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REGENERATION OF VISUAL PIGMENT IN THE BOVINE RETINA

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REGENERATION OF VISUAL PIGMENT IN THE BOVINE RETINA

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To Beverly and Stephanie

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REGENERATION OF VISUAL PIGMENT IN THE BOVINE RETINA

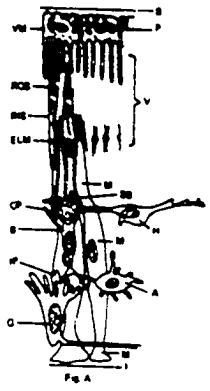
CHAPTER I

INTRODUCTION

Visual pigment, a lipoglycoprotein containing 11-cis retinaldehyde as a prosthetic group, is contained within the rod outer segment (ROS) portion of a retina receptor cell. Fig. 1-A represents a diagrammatic cross-section of the vertebrate retina in which the ROS consists of a linear array of stacked membranous disks enclosed by a plasma membrane thus forming an elliptical structure oriented perpendicular to the longitudinal axis of the pigment epithelium. The photosensitive pigment giving rise to the visual response is located within the stacked disks and must therefore be considered as a constituent of a biological membrane.

Biological membranes are characterized by lipids and proteins bonded together by noncovalent hydrophilic and hydrophobic associations. These associations are critical to the maintenance of both structural and functional integrity of the membranous units. This point is especially important when it is considered that these associations allow each membrane to function in certain ways specific to it alone.

In 1935 Danielli and Davson proposed their classical hypothesis that membrane lipids were arranged in a bilayer and that proteins were



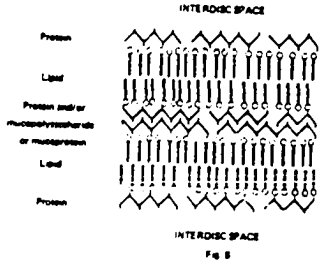


Figure 1. A - Diagramatic cross-section of vertebrate retina taken from Cohen. B, Bruch's membrane; P, pigment epithelium; VM, Verhoeff's membrane; ROS, rod outer segments; RIS, rod inner segments; SB, synaptic bases; ELM, external limiting membrane; V, ventricle lumen; M, Muller cells; OP, outer plexiform layer; H, horizontal cells; B, bipolar cells; IP, inner plexiform layer; A, amacrine cells; G, ganglion cells; I, inner limiting membrane.

B - Classical diagram of part of an outer-segment disc. Open circles represent the polar ends of lipid molecules.

associated with the polar groups of lipids on both sides of the membrane (14). After 36 years of research designed to either support or reject this hypothesis, very little is yet known about the molecular assembly of membranes. Perhaps the best line of evidence supporting the concept of Danielli is X-ray diffraction data showing high and low electron density regions in a membrane cross-section (5). The low regions are supposedly due to hydrocarbon chains of fatty acids esterified to phospholipids while high regions are presumed to be due to polar end-groups of phospholipids and protein.

In recent years, studies from several laboratories have shown that proteins can be distributed in conformations other than simply being linearly extended over the surface of a lipid bilayer which is represented by Fig. 1-B. Circular dichroism and optical rotatory dispersion results have indicated that many proteins associated with membranes have some α-helical structure (22). These membrane-bound globular proteins have diameters of 30-40 A, while the thickness of the membrane is approximately 75 A. It then follows logically that globular proteins cannot be totally contained on the surface and, at least, some of it must be distributed within the membrane (4). The protein molecules would thus be found in the interior of membranes where hydrophobic bonds could be found between lipid and non-polar regions of the protein. Recent X-ray diffraction data by Wilkens indicates that rod outer segment (ROS) membranes are composed of globular proteins half-submerged in a lipid bilayer (37).

If proteins are to be found in globular form within membranes it would be necessary for purposes of structural and functional integrity that non-covalent association occur between lipid and protein. Whether

these associations are hydrophobic or hydrophilic is of paramount interest. Hydrophilic associations would be between the polar groups of phospholipids and those of the protein, while hydrophobic attachments would be between the hydrocarbon chains of fatty acids esterified to glycerol phosphate and the nonpolar amino acids of the protein.

Hydrophilic bonding has been observed in cytochrome c of mitochondrial membranes (15, 32). Delipidated protein was found to be insoluble in organic solvents whereas addition of phospholipid restored solubility in iso-octane. The electrostatic bonding was interrupted by high salt concentrations (15). The ability to transfer electrons was lost upon removal of phospholipid but was restored upon readdition (32).

Indications of hydrophobic associations have come from studies subjecting an intact membrane to hydrolysis with phospholipase C (10). It was found that the phosphoryl-base of these membrane phospholipids could be removed without destroying membrane integrity as seen by electron microscopy.

It has been determined that inactive cytochrome P-450 absorbing at 415 nm undergoes a spectral shift to 390 nm in the presence of certain drugs as substrate and thus is converted to an active enzyme capable of hydroxylation of substrate (24). Coon has shown that phosphatidyl choline is specifically involved in the subsequent step of this multisequence system which functions to hydroxylate a wide variety of substrates including fatty acids, hydrocarbons, and drugs (11).

In recent years, controversy has been generated in the literature concerning the physical involvement of phospholipid in ROS membranes with retinal at the chromophoric site. In metarhodopsin II, an inter-

mediate in the photoinitiated, thermal bleaching sequence, an aldimine linkage of retinal to the ε-amino group of lysine was demonstrated by aqueous reduction of this intermediate with sodium borohydride followed by degradative analysis. However, "photointact" rhodopsin could not be reduced leaving some doubt as to whether this linkage was present at the chromophoric site (2, 7). Poincelot extracted N-retinalidine phosphatidyl ethanolamine quantitatively from rhodopsin with acidic methanol in the dark while confirming the ε-amino linkage to lysine in metarhodopsin II (26). Thus, he concluded that retinal was linked to phosphatidyl ethanolamine in an aldimine form and is transiminated to lysine in the photolysis sequence.

This conclusion has been disputed by other investigators (3, 6, 13). Anderson and Maude found that acidified chloroform-methanol extracts of ROS yielded a phosphatidyl ethanolamine-retinal aldimine linkage less than quantitatively, based upon a molar absorbance for rhodopsin of 42,000 (3).

In a recent publication, Borggreven, Rotmans, Bonting and Daemen treated rod outer segment preparations with phospholipase C and reduced the molar ratio of lipid phosphorus to retinal bound in the chromophore from 87 to 4 without altering the molar absorbance of rhodopsin determined from the absorbance at 500 nm as a function of released retinal concentration (6). Moreover, they report that hexane extraction of phospholipase-treated material (a) removes diglyceride hydrolysis products, (b) does not further lower the lipid phosphorus-retinal ratio and (c) has no further effect on the molar absorbance of the 500 nm band. By means of automatic amino acid analysis they determined the ratio of phos-

phatidyl ethanolamine to chromophore-bound retinal at 0.2 - 0.4 and concluded that the ratio was too low to justify retinal-phosphatidyl ethanolamine as the chromophoric linkage. In a simultaneously released paper, Daemen, Jansen and Bonting arrived at the same conclusions by comparing methanol and acidified methanol extractions of rhodopsin in lyophilized outer segment preparations to transimination potential of model compounds in the same solvents (13).

Most recently, a controversy centering around the independent results of Zorn and Futterman and those of Shichi concerning the role of phospholipids in the regeneration of visual pigment has appeared in the literature (31, 39). Zorn and Futterman have found that regeneration is specific to phosphatidyl ethanolamine upon adding lipid back to delipidated outer segment preparations extracted with detergent (39). Shichi was unable to demonstrate this specificity but admits to possible phosphatidyl ethanolamine impurity in the high concentrations of phospholipid employed (31). Moreover, Shichi proposed that phospholipid may be involved in stabilizing a native preferred conformation of opsin which allows regeneration to occur. Neither group achieved regeneration of visual pigment from all-trans retinal without introducing an isomerizing agent. Shichi incubated all-trans retinal added to pigment before photobleaching such that illumination, resulting in π,π^* transitions of the polyene retinaldehyde chain, gave rise to photoisomerization. The importance of Shichi's results is that he was able to regenerate true rhodopsin to a limit of 56% to the apparent exclusion of isorhodopsin, a pigment generated from the thermally more stable isomer, 9-cis retinal (18). On the other hand, Zorn and Futterman employed EDTA and dithiothreitol

(DTT) as dark isomerizing agents for added all-trans retinal and claimed 95% regeneration as compared to a separate control.

In a much earlier paper, Hubbard found evidence for an enzyme, which she called retinal isomerase, in the soluble fraction of retina homogenates (19). It was claimed that retinal isomerase resulted in the stereospecific isomerization of added all-trans to 11-cis retinal up to values of ∿ 30% in the presence of light which was interpreted as supplying free energy to the conversion process. However, it was also claimed that the "isomerase" would catalyze the conversion of either all-trans or 11-cis retinal to an equilibrium mixture of isomers containing 2-9% of the 11-cis form.

This latter publication then provided the stimulus to initiate the current investigation of regeneration occurring in ROS solubilized by digitonin because (a) the postulation of an isomerase affords an acceptable explanation for the phenomenon of dark adaptation and (b) a stereospecific isomerase would insure the conversion of all-trans retinal, no matter how slowly the process would occur, such that complete visual pigment regeneration could result, assuming that no alternate metabolic routes for all-trans retinal are present in the ROS. Moreover, 11-cis retinal would be the exclusive product of the isomerase reaction such that other isomers found in nature (9-cis, 13-cis, 9,13-dicis) would not be formed in the ROS. The structures of vitamin A and the various retinaldehyde isomers are illustrated in Fig. 2.

The purpose of this research was then to discover and describe the roles of enzymes participating in the regeneration process.

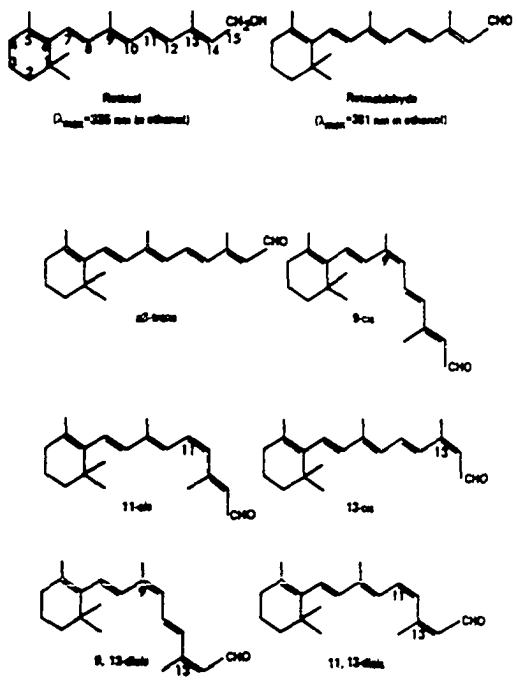


Figure 2. Structures of all-trans vitamin A (retinol) and the various stereoisomers of retinaldehyde.

CHAPTER II

METHODS AND MATERIALS

Preparation of Visual Pigment

Bovine eyes from freshly slaughtered cattle were obtained as a generous gift of a local abattoir[1] and were immediately placed in a darkened ice-water bath for transportation to the laboratory. Retinas were removed under a deep red light and either immediately used or stored frozen at -20°C. A given quantity of retinas were thawed and gently homogenized at 3°C in 66 mM phosphate buffer, pH 6.8 (0.5 ml/retina). The homogenate was allowed to remain in the cold for 30 min. and was then centrifuged at 3°C (25,000 x g) for 20 min. The pellet was resuspended in buffer, stirred 30 min. and recentrifuged to rewash the ROS; a procedure that was repeated 2 to 6 times. The pellet was then suspended in 2% digitonin (Sigma), cetyltrimethylammonium bromide (CTAB, Sigma) or, Triton X-100 (Sigma), each in phosphate buffer pH 6.8 (0.5 ml/retina) and was stirred 18 hours at 3°C. The stirred preparation was centrifuged at 45,000 x g and the supernatant solution was obtained as crude pigment preparations. None of the detergents employed were capable of removing all the pigment from ROS in the first extraction, however, the quaternary ammonium salt (CTAB) and Triton were much more efficient than

[1]The Wilson Meat Packing Company, Oklahoma City, Oklahoma.

digitonin.

Partially purified ROS pigment was obtained by fractionation with ammonium sulfate directly from digitonin solutions. The sedimented pigment obtained was resuspended in the quantity of detergent solution required to achieve the absorbance at various wavelengths desired. In this procedure, pigment solutions in digitonin were first brought to 10% saturation with ammonium sulfate and stirred 120 min. at 3°C in the dark to insure that equilibrium was achieved. The resulting slurry was centrifuged (12,000 x g for 20 min. at 3°C). The supernatant solution was then brought to 20% saturation, stirred for another 120 min. period and again centrifuged. The pellet was found to contain about 70% of the total absorbance at 500 nm and was subsequently resolubilized in an appropriate volume of 0.2% digitonin in 66 mM phosphate buffer, pH 6.8. The volume of the digitonin solution employed was that required to make the α-band of visual pigment (due to aromatic amino acid absorption) readable on the 0.0 - 1.0 optical density scale.

Preparations of visual pigment were also made by grinding frozen retinas to a homogeneous slurry in a mortar and subsequently washing with phosphate buffer. This suspension was centrifuged at 25,000 x g for 20 min. after which the pellet was stirred into a solution of 40% sucrose in 66 mM phosphate buffer (0.2 ml/retina), and centrifuged again at 2000 x g for 20 min. The supernatant solution was collected, layered under an equal volume of phosphate buffer, and subsequently centrifuged at 45,000 x g for 20 min. The ROS material collected at the interface between sucrose and buffer layers was removed and solubilized in 0.2 - 2.0% digitonin, CTAB, or Triton X-100 in phosphate buffer, pH 6.8.

