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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6 Static Test Data Pertinent to Vision Research [1]

6.1 Introduction

This chapter will review the existing and needed experimental data related to the basic physics and chemistry associated with the individual mechanisms of vision. It will not address the functional relationships between these materials. These aspects will be addressed in subsequent chapters.

6.1.1 A fundamental overview of the visual process

The original purpose of this chapter was to assimilate all of the relevant data concerning the role of the retinenes in vision. However, as the work progressed, it was found that **the retinenes play no role in the actual process of vision**. While the retinenes are precursors or chromogens to the chromophores of vision and play their normal metabolic role in the processes relative to cell growth and operation, it is the retinines, a related but distinctly different chemical family, that are the chromophores of vision. Even these materials only exhibit their unique spectral absorptions, and therefore act as chromophores, when in the liquid crystalline state. When in this state, they exhibit a highly anisotropic absorption spectrum containing two components, an intrinsic (isotropic) absorption characterized in the visual region by their peak absorption near 500 nm due to molecular-resonance mechanisms. Simultaneously, they exhibit an additional (and highly anisotropic) absorption band due to a distinct quantum-mechanical-resonance mechanism. This quantum-mechanical absorption, due to a unique slow-wave phenomenon, is characterized in the visual region by a narrow individual peak absorption defined by the conjugation level between the two oxygen atoms of the material. It is this extrinsic, highly anisotropic quantum-mechanical resonance absorption that is key to the visual process.

> **Photodetection in Vision relies upon the chemistry of the liquid-crystalline state. This state of matter is largely limited to the biological temperature range. Within the liquid-crystalline state, photodetection relies upon conjugated dipolar molecules of a simple retinoid and oxygen to form the chromophores. These molecules are excited by a unique slow-wave mechanism. They are subsequently de-excited in-vivo by a direct neural mechanism. Absent this mechanism, an alternate de-excitation mechanism must be provided by the experimentalist.**

In summary, the focus of the vision community on collecting data for materials in dilute solution has prevented it from obtaining actual spectral data related to vision. The process of photon detection used in vision is dependent on material present in the liquid crystalline state of matter. While the retinenes play a major role in the metabolism and growth of animals, they play no functional role in vision. It is the retinines, easily formed from retinol, that are the critical chromophores of vision.

Although the initial intent of this chapter was to show the progression from the spectral characteristics of the retinenes to the spectral characteristics of the retinines (note the i), this turned out to be inappropriate. The spectra of the retinines are fundamentally different from the spectra of the retinenes. The retinenes are conjugated hydrocarbons with one polar group and spectral features in the ultraviolet related primarily to their individual chemical groups. They exhibit no visible band absorption comparable to that observed in vision. The Rhodonines and other retinines are conjugated hydrocarbons that contain two polar groups. They also exhibit an overlay carbon conjugation between the two polar groups. It is the resonant absorption spectrum in the visual spectrum, due to this overlay conjugation, that characterizes these materials. The longer wavelength is due primarily to the slow wave structure exhibited by these unique materials, especially in the liquid crystalline state. This structure makes these molecules quantum-mechanically resonant at the wavelengths of visible light.

Because of the above situation, this section will merely provide a list of relevant sources for the available retinene data. Because the Rhodonines, and the retinine family, have been defined as a part of this work, there is little

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[1] Released: April 30, 2017

explicit data on these materials in the previously published literature. However, a number of investigators have encountered a retinol-like material that they could not identify. The work of Chen & Heller is reviewed in **Section 6.4.2.1**. They have isolated and provided graphical data on a "retinol-like material" that appears to be Rhodonine. It was found in the void space of their centrifuge product instead of in the precipitate.

6.1.2 Historical perspective

An immense amount of empirical data can be found in the Vision literature. Much of it, and particularly most of the spectral data, does not actually relate to the process of vision in animals. This unusual situation is due to several important factors.

The most important factor is the unwillingness of most of the community, down through the years, to use theoretical models. Traditionally, the leaders of the community have deprecated the use of models; possibly because of poor experience with "floating models," i.e., less than adequate models addressing only a small part of the overall vision process.

A related factor has been the common problem of many scientific communities. They have resisted including scientific discoveries originating outside their circumscribed arena. This has forced the community to limit the scope of their understanding of the visual process. It has also caused them to rely on intuitive theories instead of the firmer foundation available within the larger scientific community.

The most important oversight has been the lack of appreciation of the sophistication of the chemistry used in animal vision. As will be shown below, experimental design has suffered immensely in the vision community from a lack of precision brought on by a lack of knowledge. The result has been the collection of immense amounts of data that does not relate directly to the problem of understanding vision.

The consequence of the above situations is obvious in hindsight, and has been for a long time. As the scientific revolution progressed during the 1930-50's, the collective vision community accepted a specific hypothesis concerning the role of Retinol in vision. They then augmented this theory with a set of corollaries based primarily on intuition. This was due to a lack of familiarity with work going on in other communities (specifically the photographic community). As a result, many experiments were designed based on an inadequate foundation.

The result has been a reliance on Retinol in chemical combination at the molecular level with a protein, Opsin, as the foundation of the chemical theory of vision. The resulting conceptual material, Rhodopsin, and Retinol itself have been studied endlessly. Yet, these studies have not produced a satisfactory description, much less model, of the visual process.

The key problem in achieving a satisfactory understanding of the visual process has been the reliance on Retinol, a retinene, instead of the more sophisticated Rhodonines as the chromophores of vision. The Rhodonines are related to the retinenes and Retinol but exhibit an additional property not found among the retinenes, dipolar resonance. This property is only realized when the Rhodonines are in the liquid crystalline state. Chen & Heller[2] and DeLuca[3] have encountered this material in their HPLC studies but have not successfully related it to vision (see **Section 6.4.2.1**). They defined the Rhodonine material they encountered as "the retinol-like metabolite of retinoic acid (metabolite 'X') in pigmented epithelium and other tissues." A number of other investigators have encountered a "metabolite of retinoic acid" as described in **Section 6.4.1.3**. Some of these materials could be waste products derived from Rhodonine. However, it appears most of the Rhodonine in the outer segments and the RPE are recycled endlessly, and at a remarkably rapid rate (measured in a few days to a very few weeks).

Since attempts to relate Vitamin A to the chromophores of vision has been unsuccessful, most of the work since the late 1960's has related to its metabolic activity.

6.1.3 The Experimental design environment

An exhaustive study of the environmental elements impacting the process of animal vision leads to a large list of environmental variables that are critical to the design of experiments related to vision. This list of variables extends

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[2]Chen, C-C. & Heller, J. (1977) Uptake of retinol and retinoic acid from serum retinol-binding protein by retinal pigment epithelial cells. *J. Biol. Chem.* vol. 252, pp 5216-5221

[3]DeLuca, L (1977) The direct involvement of vitamin A in glycosyl transfer reactions of mammalian membranes. *Vitam Horm.* vol 34, pp 1-57

far beyond those usually controlled, or even recorded, in vision experiments. From a conceptual perspective, the list is multidimensional. This makes it difficult to describe all of the parameters in one paragraph. The following listing attempts to describe the experimental environmental involved in animal vision experiments. These sample conditions are obviously met in *in-vivo* experiments. However, even in these experiments, it is common to find difficulties related to technique.

The initial consideration in approximate hierarchal order are listed below. They apply equally to physical, electrophysical and psychophysical experiments.

A. Does the specimen under study represent the actual or an assumed actual material?

B. Is the phylogenic position of the specimen understood?

C. Is the genetic response to the environment of the specimen understood, i.e., is it saline or freshwater based?

D. Is the experiment performed *in-vivo*, quasi-*in-vivo* or *in-vitro*?

E. Do *in-vitro* or quasi-*in-vivo* experiments properly simulate the *in-vivo* physical state and environment?

F. Is the quality of the instrumentation adequate for the purpose intended?

Continuing for neural materials,

G. Does the experiment employ the computational powers of the specimen or is it less comprehensive?

H. Does the experiment isolate an individual signaling channel within the visual spectrum of the specimen?

I. Does the experiment control all other inputs to the signaling channel under observation?

J. Does the experiment encompass non-linear elements in the signaling channel?

J. If non-linear circuit elements of the specimen are included in the experiment, are they recognized?

Some of these questions may seem abstract. However, they are critically related to the validity of the experiment.

> As an example, if a spectrophotometric test is made quasi-in-vivo but presents the illumination transverse to the axis of the Outer Segment in saline based *Chordata*, the observed spectrum represents the conjugated spectrum of the chromophore present instead of the resonant spectrum. The result is always a spectrum with a peak at P502 regardless of the chromophore present. In such a test, all photoreceptors of the animal will exhibit the P502 type spectrum.
>
> Conversely, an identical test with the illumination presented parallel to the axis of the Outer Segment will produce a spectrum indicative of the resonant spectrum of the chromophore present. The P502 peak will be suppressed or absent.

6.1.3.1 Rules of biochemical synthesis

When the experiment calls for the synthesis of a biochemical material, distinguishing between a true "total synthesis" and a reconstitution is important. Shapiro has provided examples differentiating between a "total synthesis" and a reconstitution[4]. He defines a total synthesis as achieved by only using materials that have been previously synthesized in the laboratory. He describes reconstitution as arising from the use of material where one or more of the constituents had not been synthesized previously (according to the above definition). The use of a breakdown product in a synthesis is normally defined as a reconstitution and not a synthesis. The experimenter is never completely sure what material he used in the reconstitution. This is particularly important in complex organic chemistry where some materials may "indicators" that change their appearance due only to changes in pH or other parameters of the chemistry employed. A similar situation relates to proteins. If they have been denatured, how does one know they have been reconstituted to their original biochemical state?

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[4]Shapiro, R. (1999) Planetary Dreams: The Quest to Discover Life Beyond Earth. NY: John Wiley, pp 110-111

6.1.3.2 Solutions versus suspensions and gels

The biochemistry literature of vision contains frequent reference to solutions using the vernacular. Typically, the material being examined is not a true solution. A true solution is a mixture of two or more substances that will not separate by settling, cannot be separated by filtration, and is homogeneous when mixed in any proportions of the individual constituents (down to molecular sizes). A suspension is a mixture that does not meet one or more of these requirements. Many organic molecules aggregate when placed in a solvent, even after they have been subdivided into very small particle sizes. A lack of homogeneity is frequently indicated by an iridescent appearance of the material. This iridescence is due to scattering of light by the presence of particles in the mixture that have sizes approximating that of the wavelength of light. If the aggregation continues, the mixture may become a gel, where one constituent exhibits a quasi-crystalline structure. This structure may become very large relative to the size of its individual molecules. These large structures are frequently labeled gels.

6.1.4 Initial evaluation of the data base

There is a great deal of data in the vision literature which have been collected over a very long time. It has been collected during a period of unimaginable advances in the physical sciences. Because many of these great advances in the physical sciences occurred after the data was collected, much of the data has been collected and interpreted without having available the best tools. In addition, it is now known that many experiments were not formulated adequately and the options available during interpretation were limited. This has been a particular difficulty in photochemistry. The photo-detection problem in animals involves such a highly integrated series of processes and such unique processes that the experimentalist has had great difficulty teasing apart the necessary components of the process. Determining the functional details of the Inner Segment has been a prime example. He has also had very great difficulty understanding and isolating the mechanisms of the initial basic processes involved in photon detection, many of which were not understood, even partially, before the 1960's-80's. The unique combination of properties exhibited by the actual chromophores have made precise experimentation extremely difficult and provided many false leads. The fact that the chromophores are excited almost instantly but once excited do not relax through quantum means has made their technical description through experimental means extremely difficult. As pointed out by Witteman[5] and others, highly symmetrical molecules with heavy ion groups on their extreme ends do not de-excite through dipole radiation; they normally de-excite through collisions with other molecules or with the walls of a container. In the case of vision, they de-excite primarily through energy transfer to the neural system. The fact that they only exhibit their true spectral characteristics when in the liquid crystalline state has caused almost all *in-vitro* and *ex*-IPM spectral testing to fail. Because of this situation, there has been no experimental isolation of the actual chromophores involved or verification of their spectra reported in the literature. The unique mechanism (shared with the field of photography) associated with the long wavelength photoreceptor is a case in point. Without recognizing this mechanism, one cannot hope to define an adequate experiment accidently.

Based on the above discussion of the relevant experimental ground-rules, separating the existing experimental data in the vision literature concerning steady state operations into two broad categories is possible. Category I includes data collected under conditions preserving the integrity of the visual process being investigated. Category II includes all other data.

6.1.4.1 Axioms applicable to biochemistry of vision

> **It is axiomatic that in-vitro experiments relating to materials in dilute solution do not relate to the chromophores of vision. The chromophores only exhibit their resonant properties when in the liquid crystalline state.**
>
> **It is axiomatic that *in-vivo*, quasi-*in-vivo* or *in-vitro* experiments are irrelevant if they do not preserve the geometric relationship between the incoming illumination and the axis of the Outer Segments in Chordata.**

These two axioms alone place more than 90% of the steady state experimental data in the vision literature in Category II, irrelevant with regard to understanding the vision process. The challenge is to identify and memorialize the important relevant data. The Category II data may represent excellent science, but it is not directly related to the vision process.

> **It is axiomatic that the separation of the liquid crystalline chromophore from the required electrical environment (interface) destroys its steady state (in-situ) performance.**

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[5] Witteman, W.. ibid

This axiom shows why experiments involving the separation of the Outer Segments from the electrical environment, represented by the signal path through the Calyx and the return path through the IPM, do not provide useful results. The performance of the chromophores under these conditions will be discussed in the Chapter addressing transient experimental data. The chromophores become totally excited (bleached) under illumination and do not de-excite (unbleach) for many hours following illumination due to the triplet state of the excited electrons. The required electrical environment can be simulated in the same manner it is in many liquid crystal applications.

6.1.4.2 Experimental care required in the biochemistry of vision

The four chromophores applicable to either the saline-based animals or the four applicable to the freshwater-based animals, primarily freshwater fish and amphibia, share very similar steric geometry. They also occur in pairs with the same molecular weight (with differences in molecular weight of less than 6% between the pairs), and absolute molecular weights of less than 300. This makes their separation a different problem than that found in protein separation. The literature does not present any results of chromophoric separation of the Rhodonines. Because of the unique steric characteristics, re-crystallization appears to be the most viable separation technique.

There is a considerable problem with terminology in this area, related to the time period when the data was collected. Many official changes in name conventions have occurred in the last fifty years for the retinoids. In addition, many speculative names were defined before the availability of definitive test methodologies. Many of these speculations related specifically to speculations about what moieties are responsible for what region of a spectrogram.

The title rhodopsin is used in a variety of different contexts. During the 1800's, rhodopsin was a generic name for a material (of unknown characteristics) thought to be a chromophore of vision. During the first half of the 1900's, it was used to describe only the achromatic (or rod-based) chromophore of scotopic vision. Sometimes, rhodopsin was used to describe the putative middle wavelength chromophore of photopic vision. The other (usually narrow spectral band) chromophores were frequently given one or more other names, iodopsin being a prominent one. Porphyropsin, with an absorption peak at 532 nm, is another[6]. It will be shown that the latter is the M-channel chromophore of vision, Rhodonine(7). Cyanopsin is less commonly used to describe the L-channel absorber, containing the proposed Rhodonine(5). See **Section 6.3.4.2**.

In the latter half of the 1900's, rhodopsin was identified as a protein opsin combined with a retinoid, retinol. How this union was arranged was subject to debate. During the 1990's, it became possible to confirm the candidate material consisted of a retinoid attached to a specific amino acid of the protein and apparently via a Schiff base. However, to date, this configuration has not been shown to provide the required absorption spectrum to be considered a chromophore of vision. Rhodopsin has not been totally synthesized as of this date. The detailed study of the structure and properties of rhodopsin falls into Category II.

At this time, there is excellent evidence that the chromophores of vision do not include rhodopsin. While rhodopsin contains a ligand of retinol, it appears to act only as a substrate for the disks. The chromophores are the Rhodonines when present in the liquid crystalline state.

A major problem has arisen in the methodology used in investigating the chromophores of vision.
Part of this may be due to commercial pressures:

+ An interesting example is the use of a Red safe light, typically a Kodak #1 Safelight filter and a 15 watt incandescent bulb, during dissection and other procedures. The implication is that the resulting “safe light” will not affect the material under examination. In fact, the #1 Safelight filter has a shortwave cutoff (10% response) at 620 nm and is designed for use with non-color-sensitized photographic emulsions with a long wavelength cutoff at 470 nm. They are used in photographic dark rooms only after the longer wavelength color sensitive absorbers have been destroyed or removed from the emulsion during the development process. Such safe lights concentrate their visual illumination in the very region of the spectrum where the L-channel chromophore is most sensitive. A Red safe light is very effective at bleaching the L-channel chromophore and thereby distorting the overall test results.

+ Papermaster[7] reviewed the conventional method of preparation of rod outer segments. The description shows how aggressive the technique is with regard to materials with bandgaps of less than 1.5 electron volts. He only briefly discusses the aggregation of the material as a problem.

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[6]Dawson, M. & Okamura, W. (1990) Op. Cit. pg 28

[7]Papermaster, D. (1982) in Methods of Enzymology, vol 81. NY: Academic Press pg. 48-52

+ Another problem is in obtaining spectrographic data. Finding specific detailed information in the literature about how the data was collected is difficult. It is common for an investigator to publish his best (in some case, most pleasing appearing) spectrogram. The caption and text are frequently inadequate in describing how the data was obtained. Examples of this will be developed below.

There is also a problem with the wide range of solvents used while making measurements on photoreceptor cells and their components. The spectral absorption of the retinenes is very sensitive to the solvent. Sporn et. al. have shown that merely changing from acidic methanol to alkaline methanol solutions will shift the curve by 17 nm. The Rhodonines are expected to show the same sensitivity. Possibly of greater importance, Hamer[8] has indicated that for the cyanines, the reversible aggregation of these molecules may only occur in aqueous solutions. It is suggested here that all measurements should be made in a Ringer's solution matched to the Inter-Photoreceptor Matrix (IPM) of the animal if accurate and repeatable data is to be obtained.

Because of the unique nature of the chromophoric materials of vision, knowing the initial specific molarity of the test solutions and their molarity as the chromophores begin to aggregate is important. Noting the appearance of anisotropic absorption, if any, is also important. This causes a problem because so much of the data in the literature is presented on the assumption that the material under test is isotropic and that its absorption coefficient is a linear function of its concentration. The area of specific interest in vision involves aggregated material exhibiting a non-isotropic absorption coefficient.

Some data is presented in unique ways. It may be plotted with respect to energy/unit wavelength or with respect to photon flux/unit wavelength. It is important that this information be documented explicitly.

This section will attempt to define the necessary and sufficient conditions for isolating and describing a variety of the (yet to be performed) experiments. This defining process is necessarily based on the model developed above. Without an adequate model, the experiments cannot be defined properly and the experimental results will always be subject to reinterpretation.

6.1.5 The top-level flow diagram of the retinoid chemistry of vision

Figure 6.1.5-1 presents a top-level flow diagram of the retinoids used in vision. All retinoids are initially ingested, primarily as carotene, although many prepared foods also contain Vitamin A_1. Vitamin A_1 is used by saline-based chordates, marine animals and many members of arthropoda. However, fresh water-based animals use Vitamin A_2 and some scavenging members of arthropoda use Vitamin A_3 as their primary retinoid. After ingestion, the bulk of the retinoids is stored in the liver. As required, the retinoids are withdrawn from the liver and transported through the blood stream, mostly as retinoic acid, for purposes of growth and routine metabolic purposes. There is a separate transport path for the retinoids used to form the chromophores of vision. These materials are withdrawn as retinol and transported through the blood stream in a specially designed cannister. This cannister protects the retinol from degradation and probably participates in the conversion of the retinol (a retin<u>e</u>ne) into the final chromogens of vision, the Rhodonines (a family of retin<u>i</u>nes). The Rhodonines are stored in the retinal pigment epithelium, RPE, cells until required. They are then transported to the disks of the outer segments of the photoreceptor cells on demand.

The lower portion of the figure reviews the extraction of the Rhodonines in the laboratory. Conventional attempts to recover the chromophores of vision have been thwarted for many years by two factors. First, the aggressive use of sodium-based detergents has frequently destroyed the Rhodonines during the extraction process. Second, the detergent action of many chemicals has caused the unique spectral characteristics of the Rhodonines, related to their presence in the liquid crystalline state to be lost. When in the dilute state, the Rhodonines share many spectral characteristics of the retinals and other retin<u>e</u>nes. In dilute solution, all of the Rhodonines exhibit the same spectral characteristics. On the other hand, if care is taken to avoid the use of metal-based detergents, the Rhodonines can be recovered from elutes extracted from eyes without destruction or modification. The individual Rhodonines exhibit different structural characteristics. They tend to separate into homogeneous crystalline layers when encouraged to return to the liquid crystalline state. Therefore, they can be separated by a process of differential recrystallization.

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[8]Hamer, F. (1964) The cyanine dyes and related compounds. NY: John Wiley & Sons pg 717

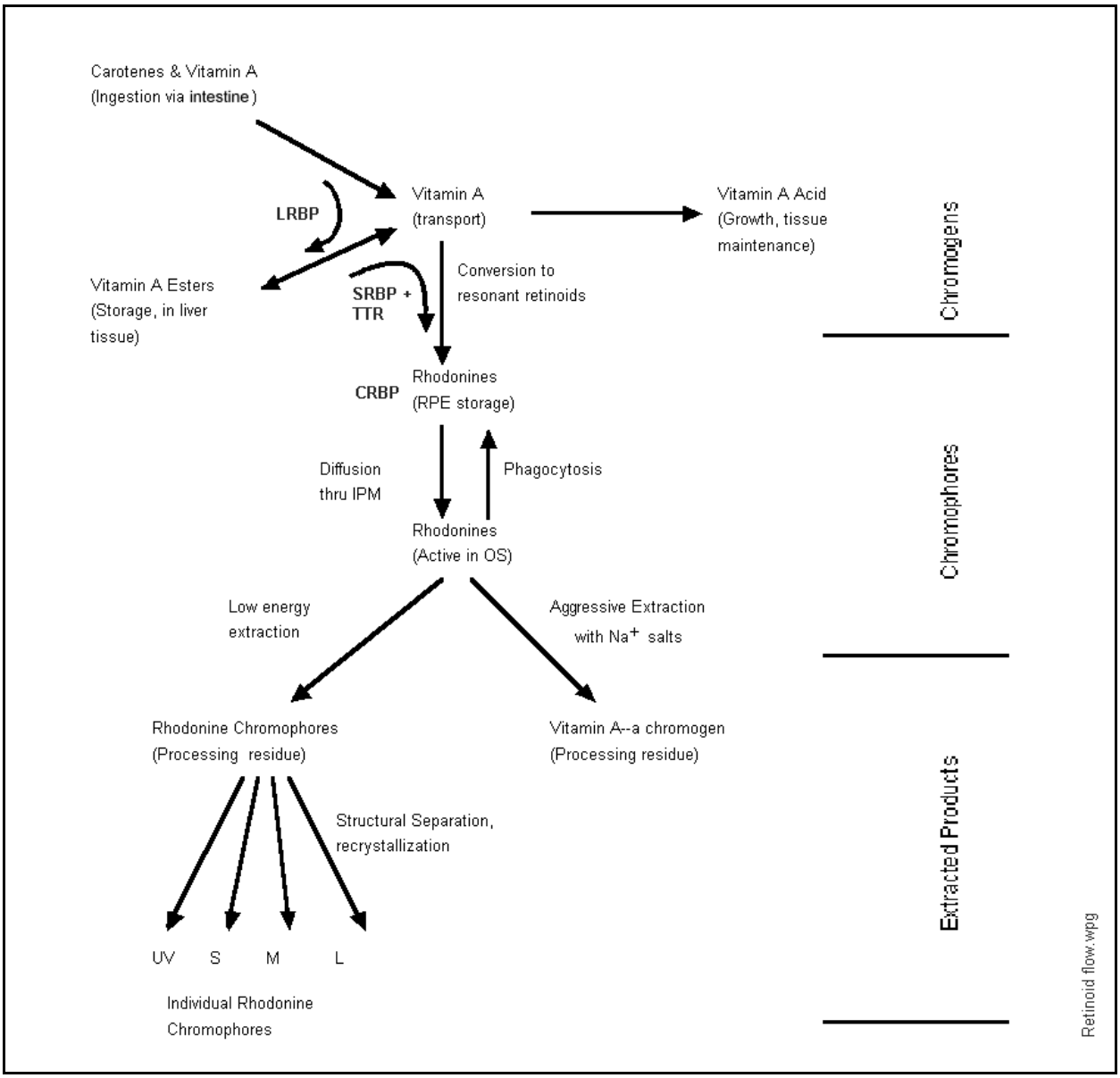

Figure 6.1.5-1 A flow diagram of the retinoids employed in vision. The retinenes are shown at the top, marked chromogens. The retinines, or Rhodonines, are shown in the middle marked chromophores. The extracted products shown at the bottom depend greatly on the methods of extraction used to recover these delicate molecules.

6.1.5.1 Initial introduction to the retinines

The retinines can be defined by a variety of their characteristics. They are retinoids based on their easily recognized terpene structure. However, they contain two heavy atoms, of either oxygen or nitrogen, separated by a conjugated carbon chain shared with most of the carbon atoms of the molecule. In all the molecules found that were related to biological vision, the two heavy atoms are both oxygens. Technical reasons for this choice will appear later in this Chapter. These members of the retinine family are known by the trademark name of the Rhodonines.

The retinines, and particularly the Rhodonines, are the long sought chemicals, frequently described as some

unknown derivatives of retinol, that are the chromophores of vision.

The retinines have not been reported previously in the literature as the chromophores of vision. Very little information concerning these materials exists in the literature. In a few cases, data has been reported when materials were prepared incidentally to another set of experiments. This data will be presented in this chapter.

With the above revelations concerning the chromophores of vision that are presented in detail in this work, the importance of the retinenes is reduced to that of interesting (Category II) chemistry. This applies specifically to the retinals associated chemically with opsin. The retinines exhibit many properties that are not shared with the retinenes. Therefore, the portion of this chapter devoted to the retinenes will be less than originally planned.

The presence of Rhodonines in a solution thought to only contain retinenes has frequently caused experimental problems, particularly when attempting spectral absorption measurements. Such measurements are highly sensitive to the aggregation level of the material in the gel (noting that the retinoids do not form true solutions is important, particularly in water-based solvents).

In the course of this work, it was discovered that there are three families of the Rhodonines that are important in biological vision. Two of these have been previously defined in terms of the biologically active hormone, Vitamin A. A third family of the Rhodonines has recently appeared and one author has suggested the existence of a fourth[9]. To maintain consistency in terminology, this situation has led to the definition of a third family within Vitamin A. This material is designated Vitamin A_3 in **Section 6.2.1** & **6.2.2**.

6.2 The Carbon chemistry of vision

Carbon chemistry, (commonly organic chemistry) is so large in scope and so complex internally that it is usually discussed based on a tree not unlike a phylogenic tree. A large portion of this tree involves the hydrocarbons. However, the portion of interest in vision includes the carbohydrates. Within this portion are many more specific families. The analogy with the phylogenic tree of the animal kingdom quickly breaks down in carbon chemistry. Where crossbreeding between families rarely, if ever, occurs in biology, it is rampant between families within the Class of carbon chemistry. Beyond the fourth level of such a tree, it becomes so differentiated (and interconnected) that it cannot be easily presented in a two-dimensional form. As a result, individual presentations usually show the pedagogical goal, or perspective, of the author.

