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PURIFICATION AND BLEACHING STUDIES OF THE BOVINE VISUAL PIGMENT, RHODOPSIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

STEPHEN JOHN GIRSCH

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PURIFICATION AND BLEACHING STUDIES OF THE BOVINE VISUAL PIGMENT, RHODOPSIN

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TO GRACE, BOTH FAMILIES, AND SIN-SIN

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS - (Continued)

PURIFICATION AND BLEACHING STUDIES OF THE BOVINE VISUAL PIGMENT, RHODOPSIN

CHAPTER I

INTRODUCTION

The primary processes of vision occur in one of the most specialized of tissues, the retina (37). Lying against the inner surface of the eye, Figure 1, the retina presents a light-sensitive surface to incoming photons. On closer inspection, a cross-section of the retina exhibits four distinct regions, as shown in Figure 2: a transparent limiting membrane containing sheathes of nerve fibers, a synaptic processes layer, a section of vertically aligned columnar structures referred to as photoreceptors, and finally the pigment epithelium, anchored to the choroid.

Structure and Molecular Architecture of the Photoreceptor Cell

In Figure 3b is shown a diagrammatic representation of a vertebrate photoreceptor cell. Structurally the cell is divisible into an inner segment and an outer segment. The former is 20-40 μ in length, containing the nucleus, ciliary

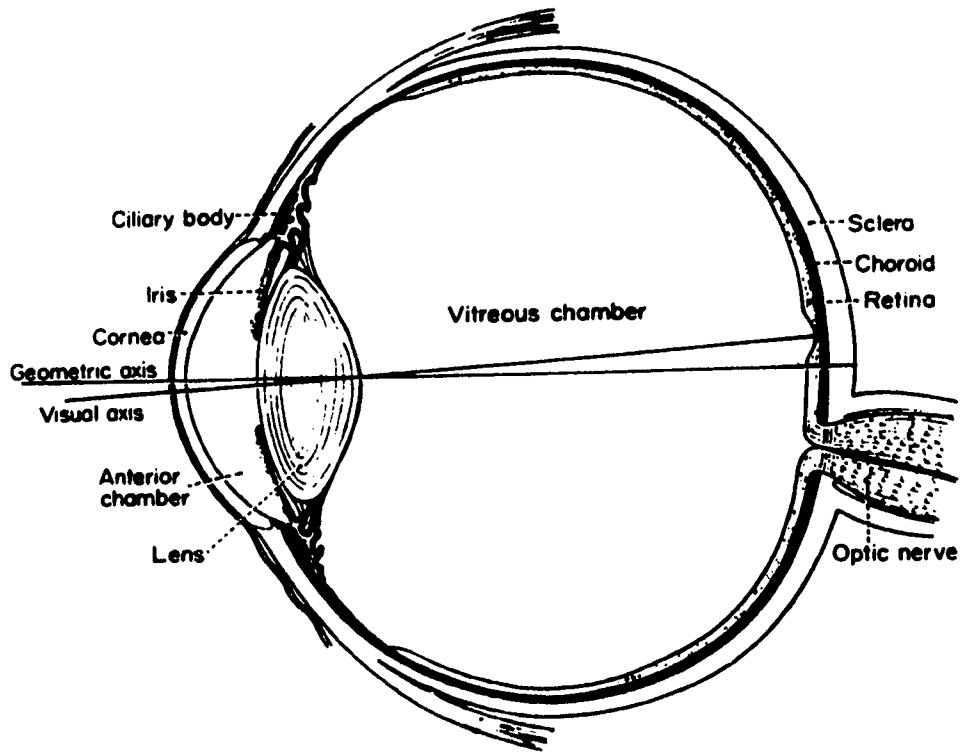


Figure 1

Vertical cross section of the eye. Light enters through the cornea and lens, passes through the transparent vitreous, and impinges on the retina, identified as the white layer in the figure.

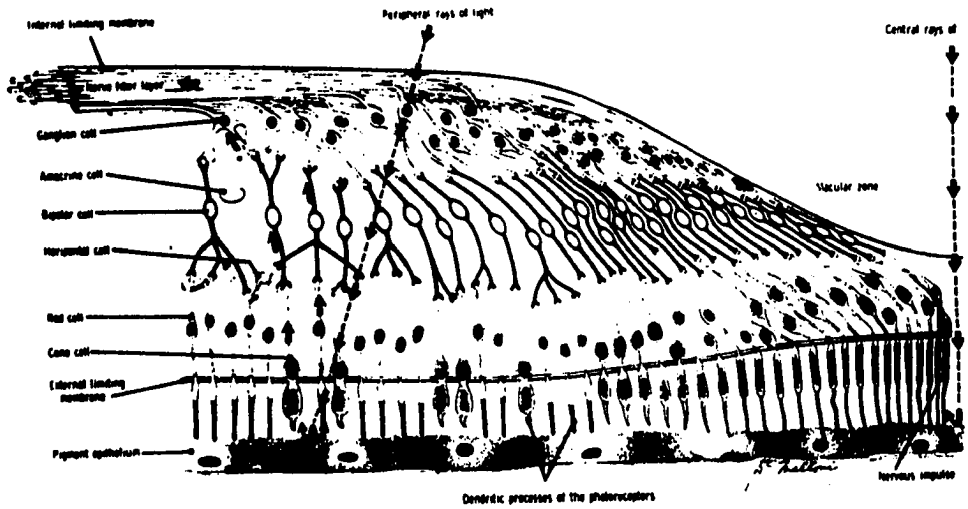


Figure 2

Diagrammatic cross section of the retina. Entering light rays must transsect the transparent layer of nerve fibers, the synaptic processes layer, the photoreceptors and finally reflect from the pigment epithelium to enter the photoreceptors.

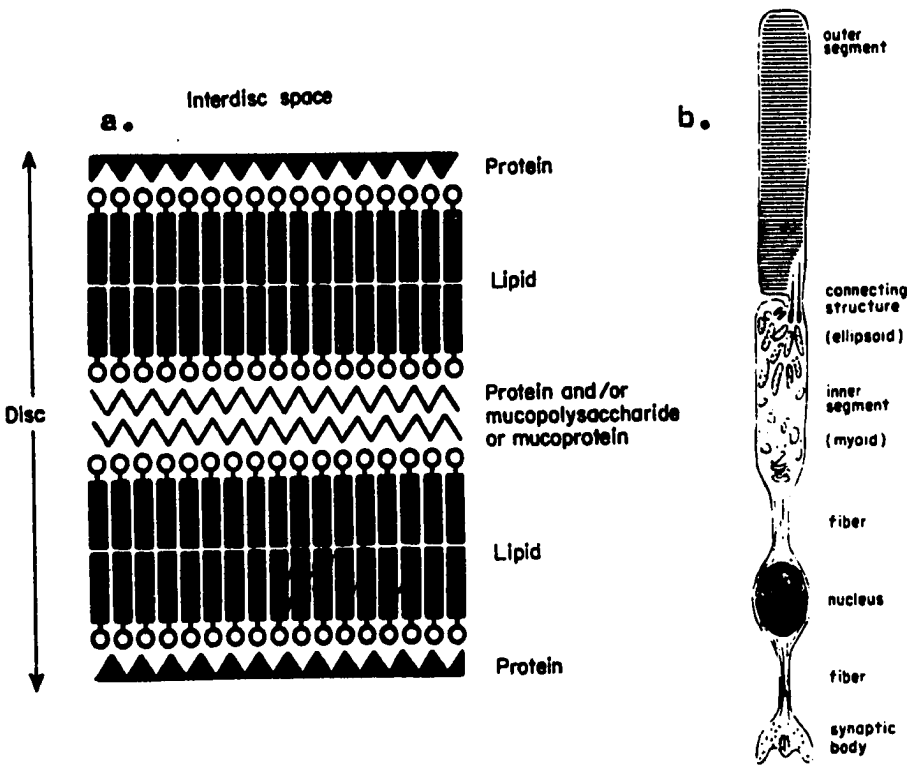


Figure 3

3a Diagrammatic representation of an ROS lamellae cross section. The disc is a double-membrane, after Danelli.

3b Diagrammatic representation of a vertebrate photoreceptor cell showing the structurally dissimilar inner segment and outer segment.

tubules, densely-packed mitochondria, and connecting fibers. The latter is 30-60 μ in length, containing light-sensitive protein complexed with lipid (63).

Electron micrographs have shown the rod outer segment (ROS) to consist of regularly-layered, double-membraned lamellae or discs (29,33,62,77). In the mammalian rod cell, approximately 500-1,000 of these discs can be detected per ROS (72). Chemically, the discs contain 60 percent by weight of lipid, 89 percent of which is phospholipid (60). Not only is this the highest percentage of lipid in a biological tissue, but the lipids themselves are more complex than those found in heart mitochondria (34). The lipids are packaged in the disc with their axes parallel to that of the rod. Conversely, the regularly-arrayed visual proteins have their axes perpendicular to that of the rod (74). The space between the discs has been described as fluid-filled but so far has not been studied extensively (30). A diagrammatic representation of the disc microstructure is shown in Figure 3a. The exact architecture of the disc remains a point of conjecture, since isolation and staining procedures differ.

The photoreceptor structure, as discussed above, provides a functional framework for the process of vision: it serves to align visual pigment within the ROS lamellae, it provides for the regeneration of visual pigment after it has been bleached by light, and it synthesizes and packages new visual pigment as the need arises. Since the structure of the rod appears to be a support facility in the functioning of visual pigment, it is not surprising that the majority of

research effort has been expended elucidating the properties of visual pigment.

Visual Pigment

History and Discovery

At the beginning of the nineteenth century, physiologists studying the eye had noted the bright red appearance of the freshly excised retina. Kühne published a book dealing with the qualitative phenomena of vision, On the Photochemistry of the Retina and Visual Purple, as early as 1878 (56). Excellent measurements of the visual process continued into the twentieth century with such notables as Arrhenius (5). However, it was not until 1934 when Wald discovered Vitamin A as the visual pigment chromophore, that the study of vision got under way (83). Most studies following this were either in vivo examinations of the whole retina or studies on the isolated chromophore, Vitamin A. It was considered impossible to extract light-sensitive visual pigment. In 1952, however, Collins made the first successful extraction of visual pigment using the natural detergent, digitonin, to solubilize the pigment (18). Since that time, the use of solutions of visual pigment has allowed application of many biochemical techniques in unraveling the mystery of this light-sensitive material.

Absorption Spectra

It has become customary to classify visual pigments by their absorption spectra. Four distinct absorption bands can be detected: the first and most important is the alpha

band which is due to the chromophore of the visual pigment. The λ_{max} for the alpha band is species-dependent and varies from 443 nm to 562 nm (12). Since few other retinal substances absorb light in this region, the alpha band is useful for following purification, and monitoring the integrity of the visual pigment. A second smaller absorption band, beta, occurs around 320 to 340 nm (13). It is postulated to be due to bound phospholipid, but no proof of this is yet available (55). A third absorption band is the customary aromatic amino acid extinction at about 280 nm, referred to as the gamma band. A near UV absorption at 220 nm is due to the peptide bonds of the visual pigment protein and is termed the delta band (80). At present, a spectrophotometric scan of visual pigment, exhibiting these four bands, is the only sure criterion for determining the type and purity of visual pigment.

Chemical Properties

Isomers. It was not until 1956 that Wald and Brown (48) discovered indirectly that 11-cis retinal, and not the all-trans isomer, was the chromophore of visual pigment. Fortunately, the two isomers are easily distinguished by their spectra. While both isomers have a λ_{max} near 380 nm, the molar extinction of 11-cis is only 0.65 times the molar extinction of all-trans, at this wavelength. In addition the cis isomer has an additional absorption occurring at about 250 nm, making it distinct from the all-trans isomer.

The viable visual pigment containing the 11-cis isomer is called rhodopsin, while the apoprotein freed of the attached chromophore is termed opsin.

It was thought that the energy-yielding cis to trans isomerization of retinal during the bleaching process might contribute to the sensitivity of the pigment to light. Recently, however, Hubbard claims to have isolated a squid visual pigment containing all-trans retinal as the active chromophore which, on bleaching releases 11-cis retinal (44). Isomerization in this case would actually require energy for bleaching to occur.

General Structure. Rhodopsin with bound lipid is believed to have a molecular weight near 40,000 (2,32,65). In contrast, the delipidized visual pigment was found to have a molecular weight of 28,000 in separate papers by Shichi, et al.,(76) and Heller (40). Heller is also credited with discovering the N-aspartylglycosylamine-linked oligosaccharide(42) which he believes may function as a surface marker in arranging visual pigment within the ROS lamellae.

Visual pigment is apparently a tightly packed sphere having a Stokes radius of 25 Å (27). The amino acid composition is high in aromatic and hydrophobic residues. This might be expected when considering the insolubility of visual pigment in the absence of detergent (11,22). Although Heller has determined an extinction coefficient of 23,000 for the native pigment (39), there is strong disagreement from other laboratories which have arrived at an extinction coefficient of

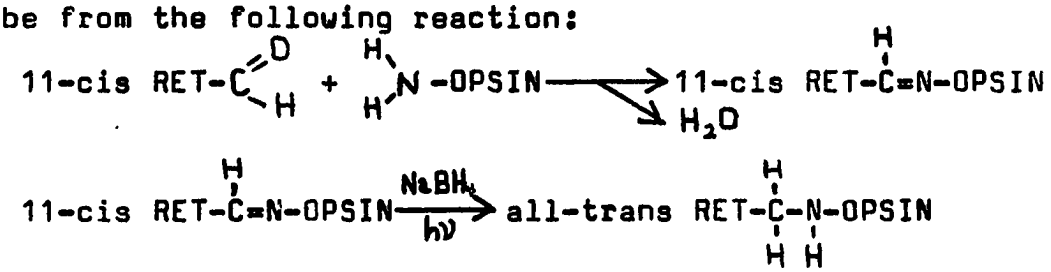
42,000 (14,23,59,84). Using the latter value, a quantum efficiency for bleaching has been calculated to be near 1.00 (41).

Stability. It has been fortunate for workers in vision that intact and unbleached visual pigment, although extremely sensitive to light, is very stable with respect to other conditions. Rhodopsin can be subjected to temperatures as high as 60°C (46), pH in the range of 4.0 to 10.0 (52, 53, 70), and ionic strengths for some salts up to 1 M (19). On the other hand, the apoprotein, once freed of its chromophore becomes sensitive to extremes in the above conditions (71). In fact, opsin alone will denature spontaneously under all conditions (49). Obviously the binding of retinal to the opsin surface is not a case of simple lock-and-key fit. The vastly altered stability of the apoprotein after the release of retinal, indicates the chromophore is bound to opsin in a unique manner. For this reason, considerable study of the chromophoric linkage has been undertaken in recent years.

Chromophore Linkage

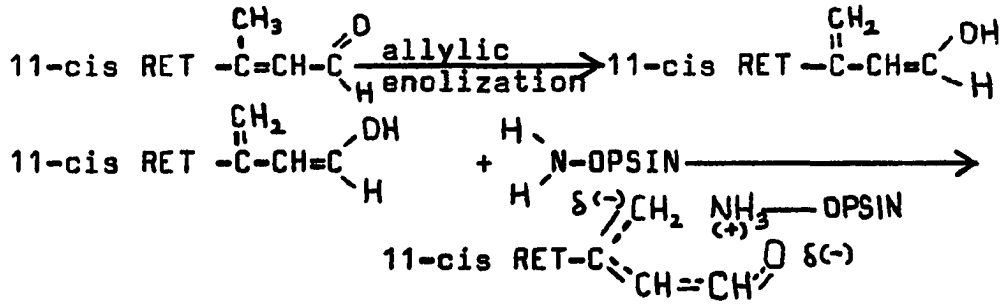
The C-N link. In early spectral studies, hydroxylamine was used to prevent the autoregeneration of visual pigment by forming an oxime with the liberated all-trans retinal (48). It was hypothesized that the imine bond formed might be similar to the *in vivo* link between the apoprotein and retinal in viable rhodopsin (17,61). Since an imine bond would be sensitive to reduction, $NaBH_4$ was used in attempts to stabilize

the linkage for study. Both Bounds, et al.,(10) and Akhtar, et al.,(4) separately isolated a reduction product conceived to be from the following reaction:



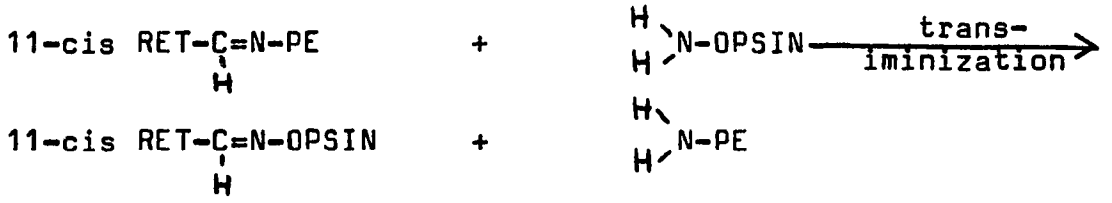
Furthermore, on isolation of the product, retinal was found to be attached to an ϵ-amino group of lysine (8). Additional work used reduction followed by mild cleavage of the apoprotein. A ten residue amino acid sequence, containing the lysine-retinal link, was isolated and found to be high in aromatic and hyrophobic residues (9).

Although the ϵ- amino lysine linkage was obtained just after bleaching, no proof has been presented for its being the linkage found in native unbleached visual pigment. Hooper and Buckser have postulated that the actual linkage is a chelate which reverts to the imine form on bleaching (43).



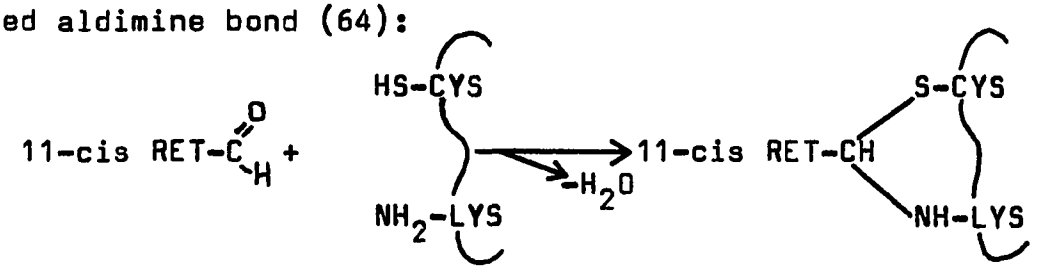
Krinsky (54) and Adams (3) first suggested that phospholipids might play a role in the binding of retinal to opsin. Poincelot identified an N-retinylidine phosphatidylethanolamine (69) in the methanol extract of an unbleached rhodopsin sample.

He concluded that at some point during the bleaching process, transfer of the retinal from phosphatidylethanolamine to lysine was feasible by a transiminization process:



Just recently, Daemen and Bonting have succeeded in preparing stable visual pigment with less than 0.1 mole of phospholipid per mole of visual pigment. They conclude that phospholipid can therefore not be implicated in binding retinal (7). Yet studies conducted by Futterman have shown that phospholipid is an absolute requirement for the regeneration of rhodopsin. He maintains that visual pigments should retain their classification as lipoproteins (35).

Participation of -SH groups. Early studies by Wald and Brown (84) indicated that two free sulfhydryl groups appear after bleaching of visual pigment. Ostroy, et al., postulated that the -SH group might be involved in a substituted aldimine bond (64):



Peskin and Lowe investigated the properties of such an aldimine bond and concluded that a secondary amine, unlike an imine, prohibited resonance making this an unlikely candidate for the

retinal-opsin linkage (66).

The microenvironment. Although the linkage binding retinal to the apoprotein is important, the contribution of the microenvironment provided by the opsin should be more closely examined. Since the union must involve interaction between a long polyene hydrocarbon and the protein, the active site must have a strongly hydrophobic character. Other forces, such as hydrogen bonds, electrostatic bonding, and van der Waals forces may play important roles in the binding of retinal (26). Unfortunately, little definitive work has been done in this area.

The Bleaching Transition

Many of the phenomena associated with the bleaching transition have been previously discussed. Among these are: isomerization of 11-cis retinal to all-trans retinal, appearance of two free -SH groups, and changes in the stability of the apoprotein after loss of the chromophore. Perhaps the most crucial change on bleaching, however, is the conformational change in the apoprotein. Heller used gel filtration to compare the size of rhodopsin and opsin (39). He has investigated conformational changes taking place in the apoprotein after bleaching. He found that although the unbleached visual pigment had a Stokes radius of 23 $\mathring{A}$, the apoprotein expanded somewhat after bleaching to 26 $\mathring{A}$ in radius. Additional work has shown that the hydrated densities of the apoprotein before and after bleaching are markedly different (40). Evidently the

bleaching process brings about marked alterations in the structure. Circular dichromism studies have been attempted to ascertain whether any changes in helical content ensue during the bleaching process. No changes have been observed (73). Even more startling were unfruitful attempts to measure dichroism by flash bleaching visual pigment with plane polarized light (38). Brown suggested that although the chromophore is indeed always located at right angles to the rod, the spherical protein itself is free to rotate with its axis parallel to that of the rod. In an elegant experiment, Brown used the bifunctional reagent, glutaraldehyde, to cross-link visual pigment, thus anchoring it within the discs. As expected, dichroism was easily produced after bleaching constrained visual pigment with plane polarized light (15).

Proposed Experimental Work

Isolation and purification. A search of the literature reveals that no simple procedure exists for purifying visual pigment. Many procedures rely on an initial sucrose density floatation to isolate whole ROS (31,39,60,85). Any pigment released during initial isolation procedures will be lost if only ROS are collected. Furthermore, every technique examined utilized at least one column step to purify visual pigment (6,11,22,59,75,76). Yields appeared to be relatively low and manipulation extensive. It was desirable to develop a simple and efficient procedure for obtaining visual pigment in bulk quantity for experimental work.

Structural changes on photobleaching. Early spectroscopic work by Wald and others elucidated a proposed sequence of steps for the bleaching process in visual pigment (2,86). By lowering visual pigment to liquid nitrogen temperatures and allowing it to warm slowly, five or six definite "spectral states" could be distinguished. Although not discussed in the text these were termed prelumirhodopsin, lumirhodopsin, etc. Unfortunately no proof is available that visual pigment in vivo undergoes this proposed sequence of steps. In fact, it is a distinct possibility that the sequence of events proposed could be artifactual.

More in line with the work of Heller (39), changes in visual pigment before, during, and after bleaching should be studied at temperatures, pH's, ionic strengths, and light intensities similar to those found in the eye. Planned research work has been to study the kinetics of bleaching of visual pigment as induced by various chemical effectors.

Visual pigment vs. rhodopsin. No distinction to date has been made between "visual pigment" and "rhodopsin." The intact lipoglycoprotein has a molecular weight of 40,000 as mentioned previously (21-24), whereas functionally deficient delipidized photopigment is said to have a molecular weight of 28,000.

