more than 80 years of intense empirical research within the community is clearly an understatement. While HUGO asserts the data is curated, that term appears to be used to indicate there is no obvious conflict between the data entries. It includes no row related specifically to the putative rod pigment(s) of vision. It includes no row relating to any ultra-violet sensory receptor (found throughout the animal kingdom and demonstrably present in human eyes, **Section 7.2.5**). After the first three rows, the chromosome listing shows no common element related to the visual modality. The entries below the first four are not demonstrated to relate to any specific function within the signal generating (stage 1) neurons of the eye or other neural tissue of the visual modality. The hyperlinks on the left lead to extensive descriptions of the source of the listings but little data suggesting the relevance of the entries.

HUGO provides another table labeled GPCR class A, orphans where “class A” refers to approved symbols for “opsin receptors.” It contains over 50 entries of G protein-coupled receptors in a sparse numerical listing unrelated to any specific function withn the visual modality.

5.5.10.7 Analysis of the connection of genetics and vision

The introduction of nearly every paper on the genetics of vision defines the Young-Helmholtz Theory of vision and the putative rhodopsin molecule as its baseline. Only recently have a number of papers concentrated on the term opsin, in lieu of rhodopsin. These papers uniformly suggest that incubating opsin and 11-cis-retinal results in the spontaneous formation of a (generally protonated) Schiff-base linkage at the highly protected e-amino group of Lysine[296] of the opsin. No justification for this position was offered except for references to earlier unverified work (see **Section 5.5.2.1**). The discussions of color abnormalities lacked a clear definition of protanopia and deutranopia, and generally lacked any discussion of tritanopia or tetartanopia (see **Section 18.1.2**). No discussion of the potential lack of a chromophore on the luminous threshold function, $T(\lambda,F)$, as compared with the chrominance threshold function, $C(\lambda,F)$, could be found among the discussions. While several papers alluded to the total absence of the M–channel chromophore in deutranopes, no justification for, or documented case supporting, this potential condition was offered. No such condition is reported in the general literature.

No paper found in the genetic literature contained any graphic describing the signal flow within the visual system. Only one paper even commented on the possibility, and the belief by others, that color vision abnormalities might arise within the neural system. Uniformly, the papers support the position that the primary source of color abnormalities is the shift in the peak absorption wavelength of one or more chromophores in response to polymorphism within the appropriate genes. No discussion of the possibility that a variation in amplitude of the signals appearing in the neural system and associated with the normal chromophores could account for the abnormal perception of color could be found.

The early papers focused on the existence of three “cone” chromophores and one “rod” chromophore in human

vision. No recognition of the ultraviolet chromophore of human vision has been encountered (see the work of Griswold & Stark in **Section 17.2.2** and Wright (1952) in **Section 17.3.2**). On the assumption that there are only three narrow spectral band chromophores in human vision, only three genes related to these chromophores have been defined. In the broader context, recent papers have described four "cone" chromophores and two "rod" chromophores among chordates. The latter are interesting because one is described as a "red rod" and the other as a "green rod." Again, no graphic, even in caricature, has appeared to explain the operational significance of these two "rods."

The current literature appears to adopt the view that the retinal portion of rhodopsin remains unchanged among the various chromophores except for the electronic impact on the protonated Schiff base between the retinal and the opsin. This impact was studied to exhaustion in the 1950's, without showing how such impact could successfully generate the absorption spectra found in biological vision. Bownds first proposed the lysyl amino acid as a possible location of the Schiff base in 1967. He was unable to demonstrate it was the junction point with the retinal moiety. With a better understanding of the sequence of amino acids in opsin, it is being studied again. The recent papers have not attempted to explore this impact, with respect to all four spectral channels, in a comprehensive, and quantum mechanical, manner (see **Sections 5.3.4.2** & **5.5.2.1**).

Recently, Ma, et. al. have investigated a gene they feel is associated with the UV sensitive chromophore of biological vision[250]. The complete gene sequence is given. The paper did not specify the precise location of the gene. However they did experiment with both the A1 and A2 variants of the retinoid. Their figure 3 can be compared to [**Figure 6.2.1-1**] and the data of Hubbard & Wald in the sections listed in the previous paragraph. The effect of pH on the absorption spectrum of the pure retinals, in the absence of any opsin, is well documented. Figure 3 appears to reflect these same spectral shifts irrespective of the incubation of opsin and retinal. Their commentary on page 398, regarding the potential of a protonated versus unprotonated Schiff base, becomes moot if the chromophores are present in the liquid crystalline form, as suggested in this work.

5.5.10.7.1 Previous genetic inferences largely unsupportable

The inferences reviewed in **Section 5.5.10.6** lack strong experimental support.

The genetics field of the 1990's was predominantly concerned with static conditions, based primarily on small portions of the genetic pool that could be used to define proteins. The field has moved forward greatly since that time period and begun to understand both more dynamic processes under genetic control and potentially how molecules other than proteins are defined within the genetic code.

Currently, the genetic database related to the visual modality remains concerned with static conditions. It does not mesh with, (1) the dynamics of the visual modality associated with the extrusion of proteins, as opsins, by the inner segment of the photoreceptor neurons, (2) the dynamics of the retinal photoreceptor epithelium (RPE) that generates the chromophore molecules (the retinines) of the visual modality that form a liquid crystalline layer as they coat the opsin discs extruded by the photoreceptor neurons. See **Section 7.1** for an understanding of these dynamic processes.

Section 3.6 develops in considerable detail the fact that the photoreceptor neurons of *Insecta* do not employ a protein, such as opsin, to support the transduction process. It also develops in considerable detail that the receptor molecules are not related to the opsin proteins and thereby labeled rhodopsins. The database of Insecta strongly suggests the receptor molecules are liquid crystalline and of the retinine family, the rhodonines. The rhodonines of *Insecta* exhibit the same spectral performance as the rhodonines of *Chordata*.

While many proteins have been identified in the literature as mediating transduction in the visual modality, there has been no confirming evidence that they are involved in the transduction process. Each sensory receptor neuron employs tens to hundreds, or even thousands of proteins that can impact the operation of the overall neuron without necessarily impacting the transduction process directly.

After 20 years since their identification, the proteins previously associated with visual transduction in the literature lack any demonstration that they exhibit the spectral performance of the *in-vivo* mechanisms of the animal visual modalities.

The putative presence of a retinene buried within an opsin and aligned with its long axis perpendicular to the

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[250]Ma, J. Kono, M. et. al. (2001) Salamander UV cone pigment: sequence, expression, and spectral properties *Visual Neurosci* vol. 18, pp 393-399

incident light has not been shown to result in a spectral absorption characteristic of any of the spectral responses of the receptors of the animal eye. Calculations of the absorption cross-section of retinenes in the putative position do not support a very high absorption coefficient based on their relatively small absorption cross-section relative to the total area of the presumed opsin substrate.

Following its stimulation, no physical mechanism has been defined supporting the release, transport, de-excitation of and return of the retinene to its original location buried within the putative opsin moiety of the receptor molecule.

While the tabulations shown in **Section 5.5.10.6.3** purport to list proteins affecting sensory neuron operation, they offer little confirmation of the inferences drawn from investigators studying the genetic code. The use of the term Opsins as a header of the tabulations appears to be drawn entirely from the conventional wisdom and not from any demonstration that the molecules listed under "approved symbols" and "approved names" by HUGO are in fact directly related to the transduction mechanism.

No genetic study has identified a complete set of receptor molecules compatible with the tetrachromatic spectral performance (UV–, S–, M– & L–) of the eyes of both *Insecta* and/or *Choradata*. No single investigator has successfully isolated the genetic codes and/or identified the actual molecules employed for even the three spectral channels supported by the prior conventional wisdom (S–, M – & L–).

5.5.10.7.2 Alternate propositions based on this work

The use of a more comprehensive model of the visual system, specifically a multipath, multistage model, brings an additional degree of clarity concerning the potential source of abnormal color vision. Such a model is presented in **Figure 17.1.1-1** of this work (and in several other places). A more detailed model appropriate to this discussion appears in [**Figure 17.1.4-1**]. The potential failure modes of color vision are developed in **Section 18.1.5**. This level of detail supports a much broader involvement of the genetic code in the list of potential degeneracies in the visual system. The recognition that the luminance and chrominance channels of vision operate in parallel beyond the pedicles of the spectrally specialized photoreceptor cells is critically important. This recognition allows the identification of the failure modes of the system with much greater precision. A particular example is useful here. The classic deutranope of the literature exhibits a loss in ability to discriminate between red and green but suffers no loss in the perception of luminance. This condition strongly suggests he has no deficiency in the spectral sensing portion of his visual system. Alternately, the classic protanope exhibits a loss in red-green discrimination capability similar to that of the deutranope and **also** suffers from a significant loss in luminance sensitivity in the red portion of the spectrum. This condition suggests a failure in the L–spectral channel of vision. However, such a loss can occur in a variety of ways totally independent of the chromophore supporting that channel. Although less well documented a similar situation arises with respect to the S–channel, resulting in either tetartanopia or tritanopia. As indicated earlier, a documented case of the complete loss of the M–channel chromophore of vision has not been reported in the literature to date.

Pugh has recently provided a "Preview" article on the features segregating "rods" from "cones," and rationalizing the existence of "green rods."[251] Interestingly, his caricature of cones is diametrically opposite to the position of both Wald and Dowling who were writing in 1965. They both thought they had the last word in this discussion (see **Section 3.1.53**). The Pugh caricature relies primarily upon a caricature by a medical illustrator working with Young in 1971. Although very photogenic, and widely reproduced, that caricature has suffered from a lack of careful confirmation. The field began to move away from it in the latter 1970's (see **Section 4.2**). It would be more useful if Pugh replaced his caricature with an actual micrograph demonstrating his morphological thesis and then proposed a more detailed electrophysiological model of his two cell types. This work does not propose or support a broadband achromatic chromophore of vision as frequently associated with a putative rod. The luminance threshold function of scotopic vision is well represented by the logarithmic summation of the S– and M–channel neural signals. There is no requirement for a broadband chromophore in biological vision.

While there is a large variation in the short wavelength region of the luminance threshold function of humans (see **Section 17.2.2** or Wright, 1952), most cases of anomalous color vision within the photopic region appear to involve errors in the neural system. The magnitude of these errors are generally linear digressions from the nominal as documented extensively by Lakowski (see **Sections 18.1.2** & **18.1.5**). Such linear digressions are not easily supported by a simple genetic explanation of their occurrence.

The material presented in **Sections 4.6.2 & 7.1** of this work focus on a specific problem in vision terminology. The problem focuses on the term rhodopsin. The term has two incarnations. In its most conceptual form, the term

[251]Pugh, E. (2001) Rods are rods and cones cones, and (never) the twain shall meet *Neuron* vol. 32, pp 375-380

rhodopsin defines the chromophores of vision, or at least of achromatic vision as typified by scotopic vision. On the other hand, the chemist describes rhodopsin as a particular compound, or closely related family of protein based compounds containing the moiety opsin combined with a retinal (a simple type of retinene) via a Schiff base. It is not at all clear that these two incarnations can be equated. Two points are becoming clear. First, the chemist's rhodopsin is not a chromophore of vision but is used as the passive element forming the disks of chordate outer segments. Second, the visual scientists rhodopsin is actually a non-protein retinoid of unique liquid crystalline configuration deposited on the passive substrate of the chemist. With its long history, the chemists name for rhodopsin is likely to survive in that domain. However, the conceptual rhodopsin of the visual scientists and pedagogue is not a protein and does not contain retinene. The light sensitive material they are speaking of is a small family of retinines, called Rhodonine, when present in a liquid crystalline form.

The work of Papermaster and others have clearly shown that the protein used in the substrates (disks) of the outer segment of photoreceptor cells is formed within those cells. The material (opsin + retinal) is secreted by those cells and formed into disks within a cavity associated with the inner segment of the photoreceptor cells. The work of Bridges, et. al. shows clearly that the retinoids used in the functional part of the visual process, the Rhodonines, do not pass through the photoreceptor cells. They are delivered to the outer segments via the RPE.

The above distinctions require a clear separation between the passive substrate material (even though it contains a retinoid) and the photon sensitive chromophores of vision (which contain a different family of retinoids). For the remainder of this Chapter, the term opsin will be used to define a complete protein that contains a ligand of retinal. This material does not participate in the functional aspects of vision. The term Rhodonine will be used to describe the functional chromophores of vision.

The above interpretation says the genetic code calling for the formation of the protein opsin has very little to do with the formation of the chromophores of vision. Study of the code to understand the manufacture of opsin is very useful. However, it does not lead to an understanding of color performance, or color abnormalities in vision. The data shows that there are a variety of genes on at least two different chromosomes that code for an opsin like material. Whether the polymorphism found in the code at these sites are significant is less clear. Many researchers have been attempting to equate this polymorphism with variations in the structure of individual opsins that result in a family of materials exhibiting spectrally distinct absorption characteristics in the visual range. This work has not been fruitful. It has not demonstrated peak absorptions for the various opsins at the wavelengths associated with vision.

To support vision throughout the animal kingdom, including in chordates, it is necessary for the genetic code to support the formation of four distinct forms of Rhodonine. These forms are the four distinct narrowband chromophores, Rhodonine(5) for the L–channel, Rhodonine(7) for the M–channel, Rhodonine(9) for the S–channel and Rhodonine(11) for the UV–channel. All four of these chromophores and channels are employed in the human visual system. The performance of the UV–channel is only restricted by the transmission properties of the physiological lens.

As discussed in **Section 4.6.2**, the creation of the non-protein chromophores of vision from retinal involve the proteins SRBP, Serum-Retinoid-Transport-Protein and IRBP, Interstitial-Retinoid-Binding-Protein. It is likely that one of these two proteins exists in four very similar forms. These forms would determine how retinal from the liver is transformed into one of the four chromophores of biological (including human) vision. Finding the genes defining these proteins could be a major step in finding the cause of protanopia and tritanopia. Alternately, another set of proteins appears to be involved in the transport and storage of retinoids within the RPE. CRBP, Cellular-Retinol-Binding- Protein and its close associates could interfere with the orderly transport of the retinoids through the RPE.