An initial tree focused on the requirements of vision is shown in **TABLE 6.2-1** beginning with the Class, Carbon Chemistry. Both the hydrocarbon and carbohydrate Orders in this Class are important.

[9]Matsui, S. Seidow, I et. al. (1988) 4-hydroxyretinal, a new visual pigment chromophore found in the bioluminescent squid, *Watasenia scintillans*. *Biochim. Biophys*. Acta. vol. 966, pp 370-374

TABLE 6.2-1
A "Phylogenic Tree" of Chemistry

Class	Carbon Chemistry (a.k.a. organic chemistry)					
Orders	hydrocarbons			|	carbohydrates	
Suborders	Aliphatic	Arenes	Aromatic	| nonhydroxy forms	—Sugars—	
Families	Alkanes		Benzenes	|	mono-	Polyhydroxy forms
	Alkenes			|		Polyhydroxy aldehydes
	Alkynes			|		Polyhydroxy ketones

As in discussions of lipids, this breakdown is not strictly by formula or structure. The left half is by structure and the right half is by constituents. Many locations in this table do not have well-recognized names.

Shepherd has provided an additional classification of interest when discussing vision[10]. He describes a bimolecular quartet consisting of fatty acids, sugars, amino acids and nucleotides.

1. Fatty acids are formed of straight chains of carbon atoms ending in a carboxyl group (– COOH).
2. Sugars (carbohydrates) are formed of straight or ring carbon chains containing a hydroxyl group (–C–OH) and an aldehyde (–CHO) or ketone (–CO–).
3. Amino acids consist of a carbon atom bound to four different moieties: a hydrogen atom (–H), an amino group (–$^{+}$NH3), a carboxyl group (–COO^{-}), and a variable side chain (–R).
4. Nucelotides consist of a pentose (5–carbon) sugar linked to a nitrogen-containing ring structure called a base.

It is the next level below these classifications that lead to an understanding of the chemistry of vision. When three fatty acids combine with a phosphate group, phospho-triglycerides are formed. These are the primary materials of cell walls within the neural system. When an arene fatty acid is further oxidized to form a conjugated straight carbon chain separating two oxygen atoms, the chromophores of vision result. When an amino acid is formed into a dicarboxylic amino acid by replacing the variable side chain by a second carboxyl group, it becomes the source of electrical energy for the entire neural system. These transformations will be discussed in detail in the appropriate sections of this work. **Section 8.2** addresses the physiological chemistry of membranes. **Sections 5.1.2** & **5.1.4** address the unique properties of the photochemistry of vision. The following sections of this chapter will address the spectral characteristics associated with the chromophores of vision. **Section 8.6** addresses the electrostenolytics of providing power to the neural system.

The alkanes, alkenes and alkynes are all "simple" alcohols. The arenes are alcohols containing both aliphatic and aromatic units. The retinenes and retinines can be described as Arenes (contain both aliphatic and aromatic subunits) based on structure and as monohydroxy or polyhydroxy- forms based on constituents. The aliphatic backbone and at least part of the aromatic subunit is conjugated. The aromatic subunit largely defines the hormonal properties of the material. For both the retinenes and the retinines, the distance between the oxygen atom and the heavy mass at the other end of the structure determines the molecular resonant frequency of the molecule. In the retinines, the distance between the two oxygen atoms along the conjugated backbone determines the specific absorption properties of the material when in the liquid crystalline state. The chromophores of vision can be categorized as resonant polyhydroxy aldehydes with a conjugated arene structure. However, many other names can be contrived to identify them. The IUPAC rules generally provide different names for different members of the Rhodonine family because of historical considerations (see **Section 5.5.12** for their complete names).

The retinenes, and in turn the retinines, are generally derived from the carotenes (and the more complex xanthophylls) formed by growing plants. The chemistry of the carotenes is quite extensive since it includes most of the natural colorants used in both the plant and animal kingdoms. Egg yolk is the color of the basic carotene. Goodwin[11] provides an extensive text on the subject of carotenes and references Isler for even more detail. Krinsky

[10]Shepherd, G. (1988) Neurobiology, 2nd ed. NY: Oxford University Press, pg 14

[11]Goodwin, T. (1980 & 83) The Biochemistry of the Carotenoids, Vol. 1 and Vol. 2. NY: Chapman & Hall

provides a more recent text using more modern and sophisticated notation[12]. A review of the terminology for presenting three dimensional molecular models on paper, and the necessary precautions to avoid misunderstanding is provided in Norman & Coxon[13]. Isler remains the source book for the carotenoids[14]. See also **Section 6.3.2.**

6.2.1 The retinenes as hormones, the Vitamin A family

Like the lipid family, the Vitamin A family is not defined by their chemical formula. They are defined by their biological impact on a living organism when present in trace amounts. Their primary feature appears to be their exterior conformation over a limited portion of the surface of each molecule. This conformation feature does not seem critical to the activity of the molecule as a chromophore or to its transport through the vascular system in conjunction with a specific serum retinoid binding protein (SRBP).

The enzymatic (or hormonal) activity of the members of the family varies considerably. There are three members of the family that are particularly important to vision. These are the simple retinols. There are three simple retinols that are chemically distinct but appear to provide similar high levels of hormonal activity. Each is associated with a different branch of the phylogenic tree. They differ only in the chemical arrangement of their ring structure.

Vitamin A_1 is the original retinol defined in the early 20th Century and shown by Wald to be critical to both the health and vision of man and many other mammals. It is used throughout most the animals who live in or have evolved from a saltwater environment. It is most effective in its all-*trans*- configuration. Because of its initial description, the name retinol is frequently used to describe the root member of the family.

Vitamin A_2 is a distinctly different member of the family. It is of primary importance in freshwater fish and has been found to be dominant in several mammals[15]. Vitamin A_2 shows much less enzymatic activity in humans. Its presence in other residents of the freshwater environment is not clearly documented. Vitamin A_2 is defined chemically as 3,4-didehydro-all-*trans*-retinol, or occasionally as simply 3-dehydroretinol.

Vitamin A_3 is a third simple retinoid that is only known to be important among the *Arthropoda*, particularly the Order *Diptera*, the two-winged flies, and some of its close relatives. Vitamin A_3 is defined chemically as 3-hydroxy-all-*trans*-retinol.

These materials contain only one heavy atom (oxygen) and show no molecular resonance with a peak absorption within the visual spectrum. **Figure 6.2.1-1** shows the form of the prototypical A Vitamins. Many additional members of the Vitamin A family can be associated with each of these simple retinols.

The naturally occurring members of the family are generally obtained from the cleaving of carotenoids, either carotenes or xanthophylls. Carotenoids that can be cleaved to form one or more of the above vitamins are called protovitamins. These carotenoids are generally dimers. Their cleavage may produce two monomers of the same vitamin A or monomers of two separate vitamin A's.

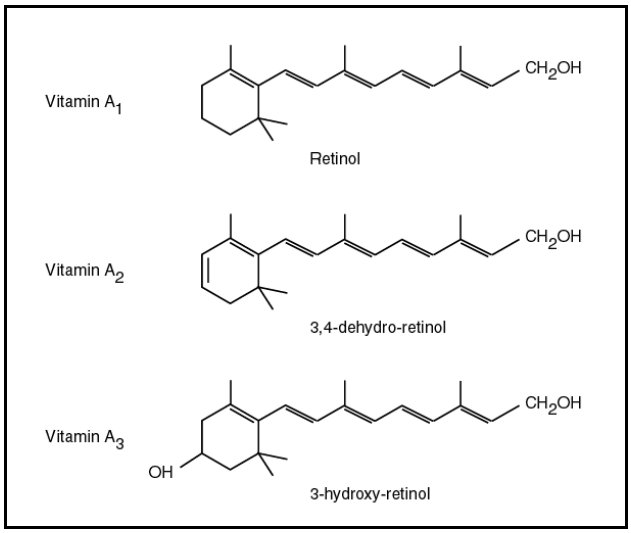

Figure 6.2.1-1 The three prototypical members of the Vitamin A family. They are transparent in dilute solution. However they are each chromogens of a family of Rhodonines.

[12]Krinsky, N. Mathews-Roth, M. & Taylor, R. *eds*. (1989) Carotenoids. NY: Plenum

[13]Norman, R. & Coxon, J. (1993) Principles of Organic Synthesis. London: Blackie Academic & Professional, of Chapman & Hall

[14]Isler, O. (1971) Carotenoids. Basle: Birkhauser

[15]Torma & Vahlquist (1988) Identification of 3-dehydroretinol (Vitamin A2) in mouse-liver. *Biochim Biophys Acta*, vol 961(2), pp 177-182

6.2.2 The retinenes as chemicals

The names applied to the retinenes have changed frequently with time. Machlin has collected all these names in a convenient table as of 1984[16]. However, the table does not include the multitude of newer retinoids defined in Sporn[17]. Neither of these sources includes any reference to the retinines.

6.2.2.1 Physical properties of some retinenes

A variety of tabulations of the properties of the retinenes appear in the literature. Unfortunately, many of the spectral properties depend on the solvent used in the measurements. Machlin has presented a set of values that are representative[18]. A more extensive Table of absorption properties of some retinoids, primarily the retinenes, appears in Sporn et. al[19]. The table does not contain data for any of the Rhodonines, the chromophores of vision.

6.2.2.2 Stereo specific forms of Retinol

Shichi notes that while the retinenes are generally considered planar molecules, this is misleading[20]. A twist of about 59 degrees is found around the C6-C7 bond. Thus, strictly speaking the all-*trans*- isomer is actually 6*S*-*cis*-retinal. This designation is usually omitted in the routine discussions of the retinenes (and Rhodonines). However, it shows up in any three dimensional models of the retinenes and retinines. In 11-*cis*-retinal, there is also a hindrance at the C12-C13 bond. Because of these out-of-plane characteristics, 11-*cis*-retinal is more completely described as 6-*S*-*cis*,11-*cis*,12-*S*-*cis*-retinal.

Because the all-*trans* forms of retinol and retinal do not exhibit absorption in the visual portion of the electromagnetic spectrum, attempts have been made to find other steric forms of these materials that do. The primary claim of Hubbard[21], apparently made without consultation with the photographic community, has been that 11-*cis*-retinal is the steric form of the molecule as it occurs in vision. This claim is generally unsupported by the dye chemistry community as represented by the following quotations[22]. 11-*cis*-retinal is a sterically hindered molecule. "Strain in a dye molecule resulting from steric hindrance may shift the color in either direction but almost always causes a loss in strength." Strength in this context refers to the molar absorption coefficient in the visual spectrum. "When an auxochrome is forced out of coplanarity by ortho substitution, its effectiveness is decreased. λ max. is shifted to shorter wavelengths and ε max. is decreased."

"In summary, conjugation is impaired when spatial effects force the atoms of a molecule out of the geometry most favorable to interaction of the mobile electrons. λ max. may be increased or decreased, as discussed, but ε max. is usually lowered." To overcome the shortcoming of 11-*cis*-retinal, it was later proposed that the required spectrum was achieved by combining the retinal with Opsin via a Schiff Base. This also failed to generate the desired absorption peak. The next proposal was that the Schiff base in the above configuration was protonated. This

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[16]Machlin, L. (1984) Handbook of Vitamins NY: Marcel Dekker, Inc pp. 4

[17]Sporn, M. Roberts, A. & Goodman, D. (1994) The Retinoids. NY: Raven Press

[18]Machlin, L. (1984) Handbook of vitamins. NY: Marcel Dekker, Inc.

[19]Sporn, M. Roberts, A & Goodman, D. (1994) The Retinoids. 2nd Ed. NY: Raven Press pg.188

[20]Shichi, H. (1983) Biochemistry of Vision. NY: Academic Press pg 68

[21]Hubbard, R. & Kropf, A. (1958) The action of light on rhodopsin. Communicated by G. Wald to Proc. Nat. Acad. Sci. of USA vol. 44 pp. 130-139

[22]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. 674-675

approach also failed to demonstrate the desired spectral peak. Ebry[23] and Honig & Ebry[24] readdressed the protonated Schiff base concept in the 1970's but with little success. They did discuss the problem in depth, reviewed the possibilities for both the cyanine and polyene family of chemicals, and noted that the half amplitude bandwidth of the chromophores was smaller in the L-channel. They noted: "It is, thus, rather disconcerting that the calculated values for λ_{max} of the isolated protonated Schiff base of retinal reported in the literature range from about 440 to 620 nm." They did provide a table of reported shifts in the absorption spectrums of the chromophores of vision in the transition from a Vitamin A1 to a Vitamin A2 based system. Unfortunately, the table contains a mixture of apples and oranges. Based on the anisotropic absorption spectrum of the S-channel chromophore, a shift in the peak absorption of about 6-10 nm was encountered. Motto, et. al[25]. readdressed the protonated Schiff base again in 1980 with little additional progress. Shriver, et. al. disclaimed the viability of the N-protonated retinylidene Schiff base model[26].

6.2.2.3 Specific characteristics

At this point, it is necessary to look at retinol and its close companion retinal separately due to nomenclature problems. Retinol ends with a "functional group" which is an alcohol. Retinal ends with a "functional group" which is an aldehyde. The term functional group is in quotation marks because it relates to the chemical functionality of a chemical and not its electronic functionality. When looking at the qualifications of a dye to achieve a high peak photon absorption in the region of the visual spectrum, it is the functionality associated with its electronic configuration that is critical. This functionality is controlled by the electronic state of the polar atoms in the molecule and not by the chemical grouping.

It was shown earlier that an effective dye requires two terminating polar atoms to achieve absorption in the desired visual spectrum. Therefore, exploring how either retinol or retinal could be modified to become a member of the polymethine family is instructive. Interestingly, this exploration will lead to the fact that retinol and retinal are both chromogens for a family of dyes that will be called the Rhodonines.

Look first at retinol. Although not a dye itself, it can be considered a chromogen for one of the simplest members of an important specific family of dyes found in the enormous field of commercial dye chemistry. This is the carbonyl family of dyes. By introducing a second polar atom at the appropriate point along its conjugated chain, it would become a member of either of two of the most prominent dye families in both the photographic and colorant fields. By introduction of a nitrogen atom, it would become a member of the amidic system of the carbonyl family. If an oxygen atom were introduced instead, it would become a member of the carboxyl-ion system of the carbonyl family (alternately called a dicarbonyl compound (or oxonol) since the chain would have an oxygen atom on both ends). It is also possible to consider introducing a nitrogen atom along the chain and then utilizing a Schiff-base to add a second nitrogen to the molecule. The family name of this configuration is cyanine. For the moment, which end is which is not important.

6.2.2.4 The Energy Band Line Spectrum

There is a myriad of energy band line spectrums in the vision literature. However, they were collected under a variety of conditions and interpreted based on a variety of backgrounds. This makes it difficult to arrive at a consistent interpretation of the overall situation. The overwhelming conclusion is that the line spectrums in the ultraviolet region of the spectrum (obtained in dilute solution) have little to do with the performance of the underlying material in vision. It is only the absorption obtained when the chromophores are in liquid crystalline form that is relevant. The spectrums obtained in dilute solution have traditionally been isotropic because of the randomness of the orientation of the molecules within the solvent. More fundamentally, the absorption of each molecule is isotropic because of its molecular structure. The literature suggests that the peak in the isotropic absorption of all of the chromophores of vison, and a great many other retinoids, occurs near 500 nm. This is true

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[23]Ebry, T. (1975) Wavelength dependence of the bandwidths of visual pigment spectra. Nature, vol. 257, pp. 823-824

[24]Honig, B. Greenberg, A. Dinur, U. & Ebrey, T. (1976) Visual-pigment spectra: implications of the protonation of the retinal Schiff Base. Biochemestry, vol. 15, no. 21, pp. 4593-4599

[25]Motto, M. Sheves, M. Tsujimoto, K Balogh-Nair, V. & Nakanishi, K. (1980) J. Am. Chem. Soc. vol. 102, letters to the editor, pp. 7947-7949

[26]Shriver, J. Mateescu, G. Fager, R & Torchia, D. (1977) Unprotonated chromophore-protein bond in visual pigments from ^{13}C-NMR spectra. Nature, vol 270, pp-271-273

whether they are based on Vitamin A_1, A_2 or A_3. In the case of the chromophores of vision when in the liquid crystalline state, the absorption is anisotropic because of the quantum-mechanical properties of the molecules. This anisotropic absorption is largely independent of the detailed configuration of the β-ionone ring.

6.2.2.4.1 Retinoids associated with Vitamin A_1

Figure 6.2.2-1 displays the energy band information for the retinoids in a different presentation. This presentation shows the wavelength of a photon having sufficient energy to excite a retinoid similar to Vitamin A aldehyde in **(a)**. The same information is shown in the lower part of **(b)**. The upper portion of **(b)** shows the wavelength of a photon having sufficient energy to excite a retinoid that also exhibits a dipolar resonance and is in the liquid crystalline state of matter.

There is general agreement in the literature[27,28,29,30] that Vitamin A aldehyde exhibits the line spectrum shown in **(a)**; this spectrum includes an absorption line in the ultraviolet near 0.16 microns, a pair of absorption lines at 0.17 and 0.29 microns due to the single carboxyl group, and finally a line at 0.39 microns. However, Stark & Tan relate the line near 0.29 microns, and labeled the γ-band, with an aromatic amino acid group in the opsin protein[31]. Wolkin (above) also associates this line with a material other than the retinoids. Retinol exhibits the same short wavelength spectral lines as retinal but the line at 0.39 microns moves to 0.325 microns. Material extracted from retinas and claimed to be Vitamin A aldehyde frequently also exhibits a line at 0.495 microns. The line at 0.495 microns is the broad absorption band frequently called P502 in the literature. It is also frequently assumed (erroneously) to be the intrinsic absorption band associated with scotopic vision. Others have mistakenly assigned it to Rhodopsin[32] although it is clearly seen in presumably protein free solutions of retinene extracted from retinas. This line is positioned to represent the molecular resonance associated with Rhodonine. The spectrum results from a conjugated carbon chain separating two oxygen atoms when in dilute solution. (Most solutions prepared in the laboratory from retinas are actually mixtures of retinol derived from Rhodonine and Rhodonine--in dilute solution).

The lines near 350 nm have been labeled β-peaks (or secondary peaks) by Govardovsii & Zueva among others. Others have chosen to call these the cis-peaks of various retinoids (quite recently by Stark and colleagues[33] quoting Tan). This is unfortunate. These peaks are clearly present in all-*trans* forms of both retinoids and carotenoids. The peaks are related to the ligands and not the backbone structure of the molecule. The precise contribution of these peaks in the visual system relates to their absorption of ultraviolet light by the lens before it reaches the chromophores of the retina. The chromophores have a wider spectral absorption than these peaks due to their liquid crystalline arrangement. These *b*-peaks actually generate a notch in the ultraviolet spectrum of the complete eye. Griswold & Stark have documented these facts meticulously. Their work shows the presence of two β-peaks, the alcohol and aldehyde peaks, simultaneously. See **Section 17.2.** This notch is a direct function of the size of the animal and therefore the thickness of the lens. This can be discerned by comparing the *b*-peak absorptions in

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[27]Barrow, G. (1961) Physical Chemistry 2nd ed. NY: McGraw-Hill pg. 354

[28]Sporn, M. Roberts, A. & Goodman, D. (1994) The Retinoids 2nd ed. NY: Raven Press pg 188

[29]Govardovsii, V. & Zueva, L. (1974) Spectral sensitivity of the frog eye in the ultraviolet and visible region. Vision. Res. vol. 14, pg. 1317

[30]Collins, F. Love, R. & Morton, R. (1952) Studies in rhodopsin. *Biochem. J.* vol. 51, pp. 292-298 & 669-673

[31]Stark, W. & Tan, K. (1982) Ultraviolet light: Photosensitivity and other effects on the visual system. *Photochem. Photobiol.* vol. 36, pp 371-380

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

[33]Stark, W. Wagner, R. & Gillespie, C. (1994) Ultraviolet sensitivity of three cone types in the aphakic observer determined by chromatic adaptation. *Vision Res*. vol. 34, no. 11, pp 1457-1459

pigeons[34] versus smaller birds[35]. Vos Hzn, et. al. report a peak sensitivity via an ERG at 366 nm. It appears this value was influenced by the *b*-peak absorption of the lenses of the animals. Their longer wavelength data shows an absorption peak near 625 nm as well. Maier gave an equivalent peak of 355 nm for a smaller bird. Maier also provided the relative absorption of the ocular media for the smaller bird between 325-700 nm. This data can be compared with that for the human in **Section 17.2**. It shows the presence of Rayleigh Scattering in this small eye but considerably less (nearly negligible) β-peak absorption.

The line at 0.495 microns is not found in the spectrum of pure retinol derived from industrial sources. It was only recorded in samples extracted from retinas or using ERG techniques.

Saari has introduced another variable into the intrinsic spectral absorption of the retinoids[36]. He noted that the retinol of Vitamin A_1 exhibited a peak in the ultraviolet at 325 nm in ethanol while that of Vitamin A_2 exhibited a peak at 350 nm. He assigned this difference to the conjugation of the basic arene structure, five for Vitamin A_1 and six for Vitamin A_2. The literature is sparse in this area. Arikawa, et. al. (1999) have shown an absorption, that they associate with the alcohol radical in retinol$_2$, at a shorter wavelength than 325 nm. The fact that the wavelength is not proportional to the conjugation level in the proposal of Saari suggests the number of variables involved in defining the peak in the intrinsic absorption of even simple retinoids. Alternately, the precise peak shown by Arikawa, without comment, may have been an error in transcription or drafting. It is likely the measured wavelength of a given ligand depends on the nature of the ligand, the location of the ligand in the molecule, the conjugation level within the molecule, the solvent, etc.

Groenendijk, et. al. have added a third complication by showing that the oxime of Vitamin A_1 (retinol$_1$) in hexane also exhibits a peak absorption at 350 nm, the same wavelength as the peak absorption as Vitamin A_2 in ethanol[37].

There have been many attempts in the literature to equate these spectral lines (other than that at 498 nm) to the absorption spectra encountered in vision with zero success.

The lower half of **Figure 6.2.2-1 (b)** illustrates the proposed line spectra of a family of retinoids, the Rhodonines, based

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[34]Vos Hzn, J. Coemans, M. & Nuboer, J. (1994) The photopic sensitivity of the yellow field of the pigeon's retina to ultraviolet light. *Vision Res*. vol. 34, no. 11, pp 1419-1425

[35]Maier, E. (1994) Ultraviolet vision in a passeriform bird. Vision Res. vol. 34, no. 11, pp 1415-1418

[36]Saari, J. (1994) in Sporn, M. Roberts, A. & Goodman, D. (1994) Op. Cit. pg. 355

[37]Groenendijk, G. DeGrip, W. & Daemen, F. (1979) Identification and characterization of syn- and anti-isomers of retinaloximes. *Anal. Biochem*. vol. 99, pp 304-310

on Vitamin A aldehyde but containing two polar groups separated by a conjugated chain and existing in the liquid

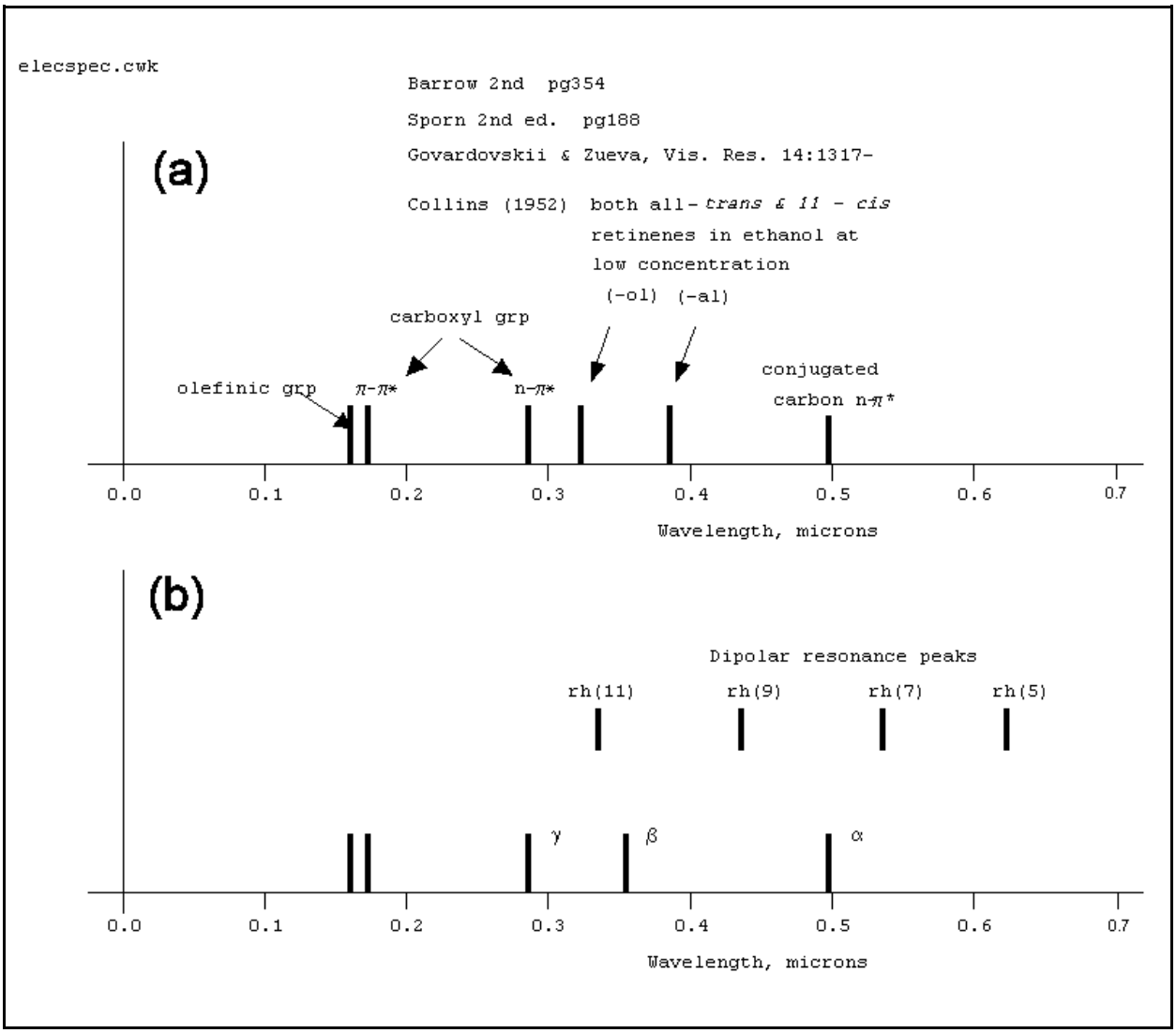

Figure 6.2.2-1 The electronic spectra of hydrocarbons. (a) various spectral peaks reported in Barrow or Sporn, 2nd ed. (b) Spectral components reported by Govardovski & Zueva in lower group. Spectral components for the four Rhodonines of this work in the upper group.

crystalline state. Below these resonant frequencies are shown the peaks in the spectra of simple retinal (but not retinol, see Section 6.3.1 xxx). The peaks have traditionally been listed beginning with the visible peak near 500 nm and proceeding to shorter wavelengths. As shown in the referenced section, the list is not in order of absorption coefficient. The gamma component exhibits considerably higher absorption than does the beta component.

The presence of the two polar groups in the Rhodonines is critical to achieving the necessary resonant structure. This structure exhibits the “slow” electron path discussed below. The liquid crystalline state is necessary to substantially enhance the absorption cross-section of the resonant band (also known as the J-band) of the material. It

also provides easy transport of the excited electrons to the edge of the crystal. As discussed in Mees & James[38], "Absorption within the J-band involves the cooperation of all the molecules in the aggregate, and absorbed energy traverses the whole aggregate. The process may be an example of exciton propagation whereby a state of electronic excitation achieved by absorption of a molecule in a closely coupled periodic aggregate is rapidly transferred from molecule to molecule in an interval less than the period of a molecular vibration."

