Extraction & Characterization of the Visual Chromophores--Rhodonine

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The appearance of the computer analysis and generation of molecular structures, particularly the appearance of Jmol and the archiving of these structures by the Royal Society of Chemistry, have introduced an entirely new ability to describe the chromophres of vision, the Rhodonines. This new capability and a review of the current framework for the choromophores will be developed beginning in **Section F.3.**

The initial portion of this appendix is a condensation of the material in Chapter 5 of "Processes in Biological Vision" and in the later portions, an expansion and update of that material. A Table of Contents, a List of Figures and an Index will be found at the end of this appendix.

F.1 Background

The conventional recipe for extracting the chromophores of vision was developed in an era when the tools available were more limited than they are today; and in fact, the concept of what was being sought was more primitive than today.

Through a variety of investigations, it was established that vision suffered in an animal if there was a deficiency in Vitamin A. Later, a chemical compound(s) was found in the retina that appeared to be related to Vitamin A. In fact, tests were performed that indicated that the chemical compound was either an alcohol or an aldehyde. Based on these tests, the compounds were initially defined as the two retinenes, retinal and retinol--an aldehyde and alcohol of vitamin A. *As noted in **Section F.3**, these designations were imprecise, the chemicals were actually resonant members of the dienes that would now be described as retinines.*

Extensive investigations were carried out to determine the spectral responses of these materials and thereby confirm that they were the chromophores of vision. Unfortunately, these tests never showed either retinal or retinol to have a spectral absorption in the visual region.

During that early period, it was found that the retinenes did not exhibit the correct energy levels to be chromophores when in their normal biological configuration, i. e. geometric isomeric state. Extensive analyses were carried out and theories developed with the most acceptable, but still energetically unacceptable, theory revolving around a cis-trans transition in a retinene.

In isolating the vitamin A related compounds of the outer segments of photoreceptors, it was also found that there was a significant amount of protein present in the outer segments. It was logical in that day to draw the conclusion that the chromophore was a composition consisting of an undetailed protein, called Opsin, and one of the retinenes. A variety of theoretical constructs for this compound were proposed and attempts were made to create the so-called Rhodopsin from the natural (uncharacterized but isolated from retinas) protein, opsin and either man made retinal or man made retinol. These attempts were also unsuccessful. The most enduring theory of how Rhodopsin was formed suggested that a coupling related to a Schiff base was involved.

In one of the most well known experiments of the day; the investigator, who was one of the principal protagonists of the isomeric transition theory, attempted to recombine a man-made retinene with a native opsin to form an active chromophore. This attempt failed and the explanation was given that the wrong isomer had been used in the experiments. As far as is known the experiment was never repeated with the "correct" isomer.

The basic problems with the above work centered around the following facts;

+ The liquid state of matter was unknown in that time period.

+ The biological community was apparently unaware of the rules of chromophore chemistry being developed simultaneously in the photographic community.

+ The biological community was apparently unaware of the rules of hydrogen bonding, frequently called Van der Waal bonding in other communities during that period.

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Because of these inadequacies in the overall knowledge of the community at that time, the search for the chromophores of vision were never successful. This was primarily due to the following conditions;

+ The community did not have a clear model of what it was looking for.

+ The community was using laboratory techniques that actually destroyed the molecule they were seeking.

+ The community was using laboratory techniques that actually changed the critical electronic configuration of the moiety that was the actual chromophore they sought.

+ The community was unable to analyze adequately the moiety they believed to be the crucial part of the compound, rhodopsin.

The situation was described by Crescitelli[2] in 1972. In summary, Rhodopsin was first used as a name for the visual photoreceptor material in 1879 by Kuhne. Wald claimed to describe it in the 1930's with mixed success. There appeared to be two different molecular systems present; rhodopsin in terrestrial animals and porphyropsin in fresh water fish. Wald's writing converge around 523 +/- 3 nm. for the spectral peak of porphyropsin in a solution of digitonin. In 1960, Wald ignored the confusing evidence from etymology and *arbitrarily* defined rhodopsin as the 11-*cis* retinal-opsin complex and porphyropsin as the 11-*cis* 3- dehydroretinal-opsin complex. (Caution is advised here as these names were changed by the IUPAC-IUB in 1960, it can only be assumed that Crescitelli made the adjustments. It will become clear below that either the retinol or retinal root could be used interchangeably in that time period.)