It should be pointed out that throughout these preparative techniques the rod outer segments present in the retina were isolated and directly solubilized in the detergent of choice. This procedure is then to be distinguished from the practice of subjecting isolated ROS to techniques such as further homogenation, organic solvent extraction or treatment with alum, which are intended to destroy their membranous integrity. In this respect, visual pigment present in ROS solubilized with the synthetic cationic detergent (CTAB) could be readily purified from solution thus indicating a loss of membrane integrity due to effects of the detergent itself. On the other hand, only limited success could be achieved toward purification of pigment present in ROS solubilized with the plant saponin, digitonin thus indicating the retention of ROS protein other than visual pigment in these preparations. This fact alone strongly suggests that considerable membranous integrity is retained when ROS are directly solubilized in this natural, nonionic detergent. The reader should bear in mind the distinction that is being made between the membranous quality of these digitonin preparations as opposed to the lipoprotein nature of purified visual pigment, such that detergents are required to bring the material into aqueous solution. The latter nature of pigment has been known for some time (21).

Preparation of "Retinal Isomerase"

Whole retinas were again removed from bovine eyes as described before except that no precautions were taken to prevent light exposure in this or any succeeding step throughout the preparation. Removed retinas were stored frozen before being further treated for periods varying from 2 days to 2 weeks. Retinas were then thawed and homogenized with

phosphate buffer (0.5 ml/retina). The homogenate was gently stirred in the cold for 20 min. and was then centrifuged at 50,000 x g for 20 min. at 3°C. The supernatant solution was decanted and taken as a first approximation of "retinal isomerase" as described by Hubbard (19).

Further attempts were made to collect a partially purified form of the "isomerase" by fractionating the supernatant with ammonium sulfate at 20, 40, 60 and 80% saturation levels. Materials collected were dissolved in the minimum amounts of 2% digitonin-phosphate buffer required for solution and were exhaustively dialyzed against phosphate buffer, in the cold.

Recording of Absorption Spectra

Absorption spectra were determined on a Carey-14 PM recording spectrophotometer or a Beckman DB-G grating spectrophotometer to which was attached a Beckman 10 inch strip-chart recorder. All spectral data presented in this dissertation in figural form are exact traces of original recording pen traces and consequently are not corrected for any source of nonspecific absorbance such as, for example, light scattering in the UV and lower visible regions of the spectrum. All data presented in tabular form are taken from original pen traces.

Regeneration Methods

Visual pigment preparations in stoppered cuvets were illuminated by exposing samples to roomlight (8 cool fluorescant lamps of total wattage 320 at a mean distance of 2M) for periods varying from 10 min. to 30 min. at temperatures of 18° or 30°C and were subsequently dark incubated at 18° or 30°C for periods varying from 10-120 min. The apparent per-

centage of regeneration was determined as extent recovery of the original chromophore by measuring

$$\frac{100\{A_{500}\ (\text{recovered}) - A_{500}\ (\text{photobleached})\}}{A_{500}(\text{unbleached}) - A_{500}\ (\text{photobleached})}$$

where A = absorbance or optical density.

Maximum recoveries of 30% were obtained by taking these measurement from spectral scans. Regeneration could be increased 5-10% by following A500 immediately after the illumination period without expending the time to obtain a photobleached scan. All additions to this system were made from µl quantities of concentrated stock solutions.

All-trans retinal (City Chemical Corporation), 9-cis retinal and 11-cis retinal (a generous gift from Hoffman-LaRoche) were each dissolved in 95% ethanol as 10^{-2}M solutions and stored in the dark at 0°C when not in use. Extents of regeneration were measured as described previously upon the addition of retinal to photobleached pigment. However, regeneration rates were determined from photobleached samples exhaustively regenerated from photoreleased retinal before 11-cis retinal was added. Rates were obtained by following A500 nm on a strip chart recorder. The absorption spectrum of 11-cis retinal in ethanol and in digitonin can be seen in Fig. 3 while its photoisomerization properties are shown in Fig. 4. CTAB was added in µl quantities from a 40% stock solution in phosphate buffer.

Retinas employed in these experiments were removed from the eyes of freshly slaughtered cattle which had <u>not</u> been previously dark-adapted and, as such, were found to contain excess empty pigment protein which was not conjugated with its 11-cis retinal prosthetic group. Thus,

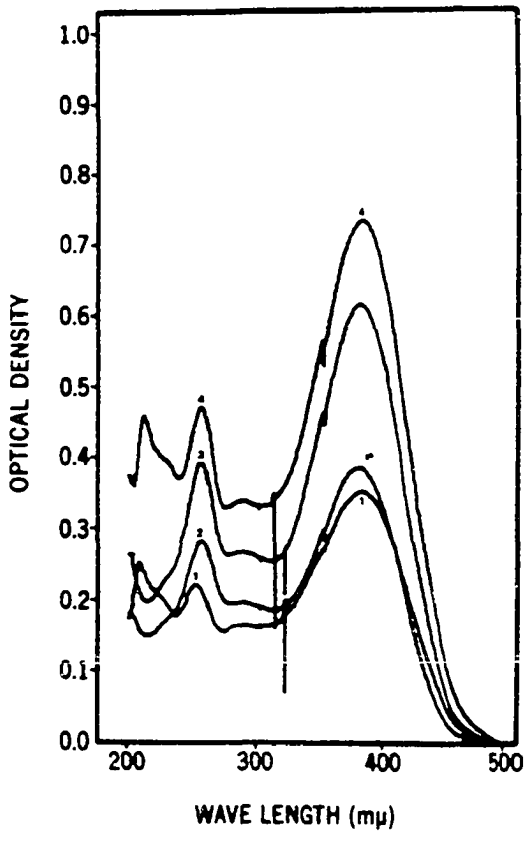


Figure 3. Absorption spectra of 11-cis retinal. 1, 1.66 x 10^{-5}M in 2% aqueous digitonin; 2, 1.66 x 10^{-5}M in 95% ethanol; 3, 2.5 x 10^{-5}M in 95% ethanol; 4, 3.3 x 10^{-5}M in aqueous digitonin.

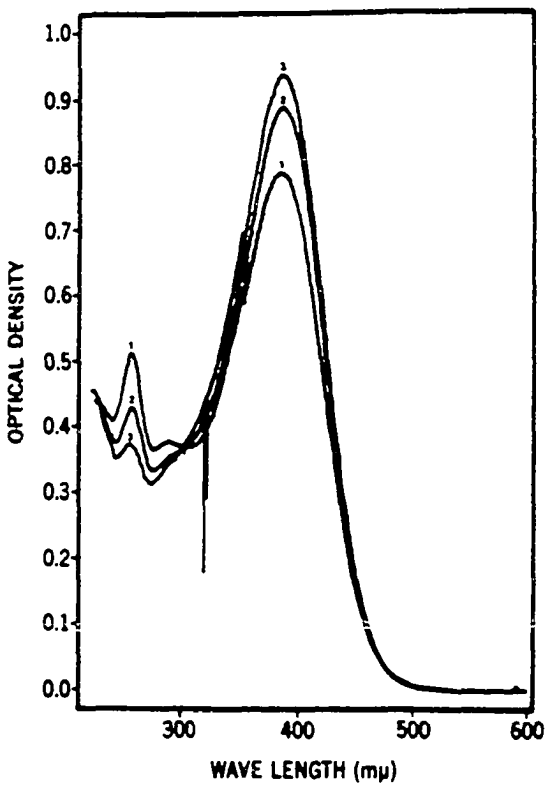


Figure 4. Photoisomerization of 11-cis retinal. 1, 3.3 x 10^{-5}M in 95% ethanol; 2, illuminated by room light 15 min.; 3, illuminated 60 min.

the term used to describe the extent of regeneration as "regeneration (apparent)" is useful only to compare the effects of certain variables upon regeneration or regenerability and, should in no way be construed to represent the absolute extent of regeneration that would be more closely approximated using dark-adapted retinas. A somewhat better approximation of the absolute extent of regeneration in these light-adapted retinas was obtained by quantitatively comparing the difference spectrum of pigment preparations regenerated with 11-cis retinal before photobleaching versus their photobleached products to those of pigment photobleached before being regenerated with 11-cis retinal versus their photobleached products. Concentrations of the various retinals and their ε_M's were calculated from their 385 nm absorbances using 40,600 for ε_M 500 nm (36).

Treatment of Visual Pigment with Phospholipase

Phospholipase A and C were obtained from Sigma Chemical Company. Phospholipase A (Vipera Russeli in 50% glycerol) was added directly (20 µl) to a cuvet containing 2 ml of visual pigment from a 20% ammonium sulfate precipitate resolubilized in 0.2% digitonin such that the amount of enzyme in the cuvet would hydrolyze 0.36 µ moles lecithin/min. at 37°C. Phospholipase C (Cl. Welchii), was first made up as a 10% solution in phosphate buffer (66 mM, pH 6.8). 40 µl of this solution was added directly to 2 ml of visual pigment in digitonin. Both of the phospholipase treated materials were allowed to incubate at 30°C for 60 min. after which they were photobleached for 15 min. The samples were then incubated in the dark for 60 min. at 30°C to determine the extent of regeneration. In the case of phospholipase C, dark incubations for periods

up to 11 hours or alternating periods of dark and light exposure for periods up to 18 hours were performed.

Determination of First Order Rate Constants

Before undertaking a kinetic study of regeneration, 10 ml of a 2% aqueous digitonin pigment preparation having an absorbance at 500 nm of 0.545 was diluted with 2% digitonin in 1 ml increments while A500 nm was measured after each addition. When the total volume reached 20 ml such that the original pigment solution was diluted 1:1, A500 nm was measured at .270. A plot of the relative concentration versus the absorbance at 500 nm yielded a straight line indicating that these preparations obey Beer's Law at 500 nm. First order rate constants were then evaluated from strip-chart recordings of ΔA 500 nm as a function of time by application of the following considerations:

We have, for a first order regeneration reaction, that

$$C_t = C^\infty (1-e^{-kt})$$

where C_t and C^∞ = the concentration of regenerated chromophore at times t and ∞, respectively. Since the absorbance increase at 500 nm may be taken to be a direct measure of the increase in chromophore concentration, we may write

$$(A_t - A_o) = (A^\infty - A_o)\ (1 - e^{-kt})$$

where A_t, A_o and A^∞ = the optical densities at 500 nm of the regenerating system at times t, o and ∞, respectively, and this may be rewritten as

$$(A^\infty - A_t) = (A^\infty - A_o)e^{-kt}$$

Thus, a plot of log $(A^\infty - A_t)$ versus the time t will give a straight line, the slope of which is the rate constant K/2.303. If the regeneration is carried out in the presence of excess 11-cis retinal, whose con-

centration may be considered to remain constant throughout the reaction, then k is in fact a pseudo-first order rate constant and must be equated to k^1R^a, where k^1 is a true rate constant, R is the concentration of 11-cis retinal and a is the order of the bimolecular reaction. A plot of k versus R will give a value to a.

CHAPTER III

RESULTS

Absorption Spectrum Properties of Moderately Purified Visual Pigment

Fig. 5 shows the absorption spectrum of a solution of rod outer segment preparations extracted with 2% aqueous CTAB. These extracts were characterized by peaks or shoulders found at 495-498 nm, 410-420 nm, 335-345 nm, 275-280 nm and 220-230 nm. These absorptions were ascribed to (a) the photosensitive α-band of visual pigment, 495-498 nm; (b) the Soret-band of a heme-protein impurity, 410-420 nm; (c) the photosensitive β-band of visual pigment 335-345 nm; (d) absorption due to the aromatic rings of the amino acids tyrosine and tryptophan incorporated in protein of visual pigment and other contaminating protein molecules, 275-280 nm; (e) transitions of the polypeptide chain, 220-230 nm. Fig. 5 reveals that illumination of these preparations resulted in the complete photodestruction of the α-band which subsequently gave rise to a photoreleased retinal species absorbing at 365 nm. Upon further incubation in the dark, the retinal species underwent a bathochromic spectral shift coupled together with a slightly decreased extinction Fig. 5. All-trans retinal solubilized in 2% aqueous CTAB absorbed maximally at 385 nm and was thus more analogous to scan 3 of this figure. The significance of this spectral shift will be discussed later in this dissertation. Fig. 6 shows

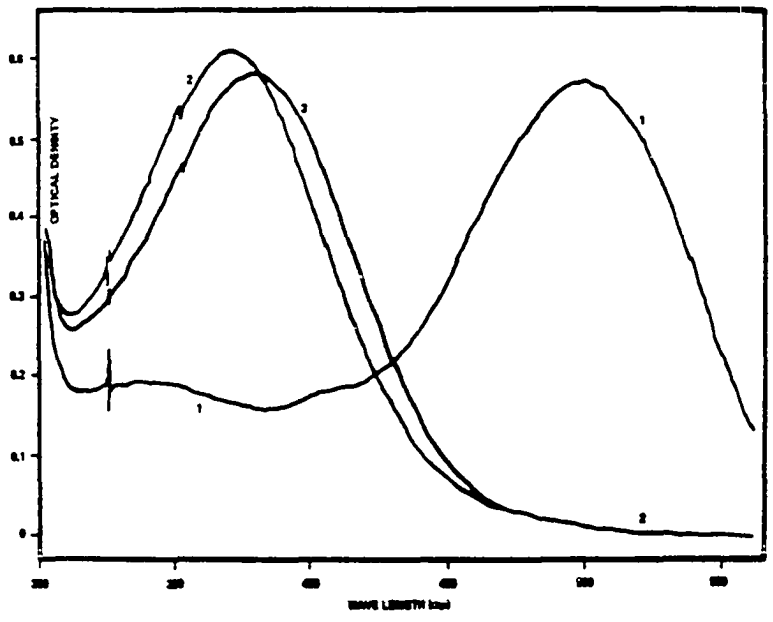


Figure 5. Absorption spectrum of highly purified visual pigment in cetyltrimethylammonium bromide. 1, unbleached; 2, photobleached; 3, dark 15 min. [Taken from Plante, E. O. and Rabinovitch, B. Enzymes in the regeneration of rhodopsin. Biochem. Biophys. Res. Commun. 46: 725-730 (1972).]

that unpurified extracts of visual pigment in CTAB do not undergo spectral shifts after photobleaching. Consequently no change in the nature of the photoreleased retinal species was observed.