A given molecule of Rhodonine exhibits one of the dipolar resonance peaks. The wavelength of the dipolar peak is proportional to the length of the conjugated carbon chain separating the two polar atoms. This distance has been called the cross-conjugation length. This cross-conjugation length need not be the same as the conventional conjugation length for the molecule as a whole.

The underlying absorption bands of the vitamin A aldehyde family remain present in the absorption spectra of the Rhodonines; however, the lines below 0.4 microns are not important to vision and the line at 0.495 microns becomes insignificant in the presence of the enhanced absorption cross-section of the resonant bands. Occasionally, the intrinsic absorption band at 0.495 microns (frequently labeled P502) can be recognized in the overall absorption spectrum of a Rhodonine. It is found far down on the skirt of the complete absorption spectrum of the overall chromophore.

Usually, the intrinsic absorption lines of the retinoids are isotropic. However, this is not true for the resonant bands. The absorption in a resonant band is related to the liquid crystalline state. If the Rhodonine of interest achieves this state during condensation from the liquid state, it may exhibit an isotropic absorption coefficient because the individual filaments of the material formed in the solute will be randomly oriented. However, if the Rhodonine is precipitated onto a substrate, the liquid crystalline material will be highly structured and follow the contour of the substrate. The absorption coefficient of the resulting structure will exhibit a distinct maximum for irradiance with a poynting vector parallel to the long axis of the conjugate chains, i.e., perpendicular to the surface of the surface.

> It must be noted for completeness that the chromophores hypothesized here do not rely on any form of isomerism to accomplish the photodetection process. In fact, again based on photographic theory, a straight, planar molecule is much to be preferred to a twisted one. A straight, planar molecule will form a more compact liquid crystalline structure. A more compact structure leads to more complete overlap of the anti-bonding orbitals, π^*, and thus the required conductivity. It will be assumed herein that the chromophores are all present in the all-*trans* configuration when utilized in the vision process. However, this is not a requirement. They could be present in any configuration dictated by other biological requirements. However, it is interesting to again note the comment of Berman[39] while working under the assumption that the so-called rods utilize the 11-*cis* form of retinal. "Except for the 11-*cis* isomers found in the retina, the retinoids in virtually all body tissues are of all-*trans* configuration."
>
> Over the years since photo-isomerization was first proposed for the mechanism of photoreceptor operation in vision, the field of photochemical reaction has matured. In Physical Chemistry, photochemistry is invariably divided into two processes. The initial (or primary) process involved is inevitably electronic photo-excitation of a molecule followed by de-excitation through many different secondary processes. In complex organic molecules, photo-isomerization is an unlikely process. The molecules have a propensity to participate in a variety of lower energy processes first, such as photolysis or photo-polymerization--especially photo-polymerization in highly aggregated molecules. In complex organic molecules in the liquid crystalline state, photo-isomerization is even less likely because of the physical distortion introduced into the structure of such closely packed molecules.

Isomerism in the retinal family is an energy intensive process compared with electronic excitation. Hubbard et. al. (1966) give the free energy of transformation of 11-*cis* to all-*trans* retinal in heptane as -1200 calories per mole at body temperature in the presence of iodine as a catalyst[40]. No mention could be found in the literature of iodine being present in the *in-vivo* eye. No mechanism has been defined in the literature for providing a significant flow of energy into the outer segments of the photodetectors. Such a mechanism would be required to support the

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[38]Mees, C. & James, T. (1966) The theory of the photographic process, 3rd ed. New York: Macmillan, pg. 245

[39]Berman, E. (1991) Biochemistry of the eye. NY: Plenum Press, pg. 48

[40]Hubbard, R. Bownds, D & Yoshizawa, T. (1966) Op. Cit. pg 301-315

continuous isomerization and de-isomerization of the chromophores.

6.2.2.4.2 Retinoids associated with Vitamin A_2

The electronic spectra and resulting spectral absorption characteristics of Vitamin A_2 are very similar to those of Vitamin A_1. The de-hydrogenation of the β-ionone ring impacts two aspects of the electronic spectra, neither of which affects the visual process. First, the signature associated with the ring at 325 nm, moves to 350 nm. This is caused by the different organization of the β-ionone ring. The second effect is a reported movement of the signature near 500 nm. associated with the conjugated chain, a few nanometers toward longer wavelength. This change is a result of increasing the length (or complexity) of the conjugated carbon chain in the molecule. Both signatures are characteristics of the material in dilute solution and are isotropic. The resonant dipole absorption characteristic, so important to vision and only present when the material is in the liquid crystalline state, is anisotropic. The peak wavelength of this absorption is controlled by the length of the cross-conjugated chain between the two heavy atoms. These resonance absorption characteristics are not significantly affected by any change in the β-ionone ring. The visual spectra of the individual chromophores of Vitamin A_1-based animals and of Vitamin A_2-based animals are identical (within current experimental errors).

6.2.2.4.3 Retinoids associated with Vitamin A_3

As developed in **Section 1.2**, there is a third form of Vitamin A besides those previously defined. Most marine life uses Vitamin A_1 based on the β-ionone ring of retinol, and most freshwater-based animals use Vitamin A_2 based on the 3,4-dehydro variant of this ring, a third form is also found in vision. Many Orders of *Insecta* appear to use this third form of Vitamin A. This is the 3-hydroxy variant. This variant has the same standing in insect nutrition and vision as the other two. It is appropriately labeled Vitamin A_3. As in the above cases, the actual wavelengths of the chromophores appear to be unaffected by this variation in the β-ionone ring. Wolf & Johnson encountered this form in 1960 (their postulated structure III) but did not pursue it extensively[41].

Whereas Vitamin A_1 is usually obtained from carotene in the marine-based animals (including most mammals), Vitamin A_3 is easily obtained from Zeaxanthin, the symmetrical dimer of the Vitamin, and from β,β-cryptoxanthin, an asymmetrical dimer of retinol and 3-hydroxy-retinol. It may also be obtained from carotene, and other sources of retinol, by hydrolysis[42].

Vogt & Kirschfeld isolated a 3-hydroxy-retinoid from the blowflies, *Diptera calliphora erythrocephala*[43]. While they described it as 3-hydroxy-retinal, this work would suggest it was actually 3-hydroxy-Rhodonine( ). As they indicated in their Fig 1, (plotting against a relative ordinate) the spectrum of this material is not that of retinol or retinal in ethanol. In dilute ethanol, it does exhibit a spectral peak near P495 (as read from their freehand sketch). However, it also exhibits a fine structure near 350 nm (with secondary peaks at 332 and 369 nm).

When they isolated components of this material that they described as the alcohol and the aldehyde, these materials did exhibit the peaks associated with $retinol_1$ and $retinal_1$ in ethanol. They provide thin film chromatography of the material along with $retinol_1$, $retinal_1$ and zeaxanthin. They also repeat, in brief, the discussion in Vogt on the nature of the material concerning zeaxanthin and other carotenoids.

Vogt made a unique proposal in 1989[44]. He proposed that the rhodopsin complex in flies consisted of two retinoids attached to one opsin molecule. One was the conventional N-cis-retinyl-opsin attached to Lysine 296. The second was an all-*trans* 3-hydroxy-retinoid attached to the outside of the opsin via two hydrogen bonds associated with the two hydroxy groups of the retinoid (at C15 and at C3). He named this putative visual pigment Xanthopsin. This form does not have a place in the Theory of this work.

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

[42]Vogt, K. (1989) in Stavenga, D. & Hardie, R. ed. Facets of Vision, NY: Springer-Verlag, Chapter 7

[43]Vogt, K. & Kirschfeld, K. (1984) Op. Cit.

[44]Vogt, K. (1989) in Stavenga, D. & Hardie, R. ed. Facets of Vision, NY: Springer-Verlag, Chapter 7

Smith & Goldsmith have broadened our understanding of the occurrence of Vitamin A_3[45]

Vogt & Kirschfeld describe two formulas for their putative chromophores in the caption to Fig. 3. The first is the "normal" chromophore based on 3-hydroxy-retinal and the second is a "sensitizing" chromophore based on 3-hydroxy-retinol. They found both materials present in the all-*trans* form. While they provide chemical diagrams (all-*trans*) for their proposed normal and sensitizing chromophores of Ultraviolet vision in *Diptera*, this work proposes the single resonant structure in **Figure 6.2.2-2**. Only the ultraviolet chromophore is shown in this figure. As shown, this molecule has a charge of -1. It can be neutralized by a single hydrogen associated with either the oxygen associated with C11 or C15. When present at C15, it can act as a hydrogen bond to a substrate such as opsin. In either case, the molecule remains chemically resonant. This single resonant chromophore will satisfy the chemical criteria used by Vogt & Kirshfeld to identify their two putative chromophores. In dilute form, it will exhibit the absorption spectrum they show in Fig 1 (with a complicated absorption near 350 nm). When adsorbed as a liquid crystal on the surface of an appropriate substrate (opsin), it will exhibit significant absorption in the ultraviolet with a peak at 342 nm. This peak will be much stronger than the complex peak associated with the chromophore in dilute solution. In contrast to the two-point adsorption suggested by Vogt, this theory suggests adsorption involving only the C15 location of the chromophore.

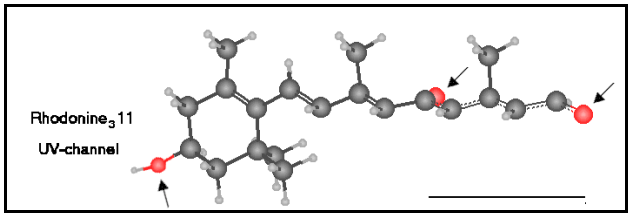

Figure 6.2.2-2 The proposed ultraviolet wavelength chromophore of the Order Diptera (and possibly Lepidoptera and other Orders). These Orders use Vitamin A_3, 3-hydroxy-retinol as their chromogen. The addition of an oxygen at C11 converts this structure to the chromophore, Rhodonine$_3$(11). The three oxygens present are indicated by the arrows. The peak spectral absorption of this chromophore is at 342 nm. The peak is due to the resonance between the oxygens associated with the C11 and C15 carbons

[45]Smith, W. & Goldsmith, T. (1990) Phyletic aspects of the distribution of 3-hydroxyretinal in the class Insecta. *J. Mol. Evol*. vol. 30, pp 72-84

6.2.2.5 Equations for the alcohol and aldehyde ligands

In preparing **Chapters 16 & 17**, the absorption spectra of both the alcohol and aldehyde ligands in the molecules of the lens of the eye play an important role. The peak absorptions for these ligands are at 325 and 357 nm respectively. There spectrums exhibit a quality factor or Q = 15. These ligands appear to be similar to those found in the retinenes and retinines under similar conditions.

Figure 6.2.2-3 shows the parameters associated with these ligands *in-vivo*.

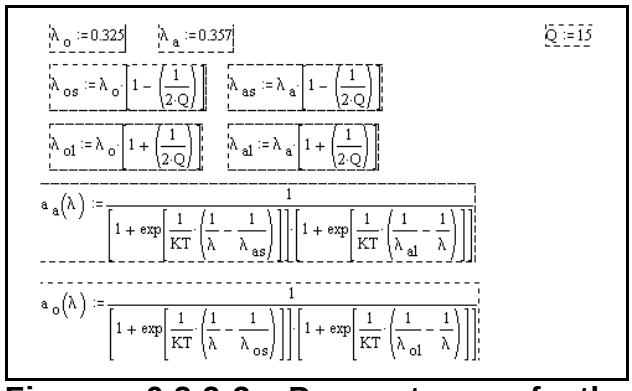

Figure 6.2.2-3 Parameters of the absorption by the alcohol and aldehyde ligands of the proteins in the physiological optics of the eye.

6.2.3 The retinenes and retinene complexes as chromophores

Major efforts have been undertaken over the last half century to demonstrate the spectral properties of a retinene in association with a protein that match the in-vivo spectra of the photoreceptors of vision. The effort has been unsuccessful. **Section 6.3.1.2** contains the basic explanation of why the putative rhodopsin is not the chromophores of vision. A retinol complexed with a simple (uncoiled) protein via a Schiff base does not exhibit absorption within the visual band. The only hope for a chromophore based on a retinal complexed with a protein via a Schiff base is the creation of a "color center" by the action of some of the charges associated with the amino acids of the protein. To date, attempts to show these interactions result in the appropriate peak absorption wavelengths (particularly at long wavelengths) have not been successful.

6.2.3.1 The putative rhodopsins

It is a proposal of this work that most of the experimental results presented in the literature related to a compound named rhodopsin must be separated into two distinct categories. In one case, the name applies to a specific chemical that combines a protein, opsin, and a retinene, generally retinol, via a Schiff base. In the second case, the name is used to describe a putative chromophore of vision based on in-vivo absorption data. Equating these two definitions in a rigorous way has been very difficult.

6.2.3.2 The metarhodopsins

Hubbard, et. al. explored how the putative rhodopsin of the time (assumed to be a compound) could generate a signal in response to the absorption of radiation within the visual band. Using an all chemical approach, they proposed that the initial absorption eventually caused an isomeric change in the structure of the molecule. However, it was not clear how this transition occurred and there was a significant energy deficit problem. They performed a series of in-*vitro* experiments at cryogenic temperatures, using an extract from photoreceptor cell Outer Segments, in an attempt to discover possible intermediate states between the initial and final isomeric states. The results of these experiments, and subsequent experiments by others[46,47], have been widely disseminated over time as the crucial steps in the photosensitivity of vision. However, the presentations have frequently omitted the fact that the experiments were carried out at cryogenic temperatures[48]. Many also omit the relaxation times associated with these different transitions. The Yoshizawa & Shichida paper provides the most complete summary of the available data. Their

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[46]Yoshizawa, T. Shichida, Y. (1982) Low-temperature spectrophotometry of intermiediates of rhodopsin. Meth. Enzymol vol. 81, pp 333-354

[47]Bridges, C. (1984) Retinoids in photosensitive systems. *in* Sporn, M. Roberts, A. & Goodman, D. (1984) Op. Cit. pg 128

[48]Chader, G. (1984) Vitamin A *in* Sears, M. *ed*. Handbook of Experimental Pharmacology, NY: Springer-Verlag. Chapter 8

figure 1 is a collage of data from many investigators. It is well known, particularly with regard to super conducting materials, that the band gaps of materials change with temperature. Accepting the assumption in these papers that the band gaps of these materials remain unchanged between cryogenic and biological temperatures is difficult. It is even more difficult to reconcile these putative reactions with those found within the liquid crystalline state of matter. It is this state that is so important to vision. This state only exists within the biological temperature range. Tests performed outside this temperature range are relegated to Category II experiments. They are interesting science with little relevance to vision.

Matthews, et. al. have presented the most comprehensive spectral data on rhodopsin as an extract[49]. The individual figures stress the variability of the spectrum of rhodopsin as a function of pH, temperature, glycerol content of solvent and other variables. However, it is not stressed that some of these parameters may change during an experiment due to secondary reactions unrelated to vision. Such changes cloud the interpretation of the data in many experiments in the literature.

In the above presentations, only the proposed initial reaction involves the absorption of a photon of light. As noted in Hubbard, Bownds & Yoshizawa, these relaxation times are such as to suggest the generation of the actual signal must occur before the generation of metarhodopsin I[50]. These authors also say the transition from the 11-*cis*- to the all-*trans*- form of the photosensitive molecules occurs in the formation of the first intermediary, pre-lumirhodopsin (apparently labeled bathorhodopsin in Yoshizawa & Shichida). All of the reactions following this event are only involved in the putative separation of the total moiety into opsin and a retinoid (labeled all-*trans*-retinal in the Yoshizawa & Shichida paper).

Bridges incorporates the interesting statement in 1984 (page 159) based on his acceptance of Hubbard's hypothesis: "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." Based on the irrelevance of this transition in the context of this work, the need for and occurrence of the *in-vivo* conversion is less clear.

The only spectral data presented by Hubbard, Bownds & Yoshizawa was for the extinction coefficient of an extract of rhodopsin in 55% glycerol at -195 C. It exhibited a molecular resonance at or near 498 nm and an additional peak absorption at a wavelength shorter than 350 nm.

Heck, Schadel, et. al. have recently presented a paper on the potential role of the metarhodopsins in vision[51].

6.3 Spectral absorption data related to the retinoids

The available data on the retinoids is extensive. However, it has frequently been collected under less than ideal circumstances. Much of the data resulting from non-biological synthesis or extraction has been evaluated when in organic solvents. Most of the data resulting from extraction of the retinoids from biological sources has been evaluated when dispersed in water. Virtually no data has been collected intentionally that relates to the retinoids when in the liquid crystalline state.

When discussing the spectral response of the chromophores of vision, both the isotropic and anisotropic absorption must be considered. The nature of the absorption depends critically on the configuration of the material at the molecular level. This configuration depends on the solvation level of the material. At high concentrations, the chromophores begin to assume the liquid crystalline state. This may involve individual strings within a solvent or the adsorption of the liquid crystal on a substrate. While the absorption of all retinoids is isotropic in dilute solution,

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[49]Matthews, R. Hubbard, R. Brown, P. & Wald, G. (1963) Tautomeric forms of metarhodopsin. *J. Gen Physiol*. vol. 47, pp 215-240

[50]Hubbard, R. Bownds, D. & Yoshizawa, T. (1965) The chemistry of visual photoreception. *Cold Spring Harbor Symposia, Vol. XXX*. Cold Spring Harbor, L.I., NY: Cold Spring Harbor Laboratory. pp-301-315

[51]Heck, M. Schädel, S. Maretzki, D. Bart,F. Ritter, E. Palczewski, K. & Hofmann, K. (2002) Signaling States of Rhodopsin: Formation of the Storage Form, Metarhodopsin III, from Active Metarhodopsin II JBC Papers in Press. Published on November 9, 2002 as Manuscript M209675200 (In /my documents)

the absorption of the Rhodonines becomes anisotropic in the liquid crystalline state. This anisotropic character consists of two components, the intrinsic molecular absorption that remains isotropic, and a more prominent quantum-resonance absorption that is highly anisotropic. While present in strings that are free to rotate within a solvent bath, the overall absorption of the Rhodonine will assume different spectral characteristics but will still appear to be isotropic. When adsorbed to a surface, the Rhodonines will exhibit a dominant absorption characteristic that is highly anisotropic for light impinging nearly perpendicular to the surface of the substrate.

The absorption of the disks of outer segments can be very complex as reviewed in **Section 5.5.11**. The investigator should differentiate between these different modes of absorption in their reports.

Much of the data for retinoids derived from biological sources has not noted clearly whether the material is derived from a marine (A_1), a freshwater (A_2) or an arthropod scavenger (A_3) based source.

The simple retinenes, containing only one oxygen atom, do not exhibit a molecular resonance. Molecular resonances are the dominant source of absorption in the visual region of the electromagnetic spectrum. Therefore, no retinene should be expected to exhibit any absorption in the visual region. The data will show they do not. Only retinoids containing two polar atoms exhibit significant molecular absorption in the visual spectrum.

Coxon & Halton identify a potentially useful characteristic of many materials in dilute solution[52]. The π-π^* and n-π^* transitions are solvent dependent. "On increasing the polarity of the solvent, the $\pi-\pi^*$ absorption generally moves to a longer wavelength (a bathochromic shift) whereas the n-π^* band usually moves to a shorter wavelength (a hypsochromic shift)."

This section will present the most relevant of the data found in the literature. The presentation is complicated by the variety of circumstances under which the data has been collected. It is particularly important to differentiate between the data collected where the retinoid is in dilute solution from that collected where it is in the liquid crystalline state.

The following sections will attempt to review the available data from non-biological and biological sources separately. However, some figures will combine the data from the two environments.

6.3.1 Data from non-biological sources of the retinenes in dilute solution

Finding specific calibrated spectral data on the various forms of Vitamin A extending from 200 to at least 700 nm remains extremely difficult. Very few spectra could be found for the materials in an aqueous solution. No spectra could be found for these materials at molar densities of 0.01 or higher in an aqueous solvent. Virtually no spectra could be found showing explicitly the variations caused by the possible *cis/trans* transitions. Therefore, assembling and rationalizing the available pieces of data is necessary. If successful, this activity can lead to a reasonable estimate of the overall spectra of the material. This will become a jumping off point for developing and comparing the spectral characteristics of the actual chromophores of vision.

6.3.1.1 The simple retinals

Kofler & Rubin provided an early summary of the absorption spectra of virtually all known isomers of the retinenes (pages 333-337) in 1960[53]. Unfortunately, the data is all in the ultraviolet. They presented no data that even hints at a visual spectrum for any of these materials.

Chader has provided a summary of the observable properties of Vitamin A and the retinoids in dilute solution[54]. Their absorption spectra when in dilute solution are entirely within the ultraviolet region. However, their precise spectra depend on the nature of the solvent in which they are suspended. He also reviews the tests used for identification prior to the arrival of NMR identification.

As will become obvious below, retinol and retinal are not chromophores of vision. They are only precursors, or

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[52]Coxon, J. & Halton, B. (1987) Op. Cit. pg 9

[53]Kofler, M. & Rubin, S. (1960)Physichemical assay of Vitamin A and related compounds *In* Harris, R. et. al. Vitamins and Hormones Volume 18 NY: Academic Press pp 315-339

[54]----(1984) Handbook of Experimental Pharmacology. NY: Springer-Verlag Chapter 8

chromogens, leading to the true chromophores.

Figure 6.3.1-1 attempts to summarize the spectral absorption data of the retinenes (families A1 and A2 only) in one figure. Their spectra in ethanol, methanol, and a mixture of chloroform and hexane are shown. The top frame of the figure is from Sebrell[55], i.e., the absorption coefficient for ***dilute*** all-*trans*-vitamin A1 in ethanol below 400 nm. It must be noted that the absorption coefficient is given in a form that assumes linearity with molarity. This is not true at levels above 0.01 molarity where the state of the retinoid begins to change. It is known to become "stringy" before forming a gel. The stringy material is indicative of aggregation and the formation of a gel is a good example of the liquid crystalline state of matter.

The middle frame shows data from Sporn. Whereas Vitamin A_1, all-*trans*-retinol, exhibits absorption peaks at 325 nm & below 220 nm in methanol according to Sebrell, Vitamin A_2, all-*trans* 3,4 didehydroretinol, exhibits two peaks, at 350 nm and at 275-280 nm in methanol. While a shift in one peak from 325 to 350 nm can be associated with the solvent, the introduction of a second peak near 275-280 nm cannot. This new peak must be related to the

[55]Sebrell, W. & Harris, R. (1967) NY: Academic Press pg.22

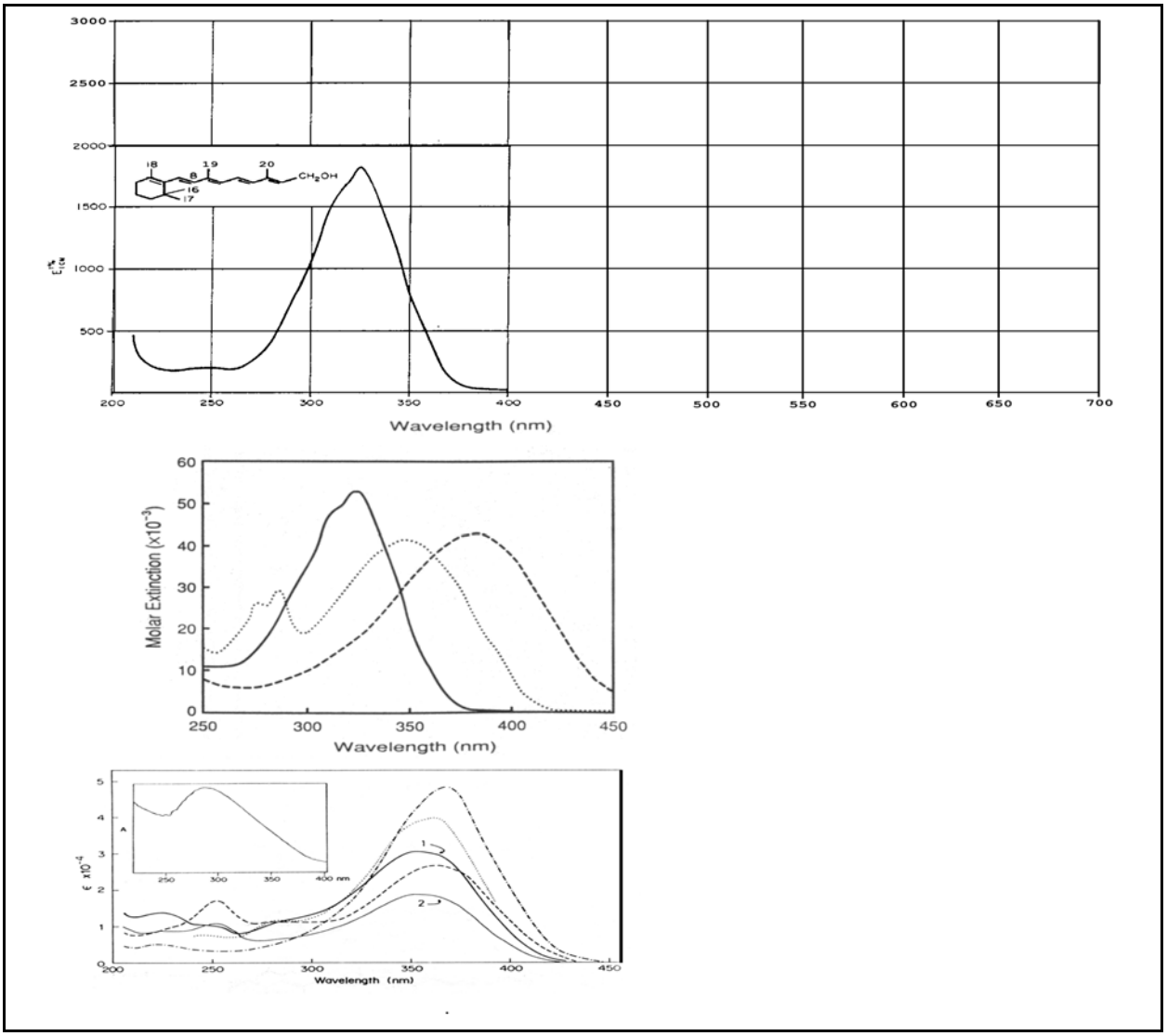

Figure 6.3.1-1 The intrinsic absorption spectra of both Vitamins A1 & A2 . Top, all-*trans*-Vitamin A_1 in dilute solution of ethanol. Adapted from Sebrell & Harris, 1967. Middle, all-*trans*-retinol$_1$ (solid line), all *trans*-retinal$_1$ (dashed line) and all-*trans*-retinol$_2$ (dotted line) in dilute methanol. Adapted from Sporn, 1994. Bottom, Absorption spectra of selected isomers of retinal in hexane (see text). From Dawson & Okamura, 1990.

extra double bond in the ionone ring of Vitamin A_2 as this is the only structural difference between the two materials. Notice that these two materials do not exhibit any absorption in the visual portion of the spectrum, although they contain a long conjugate chain of carbon atoms terminated on one end with a group containing oxygen. This conjugate chain is usually defined as a tetranene of four units length. However, looking at Vitamin A_1 closely, and including the double bond in the β-ionone ring leading to C18, the molecule is seen to be a pentanene of five units. Vitamin A_2 could similarly be defined as including a six-unit long chain of hexanene; however, this is of little interest in differentiating Vitamins A_1 and A_2. It becomes more important when exploring Vitamin A_3.