A second anomaly has arisen in regard to the reported extinction coefficient. Heller with superb technique has confirmed by three methods that 23,000 is the molar absorptivity

of pure visual pigment. Yet as mentioned previously Bridges, Wald, Daemen and Bonting, and Shichi report an extinction coefficient of 42,000. In a corresponding matter, Heller reports with great precision that only one sulfhydryl is freed after bleaching his visual pigment (39). In contrast, careful experiments by Wald and others have shown that two sulfhydryl groups are released after photobleaching. Obviously the ratio of 2/1 for these two parameters may not be accidental. There may be two chromophores per visual pigment molecule, or it is possible that phospholipid may hold visual pigments in a dimeric form, only to become a monomer in such a purified system as that used by Heller. Attempts were made to determine which is the case.

CHAPTER II

MATERIALS AND METHODS

Retinae

Bovine retinae were used exclusively in all experimental work. Whole eyes were obtained by courtesy of the Wilson Packing Company, Oklahoma City. To avoid bleaching and autoproteolysis, eyes were collected within twenty minutes of slaughter and placed in a light-tight, ice-cooled container. Storage of the collected eyes in the dark for 24 hours at $4^{o}C$ facilitated surgical removal of the retinae.

Isolation of Crude Rhodopsin

Step 1. Excision

An efficient surgical procedure was developed to excise retinae from large numbers of eyes. All operations were carried out at $0^{o}C$ and under dim red light using a Kodak Wratten Series Filter, No. 2. Using a single-edged razor blade, an incision was made at the pars plana, just distal to the forward boundary of the retina as shown in Figure 4. Diamond-edged scissors were used to cut along this boundary for the complete circumference of the eye. The frontal portion of the eye could then be lifted off and discarded. With gentle

Figure 4

An incision is made at the pars plana, just distal to the forward boundary of the retina.

compression of the remaining hemisphere the vitreous body could be expelled leaving the retina *in situ*. By swirling with blunted tweezers, as in Figure 5, the retinal tissue could be drawn upward and detached from the optic nerve. Retinae were immediately placed in a stainless steel beaker at -20°C until needed.

Step 2. Whole Homogenates

Approximately 100 frozen retinae were thawed to a slushy consistency, mixed with 100 ml of a 66 mM phosphate buffer at pH 6.8 (hereafter referred to as buffer) and decanted into a prechilled 50 ml Waring Blender. Homogenization occurred for 15 seconds at medium speed and the slurry was allowed to stand for one hour to facilitate freeing the rod outer segments (ROS).

Step 3. Washes

The slurry containing the released ROS was decanted into four 2.5 x 10 cm polycarbonate centrifuge tubes and centrifuged in a Sorvall RC2-B, rotor SS-34, at 7,500 x g for 20 minutes. The supernatant was decanted and saved for estimation of impurities.

Fresh buffer was used to resuspend the ROS pellet. After stirring for five minutes with a Teflon spatula, the suspension was centrifuged in the above manner, but at a slightly increased force of 12,000 x g. This procedure was repeated four more times, producing a clean, pinkish-white pellet. The supernatants were monitored by scanning them spectrophotometrically from 600 nm to 250 nm. Regions of interest were the heme

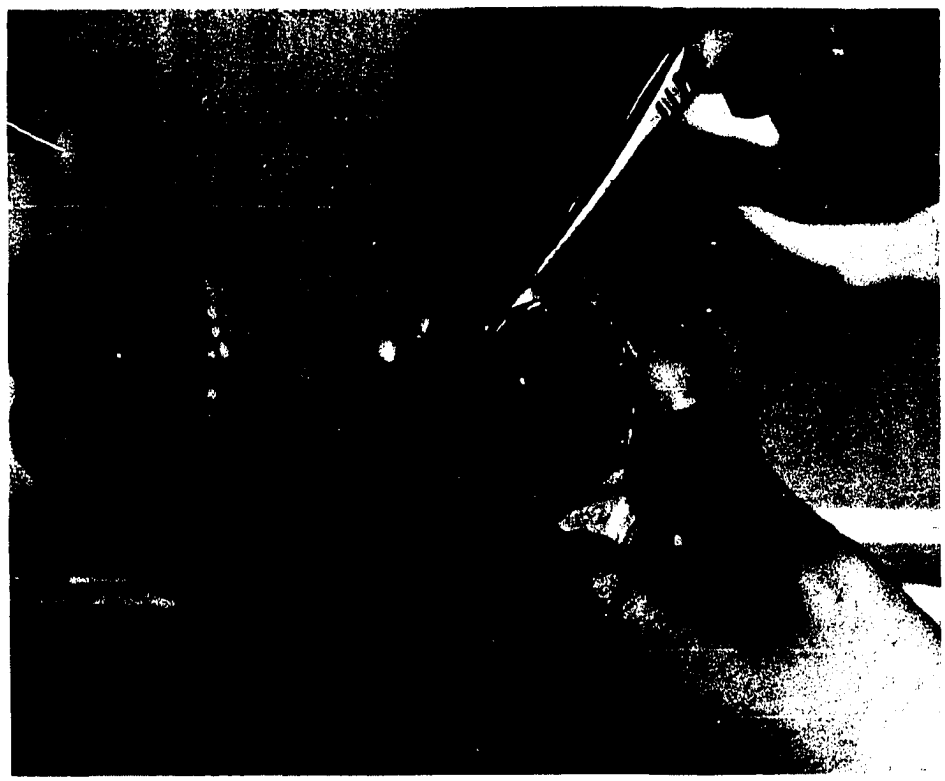


Figure 5

After cutting around the circumference of the frontal portion of the eye the hemisphere is discarded. The lens (lying left of the index finger) and the vitreous are expelled from the eye. Swirling with tweezers draws the retina up and away from its remaining site of attachment, the optic nerve.

bands at 540 and 570 nm, the alpha band near 500 nm, the Soret band at 410 nm, and the gamma band at 280 nm. The Beckman DB-G recording spectrophotometer was used in these assays.

Step 4. Extraction

The hydrophobic nature and bound lipids of the rhodopsin molecule necessitate the use of a detergent for dispersing the visual pigment in an aqueous medium. Several detergents were screened including digitonin, sodium dodecyl sulphate, Emulphogene, Triton X-100, and cetyl trimethyl-ammonium bromide (CTAB). Washed ROS pellets were mixed with 25 ml of buffer, and made one percent with respect to the appropriate detergent. The solutions were stirred overnight in the cold room, in absolute darkness, and centrifuged as above but at 48,000 x g. The supernatant containing rhodopsin was decanted and characterized by its spectrum.

Once a particular detergent was chosen for its superior extraction properties, attempts were made to optimize the amount of rhodopsin extracted per retina. These attempts included altering the following parameters: concentration of the detergent, amount of stirring time used, and whether or not sonication was helpful.

Further Purification Methods

Selective Adsorbents

It was desirable to attempt the removal of extraneous protein from the rhodopsin solution by means of selective adsorption. Adsorbents screened were aged calcium phosphate

gel, diatomaceous earth, carboxy methyl cellulose, and DEAE cellulose. The procedure used was to mix a 25 ml aliquot of crude rhodopsin solution with 10 g of the specific adsorbent. The solutions were stirred for two hours and centrifuged to separate the solid material from the solution. The supernatant was scanned spectrophotometrically while the solid was placed in higher ionic strength buffer (up to 1 M) or buffer of a different pH (4.0 or 9.0) to release the adsorbed material.

Ammonium Sulfate Fractionation

It was thought that differential solubility between rhodopsin and impurities could be used to purify rhodopsin. A 25 ml aliquot of the semi-pure rhodopsin was made 35 percent saturated with respect to ammonium sulfate (209 g/l). The solution was stirred for two hours on a cold plate (Stir-Kool model SK-11). After decanting, the aliquot was centrifuged at 48,000 x g according to the above procedure. The supernatant was collected and made 90 percent saturated in ammonium sulfate (411 g/l) and stirred as above. After centrifuging under the above conditions, a red gel-like material was collected from the surface. This was transferred and resuspended in fresh phosphate buffer.

Dialysis

To remove ammonium sulfate salt from the purified rhodopsin solution it was necessary to include a dialysis step. The rhodopsin sample was placed in a softened 2 cm diameter cellulose dialysis sack and placed in a 3,000-fold volume of

fresh buffer. After six hours the contents of the sack were poured into four 1.5 x 7.5 cm polypropylene centrifuge tubes and centrifuged in a Spinco model L, 40 rotor, for 30 minutes at 103,000 x g.

Column Methods

Calcium Phosphate Gel-Cellulose Column Chromatography

Following the procedure of Tiselius and Swingle (82) a mixture of aged calcium phosphate gel (50 percent) and cellulose (50 percent) was packed in a 2.5 x 125 cm column and washed with buffer. A 25 ml rhodopsin sample was concentrated by rotary evaporation to 2 ml, and layered on the column interface. A linear gradient eluent of 10 to 660 mM phosphate buffer, pH 6.8, was run while collecting 2 ml samples. Fractions were monitored spectrophotometrically at 498, 410, and 280 nm.

Sephadex Column Chromatography

In a similar experiment a rhodopsin concentrate was carefully layered onto an extensively washed Sephadex G-200 column, 2.5 x 125 cm. Using buffer as the eluent, 2 ml aliquots were collected and assayed spectrophotometrically as above.

Assessment of Purity

Spectral Ratios

The protein rhodopsin, unlike a typical enzyme, can not be characterized by its activity *per se*. Rather, a means

must be found to measure the absolute amount of rhodopsin in a solution. The use of spectral ratios has been partially effective in giving vision researchers a common base for comparing visual pigments. The first spectral ratio is between the gamma band and the alpha band or the $A_{280\ nm}/A_{498\ nm}$ in the case of rhodopsin. Although in one paper Heller (39) said he obtained a ratio of 1.75, most workers (12,65,69,76) feel that a value of 4-5 is indicative of pure visual pigment, since Heller made unspecified corrections for light scattering.

A second spectral ratio has been defined as the ratio between the trough in a spectrophotometric scan and the alpha band or $A_{400\ nm}/A_{498\ nm}$. Values in the range of 0.3-0.5 indicate a pure preparation. These ratios were used as initial guides in monitoring the purification of rhodopsin in many of the steps listed.

Amino Acid Analysis

As mentioned previously, rhodopsin contains a high degree of bound lipids. Before an amino acid analysis could be attempted on one of the highly purified rhodopsin preparations, it was necessary to remove the lipid without denaturing the visual pigment. A purified rhodopsin solution was taken to dryness on the Büchi rotary evaporator, keeping the temperature at 10°C. The resulting red powder was washed three times with chilled petroleum ether followed by three washes with chilled hexane. The rhodopsin was dried thoroughly under nitrogen and resuspended in fresh buffer. After addition of constant

boiling HCl, the sample was sealed *in vacuo*, and allowed to hydrolyze for 24 hours. The sample was taken to dryness and resuspended in citrate buffer, pH 2.2, and layered on the acid-neutral column of a Biocal amino acid analyzer. Ninhydrin elution patterns were recorded and the amino acid and amino sugar residues tabulated.

Disc Gel Electrophoresis

The method of Davis (28) was employed to make the disc gel characterization. Initially, the gels were prepared with 0.8 per cent CTAB but problems were encountered with the initiation of acrylamide polymerization. A riboflavin and UV light catalysis was used for the polymerization instead. Equilibration of the gels with tray buffer proceeded for one hour with a setting of 25 ma on the Canalco model 44 disc gel apparatus. One hundred µl aliquots of rhodopsin concentrate with OD near 3.0 at 498 nm were applied to the six stacking gels and electrophoresis made at a constant current of 3 ma/tube for two hours. Three gels were stained with Amidoschwartz dye and destained in acetic acid. Oil red "O," a phospholipid stain, was used on the other three gels. The gels were saved and photographed.

Ultracentrifugation

An excellent means for assessing purity and determining the micellar molecular weight is ultracentrifugation.

Sedimentation velocity. In order to determine the hydrated density of micellar rhodopsin, it was necessary to

make a series of velocity runs. Highly purified rhodopsin samples and CTAB detergent blanks were extensively dialyzed against the following NaCl densities: 1.006, 1.019, 1.039, 1.065. These equilibrated samples were placed in a Beckman Ultracentrifuge model E, titanium rotor # ANH, and centrifuged at 42,000 rpm. Schlieren optics were used to monitor peak displacement. An interference filter with λ_{max} at 436 nm was used to prevent any bleaching of the pigment sample while the run was in progress. Photographs were taken at eight minute intervals and the data used to calculate Svedberg values. The s_η values were plotted against the densities and the intercept on the ordinate gave the hydrated density of the micelles.

Sedimentation equilibrium. Using the value for the hydrated density obtained above, a rhodopsin sample of approximately 1 mg/ ml protein concentration was loaded in a similar manner for a sedimentation equilibrium run. After 24 and 72 hours at 36,000 rpm, interference optics were used to determine the protein concentration gradient. The micellar molecular weight of rhodopsin was calculated by reference to the Spinco Manual (16).

Bleaching Experiments

Urea as a Bleaching Inducer

Dark urea bleaching. Normally, rhodopsin solutions are stable in the absence of light under a variety of conditions as discussed in the "Introduction." Since urea is known

to be an inducer of conformational change, it was decided to monitor the stability of rhodopsin in the dark at various molarities of urea. A stock 10.0 M solution of reagent grade urea was used to make up the aliquots. A rhodopsin solution, 10.0 M urea solution, and buffer solution, were each allowed to equilibrate separately at 25°C in a Forma circulating waterbath, which also kept the cell compartment of the Beckman DB-G regulated to ± 0.05°. A 0.5 ml aliquot of rhodopsin was mixed with appropriate volumes of buffer and urea to make the final solution. For example, 0.5 ml rhodopsin, 0.2 ml buffer, and 0.3 ml of 10.0 M urea give a final solution 3.00 M in urea. A technique of quick mixing allowed initial readings to be taken three seconds after mixing. All of the above procedures were performed under dim red light.

Light was admitted to the cell compartment only when it was necessary to take a reading at 498 nm. Time required for this was usually 2.0 seconds. Since the chart recorder was left in the "On" position, time was kept automatically for the duration of the run. Experiments were conducted until the chromophoric absorption at 498 nm virtually disappeared. Kinetics were monitored for the following concentrations: 0 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, and 7 M.

Light-urea bleaching. In these experiments it was desirable to determine the combined effects of urea and light. The procedure used to prepare samples was identical with that listed under dark-urea bleaching, all operations being carried out under dim red light. In this series, however, the

monochromatic light source in the Beckman instrument was left on for the duration of the experiments.

Reversible-dark urea bleaching. At low concentrations of urea, the possibility of regeneration of the chromophore was considered. After monitoring the decrease of the $A_{498\ nm}$ to nearly zero, the sample was removed and dialyzed extensively against a 3,000 fold volume of fresh buffer. All operations were carried out in absolute darkness. The sample was placed back in the spectrophotometer and examined for regeneration, or more appropriately, the reappearance of the chromophoric absorption at 498 nm.

Temperature Stability of Rhodopsin

Thermal inactivation of rhodopsin. It was desirable to determine the temperature sensitivity of native rhodopsin. Aliquots of rhodopsin were incubated for ten minute intervals. A temperature range of 35° - $50^{\circ}C$, with 1° increments, was used to determine the inactivation curve. The aliquots were quickly immersed in cold water and scanned spectrophotometrically to determine the extent of thermal bleaching by plotting OD_t / OD_o versus temperature.

Temperature stability of urea-rhodopsin solutions. The relationship between temperature and urea-induced bleaching was investigated. The experimental procedure was essentially the same as that described under dark-urea bleaching. A series of runs at four urea concentrations was made at temperatures of 10°, 20°, 30°, and $40^{\circ}C$. Kinetic plots were made of the data and first order rate constants determined.

Arrhenius plots. In order to better understand the processes of urea-thermal bleaching, Arrhenius plots were constructed for each urea concentration tested. Plots of $1/^{o}K$ versus log K were made with log K = slope/ RT + log A or $\ln_e K$ + slope (2.303)/RT + $\ln_e A$ where the slope is equivalent to E_a, the activation energy, and the intercept equals log A, the steric collision factor.

Enzymatically Induced Bleaching

Phospholipase A. Phospholipase A from Vipera russeli (Sigma Chemicals) was used as a source of the enzyme. Activity of the glycerin solution was stated to be 1.0 U/mg. Both the enzyme and rhodopsin solution were equilibrated in the water bath at $30^{o}C$. To a 1 ml cuvette of rhodopsin solution was added 50 µl of enzyme. Readings were taken at 15 minute intervals to monitor possible bleaching. Release of free fatty acids was monitored by the microtechnique of Laurell and Tibling (57). The coppertriethanolamine-fatty acid complex produced a magenta color which was read at 550 nm on a Beckman DU.

Phospholipase C. Phospholipase C from Cl. Welchii (Sigma Chemicals) was used in the experiment. The enzyme was solubilized by mixing 5 mg (2.5 U/ mg) with 1 ml of buffer solution. Solutions of rhodopsin and enzyme were allowed to equilibrate at 30^{o} before mixing. A 50 µl aliquot of the enzyme was added to 1 ml of rhodopsin and placed in the spectrophotometer. Readings were taken at 10 minute intervals. Production of alpha, beta diglyceride was monitored by its

appearance on thin layer chromatographic (TLC) plates, the procedure being described in the next section.

Phospholipase D. Phospholipase D from spinach extracts (Calbiochem) was used in the study. The powdered enzyme with an activity of 5.0 U/mg was placed in buffer solution before use (3 mg/ml). The same experimental technique was used as in the phospholipase C treatment. The release of phosphatidic acid was used to monitor the activity. Identification of phosphatidic acid by thin layer chromatography is described in the following section.

Thin Layer Chromatography

Detection of Phospholipids

Isolation of phospholipids. Rhodopsin, controls, and standards were mixed with an equal volume of chilled chloroform in conical tubes and agitated for five minutes to produce a white emulsion. Centrifugation at 10,000 rpm for 10 minutes in an International preparative centrifuge served to separate the phases. The chloroform lower layer was recovered and taken to dryness by bubbling under nitrogen. The residues were re-suspended in 0.1 ml of absolute ethanol and stored at -20°C until used in thin layer chromatography.

Separation of phospholipids. Thin layer chromatography was used to separate the components. An absorbent of Brinkman silica gel "G," was used with a solvent system of chloroform/ methanol/ acetic acid: 80/20/4. Saturated filter paper in the tanks maintained a uniform solvent front. Development time

was 15 minutes to reach 80 percent of the way to the top of the front.

Identification of phospholipids. A molybdic acid spray was used to pinpoint the phosphorus-containing spots on the plate, and after five minutes a Polaroid color photograph of the plate was taken to preserve the data. The plate was inserted into a small broiler for one hour at 300°C to carbonize all spots on the plate and a second photograph was taken. Comparisons were made between standards, samples, and controls.

Standards for phospholipids. Sigma brain extracts were used for phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidic acid (PA).

Detection of Retinals

Isolation of retinals. The procedure was the same as that used for phospholipids except activated silicic acid was added to the emulsion to remove phospholipids.

Separation of retinals. Thin layer chromatography was also used to separate retinals except that the solvent system was methanol/petroleum ether: 10/90.

Identification of the samples and standards was made by spraying the plates with acidified p-amino benzoic acid, which gave dark indigo spots with retinals. UV light could also be used to locate retinal-containing spots.

Retinal standards. The 11-cis isomer of retinal was a gift from Hoffman LaRoche Pharmaceuticals, all-trans, 9-cis, and 13-cis were obtained from Sigma.

RESULTS

CHAPTER III

Isolation of Crude Homogenates

Whole Homogenates

Excision of retinae and their homogenization were conducted as described in "Materials and Methods." Six washing steps were required to free the ROS from extraneous material. Results of the spectral analyses are shown in Table 1.

Extraction of Rhodopsin

Removal of rhodopsin molecules from the outer segment saccules and their concomitant solubilization in an aqueous phase are perhaps the more crucial aspects of any isolation procedure. Choice of detergent is thus extremely important in increasing the yield of visual pigment and decreasing the solubilization of extraneous matter. Effectiveness of a particular detergent was measured by two criteria: the ratio of protein absorption to chromophore absorption, and the clarity of the solution after extraction. Each detergent trial was conducted using 100 retinae. Results are shown in Table 2. Cetyl trimethylammonium Bromide (CTAB) was found to be the

TABLE 1

PROTEIN REMOVAL IN HOMOGENATE WASHES

Supernatant Number	Soret Band OD @ 410 nm	Alpha Band OD @ 498 nm	Gamma Band OD @ 280 nm	Protein* Removed
1	2.70	0.00	23.50	2,600 mg
2	0.80	0.00	8.20	900 mg
3	0.07	0.00	1.25	82 mg
4	0.01	0.00	0.92	63 mg
5	0.00	0.00	0.45	32 mg
6	0.00	0.00	0.19	12 mg

*Assuming 1.0 mg/ml equals 1.0 $OD_{280\ nm}$ adjusted for wash volume

TABLE 2

RHODOPSIN EXTRACTION EFFICIENCY WITH VARIOUS DETERGENTS

BASED ON 100 RETINAE TO FINAL VOLUME OF 25 ml

Detergent	Gamma Band OD @ 280 nm	Alpha Band OD @ 498 nm	Spectral Ratio	Clarity
Emulphogene	8.70	0.18	48.1	slightly cloudy
Triton X-100	20.30	0.54	37.2	cloudy
Digitonin	24.50	0.32	76.5	cloudy
SDS	12.4	0.00	∞	clear
CTAB	17.1	1.60	10.7	clear

most effective detergent for the efficient extraction of rhodopsin from ROS.

After choosing a detergent, it was necessary to optimize certain parameters in the extraction process giving the highest yield and least impurities. Among the parameters altered were: concentration of detergent, duration of stirring, and use of sonication. The most efficient system was found to be 2 percent CTAB extractant, stirred for 18 hours with no sonication. A typical spectrophotometric scan of semi-pure visual pigment is shown in Figure 6. The alpha peak appears at 498 nm, the Soret band at 410 nm, and the beta peak at 330 nm.

Further Purification of Rhodopsin

Selective Adsorbents

Except for calcium phosphate gel, none of the adsorbents listed in the "Materials and Methods" section showed any tendency to selectively adsorb or desorb rhodopsin. Calcium phosphate gel, however, preferentially adsorbed rhodopsin, leaving extraneous protein and blood in solution. It was thought that exchange dynamics could be brought to bear in removing rhodopsin from the gel. Ionic strengths as high as 1.0 M phosphate, coupled with a range of pH from 4.0 to 10.0, were tried but it was impossible to remove the micellar rhodopsin from the phosphate gel.

Column Methods

Calcium phosphate gel - cellulose column. Since calcium

Figure 6

A spectrophotometric scan of semi-pure rhodopsin. The alpha band is at 498 nm, the Soret band is at 410 nm, and the beta band, or possible bound phospholipid, is at 330 nm.