Absorption Spectrum Properties of Rod Outer Segment Preparations

Fig. 7 reveals absorption spectrum aspects of rod outer segments solubilized in aqueous digitonin which were typical of laboratory preparations of limited purity. In all such preparations, a peak was readily distinguished in the area of 500 nm and was revealed to be the photosensitive α-band of visual pigment upon illumination. The non-photosensitive peak absorbing in the area of 410-420 nm was found in all the impure preparations and was ascribed to the Soret band of a heme-protein impurity. Upon further purification by solubility fractionation with ammonium sulfate or, by dilution with aqueous digitonin, an absorption peak at 275 nm due to the aromatic rings of the amino acids tyrosine and tryptophan was found. Purification or dilution also revealed a peak at 220-230 nm ascribed to the n,π^* transitions of the polypeptide chain. The photosensitive β-band of visual pigment absorbing at 335-345 nm was masked in all such preparations by light scattering. In many of the preparations, although not shown in Fig. 7, a peak at 440-465 nm was observed in the sample before photobleaching and was unassigned.

Illumination of these unpurified pigment solutions resulted in decreases of maximum extinction at 500 nm of only 60% of maximum, Fig. 7. In any given sample, an illumination time of 15 min. was sufficient to photobleach the pigment to its minimum 500 nm absorbance, and increased exposure time failed to further decrease the extinction. Maximum extinc-

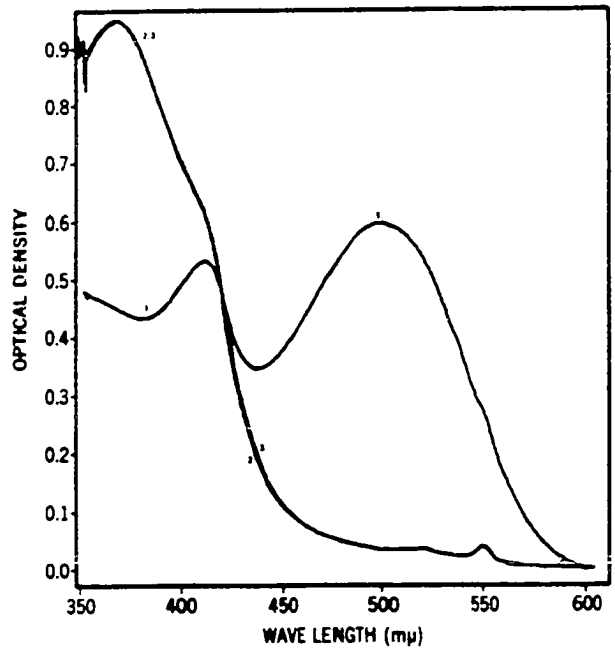


Figure 6. Absorption spectrum of unpurified visual pigment in 2% aqueous CTAB. 1, before illumination; 2, photobleached; 3, dark 15 min.

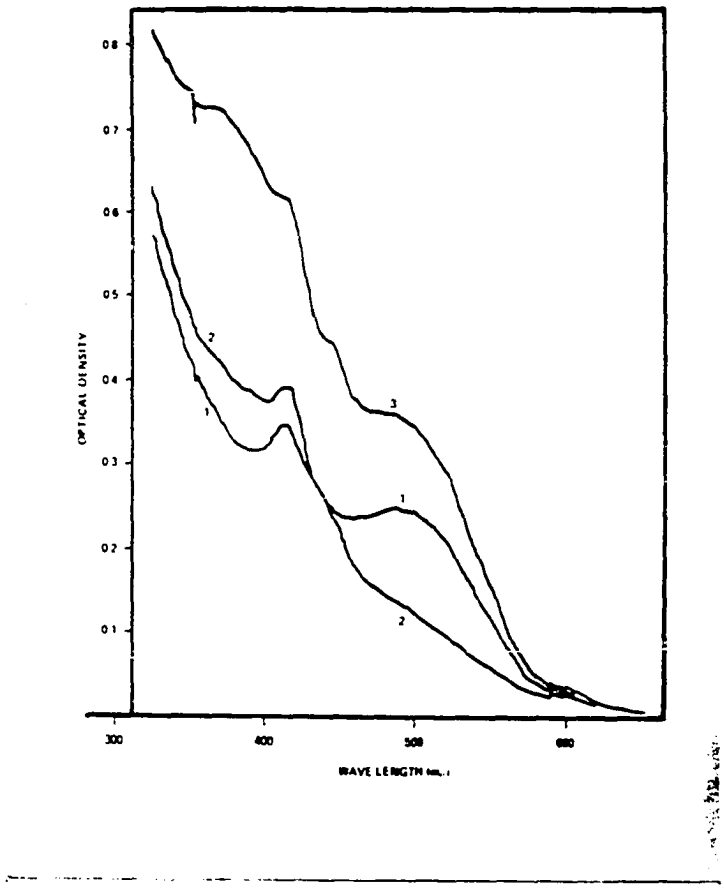


Figure 7. Absorption spectrum of unbleached, photobleached, and regenerated visual pigment in 2% digitonin. 1, unbleached; 2, photobleached; 3, regenerated in the presence of 2 x 10^{-5}M 11-cis retinal. [Taken from Plante, E. O. and Rabinovitch, B. Enzymes in the regeneration of rhodopsin. Biochem. Biophys. Res. Commun. 46: 725-730 (1972).]

tion differences between unbleached and photobleached preparations were found at ∿ 500 nm and ∿ 385 nm; the latter being presumably due to the photoreleased retinal species. Absorbance due to the released retinal was not sufficient to mask the heme-Soret band absorbing in the same general area. Illumination of these preparations manifested more clearly the species absorbing at 440-465 nm which was seen to appear as a broad shoulder of the heme-protein band at 410-420 nm. Scan 3 in this figure represents regenerated visual pigment; a subject which will be considered in detail later in this dissertation.

Fig. 8 shows the absorption spectrum of an impure preparation of visual pigment in CTAB in which the 440-465 nm absorption was identifiable before photobleaching. Scan 2 of this figure shows that illumination resulted in a complete loss of extinction in the 500 nm biological chromophore (unlike digitonin preparations) giving rise to a photoreleased retinal. Scan 3 will be discussed later.

The reader should note that peak absorbances of the different bands in visual pigment vary with the state of preparation purity and are influenced by adjacent bands. For simplicity's sake, an assigned value of the absorption peak within the wavelength range quoted will be used to characterize the band and will represent the most frequently observed value of the λ_{max}. Moreover, the photosensitive bands will be designated, P, while non-photosensitive absorbances will be assigned, NP. The following configuration for visual pigment will thus be adopted:

495-500 nm - P-500 - α band of Rhodopsin
440-465 nm - NP-465 - unidentified chromophore
410-420 nm - NP-420 - Soret band of heme-protein
335-345 nm - P-345 - β band of Rhodopsin
275-280 nm - NP-280 - γ band of Rhodopsin
220-230 nm - NP-230 - δ band of Rhodopsin

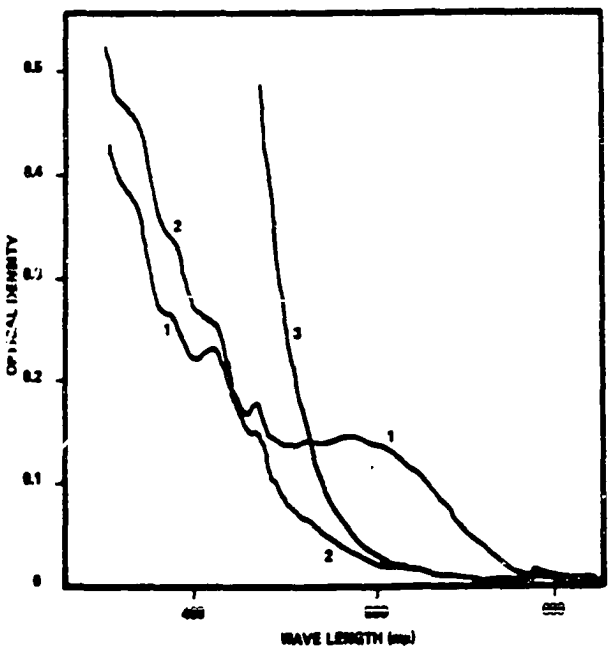


Figure 8. Absorption spectrum of unpurified visual pigment solubilized in 2% aqueous cetyltrimethylammonium bromide. 1, before photobleaching; 2, photobleached product; 3, incubated in the presence of excess 11-cis retinal.

Regenerated Pigment from Photoreleased Retinal

Illumination of visual pigment solutions in aqueous digitonin followed by dark incubation resulted in 10-40% regeneration of pigment as assayed spectrophotometrically by ΔA 500 nm. The extent of regeneration was seen to vary most prominently with the time of illumination and dark recovery of the biologically active chromophore. As seen in Table 1, longer periods of illumination and dark incubation, when coupled together, favored regeneration. A regeneration limit of 30% was achieved based upon the difference in 500 nm absorbance of unbleached and illuminated preparations. However, regeneration values of up to 40% were obtained by following the reaction course at 500 nm immediately after illumination was extinguished and, consequently without expending the time required to obtain a photobleached scan of the illuminated pigment solutions. It should be emphasized that the data presented in Table 1 was obtained from the photobleaching and regeneration of several samples of the same digitonin preparation of pigment and that, in each case, a 15 min. photobleaching time was sufficient to lower the 500 nm absorbance to its minimum value. Since additional periods of illumination beyond 15 min. did not further depress the 500 nm extinction but did increase the extent of regeneration, it seems logical to identify the resynthesis of pigment with the photoisomerization of all-trans retinal which was released from the chromophore upon photobleaching.

Table 2 shows that essentially no difference was found for illumination and regeneration procedures carried out at 30°C or 18°C thereby suggesting the lack of rate limiting enzymatic participation in the regenerating reactions.

TABLE 1

EFFECT OF ILLUMINATION AND DARK INCUBATION PERIODS ON THE EXTENT OF VISUAL PIGMENT REGENERATION IN AQUEOUS DIGITONIN SOLUTIONS

Illumination Period	Incubation Period	Regeneration (apparent)
min.	min.	%
15	10	10
15	15	15
15	30	17
10	30	14
30	10	14
30	20	20
30	30	24
30	90	29
30	120	30

TABLE 2

REGENERATION OF VISUAL PIGMENT FROM PHOTORELEASED RETINAL RESULTING FROM PHOTOBLEACHING OF VISUAL PIGMENT

	Regeneration (apparent)
VP[a] in Digitonin, 30°C	30
VP in Digitonin, 18°C	27
VP in Digitonin, photobleached at 500 nm	30
VP in CTAB[b]	0
VP in Triton X-100	0
VP in Digitonin plus VP in CTAB	0
VP in Digitonin plus 0.28% CTAB	0
VP in Digitonin plus Phospholipase A	0
VP in Digitonin plus Phospholipase C	0

[a]VP, visual pigment.

[b]CTAB, cetyltrimethylammonium bromide.

Visual pigment solubilized and purified in aqueous CTAB exhibited absolutely no tendency toward regeneration; a fact that is clearly demonstrated by Fig. 1. Moreover, unpurified CTAB extracts of visual pigment were found to be no more successful in producing pigment from its photolyzed product showing, quite clearly, that no essential ingredient(s) to regeneration are being excluded by purification in this detergent. Further experimentation revealed that mixing equal volumes of digitonin-solubilized and CTAB-extracted pigment, each in essentially the same state of purification, completely destroyed regeneration potential. These experiments demonstrate that, rather than there being present in the digitonin solution, a system that promotes regeneration, there is conversely a material in the CTAB extracts that inhibits regenerability. Furthermore, digitonin-pigment solutions made 0.28% with CTAB failed to regenerate any of the photosensitive chromophore from photoreleased retinal. Visual pigment was also found <u>not</u> to be regenerable when solubilized in Triton X-100.

In Fig. 9, a progressive sequence of illumination and regeneration events represented by the various scans reveal the nature of the regeneration process, as it occurs from the photoreleased retinal. Scans 1 and 2 were obtained for the unbleached and photobleached states of visual pigment, respectively. Scan 3 was obtained by allowing a dark incubation period of the photobleached product (scan 2). The regenerated pigment represented by scan 3 was then illuminated to obtain the product represented by scan 4. Dark incubation of this latter product produced the regenerated pigment in scan 5. This experiment was repeated many times with the same result and clearly shows that the regenerated pigment

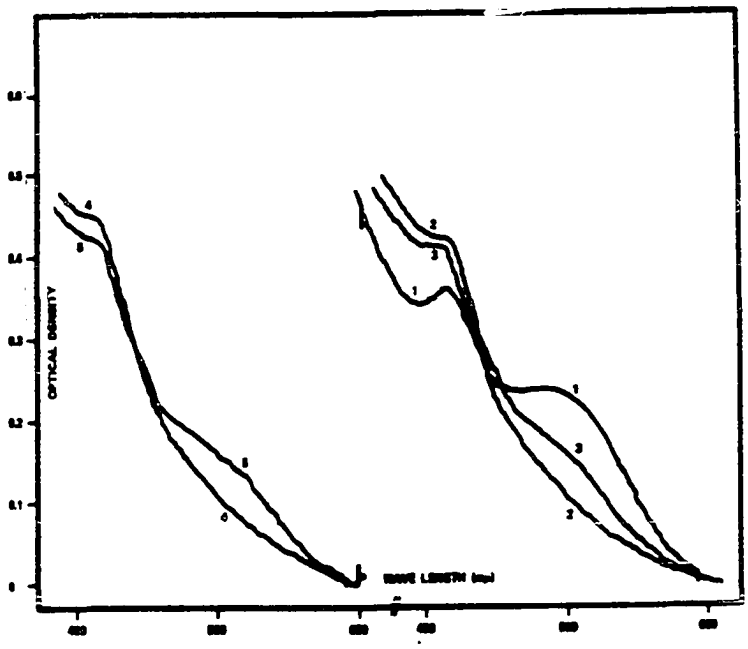


Figure 9. Absorption spectrum of unbleached, photobleached, and regenerated visual pigment in 2% digitonin. 1, unbleached visual pigment; 2, photobleached products; 3, regenerated from photobleached product; 4, photobleached products of the regenerated material; 5, regenerated again from the photobleached product in curve 4. All absorption spectra in this figure were determined against a blank of 2% digitonin.

is not partially inactivated toward regeneration resulting from some intrinsic factor of photolysis, because if this were true, an overall loss of extinction at 500 nm would be seen with each photolysis-regeneration cycle and Fig. 9 shows that this is not the case at all. Once the pigment is photobleached, it can be regenerated to the extent of approximately 30% through two successful illumination and regeneration cycles. However, this experiment does not, in itself, reveal whether in fact a change in retinal or in the lipoprotein is the cause of the loss of quantitative recovery of the biological chromophore. The absence of an isosbestic point in the illuminated and regenerated scans demonstrated the presence of a product other than retinal and visual pigment in these systems. This product can be seen forming in the 440-465 nm region of Fig. 9. Successive photobleach and regeneration steps demonstrate that once this product is formed it cannot be photobleached and consequently is non-photosensitive. In Fig. 15, C and D, difference spectra of the regenerated pigment minus the photobleached product of visual pigment reveal the normal tendency toward formation of this non-photosensitive product (C) and the minimum tendency toward formation (D). However, even in difference spectrum (D) a broadening of the absorption band in the 465 nm region can be seen. In many of these experiments spectral assays of the regenerated material after 15 min. incubation periods represented such a delicate balance in the formations of the photosensitive biological chromophore at 500 nm (P-500) and the non-photosensitive material absorbing at 440-465 nm (NP-465) that complete merging of the photobleached and regenerated spectra was observed between 500 and 440 nm. These difference spectra also show that the major pigment regenerated in

these experiments absorbs maximally at 500 nm and may therefore be equated with rhodopsin. Whether or not some isorhodopsin, a chromophore which involves 9-cis retinal and absorbs maximally at 487 nm, is formed is not known from these experiments, but if so, it is most certainly lower in concentration than the product generated from 11-cis retinal. Comparison of illuminated and regenerated scans in Fig. 9, and the difference spectra for regeneration in Fig. 15, reveal that both P-500 and NP-465 are formed at the expense of a retinal species absorbing at 380-390 nm. Free all-trans and 11-cis retinals were both found to absorb maximally at 380-390 nm in ethanol, CTAB or digitonin.