5.5.11 The anisotropic absorption of the disks of humans and other chordates

The material presented above can be combined to illustrate the absorption properties of a complete disk of a chordate photoreceptor cell; and by extension, the absorption of a complete outer segment. The overall absorption characteristic is quite complex because of the unique structure and chemistry used. It is also slightly different from the perceived absorption because some of the absorbing constituents are not in contact with the neurological signal path. [**Figure 5.5.4-1**] can be expanded to illustrate the overall situation in **Figure 5.5.11-1**. In this figure, the top half illustrates the structural and quantum-mechanical situation more completely and the bottom half illustrates the result more completely. A critical factor is the size of the two retinoids, the Rhodonines in the chromophore layer and the retinals in the opsin (or substrate) layer. They are nearly the same size, about 7 Angstrom in diameter and 15 Angstrom long. Those in the chromophore layer are closely packed, in a nominally single layer, with their long axes parallel and perpendicular to the surface of the disk. The long axes of the retinoids within the opsin molecules are parallel to the surface of the disk and separated by the pitch, in two directions. of the opsin molecules.

Because of their cross-section and the packing aspect alone, the absorption cross-section of the Rhodonines is about 50 times that of the retinals for light incident perpendicular to the surface of the disk. This ratio is suggested by the vertical diameters of the two ellipses in the bottom frame. The scales of the two axes of the figure are roughly logarithmic. Both the Rhodonines and the retinals exhibit a molecular absorption with a peak near 500 nm. The retinal is a retinoid with a single polar (nitrogen) atom but subject to a variety of other polarizing mechanisms due to the amino acids of the opsin. This electrical polarization suggests an absorption spectrum similar to that of a retinoid with two polar atoms. Because of their polar state, both types of molecules exhibit individual bulk or molecular absorption spectrums with a peak near 500 nm that are isotropic. This condition is illustrated by the small circle at the center of the lower figure. The net absorption of the array associated with each of these materials is not isotropic because of the array factor associated with each one. The apparent molecular absorption will be higher for light incident parallel to the surface of the disk because the light will encounter more absorption opportunities. This is illustrated in caricature by the elliptical shapes for the two materials.

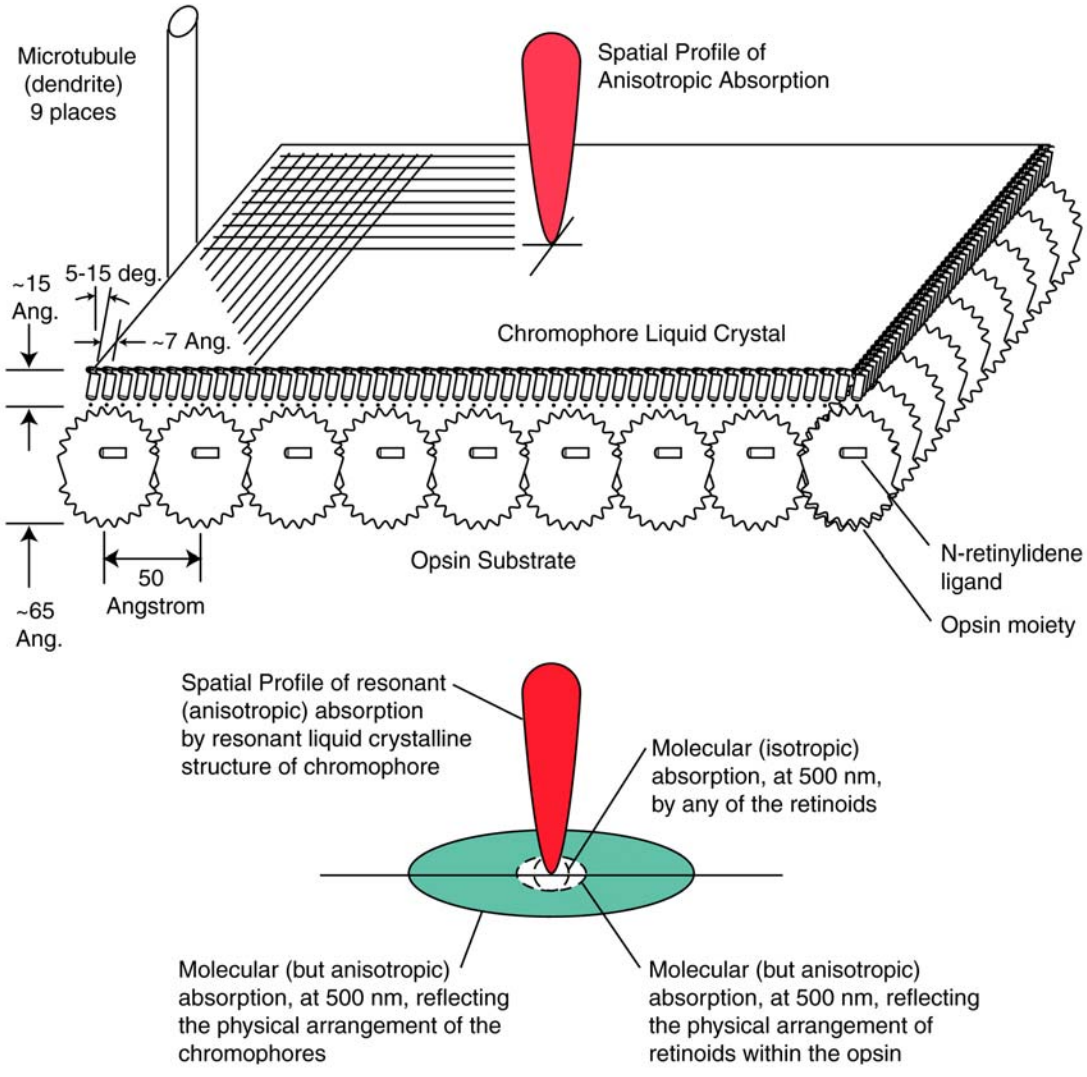

Figure 5.5.11-1 Absorption characteristics of a complete disk showing the variation in absorption properties as a function of spatial angle and absorbing species. Top; 3-D isometric view. Bottom; 2-D projection, plane contains vertical axis perpendicular to disk surface. The shared quantum-mechanical structure of the liquid crystalline chromophore(s) creates a highly focused (anisotropic) absorption profile. This structure is in quantum-mechanical contact with the microtubules surrounding the disk. The retinoids within the opsin proteins are not in quantum-mechanical contact with each other or the microtubules.

The Rhodonine of the chromophore exhibits another form of absorption because of the quantum-mechanical properties of each atom in the array. The Rhodonines have two polar atoms (most likely oxygen) which are separated by a conjugated carbon chain of variable length. This configuration creates a resonant absorption spectrum unique to these characteristics of the molecule. This resonant absorption is associated with the effective travel velocity of excitons along the length of the shared energy levels within the liquid crystalline state and the length of that path between the two polar atoms. It is the quotient (effective length over effective velocity) of these two quantities that determines the frequency of peak in the resonant absorption characteristic for each of the Rhodonines. In the case of the Rhodonines, this peak is found near 342, 437, 532 or 625 nm. In resonant absorption, the molecule is most sensitive to radiation incident to the long axis of the molecule (perpendicular to the surface of the disk). This type of absorption is negligible, compared to molecular absorption in a single free molecule. However, there is major impact when the molecules of this type are formed into a liquid crystal. The energy levels of the molecules become shared across the extent of the liquid crystal. As a result, the absorption cross-section of the array rises dramatically relative to the individual molecules. This causes the absorption coefficient for light incident perpendicular to the disk to rise dramatically. This is illustrated by one of two lobes symmetrical about the zero point of the axes of the graph. The peak in resonant absorption is typically 30-50 times the maximum in the molecular absorption of the same material (see **Section 5.5.9**).

5.5.11.1 Features and drawbacks of the complete disk configuration

The cone angle associated with resonant absorption is determined by the lateral extent of the liquid crystal and the wavelength of light. For two micron diameter disks, the cone angle is well matched to the output f/# of the physiological optics of the human eye. To optimally use this absorption cone angle, it is important that the axis of the outer segment formed from many disks point directly at the center of the exit pupil of the physiological optics (and not to the second node defined by Gaussian Optics). This is found to be the normal case (**Section 1.2.1.5**). A pathological situation is encountered if the outer segments are not pointed properly over the entire retina.

The sharing of the energy levels of the chromophores of the liquid crystal has two major impacts on the operation of the photoreceptor cell. The sharing has a major impact on the overall absorption of the structure. The Pauli Exclusion Principle says that the available number of electrons within a given energy level cannot exceed a specific number. To overcome this problem, the energy levels are broadened. This broadening accounts for the broader absorption spectrum associated with resonant absorption in the chromophores of vision (**Section 5.4.3**). Secondly, the sharing of the energy levels allows rapid transport of the excitons generated by photoexcitation to the edge of the liquid crystal and the interface with the microtubules (dendrites) associated with the photoreceptor cell inner segment. In humans, there are usually nine of these microtubules arranged in furrows at the edge of the disks. The de-excitation of the excitons at these interfaces restores the overall liquid crystalline chromophore to its original ground state without employing any change in the isomeric state of individual molecules or their removal from the chromophore. De-excitation usually occurs within a few milliseconds of photoexcitation (**Section 7.2**).

The asymmetry, in both spatial and spectral content of the absorption characteristics are particularly important when employing the suction pipette approach to measuring in-vivo photoreceptor performance (see **Section 5.5.10.3.4**). The experimental results are crucially dependent upon the incidence angle of the stimulus irradiation.

Okada, Le Trong, et. al. have found that the combination of opsin and N-retylidene in crystalline form is unstable when exposed to visible light[252]. It appears the excitation of the retinoid, or the subsequent de-excitation through an isomeric rearrangement, distorts the individual molecule and degrades the overall crystalline structure of the substrate. A similar distortion may or may not occur in the chromophore layer. It is likely the effect is more diffuse in that layer because of the shared energy levels. As noted in **Section 4.6**, the continual degradation of the substrate due to the non-reversible isomeric conversion of the retinoid ligand may be the reason for the continual replacement of the disks in the chordate photoreceptor system.

5.5.12 The Rhodonines as retinoids

The Rhodonines, as defined in this work, are the actual chromophores of vision when in the liquid crystalline state and supported by an auxiliary de-excitation mechanism. They can be placed in the chemical hierarchy in a number of

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[252]Okada, T. Le Trong, I. Fox, B. et. al. (2000) X-ray diffraction analysis of three-dimensional crystals of bovine rhodopsin obtained from mixed micelles *J. Struct Biol* vol. 130, pp 73-80

ways. Some of these are addressed by Zollinger[253] from the perspective of a dye. Others are addressed by Kennard, et. al from a crystallographers perspective[254]. At the current time, it is most appropriate to define the Rhodonines in terms of the broad retinoid family. This family also includes the retinenes which the Rhodonines resemble. The retinenes are chromogens of the Rhodonines. It is the Rhodonines which are the actual chromophores of vision. Within this family, the unique characteristic of the Rhodonines is their carboxylic-ion system.

The chromophores of vision appear to be in the all-*trans* configuration throughout their participation in the visual process. There is no need to discuss their possible *cis* configurations.

5.5.12.1 Retinoid naming systems

As the science related to the retinols has expanded in recent years, the question of terminology and naming has been repeatedly addressed. Karrer provided the original description of the retinoids following the synthesis of Vitamin A in the 1930's. As early as 1967, Frickel, writing in Sebrell & Harris[255] explored the naming of new members of the retinol family, both natural and synthetic. In 1982, the IUPAC-IUB Joint Commission on Biochemical nomenclature provided a specific definition of the retinoids based primarily on structural rules which immediately came under attack from the pharmacological community as inadequate. Frickel, writing in Sporn et. al. (1984), wrote that there were more than one thousand retinoids in their view[256]. These substances exhibited many of the biological activities of the common retinoids but bore only superficial resemblance to them from a structural perspective. In 1994, Sporn & Roberts, writing in a major expansion of their 1984 work[257], called for a new definition of the retinoids derived almost totally on pharmacological reasoning and based on the relationship with receptor cites of associated materials and not the characteristics of the retinoids themselves. The types of receptor sites discussed were limited to sites associated with metabolic processes. The types of receptor sites employed in the formation of the chromophores of vision were not discussed.

There is a broadening gulf between alternate methods of describing the retinoids. The 1982 definition is obviously inadequate as will be discussed below. The proposed 1994 definition is probably too broad unless it is subdivided into subclasses. In the above attempts at definition, the electronic configurations of the various molecules were not considered. This work highlights the importance of systematically relating the structural form and the electronic form of the retinoids involved in the visual process. It is suggested that it is time to subdivide the retinoids into at least two classes, those used in the visual process and those used in the combined metabolism and growth process.

5.5.12.2 Retinoid numbering systems

IUPAC in 1973 set down the rules for numbering the constituent atoms in the carotenoids following the work of Karrer. These rules were reaffirmed in 1982 by the IUPAC and have been used by Chemical Abstracts subsequently for the known natural carotenoids. Frickel, writing in Sporn et. al. (1984), discussed the fact that *Chemical Abstracts* does not use the Karrer notation for the more general types of retinoids but used the *more* standard "systematic system" which calls for the carbon atom bonded to the functional group (the carbonyl carbon) to be given the number "1". They then proceeded to also give the carbon atom at the junction of the β-ionone ring and the side chain a second number "1" with a number "2" assigned to the carbon in the ring with a single methyl group attached to it. Obviously, there is a difference of opinion as to which group is the functional group in even the simplest retinoids. In the more complex retinoids, the question becomes academic. It depends on the use of the material. The use of two carbons with a designation "1" in the same molecule should be avoided. Following their formal objections to the IUPAC approach, Sporn, et. al. continued to use the structural approach in their 1982 edition. In the 1994 edition, they strayed further from the orthodoxy of the IUPAC by stressing the discovery of many additional structures exhibiting pharmacological activity and many chemical receptors associated with the pharmacological retinoids. In the 1994 edition, a system of "retinoid numbering" was introduced "to simplify the description of structural analogs."