To illustrate the problem, the following is a brief description of the actual chromophore(s) of vision in all animals presented in this work.

+ The chromophore conforms to the rules of chromophore chemistry in that it contains not just one alcohol or aldehyde group, but one of each. It contains two polar atoms which are capable of existing in two adjacent states of covalency and they are joined by a conjugated chain of carbon atoms. It is most likely a carboxyl-ion system. It is a chromophore in name only since it does not yet exhibit a visual band absorption characteristic. It is a very fragile compound which is easily decomposed.

+ The above "chromophore" is deposited onto a substrate in a liquid crystalline state similar to that employed in photography. It is only in this state that the chromophoric system exhibits a long wavelength absorption band (the *j*-band) in the visual region. This absorption band is related to the shared condition of its electronic states.

+ The above "chromophoric system" will absorb photons in the visual spectrum with great efficiency up to the limit of its capability--it will bleach. To be useful in vision, this chromophoric system must be in contact with another element that can continually de-excite the photon generated electrons in the chromophoric system. This other element is normally the dendritic structure of the neuron forming the photoreceptor cell of the retina.

Based on this simple description, isolation of the chromophores of vision from a retina must be done with care not appreciated in the 1930-60's.

First, the use of a detergent to solubilize the chromophore results in the loss of the required physical and resulting electrical structure of the chromophoric system . It will no longer exhibit absorption in the visual spectrum, even though the individual molecules are still intact.

Second, use of a detergent or additional agent containing an active metallic ion, such as Na+, will cause the chromophore molecule to give up one of its auxochromes. It will no longer exhibit an absorption band in the visual range no matter what its physical configuration relative to other molecules. It will become what appears to be either retinal or retinol.

F.2 Isolation and Identification of the Rhodonines

The above considerations set the stage for the successful isolation of the chromophores of vision. However, care is required. The retinoids are very labile compounds, being sensitive to light, temperature, and oxygen as well as the

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[2]Crescitelli, F. (1972) in Handbook of sensory physiology, Vol. VII/1, Dartnall, H. ed. NY: Springer-Verlag, pp. 253-257

metallic ions of Group I & II.

1. The process of separation and purification may destroy the liquid crystalline state of the chromophores. This is acceptable as long as it is restored later before attempting spectral measurements.

2. The use of any chemical with an active metallic ion must be avoided or the chromophore will be destroyed leaving behind only a chromogen related to the retinenes, retinol or retinal. It may still differ from them depending on what atom replaced the auxochrome in the chromophore.

Identification of the chromophores of vision can now be achieved using a variety of modern analytical techniques. Probably the most definitive identification involves nuclear magnetic resonance (NMR). This technique can not only confirm the presence of the two auxochromes in the chromophore; it can also define the positions of these auxochromes in the various forms of the chromophore.

Raman spectroscopy has also been explored as a method of identifying the chromophores but it does not appear to be as useful as high frequency NMR. (Loppnow, 1989)

By re-establishing the chromophore in the liquid crystalline state, it is also possible to measure its absorption spectrum and confirm that it is the chromophore of vision. Care must be taken here because after isolation and conventional purification, it is likely that a mixture of chemically similar chromophores are present. In this case, it is necessary to carry out further isolation and purification, using techniques such as repeated re-crystallization or possibly electrophoresis, in order to separated the various forms of the chromophores present. Following this step, each of the isolated chromophores can be returned to the liquid crystalline state and its visual range absorption spectrum measured.