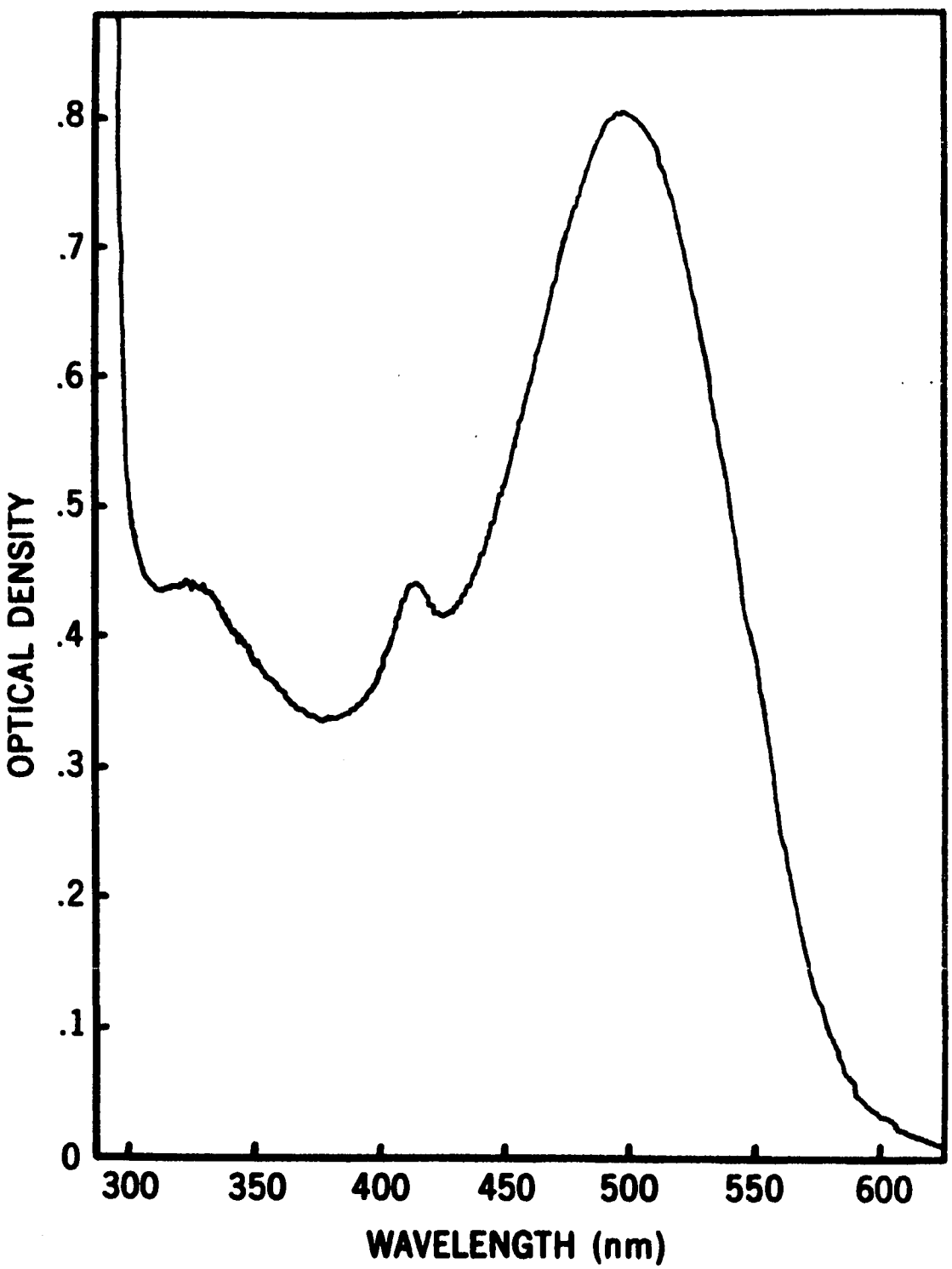

OPTICAL DENSITY
.8
.7
.6
.5
.4
.3
.2
.1
0
300
350
400
450
500
550
600
WAVELENGTH (nm)

Figure 6

phosphate gel showed such excellent rhodopsin adsorption properties it was decided to try a column of the gel with cellulose after the method of Tiselius (82), previously described in "Materials and Methods." The elution pattern for the column is shown in Figure 7. The peak due to chromophoric adsorption at 498 nm is readily apparent but slightly diffuse, probably due to detergent.

Sephadex column. It was also possible to separate visual pigment on the basis of the micellar size rather than the binding properties. The Sephadex G-200 elution pattern for rhodopsin is shown in Figure 8. Based on molar extinction of the tubes at 498 nm, only about 15 percent of the visual pigment was collected. It would appear the column may have caused bleaching or denaturation of the visual pigment.

Ammonium Sulfate Fractionation

Ammonium sulfate was found to be an extremely effective fractionating agent for rhodopsin. An unexpected benefit from the procedure was the removal of excess CTAB from the micellar system. The detergent concentration fell to 0.06 percent from the initial extraction concentration of 2.0 percent, based on a CTAB blank fractionated in like manner.

First fractionation. The pellet generated from the 35 percent cut was resuspended in 25 ml of fresh buffer and the solution spectrophotometrically scanned to calculate the amount of protein impurity removed.

Second fractionation. The supernatant from the first fractionation was taken to 90 percent saturation in ammonium

Figure 7

Elution profile for rhodopsin on a calcium phosphate gel-cellulose column 2.5 x 125 cm. Gradient Elution buffer, 10 mM to 660 mM phosphate buffer, pH 6.8, 2% CTAB. (● - ● $OD_{280\ nm}$, ▲ - ▲ $OD_{498\ nm}$)

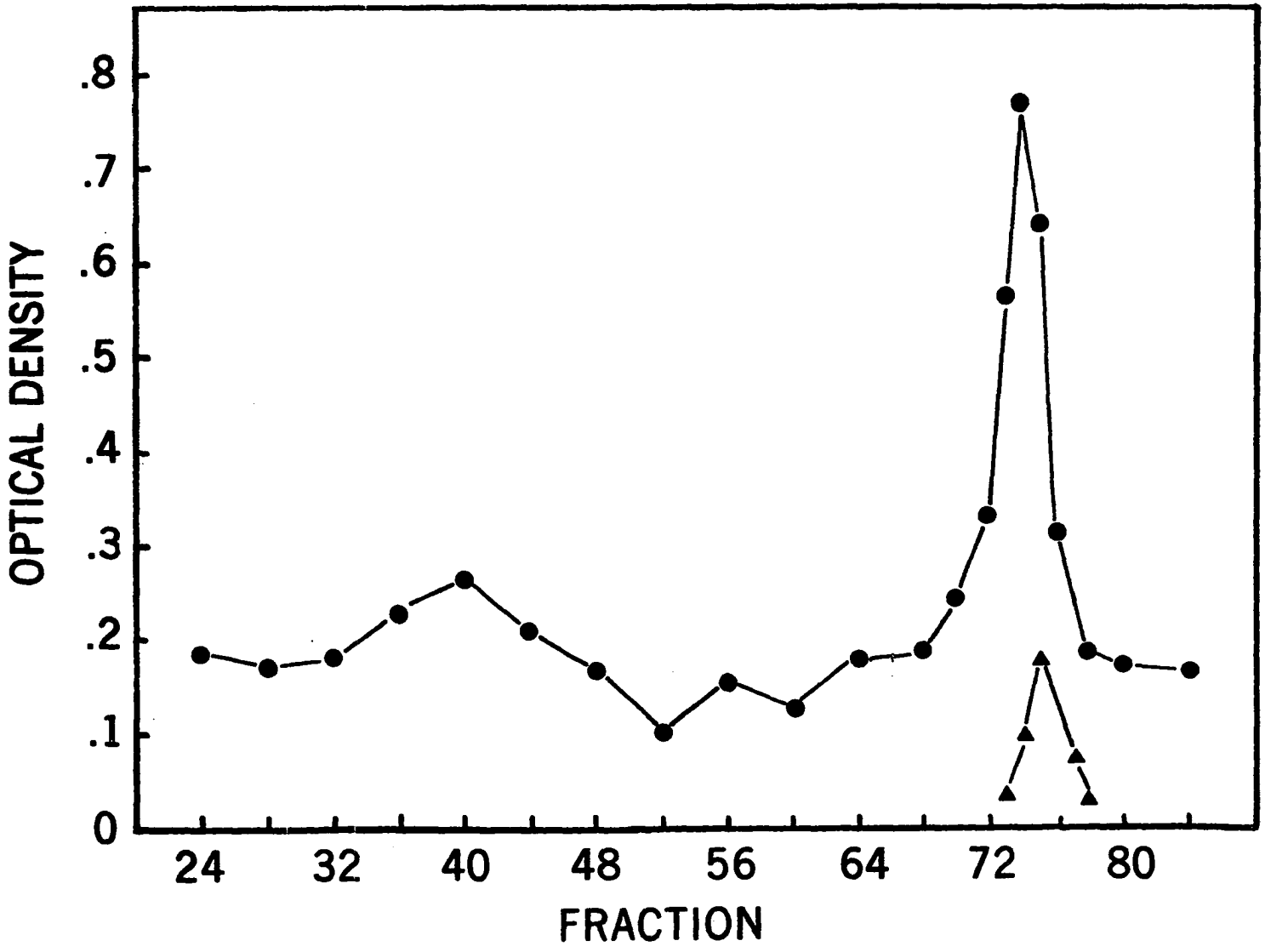


Figure 7

Figure 8

Elution profile for rhodopsin on Sephadex G-200, 2.5 x 125 cm column. The buffer used was 66 mM phosphate, pH 6.8, 2% CTAB. (● - ● $OD_{280\ nm}$, ▲ - ▲ $OD_{498\ nm}$)

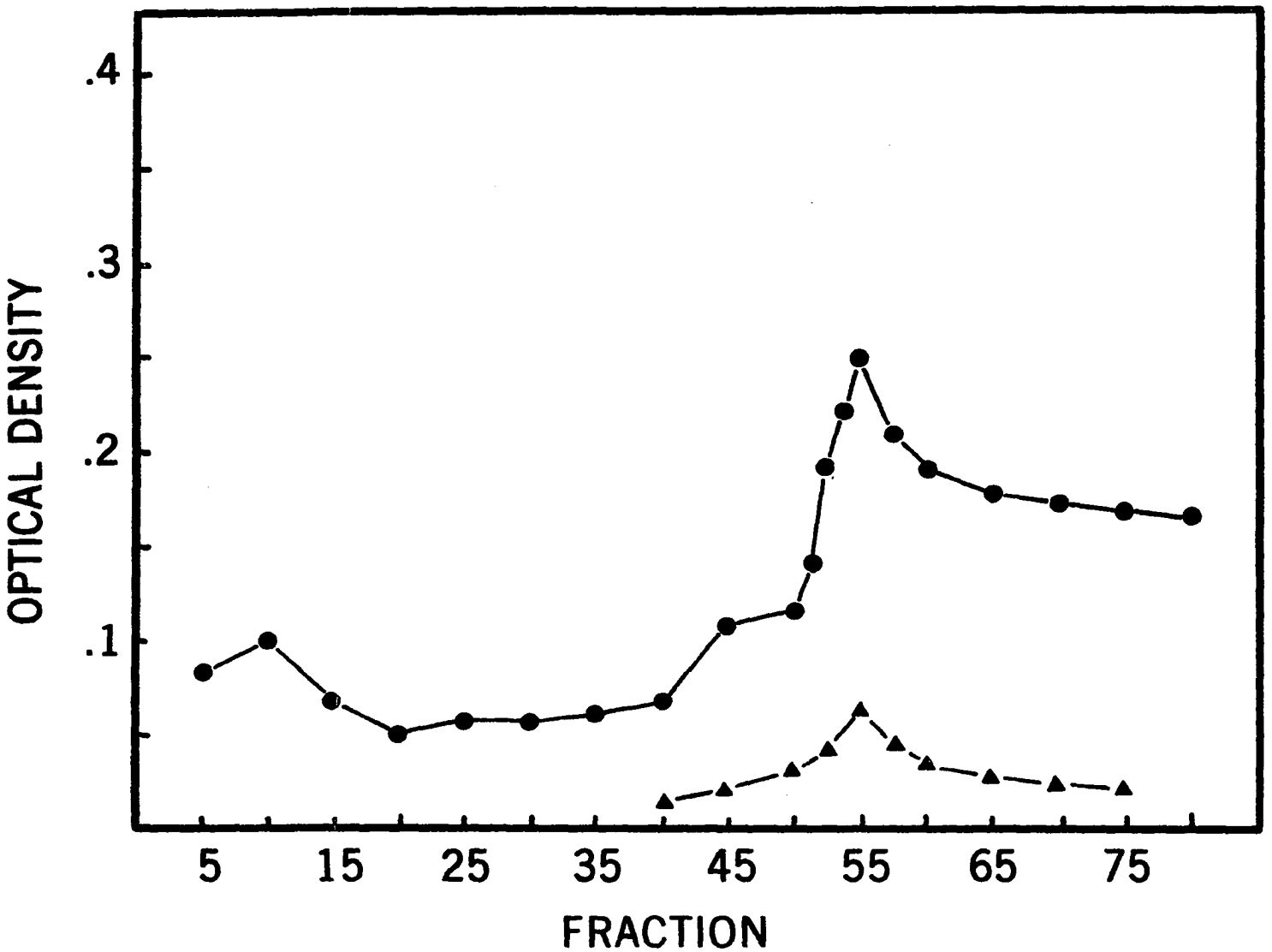


Figure 8

sulfate. After stirring and centrifuging as before, a red gel-like pellet was collected from the surface of the solutions. The pellet was redissolved in a minimal volume of fresh buffer.

Dialysis. After extensive dialysis and clarification, as described in "Materials and Methods," a spectrophotometric scan of the highly purified rhodopsin solution was taken at 1:10 dilution. The scan is shown in Figure 9. Immediately apparent is the removal of the Soret band which was very prominent in Figure 6. In addition, the spectral ratio of protein to chromophore is 6.0 indicating the visual pigment is more than 90 percent pure (65).

Assessment of Purity

Spectral ratios. Using the criteria mentioned in the "Materials and Methods" section, the following ratios were obtained: $A_{280}/A_{498} = 6.0$ and $A_{400}/A_{498} = 0.29$.

Amino acid analysis. Amino acid analysis was conducted as previously described, and the results shown in Table [illegible]. For comparison the results of Heller (39) and Futterman, et al.,(36) are shown.

Disc gel electrophoresis. Rhodopsin could be located in the center of the gels by Amidoschwartz Black, as shown in Figure 10. Since the migration occurred in CTAB, however, the appearance of a single band may not be indicative of a homogeneous protein. Oil Red "O," a phospholipid stain, failed to show any bands in gels run under the same conditions. The

Figure 9

Spectrophotometric scan of ammonium sulfate fractionated and dialysed rhodopsin. Note the disappearance of the Soret band, as well as the lowered A_{280}/A_{498} ratio. Scan taken at 1:10 dilution permitting the observation of the entire spectrum.

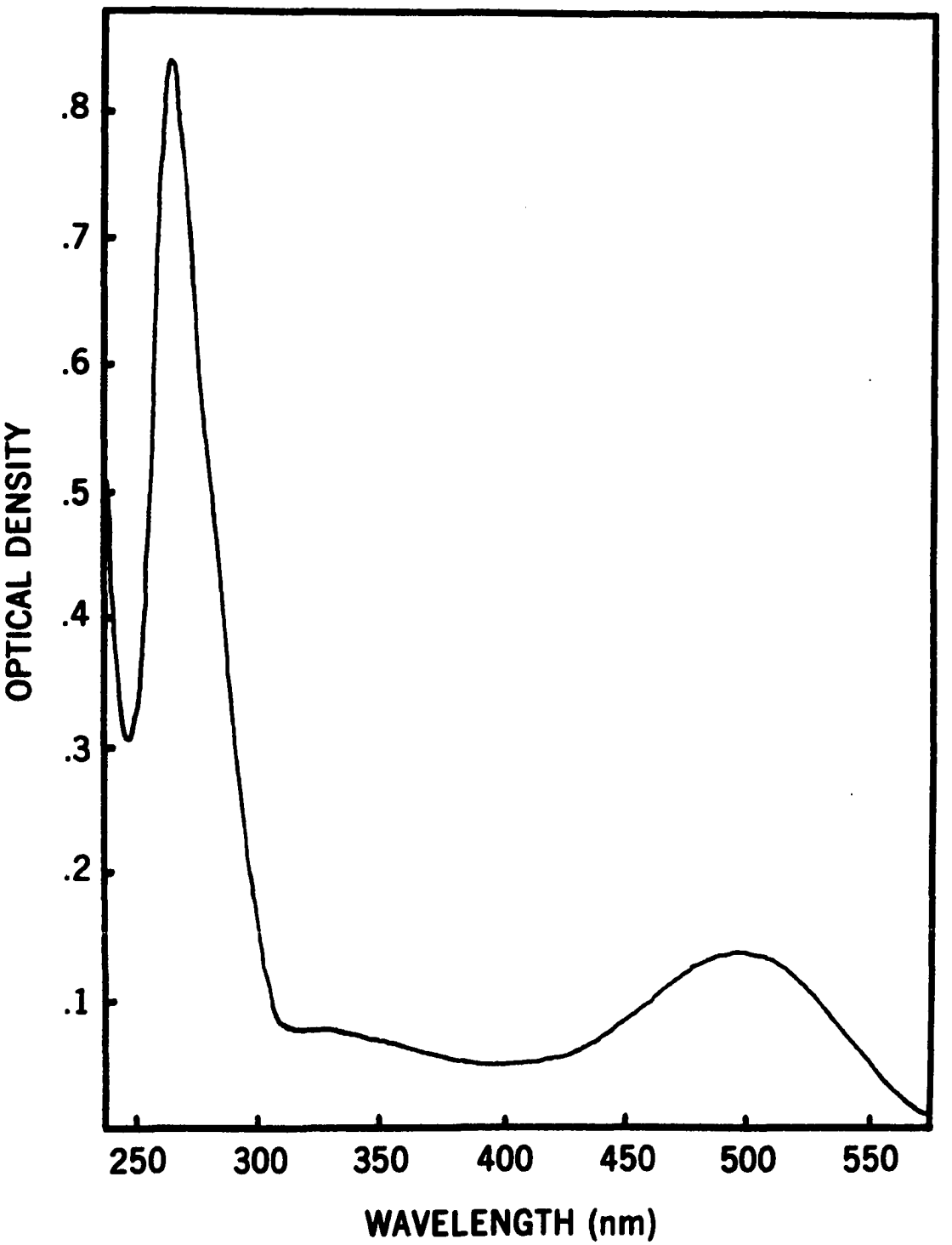

OPTICAL DENSITY
.8
.7
.6
.5
.4
.3
.2
.1
250
300
350
400
450
500
550
WAVELENGTH (nm)

Figure 9

TABLE 3

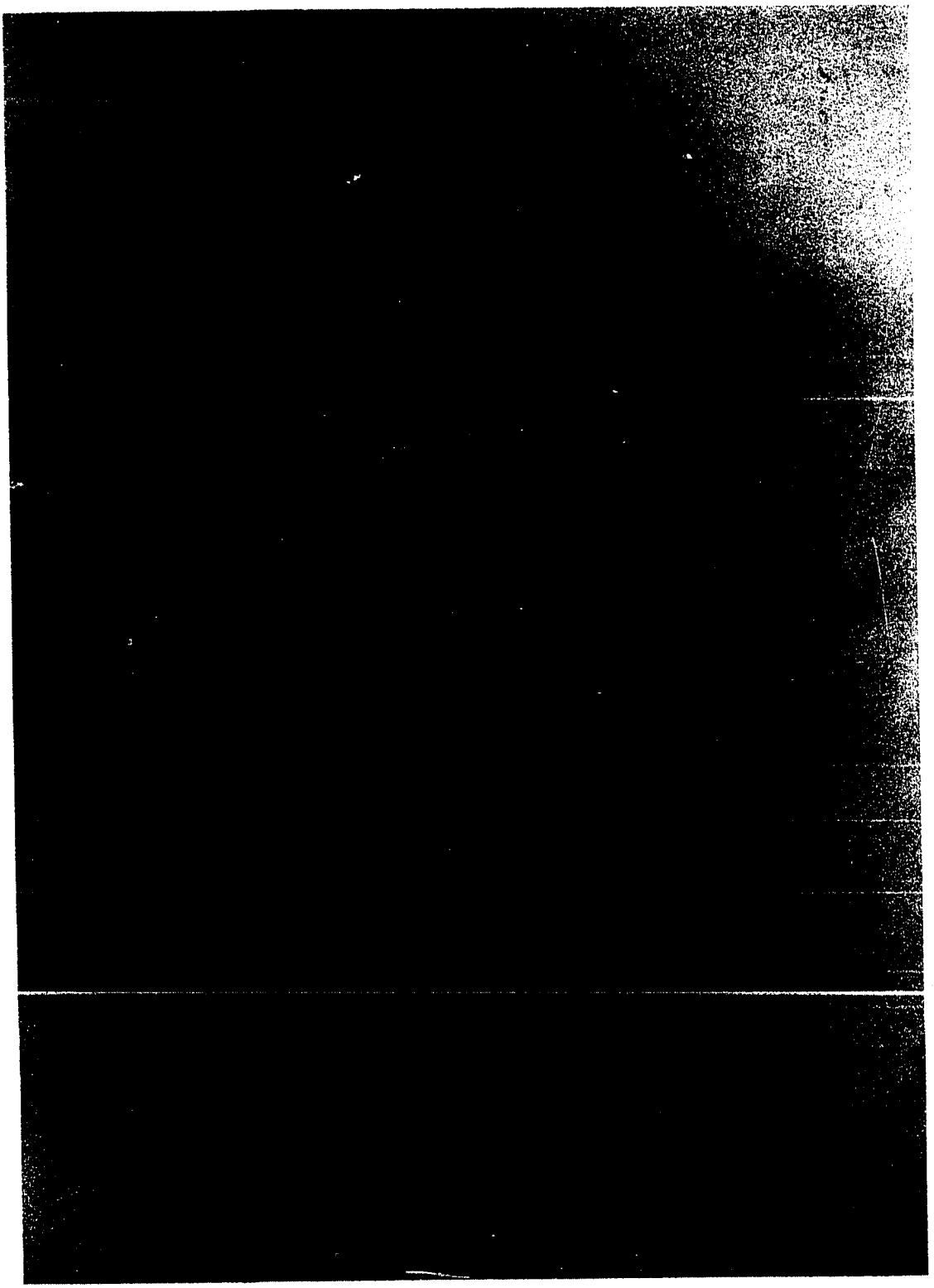
AMINO ACID ANALYSIS

Residue	Mole percent		
	Results	Heller(60)	Shichi(26)
Lysine	3.8	4.4	3.4
Histidine	1.3	1.7	1.5
Arginine	2.8	2.6	2.6
Cysteine	3.0	2.2	2.4
Aspartic	7.7	6.5	7.9
Methionine	4.0	3.5	3.1
Threonine	5.9	6.9	9.4
Serine	4.8	6.0	4.8
Glutamic	9.7	9.2	10.0
Proline	4.3	5.6	6.6
Glycine	7.3	7.1	7.5
Alanine	10.0	8.7	10.0
Valine	6.0	7.4	5.4
Isoleucine	4.1	5.0	3.1
Leucine	8.0	8.3	7.6
Tyrosine	5.2	4.8	4.4
Phenylalanine	8.2	8.3	8.8
(Glucosamine)*	4.2		

*Not included in amino acid mole percent.

Figure 10

Disc gel electrophoretic bands for rhodopsin stained with Amidoschwartz Black. Gel A was run for 2 hrs at 2.5 ma./tube and gel B was run for 1.5 hrs with the same current.