The bottom frame from Dawson & Okamura describes the absorption spectra of several isomers of retinal in hexane (with an insert showing data for all-*cis*-retinal using a solvent of 5% ether in hexane). The dash-dot curve represents all-trans; dashed curve, 9-*cis*; dotted, 11-*cis*; solid labeled 1, 9-*cis*,11-*cis*; solid labeled 2, 7-*cis*,11-*cis*. The curves

show little variation in peak wavelengths near 350-375 nm. However, they do show some differences, apparently due to structure, near 250 nm. This figure appears earlier (without the insert) in Weissmann & Claiborne. They define the structure at 250 nm as due to the 12*S*-*cis* irregularity and the 370 nm structure as due to the 12*S*-*trans* forms of 11-*cis*-retinal respectively (**Section 6.2.2.2**).

While Dyer[56] quotes Stavanga, et. al. regarding a "*cis*–peak" at 340 nm for at least one chromophore of vision, no data was provided demonstrating the existence of such a *cis*–peak in the actual chromophores of vision. On the contrary, a resonance for the Rhodonine family is found at 342 nm. This is the resonance supporting the absorption spectrum of the UV chromophore, Rhodonine(11).

Figure 6.3.1-2 is very important. It is modified slightly from the original presentation by Wolken. It shows conclusively that **pure retinal and pure retinol do not show any significant absorption near 500 nm** when in dilute solution. The three waveforms do share a common spectral peak at 280 nm. The similarity ends there. Retinal is seen to have a longer wavelength absorption peak than Retinol (Vitamin A). This is usually ascribed to the one unit longer conjugated chain found extending to include the double bond associated with the oxygen in Retinal. The third waveform labeled P495 was ascribed to Rhodopsin solubilized in the detergent Ammonyx L0 by Wolken. However, it will be seen to be a common absorption peak shared by the resonant conjugated system displayed by all of the Rhodonines, and many other chemicals of the carboxyl-ion system when in dilute solution. The minor peak in the P495 curve is at approximately 337 nm. This value does not correlate with the peaks of either Retinol(330 nm) or Retinal (370 nm) in the above figure. However, by selecting other solvents, a match might be obtained.

Dawson & Okamura provide a similar graph based on frog rhodopsin, *Rana pipiens*, under more annotated

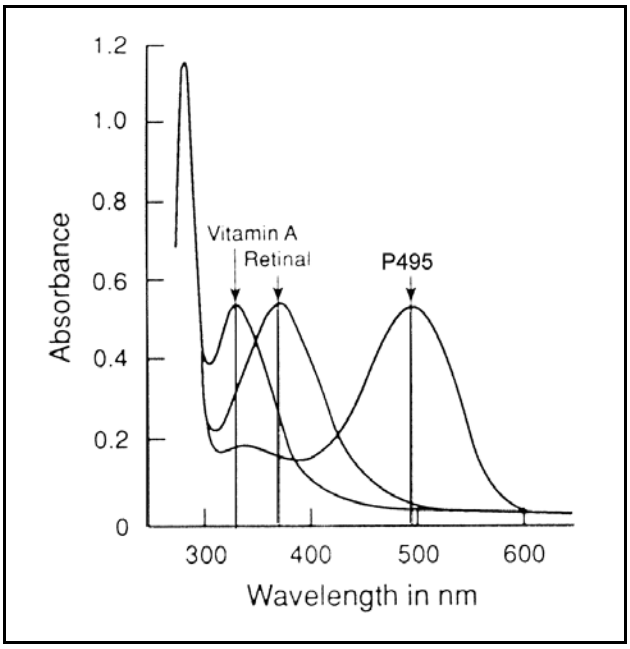

Figure 6.3.1-2 The spectral absorption of Vitamin A (retinol), retinal and a third material with peak absorption at 495 nm. Modified from Wolken, 1966.

[56]Dyer, A. (2001) Ocular filtering of ultraviolet radiation and the spectral spacing of photoreceptors benefit von Kries colour constancy *J Exp Biol* vo. 204, pp 2391-2399

conditions[57]. Vogt & Kirschfeld provide a similar graph based on the photoreceptors and chromogens of the fly, *Calliphora erythrocephala*[58]. Their graph shows a higher and more structured peak for P495 near 350 nm. This peak is even higher than the P495 peak. They stress that this peak does not correlate with either the retinol or retinal peaks. They ascribe their P495 curve to the chemical 3-hydroxy-retinol (Vitamin A_3). Assuming the material was not damaged in recovery, this work would suggest the chemical was 3-hydroxy-Rhodonine( ) in dilute form. They noted that their material was much more polar when converted to an oxime than retinal oxime.

The only proof that their material was the chromophore was the fact it was absent in carotenoid deprived flies (this is the same negation criterion used by Wald). This criterion only establishes that the 3-hydroxy-retinal was a chromogen. The material is used to form both rhodopsin (the substrate) and the Rhodonines (the chromophores) as shown in **Section 7.1.2**. P495 is not a unique spectral characteristics of the chromophores of interest. It is shared by many retinoids. The literature does not show that the simple retinenes exhibit a spectral peak near 495-500 nm as found in all chromophores from biological sources.

6.3.1.2 Schiff bases of retinol

Finding actual spectral data on the Schiff bases of retinol is difficult. Reference to a peak absorption of 443 nm for the free radical has been found in two places, without an accompanying graph. Shriver, Mateescu & Abrahamson provide several peak absorption values for protonated and unprotonated Schiff bases of retinylidene with propyliminium (**Section 6.4.1.6.1**). Their protonated materials exhibited a peak wavelength at wavelengths as long as 482 nm. This wavelength still falls short of the M– & L–channel spectral peaks. They also provided a bibliography of other similar reports related to rhodopsin.

6.3.1.2.1 The unprotonated Schiff base, N-retinylidene

all-trans-N-retinylidene is generally not found in a free state. In the recent literature, it is usually combined with a lipid, frequently as N-retinylidene-phosphatidylethanolamine. Such a configuration is not that of the conceptually or chemically defined rhodopsin of the literature. However, Shichi suggests it might participate in the preparation or reconstitution of rhodopsin[59]. This material shows a peak absorption near 460 nm. This is not a wavelength normally associated with in-vivo vision.
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6.3.1.2.2 The protonated Schiff base, $^{+}$N-retinylidene

Protonation of the Schiff base has been the favorite explanation for the putative spectral absorptions of the chemically defined material, rhodopsin. A shift of the absorption peak near 375nm to near 440 nm is usually claimed. While frequently discussed in terms of an "expanded resonance stabilization hypothesis," no generally accepted explanation of the mechanism has been found. And, no explanation of a shift to wavelengths longer than 440 nm have been offered. The smaller shift is usually explained in terms of changing the energy required for the excitation of a π-electron into the π^* state. Such analyses do not recognize the potential presence of the n-electron energy state developed in this theory. The source of the putative change in excitation energy is usually a point charge assumed to be furnished by one of the amino acids of the protein moiety. It is suggested that a point charge provided by different amino acids could account for the change in spectral absorption of the resultant different varieties of rhodopsin. This approach has been studied for more than 50 years without providing a viable theory. See Shichi, 1983 (pg 83-85) for a recent brief overview. Vreven & Morojuma have provided a recent analysis based on the premise that rhodopsin is the chromophore of vision[60]. They do introduce the possibility of a triplet excited state in rhodopsin, but they do not discuss its statistical probability or method of excitation in any detail.

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[57]Dawson, M. & Okamura, W. (1990) Op. Cit. pg 30

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

[59]Shicchi, H. (1983) Biochemistry of Vision. NY: Academic Press pp 135-138

[60]Vreven, T. & Morokuma, K. (2000) The ONIOM (our own N-layered integrated molecular orbital + molecular mechanics) method for the first singlet excited (S_1) state photoisomerization path of a retinal protonated Schiff base J. Chem Phys vo. 113, no. 8, pp 2969-2975

6.3.1.3 The Retinaloximes

The retinaloximes play a crucial role in understanding the nature of the chromophores of vision. The common wisdom has been that either retinol or retinal become chromophores when associated with opsin via a Schiff-base. Without this association, neither retinol nor retinal exhibit any absorption in the visual spectrum.

The retinaloximes are compounds of retinol or retinal with hydroxylamine. Higher retinaloximes may be formed based on more complex oximes such as the O-alkyloximes. The result is a compound of the retinoid covalently bound to a straight chain hydrocarbon by a Schiff-base. Groenendijk, et. al. have characterized this group of materials, including the special notation required to interpret the results[61]. Their molar extinction curves for both the all-trans and 11-cis isomers of retinaloxime are similar to the above figures. Van Kuijk, et. al. have extended the work to the higher oximes using HPLC techniques[62] (see **Section 6.4.2**).

The critical result gained from this work is that neither retinol nor retinal when combined with a hydrocarbon or carbohydrate via a Schiff-base exhibit any significant absorption in the visual regime of the spectrum. The peak absorption of both the syn- and anti- forms of all-trans-retinaloxime in hexane occur at 350 nm. The 11-cis- forms exhibit distinct peaks at both 350 nm and 245 nm. No absorption at 500 nm is indicated. These materials are not chromophores of biological vision. The work of Van Kuijk, et. al. was focused on nutrition and metabolic studies of the higher oximes and did not measure the spectral responses of the materials.

6.3.2 Data from non-biological sources of the retinenes in concentrated solution

At this time, no total synthesis of any chromophore of vision has been achieved. Furthermore, no unequivocal reconstitution of a chromophore of vision has been reported. (The reports of the reconstitution of the non-chromophore, rhodopsin, with its molecular absorption at 495-500 nm, will be discussed in **Section 6.3.4.4.1**.) This is primarily due to the previous lack of a definitive theory and model defining the materials. On the other hand, considerable material is available defining the properties of these materials from related technologies. The Rhodonines are not only retinoids, they are also oxonols, cross conjugated carbon molecules, supporting two polar atoms of oxygen at positions along the carbon backbone. When in the liquid crystalline state and "married" to an appropriate electronic partner, these materials are some of the most efficient chromophores within the visual spectrum. The most sensitive chromophores consist of the oxonols, the equivalent cyanines (replacing the two oxygens with two nitrogen atoms), and the merocyanines (one oxygen and one nitrogen atom in each molecule).

These classes of materials are well documented in the photography literature[63]. The properties of the Rhodonines, based on their family characteristics are discussed in **Section 5.5.10**.

6.3.3 Data for a group of resonant retinoids

6.3.3.1 Theoretical data for the Rhodonines

This subject is discussed extensively in **Section 5.5** and will not be repeated here. The proposed theoretical spectra of the Rhodonines are presented there.

6.3.3.2 Data for 11-methylmerocyanine, similar to Rhodonine(5)

Dawson & Okamura have provided a spectrum for 11-methylmerocyanine when incubated with a protein material[64]. **Figure 6.3.3-1** shows their data. This material is a resonant retinoid (two polar atoms separated by a conjugated carbon chain) similar to Rhodonine(5) or (7). Note that nitrogen, instead of oxygen, appears in place of the sixth carbon. Nitrogen typically results in a longer wavelength resonance absorption peak than oxygen. While they speak

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[61]Groenendijk, G. DeGrip, W. & Daemen, F. (1979) Identification and characterization of syn- and anti-isomers of retinaloximes. *Anal. Biochem*. vol. 99, pp 304-310

[62]Van Kuijk, F. Handelman, G. & Dratz, E. (1985) Rapid analysis of the major classes of retinoids by step gradient reversed-phase high performance liquid chromatography . . . *J. Chromatograph*. vol. 348, pp 241-251

[63]Mees, C. & James, T. (1966) The Theory of the Photographic Process, 3rd ed. NY: Macmillan

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

of the material as spontaneously forming a Schiff-base with the protein material (apparently at the nominal binding site of bacterioopsin), this work proposes an alternate mechanism for the change in spectrum observed. At zero time, the material exhibits a peak molecular absorption at slightly longer than 480 nm. With time, this absorption peak decreases in amplitude and wavelength. As the retinoid material aggregates (either on the surface of the protein material or by itself), it begins to exhibit a resonance absorption at wavelengths longer than 645 nm and reaching 662 nm after 160 minutes. This is the typical progression of a resonant retinoid when allowed to aggregate. The progression occurs in the presence of a variety of substrates, or no substrate if the molar concentration of the retinoid is sufficiently high.

Dawson & Okamura note that the absorption peak of their material narrowed to less than 50 nm at FWHM. This work does not support their assertion that their data is "strong evidence for the protonated nature of the Schiff base linkage in bacterioopsin." The same curves can be expected using a substrate other than bacterioopsin.

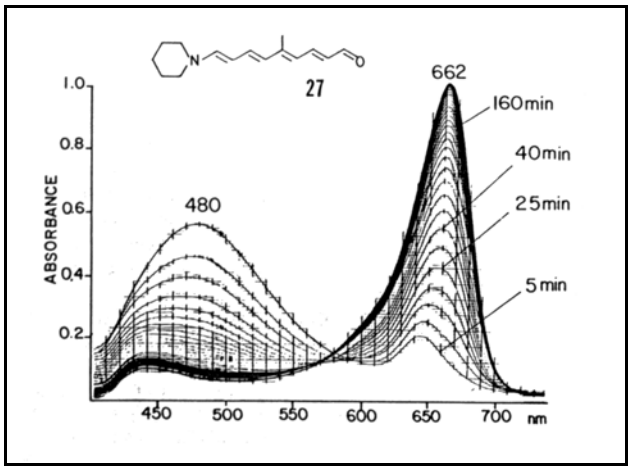

Figure 6.3.3-1 The absorption spectra of 11-methylmerocyanine with a protein material in 10mM HEPES buffer, pH 7.0, dark, 22°C. From Dawson & Okamura, 1990.

6.3.3.3 Data for a family of resonant cyanines

Figure 6.3.3-2 shows a set of spectra for a simple series of cyanines (two nitrogen atoms separated by a conjugated carbon chain) as a function of conjugation level. The family shows the typical narrow absorption spectra of the resonant absorbers at approximately equal spacing as a function of conjugation level. In this figure, the molecular absorption expected near 498 nm for any polar retinoid is to small to be presented.

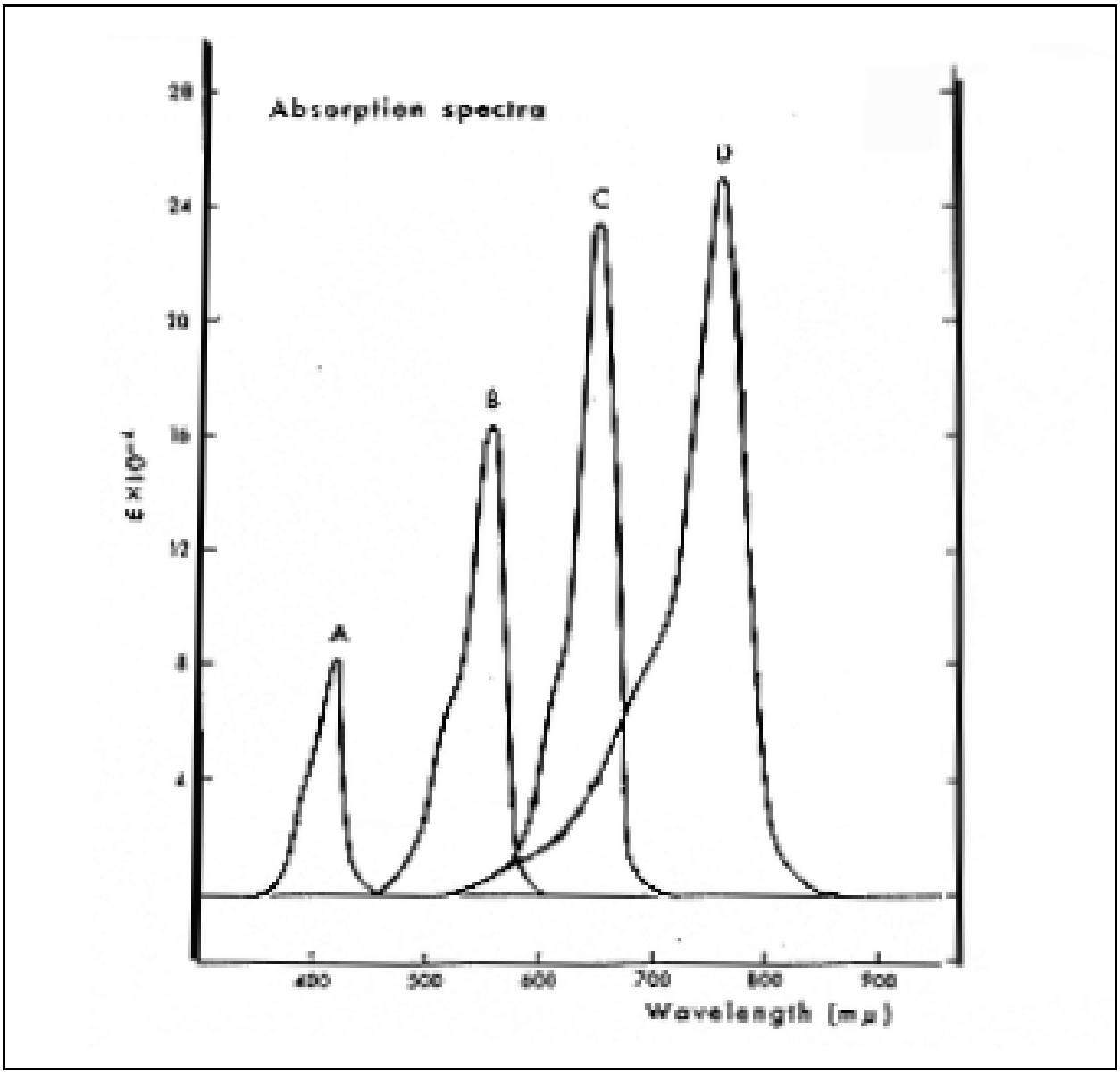

Figure 6.3.3-2 Absorption spectra in methanol for a simple cyanine. A, B, C & D describe conjugation levels of 0,1,2 & 3. From Mees & James, 1966.

6.3.4 Data from biological sources of retinoids at different levels of aggregation

It is amazing that in the year 2003, the community has still not been able to isolate any of the chromophores of vision and measure their spectra.

Several conditions can cause the above situation. Probably the most important is the lack of broad experience among many investigators, many of whom are graduate students and post-docs. Related to this condition is the limited appreciation of the importance and unique properties of materials when in the liquid crystalline state. The fact that the chromophores only exhibit their unique spectral characteristics when they are in the liquid crystalline state also contributes to the problem. Finally, the performance of the chromophores in the liquid crystalline state is further optimized when they are in a specific physical configuration.

An additional aspect of the problem is the misdirection of the community because of work in the 1950's. This work focused attention on the protein precipitate from the OS following centrifugation. It did not develop the importance of the supernatant fluid resulting from these experiments.

The above conditions are largely responsible for the lack of precise data related to the biologically derived chromophores of vision in the literature.

In centrifugal separation, it is now clear that the supernatant fluid, not the precipitate, actually contains the chromophores of vision. This fluid contains the vast majority of the retinoids in the overall extract of outer segments, with only a minor portion found combined with the protein opsin in rhodopsin[65]. Unfortunately, most of the material in the supernatant fluid was either bleached or destroyed through less than ideal handling. **Section 6.5** will address the methods, both unsuccessful and proposed for the extraction of the actual chromophores of vision.

The chromophores of vision exhibit exceedingly different spectral characteristics depending on their physical state. These characteristics change significantly with their level of aggregation within what appears at first glance to be a dilute solution. The solution may become a suspension under a variety of conditions. The resulting quasi-solution requires unique instrumentation to evaluate properly.

6.3.4.1 Methods of obtaining spectral data from biological sources

When discussing spectral data in the vision context, appreciating the variety of experimental procedures available is important. Chader has discussed these methods without referring to the *in-vivo* requirements associated with the liquid crystalline state (pg 368). Data can be obtained by using through-the-lens reflectometry, by measuring the current response within the IPM, by measuring the voltage response within the IPM, by measuring the potential at the pedicle of the photoreceptors, by making electrical measurements later in the signal chain, and by making psychophysical measurements. Only spectro-photometry applied to the OS can be considered (under minimal bleaching conditions) to generate data free of changes due to the electronic circuitry of the remainder of the optical path. Measurements at later points in the system involve at least logarithmic compression (if a voltage measurement) and most likely a considerable degree of exponential algebra and amplifier gain adjustments. Nevertheless, in recent years, all these techniques have confirmed the spectral absorption peaks of chordate vision are near 342, 437, 532 and 625 nm (within an error of less than ±5 nm).

6.3.4.1.1 Complete versus effective absorption spectrum

Figure 6.3.4-1 shows both the complete absorption spectrum and the effective absorption spectrum of a retinoid in various states of matter. The latter spectrum is related strictly to the resonant absorption spectrum of the molecule that interfaces with the neural system. The former spectrum consists of three parts. Part one is the first order resonant absorption spectrum that is used in vision. Part two is the first order molecular absorption spectrum that is not used in vision. Part three is the secondary, or second order, absorption spectrum that is not used in vision. The caricature has been modified from Rubin & Walls.[66] The figure is interesting in that it shows the variety of spectra that can be measured from the same apparent material. However, the vertical scale is not calibrated. The solid line purports to represent the complete absorption of a rhodopsin, with the caveat that it is intact. The label “intact” must be further definitized. Does it mean an intact and isolated molecule? Alternately, does it mean many intact molecules still configured as to form chromophores of vision. No measurements have ever recorded an absorption peak in the region of 500 nm for an isolated (and intact) rhodopsin molecule. Similarly, no measurements have ever recorded a 500 nm peak for a group of such molecules in dilute solution. Such molecules do show the absorption peaks at 278 nm associated with various protein ligands and an absorption at 350-385 nm associated with the retinol ligand. On the other hand, intact outer segments frequently show an isotropic peak in the 495-505 nm region

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[65]Wolf, G. & Johnson, B. (1960) Metabolic transformations of Vitamin A *Vitamin Hormon* vol. 18, pp 403-415, pg 411

[66]Rubin, M. & Walls, G. (1969) Fundamentals of visual science. Springfield IL: Charles C. Thomas. pp 57-67

(depending on the instrumentation). This is the isotropic molecular absorption of the retinoid. It is not the absorption spectrum used in biological vision. If the chromophore material is present in the liquid crystalline form, is deposited on a flat substrate, and is examined perpendicular to that substrate, the resonant absorption spectrum of the chromophore can be measured. This is the spectrum shown by the dashed line on the right peaking, for the long wavelength chromophore Rhodonine(5), at 625 nm. Alternate visual chromophores would show peaks at 342, 437 or 532 nm. These last four peaks are the associated with the chromophores of biological vision. They originate from resonant absorption by the chromophores. This resonant absorption generates excited electrons in the π^* energy band of the liquid crystal. These appear to be the only excited electrons that transfer their energy to the neural system.

Various investigators have recorded all of the spectra appearing in the above figure depending on their instrumentation. Largely unknowingly, many investigators have only provided a cursory description of their measurement apparatus and the sample measured. Many of the spectra have been recorded *in-vivo* where the structure of the material examined is not thoroughly understood. Many investigators have also concentrated on non-invasive techniques as a matter of practical convenience. While the investigator has frequently "calculated out" the contribution he believed to be due to the lens, the vitreous humor and sometimes the macula lutea, it has been left to the reader to differentiate between these recorded spectra. The empirically oriented vision community has frequently measured a composite spectrum extending from about 400 nm through the visual spectrum. This is particularly true of those using microspectrometry techniques. Such spectra are usually quite complex. They include portions of (1) the ligand absorption near 350-385 nm, (2) the resonant absorption of one or more spectrally distinct chromophores, (3) the molecular absorption of the chromophores near 500 nm, and (4) any secondary absorption of the chromophore molecules between 350-385 nm and either the 500 nm or the resonant absorption peak. Such secondary absorption is due to localized bending of the energy bands of the molecule caused by the geometrical arrangement of the ligands of the molecule. The ligands attempt to achieve a minimum overall energy state for the complete molecule.

The resonant absorption spectrum shown by the dashed line on the right is well described by the symmetrical Fermi-Dirac Equation discussed in **Section 5.5.10.1**.

Many investigators have attempted to fit empirical equations to data measured non-invasively. These techniques include reflective microspectrometry and simple ERG methods. These fittings have invariably tried to account for not only the absorption spectra related to vision, but also the secondary absorption found between the major long wavelength absorption peak and 350-385 nm.

If electrophysiological techniques are employed instead of microspectrometry, different results are obtained. These invasive techniques include both probing of the optic nerve and probing of the retina. Probing of the retina is frequently described as local electroretinography, LERG. The technique is frequently focused on recording signals at the S-plane. However, better data is obtained if signals are recorded directly at, or adjacent to, the photoreceptor cells. Invasive recording techniques generally give more precise spectral data. The secondary spectrum does not appear in the data. The short wavelength skirt of the measured spectrum extends to much lower values relative to the peak absorption. By measuring the signals in the optic nerve of rats, guinea pigs and frogs, Granit measured the effective absorption spectrum of the chromophores of chordates long ago[67]. These measurements show a set of highly symmetrical absorption spectra.

The micro-pipette techniques of Baylor et. al. have combined microspectrometry with electrophysiological techniques. Their protocols are of critical importance in that they measured both the isotropic (inactive) and anisotropic (active) absorption spectra of a single outer segment. They have primarily measured the isotropic molecular absorption spectra of chromophores (near 500 nm) when the spectrometer light has been applied transverse to the optical axis of the outer segment. When the light has been applied parallel to the optical axis of the outer segment, the anisotropic resonant absorption associated with biological vision has been measured. See **Section 5.5.10**.

6.3.4.1.2 Vitamin A1 versus Vitamin A2 or A3 based visual spectra

In the past, discussions have occurred concerning the difference in peak absorption of the chromophores of vision between the saline based and the freshwater-based animals. The assumption has been that the marginal difference in the absorption spectra of Vitamin A1 and A2 in the ultraviolet would be found in their visible spectra as well. No correlation has been shown between these shifts in the UV spectra of these chromogens in dilute solution and any

[67]Granit, R. (1943) A physiological theory of colour perception Nature, vol 151, pp 11-14 *also* Davson, H. (1990) Physiology of the Eye, 5th ed. NY: Pergamon Press pg 416

putative shift in the visual performance of the actual chromophores of vision when in the liquid crystalline state.

Wald provided the UV spectra of both of the retinols and retinals in dilute solution in 1939[68]. This material has been reproduced in Shichi, et. al. in 1969, in Chader in 1984 and in more recent sources shown in **Figure 6.3.1-1**). While the difference in these pairs of spectra is about 29 nm for the retinols and 16 nm for the retinals, these spectral features are not directly related to the structures of the molecules used in vision. The β-quinone ring where these shifts originate is not part of the conjugated backbone of the chromophores of vision.

6.3.4.2 Expected characteristics of the biological chromophores

The chordate animals have evolved along two parallel paths, one based on a salt water environment and the second based on fresh water. Animals that evolved in the salt water environment use Vitamin A_1 as a mainstay of their existence. Animals that evolved in fresh water use Vitamin A_2. Since these materials are chromogens for the chromophores of vision, their impact must be recognized in this work.

The resonant absorption spectra of the chromophores of vision are largely independent of the precise structure of the b-ionone ring present in the molecule. Thus, the individual functional spectra of the saltwater and freshwater-based animals vary in peak absorption by only a few nanometers. The situation is similar for the molecular absorption spectra occurring near 495-502 nm. However, when considering the ligand absorptions in the ultraviolet (the absorption frequently examined as a matter of convenience), the situation is quite different.