The retinoids involved specifically in vision do not relate to the same chemical receptors as those found in the nutrition aspects of pharmacology. There is a different set of chemical receptors involved in the transport of retinol

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[253]Zollinger, H. (1991) Op. Cit. pp. 56-58
[254]/ Kennard, O. Allen, F. & Watson, D. (1977) Molecular structure and dimensions : guide to the literature 1935-1976, organic and organometallic crystal structures. Utrecht : Bohn, Scheltema and Holkema
[255]Sebrell, W. & Harris, R. (1967) The Vitamins, 2nd ed. vol. 1. NY: Academic Press, pg. 12.
[256]Sporn, M. Roberts, A. & Goodman, D. (1984) The Retinoids, in 2 volumes. NY: Academic Press, vol. 1, pg. 11
[257]Sporn, M. Roberts, A. & Goodman, D. (1994) The Retinoids, 2nd ed. in one volume. NY: Raven Press

from the liver to the RPE cells of the retina. These receptors are found on the surface of the RPE cells and are specific for the retinoid binding proteins (RBP's) that transport the retinoids to the RPE. These RBP's play a unique role in the chromophore forming process that is not shared with the transport of retinol for purposes of metabolism and growth. Machlin says there are as many as 50,000 RBP receptor sites on the exposed surface of each RPE cell[258].

Because the molecules employed as chromophores in vision are relatively complex and include many subgroups defined in the literature, there is a confusing array of diagrams used to represent members of the Retinol Family. Most of them are used to aid in the process of fabrication or in teaching. They frequently define a "functional group" at the end of the conjugated chain that includes the last carbon atom of the chain. **Figure 5.5.12-1** will be used to develop the preferred nomenclature for describing the chromophores of vision. Many diagrams found in the literature stress the conjugated nature of the side chain in its relationship to the isoprene groups. While the isoprene structure is prominent in the formation of the natural chromophores of vision, it is not a required structure. The methyl groups associated with the isoprene unit are not required for vision. This work will stress the conjugated nature without reference to the methyl groups by referring to the vinylene (or even simpler methine) groups.

Frame **A** of the figure shows the numbering system developed by Karrer in the 1930's following the cleaving of Carotene to form Vitamin A (retinol). The nomenclature was based on a two dimensional representation of retinol without regard to its actual stereometric form. The carbons were numbered in order beginning with the carbon sharing two methyl radicals and initially progressing counterclockwise around the ionone ring. This is the numbering scheme adopted by the IUPAC for the carotenoids beginning in 1973 and reaffirmed in 1982. It is the scheme used by *Chemical Abstracts* for the simple retinoids.

> Over the years, the form shown in frame **A** has been loosely defined as all-*trans* retinol. However, it is important to note the fact illustrated by Birge[259], that the version shown, and all of the retinenes, actually have a distorted linkage between C6 and C7. Figures displaying carbon C16 & C17 at the top of the drawing are technically described as "all-*trans*, 6-s-*cis*" retinol although the 6-s-cis descriptor is usually omitted. To be precise, only the form shown in frame **C** is truly all-trans retinol according to Birge. The form normally labeled 11-*cis* retinol is more formally labeled 11-*cis*, 6-s-*cis* retinol.

Frame **B** shows an alternate system that separates the carbon count into two parts. The carbons of the ring are counted beginning at the point of the connection to the backbone and progressing clockwise around the ring. The count reaches the carbon sharing two methyl radicals last. The carbon count of the backbone begins with the carbon at the extreme end of the structure and progresses toward the ionone ring. This is the so-called systematic numbering scheme proposed by Frickel in the 1982 edition of Sporn, et. al. and repeated in the 1994 edition.

Frame **C** shows a third system also found in the literature. This scheme begins with the carbon at the extreme end of the backbone and counts up until it reaches the ionone ring where the count proceeds counterclockwise around the ring, ending at the carbon sharing two methyl radicals.

As Frickel has pointed out, *Chemical Abstracts* is inconsistent in using the Karrer nomenclature for simple retinoids derived from carotene but not for other retinoids of similar complexity. In the case of a simple retinoid containing a five-carbon ring, such as at least one carboxylic acid, it uses the systematic notation of frame **B**.

A more important physical aspect of the problem relates to the actual stereometric structure of the molecules and two features related to that structure. First, it is important in the transport of the retinoids through the bloodstream that the carbon of the ionone ring in contact with only one methyl group is as exposed as possible. Second, the formation of liquid crystalline structures by the simple retinoids is critically dependent on the location of the carbon of the ionone ring sharing only one methyl group. As will be shown later, the stereographic arrangement of the retinoids of vision can be equally well represented on two-dimensional paper by the configuration of frame **A** or **B**.

Frame **D** uses the IUPAC nomenclature. This frame shows the single methyl group replaced by an oxygen atom for three reasons. It stresses the "carboxylic ion system" character of the molecule, with conjugation shown extending between the two oxygen atoms (This feature will be discussed in the next paragraph). It stresses the fact that, in this retinoid, the terminal, or chemically functional, groups do not include the "last carbon." The "electronic functional group" of interest here consists of a polar atom, either an atom of nitrogen or an atom of oxygen at each of two

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[258]Machlin, L. (1984) Op. Cit. pg. 15

[259]Birge, R. (1981) Photophysics of light transduction in rhodopsin and bacteriorhodopsin. *Ann. Rev. Biophys. Bioeng*. vol. 10, pp 315-354

critical locations along the conjugated backbone. This backbone is defined as extending from carbon #5 to carbon #15 in the case shown. When both of the polar atoms are oxygen, this case corresponds to the long wavelength, or L– channel, chromophore of vision. This molecule is named Rhodonine(5) to indicate its particular structure and derivation from retinol when based on the IUPAC coding. It is one of the four chromophores used in the visual system of all animals. Finally, frame **D** illustrates the physical (quantum-mechanical) resonance that exists between the two oxygens but is not shown to scale. It does not stress the effective physical distance in three dimensions between the two oxygen atoms. The vectorial distance between these oxygen atoms that is critical to the coupling between the stimulating photons and the chromophore. Variations between this vector and the trajectory of the photons is reflected in the Stiles–Crawford Effect of the 1st kind (Section xxx). These are key features in understanding the chemistry of the chromophores of vision.

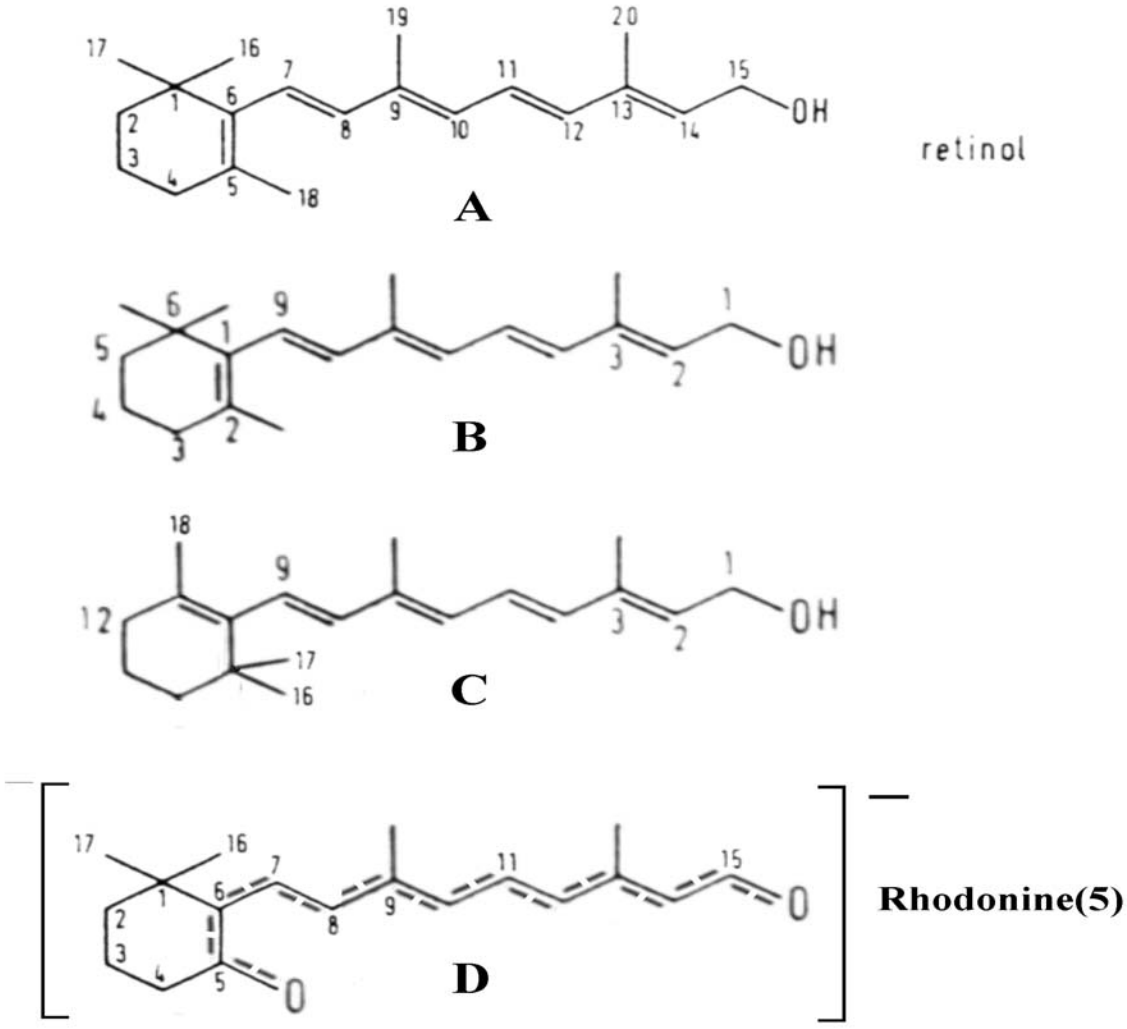

Figure 5.5.12-1 Alternate nomenclatures for the retinoids associated with vision. A; the Karrer based nomenclature adopted by the IUPAC. B; the systematic nomenclature proposed by Frickel and others. C; a second systematic nomenclature found in the literature. D; the modified (2nd order) pictograph of the IUPAC nomenclature used in this work.

Frame **D** also stresses the methine chain aspects of the molecule in a somewhat different manner than other presentations. The presence or absence of the methyl groups along the length of the side chain are not significant in vision. Similarly, whether the ionone ring is that ascribed for Vitamin A1, A2 or A3 is of little importance in

vision.

Frames **A** and **D** are equally appropriate representations of a molecule where the ionone ring is not in the plane of the paper relative to the backbone. However, the "modified IUPAC notation" of frame **D** is considerably more instructive pedagogically.

Karrer was an industrial chemist and his scheme reflects his view of how to manufacture the simple retinoids from a ring structure and a side chain. Frickel exhibits a more theoretical approach but does not reflect the significance of the electronic configuration of the materials. The proposed alternative stresses the electronic and chromophoric properties of the molecules, especially when modified for the purposes of vision.

In the top view, IUPAC stressed that "retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner; all retinoids may be formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional terminal group at the terminus of the acyclic portion." While Sporn and Roberts objected to this definition because compounds have been found that exhibit significant pharmacological activity without incorporating a poly-isoprenoid structure, this work suggests that the conventional presentation of both variants are arbitrary and awkward for purposes of vision. This is partly due to the desire to draw a three-dimensional structure on a two-dimensional medium. The bottom frame shows the cyclic element rotated to emphasize the conjugated portion of the molecule. This view clearly shows that there are five (not four) isoprenoid units in the common retinenes. The fifth unit plays a critical role in the formation of the long wavelength chromophore of vision. To avoid the introduction of two carbons bearing the designation (1), the proposed "serial systematic approach" numbering sequence continues sequentially as in the IUPAC (structural) approach but it starts at the carbonyl carbon as in the Frickel (systematic) approach. It proceeds around the cyclic portion and then numbers the more remote groups.

This work will continue to use the IUPAC notation to avoid adding to the confusion in the literature.

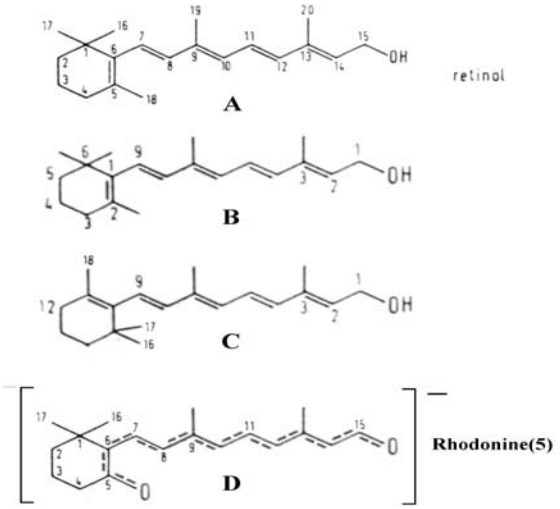

Figure 5.5.12-2 Three numbering schemes for the retinoids. Top, carotenoid based structural scheme used by the IUPAC. Middle, systematic scheme of Frickel. Bottom, proposed chromophore based "serial systematic" scheme. See text.

5.5.12.3 Description of the Rhodonines as retinoids

The Rhodonines appear to be a unique family of retinoids derived from retinol. However, contrary to the simple retinenes (that include retinol) which are polyenes, the Rhodonines are polymethines. The complete family consists of eight members, four of which are based on $retinol_1$ and four of which are based on $retinol_2$. They all exhibit the same basic structure as retinol except they incorporate two oxygen atoms at specific locations along the "side chain."