A B

Figure 10

concentration of phospholipid in the pure rhodopsin sample may have been too low for detection.

Ultracentrifugation

Sedimentation velocity. In the velocity runs to determine hydrated density, rhodopsin micelles appeared to produce a single peak, as shown in Figure 11. The peak was symmetrical and remained so throughout the run, whether it was floatation or sedimentation density. It was thought that the observed peak might be an artifact produced by the detergent micelles alone. A control consisting of CTAB and buffer dialyzed against the same salt gradients gave no discernible peak.

From the four densities of rhodopsin solutions run, Svedberg values were calculated and the results plotted against density as shown in Figure 12. The intercept inverse gave a micelle hydrated density of 0.9699 ml/g.

Sedimentation equilibrium. Using the value calculated for the hydrated density, a sedimentation velocity run was conducted at 36,000 rpm. Interference photographs were taken at 24 and 72 hours. Vertical displacement of the interference lines was used to calculate the micellar molecular weight. An average value of 310,000 MW was calculated for the micelles.

Bleaching Experiments

Urea as a bleaching inducer. Loss of activity in the visual pigment rhodopsin was measured by monitoring the disappearance of all chromophoric absorption at 498 nm, and the appearance of released retinal absorbing at 380 nm. Urea was found to be an effective bleaching agent of rhodopsin, more effective

Figure 11

Sedimentation Velocity of rhodopsin samples at various densities. Photographs taken at 8 minute intervals from right to left. Row A is 1.065 g/ml, row B is 1.039 g/ml, Row C is 1.019 g/ml, and Row D is 1.006 g/ml. Rows A and B illustrate sedimentation of rhodopsin, while rows C and D illustrate floatation.

Figure 11

Figure 12

Plot of rhodopsin solvent density versus observed Svedberg X viscosity. The intercept, where S_o equals zero, gives the inverse of the hydrated density.

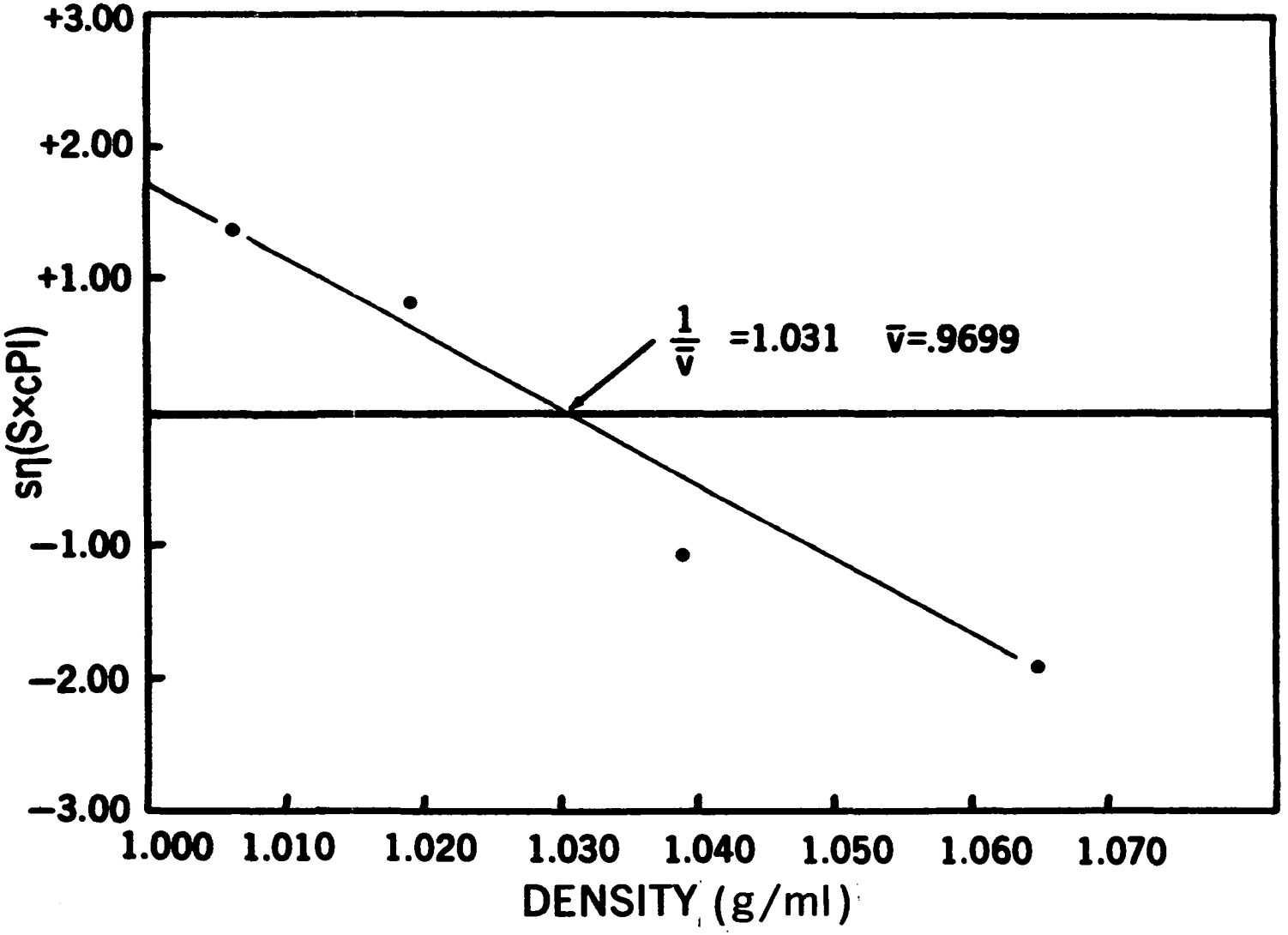

+3.00
+2.00
+1.00
−1.00
−2.00
−3.00
sη(S×cPI)
1/v̄ =1.031 v̄=.9699
1.000 1.010 1.020 1.030 1.040 1.050 1.060 1.070
DENSITY (g/ml)

Figure 12

even than light. At higher concentrations of urea, 5-7 M, the disappearance of all chromophoric absorption took place in seconds in the absence of light. Comparably, a rhodopsin control would take hours to bleach to completion when the monochromatic light source of the spectrophotometer bathed the sample cell continuously. ($t^{\frac{1}{2}}$ = 16.1 hours, k = 1.19×10^{-5} sec^{-1} as plotted from Figure 15.)

At lower concentration of urea, 2-4 M, bleaching in the darkness was a slower process, and in certain cases produced a phenomenon termed half-bleaching, to be discussed later. A plot of the bleaching kinetics observed at higher concentrations of urea is shown in Figure 13. Note that the slopes are not straight lines as would be expected in a simple first order process when log OD is plotted against elapsed time. When the data are replotted, however, assuming two chromophores per visual pigment molecule, only one of which is initially being eliminated in the dark-bleaching process, the plot shown in Figure 14 is obtained. The ordinate is determined as follows:

let $OD_o = \frac{OD}{2}o + \frac{OD}{2}o$

replot taking the log $(OD_t - \frac{OD}{2}o)$ for the ordinate

By this manner, the dark-bleaching process can be expressed in simple first order terms. The following data were obtained from the kinetic plot in Figure 14.

4 M dark-bleaching, k = 4.5×10^{-4} sec^{-1}

5 and 6 M dark-bleaching, k = 1.95×10^{-3} sec^{-1}

Figure 13

Plot of log optical density versus time for the loss of alpha band absorption in rhodopsin subjected to 4, 5, and 6 M urea in the dark.

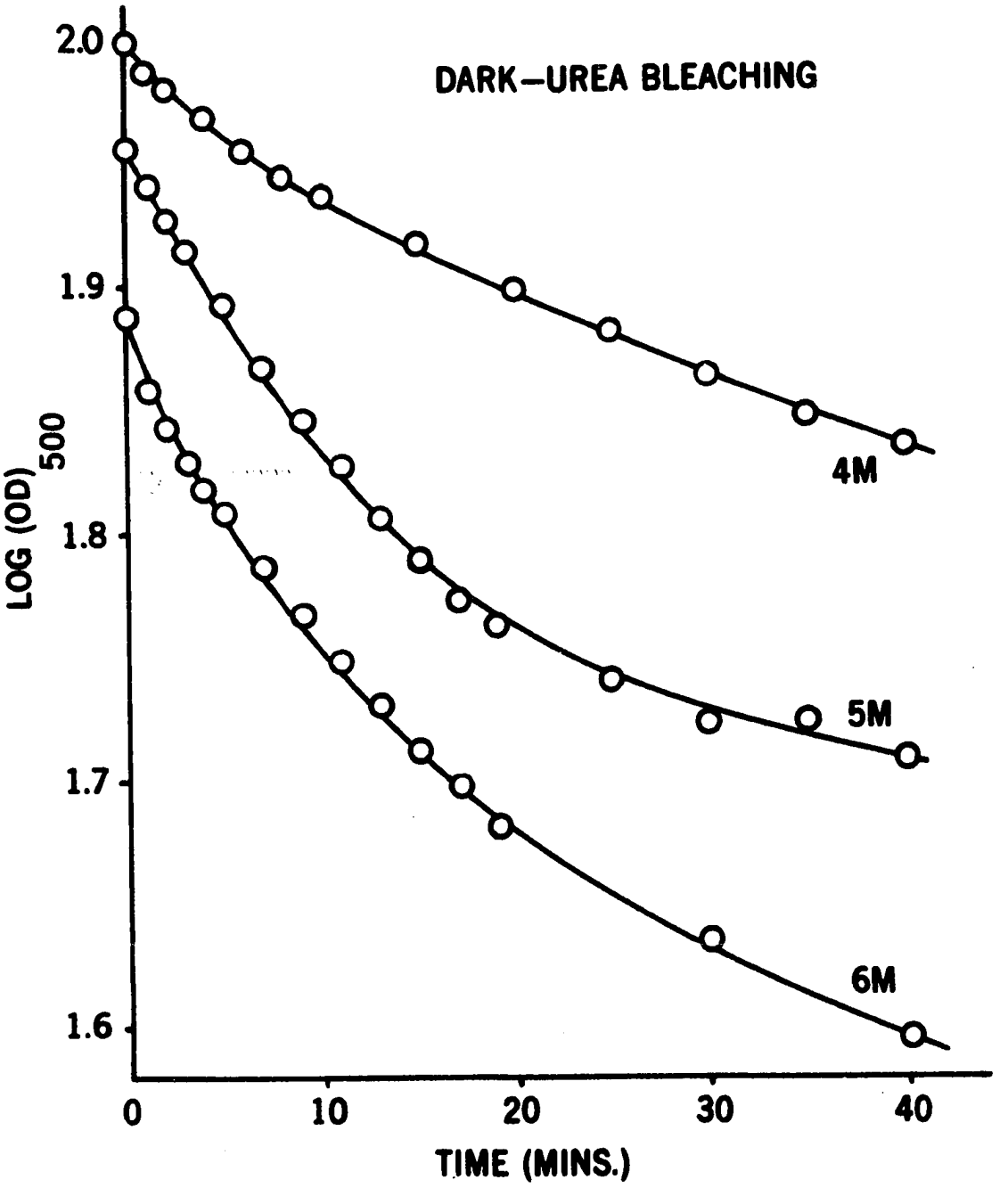

DARK–UREA BLEACHING
2.0
1.9
1.8
1.7
1.6
LOG (OD)500
4M
5M
6M
0
10
20
30
40
TIME (MINS.)

Figure 13

Figure 14

Plot of log ($OD - OD_0/2$) versus time for the loss of alpha band absorption in rhodopsin subjected to 4, 5, and 6 M urea in the dark.

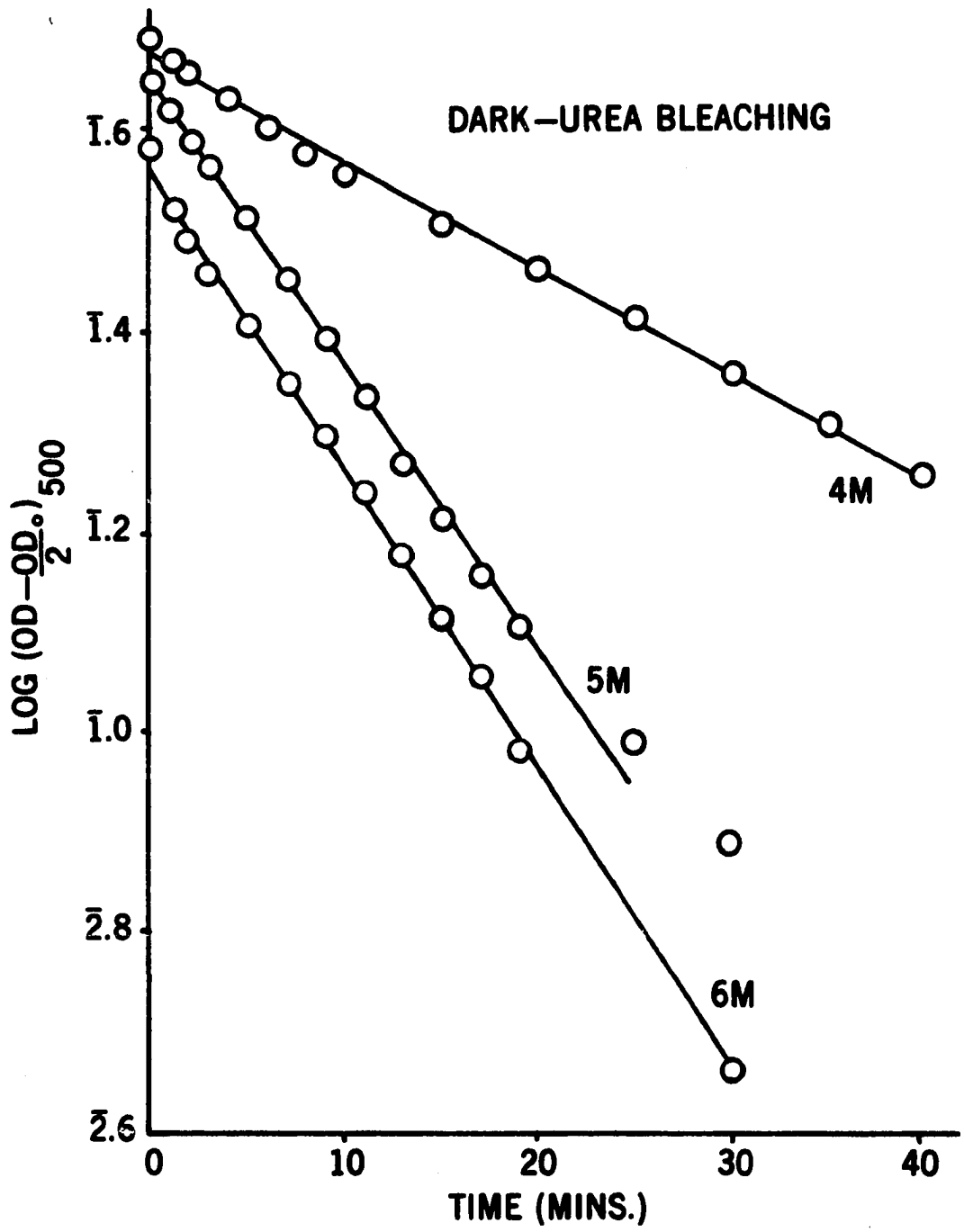

DARK—UREA BLEACHING
4M
5M
6M
LOG (OD—OD∘/2)500
1.6
1.4
1.2
1.0
2.8
2.6
0
10
20
30
40
TIME (MINS.)

Figure 14

Having discovered the phenomenon of bleaching a light-sensitive photopigment in the absence of light, it became necessary to look for the mode of action of urea in bleaching. It was possible that urea could be effecting the chromophore directly. Solutions of 6 M urea with both 11-cis and all-trans retinal standards showed no spectral changes after 24 hours. The results could also be explained by direct denaturation of the opsin apoprotein by urea with the concomitant loss of chromophoric absorption. A plot was made of log urea concentration versus log k, the first order bleaching constant. The pseudo first order rate constant could be represented by the following:

$$k = k_1 (\text{Urea})^{\alpha}$$

where (Urea) is the concentration of urea and α is the slope of the curve as shown in Figure 15. The slope is shallow giving alpha a value of 2.4. This order of magnitude for the exponent of the urea concentration indicates the urea dark-bleaching of rhodopsin may be a chemical process, rather than a conformational process in which alpha would be several times larger. Urea may be affecting the chromophoric linkage as does light itself.

Light-urea bleaching. Measurements were made to evaluate the bleaching of rhodopsin in the presence of both light and urea. Experiments were conducted as described under dark-bleaching with one exception: the cell compartment was illuminated by the instrument's dim light source for the duration

Figure 15

A plot of log k versus log urea concentration for the dark urea-bleaching of rhodopsin. The slope, α, is the exponent of the urea concentration relating the bleaching rate to the urea concentration.

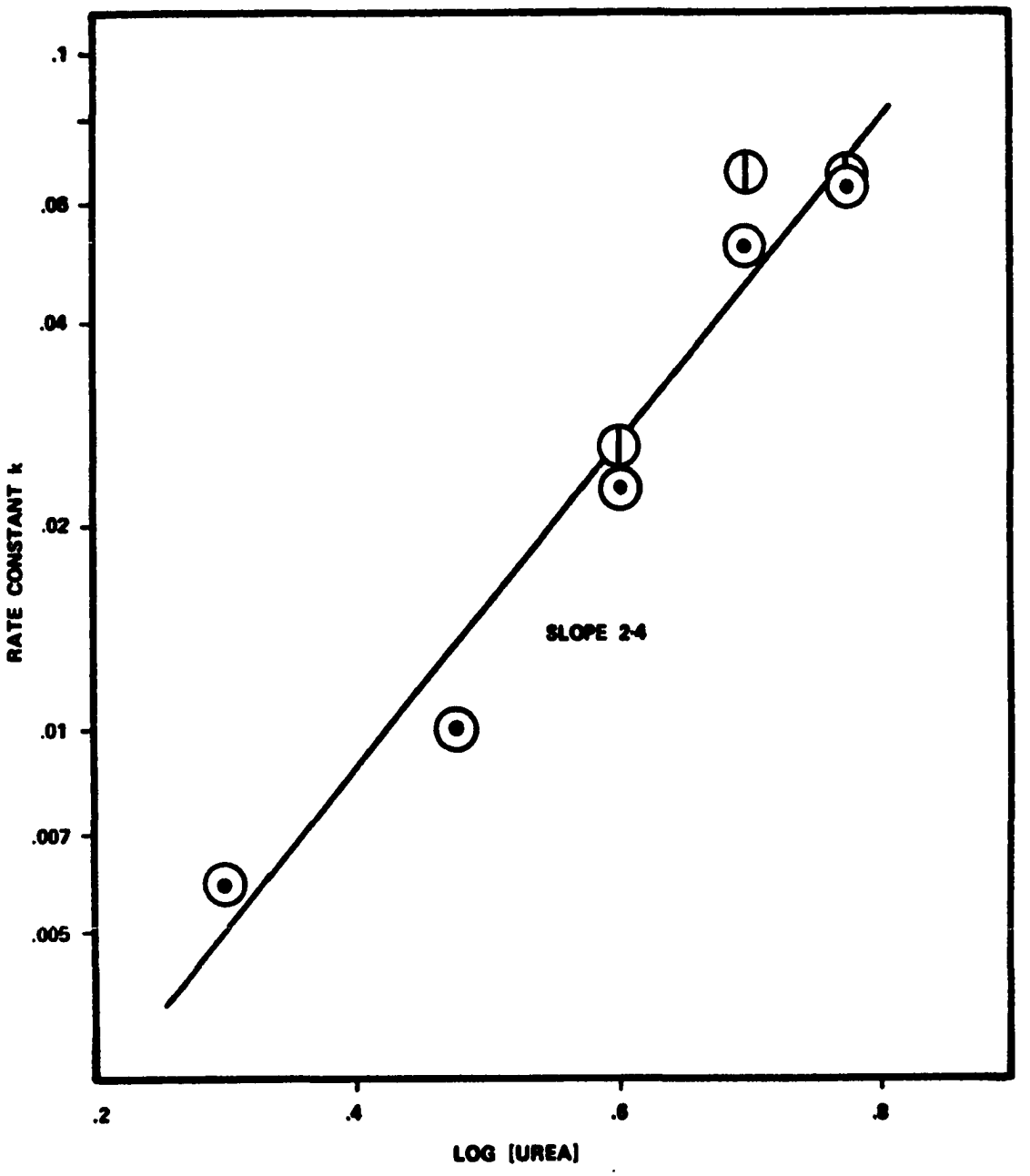


Figure 15

of the experimental run. The disappearance of rhodopsin measured by loss of absorption at 498 nm is as follows:

$$\frac{-d\,(Rh)}{dt} = k'\,(Rh)\,(I) + k''\,(Rh)\,(U)$$

where (Rh) is the initial concentration of rhodopsin, (U) is the concentration of urea, and (I) is the light intensity. In any given bleaching experiment, the above parameters are constants allowing the equation to be written with pseudo-first order rate constants k_1' and k_1'' such that:

$$\frac{-d\,(Rh)}{dt} = k_1' + k_1'' = k_1'''$$

In more simple terms the first order rate constant defining the light-urea bleaching of rhodopsin should be equal to two pseudo-first order rate constants, the first dependent on the presence of light, and the second dependent on urea. It was discovered, however, that rhodopsin bleached with markedly different kinetics and with a rate at least one order of magnitude greater when compared with similar curves from the dark-bleaching study. In Figure 16 are shown the results obtained when rhodopsin is bleached with both light and urea. The dependence on urea concentration for the rate of bleaching appears more complicated. An axis with two time scales must be used to visualize the expanded range of bleaching rates. Using the initial slopes of the light-bleaching curves, first order rate constants and half-lives were calculated for each urea concentration and are shown in Table 4.

It was desirable to examine more closely the relationship

Figure 16

A plot of optical density versus time for the bleaching of rhodopsin in 0, 2, 3, 4, 5, 6, and 7 M urea. The monochromatic light source in the instrument was allowed to pass through the cell compartment during the light-urea bleaching experiment.