It has been proposed in this work that Vitamins A_1 & A_2 are only chromogens of the chromophores of animal vision and that the chromophores consist of molecules containing two polar groups terminating a cross conjugated carbon chain. Following this proposal, an isotropic molecular absorption spectrum common to the chromophores based on both the above chromogens can be sketched. The molecular absorption spectra are common in the visual region because the two resonant polar chains are identical in the two cases; i. e., the same resonant chain is found in both marine and freshwater based chordate animals.

It is further proposed that if a mixture of the Rhodonines, based on either Vitamin A_1 or Vitamin A_2, is tested for spectral absorption as a function of molarity, the P502 waveform will appear at low concentrations. The observed spectrum will begin to be corrupted as the molarity of the Rhodonines is raised to about 10^{-2}. This corruption will be due to the formation of “strings” of Rhodonine material in the solvent. These are aggregates forming without benefit of a substrate. The aggregates will begin to exhibit an additional spectral component due to resonance absorption. These spectral components will initially be quite narrow. However, as the degree of aggregation increases, the spectral widths will broaden. Lacking any substrate, the aggregates will be randomly oriented and the resonance absorption spectra will remain isotropic relative to the solvent container. However, if there is a suitable substrate present, which may be the wall of the container, the material will proceed to aggregate into one or more regions on the substrate. Each aggregate will be organized as a single layer of molecules constituting a liquid crystal. As a result, the new resonant absorption component will exhibit a strong anisotropic component that may be missed by the investigator. It will appear perpendicular to the surface of the aggregate.

If one of the above regions of liquid crystal on a substrate is now subjected to spectral absorption measurements, the absorption will be found to be highly anisotropic and exhibit a peak at a unique wavelength. The peak wavelength will be either 342, 437, 532 or 625 nm. depending slightly on the solvent used. These values are for the aggregate material in a Ringer’s solution, both matched to the IPM of, and at the physiological temperature of, the animal.

It should be noted that the anisotropic spectrum at 342 nm is only coincidentally similar to the isotropic ligand absorption peaks near 325 or 350 nm and representing structural features of the Vitamin A appropriate to the animal species involved.

The peaks at 437, 532 and 625 nm will generally be higher than the P502 peak for the same concentration and

[68]Wald, G. (1939) J. Gen. Physiol. vol. 22, pgs 391 & 775

measured using the same equipment and container. More important, these spectral peaks are in good agreement with the literature where techniques isolating individual spectral channels were used;

+ Marks[69] in 1963 for the goldfish, although the data is quite noisy
+ MacNichols[70] in 1964 for goldfish, with peaks measured individually at 450, 550 and 655 nm using single photoreceptor spectro-photometer. (450, 530 and 625 nm after correction for "progressive bleaching")
+ Tomita et. al.[71] showing peaks at 462, 529 and 611 nm along with standard deviations for the individual curves
+ Neumeyer[72] in 1988 using data from Harosi & Hashimoto[73] (1983) for the freshwater dace, *Tribolodon hakonensis*, and van Dijk & Spekreijse[74] (1984)

6.3.4.2.1 Differences, aggregated proteins versus aggregated chromophores

The stereo-metric assembly of organic materials differs significantly between protein material (such as rhodopsin), and the retinoids when standing alone. In the case or the retinoids, their stereo-metric assembly results in a sharing of quantum-mechanical energy levels between the individual molecules. While this is not too important in retinoids containing only one polar atom, it becomes extremely important in retinoids containing two polar atoms in a conjugated relationship. The relationship causes the appearance of a new resonant absorption spectral band.

In the case of the aggregation of rhodopsin, the situation is more complex. The aggregation is controlled by the steric form of the overall protein and the retinoid within the molecule contains only one polar atom, nitrogen. That nitrogen is electronically and physically shielded from the region outside of the complete molecule. Because of these considerations, the aggregation of rhodopsin does not result in the quantum-mechanical sharing of energy bands between the retinoid ligands of adjacent molecules. Furthermore, the retinoids have only one polar atom per ligand. Lacking another mechanism (usually proposed as a protonated Schiff base), the molecules would not exhibit a resonant absorption spectral band even if they did share quantum-mechanically.

Rhodopsin does not exhibit the resonant absorption bands associated with the chromophores of vision under any conditions.

6.3.4.3 Critical importance of determining the actual material being examined

Because of the complexity of the spectra related to the presence of a specific chromophore and its physical state, it is critically important to determine precisely the material being examined. As indicated above, rhodopsin does not exhibit a resonant absorption spectra under any conditions and cannot be considered a chromophore of vision.

The Rhodonines will exhibit resonant spectral absorption when in the appropriate physical state. However, unless separated into aggregates of only a single Rhodonine, the overall spectrum of the sample may be very complex (similar to [**Figure 6.3.3-2**]) . If the sample is prepared so that only one Rhodonine is present, the spectral response of the sample will be easily interpreted, although it will still vary as a function of the concentration (aggregation) level of the sample.

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[69]Marks, W. (1963) Difference spectra of the visual pigments in signle goldfish cones PH. D. Thesis, Johns Hopkins University, (cited by MacNichols, E. (1964) Retinal mechanisms of color vision *Vision Res*. vol. 4, pp 119-133)

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

[71]Tomita, T. Kaneko, A. et. al. (1967) Spectral response curves of single cones in the carp Vision Res. vol. l7, pp 519-531

[72]Neumeyer C. (1988) Das Farbensehen des Goldfisches. Thieme

[73]Harosi, F. & Hashimoto, Y. (1983) Ultraviolet visual pigment in a vertebrate: a tetrachromatic cone system in the dace *Science* vol. 222, pp 1021-1023

[74]van Dijk, B. & Spekreijse, H. (1984) Color fundamentals deduced from carp ganglion cell responses *Vision Res*. vol. 24, pp 211-220

6.3.4.4 Data from saline-based (Vitamin A_1) chordates

6.3.4.4.1 Data from dilute solutions

Figure 6.3.4-1 is representative of the spectral response obtained from any retinoid in dilute solutions. They show only molecular absorption in the visible band and ligand absorption in the ultraviolet. Note that a shift of the type seen in the visual band can frequently be observed by merely changing the pH of the solution. The same type of shift can also be seen due to the quantum-mechanical relaxation of the sample following strong bleaching. These two mechanisms for changing the spectral response of the sample were not addressed in the reports of Wald concerning his 1950's experiments to reconstitute rhodopsin. He mixed a solution containing bleached rhodopsin, from a biological source, with a second solution containing an industrially manufactured Vitamin A (undoubtedly in a solvent).

Section 6.3.1.1 references many sources of data collected from biological sources of rhodopsin in dilute solution. These generally show only a single visual band absorption peak near 495-502 nm.

Finding composite figures showing peaks at different wavelengths is also common. In these figures, the different materials are frequently labeled porphyropsin, iodopsin and cyanopsin. Many of these figures were developed for pedagogical purposes and do not have a calibrated vertical scale.

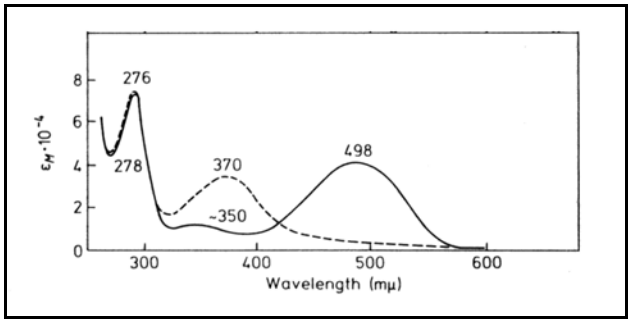

Figure 6.3.4-1 Absorbance of bovine rhodopsin extracts in digitonin. Solid line, dark adapted. Dashed line, light bleached. From Shichi, 1969.

6.3.4.4.2 Data from in-situ experiments

Going one step further, the four peaks predicted above are the same as the four peaks presented by Neumeyer and Arnold[75]. These predicted peaks account completely for their observed tetrachromatic color vision of the goldfish. They also agree with the data of Douglas, Bowmaker and Kunz[76] representing the four visual chromophores in the fresh water cyprinid fish, roach *Rutilus rutilus*, see **Figure 6.3.4-2**. This fish is Vitamin A_2 based. Neumeyer and Arnold note the long wavelength spectral channel disappears under inadequate stimulus intensity[77]. This work shows that this loss is a result of the "square-law" de-excitation process associated with the neural system. It is not directly attributable to the chromophore alone.

Douglas et. al. also show how noisy the raw data was in their figure 2. It required that they acquire multiple spectral tests. The results were then averaged for each of several animals and then the results from all of the animals must be combined to give an overall best estimate of the characteristic spectra for the species. The results show the impact of the central limit theorem and the presence of a ligand absorption peak in the 325-375 nm region. The responses do not have the shape of the Fermi-Dirac Function that defines the individual responses. The solid lines are the result of fitting templates to the data. The dashed line is shown on the left based on the spectral response of the UV chromophore, Rhodonine(11) (**Section 5.5.8**). It suggests the peak for this chromophore is at 342 nm. Additional comments about this spectrum will be presented below based on the rainbow trout. Those comments will show that the caption of figure 1 in the Douglas, et. al. paper needs modification. The Douglas, et. al. experiments were behavioral. The eyes of the fish were intact. The caption should read, "Mean absorbance spectra of the four roach cone visual pigments *as modified by the absorption of the lens*."

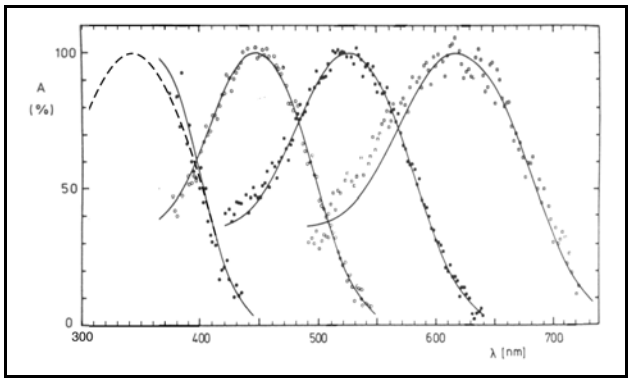

Figure 6.3.4-2 Normalized spectra of four chromophores of vision in the Vitamin A2 based fish, Roach. The instrumentation of Douglas, et. al. only extended to 370 nm. The graph has been extended to show the predicted peak in the UV based on this work (dashed line). Expanded from Douglas, et. al. 1987

Hawryshyn & Harosi provided a tetrachromatic spectra for the rainbow trout, *Oncorhynchus mykiss*, in 1994. The data is very similar to the above figure except they extended their readings down to 320 nm. Their UV response is narrower than their S–channel responses and suggest that their measurements include but do not identify the absorption of some energy by material containing an alcohol radical, –ol.This radical has a maximum absorption near 330 nm. This material may be the disk substrates based on the protein opsin. When the absorption of this radical is superimposed on the theoretical absorption of the UV chromophore, Rhodonine(11), the composite curve is shown in **Figure 6.3.4-3**. This graph has been modified to extend to 275 nm. Hawryshyn & Harosi did not explain the meaning of negative absorbance values on their curves. It suggests a value due to another material has been subtracted from their data. The Rhodonine(11) response has its normal bandwidth of about 95 nm at half amplitude. The fit of the composite curve to the data points of Hawryshyn & Harosi is better than their smoothed line through the data. Both curves suggest the data point at 0.006 O. D is an outlier.

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[75]Neumeyer, C. & Arnold, K. (1989) Tetrachromatic color vision in the goldfish and turtle *In* Kulikowski, J. Dickinson, C. Murray, I. *eds*. Seeing Contour and Colour. Oxford: Pergamon Press pp 617-631, pg 624

[76]Douglas, R. Bowmaker, J. and Kunz, Y. (1987) Ultraviolet vision in fish *In* Kulikowski, J. Dickinson, C. & Murray, I. Seeing Contour and Colour. NY: Pergamon Press pg. 602-603

[77]Neumeyer, C. & Arnold, K. (1989) Tetrachromatic color vision in the goldfish becomes trichromatic under white adaptation light of moderate intensity Vision Res. vol. 29, pp 1719-1727

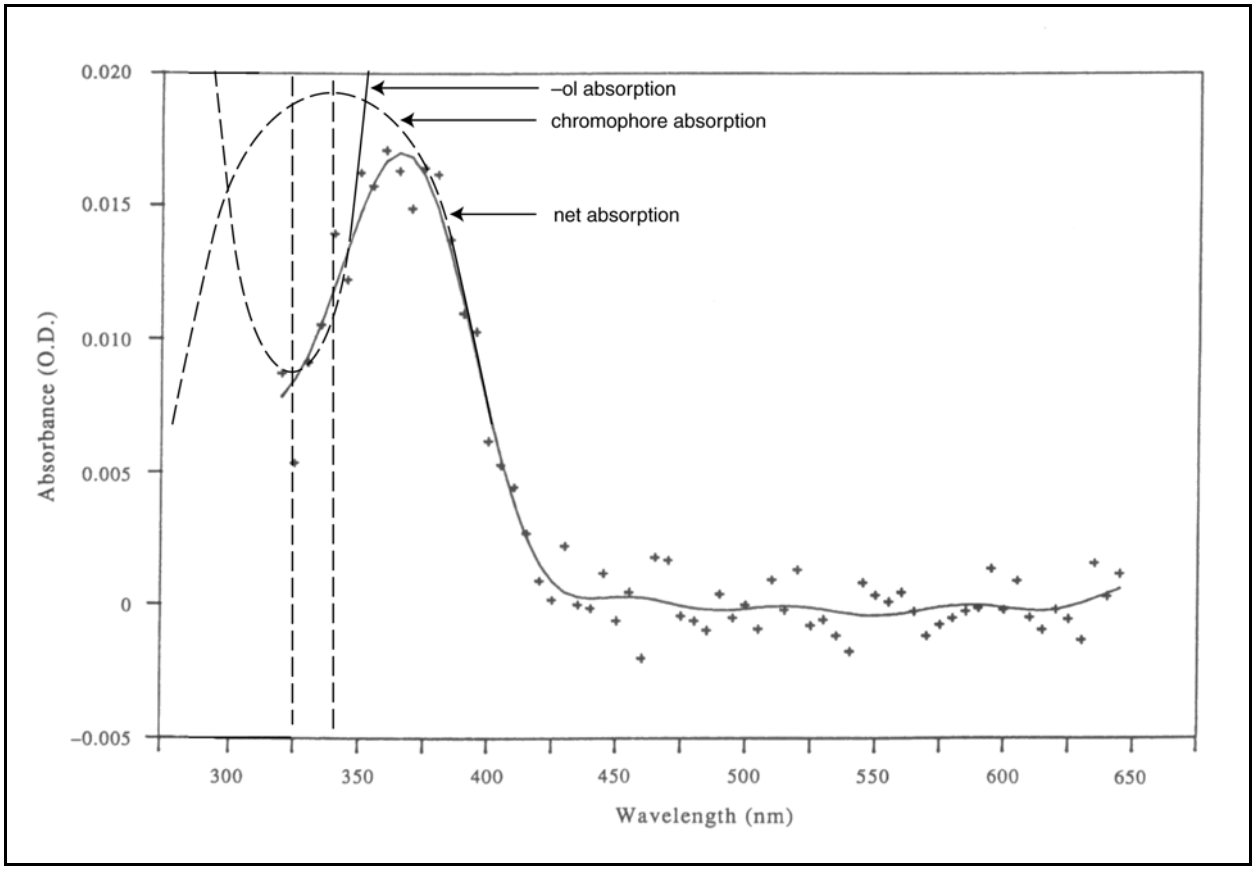

Figure 6.3.4-3 Average absorbance spectrum from rainbow trout "UV-absorbing" single cones in the presence of an alcohol radical containing material. Data points and solid line from Hawryshyn & Harosi. Dashed lines show theoretical Rhodonine (11) absorption (peak at 342 nm) and alcohol radical absorption (peak poorly defined but near 325-330 nm). Modified from Hawryshyn & Harosi, 1994.

Based on a set of templates, Hawryshyn & Harosi determined the peak absorption wavelengths for their rainbow trout were 365 ± 5, 434 ±5, 531 ± 6 and 576 ± 3 nm. However, the correction to their UV value would suggest a number closer to 342 nm and the very limited number of "red absorbing" chromophores (5) is of concern. They noted individual red absorbing chromophores at 615 and 596 nm.

Neither Douglas, et. al. or Hawryshyn & Harosi suggested that the UV chromophore was only the β absorption of a simple retinal as suggested by Stavanga and supported by Dyer (**Section 6.3.1**).

See **Section 5.7** for a discussion of the use of templates to fit measured data. Although the use of templates may be handy conceptually, and provide some rationale for the location of a set of peak responses, they do not describe the short wavelength skirts of an individual response well.

Interestingly, Naka & Rushton[78] in 1966 presented a variant of Dartnall's nomogram for three chromophores centered near 540, 610 and 700 nm. If there was an animal with such spectral channels, it is compatible with the theory developed here, but only in Vitamin A_2 based organisms. A possibility exists in that system for a chromophore, Rhodonine(3), that would correspond with the spectra labeled G_3 in that work.

[78]Naka & Rushton (1966) pg 538

The literature frequently struggles with the peak wavelength of the long wavelength chromophore. This is due primarily to the premature claim by Wald that the long wavelength chromophore exhibited a peak at 565 nm. The peak at this wavelength is an artifact due to his inadequate chromatic adaptation protocol. Peaks are frequently reported in the 565 -585 nm region due primarily to inadequate test protocols. If the absorption data from two photoreceptors is combined, a peak at a wavelength between 532 and 625 nm is to be expected. Similarly, if psychophysical data exhibits a peak near 565 nm, this is usually due to inadequate suppression of the M-channel during the tests. The resulting peak is due to the logarithmic summation of signals from both the M–channel and L–channel photoreceptors. This peak is known as the Purkinje Peak in the literature.

Parkyn & Hawryshyn have recently summarized the spectral peaks of the chromophores measured in salmonids. They were obtained by a variety of investigators using different techniques[79]. None of the techniques obtained measurements directly from the sensory neurons. Three of the eight teams showed peak L–channel response in the 620-630 nm region. The others showed long wavelength peaks at 580, 580-600, 560, 600, 598 & 576. Parkyn & Hawryshyn speak of pseudo-peaks without carefully defining the true L–channel peak.. However, the sources they quote show distinctive peaks at 610 nm and 625 nm. In fact, the three teams including Hawryshyn's name found peaks at 576, 620 & 625 nm. There are clear protocol problems in this area.

6.3.4.4.3 Interpreting the difference in half-width of spectra

A considerable difference is found in the half-width of spectra from measurements taken in-situ (**Figure 6.3.4-2**) and for aggregated suspensions (**Figure 6.3.1-2**). These differences are due entirely to the degree of aggregation of the specimens combined with the multilayer construction of the outer segments in the *in-situ* case. Aggregation results in a broadening of the resonance absorption spectra due to the Pauli Exclusion Principle.

6.3.4.5 Reviewing and interpreting the properties of rhodopsin

6.3.4.5.1 The reported reconstitutions of Rhodopsin

6.3.4.5.2 Definition of the spectrum of the chemical Rhodopsin

Striking inconsistencies are found in the literature concerning the actual absorption spectrum of Rhodopsin, particularly with respect to the visual portion of the spectrum. Wald and Hubbard both consistently give the peak absorption wavelength of Rhodopsin as near 500 nm. Hubbard & Kropf[80] specifically label the peak as 493 nm. *in-vivo* in Squid. Wald in many articles[81] places it at 498 nm in-vivo. Goldsmith, an ex-student of Wald, and many others plot and speak of that peak being near 530 nm (with a related peak for meta-rhodopsin near 500 nm). Furthermore, there is a great disparity in the shape of the absorption waveform near 500 nm when measured *in-vivo* and *in-vitro*. The *in-vitro* data of Hubbard & Kropf for Squid gives maximum absorption at 5°C of 493 nm and a FWHM of 100 nm in 2% aqueous digitonin.

Schwemer & Paulsen[82] may have presented the data to solve this dilemma. **Figure 6.3.4-4** shows the absorption spectra for the three chromophores of *Arthropoda*, drawn from their work, along with three separate but related waveforms. Those authors were careful to caveat their work as unfinished in the area of the related waveforms. Note the different sequence in the labeling between the upper and lower waveforms. This work proposes that the three absorption spectra at 345, 440, and 520 nm. are the anisotropic spectra due to the resonant conjugate molecules, defined as Rhodonine 11, 9 and 7 respectively, when in the liquid crystalline state and illuminated by light perpendicular to the surface of the liquid crystal--as found *in-vivo*. It also proposes that the three spectra near 495 are the equivalent isotropic spectra due to the conjugate, *but not resonant conjugate*, absorption of the molecules defined as Rhodonine 11, 9 and 7. These would be the spectra expected when the materials were illuminated by light parallel to the surface of the crystal *or* when the molecules are in dilute solution. Noting that individual excited molecules of Rhodonine are unable to absorb additional photons by dipole resonance is important. They may still be

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[79]Parkyn, D. & Hawryshyn, C. (2000) Spectral and ultraviolet-polarization sensitivity in juvenile salmonids: a comparative analysis using electrophysiology *J Exp Biol* vol. 203- pp 1173-1191 Table 1

[80]Hubbard, R. & Kropf, A. (1958) The action of light on Rhodopsin. *Proc. Nat. Acad. Sci*. vol. 44 pp. 130-139

[81]Wald, G. (1968) The molecular basis of visual excitation. Nature vol. 219, pg. 804

[82]Schwemer, J. & Paulsen, R. (1973) Three visual pigments in Deilephila elpe\nor (Lepidoptera Sphingidae) J. Comp. Physiol. Vol. 86, pp. 215-229

able to respond to photons by monopolar excitation. This monopolar excitation is likely to exhibit a spectral peak near 495 nm since it involves the same molecular excitation mode as for the isotropic absorption of the unexcited Rhodonine molecule.

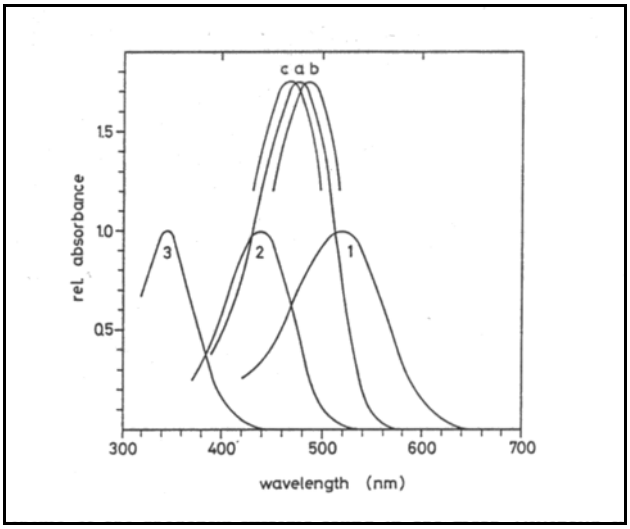

Figure 6.3.4-4 Proposed isotropic and anisotropic absorption spectra for *Arthropoda*. The peak absorptions for the chromophores are given as 345, 440 and 520 nm. With the corresponding upper waveforms listed as the corresponding acid *meta*-rhodopsins. From Schwemer and Paulsen, 1973

If the above proposal is correct, each photoreceptor of animal vision would exhibit a spectral characteristic described by one anisotropic absorption peak related to the dipole resonant conjugate of its specific chromophore and a related anisotropic monopole absorption peak near 495 nm. This second peak could be detected whether the molecule *was or was not* in its excited state relative to dipole resonance.

The important conclusion is that the spectral signature at or near 495 nm is due to the conjugate structure of the entire molecule. As such, it is shared by a great many retinoids including both the retinene and retinine (Rhodonine) families, and specifically including both the unexcited Rhodonines and the Rhodonines exhibiting dipole resonance excitation. It can be recorded isotropically for these materials when in dilute solution or the liquid crystalline state. Thus, this signature is not evidentiary of a specific molecule within the retinoid family and is likely to be associated with many molecules in the phthalein and carboxyl-ion families as well.

In spectrophotometric measurements of chromophoric material removed from a retina but not processed further to separate the individual chromophores, the measured peak near 495 nm would actually be a summation of the peaks of the three chromophores present. The result is likely to be a broader overall waveform depending on the exact content of the mixture. This condition appears to be consistent with the experimental literature and stresses the need for very careful experimental design before beginning laboratory work.

6.3.4.6 Collecting relevant data for the Rhodonines

The Rhodonines have yet to be isolated individually from biological material for the reasons developed in **Section 6.3.4.1**. A program is currently being developed to accomplish these isolations (See **Section 6.5.2**).

There are many precautions and recognitions required in the collection of spectral response data.

Such data is frequently collected at threshold conditions but it is also collected in a differential illumination mode. In both cases, using "lights" of different color to condition the retina or photoreceptor cell under test is common. Because of the considerable overlap between the spectra of the individual chromophores of vision, choosing the spectral characteristics of these "lights" carefully is important. Clearly, the optimum "light" is a single wavelength source causing minimal excitation of the chromophore and channel under test while maximally bleaching the other channels. In any case, the investigator must specify in detail the spectral characteristics of the "lights" used.

If the data is collected at the photoreceptor output or more proximal point in the signal chain, it must be appreciated that the output may represent a signal that has been manipulated. Its amplitude characteristics may not represent the characteristics of the chromophoric absorption process alone. Furthermore, if the output is collected at a point proximal to the computational optics region, the total signal may not represent merely a single sensory channel. It may be a function of the spatial location where the illumination is applied to the retina. This is clearly the case when the output is taken at such a level that only the b-wave, of the ERG, is recorded.

Investigators typically acquire difference spectra based on voltages and then manipulate the spectra using simple linear algebra. This is a highly questionable practice since such data already includes a logarithmic conversion of the original current signal waveform (assuming no other saturation or adaptation effects have occurred). The use of

linear algebra accounts for many of the difficulties found in discussions of the spectral characteristics of human vision.

Davson[83], based on the information of Hough, 1968, says that a given mesopic luminosity curve cannot be constructed by linear addition of the pure scotopic and photopic curves. While the statement is technically correct, it will be shown in **Chapter 15** that it does not apply to the biological vision system. The scotopic, mesopic, and photopic (along with the hyperopic) luminosity curves can all be constructed from the underlying chromophoric absorption spectra using logarithmic addition.

Tracing the many curves in the literature purporting to show the absorption characteristics of various biological samples to their ultimate experimental source is difficult. Some represent *in-vivo* measurements. Others relate to measurements of extracts in dilute solutions. The latter generally do not exhibit the resonant spectral absorption characteristics of the chromophores of vision. They typically show the molecular resonance at 495-502 nm and long associated with the label rhodopsin. Many measurements presented are not direct measurements but difference measurements. Some of these difference measurements rely upon the CIE luminous efficiency function as a reference. Others assume linear operation of the visual system. Such measurements must be examined closely for relevance to the actual situation. Much of the data, particularly the data for humans, is suspect because the state of adaptation of the system is not defined for each set of data collected.

Some of the reported data suggests that the spectral width (FWHM) of the individual chromophores is not constant. This can be due to two distinct parameters. The diameter of a photoreceptor disk can impact the spectral width of the associated chromophore due to the Pauli Exclusion Principle. The number of disks present can also impact the cumulative spectral width of the complete outer segment. Finally, the state of excitation of the chromophores as a group can impact the measured spectral width. If the spectra are measured by psychophysical means, the operating state of the amplifiers within the photoreceptor cells can also impact the results.

The literature often attempts to account for the cumulative absorption of the chromophores on multiple disks using the concept of "self shielding." This appears to be a poor concept. The photons absorbed initially are just as effective in exciting the neural system as any other photons. They do not shield the latter chromophores in any meaningful way.