Unfortunately, the Rhodonine family defined here is found to cross a very arbitrary boundary suggested by Sporn et. al. Whereas three members of the family would fall in the category labeled "Chain Modifications," the fourth member would fall in the category labeled "Ring Modifications." This may or may not be significant in terms of the industrial synthesis of the Rhodonines. However, it is not significant in the natural formation of the molecules. If the number 11 carbon of the suggested "serial systematic" notation (number 5 carbon of the β-ionone ring using the structural notation of the IUPAC) is considered a member of the isoprene chain for the purposes of synthesis, the material in Sporn et. al. relative to modifications to the "Chain" is probably useful.

The complexity of the chemical structure of the Rhodonines is such that they can be described using a large variety of chemical terms, aldehydes, alcohols, ketones, carbonyl carbon, etc. Their electronic resonance adds an additional level of complexity to even these designations. In general, the reactivity of the Rhodonines is not of interest in vision. However, their structure does suggest how the materials must be protected from undesired reactions.

5.5.12.4 Structural description of the Rhodonines

The four principal chromophores of vision based on Vitamin A1 presented in [Figure 5.5.8-2], the Rhodonines are shown again in **Figure 5.5.12-3** along with a chromogen, Vitamin A1 aldehyde or retinal$_1$. The auxochromes (oxygen atoms) of the Rhodonines are shown in red. A horizontal line is shown below each Rhodonine to illustrate the relative length of its resonant structure. It is this length that determines the center wavelength to which each chromophore is quantum-mechanically sensitive.

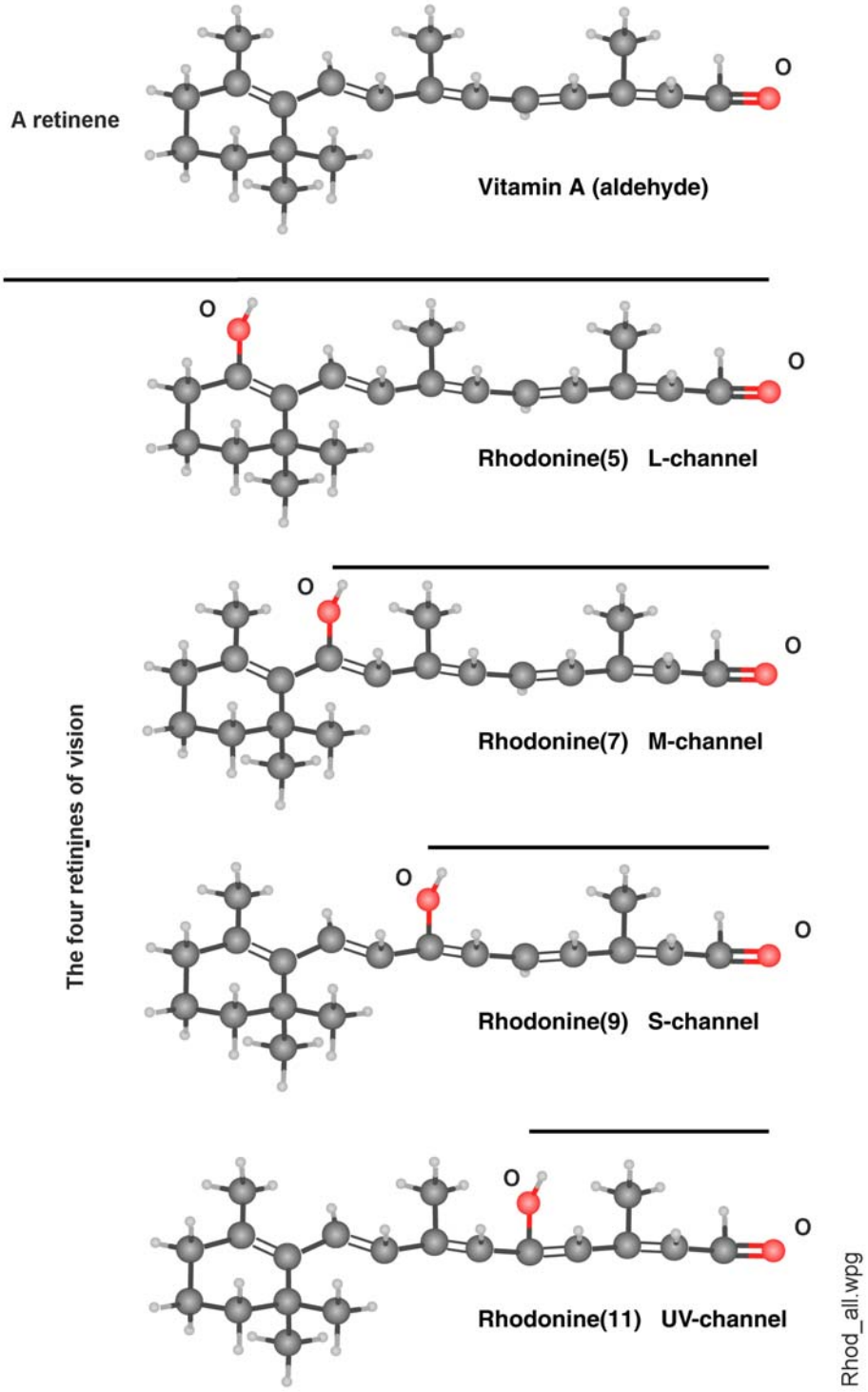

Figure 5.5.12-3 The four chromophores of vision, the Rhodonines. Retinal (Vitamin A1) is shown for reference in aldehyde form. The oxygen atoms are shown in red. The horizontal line above each molecule shows the relative length of its resonant structure.

5.5.12.5 IUPAC naming of the Rhodonines

On the assumption that the Rhodonines of vision are as described above, the Chemical Abstract Service of the American Chemical Society provided systematic names for these chromophores using the IUPAC 1982 notation. The names, expressed as ions based on Vitamin A_1 and provided in 1998 were:

Rhodonine(5), the long wavelength chromophores

> 2,4,6,8-Nonatetraenal, 9-(2-hydroxy-6,6-dimethyl-l-cyclohexen-1-yl)-3,7-dimethyl-,ion(l-), *(2E,4E,6E,8E)-*

Rhodonine(7), the medium wavelength chromophores

> Retinal, **7**-hydroxy-, ion(l-)

Rhodonine(9), the short wavelength chromophore

> 2,4,6,8-Nonatetraenal, 7-hydroxy-3-methyl-**9**-(2,6,6-trimethyl-l-cyclohexen-1-yl)-, ion(l-), *(2E,4E,6E,8E)-*

Rhodonine(11), Ultraviolet wavelength chromophores

> Retinal, **11**-hydroxy-, ion(l-)

Since Rhodonine(7) and (11) only differ from the retinenes in the replacement of a hydrogen by a hydroxyl group, they were named by using the short hand of retinal in the systematic names. In the case of Rhodonine(5) and (9), no shorthand notation was available. The systematic names for the chromophores based on Vitamin A_2 and A_3 will be found in the Appendices.

5.5.13 The Rhodonines as indicators

To avoid problems in the laboratory, it is important to recognize that the Rhodonines are members of the indicator family of chemistry, related to phenolthalein. In dilute solution, the absorption spectrum is a function of the environment due to the chemical resonance associated with its structure. This feature requires that the parameters of the solution be specified when recording spectra of the Rhodonines.

5.5.14 Laboratory fabrication of the Rhodonines

The Rhodonines share the extreme sensitivity of the retinoids to heat, light, oxygen and strong chemical reagents. In fact ***their sensitivity to destruction exceeds that of the retinenes***. Therefore, both their extraction and preparation require special care. Because of the unique resonant character of the Rhodonines defined in this work, there has not been any previous planned effort to fabricate these materials in the laboratory. Most of the previous work appears focused on maintaining the hormonal character of the resulting material. This has called for maintaining the methyl groups at C-5 and C-9 as a minimum. This focus insures that the chromophores of vision will not be synthesized. However, the general methods for the fabrication of similar retinoids have been developed.

Kaegi writing in Sporn et. al. (1984) [particularly page 169] and Dawson & Hobbs writing in Sporn et. al. (1994) provide a broad overview to the synthesis of the retinoids (utilizing the broadest definition of a retinoid). They discuss the synthesis of various modifications to ring structures, including the attachment of an oxygen to the carbon at position 18 of retinoic acid, but not the replacement of the entire methyl group at that position by an oxygen atom (8 in Fig 3.). The Rosenburger team at Hoffmann-La Roche[260] has provided data on the synthesis of many individual retinoids. Their structure #7 in Sporn is very close to Rhodonine(5), but involves an addition rather than a substitution. It is described as (E)-4-oxoretinoic acid. Aig, et. al. have provided a detailed synthesis procedure for

[260]Aig, E. et. al. (1986) A European patent application, See Sporn, et. al. (1994) pg 7 & 8.

a group of metabolites of retinoic acid[261]. However, these metabolites did not include any of the Rhodonines. The ability of experimenters to create these similar materials assures the feasibility of creating the Rhodonines in the laboratory or extracting them from biological material.

Redfern has prepared a group of protocols for the retinoids[262]. He also does not address the liquid crystalline state of these materials or any family similar to the Rhodonines. The Rhodonines are even more sensitive to degradation than he suggests for the other retinoids.

Table 2 on page 21 of Sporn (1994) shows the extent that laboratory research has explored the retinoids related to vision (with one exception). That particular table involves retinoids containing a complete benzene ring rather than an ionone ring. A similar but simpler organization more directly related to vision is shown in **Table 5.5.14-1**. The conjugate structure associated with quantum-mechanical resonance and the resulting spectral absorption only extends between the two oxygen atoms present.

Dawson & Okamura have provided a more extensive text on synthetic retinoids[263]. It does not address any family with the resonant dipolar characteristics of the Rhodonines, nor does it address the liquid crystalline state of matter. Balogh-Nair & Nakanishi provide information on one material that is functionally very similar to the Rhodonines except it contains one additional methine group, formula 27 on page 169 of that text. The report is based on Derguini, et. al[264]. They synthesized a series of merocyanines, consisting of a ring and a side chain, that resemble retinaldehyde. However, they do not appear to be derivable from retinaldehyde. The material is an amidic system (merocyanine) instead of a carboxylic-ion system. At 10 mM and a pH of 7.0, it exhibited a dipolar molecular absorption at 480 nm and a *j*-band (resonance absorption band) at 662 nm following incubation with an apomembrane (?) of *Halobacterium halobium*. The character of the resonant absorption changed with incubation period. Note the variation in FWHM for this material as a function of time in their experiments. Although Derguini, et. al. discuss the half-bandwidths of the enhanced spectrums as narrow, this parameter is seen to be a clear function of the aggregation level in their figure. It is suggested here that the appearance of the *j*-band was due to aggregation of the material without actual chemical combination with the apomembranes. It should also be noted how the growth in the absorbance of the enhanced spectrum is at the expense of the 480 nm spectrum. The suggestion of those authors that this merocyanine *could* combine with a protein to form a cyanine is probably true. However, there is no data to support the presence of nitrogen-based cyanines (amidinium-ion systems) within the visual system in place of the oxygen-based Rhodonines.

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[261]Aig, E. Focella, A. Parrish, D. Rosenberger, M. Scott, J. & Zenchoff, G. (1987) Synthesis of the major metabolites of . . . nonatetraenoic acids. *Synthetic Communications*. Vol 17(4) pp 419-429

[262]Redfern, C. (1998) Retinoid Protocols. Totowa, NJ: Humana Press.

[263]Dawson, M. & Okamura, W. (1990) Chemistry and Biology of Synthetic Retinoids. Boca Raton, FL: CRC Press.

[264]Derguini, F. Caldwell, C. Motto, M. Balogh-Nair, V. & Nakanishi, K. (1983) Bacteriorhodopsins containing cyanine dye chromophores. J. Am. Chem. Soc. vol. 105, pp 646-648

TABLE 5.5.14-1
Table of Rhodonine Ligands Critical to Vision

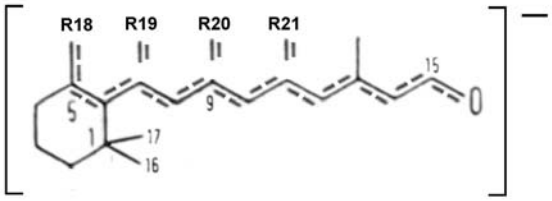

Figure 5.5.14-1 Ligand binding sites

Chromophore	Spectral Range	Loc. Grp.	C5 R18	C7 R19	C9 R20	C11 R21	Functional Group @ C15
Oxygen @ C-15							
Rhodonine (5)	**L**		**÷O**	**H**	**Me**	**H**	**÷O**
Rhodonine (7)	**M**		**Me**	**÷O**	**Me**	**H**	**÷O**
Rhodonine (9)	**S**		**Me**	**H**	**÷O**	**H**	**÷O**
Rhodonine (11)	**UV**		**Me**	**H**	**Me**	**÷O**	**÷O**

Dawson et. al[265]. have provided a large work on the synthetic methodology for constructing a wide range of retinoids involving chain modifications. While the study of more complex retinoids can be useful, the unique stereo-chemistry of the chromophores is very important. Large ancillary ligands are probably not compatible with the transport of chromogens within the visual system. It is also important to note that the chromophores of vision only exhibit their unique spectral characteristics when assimilated in the liquid crystalline state.

Table 4 of Dawson & Hobbs in Sporn et. al. includes the replacement of the methyl group at position 20 in retinol by bromine and figure 44 illustrates the synthesis of a retinoic acid with bromine replacing the methyl group at location 19. Figure 45 shows a configuration with an oxygen replacing the methyl at position 20 with an oxygen in a compound very close to retinol. With only slight modification, this compound would correspond to Rhodonine 13. Its spectral absorption would be in the ultraviolet region. The remainder of that work goes into ever more complex variants of the broadly defined retinoid family, many of them non-planar.

pg 94 of Sporn (1984) begins a discussion of structures simlar to that of the Rhodonines.
+ eq. 469 shows the synthesis of an analog of Rhodonine 9. It replaced oxygen with bromine.