Regeneration from An Added Source of All-Trans Retinal

In Table 3, the regeneration results obtained by alternately illuminating and regenerating a sample of visual pigment in digitonin, alone, and with added all-trans retinal are presented as a progressive sequence of events. The sample of unbleached pigment was first illuminated for 15 min. and allowed to regenerate from photoreleased retinal. The extent of recovery of absorbance at 500 nm was measured at 10 min. intervals throughout the dark incubation phase and reached a maximum of 25% after a period of 40 min. This regenerated pigment was then photobleached during another 15 min. illumination period and subsequently incubated in the dark with added all-trans retinal (6×10^{-5}M). A recovery value of 24% was recorded in 30 min. and was observed to be clearly reaching a maximum at that point showing that little, or no isomerization to the 11-cis form occurs when all-trans retinal is incubated with opsin. This regenerated product was then photolyzed as described before

TABLE 3

REGENERATION OF VISUAL PIGMENT FROM PHOTOISOMERIZED RETINAL

	Incubation	Regeneration (apparent) %
VP in 2% Digitonin	10	13
	20	18
	30	22
	40	25
VP in 2% Digitonin + All-Trans Retinal (Dark)	10	17
	20	21
	30	24
VP in 2% Digitonin + All-Trans Retinal (Light)	10	80
	20	100
	30	114
VP in 2% Digitonin + All-Trans Retinal (Light) Photobleached	10	49
	20	63
	30	73
	40	76
	50	77

Regeneration of visual pigment from photoreleased retinal, added all-trans retinal, and photoisomerized all-trans retinal, as measured by A 500 nm. Conc. of rhodopsin is 1×10^{-5}M; conc. of all-trans retinal is 6×10^{-5}M.

and allowed to reform in the dark. A regeneration value of 114% as measured by ΔA 500 nm was found after 30 min. dark incubation. This product was allowed to stand in the dark for 18 hrs. to insure complete formation of the chromophore and was then illuminated again. Subsequent dark incubation produced the surprising result of a decreased rate and extent of recovered pigment formation. The value of 114% produced by photolyzing regenerated pigment in the presence of all-trans retinal is not so surprising in view of the fact that all-trans retinal was present in sufficient excess to provide even a slight excess of cis isomer formed as a photoisomerization product. Apolipoprotein (opsin), present in the original retinas or arising from thermal bleaching or unsuspected photobleaching during preparative procedure, could easily account for the slightly better than quantitative yield of regenerated pigment. However, since visual pigment is not partially inactivated toward regeneration by the act of photolysis itself, the depressed regeneration resulting from the 2nd illumination of pigment in the presence of all-trans retinal can only be explained by a decrease in the amount of one or both reacting species.

In separate experiments, visual pigment was regenerated many times from photoisomerization products of all-trans retinal (Fig. 10). Spectra of the regenerated samples revealed an absorption maximum at 470 nm which was too low for either rhodopsin generated from 11-cis retinal (500 nm), or, isorhodopsin generated from 9-cis retinal (487 nm). Photobleaching of these regeneration pigments resulted in a decrease in extinction in the 500 nm area and a simultaneous shift of the peak absorbance in the product to 465 nm. This same pattern for the regeneration

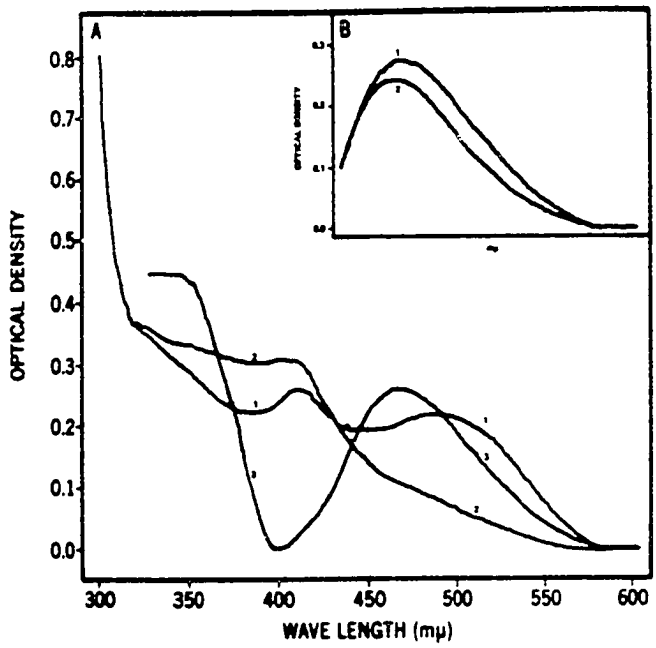


Figure 10. Regeneration of visual pigment from photoisomerized all-trans retinal. 1, unbleached; 2, photobleached; 3, regenerated 60 min. from 1.66 x 10^{-5}M all-trans retinal illuminated 15 min. in both sample and blank.

Insert - photobleaching of the regenerated material. 1, before photobleaching; 2, photobleached 15 min.

was obtained regardless of whether all-trans retinal was photoisomerized in situ with the photobleached pigment or photoisomerized before addition. These results show that a photosensitive chromophore absorbing maximally in the region of 475-525 nm is produced from a photoisomer of all-trans retinal during regeneration while a nonphotosensitive chromophore absorbing at 465 nm is formed from the same or some other photoisomer of all-trans retinal. As a "note of proof" of the nonphotosensitivity of the 465 nm chromophore, both the unbleached and photobleached regenerated pigments were observed to be intensely orange in color, while that of native rhodopsin is pink or purple.

Regenerated Pigment from An Added Source of 11-Cis Retinal

Fig. 7 shows that absorbance at 500 nm can be recovered far in excess of the quantitative amount based upon the 500 nm absorbance of pigment before photobleaching, by the simple addition of 11-cis retinal. The concentrations of visual pigment and added retinal were 6×10^{-6}M and 1.67×10^{-5}M respectively such that an excess of 11-cis retinal was provided. An equal measure of 11-cis retinal provided to the blank demonstrated spectral properties near identical to those observed with the photoisomerized all-trans retinal system (Fig. 10). A peak absorbance at 470 nm which shifted to 465 nm upon photobleaching was observed, thus, repeating the same pattern observed for regeneration from photoisomerized all-trans retinal (Fig. 10). It then appears that the nonphotosensitive chromophore absorbing at 465 nm (NP-465) can be generated at the expense of 11-cis retinal but not from all-trans retinal. This point is demonstrated most unequivocally in Fig. 11 where the scans were obtained for

a visual pigment preparation that had been fractionated with ammonium sulfate and diluted in 0.2% digitonin. Scan 1 represented a 3 hr. incubation of all-trans retinal (2.5×10^{-6}M), which had been added to both the sample and the blank. Scan 2 was produced by adding 11-cis retinal (2.5×10^{-6}M) to both sample and blank and allowing a dark incubation period of 1 hr., whereas scan 3 represented the readdition of 11-cis retinal (2.5×10^{-6}M) followed by a 4 hr. dark incubation period. Addition of 11-cis retinal or photoisomerization of all-trans retinal produces the same general pattern as observed in Fig. 11.

The emergence of spectral changes resulting from the dark incubation of 11-cis retinal with outer segment preparation solubilized in aqueous digitonin can best be observed in Fig. 12-A. Scan 1 was obtained for a visual pigment preparation which had been ammonium sulfate fractionated and diluted with 0.2% digitonin. Scans 2 and 3 were obtained 1 min. and 45 min. after adding 11-cis retinal (2.5×10^{-6}M) respectively and show that extinctions were increased in the 470 nm and 320 nm areas at the expense of a decrease in extinction in the 380-400 nm area (retinal). Scans 2 and 3 represented additions of 11-cis retinal to the sample only whereas scan 4 was produced by also making the blank 2.5×10^{-6}M with 11-cis retinal after the sample had been incubated for 60 min. Scan 4 can be compared with scan 1 to obtain the same general pattern that was seen to be developing in 2 and 3.

The scans presented in Fig. 12-B represent further progressions of the same experiment. Thus, after a dark incubation period of 4.5 hrs., scan 1 was produced. Scan 2 was obtained as the photobleached product of this regeneration. This latter product was placed in the

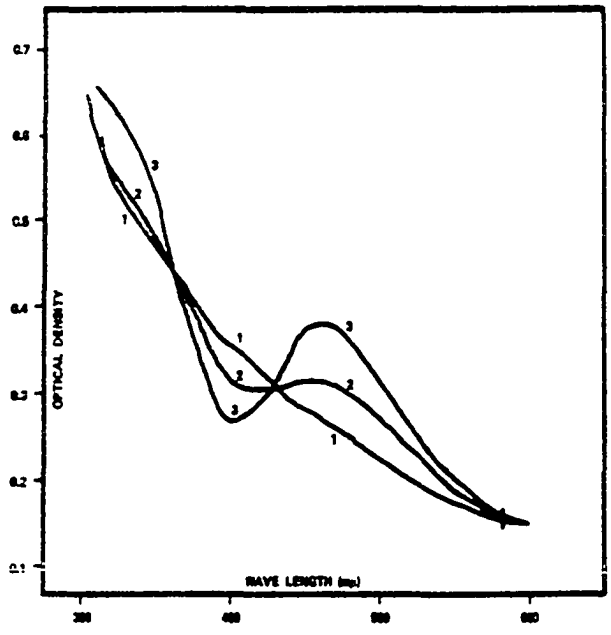


Figure 11. Absorption spectrum of visual pigment diluted with 0.2% digitonin, incubated initially with all-trans retinal and subsequently with 11-cis retinal. 1, three hr. incubation with all-trans retinal; 2, one hr. incubation with 11-cis retinal; 3, four hr. process involving photobleaching and readdition of 11-cis retinal.

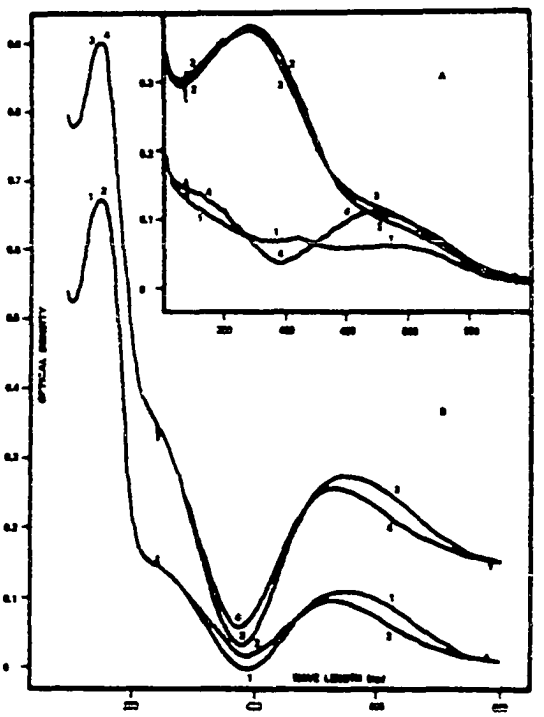


Figure 12. A - Absorption spectrum of visual pigment diluted with 0.2% digitonin and made 10^{-5}M with 11-cis retinal. 1, diluted visual pigment; 2, one min. incubation with 11-cis retinal; 3, forty-five minute incubation with 11-cis retinal; 4, blank of 0.2% digitonin also make 10^{-5}M with 11-cis retinal.

B - Absorption spectrum of diluted visual pigment regenerated and photobleached in the presence of 10^{-5}M 11-cis retinal added to the sample as well as the 0.2% digitonin blank. B-1, regenerated 4.5 hrs.; B-2, photobleached product of the 4.5 hr. regeneration; B-3, regenerated 40 hrs.; B-4, photobleached product of the 40 hrs. regeneration. Displacement on the optical density axis is artificial.

dark for 35.5 hrs. and scan 3 was determined while 4 was the photobleached product. Displacement along the optical density axis was necessary to obtain the minimum at 390 nm for 3 and 4. It is seen that the formation of photosensitive chromophore had reached its limit, whereas the generated absorptions at 465 nm and 320 nm were not so limited. Moreover, the absorption band at 320 nm, it should be noted, was also non-photosensitive.