The above procedures will confirm;

+ the existence of up to four chromophores of the rhodonine family in the retina of a given animal

+ the spectral absorption characteristic of each rhodonine matches the *in-vivo* microspectrometry of one of the photoreceptor outer segments (or equivalent structure in arthropods and molluscs) of animal vision.

Because of the physical and electronic nature of these chromophores, it is possible to use them in the coating of silver halide crystals in order to fabricate photographic film. Such an emulsion will be sensitive to the same spectra as the individual chromophores (as long as a small amount of silver sulphide is present to activate the silver halide).

The name rhodonine is derived from the rose color historically associated with the chromophores of vision, rose being the spectral compliment of the dominant chromophore absorbing in the yellow-green (peak at 532 nm.) portion of the spectrum. The suffix is from the chemical class of the material, a hydrocarbon containing two polar atoms separated by a conjugated carbon chain. In this application and with respect to vision, the quinone ring in the molecule is only used for stabilizing purposes.

The actual absorption spectra of each chromophore are the same as the “dyes” used in the color separation printing industry as they have the same electronic configuration as the magenta, cyan and blue dyes used in those films. [see Stenstrom, pg. 175 for a useful sketch]

F.3 The redefinition of the Rhodonines in the computer age (ca. 2014)

After a very prolific career in the biology of vision over 30 years, Wald essentially withdrew from the field and became an activist opposing the Vietnam War. He refused to discuss his work further and essentially withdrew from the biological community around Boston (although his wife, Ruth Hubbard remained active in her ancillary area). His concepts related to the photoreceptors of vision were never seriously challenged or verified during the following 40 years. While extensively repeated in all relevant textbooks to this day, it is now clear that his concept, and the protocols used to develop them were seriously flawed.

• Wald’s aggressive protocol for separating the disks of the photoreceptors from the remainder of the sensory neurons (using detergents specifically and centrifugation) resulted in the liquid crystal coating of the disks being decanted away and released down the chemical sink drain.
• The presence of a retinal moiety within the opsin protein forming the disks was never disputed but is was never shown to present the required spectral absorption properties either.
• The method of photoreceptor cell formation was never developed during Wald’s time.
• The mechanism of photon absorption, also used in photographic emulsions, was not elucidated until 1956 by Pratt.
• The depiction of the basic form of the four chromophores was first conceptualized in the 1960's by Fulton

• The liquid crystalline structure of the chromophores forming the coating on the exterior of the disks was not appreciated until the 1980's.
• The 3D description of the chromophores has only recently been defined using computerized depictions.

The preferred method of chromophore extraction is simpler than that of Wald and is proposed to give superior results. It is described in **Section 5.15** and involves separation of the chromophores from the photoreceptor substrates without the employment of oxidizing detergents and the differential re-crystallization of the chromophores (if a mixture of photoreceptors form the starting material).

F.3.1 Requirements on the chromophores of mammalian vision

The necessary properties of the chromophores in order to be effective in vision have now become much clearer:

• To be an effective chromophore in the visual spectrum two orbitals separated by a conjugated chain are required.
• To be an effective chromophore, the aliphatic chain must be in *all-trans* form (straight), resulting in delocalized (shared) electrons.
• To be an effective absorber, the chromophore must be stimulated by photons traveling parallel to the aliphatic axis.
• To be an effective absorber, the chromophores must form a dense monolayer perpendicular to the incident photons.
• A liquid crystalline (nematic) monolayer offers a uniform surface of delocalized (and excitable) electrons at maximum density.
• To be an effective photoreceptor cell, the cell must place the active surface of the disks of the outer segment perpendicular to the input photon stream.
• To be maximally effective, the outer segment of the PC must place multiple active surfaces perpendicular to the input photon stream.
• To be maximally effective, the retina of the eye must exhibit a filled array of photoreceptor cells with no appreciable space between the outer segments of the PC's.
• To be maximally effective, the entrance aperture of the outer segment of each photoreceptor of the retina must be maintained in and perpendicular to the Petzval plane of the optical system.