+ eq. 491displays a variant that could be described as Rhodonine 10 (except for an extra double bond). This structure does not exhibit conjugation extending between the two oxygens.

+ eq. 502 could be interesting.

+ eq. 526 thru 530 are interesting for synthesis.

In summary, the Rhodonines are retinoids consisting of a β-ionone ring, an isoprenoid chain and TWO polar end groups (frequently described as R-groups) containing oxygen. The two oxygen atoms form the terminal groups of a

[265]Dawson, M. & Okamura, W. (1990) Chemistry and Biology of Synthetic Retinoids. Boca Raton, FL: CRC Press

resonant conjugated structure (derived from a polyene chain). The physical distance between these two oxygen atoms incorporated into the polar end groups is the distinguishing feature of each Rhodonine and defines its peak spectral absorption when in the liquid crystalline state (the absorption is typically anisotropic when in this state). The β-ionone ring stabilizes the molecule against thermal deterioration. All other molecular groups and features associated with the molecule are merely decoration. When in dilute solution, the (isotropic) spectral absorption characteristic of these molecules (peak absorption near 500 nm) appears to be determined by the length of the conjugated system formed by the β-ionone ring and the oxygen atom associated with C_{15}.

5.5.14.1 Structural features of the Rhodonines

[**Figure 5.5.6-1**] is repeated here as **Figure 5.5.14-2** for convenience. It provides a description of the important features of the Rhodonines from the perspective of a physical or "radiation" chemist. The description derives from a similar description of the retinaldehyde from the perspective of a "reaction" chemist. (a) is a diagram similar to one for retinol presented by Ganguly[266]. Berman[267] has presented a similar family of diagrams for other members of the retinene family. Fatt[268] has also presented a similar diagram. Sebrell[269] speaks of this diagram as illustrating the S-*cis* form of the retinol molecule relative to the 6-7 bond. The presentation is a combination of a structural diagram and a formula. It is designed to show the functional chemical groups that might be important in forming the molecule and in understanding its solubility. It indicates a carbon conjugation level of four since C15 is considered part of the polar end group. (b) presents an alternate view formed by rotating the above conventional 2-dimensional structural presentation without making any change in the actual molecule. Sebrell speaks of this figure as representing the S-*trans* form of the molecule relative to the 6-7 bond. Clearly, the distinction made by Sebrell is unnecessary. The actual molecule did not change, only the human method of presenting it on paper.

In (b), the leftmost dotted line is shown bifurcated to indicate an additional level of conjugation if the carbons of the ionone ring are considered. This view stresses the fact that the conjugation level of the molecule can be considered to be either five or four. (c) provides a pure structural diagram of the same molecule. The terminal oxygen is shown connected to the end carbon by a double bond. This view is drawn to stress the ***total*** conjugation level of the molecule. Note the level of conjugation is now clearly six.

> It is interesting to note, but of little significance to vision, that the form of retinaldehyde related to Vitamin A_2 contains an additional double bond within the ionone ring. This bond is located between C3 and C4 using Karrer's notation. Overall, this molecule exhibits a conjugation level of seven. However, the conjugated backbone is no longer straight and the absorption spectrum of retinaldehyde$_2$ is not shifted as much as would be expected relative to retinaldehyde$_1$. It is shifted a fractional amount as seen in **Section 5.5.3.4**.

(d) shows a second oxygen atom added to the molecule by substitution at C5. The molecule is no longer a retinene, i.e., a neutral molecule based on a conjugated isoprene structure. It has become a retinine, in this case, Rhodonine(5), a negatively charged ion with a resonant structure terminated by an oxygen at each end. This molecule can be described as a carboxyl-ion system. However, this description is awkward due to the distance

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[266]Ganguly, J. (1989) Biochemistry of Vitamin A, Boca Raton, FL: CRC Press pg. 102
[267]Berman, E. (1991) Biochemistry of the eye. NY: Plenum Press, pg. 48
[268]Fatt, I. & Weissman, B. (1992) Physiology of the eye. Boston, MA: Butterworth-Heinemann. pg. 218
[269]Sebrell, W. *In* Sporn, et. al. (1994) The Retinoids. NY: Raven Press. page 195

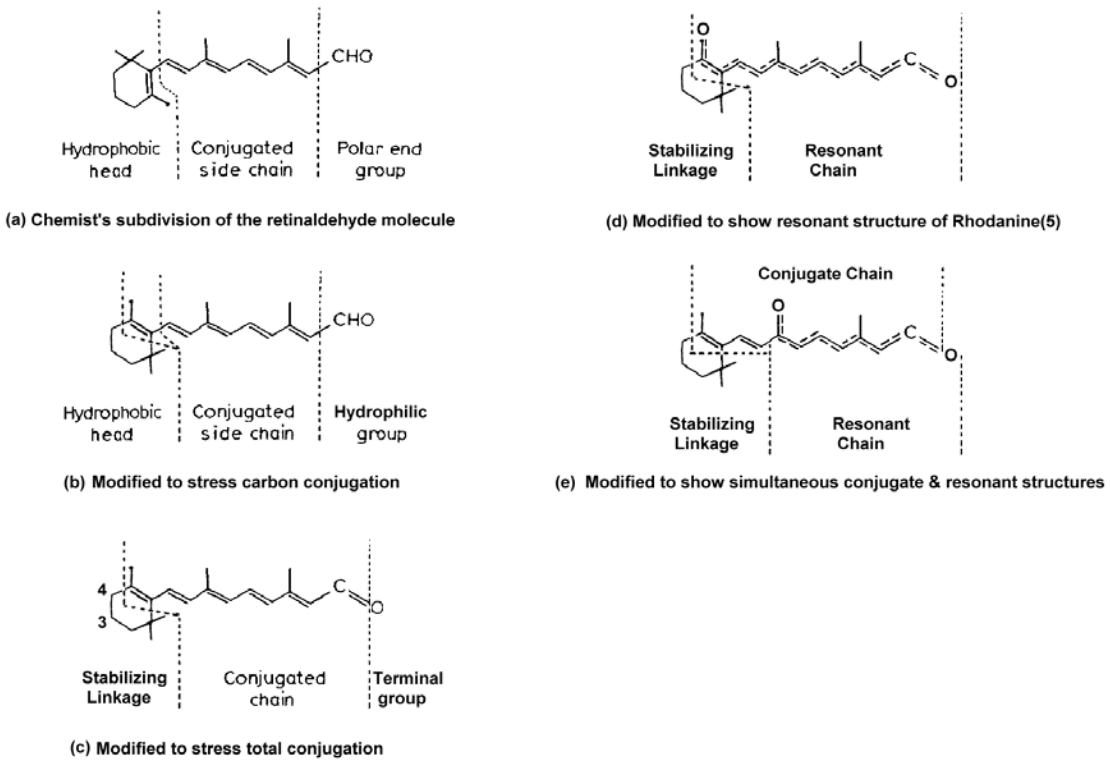

Figure 5.5.14-2 Comparing the Rhodonines to the retinenes. (a); Retinaldehyde as conventionally displayed. (b); presentation rotated to stress length of conjugated carbon chain. (c); presentation modified to stress total conjugation. (d); presentation modified to illustrate combined conjugation and electronic resonance over the maximum length of the molecule. (e); presentation modified to show the potential separation of the conjugated structure from the electronically resonant structure. See text for details.

between the =O and -OH units. It can also be described as a member of the phthalein family. Under laboratory condition, this ion will normally be associated with a hydrogen ion taken from the solvent bath. However, the location of this hydrogen ion is indeterminate.

When the hydrogen ion is considered to be attached to the rightmost end of the Rhodonine ion, Rhodonine will appear to be an alcohol and respond to simple chemical tests almost identically to Retinol. When the hydrogen ion is considered to be attached to C5, Rhodonine will appear to be an aldehyde and respond to simple chemical tests almost identically to retinaldehyde. The chemical ambiguity in the properties of the Rhodonines due to this resonance condition is the principle practical reason for the confusion in the literature concerning the chemical properties of the chromophores of vision. The principal conceptual reason is the prior failure of the community to recognize the existence of the resonant form of the retinoids, the Rhodonines.

It should be clear that when the terminal group of any Rhodonine responds as an alcohol, the oxygen and carbon associated with the side chain will respond in a manner similar to an aldehyde. The converse is also true.

Under the proper conditions, Rhodonine(5) will exhibit a long wavelength absorption peak at P625. The presentation now stresses the resonant chain extending from one oxygen to the other. The stabilizing linkage associated with the ionone ring is still present. However, the ring plays no significant role in the absorption characteristics of the molecule. It does play a part in stabilizing the ring relative to thermal degradation. Because of the chain being terminated by two oxygen atoms, the molecule is able to exhibit quantum-mechanical resonance under proper conditions.

(e) is presented to demonstrate two additional features.

+ By introducing the second oxygen by substitution for the methyl group at C9, the molecule becomes Rhodonine(9). Rhodonine(9) exhibits both the resonant structure extending between the two oxygens, and the conjugate structure between the methyl group attached to C5 and the rightmost oxygen. The conjugate structure and the resonant structure respond independently to radiation. Under the proper conditions, the molecule exhibits either two separate and essentially independent absorption peaks or one composite waveform. The two separate and distinct peaks in its long wavelength absorption spectrum occur at P502 for the conjugate peak and at P437 for the resonant peak.

+ All of the Rhodonines exhibit the P502 spectral absorption peak when interrogated while in dilute solutions. This peak is due to the conjugated structure these molecules share with the retinenes.

By introducing the second oxygen by substitution for the hydrogen at C7, Rhodonine(7) is formed. This molecule also exhibits two separate long wavelength absorption peaks, one at P523 due to resonance and one at P502 due to conjugation. It should be noted that the absorption peaks in the spectra of these molecules do not depend on the presence or absence of the various methyl groups. Although the presence of these groups plays an important role in the natural formation of the retinoids, they play no significant role in the visual application of the Rhodonines. The long wavelength spectral characteristics of the Rhodonines depend only on the conjugation of the carbon atoms to and including the two terminal oxygens, the resonant condition. Because of this fact, the conjugated chain can be considered as a series of vinyl groups, occasionally modified by the presence of a methyl group attached to one or more of the carbons. Similarly, the stabilizing linkage related to the ionone ring plays no significant spectral role.

5.5.14.2 Testing for the Rhodonines

The above figure illustrates diagrammatically the functional features of the Rhodonine family. Continuing to assume the actual chromophore is a dicarboxyl as opposed to an amidic system, it is clear that many tests for simple functional groups will return positive results when applied to this chromophore. Between its two polar components, it can exhibit properties of an aldehyde, an alcohol, a carbonyl group, a dicarbonyl, a ketone and many other functional identifiers. It may exhibit one or more of these functional groups depending on the environment in which it is interrogated. It will also test positive for a vinylene chain, a terpene or isoprene chain and a cyclohexene ring. Because of this ability to display many functional characteristics, it will require more sophisticated testing than used currently to distinguish the Rhodonines from their simpler precursors, the retinenes. Stated more explicitly, each of the Rhodonines will test positive using most of the gross tests used currently to identify the retinene family--in fact they will each test positive for both retinal and retinol. ***However, they are not retinenes***.

Wolf & Johnson[270] tabulated some of the more conventional tests for the various functional groups present in both the retinenes and the Rhodonines. None of these tests seeks to identify the resonant structure of the Rhodonines and thereby differentiate between the retinenes and the Rhodonines. These authors do refer to the “alleged hidden forms” of Vitamin A suspected in the 1960's.

To test for the Rhodonine family by chemical means, it is necessary to test for the simultaneous presence of both auxochromes and the conjugated carbon chain. To test for the individual Rhodonines, even more sophisticated procedures will be required than that to identify the family characteristic. The difference between the Rhodonine with a polar atom at position C7 and the Rhodonine with a polar atom at C11 is quite small. Fortunately, as it will be shown later, the C11 form is only found in the insect world. The differentiation between the C5 and C9 form can probably be achieved by testing for the unique cyclohexene ring structure associated with the carbonyl group at C5. It is most useful to utilize an NMR test to explicitly determine the specific retinoid present. This test is highly deterministic and virtually foolproof. The details related to NMR testing appear in **Chapter 6**.

Note that when a biologist or other researcher attempts to separate the chromophores from a retina, he typically uses detergents as solvents. These harsh agents will cause the chromophores to go into solution and no longer display their molecular system properties; thereby hiding the very property the researcher is seeking to use as a method of separation and/or confirmation. They will not exhibit their individual high absorption coefficients in the visual spectral region. Further, until they are separated through special techniques, probably through liquid crystallization on different areas of a substrate, they are almost impossible to separate through simple chemical techniques. This accounts for why earlier researchers have not been able to isolate the individual chromophores from the fluids decanted from the retinal extract in the process of isolating the protein material opsin. It also explains why opsin has never been found to display the sought after chromophoric properties.

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[270]Wolf, G. & Johnson, B. (1960) Metabolic transformations of Vitamin A. In Vitamins & Hormones, vol. 18

5.5.14.3 Fabrication from "feedstock"

If the Rhodonines are members of the Carboxyl family, their fabrication in pure form in the laboratory is conceptually straightforward. Beginning with the chromogen, retinol, substitute an oxygen for the element or group associated with the carbon at either IUPAC location 11, 9, 7 or 5. If there is adequate control to insure the substitution only occurs at one location, an essentially pure chromophore will be obtained. If a mixture is obtained, further steps will be needed to separate these chemically different but quite similar chromophores. **Figure 5.5.10-2** illustrates the spectra of the resulting four chromophores. Note their color will be quite intense when prepared in solutions at high molar concentration or when precipitated individually on a substrate. However, as indicated in **Section 5.4.4**, the color may be transient if the chromophores are unable to be de-excited after excitation (bleaching). The C-7 chromophore will absorb strongly in the yellow-green and appear purple (the "visual purple" described in the literature). The C-5 chromophore will absorb strongly in the red and appear greenish. The C-9 material will absorb strongly in the blue and appear yellowish. The C-11 material will absorb strongly in the UV but not in the human visual spectrum, so this material will appear essentially colorless to human eyes (it will absorb in the shortwave region of the insect eye and appear as the complimentary color to the insect).