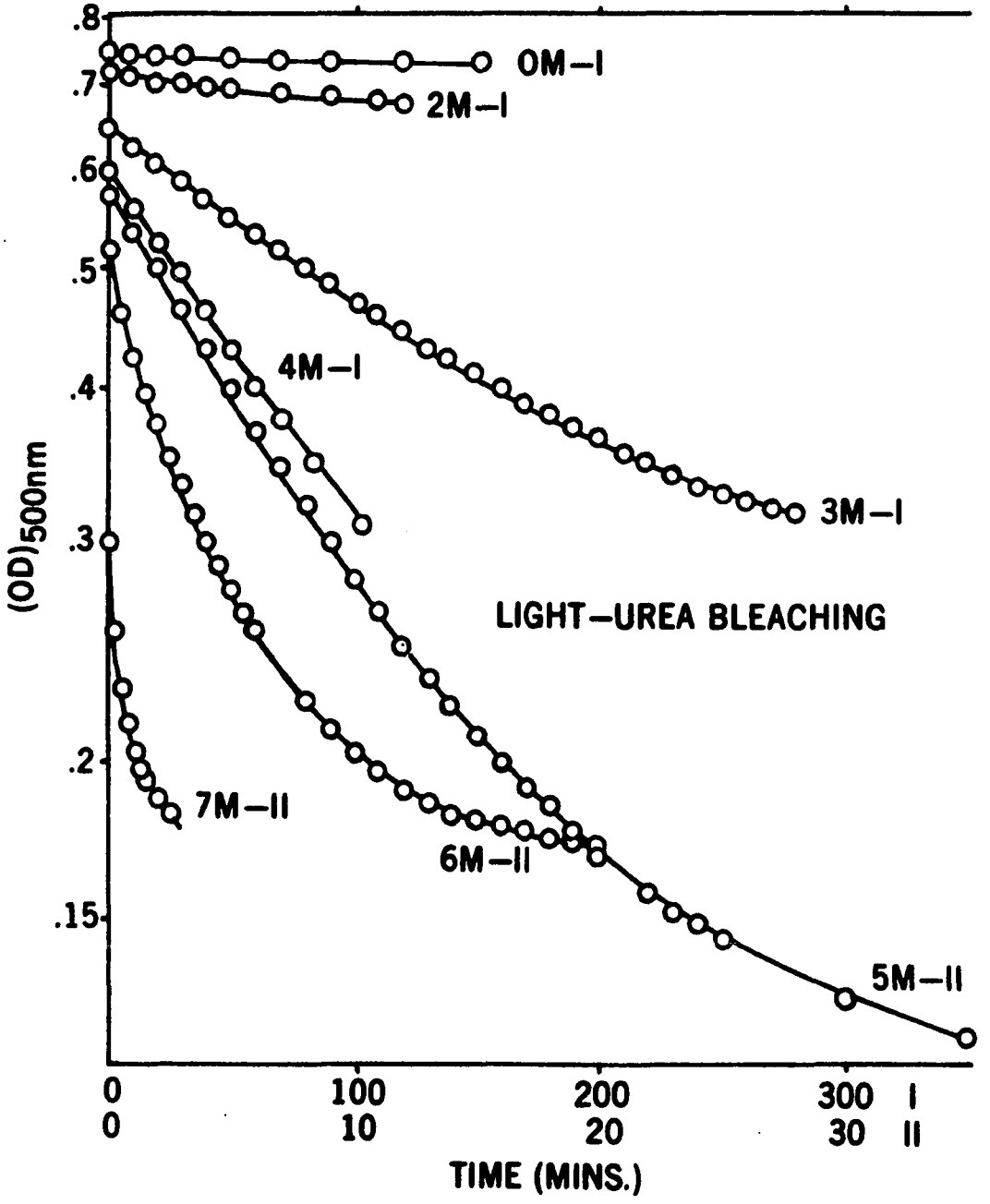

.8
.7
.6
.5
.4
.3
.2
.15
(OD)500nm
0M–I
2M–I
4M–I
3M–I
LIGHT–UREA BLEACHING
7M–II
6M–II
5M–II
0
0
100
10
200
20
300
30
I
II
TIME (MINS.)

Figure 16

TABLE 4

THE BLEACHING KINETICS OF RHODOPSIN WITH LIGHT AND UREA

Urea Molarity	Pseudo First Order Rate Constant	Half-life
0	1.19×10^{-5} sec^{-1}	16.1 hr
2	1.59×10^{-5} sec^{-1}	12.1 hr
3	5.85×10^{-5} sec^{-1}	3.82 hr
4	1.01×10^{-4} sec^{-1}	1.92 hr
5	8.45×10^{-4} sec^{-1}	13.7 min
6	2.56×10^{-3} sec^{-1}	4.50 min
7	7.54×10^{-3} sec^{-1}	1.60 min

between urea concentration and bleaching rate. A plot was constructed of log (Urea) versus log (k). The results are shown in Figure 17. At higher concentrations of urea the calculated value of alpha is 7.8. At lower concentrations of urea the slope becomes shallower reverting to a value similar to that observed in the dark-bleaching studies as shown in Figure 15. Light-urea bleaching would appear to consist of two processes: a low-urea concentration process indicative of a simple chemical reaction at the chromophoric site, exhibiting a urea concentration exponent of 2.4, and a high-urea concentration process involving gross conformational changes exhibiting a urea concentration exponent of 7.8.

Reversible dark-urea bleaching. Both Snodderly (79) and Plante, et al.,(68) have demonstrated that rhodopsin solubilized in CTAB looses its ability to regenerate after photobleaching. Since urea-induced bleaching is nonphotochemical it was of interest to test dark-urea bleached rhodopsin for regenerating ability. Samples of rhodopsin were made 3 or 4 M in urea and scanned spectrophotometrically. After 4 hours in the dark a second scan was taken to measure the amount of chromophore lost in the dark-bleaching process. Samples were carefully dialyzed against a thousand-fold volume of fresh buffer for one hour and the sample scanned a third time. The results of the experiment are shown in Figure 18. Regeneration was 33 percent complete and indicates that urea-bleached rhodopsin retains its ability to regenerate once the urea has been

Figure 17

A plot of log k versus log urea concentration for the light-urea bleaching of rhodopsin. The slope, α, is the exponent of the urea concentration relating the bleaching rate to the urea concentration.

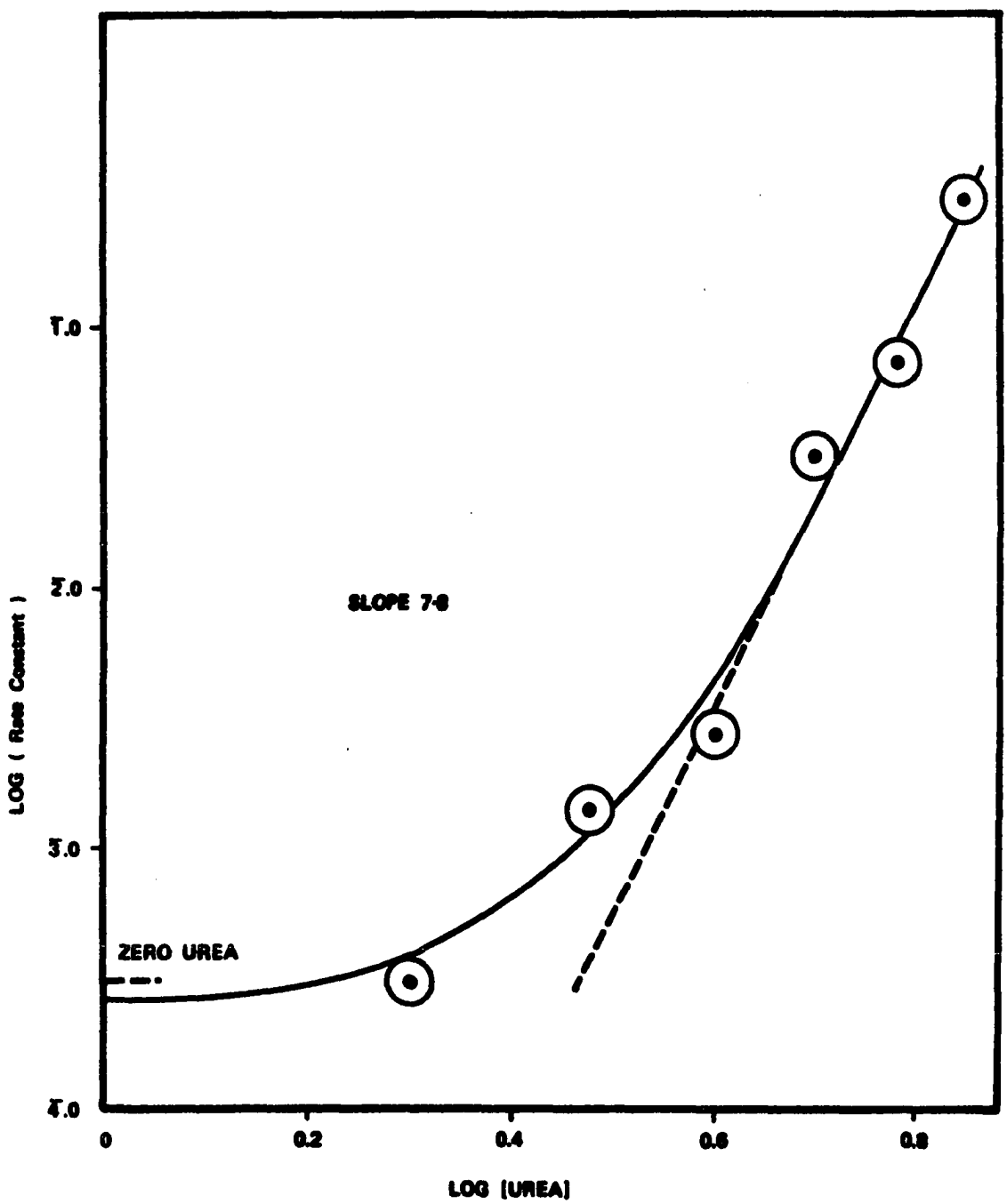

LOG (Rate Constant)
7.0
2.0
3.0
4.0
SLOPE 7-8
ZERO UREA
0
0.2
0.4
0.6
0.8
LOG [UREA]

Figure 17

Figure 18

Curve 1 is the spectrophotometric scan of a rhodopsin sample. Curve 3 is the same sample made 3 M in urea and allowed to stand 4 hours in absolute darkness at 20°C. Curve 2 is the same sample after extensive dialysis in the dark to remove urea. Regeneration of the peak is 33% efficient.

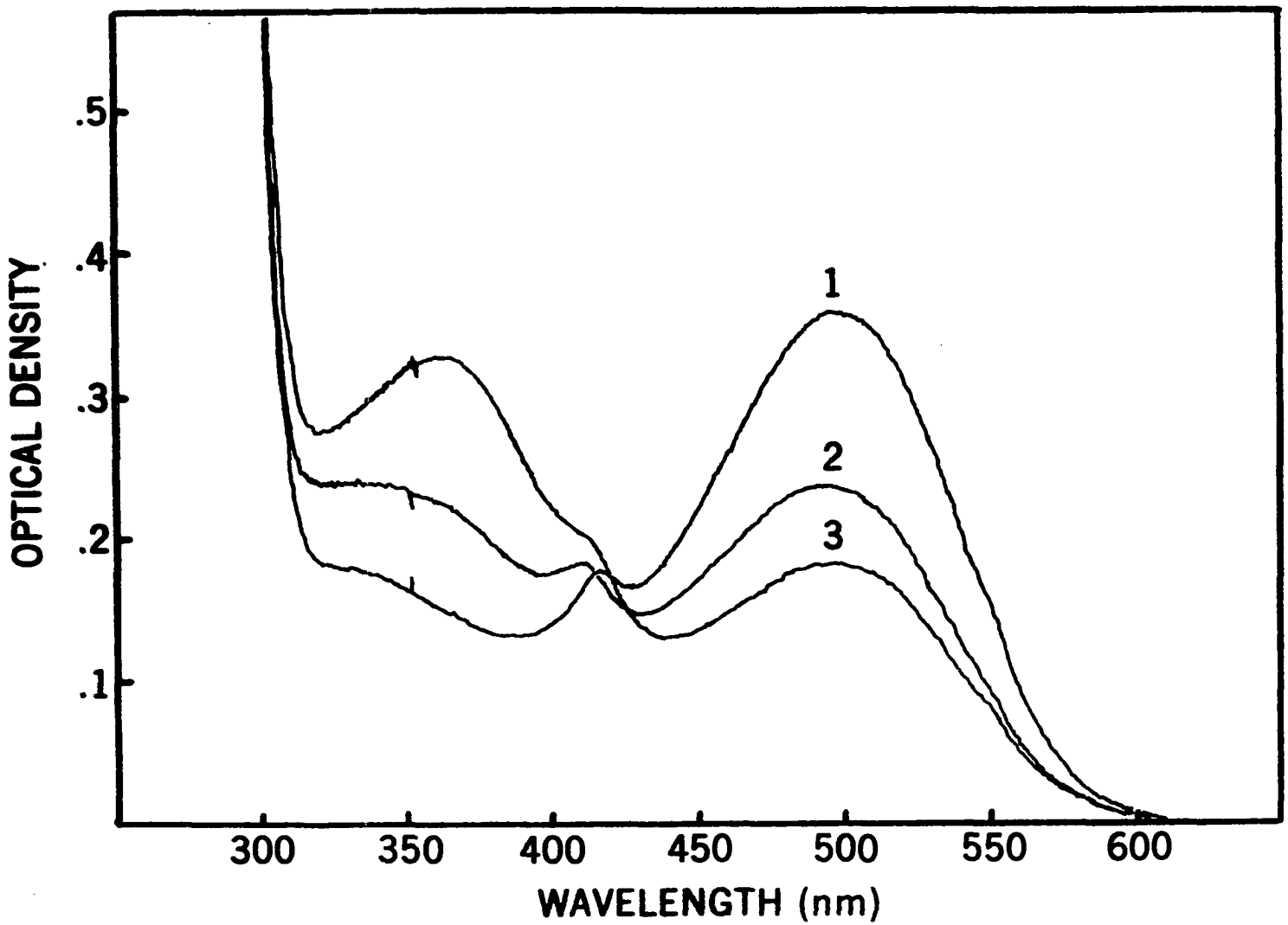

OPTICAL DENSITY
.5
.4
.3
.2
.1
1
2
3
300
350
400
450
500
550
600
WAVELENGTH (nm)

Figure 18

removed from the system. A figure of 36 percent regeneration has been cited recently for light-bleached Rhodopsin in CTAB (68). This is further proof that at lower concentrations urea is not involved in denaturation or gross conformational changes. Note also that the increase in OD_{365nm} is exactly equal to the decrease in OD_{498nm}. This would indicate that all-trans retinal results from the half-bleaching of rhodopsin at lower concentrations of urea.

Half-bleaching of rhodopsin. While conducting experiments in reversible bleaching, it was noted that at lower concentrations of urea the absorption at 498 nm could only be reduced by half, where $OD_{final} = OD_o / 2$. Moreover, this particular meta-stable state of rhodopsin was especially sensitive to light. If light were admitted to the cell compartment, the rhodopsin sample began bleaching at a faster rate than if urea and light had initiated the reaction. Clearly, a synergism exists between light and urea. Figure 19 shows an example of dark-urea bleaching with the decrease in chromophore approaching a value equivalent to $OD_o/2$. At the position indicated on the Figure, the light source in the instrument was switched on. The bleaching of rhodopsin proceeded at a new rate and went to completion.

Difference spectrum. If the effects of light or urea on the visual pigment, rhodopsin, are similar, the products of the two bleaching processes should be similar. Two samples of rhodopsin were bleached separately: the first by exposure to light, the second by 5 M urea in the dark. The urea-bleached

Figure 19

The phenomenon of half-bleaching illustrates the attainment of a meta-stable state where OD equals $OD_o/2$. An increased rate of bleaching is observed when light is admitted to the cell compartment.

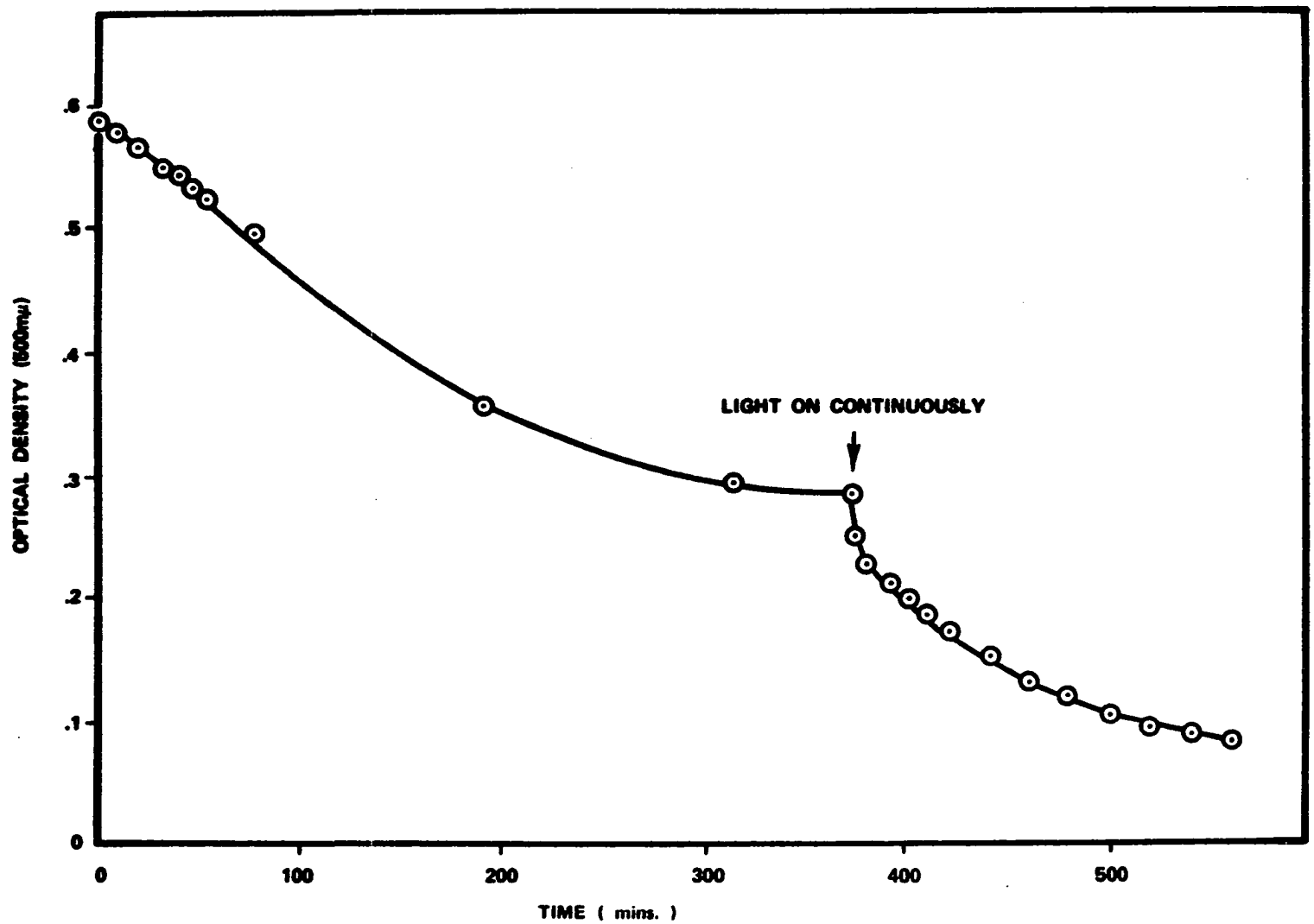

OPTICAL DENSITY (500mμ)
.6
.5
.4
.3
.2
.1
0
0
100
200
300
400
500
TIME (mins.)
LIGHT ON CONTINUOUSLY

Figure 19

sample was placed in the sample compartment while the light-bleached sample was used as the reference cell. The spectrophotometric scan recorded is shown in Figure 20. Differences can be seen around the gamma band. A shift in aromatic residues may have produced the observed peak at 245 nm. A similar difference spectrum was observed by Cox when lipoproteins in guanidine HCl were compared with native lipoproteins (21).

Thermal Stability of Rhodopsin

Effects of temperature on the stability of rhodopsin. The thermal stability of rhodopsin with increasing temperature was monitored by the procedure listed under "Materials and Methods." Aliquots of a purified rhodopsin preparation were subjected to a series of increasing temperatures for a period of ten minutes. The samples were cooled in ice and the optical density at 498 nm recorded. A plot was constructed of temperature versus relative optical density and the results shown in Figure 21. The inactivation of rhodopsin may be a two-stage process since an inflection point appears in the curve at a value equal to $OD_0/2$. These data are consistent with the two-stage bleaching of rhodopsin observed at lower concentrations of urea.

A comparison of light, temperature, and urea. One sample of rhodopsin was divided into three aliquots. The first was exposed to room light and allowed to bleach to completion. The second was made 5 M in urea and placed in absolute darkness at 20°C for four hours. The third was allowed to incubate for one hour at 50°C in total darkness. Each sample was scanned

Figure 20

Difference spectrum comparing a totally light-bleached rhodopsin sample with a totally urea dark-bleached sample. There appears to be a difference in the beta, gamma, and delta bands of rhosopsin.

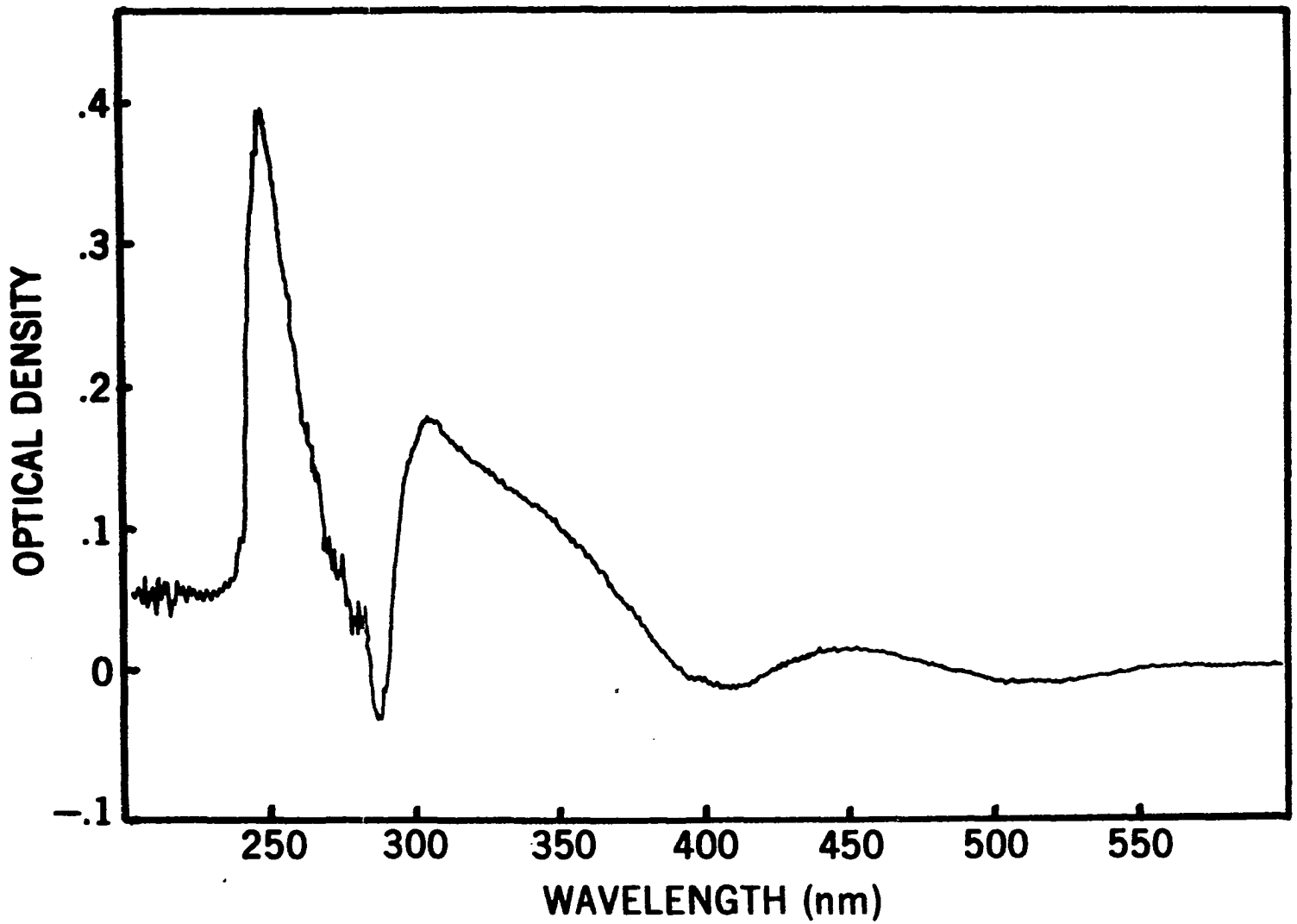

OPTICAL DENSITY
.4
.3
.2
.1
0
−.1
250
300
350
400
450
500
550
WAVELENGTH (nm)

Figure 20

Figure 21

The thermal stability of rhodopsin subjected to increasing temperatures for a duration of 10 minutes. Loss of the chromophore appears to take place by a two-stage process occurring at a value equal to $OD_o/2$.