F.3.2 Features related to the chromophores and their neural interface

The disks, and their liquid crystal coating of chromophore is replaced on a weekly basis in humans (and presumably most other mammals (if not all animals). See **Section 4.3**.

New disks are extruded from the inner segment of the PC before being stacked in the outer segment for utilization. There is no lemma surrounding the outer segment stack of any type PC. The outer segments exhibit an absorption efficiency of 100% to light applied axially due to the serial character of the coated surfaces (**Figure 4.3.4-2**)

The outer segments of the photoreceptors are at an angle with respect to the inner segments as a function of their retinal location (**Figure 4.3.1-2**).

To maintain the liquid crystal coating on newly formed outer segment disks, a reservoir of the necessary chromophores is maintained within the retinal pigment epithelium (RPE), see **Figure 4.5.1-1**. The RPE is known to provide all four of the chromophores of mammalian vision to the newly extruded disks of the outer segment through vents in the extrusion cup of the inner segments (**Figure 4.6.2-6**).

The typically nine dendrites of the PC form a candelabra like structure (where the dendrites are the candles, **Section 4.3.5.1**) that embrace the disk stack of the outer segment of each cell. The dendrites are present in notches in the surface of the stack corresponding to notches around the edge of each disk. The phono-elastic energy created by the first step in the transduction process is translated into an electrical current at this liquid-crystalline-dendritic interface.

F.3.3 Identifying the specific structure of the Rhodonines

The Rhodonines have recently been described at the 3D molecular level using the ArgusLab's Builder Tool within ArgusLab version 4.0.1. They were developed by modifying retinal2_444397, the only totally *trans*- form of retinal or retinol found in the ChemSpider data base (ca. Jan. 2014). Several forms exhibit a vernacular title including the word *trans*- but do not conform to that description in their formal name (i.e., retinol2_4940721, all-*trans*-3,4-didehydroretinol_8192325 is actually (9cis)-3,4-didehydroretinol, and all-*trans*-retinol acetate_8421459 is actually (9cis)-O^{15}-acetylretinol, etc.).

The modification involved replacing the appropriate hydrogen or methyl group with a hydroxyl group at the positions listed below (using the numbering adopted within the Jmol protocol.

Chromophore	Formal Name	Replaced structure	New structure	Hybrid	Wavelength
Rhodonine(5)		Methyl at C11	hydroxyl (O11)	sp3	Long
Rhodonine(7)		Hydrogen at C10	hydroxyl (O22)	sp3	Medium
Rhodonine(9)		Methyl at C14	hydroxyl (O14)	sp3	Short
Rhodonine(11)		Methyl at C16	hydroxyl (O27)	sp3	Ultra-violet

The resulting chromophore structures are available in Jmol format under “download chapters” on the left navigation panel of the website, http://neuronresearch.net/vision . A suitable visualizer will be needed to view these files. The free version of the DS visualizer by Accelrys is more than adequate for this task. Open the visualizer and then locate the desired Jmol file in the above folder.

The use of a building tool to create representations of a given molecule involve the use of many parameters (such as bond lengths) not explicitly shown to the investigator during the modeling. One must take it on faith that the correct bond lengths, etc., are used in the modeling process.

The ArgusLab’s program and the majority of the forms found on ChemSpider show a confirmation with the single methyl group of the ring structure displaced from the axis formed by the all *trans*- aliphatic chain, **Figure F.3.4-1**. These representations are 3-dimensional but the aliphatic chain is planar and the aromatic ring is only slightly puckered and out of plane. The symmetry between the molecules is evident with the two oxygen atoms on the same side of the aliphatic chain except for Rhodonine(5) where the oxygen atom is on the opposite side. This condition is easily remedied if the aromatic ring is rotated about the single bond attaching it to the aliphatic chain. This modified configuration is shown in [**Figure 5.5.8-2]** of the main work.