If the Rhodonines are members of the β-ionone family, their fabrication in pure form in the laboratory is also conceptually straight forward using the same steps as are used in the production of Vitamin A.

Frickel[271] has provided a comprehensive review of the chemistry of the retinoids which shows that most of the literature relates to molecular modifications aimed at pharmaceutical purposes. It has been rare to find a retinoid designed to include a polar element at a side chain location, although some work has been done on substituting fluorine into the side chain. Dawson & Hobbs[272] updated Frickel's review to 1994 and noted some additional work in introducing halogens into the side chain. If the work of Motto & Nakanishi[273] could be repeated with the intent of introducing oxygen instead of bromine in place of the methyl group attached to C-9, it should be possible to obtain Rhodonine (9) in the laboratory (i.e., non-biologically).

Sebrell & Harris[274] has provided a comprehensive review of the starting materials for Vitamin A synthesis, **Figure 5.5.14-3**. Many of these materials could be modified before further processing to obtain any one of the Rhodonines.

Tokunaga, et. al. have provided details concerning the fabrication of a variety of retinal analogs that might provide suggestions on how to fabricate the Rhodonines[275]. Zingoni, et. al. have provided similar material[276]. However, it is important to remember, the chromophores sought are the Rhodonines and not analogs of the retinols.

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[271]Frickel, F. in Sporn et. al. (1984) pg. 91
[272]Dawson, M. & Hobbs, P. Chap. 2 in Sporn et. al. (1994) 2nd ed.
[273]Motto, M. & Nakanishi, K. (1982) Heavy atom labeling of retinal in bacteriorhodopsin. Methods n Enzymology, vol 88, pp 178-19
[274]Sebrell, W. & Harris, R. (1967) The Vitamins. NY: Academic Press pg. 25
[275]Tokunaga, f. Ebrey, T. & Crouch, R. (1981) Purple membrane analogs synthesized from C17 aldehyde. *Photoehem. Photobiol* vol 33, pp 495-499
[276]Zingoni, et. al. (1986) Op. Cit.

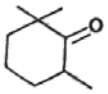
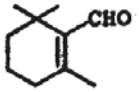
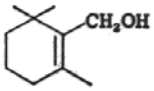
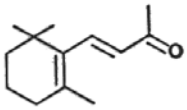
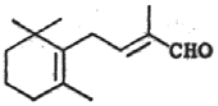
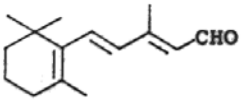
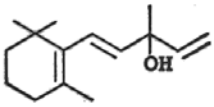
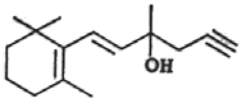
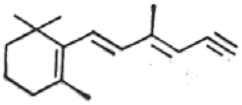
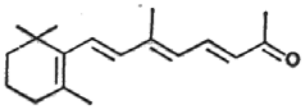
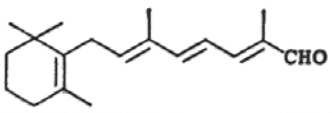
Starting material			Heading of subsection
Number of carbon atoms	Synonym	Formula	
9	2,2,6-Trimethyl-cyclohexanone		$C_9 + C_{11} = C_{20}$
10	**β-Cyclocitral**		$C_{10} + C_{10} = C_{20}$
	β-Cyclogeraniol		$C_{10} + C_{10} = C_{20}$
13	β-Ionone		$C_{13} + C_7 = C_{20}$
14	β-C_{14} aldehyde		$C_{14} + C_6 = C_{20}$
15	β-Ionylidene-acetaldehyde		$C_{15} + C_5 = C_{20}$
	Vinyl-β-ionol		$C_{15} + C_5 = C_{20}$
16	β-C_{16} acetylenic carbinol		$C_{16} + C_4 = C_{20}$
	β-C_{16} hydrocarbon		$C_{16} + C_4 = C_{20}$
18	β-C_{18} ketone		$C_{18} + C_2 = C_{20}$
19	β-C_{19} aldehyde		$C_{19} + C_1 = C_{20}$

Figure 5.5.14-3 Starting materials for the synthesis of the Rhodonines based on the synthesis of Vitamin A. From Sebrell, 1967

5.5.14.4 Relationship between Opsin and the Rhodonines

The Rhodonines, when in the liquid crystalline state, are complete chromophores. They need not be associated with any other material to accomplish the absorption process. When still suspended in a liquid medium, they will not, as a large group, display any unique polarization properties. If they are brought to an ever higher level of concentration in a liquid, they will form a gel which will exhibit preferred polarization planes. They can also be precipitated onto a surface after which they will exhibit preferred planes of polarization. They can be precipitated onto glass as well as a protein substrate. They must, of course, be associated with some type of nervous tissue in order to generate the necessary nerve signals. This is clearly not a role for opsin, defined as a protein, to play.

There are records in the literature of recombining the opsin extracted in the laboratory with the previously decanted material resulting after a period of time in a material that does absorb light in a manner described as similar to the original in-vivo or in-vitro process. This effect is reported without reporting any chemical activity involving any change in energy. This data could be interpreted as supporting the proposition that;

+ the chromophores are recombining on the surface of the substrate opsin in a liquid crystalline form and
+ the chromophores are in fact attached to the substrate opsin by very weak forces and not strong chemical interaction.

Based on the requirement that the chromophore be able to form a planar liquid crystal with its peers, it is very unlikely that Opsin and Rhodonine can be attached at the C5, C7, C9 or C11 positions. It is possible that they could be attached at the C15 position as long as the conjugation requirements are met. It is clearly more likely that they are not attached at all in the full bonding sense. It is more likely that they are associated with each other in the sense of a substrate host to an overlaying precipitate. If this is true, opsin is relegated to the role of a passive physical structure.

5.5.15 Extraction, Separation and Chemical Analysis of the Rhodonines

The Rhodonines share the extreme sensitivity of the retinoids to heat, light, oxygen and strong chemical reagents. In fact their sensitivity exceeds that of the retinenes. Therefore, both their extraction and preparation require special care. Groenendijk, Jansen, Bonting & Daemen noted the extreme risk of contamination when extracting retinoids from rod outer segments, noting their low initial concentration (0.5%) relative to the associated phospholipids[277]. Groenendijk, De Grip & Daemen also noted the difficulty of extracting any retinoid from rod outer segments using the best techniques available in 1980[278]. Their attempts to achieve significant extraction using solvents were unsuccessful. They chose to convert any retinoid present to the oxime before further analysis. Using this technique, they continued to assume that the chromophore present was retinal. Somewhat later, Packer contains many papers on the nature and processing of carotenoids by man[279]. An entirely new protocol is needed to replace those used previously for the extraction of the retinoids within the retina[280]. In those protocols, it appears that the chromophores of vision were routinely and unknowingly reduced back to retinenes (or retinene like residues). Even if still present as chromophores, they were evaluated in dilute solution and only exhibited their intrinsic, isotropic absorption spectrums. Previous protocols and procedures have led to glaringly inappropriate conclusions concerning the chromophores of vision.

If the actual chromophores of vision were present in the above experiments as the oximes of Rhodonine, the data representing them may have been looked upon as due to artifacts. There is the possibility of two distinct oximes being present due to the chemical resonance between the two oxygens of Rhodonine and two distinct isomeric configurations for each oxime. The molecular weights of these oximes would be higher by the weight of one oxygen than expected for oximes of the retinenes.

5.5.15.1 Conventional methods of extracting the retinoids

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[277]Groenendijk, G. Jansen, P. Bonting, S. & Daemen, F. (1980) Analysis of geometrically isometric Vitamin A compounds. Methods Enzymol. vol. 67, pp 203-220
[278]Groenendijk, G. De Grip, W. & Daemen, F. (1980) Quantitative determination of retinals with complete retention of their geometric configuration. Biochim. Biophysica Acta, vol 617, pp 430-438
[279]Packer, L ed. (1990, vol. 213, and 1993, vol 214) Methods in Enzymology: Carotenoids, NY: Academic Press
[280]Redfern, C. (1998) Retinoid Protocols. Totowa, NJ: Humana Press. Pp 6-7

Berman discusses the difficulty of extracting the proteins of vision from the retinal outer segments based on the assumption that the chromophores of vision are an integral part of the protein rhodopsin[281]. She discusses the various extraction techniques based on this assumption and the resulting percentage compositions reported in the literature.

Frolick & Olson describe the conventional methods of extracting retinoids from biological samples[282]. They stress the delicacy of the retinoids and describe two important points to consider, (1) whether the method chosen will result in complete extraction of the retinoid of interest, and (2) whether it will contribute to the production of artifacts. Unfortunately, they do not consider whether the product itself could be degraded. They do stress the sensitivity of these labile compounds to both hydrolysis and oxidation. They also address the use of fluorescence in assays. This work points out that the use of fluorescence is a negative test for the retinoids of vision when in liquid crystalline form. They point out that mass spectrometry is one of the most specific forms of assay for the retinoids. The utility of this technique will be discussed in **Chapter 6**. They do not describe how the techniques mentioned are able to remove a retinoid from within the putative rhodopsin molecule, or how to do in without damage to the retinoid.

5.5.15.2 Special methods for extracting the Rhodonines

Many of the steps discussed in the literature for obtaining retinol from biological samples are not applicable to the Rhodonines. These steps inadvertently destroy the chromophores being sought, the Rhodonines, and produce retinol by default. Most of these steps will insure that the end product is in fact retinol because of the sensitivity of the Rhodonines to hydrolysis, saponification, etc. Furthermore, the Rhodonines exhibit the same ultraviolet spectra as their chromogens, the retinenes and many other retinoids including retinyl esters and retinyl ethers; only their visible spectra are different. Whereas all of the Rhodonines, as members of the Vitamin A Group, exhibit an isotropic visible spectrum peaking at or near 495 ± 5 nm. in most dilute solutions, their individual enhanced absorption profiles will only appear under the proper conditions. Therefore, confirmation of the presence of a specific Rhodonine requires very careful methods. When in the liquid crystalline state of matter and deposited on a planar substrate, the enhanced absorption profile is highly anisotropic and peaks at 437, 532 or 625 ±3 nm.

Recovery of the individual Rhodonines from biological sources is not difficult if the proper methodology is adopted. The methods and suggestions of Papermaster[283] are not supported.

Sporn, Roberts & Goodman give a thorough review of the difficulties of handling the retinoids in order to obtain repeatable results. However, they do not recognize or discuss the additional precautions that must be taken with regard to liquid crystalline materials. Basically, if a liquid crystalline material is converted to a different state of matter, its characteristics can be assumed to change. Therefore, one cannot expect to characterize the chromophores of vision that involve the liquid crystalline state of matter (as when present in the disks of the retina) by using material that has been converted to another state of matter during the extraction and separation process. In addition, these chromophores are very delicate molecules from an energy state perspective and should not be subject to "detergents" and other reactants such as sodium based complex salts. Although, it is possible (and actually easy) to return the chromophores of vision to the liquid crystalline state after extraction and purification, ***it is not easy to prevent conversion of the chromophores into their chromogens or similar retinoids.*** These decomposition products do not exhibit the resonance phenomena (and resulting spectral characteristics) no matter what state of matter they are in.

Of the two primary methods of isolating the retinoids, saponification and direct extraction, saponification is almost bound to destroy any actual resonant retinoids present. Direct extraction promises more success. However, even in this method, care must be taken to avoid oxidation, or other modifications (including rearrangements) of the chromophores. Roberts, et. al.[284] has shown that significant levels of hydrolysis occurred in both the direct extraction method, using the sodium sulfate procedure of Ames et. al., and the lyophilization method of Ito. It appears that hydrolysis leads to retinol as an end product. This accounts for the common assumption that retinol (which was found in the final residue) must have been the original chromophore (with or without an associated opsin).

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[281]Berman, E. (1991) Biochemistry of the Eye, NY: Plenum pp 332-333
[282]Sporn, M. Roberts, A. & Goodman, D. (1984) The retinoids, vol. 1. NY: Academic Press, pp. 189-225
[283]Papermaster, D. (1982) Preparation of retinal rod outer segments. Methods in Enzymol. Vol. 81, pp. 48-53
[284]Roberts, A. Nichols, M. Frolik, C. Newton, D. & Sporn, M. (1978) in Cancer Research, vol. 38. pp. 3327-3332

It must also be recognized that after suitably isolating the chromophores, their resonant configuration and polar end groups make their identification by chemical tests difficult. If the chromophores are members of the oxonol family, as proposed here, they can exhibit both an aldehyde moiety and an alcohol moiety at the same time. Because of this, conventional colorimetric testing, as used for the non-resonant natural retinoids, is not likely to be sufficiently precise to identify the resonant retinoids--either as a group or individually. If the chromophores of vision are actually members of the merocyanine family instead of the oxonol family, they may still give unusual results in chemical tests because of their resonant properties involving the polar group containing oxygen. NMR testing is a more suitable and definitive test to identify the individual chromophores of the Rhodonine family.

It should be relatively easy to separate the individual chromophores of the Rhodonine family by re-crystallization. Because of their liquid crystalline properties, they will tend to crystallize out in separate domains, each exhibiting a different color (apparently as they do when stored in the RPE of the eye).