Figures 13, 14 and 15 contain comparisons of difference spectra obtained for a given preparation of ROS solubilized in digitonin. Fig. 13 compares the photobleaching of native material to that of the same preparation which had been incubated with 11-cis retinal before photobleaching. This experiment demonstrated quite definitely that bleached visual pigment was present in the original preparation and that it was still viable in reforming pigment with 11-cis retinal. Thus, the recovery of pigment beyond the theoretical amount seen in Fig. 7 is not exclusively due to generated NP-465. Fig. 14 compares the photobleaching of a 11-cis retinal regenerated material to its regeneration, before it was photobleached. In this experiment, visual pigment was first photobleached (1), and subsequently regenerated with 11-cis retinal (2). This regenerated material was then photobleached to obtain (3). Difference spectrum A was then obtained by (2) - (3) whereas B was determined by (2) - (1). Therefore, the observable difference in extinction of the positive peak that exists between these two difference spectra reflects the synthesis of NP-465. Fig. 15, A and C reveal more clearly the bichromophoric synthesis in the 450-550 region at the expense of retinal. Both C and D show that, in the presence of excess opsin and limited

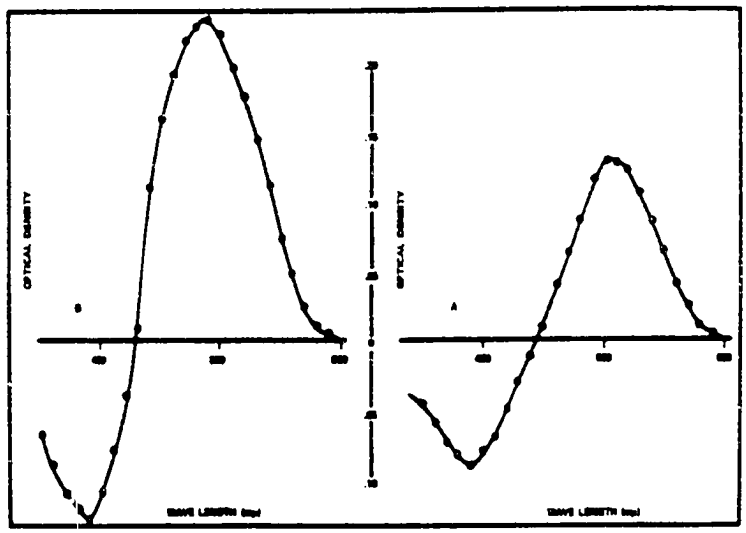


Figure 13. Properties of visual pigment in 0.2% digitonin as revealed by difference spectra. A, unbleached visual pigment - photobleached product; B, unbleached visual pigment regenerated in the presence of 2×10^{-5}M 11-cis retinal - the photobleached product of this regeneration.

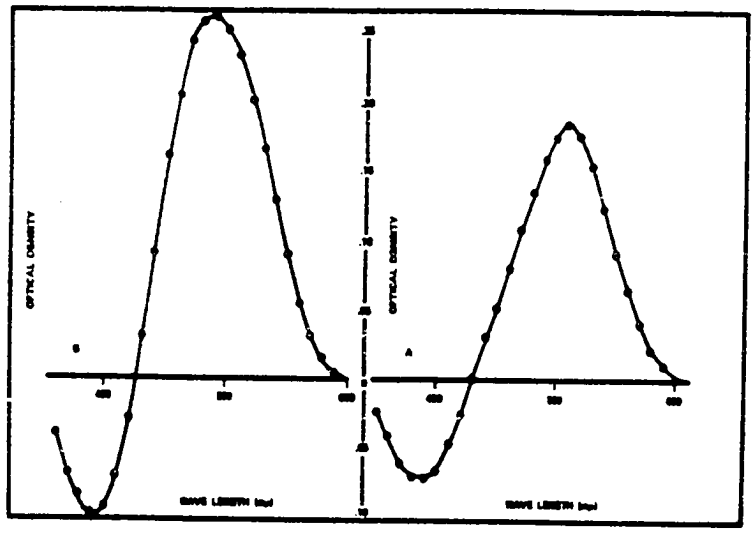


Figure 14. Properties of visual pigment in 0.2% digitonin as revealed by difference spectra. A, photobleached visual pigment regenerated in the presence of 2 x 10^{-5}M 11-cis retinal - the photobleached product of this regeneration; B, photobleached visual pigment regenerated in the presence of 2 x 10^{-5}M 11-cis retinal - the photobleached product of unbleached visual pigment.

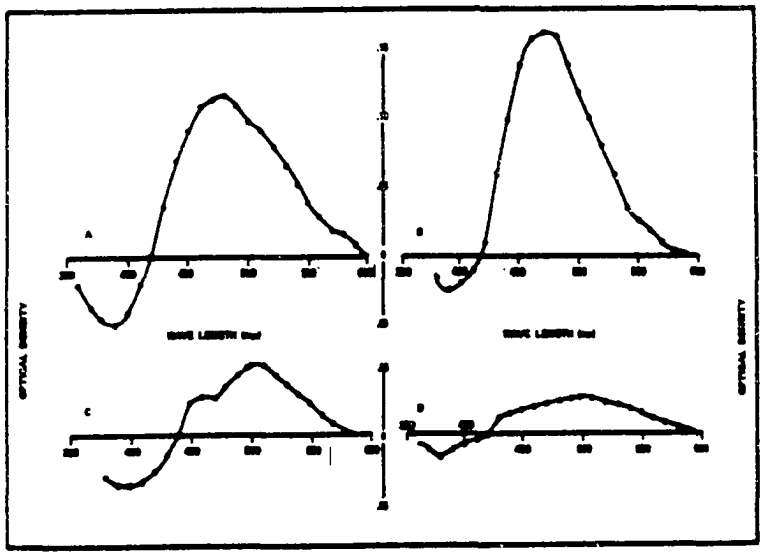


Figure 15. Properties of visual pigment in 0.2% digitonin as revealed by difference spectra. A, unbleached visual pigment regenerated in the presence of 1 x 10^{-5}M 11-cis retinal vs. unbleached visual pigment; B, photobleached visual pigment regenerated in the presence of 1 x 10^{-5}M 11-cis retinal vs. unbleached visual pigment; C, photobleached visual pigment regenerated 60 min. in the presence of photoreleased retinal from unbleached pigment vs. the photobleached product of unbleached visual pigment; D, photobleached visual pigment regenerated 15 min. in the presence of photoreleased retinal from unbleached pigment vs. the photobleached product of unbleached visual pigment.

amounts of 11-cis retinal, P-500 is clearly regenerated in preference to NP-465. However, neither of these difference spectra can reveal with certainty whether the preference for P-500 is a kinetic one or is merely reflecting a difference in ε_M between these two chromophores. Regardless of whether the preferred regenerated chromophore (P-500) is a kinetic expression or simply has a larger ε_M, the upper limit of absorption of 500 nm in difference spectrum A indicates saturation of the photosensitive α-band of the excess opsin in these preparations which is still viable to regeneration when combined with 11-cis retinal. Yet another aspect of the bichromophoric synthesis can be seen by comparing A and B of this figure with regard to their difference in shape in the 500 nm region. This comparison suggests that, once the pigment preparation is photobleached (B) and regenerated in the presence of 11-cis retinal, some net synthesis of NP-465 occurs at the expense of some loss of the photosensitive band (P-500). The possible meaning of this result will be considered in more detail in the discussion section.

In Table 4 are listed some of the regeneration properties of visual pigment in the presence of excess 11-cis retinal. Regeneration was seen to occur rapidly and completely at 30° and at 18°C. However, pigment at the same level of purification in CTAB (Fig. 8), or in Triton X-100 showed no tendency to regenerate in the presence of 11-cis retinal. Moreover, pigment in digitonin combined with pigment in CTAB, and also pigment in digitonin to which only 0.28% CTAB was added did not regenerate (Fig. 16). Rate measurements of regeneration revealed that as little as .07% CTAB resulted in some inhibition (Fig. 17). These results coupled together with those obtained for regeneration from photoreleased

TABLE 4

REGENERATION OF VISUAL PIGMENT FROM AN ADDED EXCESS OF 11-CIS RETINAL

	Regeneration (apparent)
VP[a] in Digitonin, 30 C	100+
VP in Digitonin, 18 C	100+
VP in CTAB[b]	0
VP in Triton X-100	0
VP in Digitonin plus VP in CTAB	0
VP in Digitonin plus 0.28% CTAB	0
VP in Digitonin plus Phospholipase A	100+
VP in Digitonin plus Phospholipase C	100+

[a]VP, visual pigment.

[b]CTAB, cetyltrimethylammonium bromide.

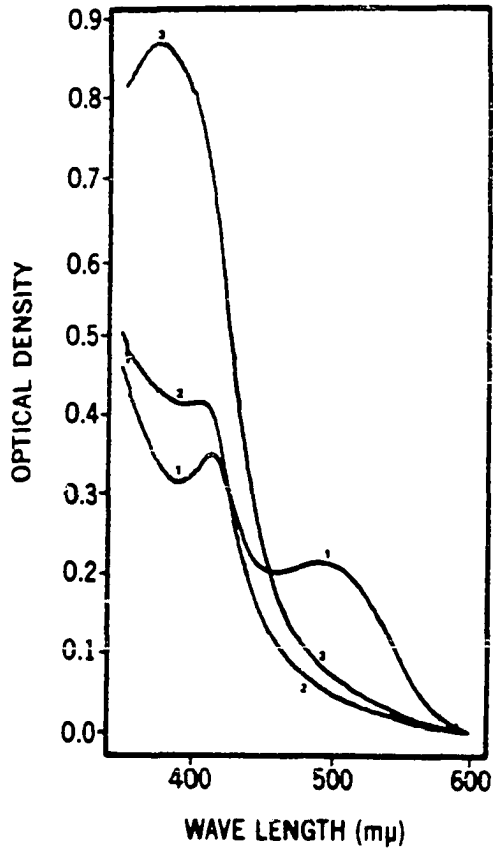


Figure 16. Regeneration of unpurified visual pigment in 2% aqueous digitonin from an added source of 11-cis retinal and CTAB. 1, before photobleaching; 2, photobleached; 3, addition of 1.66×10^{-5}M 11-cis retinal and 0.28% CTAB.

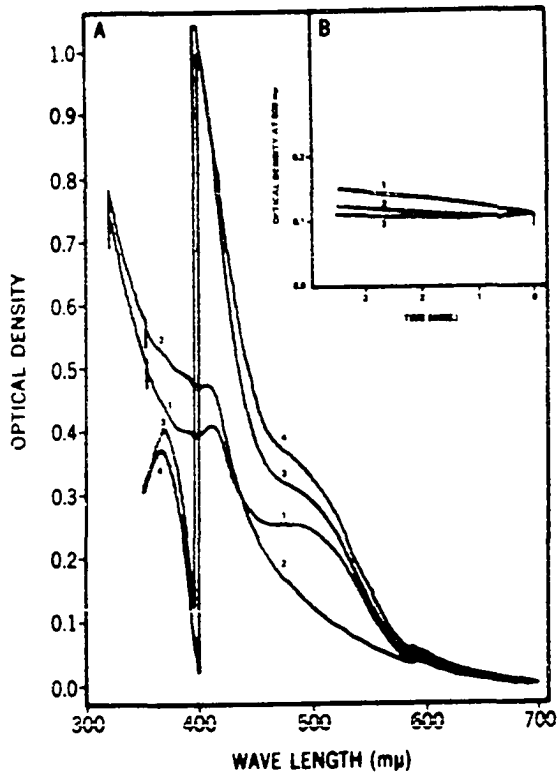


Figure 17. Regeneration of unpurified visual pigment in 2% aqueous digitonin from an added source of 11-cis retinal and CTAB. 1, before photobleaching; 2, photobleached; 3, regenerated 30 min. from 3.33×10^{-5}M 11-cis retinal and 0.07% CTAB; 4, incubated 24 hrs.

Insert - rate recovery of 500 nm absorption as function of CTAB concentration. 1, .07% CTAB; 2, .14% CTAB; 3, .28% CTAB.

retinal in which CTAB was present (Table 2) serve to demonstrate the inhibitory character intrinsic to CTAB.

Regenerated Pigment from An Added Source of 9-Cis Retinal

Fig. 18-A shows that an added source of 9-cis retinal produced the same pattern of spectral change as that seen to emerge upon addition of 11-cis retinal (Fig. 12-B). Absorption was seen to increase in the 420-520 and 320 nm areas at the expense of 9-cis retinal absorbing at 380-390 nm. The results obtained by photobleaching the pigment generated from 9-cis retinal are seen in Fig. 18-B. The maximum difference between scans 1 and 2 is clearly displaced below 500 nm and is consistent with the production of isorhodopsin λ_{max} (487 nm). Comparison of the cross-over point between the expended and generated chromophores upon photobleaching in this figure (λ 410 nm) with that of Fig. 12-A (440 nm) suggests that the nonphotosensitive absorption generated from either 11-cis or 9-cis retinal are similar if not identical. Thus, the 11-cis and 9-cis retinal both appear to generate NP-465.