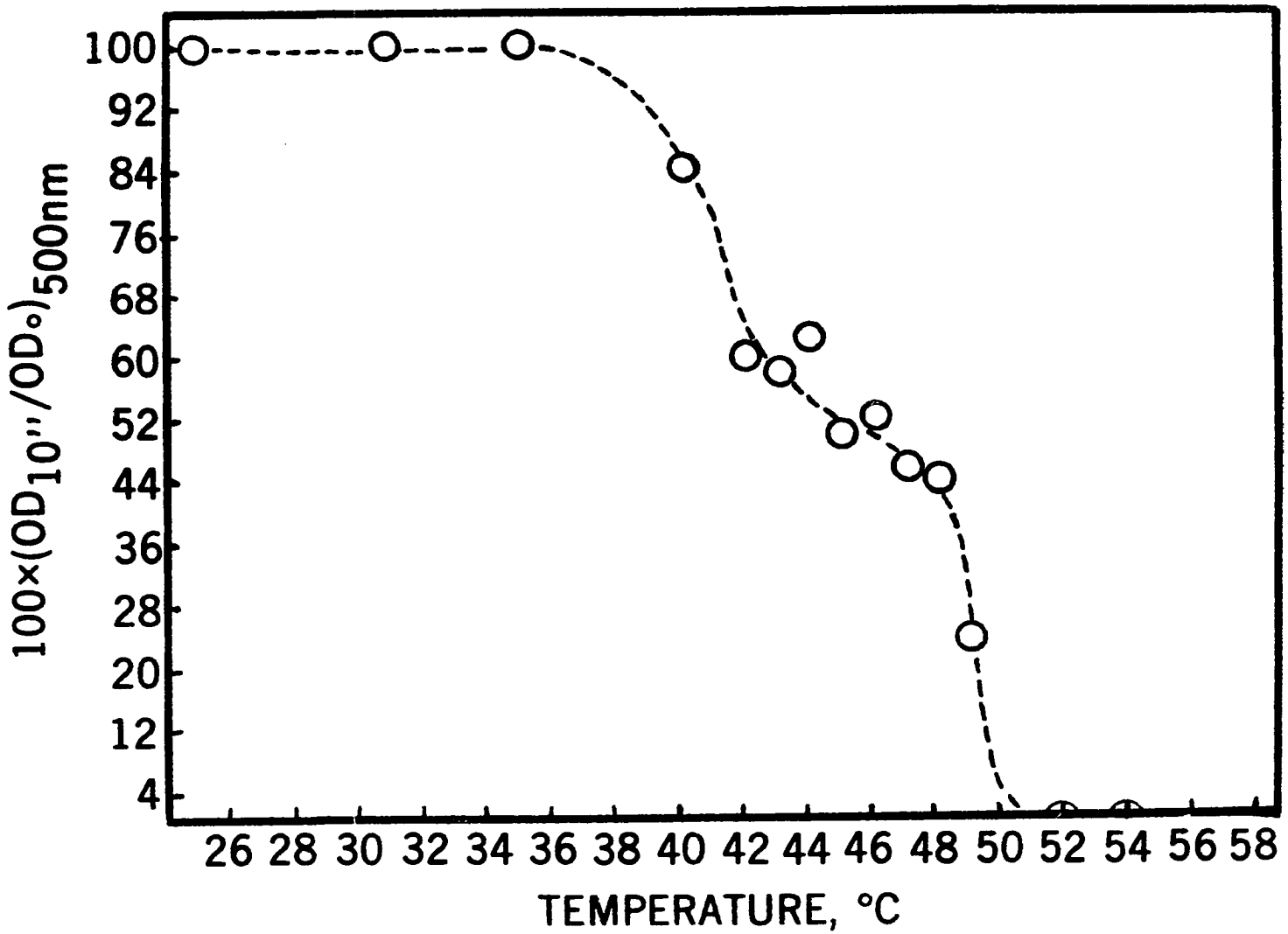

100
92
84
76
68
60
52
44
36
28
20
12
4
100×(OD10''/OD°)500nm
26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
TEMPERATURE, °C

Figure 21

at 20°C after the bleaching process and the results shown in Figure 22. The urea bleached sample, curve 4, verified the results from the previous difference spectrum. The thermally bleached sample, curve 3, is apparently identical with the photobleached sample, curve 2. The ratio of increase at A_{365nm} to decrease at A_{498nm} is 0.77. This would indicate that equimolar amounts of all-trans and 11-cis retinal were produced in either light-bleaching or dark urea-bleaching.

Combined effects of urea and temperature change. To further understand the urea-induced dark bleaching of rhodopsin it was decided to compare the bleaching efficiencies of urea at various temperatures. Purified samples of rhodopsin were mixed with the appropriate buffer and 10 M urea to give the desired molarity. A complete scan of the urea-rhodopsin preparation was made at 10 minute intervals, at the fastest scanning speed of the spectrophotometer. A typical spectrophotometric scan is shown in Figure 23 for 5 M urea at 30°C. Note the logarithmic decrease in chromophore at 498 nm, the appearance of retinal extinction at 365 nm, and the presence of a distinct cross-over point for the duration of the experimental run. This isosbestic point indicates that protein denaturation and gross conformational changes are not involved in the dark-urea bleaching of rhodopsin. Moreover, the cross-over indicates that the type and amount of product appearing in the cuvette is directly proportional to the disappearance of reactant, rhodopsin. As before the ratio of increase at A_{365nm} to decrease at A_{498nm} is 0.77 at the termination of the

Figure 22

A comparison of the products resulting from three different bleaching processes. Curve 1 is unbleached rhodopsin, curve 2 is totally light-bleached, curve 3 is totally thermal-bleached, and curve 4 is totally dark urea-bleached.

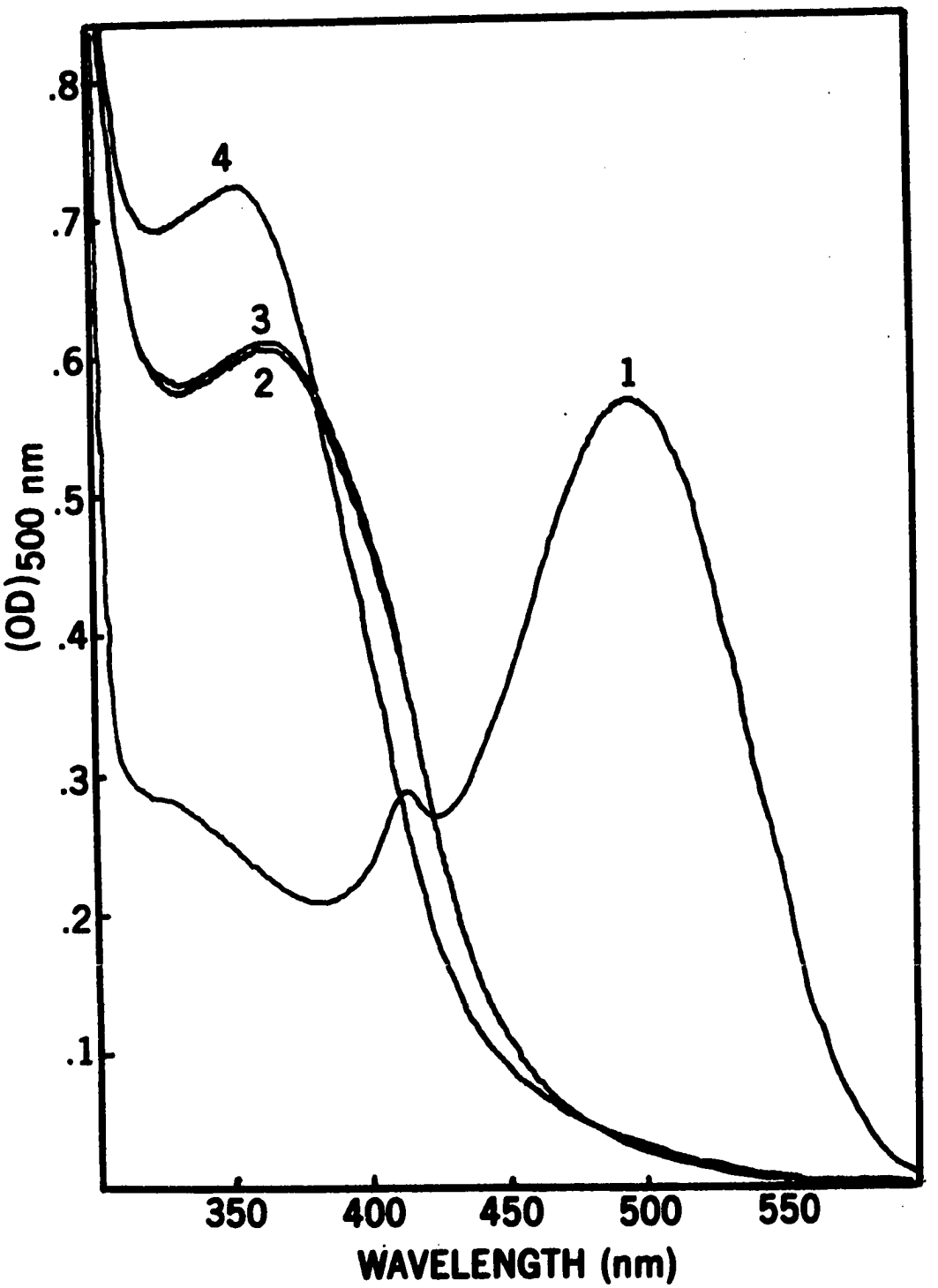


Figure 22

Figure 23

An illustration of the dark urea-bleaching process. The sample is rhodopsin in 5 M urea at 30°C. Successive spectrophotometric scans were made of the sample at 15 minute intervals. The single isosbestic point is prominent in the bleaching process.

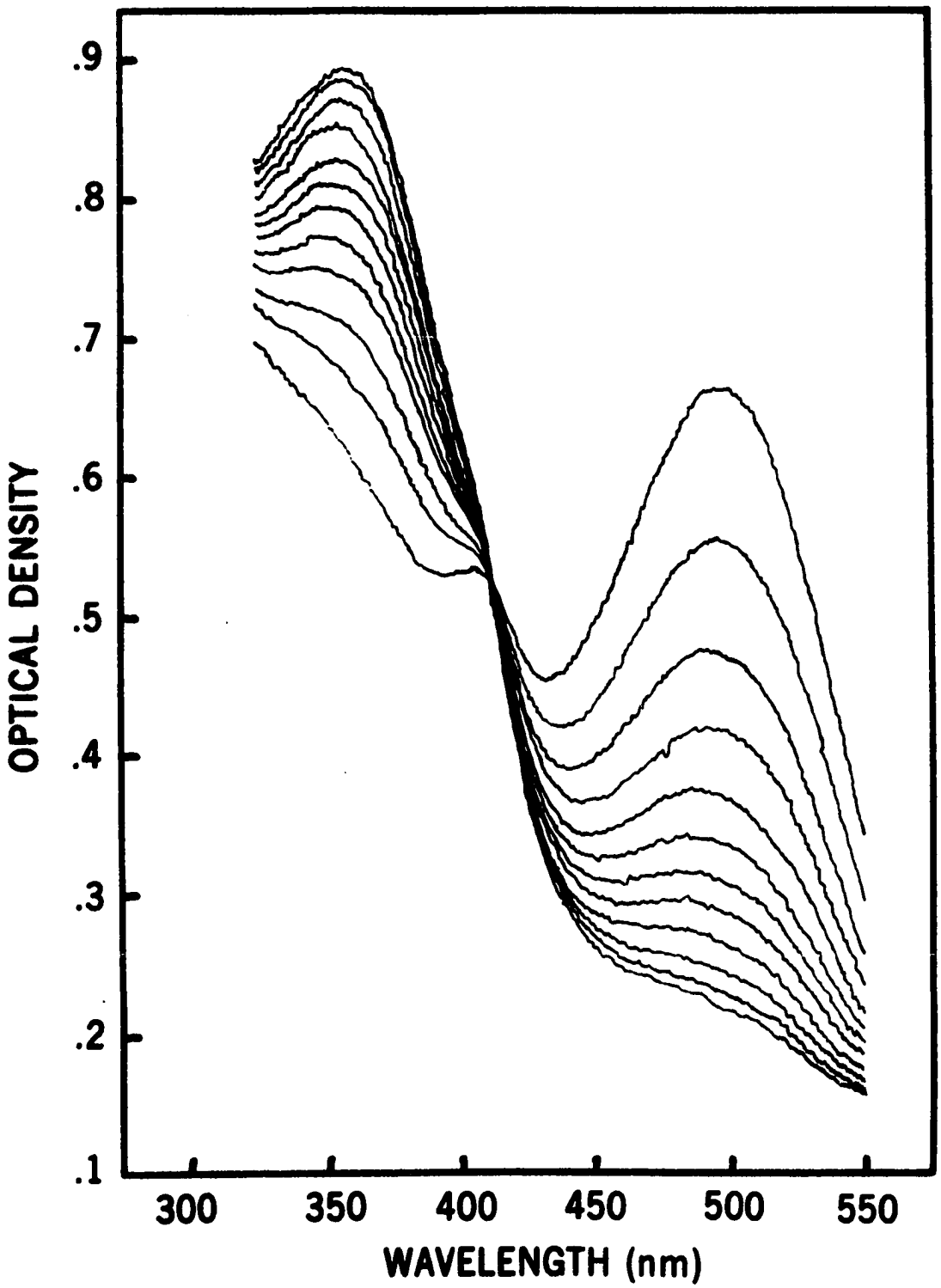

.9
.8
.7
.6
.5
.4
.3
.2
.1
OPTICAL DENSITY
300
350
400
450
500
550
WAVELENGTH (nm)

Figure 23

experiment. The data resulting from the bleaching of rhodopsin at the molarities of urea and temperatures studied were carefully tabulated. Figures 24 to 27 show the results of plotting the data for the four temperatures studied, 10^{o}, 20^{o}, 30^{o}, and 40^{o}C. From the slopes of the curves it is possible to calculate the pseudo first order rate constant for each process. In Table 5 is shown a tabulation of pseudo first order rate constants by molarity of urea at a given temperature. Also listed are the log k values for each rate, the temperature in ^{o}C and the reciprocal temperature in ^{o}K. An Arrhenius plot was constructed using the log k and $1/^{o}K$ values for each urea concentration. Figure 28 shows the results of the Arrhenius plot. From the previous equation for the pseudo first order rate constant:

$$k' = k(U) = (U) \cdot A \cdot e^{-E_a/RT}$$

$$\text{therefore} \quad \log k' = \log A + \log(U) - E_a/2.303\ RT$$

in which case a tangent to the Arrhenius curve at the desired temperature gives E_a, the activation energy, for the process at that particular temperature. Using slopes at 30^{o}C, the following activation energies were calculated for the dark-urea bleaching processes: 1 M = 21.6 Kcal/mole, 3 M = 17.4 Kcal/mole, and 5 M = 15.2 Kcal/mole.

Enzymatically Induced Bleaching

Phospholipase A. This particular enzyme from Vipera russeli was found to be an ineffective bleaching agent for the dark-bleaching of rhodopsin. A standard curve for fatty acids was constructed using the colorimetric microassay

Figures 24-27

Kinetic plots for the bleaching of rhodopsin in 1, 3, and 5 M urea at temperatures of 10^{o}, 20^{o}, 30^{o}, and 40^{o}C. Data is plotted as $OD-OD_{o}/2$ versus time at the four listed temperatures.

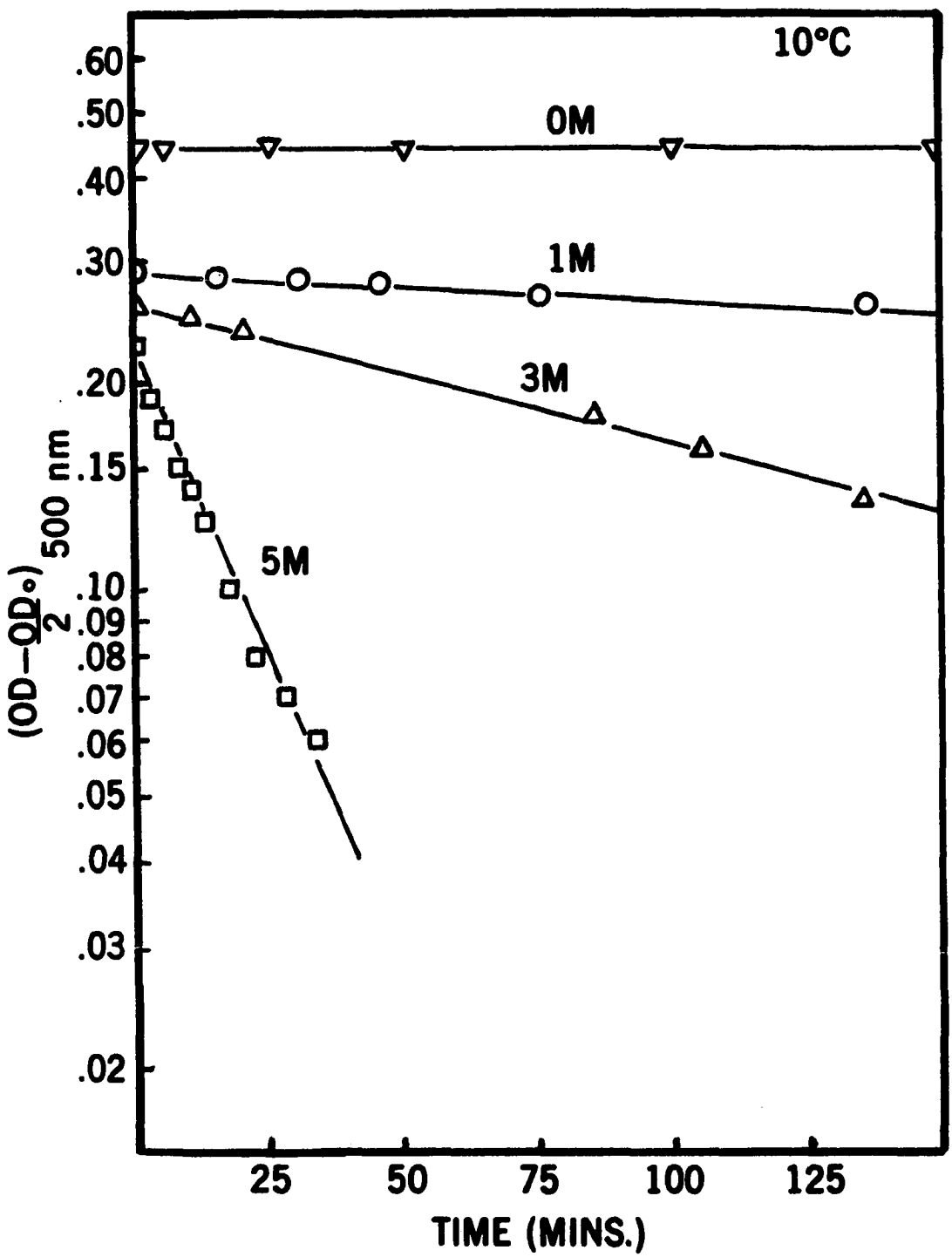

10°C
0M
1M
3M
5M
.60
.50
.40
.30
.20
.15
.10
.09
.08
.07
.06
.05
.04
.03
.02
(OD−OD°) 500 nm
25
50
75
100
125
TIME (MINS.)

Figure 24

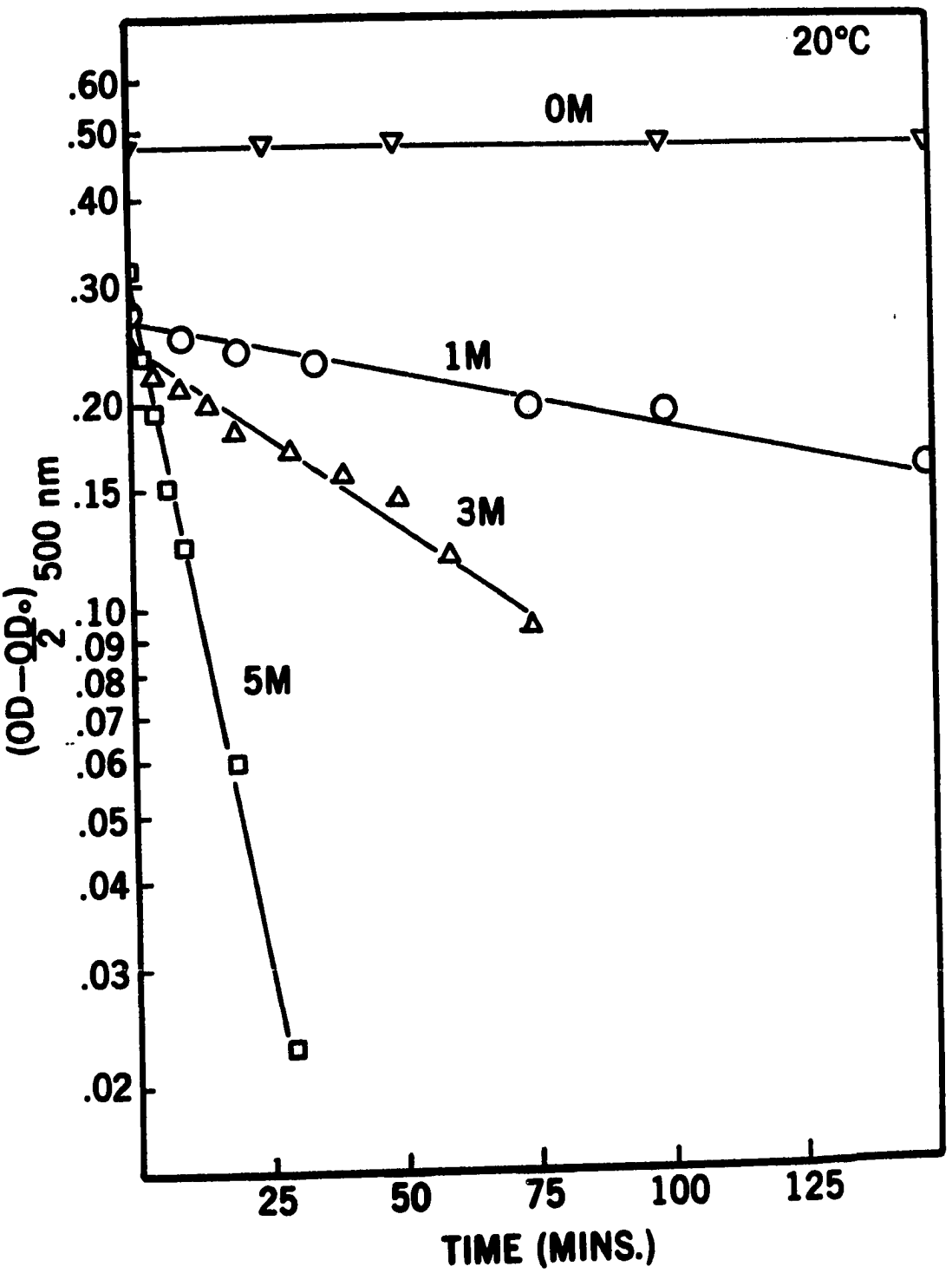

20°C
0M
1M
3M
5M
.60
.50
.40
.30
.20
.15
.10
.09
.08
.07
.06
.05
.04
.03
.02
(OD − OD°) 500 nm
25
50
75
100
125
TIME (MINS.)