When attempting to extract and characterize the retinoids of vision, it is best to consider the above factors and then to follow Sporn, Roberts & Goodman's advice[285]: "In conclusion, before any extraction procedure is applied to the removal of retinoids from tissue samples, it is essential that the method be thoroughly examined with the appropriate controls. Without this close examination, of methods used, the data obtained could be both difficult to reproduce and unrelated to physiological events." More specifically, the data will probably only apply to the residue, retinol, resulting from excessively aggressive extraction techniques. At best, it will apply to the Rhodonines in dilute solution.

Arikawa et al. (1999) claim to have successfully isolated and defined the ultraviolet chromophore of vision in *Papilio*[286] They performed the re-crystallization of the liquid crystalline material by extracting it from the retina using the formaldehyde method (section 2.4) and precipitating it on a microscope slide substrate and then observed its emission spectra through a light microscope-attached photodiode array (USP-410, Unisoku), equipped with an image intensifier (V1366U, Hamamatsu photonics). The glass of the microscope slide and the optics of the microscope/image intensifier probably limited the measurements (**Section 3.6.4.1**)

They identified the chromophore as a retinene, 3-hydroxyretinol, but immediately qualified their remarks by suggesting it might be the form 3-hydroxyretinal (paragraphs 2.4 & 3.2). The use of formaldehyde in extraction may have introduced an unexpected complication into their molecular identifications. Their method involved a presumptive identification as they did not demonstrate the character of the atom or group attached to Carbon 5 of these (potentially conjugated diol, and therefore resonant) arenes. This work demonstrates these two molecules are both conjugated diols with an oxygen atom at positions 11 and 15 (or 1 and 5 depending on the convention employed) of the aliphatic chain. As a result, the molecule is more appropriately named Rhodonine(11), a retinine where the underlined "i" indicates the diol form of this family.

The prefix 3-hydroxy- indicates this retinine family is based on vitamin A_3 as described in **Section 5.3.3**.

It is now appropriate for some adequately equipped laboratory to isolate all four of the chromophores of animal vision. The protocol used must respect the resonant structure of each chromophore and not cause a reduction of the resonant chromophores (retinines) to retinenes (of either the alcohol or aldehyde form). Such a reduction will result in the loss of the (directionally sensitive) absorption band required in vision. It will be found that the protein-based opsin plays no active role in transduction and is not even associated with the eyes of *Insecta*, and probably *Mollusca* as well. Isolating these chromophores will have a major impact on the current direction of genetic research. Peak absorption of the retinenes occurs for light traveling parallel to the resonant axis or the retinine. This absorption is in addition to the isotropic absorption (centered at 495 nm) associated with the non resonant structure.

5.5.15.3 Additional comments leading to an extraction protocol

The following script was prepared in discussing the extraction problem with a potential investigator. It incorporates many of the comments in the discussion above.

1. No one has ever successfully isolated the chromophores (or "photopigments") of chordate vision for two reasons. First, they are highly reactive free-radicals. Second, they are only viable as chromophores when they are in the liquid crystalline state of matter. As noted above, Arikawa et al. (1999) claim to have isolated

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[285]Sporn, et. al. (1984) Op. Cit. pp. 193-198

[286]Arikawa , K. Mizuno, S. Scholten, D. Kinoshita, M. Seki, T. Kitamoto, J. & Stavenga, D. (1999) An ultraviolet absorbing pigment causes a narrow-band violet receptor and a single-peaked green receptor in the eye of the butterfly Papilio *Vision Res* vol 39, pp 1-8

the UV chromophore, Rhodonine(11). However, they did not provide any sensitivity spectrum and relied upon the fluorescence of their reconstituted “chromophore” deponsited on a glass substrate.

2. Most laboratory efforts have involved separating the outer segments of the photoreceptors by solvation using relatively strong detergents (particularly those containing sodium ions). The goal has been to recover the large opsin molecules instead of the chromophore molecules that rapidly separate from the complex. Most researchers have unknowingly poured the solvated chromophores (now transparent chromogens) down the drain by mistake.

3. Like most water soluble inorganic ions, sodium ions interact with the delicate color chromophores which have a bandgap of less than 2.0 electron volts. The critical covalent oxygen is removed or replaced very easily. One electron volt is a minuscule amount compared to the formation energy of a typical molecule (1 ev = 1.6×10^{-19} Joules).

4. The separation of the chromophores can be accomplished by dissolving only the chromophores of the outer segment in alcohol (or some ethers). The key is extracting them from the interphotoreceptor matrix (IPM) without allowing them to be attacked by other chemicals. They must not come in contact with other materials present in the blood stream or interneural matrix (INM).

5. Once extracted and in solution, they can be re-crystallized in a protected (absolutely dark) environment to form islands (films) of pure liquid crystalline chromophore. The spectral properties of the material can then be measured. As I mentioned, the center wavelengths of the chromophores can be easily measured. The spectral width of the response will depend on how large the liquid crystalline domains are. If uncontrolled light strikes the material, the chromophore will become transparent.

More analysis of how to handle the chromophores is needed. However, one promising approach is to deposit the material on a "half-silvered mirror" surface made of actual silver and not mercury. The result would be similar to a conventional photographic emulsion.

6. When the material is removed from a retina, and no longer de-excited by the neural system, its relaxation time will go up immensely. Thus great care must be taken to avoid bleaching the material beyond recovery during the spectral measurements. Use of a dark-room red "safelight" is not adequate. If your eye can see the light, so can the material you are trying to investigate.

5.5.15.4 History of trying to isolate the chordate chromophores

Attempts to isolate the chromophores of vision were first undertaken seriously in the 1930's. However, at that time the understanding of the character of the chromophores was minuscule. Resonance was a little known subject in organic chemistry, particularly with respect to the photo-chemistry of organic chemistry. As a result, it became common to consider isomerization to be the key mechanism of transduction (although it was never shown how isomerization could accommodate four different spectral absorption regimes.

Attempts to isolate the chromophores of vision began with very brutal chemical methods. Many of these methods such as the use of high-g centrifuges, resulted in the chromophores and/or their residues going into solution and being decanted off and discarded.

Seki et al. employed particularly brutal chemical techniques (both physically shaken and energy-state wise) to process insect chromophores with decomposition energies of less than two electron volts[287]. They did not include any spectral data in their paper of 1994 to demonstrate any of their materials exhibited the spectra of in-vivo chromophores. Their focus was on exploring the role of stereographic state of the retinenes (with an *e*) among the potential chromophores. They did not consider the possibility that any of the chromophores might be resonant and belong to the retinines (with an *i*). Nor did they consider the possibility that the in-vivo chromophores might be present in the liquid state of matter. They extracted their residues using a “modified formaldehyde” method traceable to Suzuki et al. (1986)[288]. Suzuki et al. focused all of their activity on preserving the presumed 11-*cis* retinal configuration of the chromophore(s) of vision. No spectral data was provided to support this reliance. They

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[287]Seki, T. Isono, K. Ito, M. & Katsuta, Y. (1994) Flies in the group Cyclorrhapha use (3S)-3-hydroxyretinal as a unique visual pigment chromophore *Eur J Biochem* vol 226, pp 691-696

[288]Suzuki, T. Fujita, Y. Noda, Y. & Miyata, S. (1986) A simple procedure for the extraction of the native chromophore of visual pigments: The formaldehyde method *Vision Res* vol 26(3), pp 425-429

were unaware of the necessity of resonant material covering the four spectral bands of interest required the chromophores be present in all-*trans* form. They employed formaldehyde at a concentration of 6M and a phosphate buffer at a concentration of 0.1M. They noted the "Addition of antioxidant was not necessary during the incubation and the extraction, because formaldehyde itself has a strong reducing power." The presence of a reducing agent is not compatible with resonance in the chromophores.

5.6 Physical configuration of the chromophores

5.6.1 Adsorption versus reaction between the chromophores and opsin

There are at least three methods by which the chromophores of vision could be associated with the substrate proteins. It has generally been recognized that the actual method should be nearly energy neutral or slightly exothermic.

5.6.1.1 Historical Background

Synopsising the relevant history concerning the association of opsin with the chromophores presented by Rubin & Walls[289],

> Kuhne first extracted a photosensitive material from the retina in 1877 and called it *Rhodopsin.*
>
> Krause in the 1930's proposed that the chromophore of rhodopsin was similar chemically to the polymethine compounds known as "cyanine dyes" which are also used to sensitize photographic emulsions to the longer wavelengths of the visible spectrum. He proposed that the chromophore was bound to a very large lipid/protein complex with a molecular weight near 800,000.
>
> Wald took a different approach than Krause and considered the chromophore a carotenoid which he named retinene. He associated this chromophore directly with a protein resulting in a molecular weight on the order of 25,000-50,000.
>
> Kuhne proposed that the association of the chromophore and the substrate occurred spontaneously and he called the process *anagenisis.*
>
> Collins proposed that the chromophores were attached to the opsin through a Schiff base formed by substitution for the oxygen atom of the retinene.
>
> Wald claimed to have separated the chromophore from the substrate and found that they recombined in the dark to form the original rhodopsin. See **Section 5.5.2.2.**
>
> Wald speculated that the association was by means of a sulfhydryl group (-SH), the sulfhydryl radical being one of the most "active" ones known to biochemists at the time.

The sulfhydryl proposal did not gain support and has disappeared from the literature. More recent investigations have generally assumed the association is by way of a Schiff Base reaction.

Wolken has worked with similar materials and converged on a molecular weight between those proposed by Krause and by Wald. He has proposed a molecular weight of 275,000 to 295,000 for the putative rhodopsin.

It is worth noting that the proposed chromophores are minuscule compared to the molecular weight of the protein. This difference makes it difficult to demonstrate the chromophore is present in the molecules being evaluated.

All of the above experiments and proposals assumed the opsin and the chromophore were firmly linked through a substantial chemical bond. Another possible association is by way of a process poorly understood in the 1950's. This coupling appeared under a variety of names initially but it is now called the 'hydrogen bond'.

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[289]Rubin, M. & Walls, G. (1967) Fundamentals of Visual Science. Springfield IL: Charles C. Thomas pg. 57-82

Most materials that precipitates onto a surface are now known to utilize hydrogen bonding as the "glue" holding the composite together. These forces are quite weak but adequate for the purpose. Hydrogen bonding does not involve any true chemical reaction. It does not impact the electronic configuration of either of the materials involved.

The simplest and most direct method of association between the chromophore and the substrate, and the one most compatible with what is known about the coating of the substrate in the IPM region, is the process of hydrogen bonding. This method has no significant effect on the spectral characteristics of the chromophore, involves very small energy changes and is completely compatible with the results (but not the interpretation) Wald reported.

5.6.1.2 Current status

The adsorption of dyes to proteins has been studied extensively in the textile industry[290]. A critically important fact is that acid dyes do not dye cotton[291]. They can dye wool, silk and other natural proteins. Rattee & Breuer have provided considerable information concerning the mechanism of dye interaction with proteins, including amphoteric proteins. Their Chapter 5 discusses the molecular nature of proteins. They have also addressed the subject of hydrogen bonding between dyes and proteins.

It is well known that a chromophore or other dye will self assemble into a liquid crystalline monolayer on both a protein substrate and a silver halide substrate. The process is known by various names, stacking, aggregation, etc[292]. but the result is the same. Wolken noted this aggregation when a precipitate formed in a solution of retinene$_1$ and a variety of proteins that were dissolved in acetone and shaken[293]. In the photographic field, the progression of the process is monitored until maximum sensitivity is reached. At that point, no more deposition is supported. Under examination, the resulting film is always found to consist of a monolayer covering the entire surface of the substrate. This is apparently the same procedure used by Balogh-Nair & Nakanishi[294].

5.7 The use of templates

Templates have played a significant role in the early understanding of animal vision. In 1953, Dartnall provided a series of templates that many researchers found useful[295]. Unfortunately these early templates were based entirely on empirical data--with some attempts to align them to putative mathematical equations that were in themselves inappropriate. If possible, a template should represent the form dictated by the underlying physics whether based on Gaussian, Fermi-Dirac or other statistics. Many of the templates have relied upon the linearity of the visual system to support their legitimacy.

Partridge & De Grip[296] have provided a review of the different templates used in vision research over the last 50 years and suggested a new one. They indicate clearly *some* of the problems with the different templates. In general, their proposed template completely ignores the ultraviolet spectra of the chromophores. Therefore, they are attempting to scale only the visual portion of the overall absorption curve. Because of the difference in wavelength between the UV and visual peak for each chromophore, the short wavelength sides of the absorption spectrums are asymmetrical with respect to the long wavelength sides. Partridge & De Grip utilized the long wave side of the visual peaks in their calculations. They also stress that their template only applies over a limited ratio of λ_{max}/λ (where λ_{max} = 497.63 nm) *and only to the peaks of the spectral waveforms*. They also point out that their work was carried out under dim red light (λ greater than 620 nm). Unfortunately, this level of illumination has the potential of significantly bleaching the L–channel chromophore in an uncontrolled manner.