Fig. 19 shows that the nonphotosensitive absorptions in the 465 and 320 nm spectral regions can also be formed in the presence of light. Scan 1 was obtained for a 60 min. dark incubation of ammonium sulfate fractionated and diluted visual pigment in 0.2% digitonin with 11-cis retinal. The sample was then continuously illuminated for 20 hrs. immediately after which scan 2 was determined. Thus, in the 20 hr. light incubation period which served to "photosuppress" formation of P-500, any net increase in absorbance of NP-465 or NP-320 would have to occur (a) independently of P-500 and (b) in a constant field of illumination

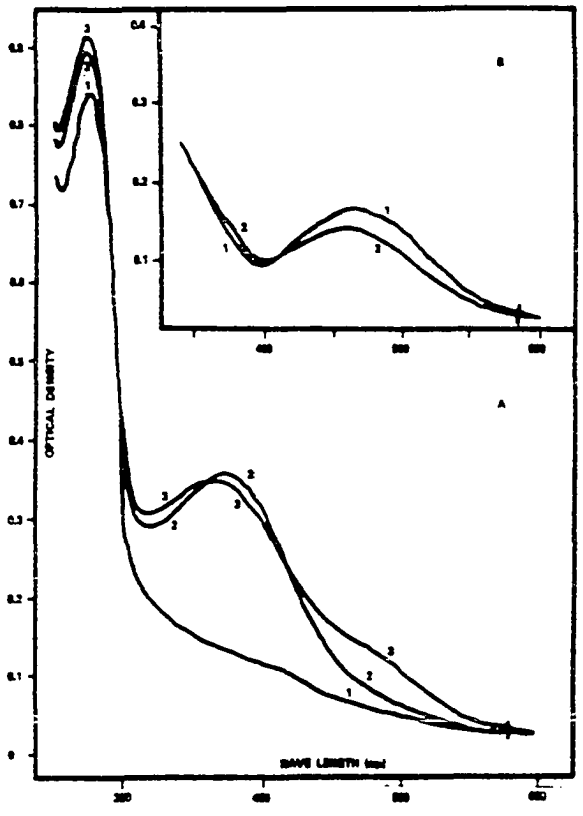


Figure 18. A - Absorption spectrum of visual pigment diluted with 0.2% digitonin and made 10^{-5}M with 9-cis retinal. A-1, diluted visual pigment; A-2, one min. incubation with 9-cis retinal; A-3, forty-five min. incubation with 9-cis retinal.

B - Absorption spectrum of diluted visual pigment regenerated and photobleached in the presence of 10^{-5}M 9-cis retinal in both the sample and the blank. B-1, regenerated 20 hrs.; B-2, photobleached product of the 20 hr. regeneration.

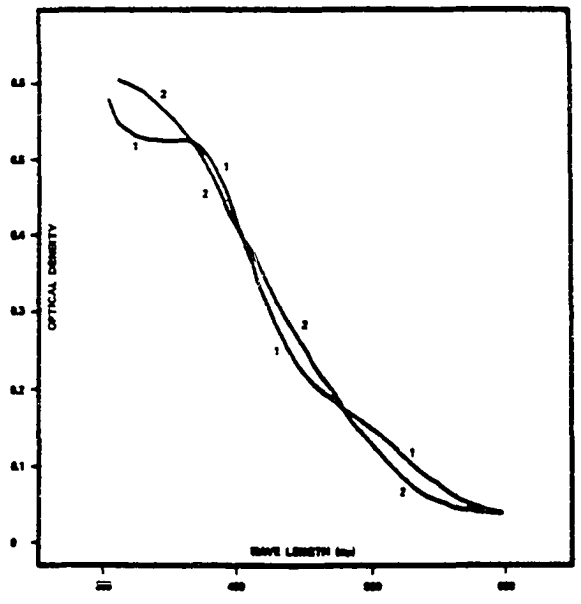


Figure 19. Absorption spectrum of diluted visual pigment regenerated and photobleached in the presence of 10^{-5}M 11-cis retinal added to the sample only. 1, regenerated one hr.; 2, continuously photobleached twenty hrs.

serving to photoisomerize the added 11-cis retinal to an equilibrium dispersion of isomers including among others, all-trans, 11-cis and 9-cis retinals. Limited decrease in absorbance at 380 nm (retinal) was observed in the difference spectrum because 11-cis retinal ($\varepsilon_M \sim 27,000$) is predominately photoisomerized to the all-trans form ($\varepsilon_M \sim 41,000$) upon illumination.

Assignment of Retinal Isomers to NP-465 and NP-320

As shown in Table 5, all-trans, 11-cis and 9-cis isomers of retinal were equally capable of generating the increased absorption at 320 nm. On the other hand, only the 9-cis or 11-cis forms were capable of forming NP-465. Fig. 20 shows that added all-trans vitamin A alcohol failed to generate P-500 or NP-465. Illumination of these samples also failed to increase extinction in the 450-550 spectral region. However, it must be stated that the availability of photons required for photoisomerization from the illuminating source at this wavelength (325 nm) is limited due to a rapid decrease in intensity below 350 nm.

Original Presence of NPS-465 in Visual Pigment

Fig. 21-A demonstrates that the nonphotosensitive absorption at 465 nm is originally present in ROS preparations solubilized in aqueous digitonin. The spectrum of visual pigment precipitated from 20% ammonium sulfate saturation, washed and resolubilized in 0.2% digitonin was obtained as scan 1. Scan 2 was obtained for the photobleached product while scans 3 and 4 were seen when all-trans retinal was added to the blank in concentrations of 2.5×10^{-6}M and 5×10^{-6}M respectively. The concentration of photoreleased retinal in the sample was determined to

TABLE 5

ASSIGNMENT OF ADDED RETINAL ISOMERS TO GENERATED ABSORPTION BANDS OF ROS SOLUBILIZED IN DIGITONIN

Absorption Band	Retinal Isomer Involved in Synthesis
P-500	11-cis
P-487	9-cis
NP-465	11-cis or 9-cis
NP-320	all-trans, 11-cis and 9-cis

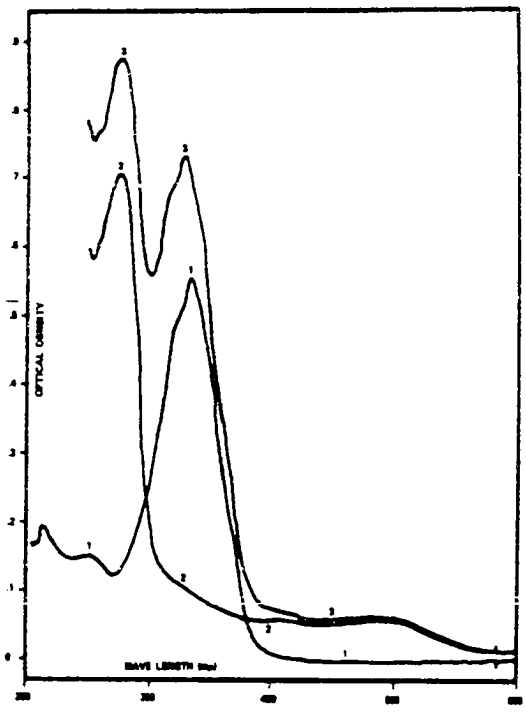


Figure 20. Absorption spectra of 1.25 x 10^{-4}M all-trans vitamin A and diluted visual pigment incubated with 1.25 x 10^{-4}M all-trans vitamin A. 1, vitamin A in 0.2% digitonin; 2, diluted visual pigment; 3, one hr. incubation with vitamin A.

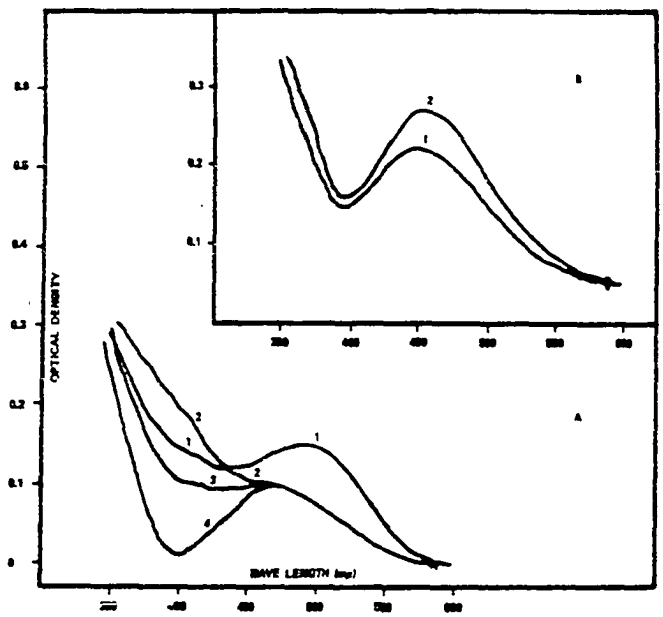


Figure 21. A - Absorption spectrum demonstrating the original presence of NPS-465 in visual pigment solubilized in aqueous digitonin. 1, before photobleaching; 2, photobleached product; 3, blank made 2.5 x 10^{-6}M with all-trans retinal; 4, blank made 5 x 10^{-6}M with all-trans retinal.

B - Absorption spectrum demonstrating the progressive synthesis of NPS-465. 1, twenty hr. incubation of visual pigment with 11-cis retinal and subsequently photobleached; 2, three hr. incubation with additional 11-cis retinal and subsequently photobleached.

be 2.5 x 10^{-6}M such that scan 3 presumably represents a balance of absorption due to retinal. Fig. 21-B shows the progressive synthesis of the "photoimmune" chromophore as a basis for comparison.

As opposed in preparations in CTAB, illumination of ROS solubilized in digitonin failed to produce the pure yellow coloration seen when retinal is added to the blank. The sample was amber and became increasingly orange with the progressive formation of NP-465 from either 11-cis or 9-cis retinal. These observations are consistent with the nonphotosensitive absorption at 465 nm.

Phospholipase Treatment of Pigment Solubilized in Digitonin

Incubation of either phospholipase A or C with ROS solubilized in digitonin micelles resulted in no change in extinction of the photosensitive chromophore (P-500) but, increased the absorbance seen to occur in the area of 380-480 nm. Illumination of pigment preincubated 1 hr. with phospholipase A or C "photodestroyed" P-500 as expected (Fig. 22-A, Scan 2). However, dark incubation to obtain regenerated pigment from photoreleased retinal failed with both phospholipases. In fact, preincubation with either phospholipase resulted in a slight further loss of extinction at 500 nm after the dark regeneration period (Scan 3). Most surprisingly, addition of excess 11-cis retinal at this point resulted in regeneration for both phospholipase A- and C- treated materials, but, at an observed decrease in rate (Table 4).

Fig. 22-B shows that photosensitivity at 500 nm is renewed in photobleached preparations preincubated with phospholipase C after 10 hr. dark incubation period. Scan 1 was obtained for a photobleached prepara-

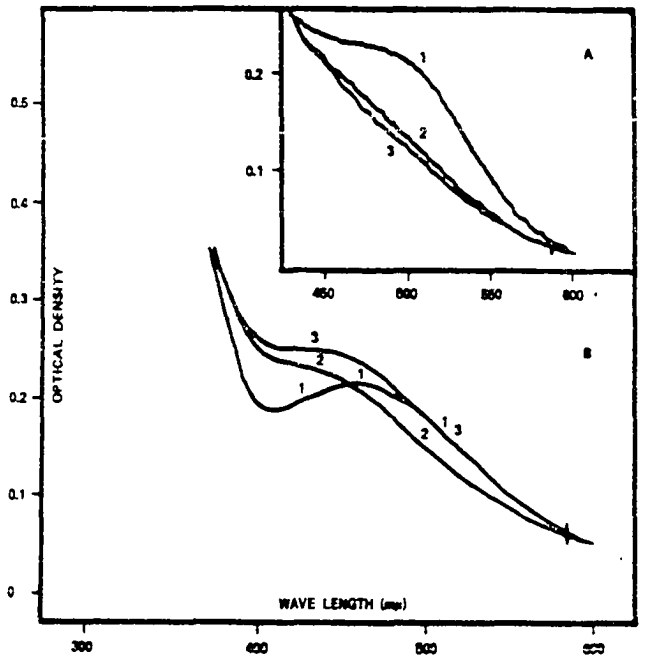


Figure 22. A- Effect of phospholipase C upon regeneration of visual pigment from photoreleased retinal. 1, unbleached visual pigment incubated 60 min. with phospholipase C; 2, photobleached product; 3, incubated in the presence of photoreleased retinal one hr.

B - Progressive effect of phospholipase C upon photobleached visual pigment. 1, ten hr. incubation of photobleached visual pigment with phospholipase C; 2, three successive one hr. incubations (dark) interspersed with fifteen min. photobleaching steps terminating with a photobleaching step; 3, eighteen hr. dark incubation.

tion of pigment on which the absorption due to photoreleased retinal was balanced by adding all-trans retinal to the blank. Scan 1 was made after dark incubation with phospholipase C for a period of 10 hrs. Scans 2 and 3 show the effects of photobleaching and subsequent incubation in the presence of phospholipase. The effects of phospholipase added directly to pigment solubilized with digitonin are then to (a) decrease the rate (but not the extent) of regeneration from either photoreleased retinal or added 11-cis retinal and (b) continuously increase with time the absorbance at 465 nm in either the unbleached or photobleached state.

Regeneration Rates of Photobleached Pigment

Fig. 23-A is a direct trace from the strip-chart recorder of the recovery of absorbance at 500 nm for a pigment preparation in digitonin illuminated 10 min. Longer illumination periods increased both the rate and extent of recovered absorbance. Rate 2 was produced by adding excess all-trans retinal of which little precaution had been taken to prevent cis isomerization in storage. When fresh samples of all-trans retinal were employed, little difference in rate and extent of regeneration from added all-trans or photoreleased retinal was observed as indicated previously in Table 3. Rate 3 was recorded using the same sample of all-trans retinal that produced rate 2, together with "retinal isomerase". When compared to 2, the reduced rate in 3 reflected a slight dilution by the "isomerase". Various ammonium sulfate fractions of the "isomerase" preparation also failed to increase the regeneration rate.

In Fig. 23-B, rates 4, 5 and 6 were obtained by adding excess 11-cis retinal. Increased concentrations of retinal resulted in increased regeneration rates.