The conformations shown may be the true confirmations of the molecules under a set of unstated conditions (probably for the isolated molecule, potentially in a vacuum). The conformation of Rhodonine(5) may be rotated from the typical Jmol representation in order to maintain the “straight” hetro-conjugated requirement described by Platt. This preferred conformation is with the C5 methyl group in line with the all *trans*- aliphatic chain before and/or after hydroxylation at that location. This is readily achieved by rotation about the single bond between C6 and C7. It is also possible that following hydroxylation, the formation of the most stable liquid crystalline arrangement involves stereoselective conformation of the chromophore on the substrate surface. The role of stereoselection could be resolved by comparing the conformation of the chromophore when present in the liquid crystalline form on a disk to its conformation when stored in the RPE or during transit through the inter-photoreceptor matrix (IPM), see **Figure 4.5.1-1**.

Using the modified conformation for Rhodonine(5), the peak in the spectral absorption of each chromophore is a direct function of the length (L) of the conjugated chain extending between the two

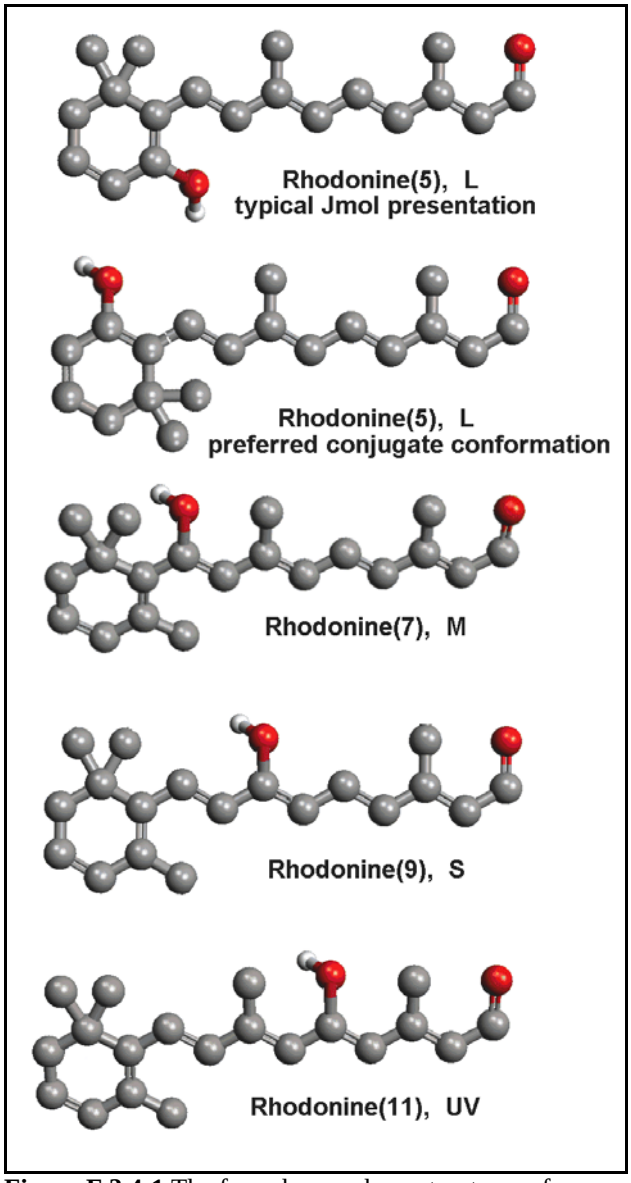

Figure F.3.4-1 The four chromophore structures of mammalian vision based on Jmol representations. Rhodonine(5) is shown in two conformations. The lower conformation preserves the hetro-conjugate structure of the molecule. Compare with the alternate conformation of Rhodopsine(5) shown in **Figure 5.5.8-2**. The nominal lengths (L) of the resonant structures are also shown in that figure.

oxygen atoms. Conjugated chains of hetero-atoms are not normally addressed in chemistry textbooks, even physical chemistry texts, but the subject is critically important to understanding the chromophores of vision.