6.3.4.7 Resolution of inconsistencies, & definitizing the terms used, in the literature

By reinterpreting the literature based on this work, the following conclusions can be drawn:

+The spectrum usually associated in the literature with the name rhodopsin is actually the isotropic spectrum of any of the Rhodonine chromophores (or many other retinoids). It may be measured in dilute solution or in the liquid crystalline state.

+The spectrum associated in the literature with porphyropsin is actually the anisotropic spectrum of Rhodonine(7).

+The spectrum associated with the name cyanopsin is actually the anisotropic spectrum of Rhodonine(5).

+Rhodonine(9) is the chromophore of the S-channel, frequently called the blue sensitive chromophore.

+Rhodonine(11) is the chromophore of the UV-channel in animals employing that chromophore.

As shown in Chapter 17, the broadband spectra associated with the scotopic visual function is actually the (exponentially based) sum of the S- and M-channels in the visual system. Similarly, the photopic visual function is the (exponentially based) sum of the S-, M-, and L-channels. The scotopic visual function is a subset of the photopic visual function due to the unique "square law" aspect of the L-channel.

6.4 Non-spectral data for the retinoids

6.4.1 Non-spectral data from non-biological sources of retinoids

[83]Davson, H. (1990) Physiology of the eye NY: Pergamon Press pg. 442

Kofler & Rubin have summarized a large range of non-spectral data related to the retinenes and their isomers[84]. However, this database is quite old. Recent developments in chromatography have allowed the identification of complex chemicals based on comparison with a known sample. However, there are no known and verifiable samples of the chromophores of vision at this time. Until recently, there have been no truly quantitative tests with sufficient specificity to identify a specific member of the retinoid family because of the complexity of (and redundant structures in) these molecules. The recent change has involved the development of Nuclear Magnetic Resonance (NMR) techniques and X-Ray crystallography techniques aimed at analyzing crystalline forms. These techniques can also be used to analyze the liquid crystalline forms of the retinoids.

6.4.1.1 Simple bench tests

6.4.1.1.1 Tests stable with time

Ref: Wolf & Johnson (1960)

6.4.1.1.2 Transient tests--Carr-Price reaction family

Carr and Price developed a colorimetric test designed to assay for retinol in 1926. It was intended to be quantitative but it showed little precision. The tests utilized antimony trichloride and involved test solute exhibiting a transient color. The maximum wavelength of the color was believed to be characteristic of the retinoid present, retinol, retinal, or retinoic acid (essentially all of the retinoids known at that time). The test was redesigned later to utilize a variety of Lewis acids, most frequently trifluoroacetic acid and trichloroacetic acid.

The test was used by Wald, et. al. to confirm that retinol, when combined with opsin, was the chromophore extracted from animal photoreceptors. Such a test result would not be accepted today. It has not been shown to separate the retinenes from the retinines. What result would be obtained in testing a carboxyl-ion system, such as a Rhodonine, is not clear. Such systems contain both an aldehyde and alcohol ligand.

It is now recognized that the test is not specific for the retinene family of retinoids and cannot be used as confirmation of any chemical beyond its classification as a retinoid. In fact, how the mechanism of the test is still not understood. Frolik & Olson[85], writing in Sporn, et. al. say: "The interaction of Lewis acids with retinol initially involves the extraction of the hydroxyl moiety, leaving a retinylic cation (λ_{max} 586 nm), which forms a complex with $SbCl_3$ at the C-4 position (λ_{max} 619 nm) **or** at the C-15 position (λ_{max} 586 nm). De-protonation of the retinylic cation **or** dissociation of the latter two complexes gives anhydroretinol." They go on for a page to discuss what **may** happen and then give a long paragraph on the disadvantages of the test, including:

1. the accurate measurement of the transient blue color complex is not always easy.
2. the absorption of the complex has been measured at a specific time after mixing, at the peak or "pause" point, or by extrapolating values obtained at given times back to the time of mixing.
3. the method is not particularly specific, inasmuch as **most polyenes**, including the carotenoids, **react with these reagents** to give colored complexes.
4. For some unknown reason, not all batches of trifluoroacetic acid and trichloroacetic acid prove to be suitable for color development even though they appear to be pure and anhydrous.

Kofler & Rubin[86] further point out that:

1. the test does not distinguish between isomers of a retinoid
2. Since Beer's law does not hold with large changes in concentration, calibration curves are necessary.
3. The reactions are not specific for Vitamin A (i.e., retinol) and give positive results with many other substances in particular the polyenes.

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[84]Kofler, M. & Rubin, S. (1960)Physichemical assay of Vitamin A and related compounds *In* Harris, R. et. al. Vitamins and Hormones Volume 18 NY: Academic Press pp 315-339

[85]Frolik, C. & Olson, J. in Sporn et. al. (1984) 1st edit. pg. 218

[86]Kofler, M. & Rubin, S. in Vitamins and Hormones (1960) Vol. 18 edited by Harris, R. & Ingle, D. NY: Academic Press pg. 319-329

4. The active species are traces of $SbCl_5$.

Machlin[87] updates the material in Kofler & Rubin to stress the Lewis Acid aspects of the test. He essentially rephrased Kofler & Rubin's remarks in his handbook of 1984.

Based on the above commentary, the best that can be said is that Wald justified his deduction that Vitamin A (retinol) was the chromophore of vision on the fact that animals needed to ingest Vitamin A (usually via the carotenes) in order to maintain healthy vision, particularly at night, and a Carr-Price test indicated the presence of a polyene which he assumed had to be Vitamin A (retinol). His facts are correct as far as they go. However, these facts only show Vitamin A is a chromogen.

The Carr-Price type of colorimetric test is no longer used in precise scientific investigations and cannot be relied upon in vision research. Machlin stresses the utility of the absorption spectrum in the ultraviolet region as a method of identifying Retinol and its close relatives. However, as shown above, the absorption peak at or near 325 nm. is not characteristic of just the retinenes. It is characteristic of a wide variety of polyenes containing a alcohol ligand. Such a test can only be used in conjunction with other tests to narrow the identity of the unknown material.

6.4.1.1.3 Reaction tests–with dichloropropanol

A test used to confirm the presence of retinol involves reacting the suspected material with dichloropropanol in a solution of chloroform. The residue of the reaction exhibits a skewed absorption in the visual region centered on 550 nm[88]. The test is confirmatory but not diagnostic. To obtain a molecular absorption at this wavelength suggests that the retinol reacts with the dichloropropanol to form a retinol chloride. The heavy atoms of chlorine and oxygen are sufficient to cause a molecular resonance. Since chlorine is heavier than a second oxygen atom, retinol chloride would be expected to have a peak absorption wavelength beyond the 500 nm found for a molecule of Rhodonine containing two oxygen atoms.

If dichloropropanol was to react similarly with Rhodonine, the resulting molecule might show a complex molecular absorption spectrum unless it merely replaces one of the oxygens with a chlorine. In that case, a peak molecular absorption near 550 nm would be expected.

6.4.1.1.4 Circular Dichroism

Dichroism has been used to attempt to differentiate various species of chromophores and chromogens in vision[89]. However, the technique is marginally useful, particularly *in-vivo* and/or when the material is in the liquid crystalline state. Under either of these conditions, the dichroic parameters of the material may be influenced by other structural or substrate parameters.

Shichi, 1983 (pp 87-89) includes a few remarks on the subject and provides a few references. No analytically useful and unequivocal data has been located based on this technique.

6.4.1.1.5 Colorimetric methods

Until recently, the identification of the chromophoric material has generally relied on colorimetric methods dating from the 1920's. The initial tests used antimony trichloride and a solvent such as ether. More recent tests have used a Lewis Acid and a similar solvent. The original test was to identify Vitamin A in foods and body tissue, etc. Although the method is frequently described as quantitative, it involves the observation of a transient state exhibiting a "blue" color. The spectrum of the color is quite broad and generally has a long wavelength limit between 555 and 620 nm. The test is not specific; a flash being observed for any natural retinoid in the literature, and presumable many man-made retinoids. Sporn has provided a good summary of the results by different investigators and appropriate references. The spread in results is quite enlightening.

Sporn includes two pages of discussion about what is believed to occur in these tests. The reading is not very

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[87]Machlin, L. (1984) Handbook of vitamins. NY: Marcel Dekker, Inc. pg. 9

[88]Henry, R. Cannon, D. & Winkelman, J. *eds*. (1974) Clinical Chemistry, 2nd ed. NY: Harper & Row Pg 1375

[89]Yoshizawa, T. & Shichida, Y. (1982) Low-temperature circular dichroism of intermediates of rhodopsin. *Meth. Enzymol*. vol. 81, pp 634-641

satisfying. As an example, on page 218, "The interaction of Lewis acids with retinol initially involves the extraction of the hydroxyl moiety, leaving a retinylic cation (λ_{max} 586 nm.) , which forms a complex with $SbCl_3$ at the C-4 position (λ_{max} 619 nm.) or at the C-15 position (λ_{max} 586 nm.). Since all of the Rhodonines are retinoids exhibiting a hydroxyl moiety and a region around the C-4 position identical to all known natural retinoids, they will all satisfy this test. **Clearly, the colorimetric tests developed from the original Carr-Price procedure cannot be used to specify the retinoid found in the disks of the photoreceptors of the animal eye.** It is not specific to retinol, or any other retinene. It is not even certain that the tests are specific to the retinoid family. Machlin[90] also discusses the shortcomings of this test in detail.

6.4.1.1.6 Fluorescence

Fluorescence has not provided an important analytical test for the chromophores of vision. Retinol and its naturally occurring esters are known to fluoresce in the laboratory environment, although many of its Vitamin A_2 related cousins do not. This is particularly true of the cross-conjugate molecules containing two oxygen atoms.

The Rhodonines, the actual chromophores of vision do not fluoresce because of their unique quantum chemical arrangement and the special properties of the oxygen atom. The Rhodonines are dipole molecules that do not normally fluoresce. The oxygen atom is almost unique in its electronic arrangement. Its unpaired electrons are in the triplet, instead of the singlet state. This state is not conducive to fluorescence following excitation. Finally, *in-vivo*, the excited molecules are normally de-excited by the neural system before they have a chance to fluoresce.

The Rhodonines do not fluoresce *in-vitro* because of the initial excitation of the n-electrons into a triplet state. Decaying by fluorescence into the readily available singlet ground states associated with most of the atoms of the molecules is not easy for these electrons.

The observation in Sporn, et. al[91]. that the combination of the serum retinoid binding protein and the retinoids found in the blood stream does fluoresce is interesting because it may aid in determining precisely when the retinoid is converted into a Rhodonine.

6.4.1.1.7 Ultraviolet absorbancy

All of the naturally occurring retinoids, and presumably many man-made retinoids, absorb strongly at 325-375 nm. in the ultraviolet. As shown in **Section 6.3.1**, such absorption is characteristic of the alcohol and aldehyde ligands associated with retinoids. These absorptions are not specific to the chromophores or chromogens of vision.

6.4.1.2 Chromatography

Chromatography offers advantages in the separation of the chromophores of vision due to their different chemical formula but very similar molecular weights. Since they are temperature sensitive organics, the method of choice is liquid chromatography. The technique includes many variants shown to be useful in differentiating various types of compounds. Both straight-phase and reverse-phase techniques are used in both conventional and high pressure liquid chromatography (HPLC). A good explanation of the liquid chromatography technique is available in Ewing[92]. Results of a test sample containing many components found in the electrostenolytic mechanisms of a neuron are presented in his figure 20-10. It is the result of using the ion-exchange approach to liquid chromatography. Livrea & Packer develop the options associated with the use of chromatography with the retinoids[93].

The differentiation depends more on the electrical polarity of the molecules than on any other individual factor. While of great value, use of the various techniques requires a great deal of experience. Based on this experience, the experimenter frequently optimizes the procedure in subtle ways to provide optimal graphic results.

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[90]Machlin, L. (1984) Handbook of Vitamins. NY: Marcel Dekker, Inc. pg. 9

[91]Sporn, M. Roberts, A. & Goodman, D. (1994) The Retinoids, 2nd ed. NY: Raven Press, pg 201

[92]Ewing, G. (1975) Instrumental Methods of Chemical Analysis. NY: McGraw-Hill. Chapter 20 *In my personal library.*

[93]Livrea, M. & Packer, L. (1993) Retinoids. NY: Marcel Dekker, Inc. chapter 37

There is a great deal of chromatography data on the retinenes (and other simple retinoids). However, this data is largely irrelevant to the visual process. Its value is in pharmacology and nutrition. Chapter 3 of Sporn, et. al. provides a broad review of the available results in the literature[94]. Volume 67 of Methods in Enzymology also provides considerable data. McCormick, Napoli & DeLuca provide a graph of the results obtained for retinoic acid, retinol, retinal and retinyl palmitate[95]. The number of test parameters listed in the caption suggests the experience needed to obtain the desired results. The materials do not appear in order of molecular weight. They also note that "The detailed mechanism of retention in reverse-phase HPLC is not understood." They present their data using an unusual horizontal scale. The scale is in units of volume of flow through the column rather than the more conventional time before the component reaches a typically ultraviolet sensitive photodetector. Van Kuijk, et. al. provide a similar graph extended to include retinal-O-ethyloxime[96]. Barua has provided a graph of a variety of retinoids (other than the chromophores of vision) described as standards and based on a complex form of liquid chromatography[97]. Groenendijk, et. al. have provided examples of varying one parameter at a time to optimize the resulting graphs plotted as a function of time[98]. Taylor & Ikawa present a large amount of data on many carotenoids and include a large glossary of carotenoids (both trivial and semi-systematic names)[99]. However, no data is presented for the Rhodonines, the chromophores of vision.

The Rhodonines, the actual chromophores of vision, are all used in the all-*trans* form in vision. Thus, their potential stereochemistry is not important. However, their detailed steric structure (the location of the second oxygen) does vary to provide sensitivity to different wavelengths. It is the difference in polarity, and possibly the adsorption at sites within the chromatography column, that should be definable with liquid chromatography techniques.

6.4.1.3 Radiation Chemistry RESERVED

A table of available neucleotides used in biological research, and their lifetimes, is available in Ewing[100].

6.4.1.4 Mass Spectrometry

Mass spectrometry offers an excellent method of determining the precise constituents of an organic material. Groenendijk, et. al. have provided useful data for both syn- and anti- all-trans-retinaloxime as an example[101]. They also provide NMR data on the same compounds for comparison.

6.4.1.5 X-Ray crystallography

Dunitz has provided a theoretical reference on X-ray analysis of organic molecules[102]. He also references the

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[94]Sporn, M. Roberts, A. & Goodman, D. (1994) The Retinoids, 2nd ed. NY: Raven Press. Chapter 3

[95]McCormick, A. Napoli, J. & DeLuca, H. (1980) High pressure liquid chromatography of Vitamin A metabolites and analogs. *Methods in Enzym*. vol. 67, pp 220-233

[96]Van Kuijk, F. Handelman, G. & Dratz, E. (1985) Op. Cit. pg 245

[97]Barua, A. (1990) Analysis of water-soluble compounds: glucuronides. *Methods Enzymol*. vol. 189, pp 136-145

[98]Groenendijk, G. Jansen, P. Bonting, S. & Daemen, F. (1980) Analysis of geometrically isomeric Vitamin A compounds. *Methods in Enzym*. vol. 67, pp 203-220

[99]Taylor, R. & Ikawa, M. (1980) Gas chromatography, gas chromatography– mass spectrometry, and high pressure liquid chromatography of carotenoids and retinoids. Meth. Enzym. vol. 67, pp 233-261 *with Groenendijk set in personal file.*

[100]Ewing, G. (1975) Op. Cit. pg 457

[101]Groenendijk, G. DeGrip, W. & Daemen, F. (1979) Identification and characterization of syn- and anti-isomers of retinaloximes. *Anal. Biochem*. vol. 99, pp 304-310

[102]Dunitz, J. (1979) X-ray Analysis and the Structure of Organic Molecules. Ithaca, NY: Cornell Univ. Press

Cambridge Crystallographic Data Centre as a source of information[103]. Kofler & Rubin have provided excellent examples of the x-ray patterns of a variety of retinoids, but not for the chromophores of vision[104]. Hamanaka, et. al. have provided a numbering scheme for all of the atoms (including hydrogen) in retinal[105]. Unfortunately, many hydrogen atoms have a different designation than their associated carbons. Furthermore, the nomenclature does not provide for the hydrogen associated with the oxygen of retinol. An alternate notation will be defined in **Section 6.5.2.4**. They do provide considerable data on bond angles.

The crystalline structure of the retinals has been determined by Gilardi, et. al.[106]. They presented the following data based on X-ray analysis of retinal crystallized from petroleum ether:

Material	Space group	*a*	*b*	*c*	β	molecules per cell
All-*trans* retinal	$P2_1/n$	14.961	8.279	15.316A	104.87°	4
11-*cis* retinal	$P2_1/c$	7.540	10.666	22.102A	95.19°	4

They say their data agrees with Kuwabara et. al.[107]. The work also confirmed the assumed structure arrangement of retinal; the all-*trans* version being planar with the six-member ring inclined to the plane of the chain by the torsional angle given by the 5-6-7-8 segment. This angle measures 59°. The 11-cis retinal was found to be non-planar, in fact it includes two breaks in the planarity. One break associated with the 10-11 or the 11-12 bond and a second break associated with the 12-13 bond. As a result, the C(13)=C(14) and the C(15)=O double bonds are no longer parallel to the conjugated double bonds of the other segment. They provided details on the bond angles and bond lengths for the material. They also pointed out that the 11-cis isomer was unstable in solution and rapidly isomerized at room temperature.

Hamanaka, et. al have provided detailed information on the structure of all-trans-retinal$_1$ [108].

Okada, Le Trong, et. al. have provided recent data on three-dimensional crystals of bovine rhodopsin[109]. They took care to protect the crystals from light because they observed rapid deterioration in the structure of the crystals under visible illumination. They did not degrade under dim red light.

6.4.1.6 Nuclear Magnetic Resonance (NMR)

NMR is currently the method of choice for the determining the detailed chemical structure of molecules up to a molecular weight of 400-1000, provided relatively pure samples are available. The technique depends on a quantum level asymmetry in the electronic structure of atoms within molecules of interest. Chamberlain has provided a good

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[103]Kennard, O. Watson, D. et. al. (series) Molecular Structures and Dimensions, Bibliography. Utrecht: Bohn, Scheltema & Holkema

[104]Kofler, M. & Rubin, S. (1960) Op. Cit. pp 329-333

[105]Hamanaka, T. Mitsui, T. Ashida, T. & Kakudo, M. (1972) The crystal structure of all-trans-retinal$_1$ Acta Cryst vol. B28, pp 214-222

[106]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

[107]Kuwabara, M. Hamanaka, T. & Mitsui, T. (1968) J. Phys. Soc. Japan, 25, 920

[108]Hamanaka, T. Mitsui, T. Ashida, T. & Kakudo, M. (1972) The crystal structure of all-trans-retinal$_1$, *Acta Cryst*. vol. B28, pg 214-222

[109]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

tutorial on NMR techniques[110]. Becker & Fisk have provided a good discussion of the use of NMR techniques in biological research[111]. Wray has provided a similar discussion of the technology[112]. **Figure 6.4.1-1** provides some basic parameters related to the available nuclei.

Nucleus	Spin	Magnetogyric Ratio MHz/T	Sensitivity	Natural Abundance %	Relative Sensitivity
1-H	1/2	42.57	1.00	99.98	100
13-C	1/2	10.71	1.59×10^{-2}	1.11	1.76×10^{-2}
14-N	1	3.08	1.01×10^{-3}	99.63	1.01×10^{-1}
15-N	1/2	4.31	1.04×10^{-3}	0.37	3.85×10^{-4}
17-O	**5/2**			**0.004**	**0.001**
19-F	1/2	40.06	0.83	100	83
23-Na	3/2	11.26	9.25×10^{-2}	100	9.25
31-P	1/2	17.23	6.63×10^{-2}	100	6.63

Figure 6.4.1-1 Magnetic nuclei of significance in vision research. Modified from Wray, 1991.

Although no NMR spectra are available for the Rhodonines, there are a variety of spectra for the various forms of Vitamin A. Because of the specificity of the technique, an expert in the technique can describe the putative spectra for each Rhodonine in considerable detail based on the available Vitamin A spectrum. An expert is required because of the differential methods used to optimize the technique. The scope of the technique is illustrated in the next section. Examples of the signature definition available and the definition of the expected spectra of the Rhodonines are presented following that introductory material.

Nuclear Magnetic Resonance (NMR) techniques are powerful tools in the definitive description of complex molecules. Since 1980 Nuclear Magnetic Resonance spectroscopy has been the method of choice for examining uncommon retinoids and establishing unequivocally their structure. A variety of techniques within the category of nuclear magnetic resonance spectroscopy are available.

By selecting the appropriate test conditions, every nucleus in the molecule can be located and explicitly identified. Describing the association of every nucleus with its nearby neighbors is also possible. For a complete analysis, all of the hydrogen atoms must also be enumerated. Hamanaka, et. al[113]. have provided a numbering system that is consistent with that of Karrer. Locating the individual hydrogen nuclei usually involves NMR using a nominal frequency of 200-250 MHz. However, NMR at an oscillator frequency of 60 or 100 MHz is more common due to the simpler and cheaper equipment involved. These are the center frequencies commonly used in NMR to accentuate different details in the spectrum of the same material. By combining data collected using both excitation oscillator frequency ranges, every structure in the molecule can be specifically identified.

6.4.1.6.1 Background

The Becker & Fisk paper provides a simplified discussion of the mechanisms creating NMR spectrums and a list of more complete sources. They also discuss the relative application and importance of the different species used in biology, hydrogen, carbon, phosphorous, oxygen and nitrogen. Applications of NMR in biology have been

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[110]Chamberlain, N. (1974) The practice of NMR spectroscopy, with spectra-structure correlations for hydrogen-1 NY: Plenum Press

[111]Becker, E. & Fisk, C. (1987) NMR: Physical principles and current status as a biomedical techniques, *In* Cohen, S. *ed*. Physiological NMR Spectroscopy: From Isolated Cells to Man. NY: *New York Acad. Sci*. vol. 508, pp 1-9

[112]Wray, S. (1991) Biomedical muclear magnetic resonance spectroscopy and transport. In Yudilevich, D. Deves, R. Peran, S. & Cabantckik, Z. Cell Membrane Transport NY Plenum Press Chap. 15

[113]Hamanaka, T. Mitsui, T. Ashida, T. & Kakudo, M. (1972) The crystal structure of all-*trans*-retinal$_1$ Acta. Cryst. vol. B28, pp. 214-222

expanding at an exponential rate since the 1970's. The species of most interest in vision are hydrogen, an isotope of carbon, and an isotope of oxygen.

Proton-based NMR studies

Shriver, Mateescu & Ababrahamson have performed a proton NMR study of a protonated 11-cis retinal Schiff base to provide an analog of rhodopsin[114]. Both N-(all-trans- and N-11-cis retinylidene)propylimine acidified with a fivefold excess of trifluoroacetic acid at -55 Centigrade were explored using 100 MHz excitation.

Rowan, Warshel, Sykes & Karplus have provided NMR spectra for all-*trans*-retinal at 100 MHz in solution[115]. These spectra provide more resolution of the individual spin components than at 60 MHz. The signatures associated with C5, C9 & C13 are particularly well defined in **Figure 6.4.1-2**. Rowan, et. al. also provide data on the chemical shifts, the coupling constants and other NMR parameters related to all-*trans*-retinal.

Carbon-13 NMR studies

Becker, Berger, et. al. have studied the carbon-13 NMR of several retinal isomers in solution[116]. They provide tabulations and correlation diagrams showing the impact of isomerization on the NMR spectrums of the retinals.

Shriver, Mateescu & Ababrahamson have performed a carbon-13 NMR study of several protonated 11-cis retinal Schiff bases in solution to provide an analog of rhodopsin. They also provide a comprehensive bibliography.

Stothers has examined the carbon-13 NMR spectroscopy of the constituents of vitamin A, the quinine ring and a variety of terpenes[117]. He also addresses some subtle features associated with biological NMR measurements. Rowan & Sykes have also presented carbon-13 NMR of the simple retinenes, and noted some potential

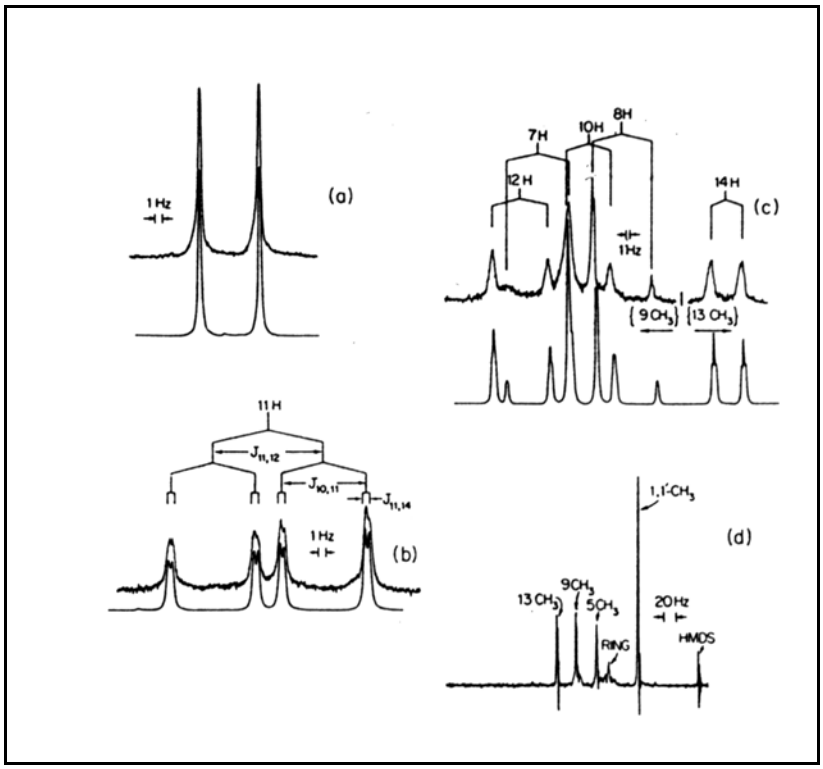

Figure 6.4.1-2 Observed and simulated 100 MHz nmr spectra of all trans-retinal. See Rowan, et. al. 1974 for interpretation.

[114]Shriver, J. Mateescu, G. & Abrahamson, E. (1979) A proton and carbon-13 nuclear magnetic resonance stereoscopy study of the conformation of a protonated 11-cis-retinal Schiff base *Biochem* vol. 18, no. 22, pp 4785-4792

[115]Rowan, R. Warshel, A. Sykes, B. & Karplus, M. (1974) Conformation of retinal isomers *Biochem* vol. 13, no. 5, pp 970-980

[116]Becker, R. Berger, S. Dalling, D. et. al. (1974) Carbon-13 magnetic resonance investigation of retinal isomers and related compounds *J. Amer Chem Soc*. vol. 96, pp 7008-7014

[117]Stothers, J. (1972) Carbon-13 NMR Spectroscopy NY: Academic Press 434-438

inconsistencies with prior work[118].

Oxygen-17 based studies

There has been less work in the biology of vision using the Oxygen-17 isotope. The parameters of the isotope are available at http://www.pascal-man.com/periodic-table/oxygen.html. Recent activity has been vigorous at the Univ. of Illinois at Urbana-Champagne and at Emory University under Morokuma. Some work related to vision has been included in these studies.