Figure 25

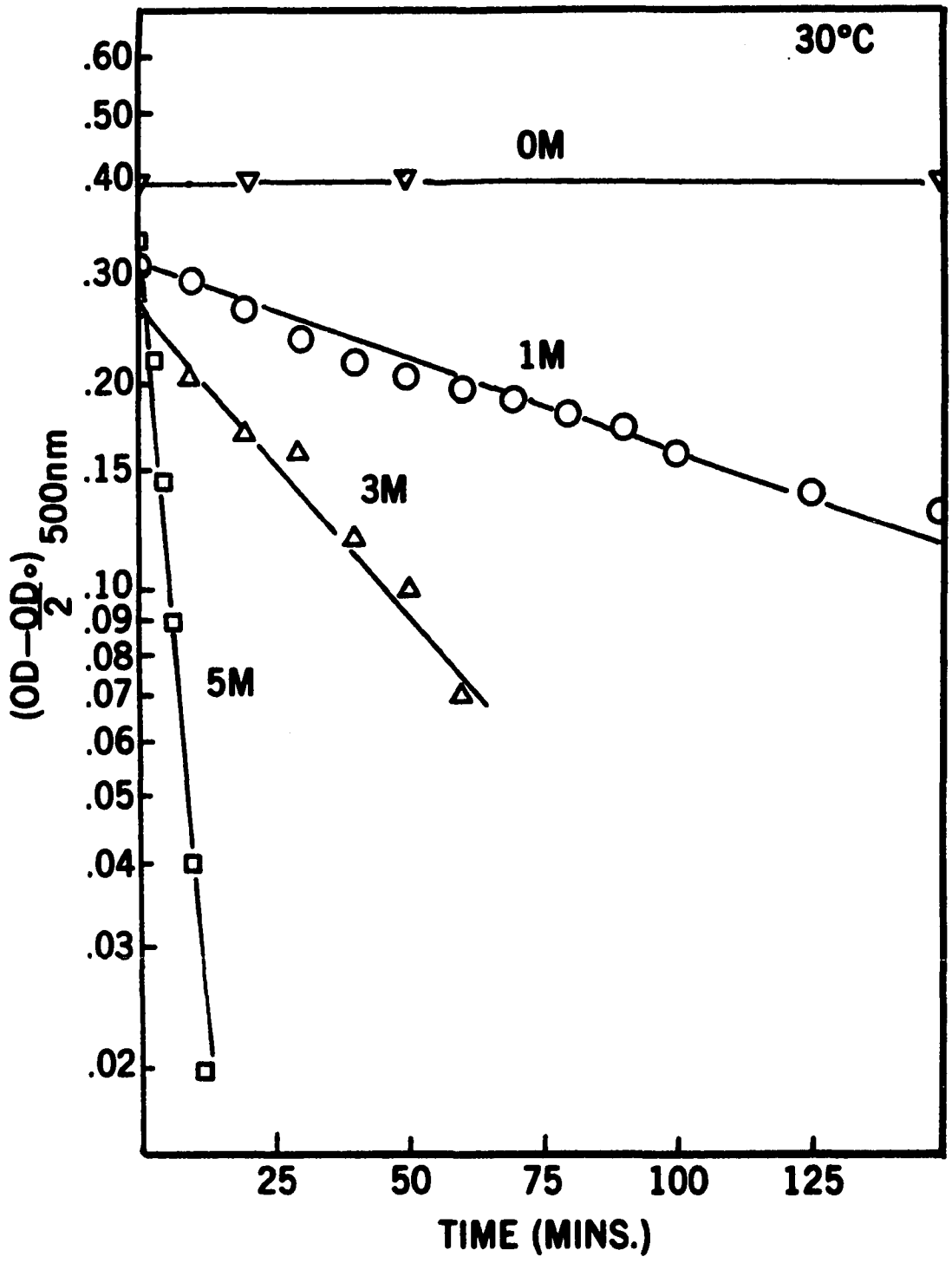

30°C
0M
1M
3M
5M
.60
.50
.40
.30
.20
.15
.10
.09
.08
.07
.06
.05
.04
.03
.02
25
50
75
100
125
TIME (MINS.)
(OD−OD°)/2 500nm

Figure 26

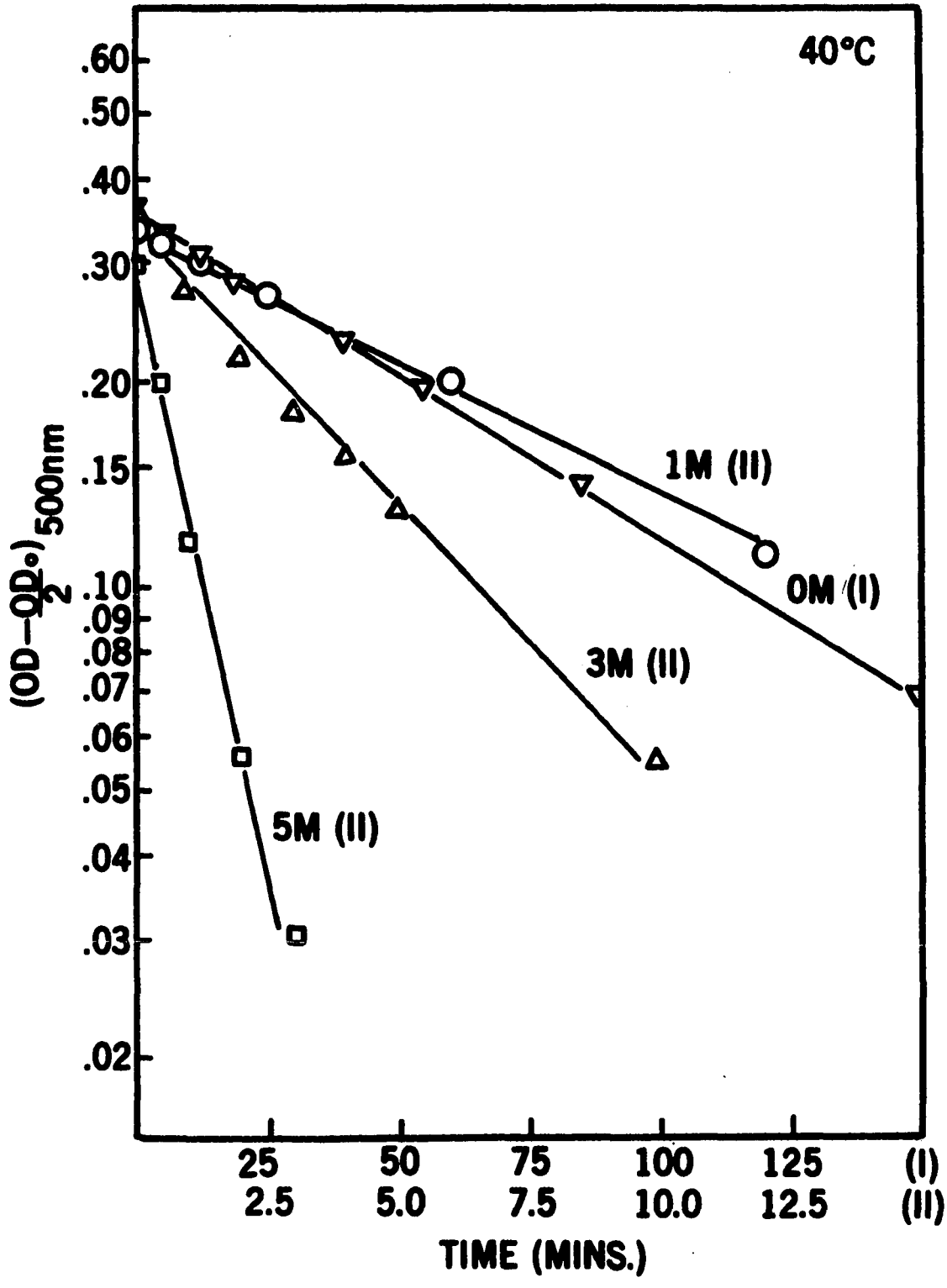

40°C
.60
.50
.40
.30
.20
.15
.10
.09
.08
.07
.06
.05
.04
.03
.02
$(OD-OD_\infty)_{500nm}$
1M (II)
0M (I)
3M (II)
5M (II)
25
2.5
50
5.0
75
7.5
100
10.0
125
12.5
(I)
(II)
TIME (MINS.)

Figure 27

TABLE 5

KINETIC DATA USED TO CONSTRUCT ARRHENIUS PLOTS

Urea Molarity	Pseudo first order rate constant, k, at temperature	log k	$1/T^{\circ}K$
1M	1.69×10^{-5} sec^{-1} at 10° C	-4.772	3.533×10^{-3}
	6.58×10^{-5} sec^{-1} at 20° C	-4.182	3.413×10^{-3}
	1.13×10^{-4} sec^{-1} at 30° C	-3.947	3.300×10^{-3}
	1.52×10^{-3} sec^{-1} at 40° C	-2.818	3.195×10^{-3}
3M	8.72×10^{-5} sec^{-1} at 10° C	-4.059	
	1.84×10^{-4} sec^{-1} at 20° C	-3.735	
	3.54×10^{-4} sec^{-1} at 30° C	-3.451	
	3.21×10^{-3} sec^{-1} at 40° C	-2.494	
5M	7.36×10^{-4} sec^{-1} at 10° C	-3.133	
	1.46×10^{-3} sec^{-1} at 20° C	-2.836	
	3.51×10^{-3} sec^{-1} at 30° C	-2.455	
	1.43×10^{-2} sec^{-1} at 40° C	-1.845	

Figure 28

Arrhenius plot of log k versus $1/^{o}K$ for the bleaching of rhodopsin in 1, 3, and 5 M urea. A tangent to the slope gives the activation energy, E_a, for the process.

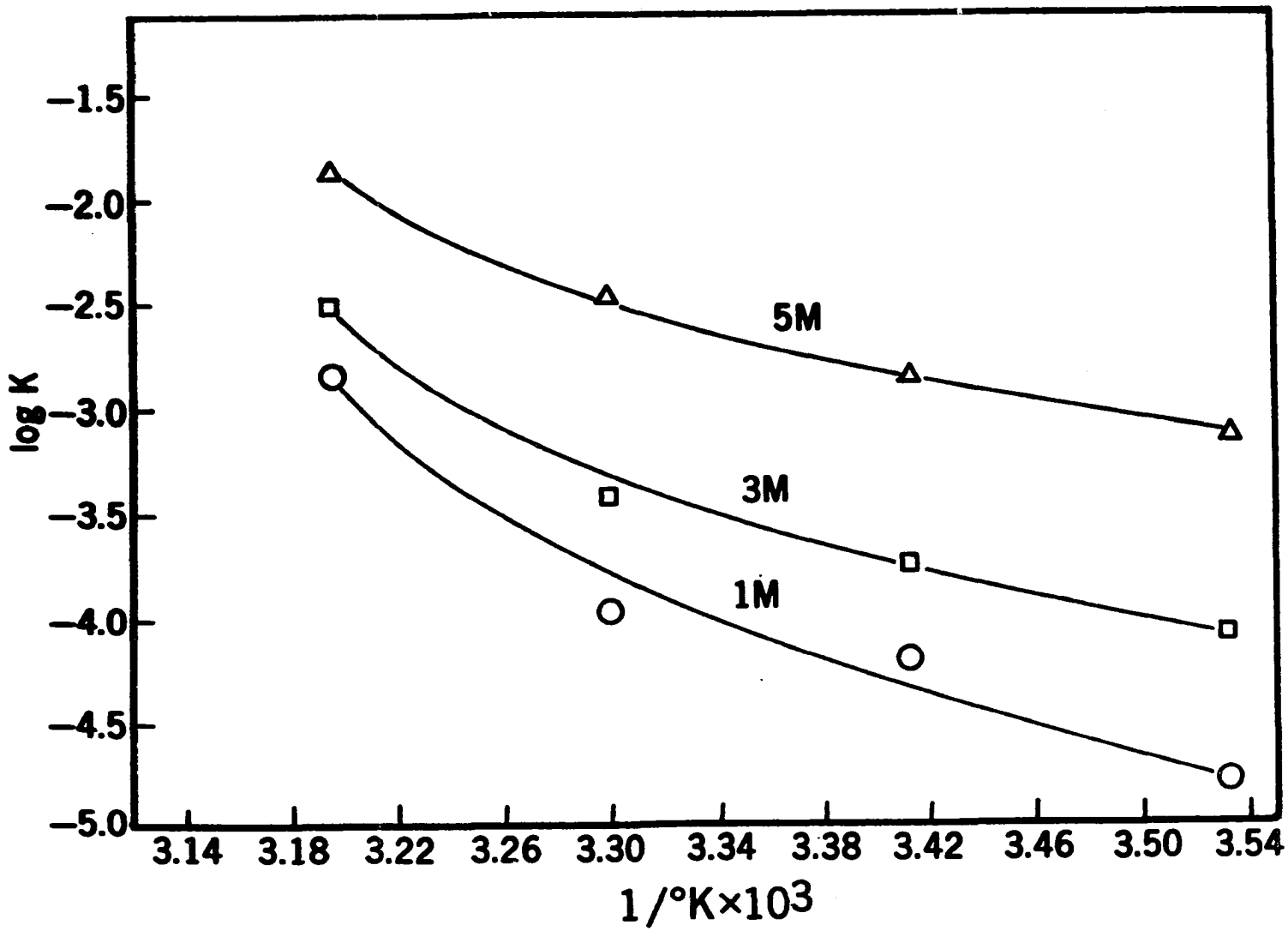

log K
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
−4.5
−5.0
5M
3M
1M
3.14
3.18
3.22
3.26
3.30
3.34
3.38
3.42
3.46
3.50
3.54
1/°K×10³

Figure 28

described in "Materials and Methods." The results are shown in Figure 29a. The slope of the curve is fairly linear with increasing concentration, but does have a blank reading which must be subtracted from observed values to find the true standard curve for the microassay. Once the assay procedure had been standardized, an aliquot of the phospholipase A was added to a sample of rhodopsin in the dark. Samples were withdrawn at 15 minute intervals and the fatty acid content determined. Although no bleaching of the rhodopsin occurred, there was some release of fatty acids as shown in Figure 29b. The change in fatty acid content is small corresponding to Δ .06 $OD_{550\ nm}$/90 minutes. Using the standard curve this reduces to 110 pmoles fatty acid released/min/ml of rhodopsin.

Phospholipase C. This enzyme was found to be a particularly effective dark-bleaching agent for rhodopsin. In Figure 30 is shown a direct tracing of the observed bleaching kinetics. Two features of the recorded data, taken at 15 minute intervals, are surprising. First, unlike the data in Figure 23 which was illustrative of the dark-bleaching process, there is no isosbestic point. Second, there is no logarithmic decline in peak height, but one considerably more linear in nature. Assuming zero order kinetics for the process, a decrease in chromophore of Δ.057 $OD_{498\ nm}$/15 min. or 90 pmoles rhodopsin/min is the rate of bleaching observed. A heat inactivated phospholipase C enzyme was found to have no effect when added to rhodopsin in the dark. The process is therefore

Figure 29

a. (O-O) The standard curve for the colorimetric microassay of fatty acids. Aliquots of palmitic acid were used as the standard. A blank reading of 0.110 is noted.

b. (Δ-Δ) Release of fatty acid from a 1.0 ml sample of rhodopsin at 15 minute intervals. Incubation is with Phospholipase A, from Vipera russeli. Rate of release of fatty acid corresponds to Δ $OD_{550\ nm}$/90 minutes or from the standard curve equals 110 pmoles fatty acid released/min/ml rhodopsin.

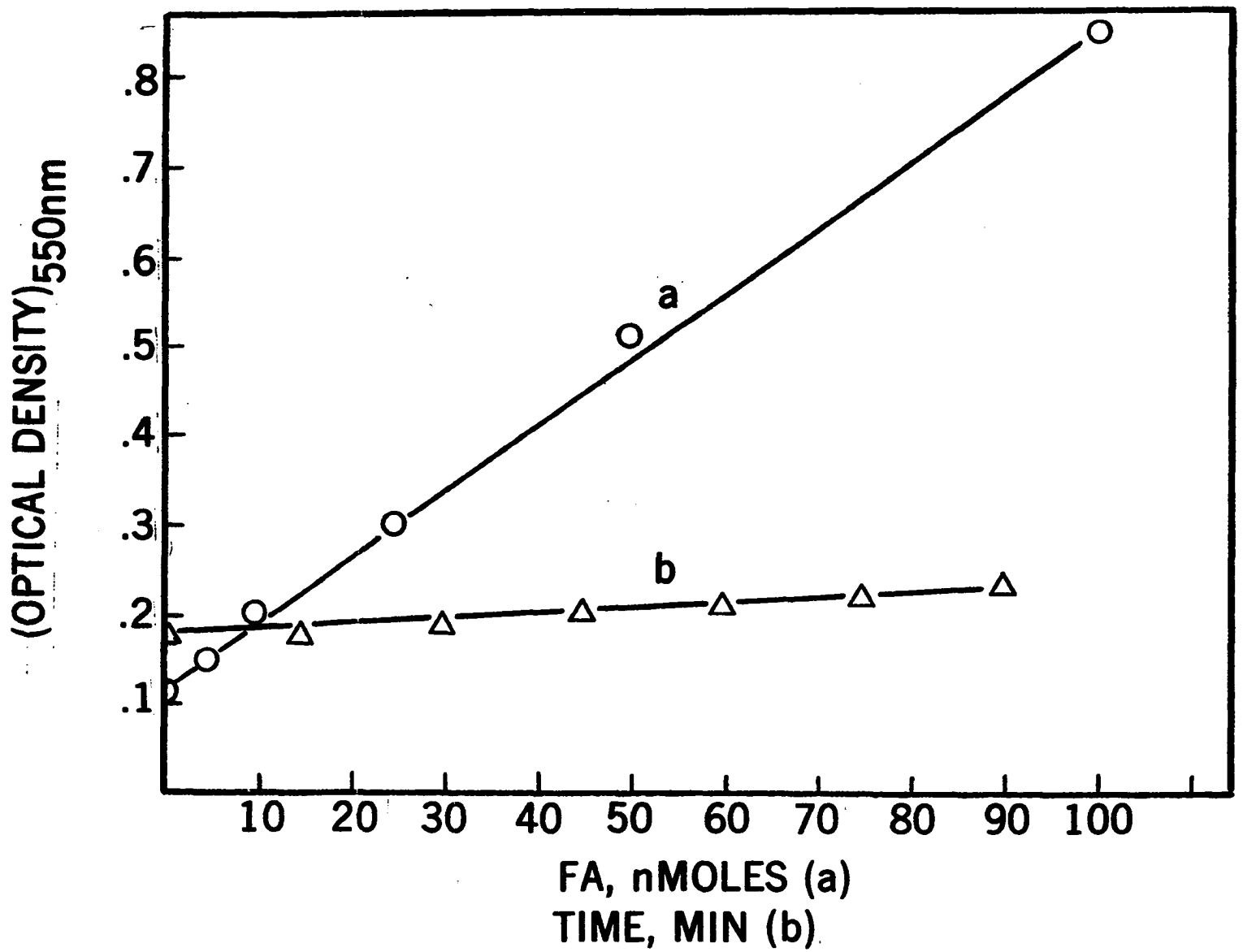

(OPTICAL DENSITY)550nm
.8
.7
.6
.5
.4
.3
.2
.1
a
b
10
20
30
40
50
60
70
80
90
100
FA, nMOLES (a)
TIME, MIN (b)

Figure 29

Figure 30

The dark-bleaching of rhodopsin induced by phospholipase C. Successive scans are taken at 15 minute intervals. There is no isosbestic point for the bleaching process.

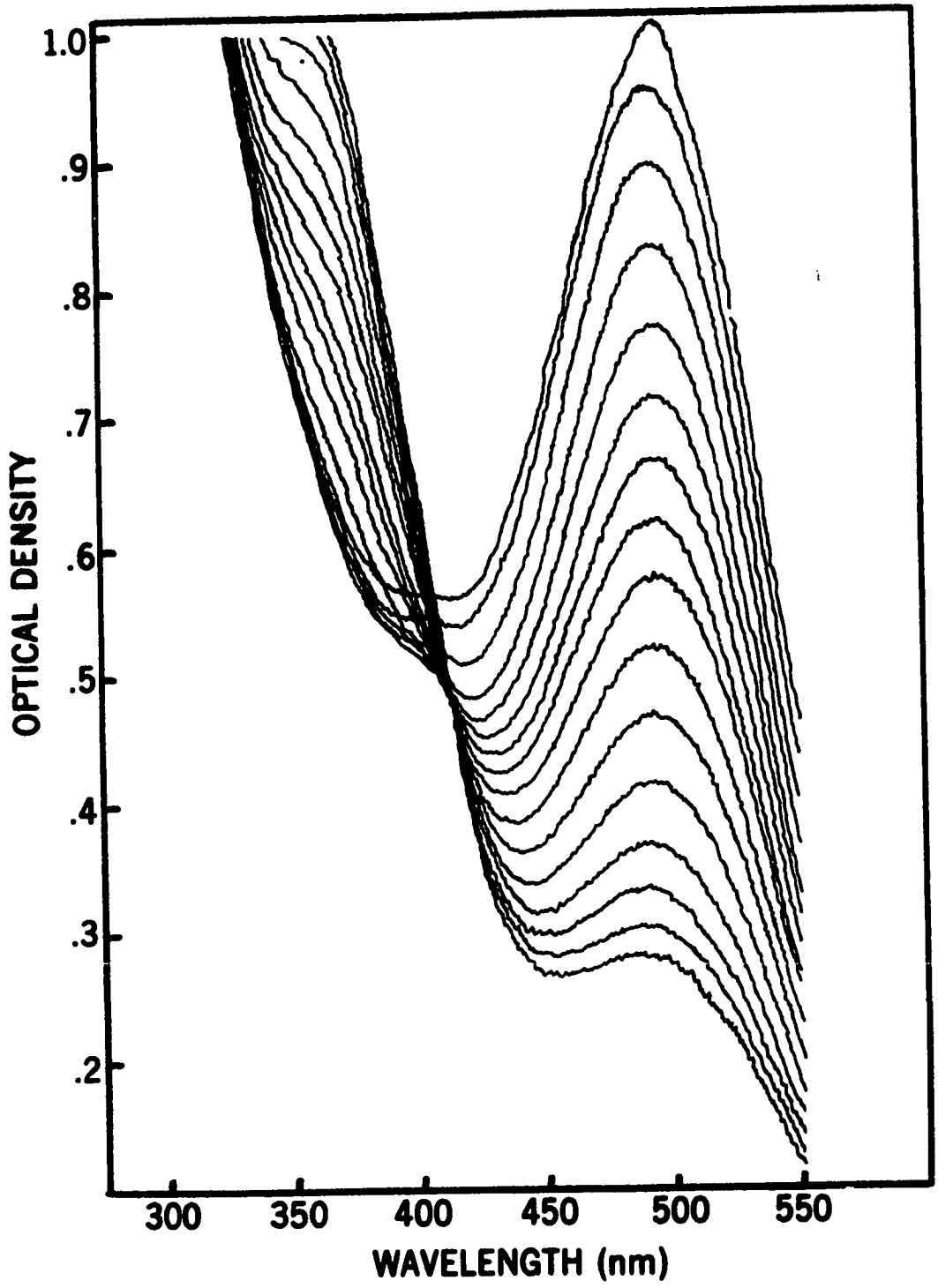

1.0
.9
.8
.7
.6
.5
.4
.3
.2
OPTICAL DENSITY
300
350
400
450
500
550
WAVELENGTH (nm)

Figure 30

thought to be a true enzymatic reaction. In addition after treating a PE standard with the enzyme, a qualitative increase in the amount of diglyceride present could be observed by thin layer chromatography.