More recently, Palacios, et. al.[297] touched briefly on each of a wide variety of templates proposed during the last ten years, including one based on a log-normal distribution. This latter distribution mandates a significant asymmetry

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[290]Rattee, I. & Breuer, M. (1974) The physical chemistry of dye adsorption. NY: Academic Press.
[291]Venkataraman, K. (1977) The analytical chemistry of synthetic dyes. NY: John Wiley & Sons. pg. 6
[292]Rattee, I. & Breuer, M. (1974) Op. Cit. Pg.152-153
[293]Wolken, J. (1966) Vision. Springfield, IL: Charles C. Thomas. pg. 45
[294]Dawson, M. & Okamura, W. (1990) Chemistry and Biology of Synthetic Retinoids. Boca Raton, FL: CRC Press. pg 169
[295] Dartnall, H. (1953) The interpretation of spectral sensitivity curves. Brit. Med. Bull. Vol. 9, pp. 24-30
[296]Partridge, J. & De Grip, W. (1991) A new template for rhodopsin (Vitamin A1 based) visual pigments. Vision Research vol. 31 no. 4 pp. 619-630
[297]Palacios, A. Varela, F. Srivastava, R. & Goldsmith, T. (1998) Spectral sensitivity of cones in the goldfish, Carassius auratus. Vision Res. vol. 38, pp. 2135-2146

between the long and short wavelength side of a given spectrum. They also hint at how the variation in bandwidth between the various absorption spectrums has played a role in shaping these templates. Stockman, et. al.[298] have also discussed various templates, discoursed on the subject of bandwidth as a fraction of center frequency (or wavelength) in various spectrums and offered their own template. Stockman offered a template based entirely on curve fitting by a computer expanding a series in even powers of the unknown. This approach is guaranteed to provide a symmetrical absorption spectrum. The approach was substantially based on data in a 1993 paper[299]. That paper suggests why an absorption spectrum is frequently reported near 576 nm. The experimentation used a 17 Hz. flicker rate. The paper presents data with a statistical variance on the order of ±1-2 nm that is consistent with the theoretical values of this work. Based on the theory of this work, obtaining a higher degree of accuracy will require measuring, and reporting, the core temperature of subjects to an accuracy of close to 0.1 Celsius.

Lamb[300] has offered a template using an empirical series based on an exponential expression. Lamb also summarizes the long wavelength slopes for a variety of photoreceptors as measured by the various authors mentioned here. Both the Lamb approach, the Palacios observation, the tabulation supplied by Lamb, and the measurements of both Stockman and Sliney are consistent with a feature of the Fermi-Dirac difference equation. The response far from, and on either side of, the absorption peak falls off exponentially with wavelength. This feature is not found in a one dimension normal (Gaussian) or a one dimension log-normal function. By using, Stockman's approach, it can be emulated to an arbitrary degree of accuracy. However, Stockman's approach has already been questioned based on the implied precision of his calculated values, eight terms (with associated arbitrary constants) and the use of 18 place numbers (twelve after the decimal) to define these arbitrary constants. With the actual Fermi-Dirac difference equation available, it can be used to rearrange Lamb's equation into the Fermi-Dirac difference equation plus a residue (which represents the transformation from a λ_{max}-based formulation back to a half-amplitude-based formulation and could also include an experimental error term).

As remarked by Palacios, et. al. when discussing templates: "Although with limited (or no) theoretical basis, such templates are nevertheless very useful in characterizing noisy MSP data or spectral sensitivity measurements made from single cells at widely separated wavelengths." This work provides a traceable theoretical foundation to the absorption spectrum of each visual chromophore and a specific theoretical equation, based on Fermi-Dirac statistics, that can be used to draw a precise template for each absorption spectrum. However, with the availability of such an equation, the term template is no longer needed. The graph is an expression of the underlying theoretical equation. The availability of the equation also highlights the fact that there is no precisely defined maximum wavelength for any of the absorption spectrums. The absorption spectrum of a given chromophore is defined by the energy (directly proportional to frequency and inversely proportional to wavelength) of its two half amplitude points. A peak wavelength can be calculated from the equation. However, it is at a very flat part of the characteristic. This peak will generally not agree with the values found in the laboratory for two reasons. The data is usually smoothed by a finite width spectrometer filter and individual spectral scans are usually combined. The resulting function is invariably a more Gaussian characteristic than appropriate in accordance with the Central Limit Theorem. An alternate method of characterizing the spectrum would be to take either the mean or median of the two half amplitude points. It is the difference between the half amplitude points of adjacent spectral responses that can be determined most precisely by calculating the theoretical luminosity function. By adjusting the theoretical half amplitude points, the best values can be obtained when the theoretical luminosity function best describes the amplitude and shape of the Brezold-Brucke peak near 494 nm and the Purkinje peak near 600 nm. This approach is used in **Chapter 17** to define the half-amplitude points to an accuracy of ±2 nm based on the experimental data.

Palacios, et. al. provide a tabulation of most of the recent work on defining the peak spectral wavelengths of the goldfish, *Carassius auratus*. Except for the short wavelength chromophore, the theoretical values presented here are within the standard deviation of virtually all of the values given in their table. These values are all based on axially illuminated photoreceptors, either using conventional microspectrophotometry (MSP) or using MSP in conjunction with the suction pipette electrode (SPE) technique. The black body temperature of the light source was not specified in the summary table and it is quite likely that the source was not operating at a high enough temperature to obtain correct experimental values for the S–channel. Palacios, et. al. also reported a UV–channel chromophore with a peak wavelength at "about 356 nm." Again considering the light source used, and the additional fact that a quartz bulb is required at this wavelength for good transmission, this value is close to the theoretical value of 342 nm proposed here.

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[298]Stockman, A. Sharpe, L. & Fach, C. (1999) The spectral of the human short-wavelength sensitive cones derived from thresholds and color matches. Vision Res. vol. 39, pp. 2901-2927

[299]Stockman, , MacLeod, D. & Johnson, N. (1993) Spectral sensitivities of the human cones. J. Opt. Soc. Am. A. Vol. 10, no. 12, pp. 2491-2521

[300]Lamb, T. (1995) Photoreceptor spectral sensitivities: common shape in the long-wavelength spectral region. Vision Res. vol. 35, pp. 3083-3091

Figure 5.7.1-1 from Hoglund, et. al. illustrates how Dartnall's templates of 1953 deviate from the actual spectrum of Insecta, including the ultraviolet portion, as represented by the moth, *Deilephilia elpenor*[301]. Note five points.
1. The skirts of the measured responses are not tracking each other.
2. The widths of the measured responses are not similar to the widths predicted by the template.
3. The above deviations are systemic, indicating the theory underlying the template is not adequate. Clearly, when extended to the S– and UV– channels, the template derived from the M–channel is less than ideal.
4. The template was apparently derived on the basis that the chromophoric spectra were related incrementally based only on spectral *frequency* rather than wavelength.
5. The measured peak wavelengths track the peaks proposed in this work, 342, 437 and 532 nm, quite well.

Stiles & Wyszecki performed a mathematical analysis in 1974 that made the following claim in its last line[302]. ". . . it is readily calculated that for the normal trichromat no visual pigment with a peak wavelength greater than 556 nm can be of the rhodopsin type." This line has frequently been quoted out of context and without describing the "rhodopsin type" defined in the paper. Their rhodopsin type was modeled on an extrapolated Dartnall template, the assumption of linearity in the visual system, and the assumption of linearity in the operation of the photoreceptors. The findings do not apply to real chromophores obeying Fermi-Dirac statistics in a logarithmically based system.

The figures and the theory developed in this work assume the spectra are based on Fermi-Dirac statistics and are related incrementally based on spectral *wavelength*. In addition, an analysis has been performed to determine if the individual chromophoric spectra show a variation in the ratio of peak wavelength to ½ amplitude wavelength difference. A similar ratio, based on frequency at the lower frequencies used in the radio spectrum, is considered a quality factor and is designated by Q. The best available estimates of the Q of the visual chromophores of the human eye appear in **Table 5.5.10-2** and in the appendix describing the Standard Eye.

The wavelength corresponding to the peak in the spectrum is directly related to the (effective) physical length of the chromophoric portion of the retinoid. The term effective is included in the above sentence to avoid confusion. The physical length of the resonant portion of the molecule is too short to be resonant at the proper frequency if the speed of propagation is assumed to be equal to the speed of light. However, these molecules exhibit a "slow wave structure" where the speed of propagation is approximately 200-250 times slower than the speed of light[303]. Thus, the effective resonance length of the molecule is approximately 200-250 times longer than the physical length. As shown elsewhere herein, this effective length can only be used in a structure containing many molecules such as a liquid crystalline structure.

The peak wavelengths in the figure appear to be within a few nanometers of the theoretical. This is the best attainable agreement without carefully specifying the temperature of the animal at the time of data collection. Note, the temperature of a nearby substrate is not sufficiently accurate unless a calibration procedure has been used to ascertain the offset between the specimen and the substrate. Whereas significant spectral changes can be measured by varying the temperature of cold-blooded animals, Brindley claimed in 1960 to have detected a similar change in warm blooded animals under thermal stress[304]. The section containing this claim did not appear in his second edition, in 1970.

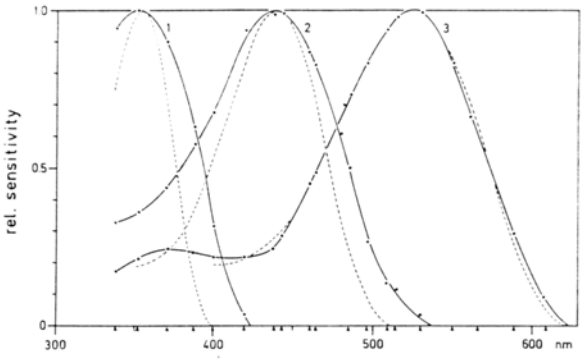

Figure 5.7.1-1 Spectral sensitivity (mass recording) of the moth, *Deilephila elpenor*, retina compared to the templates of Dartnal Individual curves were obtained during differential adaptation. From Hoglund et. al., 1973

[301]Hoglund, G. Hamdorf, K. & Rosner, G. (1973) Trichromatic visual system in an insect and its sensitivity control by blue light. J. Comp. Physiol. vol. 86, pp. 265-273
[302]Stiles, W. & Wyszecki, G. (1974) Colour-matching data and the spectral absorption curves of visual pigments *Vision Res* vol 14, pp 195-207
[303]Platt, J. (1964) Systematics of the electronic spectra of conjugated molecules. NY: John Wiley paper 20, pp. 71-123 *originally published in* Hollaender, A. *ed*. (1956) Radiation Biology NY: McGraw-Hill
[304]Brindley, G. (1960) Physiology of the retina and visual pathway 1st ed. Baltimore: The Williams & Wilkins Co. pp. 251-252

Boynton[305] discusses the inadequacy of the available templates, with specific emphasis on their use in abnormal color vision.

Govardovskii et al. developed a new nomograph in 2000 for animals employing the Vitamin A2 based chromophores[306]. The template and the underlying data supports the predicted spectra of this work very closely as shown in **Figure 5.7.1-2**. The measured and template peaks are very near the peaks predicted by this work.

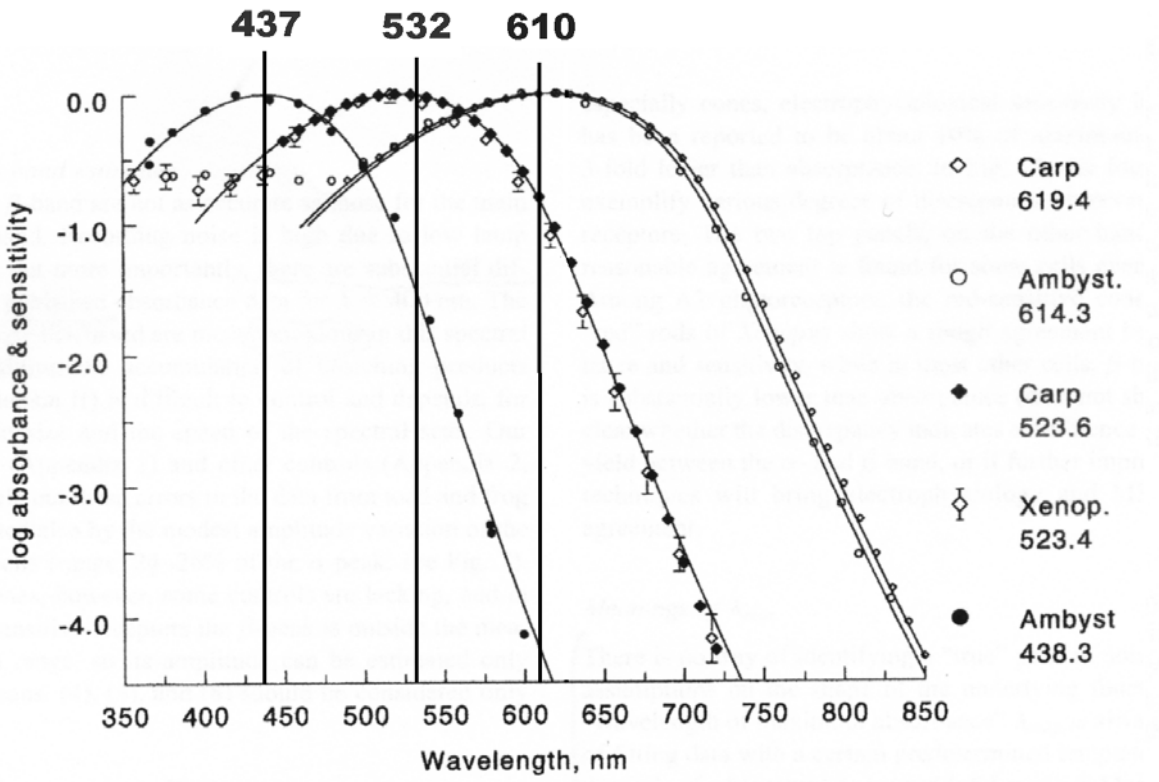

Figure 5.7.1-2 Comparison of a new template describing the absorption spectra of the A2 family of chromophores. Data is from different fish as indicated on the right. The peaks in the measured spectra agree within 1.5 nm for the S-chromophore, less than 9 nm for the M-chromophore, and within 10 nm of the peak of the broad L-chromophore. Data from Govardovskii, 2000.

With the detailed models and precise mathematical equations available from this work, the old templates found in the literature are seen to be obsolete. New templates, if desired, can be derived that provide much more accurate predictions. However, the equations themselves are available. Predictions by scaling are no longer required. Furthermore, the equations avoid the problems arising from linearly subtracting two nomographs representing processes that are not linearly related.

[305]Boynton, R. (1979) Human color vision. NY: Holt, Rinehart & Winston pp. 147-155
[306]Govardovskii, V. Fyhrquist, N. et al. (2000) In search of the visual pigment template *Vis Neurosci* vol 17, pp 509-528

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