N-retinylidene spectrums

Shriver, Mateescu & Ababrahamson have provided peak absorption values (ε_{max}) for a variety of Schiff bases (using propylimines) that are analogs to rhodopsin. Their results show the variety of wavelengths (shorter than 482 nm) available depending on the specific solvents used.

Recently a method of examining membrane proteins as solids has been developed. Watts, et. al. discuss the highlights and difficulties of this method[119]. The technique offers many new capabilities, including determining certain angles associated with the molecular structure. The method is compatible with proteins of high molecular weight, however, interpreting the results is complicated because of all of the nucleotides present. Grobner, Choi, et. al. presented the follow-up paper on this technique[120]. This work continues to show the long axis of the retinal parallel to the surface of the disk membrane and perpendicular to the incident light.

In 2000, Palczewski, et. al. presented a detailed study of crystallized rhodopsin using solid state NMR was presented[121]. The crystals were recovered from bovine micelles by solubilization and centrifugation, were formed by vapor deposition, and were then soaked in mercury acetate for a period of months. No details of the changes resulting in the mercury derivative were provided. A limiting resolution of 2.8 Angstrom was stated. The crystals formed contain two molecules in an asymmetrical pairing. They provided a three-dimensional drawing of the seven helices and used a computer program to introduce the retinal component. Additional drawings show particular views of the molecule. Although they speak broadly of the conventional wisdom of the photoexcitation process, speaking of wavelength shifts due to potential interaction between the retinoid and various amino acids, they provide no data on the absorption spectrum for any of their crystals. It is noted here that the retinal is completely enclosed by the seven helices. It has little access for extraction or reinsertion during any proposed isomeric shift and removal for re-isomerization outside of the molecule. The degree of access was discussed by Stenkamp, Teller & Palczewski in 2002[122]. They also provided additional perspective views of the complete molecule, including the short helix number VIII. Their analyses showed that the individual helices were not straight as generally assumed but exhibit kinks of as much as 30 degrees. They report as follows. “The retinal is located between the helices towards the extracellular side of the protein and is completely buried within the protein. The transmembrane helices block ready access to the hydrophobic region of the bilayer, and the β strands and extracellular loops shield the chromophore from the aqueous environment on the extracellular face. The binding site is some distance from the cytoplasmic surface and amino acid side chains from the transmembrane helices block access to that surface.” They go on. “The retinal site is not accessible to the hydrophobic environment within the membrane either. No gaps between the helices present a path for the retina to exit the binding site. Given the need for ligands to move in and out of the binding site and for retinal to do likewise in the regeneration of the photopigment, alterations in the published structure must occur in the functional protein.” Translating, our present knowledge of the structure of the rhodopsin molecule does not support

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[118]Rowan, R. & Sykes, B. (1974) A carbon-13 nuclear magnetic resonance study of the visual chromophores and model compounds *J. Amer. Chem. Soc*. vol.96, no. 22, pp 7000-7008

[119]Watts, A. Burnett, I. Glaubitz, C. et. al. (1999) Membrane protein structure determination by solid state NMR *Nat. Prod. Rep.* vol. 16, pp 419-423

[120]Grobner, G. Choi, G. Burnett, I. et al. (1998) Photoreceptor rhodopsin: structural and conformational study of its chromophore 11-cis retinal in oriented membranes by deuterium solid state NMR. *FEBS*, vol. 422, pp 201-204

[121]Palczewski, K. Kumasaka, T. et. al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor Science vol. 289, pp 739-745

[122]Stenkamp, R. Teller, D. & Palczewski, K. (2002) Crystal structure of rhodopsin: a G protein-coupled receptor *ChemBioChem* vol. 3, pp 963-967

the concept of re-isomerization of the retinoid outside of the molecule.

6.4.1.6.2 NMR spectrum of the retinenes

The NMRs of several carotenes are illustrated in **Figure 6.4.1-3** from Sebrell & Harris[123]. The image is a bit complex; it consists of the spectrum of the full molecule in the center. The spectra on each side only include the area between 40 and 140 Hertz. Note how each atomic nucleus of the molecule can be specifically identified. The subtle differences in the molecular structure of α-, β-, and γ-carotene (in $CDCl_3$) are shown. Note the separation of the spectrum due to $CH_3$18 and $CH_3$18' in the left spectrum compared with the center because of the relocation of one bond in the primed β-ionone ring. Similarly, note the separation in the spectrum due to $CH_3$16,17 and $CH_3$16',17' in the right frame compared with the center because of the extra bond in the primed β-ionone ring. The magnetic field was adjusted for a nominal 60 MHz spectrum.

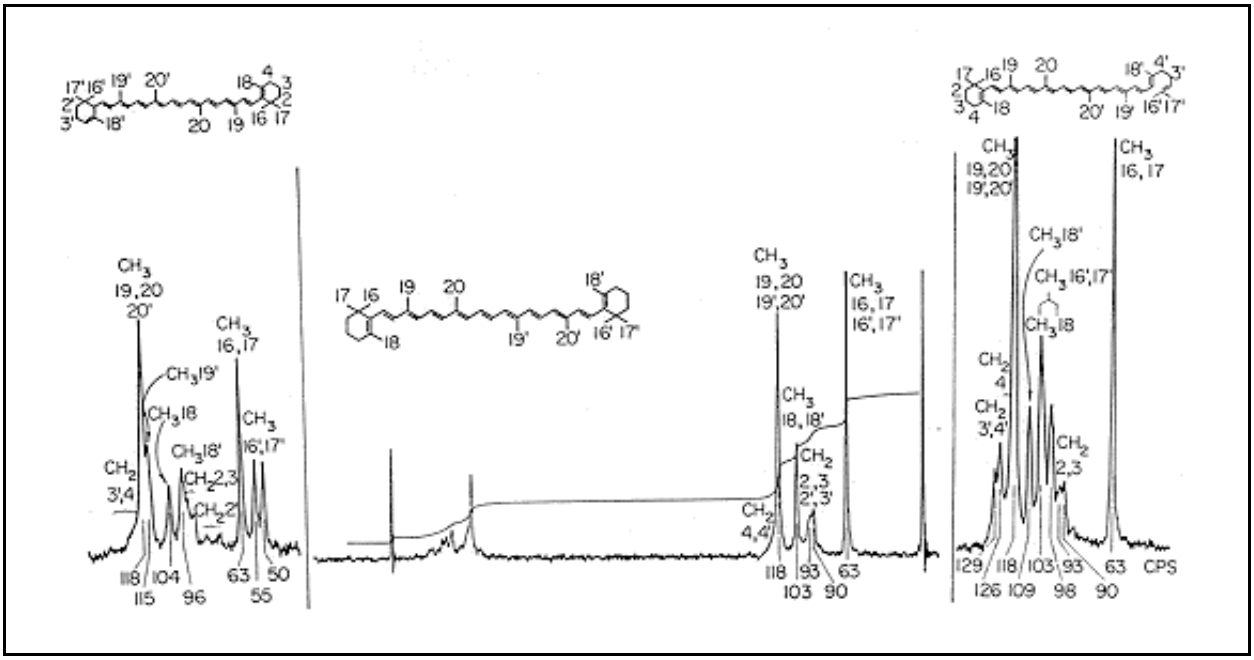
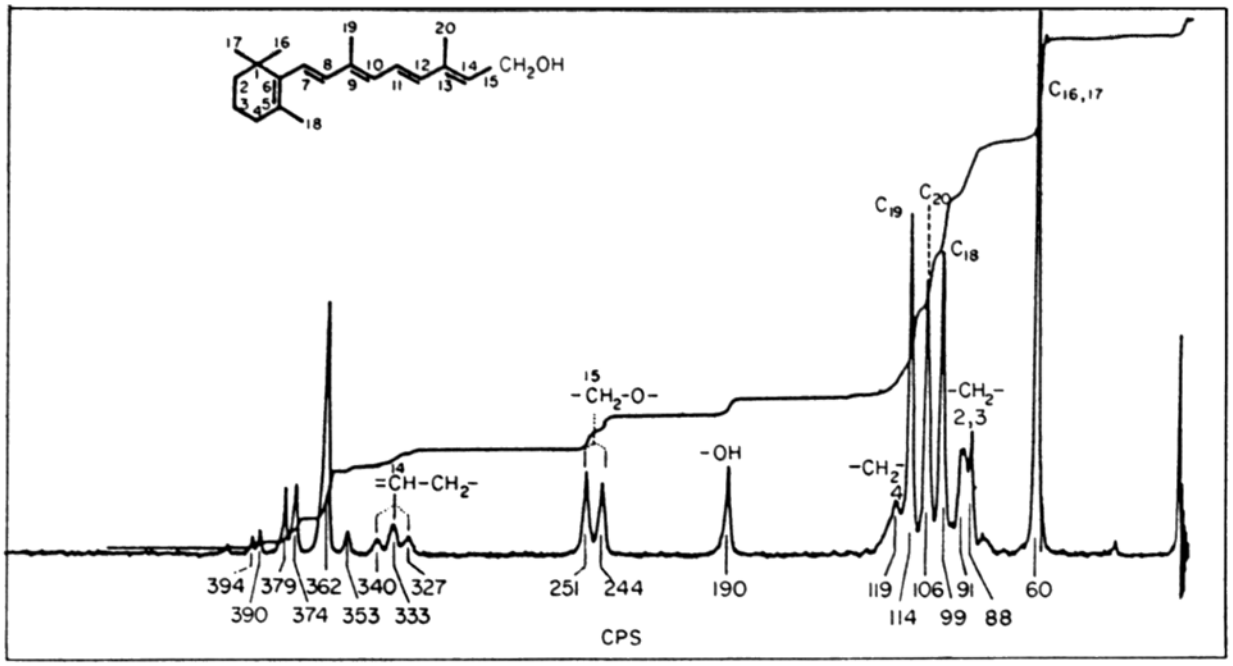


Figure 6.4.1-3 Nuclear magnetic resonance spectra (60 MHz) of α-, β-, & γ-carotene (in $CDCl_3$). Labels 19' & 20' appear to be interchanged in this figure. From Sebrell & Harris, 1967.

Figure 6.4.1-4 also from Sebrell & Harris, provides more detail for all-*trans*-Vitamin A (also in $CDCl_3$). Both the differential and cumulative response are shown relative to the zero reference on the right. Sebrell & Harris identified the individual features in the table below the figure. The spectral line associated with the pair of methyl groups, C_{16} and C_{17} are seen clearly at 60 Hz with the other methyl groups clearly shown at C_{18}, C_{19}, & C_{20}. If the ring structure is rotated in the symbol at upper left to place C_{18} at the top of the figure, the molecular presentation is in better agreement with the NMR signature. Note the resonance of the -OH group by itself at 190 Hz and the resonance of the -CH_2-O- structure, associated with carbon 15, in the region of 244-251 Hz. Note also the additional detail at higher frequencies. Kofler, et. al. did not define the other lines in the spectrum individually. It is likely that C8, C10, and C12 signatures are located in the complicated structure between 353 and 394 cycles. Their specific locations are not important here. What is important is to determine what methyl groups would not be present in the

[123]Sebrell, W. & Harris, R. (1967) NY: Academic Press pg. 24-76

various putative Rhodonines.



Frequency	Carbon Location	Group	Frequency	Carbon Location	Group
94	C-2	------- groups in the β- ionone ring	60	C-16	Methyl groups @ C-1 on the ring
88	C-3			C-17	
119	C-4				
327-333-340	C-14	Isoprenyl group	99	C-18	Methyl groups on the conjugate chain
244-251	C-15	carboxyl group	114	C-19	
			106	C-20	

Figure 6.4.1-4 NMR spectrum (60 MHz) of all-*trans*-vitamin A (in $CDCl_3$). Tabulation describes the major groups generating the responses. The similarity of the response due to C18, C19 & C20 suggests the stick model should be rotated to show C18 at the location of C16-C17. See text for more details. From Sebrell & Harris, 1967.

The 60 MHz NMR spectrum for all-*trans*-Vitamin A (in $CDCl_3$)[124] shows clearly every individual group in the molecule. However, all groups are not listed in the tabulation within the figure. C8, C9 & C10 are associated by only one hydrogen nucleus when the conjugation of the molecule is ignored. C8, C9 & C10 are represented by the splits at 353-361, 374-379 and 390-394 but not in any specific order. These splits appear to illustrate the resonant nature of the overall structure. Note the signatures of C18, C19 & C20. Grouping of these signatures suggests that their quantum-electronic environment are similar. This would suggest that C18 would be more appropriately shown with the b-quinone ring rotated about the C6-C7 bond to the top of the carbon backbone. Since this molecule is not

[124]Sebrell, W. Jr. & Harris, R. (1967) The Vitamins. NY: Academic Press pg. 24

planar (there is a 59-degree out of plane rotation at the C6-C7 bond), the alternate description remains compatible with the actual molecule. The signature at 362 CPS was not discussed by the authors. The signature, and its expected source, is documented at 337 CPS in the following figure by Kofler, et. al.

Note that for the Rhodonines, the C-19 group would be missing for Rhodonine(9) and the C-18 group would be missing for Rhodonine(5); other groups indicative of hydroxyl or aldehyde would appear. Unfortunately, the location of a doubly bound oxygen, =O, cannot be defined using 60 MHz excitation.

As shown below, Rhodonine (7) and Rhodonine(11), would exhibit additional groups in their NMR similar to the groups shown at 190 & 244-251 cps. It should be noted that Sporn says: "Owing to high background noise, it is difficult to use 13-C-NMR spectroscopy directly for the investigation of the pigments rhodopsin and bacteriorhodopsin." This may be due to the inadequate separation of the individual chromophores from the retinal extract and the assumption that all of the retinoid in the extract is of a single molecular structure. Shriver[125] has reported a spectrum for rhodopsin using a retinaldehyde labeled with the 13-C isotope in position C-14 or C-15. However, there are clearly difficulties with this spectrum;

+ it has a very low signal-to-noise ratio

+ the sample is that of an "enriched" rhodopsin

+ the labels assigned to the components by Shriver do not agree with the labels assigned by Kofler and by Sebrell. The differences are gross.

To obtain a meaningful NMR for the chromophores of vision, it is necessary to insure that only one chromophore is present at a time. This probably requires the use of re-crystallization techniques, and the use of the techniques of Kofler and/or Sebrell to insure adequate component definition.

6.4.1.6.3 NMR spectrum of the retinaloximes

Groenendijk, et. al. have provided useful NMR data on two stereo-isomers of retinaloxime[126]. **Figure 6.4.1-5** presents part of their data. They provide a useful table comparing the frequency shifts for many of the ligands of several retinals and the retinaloximes. They show that the syn- and anti- forms of the oximes can be readily identified using NMR techniques. The ratio of the constituents of such mixtures can also be readily determined. For unexplained reasons, their spectra do not identify the -OH radical to be expected at 3.16 parts per million or any value for the combination of N-OH, the analog of the CH_2-OH found in the retinene spectra above. Identification of these two components should have been a major goal of their study. No explanation was found as to why their integral function was not continuous. Since their signal-to-noise ratio appears quite adequate, another explanation is needed for the absence of these signatures.

De Leenheer & Ruyter have provided NMR spectra for the syn- and anti- isomers of retinalmethoxime[127]. Their data. Their spectra are very similar to those of Groenendijk, et. al. but do show a response at 3.9 PPM attributable to the O-CH_3 component. They note the spectral absorption peak for these materials occurs at 362 nm (syn-) and 365 nm (anti-).They provided no visible band absorption spectrum.

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[125] Shriver, J. Mateescu, G. & Abrahamson, E. (1980) in "Methods in Enzymology" Packer, L. ed. NY: Academic Press Vol 81 pp. 698-703

[126]Groenendijk, G. DeGrip, W. & Daemen, F. (1979) Identification and characterization of syn- and anti-isomers of retinaloximes. *Anal. Biochem*. vol. 99, pp 304-310

[127]De Leenheer, A. & Ruyter, M. (1975) Identification of the Syn and Anti geometric isomers of retinalmethoxime *Anal Biochem* vol. 63, pp 169-174

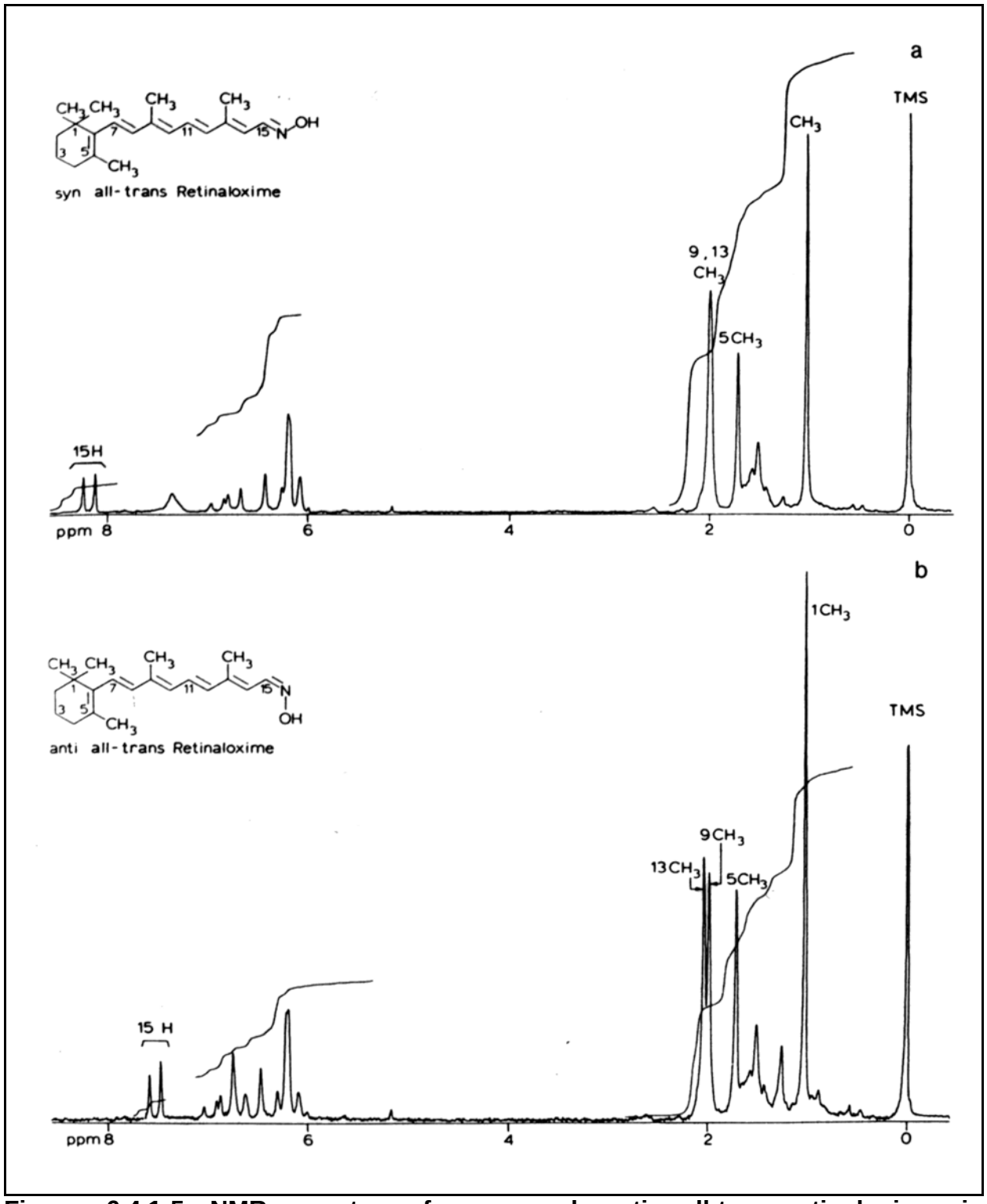

Figure 6.4.1-5 NMR spectra of syn- and anti- all-trans-retinaloxime in deuterochloroform. 90 MHz excitation. From Groenendijk, deGrip & Daemen, 1979.

Figure 6.4.1-6 from Kofler et. al.[128], provides two NMR spectra comparing two isomers, all-*trans*-Vitamin A with neo-a-Vitamin A. Although he used a different magnetic field, causing the baseline frequency to be at 56.4 MHz instead of 60 MHz (and the individual frequency deviations to be scaled accordingly), they clearly show that the different isomers exhibit different spectra. They also show that the various cis- points can be individually recognized if present. They also use a variant of Karrer's numbering system, describing the various locations of the Methyl groups by using the carbon number where they attach to the backbone of the molecule instead of assigning them a separate carbon number. This numbering system is compatible with the identification of the hydrogen nuclei as will be seen below.

[128]Kofler, M, & Rubin, S. (1960) Physicochemical assay of Vitamin A, Vitamins and Hormones. vol. 18 pg. 315-335

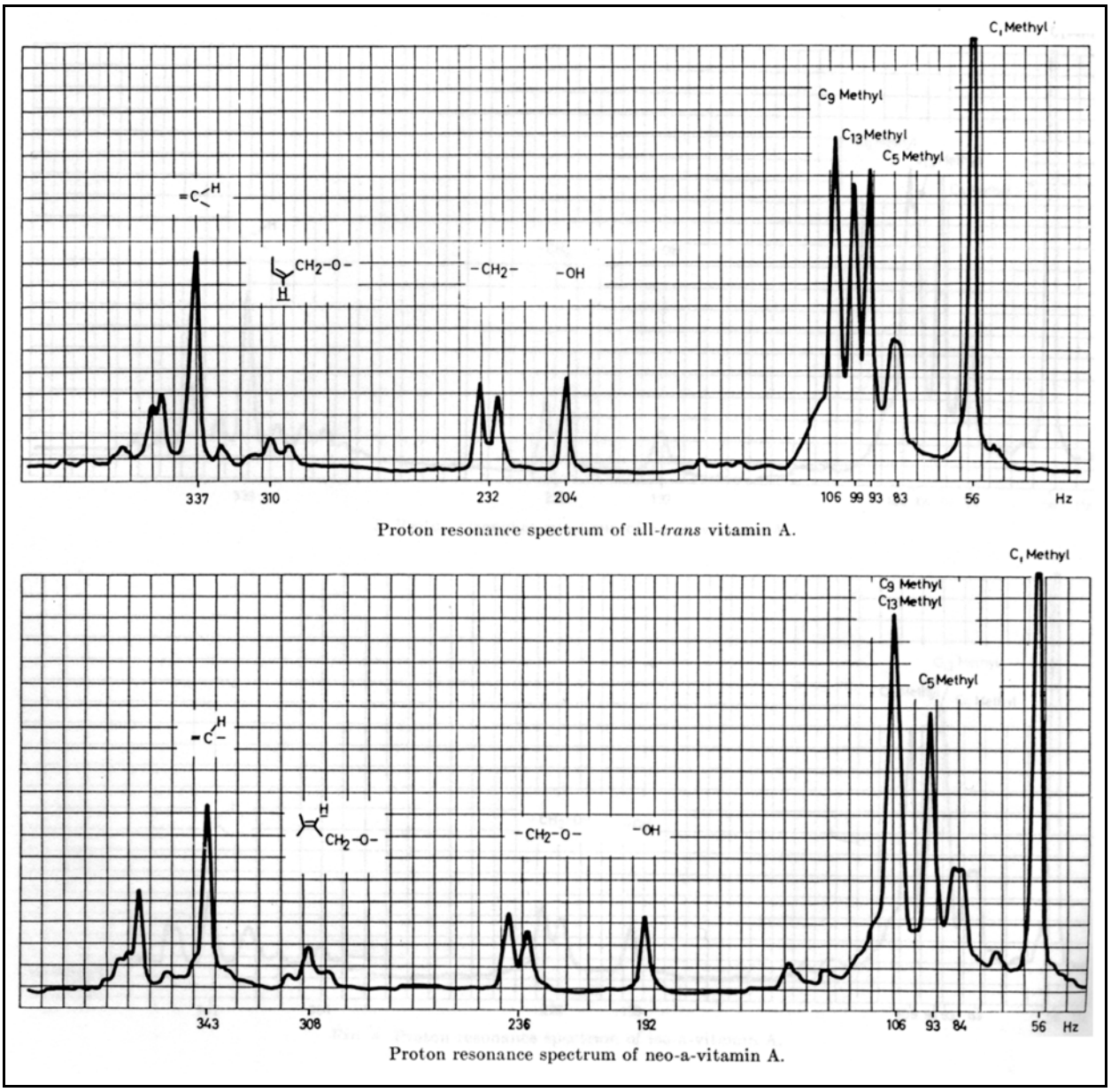

Figure 6.4.1-6 Two proton resonance spectra for isomers of Vitamin A. Top, all-trans- form. Bottom, neo-a- form. The high peak for C_1 methyl is indicative of two ligands. Frequencies are in Hz with excitation at 56.4.1 MHz. Kofler, et. al. 1960

Some authors use a ratio to describe the deviation of the frequency of each component of the spectrum relative to the carrier frequency. The resulting coordinates are independent of the carrier frequency. As an example, the C_{16}, C_{17} location is given as 60 Hz/60 MHz= 1.0 x 10-6 in Sebrell, et. al. 56 Hz/56.4.1 MHz in Kofler, et. al. gives the same value to two digit accuracy.

Rowan, et. al.[129] provide additional data for all-*trans*-retinal, using both 100 & 250 MHz nominal frequencies to accentuate the location of the hydrogen nuclei.

Zingoni, et. al. have provided tabular data for the NMR of a series of retinol analogs[130]. No values were given that relate to the oxygen in the molecules.

6.4.1.7 Electron Spin Resonance spectroscopy (ESR)

Electron spin resonance is another tool of interest in evaluating the chromophores of vision. This is a higher frequency technique, usually involving frequencies near 10,000 MHz. While it allows the identification of both diamagnetic and paramagnetic materials, it requires hardware that has only become available to academia. Coxon & Halton provide an overview of the techniques used. Oxygen is unique in the paramagnetic properties of its unpaired electrons when otherwise bound in a molecule. The signature of these unpaired electrons is frequently diagnostic for the presence of oxygen. The excitation of their unpaired electrons results in electrons in the triplet excited state. Gueron has discussed the signature of the triplet states in ESR and the equipment required[131]. He also notes the mobility of electrons excited into the triplet state within liquid crystalline forms of matter. The use of this variation on the ESR technique, the electron paramagnetic resonance technique (EPR), is complementary to the NMR technique in vision since NMR cannot normally identify oxygen when it is present alone (not in a hydroxyl group).

A new technique of Fourier Transform ESR is now emerging[132]. This technique offers considerable capability in the future.

6.4.1.7.1 Electron paramagnetic resonance

A technique uniquely suited to the exploration of chemicals containing oxygen atoms with unpaired electrons, and becoming more common in the laboratory, is EPR. An introduction to the technique and a list of references up to that time has been provided by Bersohn & Baird[133]. Although that work addresses both the crystalline and liquid states, it does not address the case of the liquid crystalline state. It does explain how the Pauli Principle is circumvented by these atoms and defines the triplet state (pgs 13-14 & 108). It also addresses the long time constants associated with atoms and molecules exhibiting a triplet state. Alger provides an early cookbook on the test configurations used in EPR, although it presents a view in conflict with later data concerning oxygen on page 470[134]. Saifutdinov, et. al. have provided a more current work on the subject[135]. As in NMR, the measured response involves a high magnetic field and an electrically oscillating field. It also involves a coarse and a fine structure. Not all test sets are capable of measuring both for oxygen compounds.