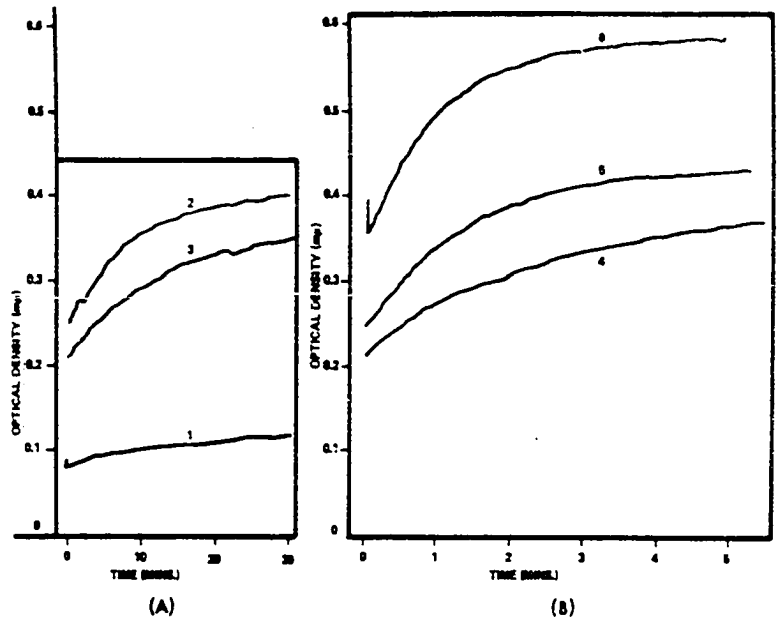


Figure 23. Rates of regeneration of photobleached visual pigment at 500 nm. 1, in the absence of any added retinal; 2, with all-trans retinal; 3, with all-trans retinal plus "retinal isomerase"; 4, with added 11-cis retinal, concentration 3.33×10^{-5}M; 5, 6.66×10^{-5}; 6, 10×10^{-5}M.

Kinetics of the Regeneration Process

Addition of 11-cis retinal to ROS solubilized in digitonin clearly raises the extinction of two bands absorbing close together in the visible spectrum. A kinetic examination then becomes difficult due to the contribution made by each band to the absorbance at any particular wavelength in this spectral region. However, as seen by the difference spectrum in Fig. 15-C, the photosensitive chromophore (P-500) either is favored kinetically or has a much higher ε_M compared to NP-465. In either event, initial velocities taken in the first few minutes after adding 11-cis retinal should reflect largely the regeneration rate for P-500.

Fig. 24 shows first order nature of the regeneration rates for product formed at 500 nm, and serves to illustrate scatter in the data obtained for several sample measurements. Pseudo first order rate constants were evaluated from plots such as these and were subsequently plotted against retinal concentration to develop the linear dependence seen in Fig. 25. It is obvious that the regeneration process remains strictly first order throughout the reaction. Enzyme kinetics demands that the product formation rate be first order only initially, reducing to zero order as the enzyme becomes saturated with substrate shifting the dependence from substrate concentration to enzyme concentration. It is then very clearly indicated that the coupling process of 11-cis retinal to opsin is of a nonenzymatic nature. However, the reader is cautioned against an unequivocal assessment of this conclusion since some biological enzymes have Km's of the order 10^{-3}M whereas the maximum concentration of retinal practical to employ in these experiments was

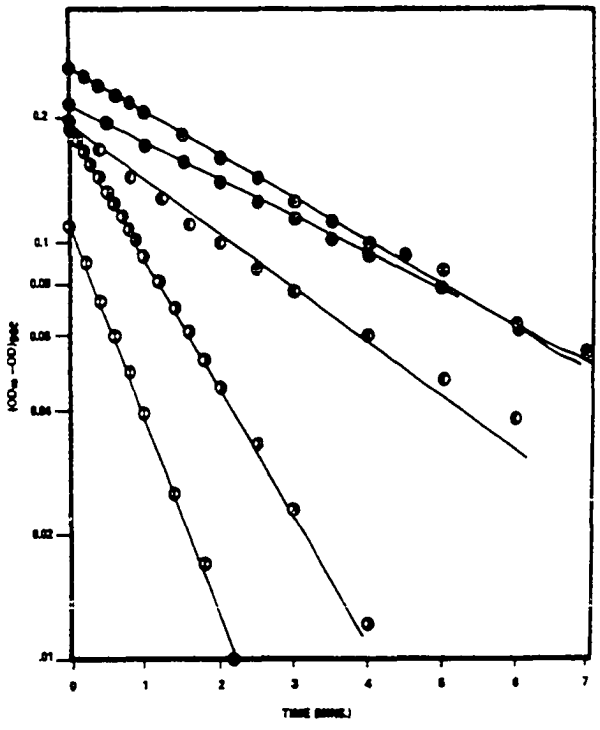


Figure 24. First order nature of regeneration rates with respect to product formation at 500 nm. ⊖, concentration of 11-cis retinal 6.66×10^{-5}; ●, 6.66×10^{-5}M; ◐, 6.66×10^{-5}M; ◑, 13.3×10^{-5}M; ◒, 13.3×10^{-5}M.

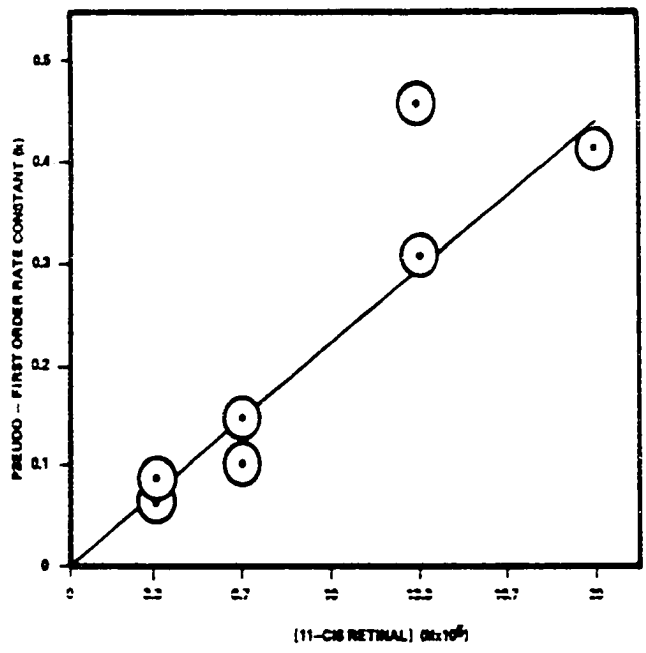


Figure 25. Dependence of the pseudo first order rate constant for the regeneration of visual pigment in several essentially constant concentrations of 11-cis retinal. [Taken from Plante, E. O. and Rabinovitch, B. Enzymes in the regeneration of rhodopsin. Biochem. Biophys. Res. Commun. 46: 725-730 (1972).]

2×10^{-4}M. Nevertheless, it does seem logical that some deviation from the linear relationship would be seen at the lower concentrations in the case of an enzymatically controlled coupling process.

CHAPTER IV

DISCUSSION

Regeneration and Conformational Change of Visual Pigment

An acceptable explanation of the continuous availability within the viable eye of unbleached visual pigment in a continuing moderate intensity light source, as well as the phenomenon of dark adaptation, demands that the photosensitive chromophore regenerate in both the presence and absence of light. Moreover, the regeneration process must necessarily result in essentially 100% chromophore recovery. These assumptions are based upon the thesis of a very simple photochemical interpretation of dark and light adaptation. Partial inactivation of pigment resulting from photolysis is a condition that the eye cannot tolerate, for the biosynthetic turnover cycle is too slow (7-9 days as measured by Young (38)) to maintain an adequate pigment supply. Thus, the eye, constantly bathed in light, must achieve for itself a delicate balance of the photolysis and regeneration processes. Assuming for the moment that the only retinal available for regeneration is that present in the active site of visual pigments, a purely photophysical scheme involving the reactions,

1) Rhodopsin $\xrightarrow{h\nu\ (k_1)}$ Rhodopsin*

2) Rhodopsin* $\xrightarrow{\Delta H\ (k_2)}$ Opsin + all-trans retinal

3) All-trans retinal $\xrightarrow{h\nu\ (k_3)}$ 11-cis retinal

4) 11-cis retinal + opsin $\xrightarrow{\Delta H\ (k_4)}$ Rhodopsin

where rhodopsin* is an electronically excited state of rhodopsin can satisfy the demands imposed upon the retina only if certain specific restrictions are imposed. These restrictions center about reactions 3) and 4) in this sequence for, it is clear that these reactions forming pigment must compete with reactions 1) and 2) which destroy pigment in the light exposed eye.

The necessary conditions that must be satisfied in the eye are:

a. incomplete pigment depletion during all ordinary illumination, since blindness does not result,

b. a relatively slow regeneration of rhodopsin during dark adaption,

c. total recovery of rhodopsin during dark adaption, that is, the absence of free regenerable opsin in the totally dark adapted eye,

d. little or no free all-trans retinal in the light adapted eye since the unexposed retina from such an eye is clearly purple.

To meet these conditions we must postulate, for this purely photophysical scheme,

i. no irreversible changes in opsin during photolysis, otherwise neither a. nor c. are met

ii. a quantum yield for step 3 much greater than for step 1, otherwise neither c. nor d. are met

iii. a much slower step 4 than step 2, otherwise b. is not met.

Postulate i. is acceptable since the turnover time for opsin is

known to be too long to explain dark-adaptation. Postulate iii. is reasonable since it is well known that the decay of electronically excited states to the ground state is rapid, at worst in the order of 10 seconds, as well as the fact that step 4 must be the rate determining step. To consider postulate ii.; the relative quantum efficiency requirements, the following steady state rate equations may be examined in which the stationary concentrations are given by:

1. $\frac{dRh^*}{dt} = k_1, (I)(Rh) - k_2Rh^* = 0$

2. $\frac{dtrans}{dt} = k_2Rh^* - k_3I.\ (trans) = 0$

3. $\frac{d(11)}{dt} = k_3I.\ (trans) - k_4\ (11)\ (OP) = 0$

4. $\frac{-dRh}{dt} = k_1\ (I)\ (Rh) - k_4\ (11)\ (OP) = 0$

where I = incident light intensity;
Rh = rhodopsin;
Rh* = an excited state of rhodopsin;
trans = all-trans retinal;
OP = the apoprotein opsin; and
11 = 11-cis retinal

Thus, it follows that

5. $\frac{Rh}{(11)(OP)} = \frac{k_4}{k_1,1}$

6. $\frac{Rh^*}{Rh} = \frac{k_1,1}{k_2}$

7. $\frac{(trans)}{Rh^*} = \frac{k_2}{k_3 1}$

8. $\frac{(11)(OP)}{trans} = \frac{k_3 1}{k_4}$

substituting (7) into (6) or (8) into (5), we obtain

9. $\frac{k_1}{k_3} = \frac{\text{trans}}{\text{Rh}}$

But since we know Rh >> trans, it follows that $k_3 >> k_1$, and postulate ii. would appear to be proven.

In the in vitro situation, reaction (3) fails the requirements for the photolysis-regeneration processes of this particular model by inefficient photoisomerization to 11-cis retinal. Although a limit of 40% regeneration is obtained from photoisomerization of retinal released by photolysis of the chromophore this level of recovery is clearly not good enough to explain dark adaptation. That reaction (3) is very significant in the regeneration of pigment is affirmed by the 40%, or for that matter, even 30% recovery of the chromophore absorbing maximally at 500 nm. 11-cis retinal is the least favored isomer, thermodynamically (25), and consequently exists at a higher energy state than any other retinal isomer including the 9-cis form which regenerates isorhodopsin absorbing maximally at 487 nm (2, 18). Upon photoexcitation of the π electrons in the retinal polyene chromophore to their corresponding anti-bonding orbitals the imposition of restricted rotation around the double bond is removed and, in the eye, this momentary unrestricted rotation, before decay to the ground state, must in some way be channeled toward the 11-cis form. This process is to be distinguished from that of a normally occurring equilibrium dispersion of stable retinaldehyde photoisomers which would result in the production of some 9-cis retinal forming isorhodopsin in the ROS which is itself not found in the normal seeing eye (9, 27). The quantitative regeneration of chromophore must then reflect a more efficient manifestation of the light induced steric mechanism, operating in reaction 3. To deny this fact is to call upon an unidentified

source of 11-cis retinal which (a) bathes the ROS membranous discs but (b) is "photoimmune" to π,π^* transitions producing photoisomers other than the 11-cis form. This latter concept is, in the first place, inconsistent with the fundamental nature of membranous involvement for functional proteins which is to eliminate the element of substrate diffusion. In this respect, for an efficient photolysis regeneration cycle, assuming the only retinal available is that present in the chromophoric site, it is illogical that photoreleased retinal could escape the confinement of the particular membranous disc from which it arises in the in vivo condition. To insure against substrate diffusion, the photoreleased retinal may never be hydrolyzed from its Schiff base linkage to protein but remains as N-retinylidine (all-trans) opsin (N-R opsin) stabilized by an intimately associated phospholipid in which the 22:6 docosohexanoic acid (all-cis) (3) in the 2 position participates to direct the photolyzed prosthetic group back to its original 11-cis chromophoric state by forming a stable association complex. Fig. 5 indicates that the immediate chromophoric product of photolysis in CTAB (purified preparation) is one absorbing at approximately 365 nm and could represent N-R opsin (λ max, 367 nm). In the 15 min. interval following photolysis, this photoproduct is hydrolyzed to free retinal absorbing maximally at approx. 380 nm. The first product is then taken to be the unprotonated form of a retinaldehyde Schiff base which has become vulnerable to hydrolysis after photobleaching. Unpurified extracts of visual pigment in CTAB (represented by Fig. 6), decay to the retinaldehyde imine linkage (365 nm) which is the stable end product of photolysis, even though these preparations do not regenerate, indicating considerable protection

against hydrolysis is present in the unpurified form. However, digitonin solubilized ROS preparations at least partially photobleach to a retinal end product absorbing maximally at 380-390 nm. Free retinal in aqueous digitonin has its absorbance maximum at 380 nm. It may well be that the use of the detergent digitonin results in weak lipid-protein associations in the membranes such that some hydrolysis of N-R opsin occurs relegating a portion of the released retinal to diffusion chemistry within the digitonin micelle. However, once hydrolysis occurs, only the 11-cis or 9-cis isomer can reform N-R opsin because Fig. 5 shows that the apoprotein does not recognize added all-trans retinal, but readily reacts with added 11-cis retinal.