The Rhodonines are dienes, the suffix -al or -ol used to define aldehydes and alcohols do not apply to these chromophores. The hydrogen shown attached to the hydroxyl oxygen is released when each of the molecules shown is in the hetro-conjugated state. The two oxygen atoms and the conjugated chain between them exhibit de-localized electrons found along the length of the structure. When in a larger monolayer liquid crystalline structure, the de-localized electrons exist in a cloud within the planar dimensions defined by the liquid crystal and the parameter L.

The dimension L is defined by the width of the potential energy well described by Platt[3] using the "free-electron molecular-orbital method." This method is generally equated to, but is a bit more precise than, the linear combinations of atomic orbitals (LCAO) method. This dimension multiplied by a nominal value of 460 equals the wavelength of the photons maximally exciting that specific chromophore. The relationship recognizes that the delocalized electrons within the chromophore move at a much lower velocity than photons in a vacuum.

The spectra observed is known as the *J*-band of the chromophore in the science of color photography in order to distinguish it from the lower sensitivity M-band obtained when the incident light is not parallel to the axis of the aliphatic chain. The *J*-band is associated with the *J-aggragate* structure or liquid crystalline structure of the coating of the chromophore on a substrate.

F.3.4 Means of identifying individual chromophores of animal vision

There are multiple ways to distinguish between and confirm the spectral response of the chromophores, depending largely on the facilities available to the investigator.

F.3.4.1 *In-vivo* identification of individual photoreceptor chromophores

The *in-vivo* approach involves the fewest parameters to control via the test protocol but requires a patch clamp configuration involving the axoplasm of a single photoreceptor neuron. With such a configuration, the light from a spectrometer can be used to stimulate the photoreceptor through the natural optical system of the eye. Under minimum stimulus intensity, the analog potential measured via the patch clamp will faithfully represent the spectral response of the photoreceptor (limited only by the spectral admittance of the optics of the eye (nearly negligible for all except the photoreceptor containing the Rhodonine(11) UV chromophore). See **Section 2.4.2** for the correction required.

Conventional psychophysical experiments, including those using microspectrophotometry, can be used to describe the spectral performance of a specific class of chromophores but efforts must be taken to suppress (through spectral adaptation) the adjacent photoreceptors.

F.3.4.2 *In-vitro* identification of individual photoreceptor chromophores

In the *in-vitro* approach, the patch clamp configuration involving the axoplasm of the photoreceptor neuron is preferred. The stimulating light must be applied axially to the photoreceptor to avoid the Stiles-Crawford Effect and avoid contamination of the response by the lower sensitivity spectrum associated with the m-band of the chromophores. It was the m-band that Baylor and colleagues measured in the 1970's using stimulation perpendicular to the axis of the aliphatic portion of the chromophores *in-vitro* (**Section 5.5.10**).

In 1987, Douglas, Bowmaker & Kunz identified the four actual spectra of the roach (a fish) using axial illumination (**Figure 5.5.10-9**).

F.3.4.3 Electron microscopic examination of individual disks *in-vitro*

Since the distance between the two heavy oxygen atoms of the chromophores is well characterized by molecular modeling, it is possible to observe the distance between the oxygen atoms of the various chromophores when present on the surface of the disks using electron-microscopy in order to determine their spectral properties. See **Section 4.3.5** and **Figure 4.3.5-8**. The small structural deviations from a theoretical liquid crystalline array may be a reason for the weekly replacement of individual disks in a given disk stack. More careful examination of the electron microscope imagery may allow the determination of the conformation of the individual chromophore molecules when in the liquid crystalline state on the substrate.

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[3]Platt, J. (1956) Electronic structure and excitation of polyenes and porphyins *In* Hollander, A. *ed.* Radiation Biology NY: McGraw-Hill page 71

F.3.4.4 Direct chemical analysis of the chromophores

Direct chemical analysis of the chromophores of a specific spectral performance remains an option. However, the isolation of the individual types of chromophore molecules and their chemical decomposition without losing information concerning their *in-vivo* stereographic structure is a very demanding activity. All of the chromophores share a nearly identical molecular weight and nearly identical backbone.

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