EPR can resolve the question of whether the putative compound rhodopsin or the proposed compound Rhodonine does or does not contain oxygen in the triplet state. Such a determination would be very important in resolving two situations. First any oxygen remains associated with the retinol combined via a Schiff base in rhodopsin. Second,

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[129]Rowan, R. Warshel, A. Sykes, B. & Karplus, M. (1974) Conformation of Retinal Isomers. *Biochemistry*, vol. 13, no. 5, pg. 971-980

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

[131]Gueron, M. (1971) Electron spin resonance of excited triplet states *In* Lamola, A. *ed*. Creation and Detection of the Excited state NY: Marcel Dekker, Volume I, Part A, Chapter 6

[132]Kevan, L. & Bowman, M. *ed*. (1990) Modern Pulsed and Continuous-wave Electron Spin Resonance. NY: John Wiley

[133]Bersohn, M. & Baird, J. (1966) An Introduction to Electron Paramagnetic Resonance. NY: W. A. Benjamin, Inc.

[134]Alger, R. (1968) Electron Paramagnetic Resonance: Techniques and Applications NY: Interscience.

[135]Saifutdinov, R. Larina, L. Valul'skaya, T. & Voronkov, M. (2001) Electron Paramagnetic Resonance in Biochemistry and Medicine. NY: Kluwer Academic/Plunum Publishers

whether the Rhodonines, either in-situ or in-vitro contain any unpaired electrons associated with the oxygen claimed to be present in these materials when unexcited. No data specific to the retinoids has been found in the literature.

The current focus of activity employs samples cooled to cryogenic temperatures to minimize thermal motion at the molecular level. This cooling will not be required to analyze the chromophores of vision. These materials naturally form a liquid crystalline structure suitable for analysis by EPR.

6.4.1.8 Raman Spectrography

Callender, et. al. have provided information on the Raman spectra (and the resonance Raman spectra) of a series of retinal isomers, a rhodopsin and an isorhodopsin[136]. Their introduction states the conventional assumption that rhodopsin is the visual pigment in disk membrane of vertebrate rod cells. The resonance label indicates the data was collected while the material was being irradiated by light at its peak absorption wavelength. They also employed a flowing technique to replace the sample material within the irradiation zone rapidly. It was proposed that this replacement was sufficiently fast so that bleaching had negligible effect on the results. The rhodopsin data shows two bands not found in the retinene data.

Based on this theory, that the Rhodonines are the actual chromophores of vision, the relevance of this work involving the substrate rhodopsin is not clear.

6.4.2 Non-spectral data from biological sources of retinoids

6.4.2.1 HPLC data

Groenendijk, de Grip & Daemen extended their HPLC studies to include retinals extracted from the rod outer segments of eyes and bacteriorhodpsin. This technique does not offer the specificity of NMR. Their 1980 paper was not specific as to the source of their ROS[137]. They discuss the instability of their extracts and the use of NADPH in the extraction process. Their yields were low (see **Section 6.5.1**).

While no chromatography data exists as of this writing known to represent the retinines and Rhodonines, Chen & Heller[138] have provided graphical data on a "retinol-like material." They say this material was found in the RPE cells after the delivery of retinoic acid to those cells by the blood stream.

> The Rhodonines (members of the carboxyl-ion family) could easily be confused with retinoic acid (a member of the carboxylic acid family). The Rhodonines differ in having the aldehyde and alcohol ligands separated by part of the conjugated carbon backbone.

They say, based on their heuristic understanding of the nature of rhodopsin, that: "This retinol-like material is not vitamin A itself since it is not incorporated into rhodopsin *in-vivo*." They found it both free (molecular weight less than 1000) and bound to an unidentified protein (presumably an RBP from the RPE, see **Section 7.1.2**). Several others investigating rhodopsin extracts have also encountered unexpected and unknown material in the void volume of their experiments. The material in this void space appears quite significant to Heller[139]. These materials may well be the chromophores of vision, the Rhodonines.

6.4.2.2 Radiation Chemistry

While radiation chemistry could be introduced into a variety of vision related experiments, it has not been used

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[136]Callender, R. Doukas, A. Crouch, R. and Nakanishi, K. (1976) Molecular flow resonance Raman effect from retinal and rhodopsin *Biochem* vol. 15, pp 1621-1629

[137]Groenendijk, G. de Grip, W. & Daemen, F. (1980) Quantitative determination of retinals with complete retention of their geometric configuration *Biochimica et Biophysica Acta* vol. 617, pp 430-438

[138]Chen, C. & Heller, J. (1977) Uptake of retinol and retinoic acid from serum retinol-binding protein by retinal pigment epithelial cells. *J. Biol. Chem*. vol. 252, no. 15, pp 5216-5221

[139]Heller, J. (1976) Intracellular retinol-binding proteins from bovine pigment epithelial and photoreceptor cell fractions. *J. Biol. Chem*. vol. 251, no. 10, pp 2952-2957

widely in the field except for the retinoid transport studies of R. Young and others (See Sections 7.1). Ganguly summarized some work with an isotope form of retinol as of 1989[140]. The material is described as "Vitamin A-2-^{14}C." He notes the following. "Following i. p. injection of a colloidal dispersion fo the radioactive Vitamin A, appreciable amounts of the radioactivity had appeared in the urine of these rats in a water soluble form. One of these materials was both ether and water soluble (WES, 33%), the other ether insoluble and water soluble (WS, 67%). The water soluble fraction was described as containing a carboxyl group of an ester and a nonconjugated keto group. The material they isolated could be a Rhodonine. He also summarized work that resulted in locating radioactive "retinoic acid" in the urine as well as radioactive carbon dioxide in the respiration. The material in the urine could also be Rhodonine (a carboxylic acid system). Finally, he noted the work of Rietz, et. al. and of Hanni, et. al. Rietz, et. al. isolated four different metabolites of what they identified as retinoic acid in the urine of rats and humans[141]. Hanni, et. al. also isolated three major metabolites of retinoic acid from the urine of rats[142]. Both groups focused on metabolites in the urine that showed the ring structure modified to a cyclohexenone (a 4-oxoretinoic acid). This feature may be common to the excretion process associated with respiration of retinol.

6.5 Extraction, separation and identification of the chromophores from biological sources

6.5.1 Historical extraction, separation and identification of the chromophores

The lower right portion of [**Figure 7.1.2.1**], illustrates the conventional approach to isolating the chromophores of vision. There is great scientific interest in recovering the chromophores of vision. Unfortunately, attempts to date have not been satisfactory. Many investigations have relied upon the paper by Hubbard, et. al. in 1960 as the bible in this area[143]. However, that work was prepared when the available test instrumentation was primitive. There were many assumptions in that work. One paragraph is particularly enlightening in hindsight: "One is tempted to speculate that the 11-*cis* isomer of retinal has been selected as the chromphore of visual pigments, wherever they appear in evolution, because it is the isomer that is most readily isomerized by light." **This was precisely the case, although it is now clear that isomerization plays no role in the visual process.**

Overall, the literature presents the view that only one "pigment" has ever been recovered from the human eye and this pigment was made up of retinol and a protein, stipulated to be opsin. While the combination of opsin and a retinene has now been defined in detail through amino acid analysis, the material has not been shown to be the (or a) chromophore of vision. In fact this putative material has never been shown to exhibit the spectral response of a chromophore. In dilute solution, this material exhibits a peak absorption at 498-505 nm depending on the solvent. This peak is associated with the intrinsic spectrum of many non-resonant retinols in dilute solution. There are two specific reasons for the failure to isolate the chromophores of vision. First, the aggressive chemicals used (detergents and metallic salts) to solubilize the material of the Outer Segments destroy the liquid crystalline state of the sought after material. Second, these materials often attack the delicate chromophores, and change their chemical composition. Generally, they are returned to the state of a chromogen, e.g., retinol or retinal. Sometimes they are converted to a chlorinated or fluorinated retinene.

6.5.1.1 Extraction

The historical and generally accepted methods of extracting the chromophoric material from disks of the animal eye are harsh ones. They can be divided into two categories; those utilizing saponification and those defined as using direct extraction. They generally begin with the physical detachment of the outer segment layer of the retina from the remainder of the retina. This is followed by steps designed to separate the chromophoric material from any other material present. This generally involves the use of a detergent to help emulsify the material of the outer segments and to bring the individual molecules of the chromophore into solution. It also involves the use of a "red" light, usually specified as a "dim red light" to satisfy the investigator's visual needs. This procedure is then followed by

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[140]Ganguly, J. (1989) Biochemistry of Vitamin A Boca Raton, FL: CRC Press pp 103-105

[141]Rietz, P. Wiss, O. & Weber, F. (1974) Metabolism of Vitamin A and the determination of Vitamin A status. *Vitamin Horm*. vol. 32, pp 237-249

[142]Hanni, R. Bigler, F. Meister, W. & Englert, G. (1976) Isolation and identification of three urinary metabolites of retinoic acid in the rat *Helv Chim Acta*, vol. 59(6), pp 2221-2227

[143]Hubbard, R. Bownds, D. & Yoshizawa, T, "The Chemistry of Visual Photo-reception" *In* Symposia on Quantitative Biology vol. 30, 1965 The Cold Spring Harbor Laboratory of Quantitative Biology

several stages of centrifugation and decantation, frequently involving the introduction of complex sodium salts. The final material is usually claimed to contain primarily two constituents; the chromophoric material and a protein material, stipulated to be opsin.

The literature generally equates rhodopsin to the chromophore of vision without any data confirming this premise. The most frequently referenced method of rhodopsin extraction is that of Papermaster[144]. His focus appears to be more on the physical methods of extraction than on preserving the absorption properties of their materials. His use of NaCl and $MgCl_2$ is probably deleterious to the actual chromophores of vision. The use of a "red safe light" is definitely incompatible with isolating the L–channel chromophores of vision. Papermaster did not provide any spectral absorption characteristic for his recovered material.

The most important point in the extraction and separation procedure is to avoid changes in the molecular structure of the chromophores. Saponification clearly involves such a change. The introduction of detergents and/or strong metallic salts frequently leads to structural and/or chemical changes in biological samples. Sporn provides 2.5 pages of precautions in this area. They point out that Retinyl acetate underwent up to 92% hydrolysis when sodium sulphate was used in the extraction process but only a maximum of 24% hydrolysis when a lyophilization method was used. Both results appear unsatisfactory for recovering the Rhodonines. Most extractions in the past have involved a complex sodium salt that can easily cause hydrolysis, leading to the conclusion that all chromophores of vision use retinol, the dominant retinoid recovered following the extraction process.

It is also important to avoid isomerization, bond rearrangements and/or oxidation during the extraction process.

6.5.1.2 Separation methods

Occasionally, chromatography techniques have been used in attempts to achieve a higher level of purification through the separation of various components of a retinal extract. The results have shown limited success, possibly because of hydrolysis of many of the original constituents. Chromatography would appear to offer a method of separating the three or four Rhodonines present in a given extract from a retina. However, the very similar molecular weight and structure of the Rhodonines may not support acceptable separation using these techniques.

Contrary to the dated position of Kofler & Rubin that chromatography techniques (adsorption and partition) offer the only chance of separating the chromophores of vision (page 331), recrystallization of liquid crystalline material offers a very promising technique.

6.5.2 Proposed extraction, separation and identification of the chromophores

Sections 4.6.2.3 & **7.1** define distinctly different mechanisms and constituent flow paths for the production of rhodopsin and the Rhodonines. While it may be useful to extract both rhodopsin and the Rhodonines in pure form for purposes of analysis, only the Rhodonines qualify as the actual chromophores of vision (according to this work).

The most important issue in the extraction of the chromophores of vision is to avoid any detergents, and other chemicals, that will attack the very delicate Rhodonines. The oxygen ligands are very weakly attached to the retinoid backbone. Metallic salts should be avoided entirely. Salts of chlorine and fluorine should also be avoided. The materials are extremely light sensitive in the liquid crystalline state. Without a suitable de-excitation mechanism, they are rapidly bleached and exhibit a decay time constant measured in hours to days. Use of a "dim red light" is completely inappropriate. For accurate results, the chromophores must be not be exposed to any light at a wavelength less than 1000 nm until a suitable de-excitation substrate is provided. They normally exist in an oxygen free environment, the IPM. It is likely that they must be protected from oxygen, and other oxidizing agents, during extraction[145]. Coxon states the situation specifically, for any desired reaction to proceed from the triplet state, the sample must be protected from paramagnetic quenchers and free radical scavengers, such as molecular oxygen. They describe molecular oxygen as a ground state triplet (pg 15-16). "It requires only about 92 kJ mol^{-1} to excite oxygen into it's lowest excited singlet state." "Ground state oxygen has been shown to quench triplet acetone at almost every molecular collision. The quenching of an excited singlet state is also efficient." This feature accounts for much of the chemical activity of oxygen. Its presence may divert the course of an otherwise well defined reaction. Retinol itself is subject to breakdown in the presence of oxygen.

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[144]Papermaster, D. (1982) Preparation of retinal rod outer segments *Meth Enzymol* vol. 81, pp 48-53

[145]Coxon, J. & Halton, B. (1987) Organic Photochemistry NY: Cambridge Univ. Press pg 9

6.5.2.1 Extraction & separation from the outer segments of the photoreceptors

Section 6.5.2 discussed the delicacy of the chromophores of vision in terms of what not to do. Because of the chemical delicacy of the chromophore molecules, every effort must be taken to prevent their chemical destruction during processing. They are extremely susceptible to photo-excitation, especially when in the liquid crystalline state. When separated from their neurological interface, they lack their normal method of de-excitation. Great care must be taken to prevent their unwanted or unappreciated excitation. Otherwise, the materials will rapidly become transparent (bleach) or decompose through other mechanisms.

While in the IPM space, the chromophores of vision are protected against the actions of oxygen, a variety of other strong oxidizers and active metallic salts. When attempting to recover the chromophores in the laboratory, it is mandatory that the materials be protected from these same antagonists (antagonists in the social context). Agitation, mild organic detergents and other techniques known to transform liquid crystalline material into particles of molecular size can be used to support extraction and concentration. These activities should be carried out in an oxygen free environment. Any solvents must be free of dissolved oxygen.

While present in the outer segments, the chromophores of vision remain in contact with the de-excitation mechanism provided by the neural system of the inner segments. This contact provides a method of de-excitation in the presence of photon irradiation. When this contact is lost, the chromophores become nearly perfect photoexcitation integrators and will bleach rapidly (and semi-permanently). They will operate much like a piece of photographic film left with the camera shutter open.

When in a dispersed suspension of individual molecules, the capture cross-section of the individual molecule is very small and the sensitivity to bleaching is greatly reduced. However, as the materials are returned to the liquid crystalline state, their capture cross-section increases immensely. Lacking a de-excitation mechanism, the material will bleach rapidly in the presence of light and remain transparent until de-excited. Thus, it is extremely important that the extraction and recrystallization activities be carried out in complete darkness until a de-excitation mechanism is provided to replace that provided by the inner segments (via the microtubules/dendrites).

Centrifugation can be used to concentrate the chromophores. However, their molecular weight of less than 300 means the chromophores (Rhodonines) are found in or at the interface of the supernatant portion of the recovered material. Most of the precipitate will be the non-functional protein (rhodopsin) and other cell debris.

6.5.2.2 Extraction & separation from the RPE cells

While in the RPE cells, the chromophores of vision are protected against the actions of oxygen, a variety of other strong oxidizers and active metallic salts. However, if the RPE cell membrane is broken in order to recover the chromophores stored in the “color globules,” they become subject to the same antagonists as described above. The same precautions must be taken against chemical decomposition, or photoexcitation.

6.5.2.3 Recrystallization

The chromophores of vision are stereochemically distinct. When a mixture of chromophores is encouraged to precipitate, they will attempt to re-crystallize as segregated masses (films). Physical isolation of the individual masses should be feasible by observing them under a low power microscope and teasing them into separate regions or containers (see the next paragraph). The segregated masses can then be re-solubilized and then encouraged to re-crystallize into larger masses (films). Values of pH compatible with the in-vivo condition should be maintained during re-crystallization.

6.5.2.3.1 Critical importance of the substrate

Because of the long lifetime of the Rhodonines in liquid crystalline form, it is critically important that a suitable substrate be provided. This substrate must be in quantum-mechanical contact with the liquid crystal in order to de-excite any excitons formed by absorption of photons. Fortunately, this problem is similar to that faced by engineers working with liquid crystalline display technology. The reader is referred to the literature of that specialty. In general, a substrate must be chosen that exhibits an ability to absorb energy at the 1.0-3.0 electron-volt level and either dissipate the energy or conduct it away as an electrical charge (current).

6.5.2.4 Identification using NMR and EPR techniques

6.5.2.4.1 Putative NMR spectrum of the Rhodonines

NMR testing provides a distinct and absolute method of identifying the chromophores of vision. It does this by identifying the signature of each nucleus in the molecule. This signature reflects not only the nucleus present, but also its perturbation by adjacent nuclei. By employing both low and high frequency NMR, it is possible to obtain a unique spectrum for a given chemical structure.

Chapter 5 provides several tabulations used to define the parameters of the retinenes and the various potential Rhodonines. Section 6.4.1.6 summarizes the NMR data available for a variety of retinoids.

Providing the salient features of the predicted NMR spectra of the Rhodonines is possible by compiling the work of these investigators. However, doing this in one figure is difficult. To highlight both the Methyl groups and the hydrogen nuclei of interest, significantly different excitation frequencies are required in the NMR technique. For the following discussion, the designations in **Figure 6.5.2-1** will be used. These designations differ from those of Hamanaka, et. al. referenced above. A few numbers have been omitted to make designation of each hydrogen of the side chain correspond with its associated carbon designation. When a retinol is converted to a Rhodonine, either a hydrogen or a methyl group is replaced by an oxygen and an associated (although possibly delocalized) hydrogen. The hydrogens are then numbered as shown in the diagram above each candidate for replacement. If needed, the oxygens can be numbered to correspond to the carbon they become associated with, e. g., O5, O7, O9 & O11. The original oxygen would be designated O15.

Using the above nomenclature, each Rhodonine can be described by how it differs from the underlying retinene.

The L–chromophore, Rhodonine(5), replaces the methyl group associated with C5 with a hydroxyl group, O5 and a redefined H18.

The M–chromophore, Rhodonine(7), replaces H7 with O7 and a redefined H7.

The S–chromophore, Rhodonine(9), replaces the methyl group associated with C9 with a hydroxyl group, O9 and a redefined H21.

The UV–chromophore, Rhodonine(11), replaces H11 with O11and a redefined H11.

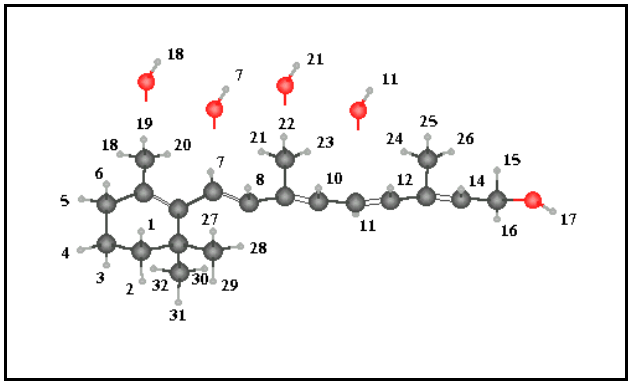

Figure 6.5.2-1 Nomenclature for each nucleus in retinol and the Rhodonines.

Figure 6.5.2-2 illustrates the putative spectra of Rhodonine (5) and (9), under the assumption that the material is all-*trans*- in structure. These are the two members of the family, where the Oxygen nucleus replaces a Methyl group. Note the absence of the C_5 Methyl group signature at 93 Hz in (a), the 56.4.1 MHz NMR spectrum for Rhodonine(5) and the absence of the C_9 Methyl group signature at 106 Hz in (b) for Rhodonine(9). In both cases, the -OH signature near 200 Hz and the CH_2-O- signature near 310 Hz can be expected to be modified and/or replaced by signatures representing the resonant Oxygens now found at both C_{15} and either C_5 or C_9.

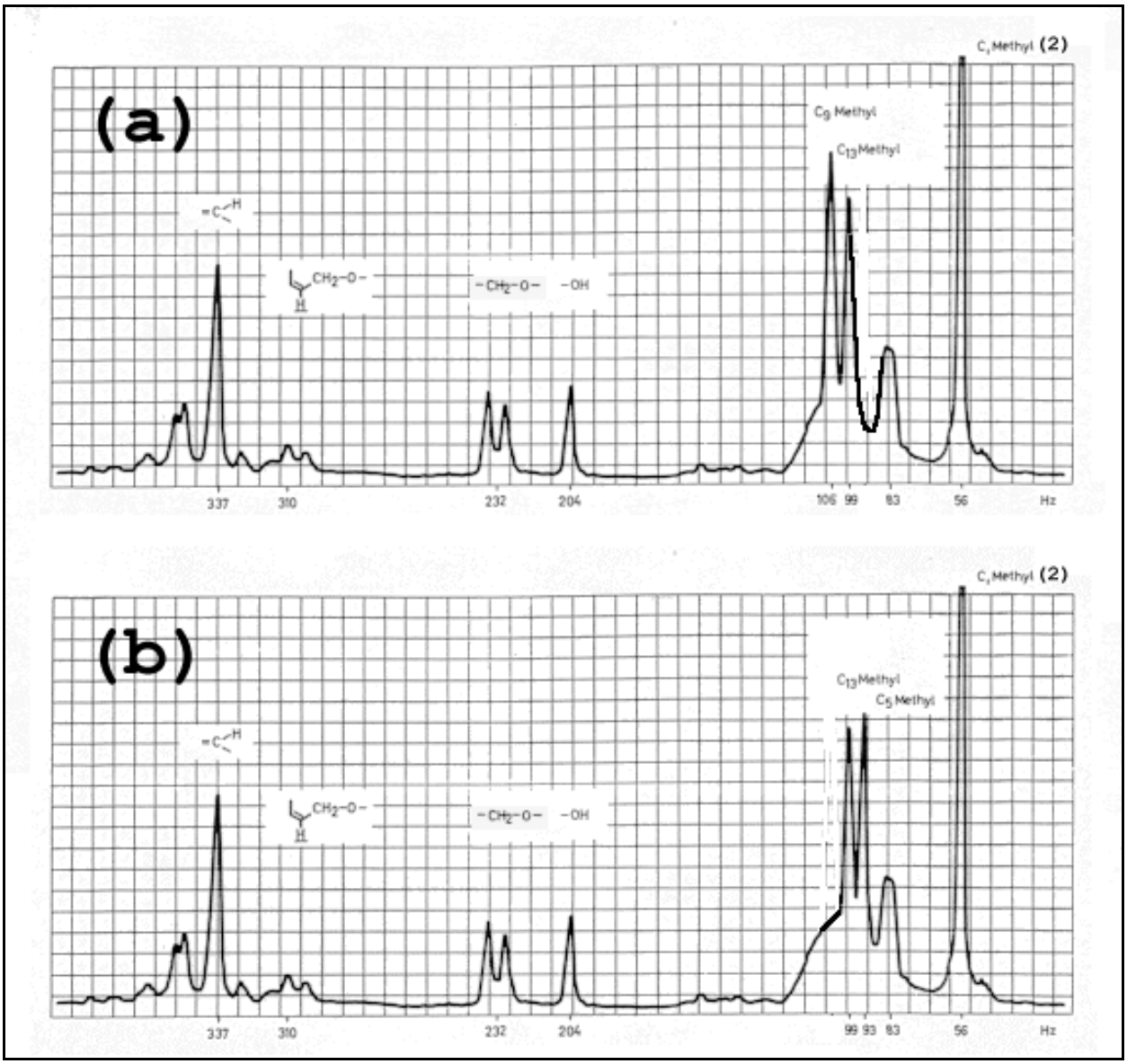

Figure 6.5.2-2 Proposed NMR spectra of (a); Rhodonine(5) and (b); Rhodonine(9) showing only the change in the spectra near 100 Hz. The changes near 200 and 300 Hz due to the presence of a resonant conjugated Oxygen structure have not be en characterized.

The changes seen in the NMR spectra of Rhodonine (5) and (9) due to the introduction of the resonant Oxygen spectra, can also be expected to be seen in the spectra of Rhodonine (7) and (11). This includes the signatures of Oxygen located at either C_7 or C_{11} respectively. However, the signatures of the Methyl groups at C_5 and C_9 will not be affected. Instead, the signatures related to the hydrogen nuclei at C_7 and C_{11} will be missing from the higher frequency NMRs of the type shown by Rowan, et. al.

Based on this cursory presentation, it can be seen that the NMR spectra of the Rhodonines will be quite similar to that of Vitamin A (retinol). However, all four Rhodonines show a distinct difference in signature. The difference is due to the presence of two Oxygens in a resonant structure instead of one Oxygen in a hydroxyl group. For Rhodonine(5), the L-chromophore, and Rhodonine(9), the S-chromophore, the absence of the Methyl group signature at either C_5 or C_9 will also be determinative. For Rhodonine(7) and (11), the absence of the respective

hydrogen nuclei will be harder to recognize.

> **Where the vision of animals using only the three longest wavelength chromophores are of interest, the chromophores extracted (with care) from the OS of the retina can be individually and definitively identified using NMR techniques. After separation and precipitation, probably through repeated re-crystallization, the three chromophores in the liquid crystalline state will all exhibit the unique signature of a resonant conjugated molecule. One chromophore will be missing the signature of the C_9 Methyl group and one other chromophore will be missing the signature of the C_5 Methyl group.**

The studies of Rowan, Warshel, Sykes & Karplus provide the foundation for a more detailed prediction of the NMR spectra of the Rhodonines.

6.5.2.5 Identification using spectrometer techniques

There is an amazing disparity in the literature with regard to spectro-photometer data for animal vision systems. The difference is striking when one compares the spectrometry data and psychophysical data. The disparity is so great that assigning it to one cause is difficult. Frequently, the cause relates to various normalizing steps, introduction of estimates in the calculations and various corrections based on linear algebra.

There is one clear cause of inconsistent data regarding the L-channel of vision. Over the years, the research community has attempted to make laboratory measurements concerning the L-channel while still operating with sufficient illumination for the investigator to see his materials. It is normal in the literature to read that the experiments were carried out using a red Kodak #1 Safelight. What is not mentioned is that the peak of the L-channel absorption spectra is within the passband of the #1 filter. Furthermore, due to the *f*/# of the optical system of the human eye, the illumination on the test sample will be approximately an order of magnitude higher than on the retina of the investigator. Therefore, such a light will bleach the L-channel chromophore and must be considered an active part of the experiment that must be controlled.

The #1 Safelight is designed for use in a dark room where only unsensitized silver halides are present. In fact, normal dark room instructions say that no light may be allowed during development until after the sensitizing dyes have been destroyed or removed. Only after this stage of development may a #1 Safelight be used. The same cautions apply to vision research. A #1 Safelight is not appropriate when evaluating the L-channel and its use is highly questionable when evaluating the m-channel. The s-channel has an absorption characteristic not unlike an unsensitized silver halide emulsion. Good research technique would require the use of no illumination at wavelengths less than about 1.0 microns. Without using an infrared illuminator and viewer, no quantitative research can be performed. When exploring the properties of the S-channel and M-channel chromophores, a #1 Safelight can be used. However, when exploring the L-channel chromophores, a #7 Safelight is required as a minimum.

Spectro-photometer frequently involves an unpolarized light source in vision experiments. However, it is important that the state of polarization at the test plane be stated since many test sets used can and do generate polarized illumination without the knowledge of the investigator. This is generally important in axial or end on illumination, especially in the UV, but especially important when using transverse illumination in which the illumination is being applied to a highly structured liquid crystalline material along an abnormal axis.

Care must be taken to avoid the introduction of extraneous pigmented cells into the path of the illumination source in both spectrophotometric and spectral response experiments. These include the retinochrome cells in the case of arthropod and mollusc animals, and their analog, Muller Cells in the case of chordate animals. These also include the RPE cells of chordates.

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Transport delay
net photoreceptor