Thus, it is concluded that, while visual pigment could be conceivably completely reformed from retinal present in the chromophore as the only source available to regeneration, the requirements for this model are indeed stringent and cannot be experimentally met using the in vitro preparations. As previously discussed, reaction 3 is the weak-link in the photolysis-regeneration scheme. This fact is well documented throughout the results section whereupon addition of 11-cis retinal results in a net synthesis of the photosensitive chromophore. In the normal state of the eye, reaction 3 must, in some way be boosted to meet its proportionate share of the burden the retina must bear in returning the concentration of visual pigment present in the ROS to its dark-adapted level. However, a source of 11-cis retinal, other than that in the intact chromophore itself, has never been demonstrated in the eye. Yet we know that there must be some cource of retinal present to be incorporated in the resynthesis of the ROS pigment which is completely

turned over from time to time, because without a source of vitamin A, the outer segments degenerate (16). The unknown source must then be all-trans retinal which should be available for regeneration as well as complete renewal of the chromophore. In this scheme, there would be an excess of all-trans retinal beyond that provided by the chromophore itself such that, in the presence of free energy supplied by light and an unknown steric entity directing the synthesis of 11-cis retinal to the exclusion of other retinal photoisomers, reaction 3 would be capable of supplying enough 11-cis retinal to form the dark-adapted level of ROS pigment.

For the active site in opsin to recognize 11-cis retinal stereospecifically, so as to regenerate true visual pigment, it should be considered to be intact; a condition which seems to be diametrically opposed to the hypothesis that, upon photolysis of retinal, thermal steps are initiated throughout which the active site becomes progressively unravelled producing a neural response (1, 20, 35). Moreover, that rhodopsin is spontaneously (nonenzymatically) synthesized stereospecifically from 11-cis retinal does not conform well to the thesis that photoinitiated thermal steps result in large changes in protein conformation involving the active chromophoric site. Then to recapitulate the argument, regeneration would require the stereospecific recognition of 11-cis retinal by an unravelled site which has resulted from photolysis and conformational changes and which must then refold itself into its native conformation, nonenzymatically. Thus, it is concluded that, while visual pigment in purified form or in strong detergents may undergo irreversible conformational changes involving the chromophoric site upon photolysis,

no such changes should be observable for the membrane-bound regenerable protein unless, of course, the conformational change occurs at an allosteric site. In these experiments, ROS solubilized in digitonin failed to reflect changes in extinction at 278 nm upon photobleaching which have been commonly observed in purified preparations (1, 20, 28, 33). However, it could be stated that the lack of effect observed here may be masked by protein absorbance at 278 nm other than that due to rhodopsin itself. But, on the other hand, that changes in extinction of this absorption band for rhodopsin actually reflect protein conformational changes must really be questioned. It is much more satisfying that Shichi was unable to detect evidence of conformational changes associated with photobleaching of membrane bound pigment by employing the method of circular dichroism, measuring changes in molecular ellipticity at 230 nm (30).

It would then appear that in digitonin pigment preparations, photoisomerization of 11-cis retinal at the intact chromophoric site produces an inherent instability in the molecule leading to hydrolysis and release of the all-trans retinal species in the in vitro preparations. However, the 11-cis retinal configuration energetically favors reformation of the retinal-opsin linkage. Whether or not rhodopsin has an energy requirement for regeneration cannot be known with certainty for this endowment could be enzymatically bestowed upon the molecule before or during photolysis and thereby escape detection since the enzyme would not be a factor in controlling the regeneration rate. For example, the pigment protein may have to be enzymatically phosphorylated before it can be regenerated and this requirement could be met either before, or, at the

time of photolysis. However, it can be argued that this is not the case since pigment can be successively photobleached and regenerated thereby rapidly exhausting the source of energy required for pigment resynthesis.

In addition, Shichi has treated ROS preparations with phospholipase A and determined that no regeneration of visual pigment occurs (31). In a previous paper, he had determined that a phospholipid is involved in stabilizing an asymmetric form of the chromophore (30). He thus assumed that other phospholipids were involved in stabilizing the native protein in a conformation required for regeneration. Pretreatment of ROS in digitonin with phospholipase (Fig. 14A) confirms that the results obtained for a 60 min. regeneration time fail to produce any recovery of P-500. However, addition of 11-cis retinal restores regeneration but, at a slower rate (Table 4) implying a more intimate requirement for phospholipid at the intact chromophoric site.

The finding of excess opsin in these preparations most likely reflects the state of the light-adapted eye, since all preparations of pigment in these experiments were made from retinas obtained from the eyes of freshly slaughtered cattle which were not previously dark-adapted. Moreover, the intact pigment in the membrane-bound form is very stable, thermodynamically, as evidenced by the fact that digitonin solubilized preparations could be stored at 3°C for periods up to 45 days with no loss in extinction or ability to regenerate upon photobleaching. It is then unlikely that unsuspected thermal bleaching could account for the excess opsin present, and, since isolation and preparative procedures were carried out in the dark or under dim red light, it is unlikely that unsuspected photobleaching could be a contributing factor.

Whatever the source of excess opsin, its presence gives rise to misleading results regarding the quantitative resynthesis of pigment when either 11-cis retinal or photoisomerized all-trans retinal is employed as the additive agent to photobleached pigment preparations. A comparison of the difference spectra in Fig. 13B and Fig. 14A exemplifies the lack of quantitative regeneration when 11-cis retinal is utilized to reform pigment. These difference spectra were obtained by identical procedures of photolysis and dark incubation in the presence of identical amounts of 11-cis retinal and identical initial concentrations of rhodopsin. The extent of regeneration can be quantitatively assessed at 76% assuming the extinction difference in Fig. 13B at 500 nm to represent the theoretical maximum value of this visual chromophore present. However, if this chromophore cannot be quantitatively regenerated after photobleaching it is illogical that it can be quantitatively regenerated before photobleaching. Thus, a somewhat higher theoretical value for the maximum absorption due to P-500 must exist and, this fact in turn would lower the true value of regenerable pigment below 76%. Further consideration of this aspect of regeneration will be given later in this section.

Prevalence and Nature of the Nonphotosensitive Bands Absorbing at 320 and 465 nm

Generation of the absorbance at 320 nm (NPS-320) is not stereospecific for a small segment of the polyene chain, as is P-500 or NP-465. This is evidenced by the fact that this band is formed from all-trans retinal, as well as, from the 9-cis and 11-cis forms. Absorption at 320 nm is consistent with the presence of the alcohols formed from these

various retinaldehydes (Fig. 20) (17, 34). The equilibrium between these oxidized and reduced species would be displaced far in the direction of the alcohol and is consistent with the formation of NP-320. However, since addition of vitamin A which has been exposed to light fails to regenerate any of the P-500 chromophore (Fig. 20), a shadow of doubt must be cast upon NP-320 as representing an equilibrium between retinaldehyde and its corresponding alcohol. NP-320 is then considered to be only a spurious reaction of the added retinaldehyde with protein in the preparations.

Since NP-465 is formed stereospecifically from 11-cis or 9-cis retinal (to the exclusion of all-trans retinal) and absorbs along with P-500 in the visible spectral region, it deserved an extra measure of consideration. Fig. 13A shows that NP-465 is either originally present in ROS membranes or arises from preparative procedures. Shichi has also published a spectrum for sonicated suspensions of ROS particles (membrane bound) in which he alludes to the 465 nm chromophore in the figure but fails to discuss it anywhere in the text (29). Shichi's spectrum is very similar in the visible region to scans 1 and 2 in Fig. 13A of this dissertation.

If NP-465 arises merely as an artefact of preparation, then, its stereospecific generation is most difficult to explain since the process requires that a protein be present in ROS membranes which is capable of distinguishing between the various retinal isomers. Stereospecificity of protein-substrate interactions is a condition imposed by a system for biological purpose. Fig. 11 demonstrates that NP-465 is formed separately from the photosensitive chromophore (P-500), so that it cannot be

an artefact resulting from the regeneration of P-500. The increasing intensity of an orange pigment observed as NP-465 generates, demonstrates that the electronic transitions occurring in this region of the spectrum are real and not the result of nonspecific absorption (light scattering) due to the addition of 11-cis retinal.

In terms of its absorption maximum, NP-465 represents an approximate 80 nm bathochromic shift from the absorption peak in retinal and could be explained possibly by formation of a protonated Schiff base between retinal and an ROS protein (absorption maximum - 440 nm) (8), but, it is difficult to explain how protonation of the nitrogen can be effected at the near-neutral pH of these solutions. Moreover, it is equally difficult to explain the extreme "photoimmunity" of NP-465 in terms of such a structure. Even if we concede that NP-465 need not be photochemically sensitive, we would expect it, as a simple Schiff base of 11-cis retinal with protein, to be photophysically sensitive. A π,π^* transition releasing the strain inherent in the 11-cis isomer (or 9-cis isomer) would manifest itself in an extinction increase as a result of the formation of all-trans isomer. No such changes are observed in NP-465 suggesting that the 11-cis (or 9-cis) configuration is indeed protected from light induced changes. Also, whereas N-R opsin is a pH indicator, NP-465 is not, since titration with aqueous base fails to lift a proton from the nitrogen, shifting the protonated Schiff base to its unprotonated form absorbing maximally at 367 nm (23). Moreover, titration with either acid or base over the pH range of 1-14 failed to result in a shift of the spectrum.

On the other hand, all the observed facts are explainable if we

take NP-465 to be the Schiff base of phosphatidyl ethanolamine, N-retinylidene phosphatidyl ethanolamine (N-RPE) which reportedly absorbs maximally at 455 nm (3). N-RPE is known to be stable only in a completely anhydrous medium, the presence of minute quantities of water leading immediately to hydrolysis (12). Thus, a hydrophobic environment would have to be supplied for the stability of N-RPE, and this could most easily derive from the hydrophobic side chains of the amino acids of the opsin in the vicinity of the chromophore (7). The alternative is to assume it to be a Schiff base of a protein in which a phospholipid covalently bound to protein envelops the retinal by intimate association of the 22:6 fatty acid. In such a hydrophobic atmosphere, not only would the Schiff base be stabilized but the aldimine nitrogen could be internally protonated by the phospholipid phosphate (12). The fatty acid would then present a potential energy barrier to free rotation such that the all-trans isomer could not be formed.

Biological Significance of the NP-465 Chromophore

The requirement for 11-cis retinal in the structure of NP-465 is analogous to that of rhodopsin (photosensitivity excluded) and it is easy to imagine a biochemical transformation resulting in an asymmetric and photosensitive configuration of the chromophore; a process requiring a mere red-shift of another 33 nm. Thus, that NP-465 is an intermediate in the path along the way to P-500 is worth considering. However, Fig. 4 shows that NP-465 is generated from photoreleased retinal as a separate and competing process to P-500, i.e., as P-500 is alternately photobleached, and regenerated, NP-465 is progressively synthesized. To further affirm that NP-465 competes with P-500 for the 11-cis isomer, the

data in Table 3 indicates that the first regeneration from photoisomerized all-trans retinal consumed a considerable amount of this polyene that was not available for the 2nd regeneration. Absorption spectra revealed that considerable NP-465 was formed in the 1st regeneration. NP-465, which is then originally present in preparations of ROS and is stereospecifically generated from 11-cis retinal appears to be curiously cast in the role of competing with P-500 for photons in the photobleaching process as well as competing for 11-cis retinal in the regeneration phase. In the in vivo system, the eye is not likely to tolerate meaningless competition. Visual pigment is then pictured as consisting of two separate and distinct chromophores involving 11-cis retinal. One such chromophore (P-500) is spontaneously regenerated from 11-cis retinal and must function to maintain a steady state concentration of photobleachable pigment in the retina whereas, the other (NP-465) captures and maintains 11-cis retinal in a photoprotected state during light exposure and is probably involved in the dark adaptive phase of pigment recovery which is not manifested by the in vitro preparations. The bovine (rod) eye would thus be adapted to function at the two extremes of light intensity presented by the terrestrial phenomenon of "night" and "day".

If the assumption of two chromophores involving 11-cis retinal in visual pigment is correct, then P-500 must be spontaneously regenerated to an approximate level of 50%. By multiplying the difference in optical density at 500 nm between Fig. 13A and Fig. 14B by a factor of 2 and adding this value to the 500 nm extinction of Fig. 13B, a regeneration extent of 54% can be calculated for the recovered P-500 in Fig. 14A. This value of regeneration was found to be typical of the results ob-

tained by difference spectra developed according to these procedures. However, the reader must be cautioned against interpreting these results to mean that there are two chromophores in one molecule of rhodopsin. An equivalent explanation would be to assume the presence of two different types of rhodopsin, or subunits, in the ROS bearing very similar, if not identical, spectral properties. One such molecule or subunit would be spontaneously regenerable and function to maintain a steady state concentration of pigment in the eye whereas the other would regenerate slowly after the illuminating source was extinguished leading to dark-adaptation.

CHAPTER V

SUMMARY

Bovine visual pigment consists of 11-cis retinal, a derivative of Vitamin A, conjugated to a protein of molecular weight, 26,000 and contains numerous phospholipids which are intimately associated with the protein. Photobleaching of visual pigment releases all-trans retinal which must then be reisomerized to the 11-cis form before the pigment can be regenerated. It was experimentally determined that light photoisomerizes some of the released all-trans retinal to the 11-cis form such that 30% of the original pigment present absorbing maximally at 500 nm could be regenerated. However, in addition to a primary photosensitive chromophore, P-500, a second, nonphotosensitive absorption band generated stereospecifically from 11-cis retinal and absorbing maximally at 465 nm was found, and therefore was termed NP-465.

Addition of 11-cis retinal to photobleached preparations of visual pigment resulted in the quantitative recovery of pigment in excess of the original amount present leading to the conclusion that some protein which had lost its retinal prosthetic group was present in the original preparations. Difference spectra based upon the total amount of pigment (both conjugated and unconjugated protein) revealed that only 54% of the biologically important, photosensitive chromophore (P-500) could be regenerated whereas considerable amounts of NP-465 were also

formed from the addition of 11-cis retinal to pigment preparations. Thus, it was concluded that visual pigment consists of two chromophores containing 11-cis retinal.

A kinetic study revealed that the photosensitive chromophore, P-500, is spontaneously, nonenzymatically synthesized by addition of 11-cis retinal to photobleached pigment preparations and consequently, must be involved with maintaining a steady state concentration of pigment in the bovine retina. It was then concluded that the nonphotosensitive chromophore, NP-465, must be involved in the dark-adaptive phase of pigment regeneration but that the full process is incomplete in the *in vitro* preparations employed in these experiments.

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