Phospholipase D. No dark-bleaching of rhodopsin was observed on addition of this particular enzyme. When added to a PE standard it did, however, effect the release of phosphatidic acid and was presumed to be active.

Thin Layer Chromatography

Detection of retinals. It was possible to separate three of the four possible single isomers of retinal by TLC. All-trans, 9-cis, and 11-cis exhibited decreasingly smaller R_f's in the solvent system used. The R_f of 13-cis retinal appeared to be identical to that of all-trans retinal. The results of the separation with standards and a mixture of the four isomers are shown in Figure 31. Similar results have been obtained by Daemen and Bonting with a different solvent system (25). The retinals could be easily seen under UV light, producing a pronounced lavender spot. In addition, the retinals formed a dark-indigo Schiff base if the developed plate were sprayed with p-amino benzoic acid in 0.1 N HCl. As the plate air-dried the spot faded to a light pink, indicating the Schiff base had gone from the protonated form to the unprotonated form as follows:

$$\text{PABA} - NH_2 + \overset{O}{\overset{\|}{H-C}} - \text{RET} \xrightarrow[-H_2O]{H^+} \text{PABA} - \overset{(+)}{\underset{H}{N}} = C - \text{RET (purple)}$$

$$\text{PABA} - \overset{(+)}{\underset{H}{N}} = C - \text{RET} \xrightarrow[-H_2O]{HCl} \text{PABA} - N = C - \text{RET (pink)}$$

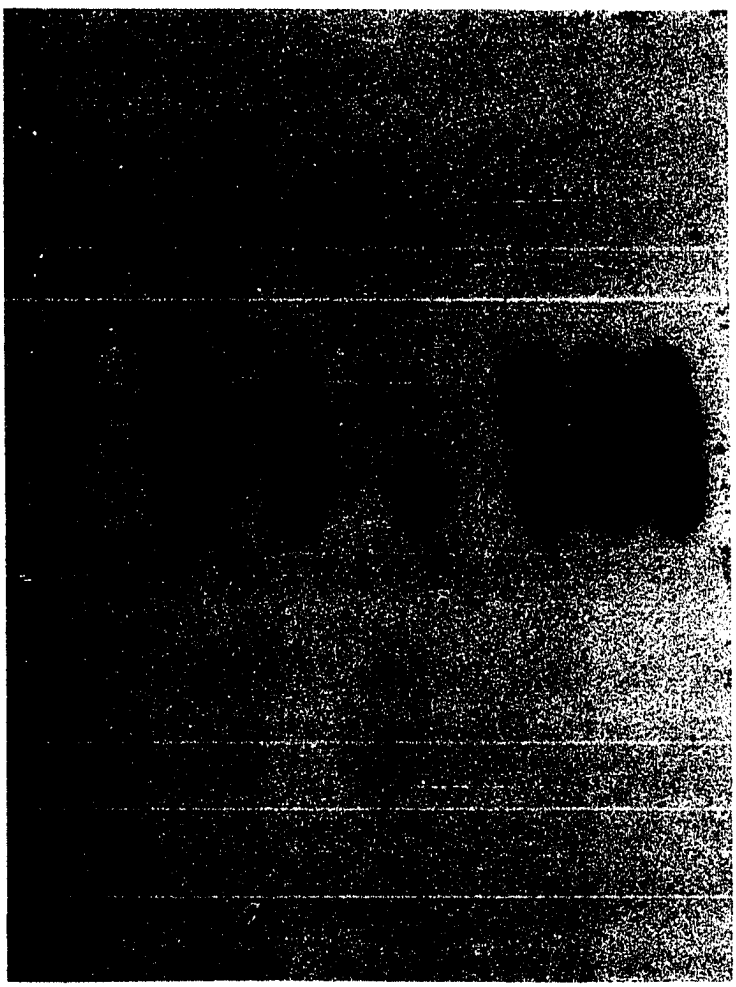


Figure 31

A B C D E F F

Separation of the single isomers of retinal. Purchased standards were: A- 11-cis, B- all samples applied, C- 13-cis, D- all-trans, E- 11-cis + all-trans, and F- 9-cis.

Aqueous samples of dark urea-bleached and light-bleached rhodopsin were spotted directly onto thin layer plates with suitable standards. Photographs of the developed plates were negative, apparently due to the low concentration of the retinals in the rhodopsin samples.

It appeared to the eye, however, that the light-bleached sample gave all-trans retinal, whereas the urea-bleached sample gave equal quantities of 11-cis retinal and all-trans retinal.

Detection of Phospholipids. Phospholipids, as well as glycerides, could be easily isolated and detected following the techniques described in "Materials and Methods." Suitable standards of PE, PA, PS, and PC were run with chloroform extracts of light-bleached and urea dark-bleached rhodopsin. The results are shown in Figure 32. In the light-bleached sample a compound with approximately the same R_f as PE, giving a molybdic acid positive reaction, and showing the characteristic UV lavender spot for retinals was observed. In like manner, the dark urea-bleached sample produced two such spots.

These two spots have been tentatively identified as N-retinylidine phosphatidylethanolamine. Since the ΔR_f of these spots is the same as the ΔR_f between 11-cis and all-trans retinal, it is possible that the spot with the larger R_f is all-trans N-retinylidine PE, while the spot with the smaller R_f is 11-cis N-retinylidine PE. No other differences in phospholipids or diglycerides were detected between urea-bleached and light-bleached rhodopsin samples.

Figure 32

A B C D E

Comparison of the phospholipids obtained from light-bleached and dark urea-bleached extracts of rhodopsin. Sample A is PC standard, B is PE, C is light-bleached, D is PC, and E is dark urea-bleached.

CHAPTER IV

DISCUSSION

Assessment of Purity

On Defining the Purity of Visual Pigment

The purification of a visual pigment imposes rather strict limitations on the investigator. Visual pigments, unlike enzymes which possess definite activity, react with photons of light and in so doing are degraded. Thus, their possession of a unique spectrum is the only yardstick currently in use to monitor the visual pigment's integrity and degree of purification. The problem is further compounded by the visual pigment's insoluble nature, necessitating the use of an accessory detergent to disperse it. Depending on the choice of detergent, the exact nature of the visual pigment under study may differ from extract to extract. In the non-ionic natural detergent digitonin, for example, the visual pigment is extracted from the ROS with a fifty percent complement of bound lipid, and even some of the membrane structure intact (50). At the other extreme, CTAB, an ionic synthetic detergent, is known to disaggregate the ROS completely, solubilizing the visual pigment into nomomeric units (78). This particular state of the visual pigment is also devoid of all

but a small portion of bound lipid. When trying to evaluate the properties of visual pigment, two immediate problems are encountered: first, little work has been done to examine the properties of visual pigment *in vivo*, and second, the investigations to date, having used different detergent preparations, make comparison and evaluation of observed visual pigment properties difficult. Though the question of purity may remain unanswered until more rigorous criteria become available, the question of nomenclature should be answered now. It is proposed that visual pigments be divided into three structural classes: the lipid-free, glycoprotein with attached retinal chromophore should be termed *rhodopsin*, the aggregated n-meric rhodopsin, with its complement of fifty percent bound lipid and showing the ability to regenerate (35) should be termed *liporhodopsin*, and finally the particulates formed of liporhodopsin with intact membrane structure should be termed *visual pigment*.

Purification and Assessment of Purity

An efficient procedure was developed for obtaining rhodopsin in bulk quantity by extensive washing and a final extraction in the ionic detergent CTAB. Further purification by columns, ammonium sulfate fractionation, followed by lyophilization and chilled hexane extraction gave a rhodopsin preparation with spectral ratio of $A_{280\ nm}/A_{498\ nm}$ of 6.0 and $A_{400\ nm}/A_{498\ nm}$ of 0.29. Based on total protein impurities removed, purification was 340 fold.

Rhodopsin at pH 9.0 gave a single band on disc gel electrophoresis as evidenced from Figure 10. This result compares favorably with that of Heller (39) who electrophoresed opsin under similar conditions. It appeared, however, that the electrophoretic process bleached rhodopsin. Even though the gels were run in absolute darkness a faint yellow band could be discerned before staining, indicating the rhodopsin had bleached during the process.

The amino acid analysis was comparable to that of Heller (39) and Shichi et al. The percent content of hydrophobic and nonpolar residues is among the highest recorded for a protein. Pro, Ala, Cys, Val, Met, Ileu, Leu, and Phe equal fifty percent of the total residues, data taken from Table 3. An aminosugar reacting with ninhydrin was present at a level equal to 4.2 percent of the residue total. It is believed glucosamine is responsible for this peak since it is the only aminosugar thus far found in the rhodopsin oligosaccharide (42).

The micellar hydrated density of rhodopsin was found to be .9699 ml/g $\pm$ .0050, giving a micellar molecular weight of 310,000 $\pm$ 30,000 Daltons. The Schlieren peaks were fairly uniform during the sedimentation velocity runs and showed little tendency to widen as shown in Figure 11. The large value for the hydrated density indicates approximately 80 percent of the weight of the micelle is due to the solubilizing detergent CTAB. This compares favorably with the results of Hubbard (44). She found a micellar weight of 290,000, 85 percent

of which was due to the detergent digitonin.

Bleaching Experiments

Urea as a Bleaching Inducer

Urea was found to be an effective bleaching agent for rhodopsin. In concentrations as low as 2 M, urea induced the loss of alpha band absorption at 498 nm. This bleaching of a light-sensitive pigment in the absence of light is a remarkable yet reproducible phenomenon. Within the range of urea concentrations used to dark urea-bleach rhodopsin, the rate of decrease of alpha band absorption increased with increasing urea concentration. When kinetic plots of the data were constructed, however, the loss of extinction at 498 nm did not follow first order kinetics. In contrast, the bleaching of rhodopsin by light has long been used as an example of a first order process. If the optical density at 498 nm were plotted as log $(OD-OD_0/2)$ instead of log (OD), versus time, the process demonstrated first order kinetics. It will be remembered that at lower concentrations of urea, 2 or 3 M, it was possible to bleach rhodopsin in the dark to a limiting value of $OD_0/2$. Moreover, this particular state of rhodopsin was indefinitely stable as long as no light was permitted to enter the solution. Thus by subtracting $OD_0/2$, as representing the concentration of nonbleachable chromophore, from each successive optical density it was possible to construct a first order plot. Even at higher concentrations of urea, where the so-called nonbleachable chromophore bleaches, the linearity of this same

plot suggests a consecutive rather than a simultaneous urea reaction. Three rhodopsin models can be presented which explain the above phenomenon: first, the rhodopsin molecule may contain two chromophores, only one of which is susceptible to dark urea-bleaching at low concentrations of urea. At a recent vision conference, Daemen and Bonting (24) hinted that a forthcoming paper will discuss this possibility. Second, rhodopsin may consist of two subunits, each of which contains a chromophore. Only one of these would be sensitive to low concentrations of urea. Third, rhodopsin may consist of two distinct protein moieties, each of which has a chromophore, and only one of which is sensitive to low concentrations of urea.

There is some basis for choosing the second model as the most probable. As mentioned in the first chapter, Heller has reported that gel-filtered rhodopsin has an extinction coefficient of 23,000. In the same paper he states that his purified rhodopsin contains only one -SH group after photobleaching. In contrast, other principal workers in the field of vision present evidence just as convincing that the extinction coefficient is 42,000 and that two -SH groups are freed after bleaching. Rhodopsin may be composed of two subunits, A and A', both of which are similar in molecular weight, amino acid content, chromophore, etc. After complexing, however, the sensitivity to dark urea-bleaching of one subunit may be different than the other.

Although the assumption that two chromophores, having different sensitivities to urea, allows the data to conform to first order kinetics, it must be stressed that this is empirical and no proof is presented that rhodopsin can be represented by any of the three models discussed.

Effects of Urea at the Molecular Level

There are several explanations for the bleaching of rhodopsin in the dark by a small molecule such as urea. Tanford (81) has investigated the general effects of urea on proteins and indicates that four distinct processes may be involved. The first of these is the disaggregation of protein; this does not necessarily involve any conformational changes. CTAB is apparently an agent which disaggregates rhodopsin without conformational change, but its effect on a hypothetical subunit structure is unknown. It certainly does not bleach rhodopsin during the disaggregation process. On the other hand a detergent such as SDS does an excellent job of disaggregating rhodopsin and bleaching it irreversibly (78). Urea could be disaggregating rhodopsin from a CTAB induced n-meric state to a monomeric state. If the CTAB induced state were already monomeric, urea could be inducing the hypothesized subunits to separate. This might cause conformational changes, exposing the anhydrous retinal-binding site to water, with cleavage of the Schiff base and loss of alpha band absorption.

The second possible effect of the urea molecule is to induce subtle conformational changes in the gross apoprotein structure, which in turn might affect the microenvironment where retinal is bound and promote hydrolysis of the Schiff base linkage as above with concomitant loss of alpha band absorption.

A third possible mode of action for urea is to affect the active site directly, somehow lifting the retinal prosthetic group free and extinguishing the absorption at 498 nm by hydrolysis of the Schiff base. It is not required that conformational changes of the protein take place during this latter process.

The fourth possible effect for urea with protein and probably the most common, is the denaturation of the protein's structure. This process may be either reversible or irreversible. Several experiments in the Results section tend to make the last process unlikely. First, it was discovered that when rhodopsin was dark-urea bleached to a limiting value of $OD_o/2$, some of the absorption at 498 nm could be recovered by dailyzing away the urea in the dark and allowing the process to reverse as shown in Figure 8. It is doubtful whether denatured opsin could reform rhodopsin so efficiently and quickly. Second, when kinetic plots were made of log urea concentration versus log first order constant, it was found that alpha, the exponent relating urea concentration to the bleaching rate, was relatively low with a value of 2.4. If complete

denaturation were in effect, alpha should have been several times larger. Third, simple examination of a bleaching curve, such as that shown for 5 M urea at 30°C in Figure 23, shows a single isosbestic or cross-over point as each successive trace is made with time. If denaturation were taking place no isosbestic point would appear as more than one product would be generated during the dark-urea bleaching process. Similar scans have been obtained by Hubbard using guanidine HCl (45) and Kito using a combination of urea and pH to bleach rhodopsin (51). There exists at present no evidence either supporting or negating the first three suggested effects of urea. The possibility that urea affects only the active site seems improbable. Simple experiments conducted with urea showed that it had no ability to isomerize 11-cis retinal, nor did it have any effect on a model chromophore of retinal thiazolidine. In addition, the breakdown products of urea, cyanate and cyanide, showed no ability to bleach rhodopsin. If circular dichroism could have been measured between the viable rhodopsin and dark-bleached rhodopsin, any difference in dichroic ratio would have implicated the second effect - subtle conformational changes - while no change in dichroic ratio would have implicated the first effect - disaggregation. The effect of urea at the molecular level on rhodopsin in the dark must remain unresolved.

Light-Urea Bleaching

The bleaching of rhodopsin in the presence of light

and urea appears to be a much more complex process than the dark-urea bleaching of rhodopsin. The decrease in absorption at 498 nm as a function of time did not adhere to any simple kinetic law. Light and urea appeared to act in a synergistic manner. The urea present in the rhodopsin solution rendered it more sensitive to light-bleaching. Since the quantum efficiency for bleaching is already near 1.00, it is possible that the light was also sensitizing the rhodopsin to urea-bleaching. In order to estimate the dependence on urea of the light-urea bleaching rate, a plot similar to that shown for dark-urea bleaching was constructed, in which log urea concentration was plotted against log pseudo first order rate constant obtained from the initial slopes. The results, shown in Figure 17, indicate the process of light-urea bleaching is more complex than the process of dark-urea bleaching. The slope, alpha, cannot be represented by a single value, as in the previous case. At higher concentrations, there appears to be a markedly larger dependence on the urea concentration and alpha has a value of 7.8. At lower concentrations, the slope becomes smaller, tending to a limiting value for alpha similar to that of the dark-urea bleaching process.

One hypothesis which might explain the apparent synergism between light and urea is based on the micellar character of rhodopsin in detergent solution. From the results shown in the present work it is known that rhodopsin in CTAB can be thermal bleached at the fairly low temperature of 45^{o}C.

Thermal Stability of Rhodopsin

Thermal bleaching of rhodopsin was shown to occur at a much lower temperature than the 62°C reported by Hubbard for rhodopsin in digitonin (46). It could be predicted that the shelf-life of rhodopsin in CTAB would be shorter than that of rhodopsin in nonionic detergents. Yet the stability of rhodopsin in CTAB appeared to be greater than six months at 4°C. By the same token, stored rhodopsin in digitonin had a much shorter shelf-life, partially bleaching and becoming turbid in a matter of weeks (67).

The thermal bleaching of rhodopsin also appeared to occur by a two-stage process, as the temperature was raised by 1° increments. Rhodopsin appeared to bleach to a limiting value of $OD_0/2$ at 45°C, while a second stage of bleaching proceeded from this temperature until 50° was reached, at which point all absorption at 498 was extinguished during the 10 minute incubation times. It is also Hubbard's assertion that the thermal bleaching process, since it is nonphotochemical, gives rise to denatured opsin and 11-cis retinal. Her experiments indicated that 70 percent of the retinal recovered from thermal bleached rhodopsin was in the 11-cis configuration. In Figure 22 a comparison is made between a rhodopsin sample (curve 1) which has been totally light-bleached (curve 2), totally thermally bleached (curve 3), and totally dark-urea bleached (curve 4). It can be seen from the figure that the light-bleached and thermally bleached curves are indentical

within experimental error. If indeed, as Hubbard predicts, 11-cis is released it would be expected that the optical density at 365 nm be reduced by almost one-half in line with the extinction coefficient of 11-cis retinal, which is approximately one-half that of the extinction coefficient of all-trans retinal.

Evidently isomerization does take place during the thermal bleaching process, at least in CTAB. In a second contrasting feature of the thermal bleaching process in CTAB, it would appear that retinal is floated free from the active site but still attached via the N-retinylidene opsin linkage, since it absorbs near 365 nm, whereas in Hubbard's study the retinal is free in solution absorbing near 380 nm. Unfortunately, a difference of only 15 nm is not enough to be conclusive when comparing the results.

Combined Effects of Urea and Temperature Increase on the Bleaching of Rhodopsin

As expected the rate of bleaching of rhodopsin in urea increased as the temperature increased. Table 5 lists the pseudo first order rate constants for the bleaching of rhodopsin in 1, 3, and 5 M urea, at temperatures of 10°, 20°, 30°, and 40°C. When a plot is made of log k versus the reciprocal absolute temperature, the activation energy for the bleaching process at the specified urea concentration can be determined from the slope of the resultant curve. By taking the tangent at 30°C the following activation energies were calculated: for 1 M urea 21.6 Kcal/mole, for 3 M urea 17.4

kCal/mole, and for 5 M urea 15.2 Kcal/mole. Kito and Takezaki (51) report a value of 28 Kcal/mole for rhodopsin in digitonin at pH 6.2 and 6 M urea. In contrast, Hubbard reports a value of 44 Kcal/mole for the thermal bleaching of rhodopsin in digitonin (46), and a value of 48 Kcal/mole has been cited for the activation energy of photobleaching calculated from the quantum efficiency of bleaching (58). That the figures cited for dark-urea bleaching are lower than other values is significant. First, the other studies used the nonionic detergent digitonin to disperse rhodopsin. Second, lower figures for the activation energy in urea and CTAB would be expected after noting the thermal bleaching temperature in CTAB was lower than the thermal bleaching temperature cited for rhodopsin in digitonin. In addition since retinal appears to remain linked to the opsin by its indicated absorption at 365 nm and not the expected absorption of 380 nm for free retinal, the activation energy for this process may be different.

Figure 28 also shows that the slopes of the lines specifying activation energy are not straight lines, but become more steep as higher temperatures are encountered. From the thermal bleaching curve, however, it is apparent that some thermal bleaching of rhodopsin is taking place at 40°C even when urea is not present. It is expected that the curves will begin to rise at 40°C and above since two processes are being monitored: dark-urea bleaching and thermal bleaching. It will be noticed that the three lower temperatures 10°, 20°, and 30°C provide a nearly linear slope for the three urea

concentrations tested, in agreement with the Arrhenius relationship.

Enzymatic Bleaching

Each enzyme was tested for activity and found to be viable either by colorimetric or thin layer chromatographic detection of product. Fortunately for the investigation the phospholipase enzymes are not susceptible to detergent inactivation. On the contrary the enzymes show heightened activity when detergent is present in the incubation mixture. Phospholipase C was the only enzyme shown to have an effect on the stability of rhodopsin in CTAB. Figure 30 shows the effect of adding a small aliquot of phospholipase C to a purified rhodopsin preparation in the dark. The disappearance of absorption at 498 nm is roughly linear with time. This is indicative of a zero order process, one frequently exhibited by enzymes with saturated substrate. This successive linear loss of OD at 498 nm contrasts with the successive logarithmic loss of OD at 498 nm obtained in the dark urea-bleaching process in Figure 23. A second notable feature of the enzymatic bleaching process is the absence of a distinct isosbestic or cross-over point. Instead the cross-over point continually shifts to shorter wavelengths and higher optical density with each successive scan. This may be due to turbidity but is more likely due to the appearance of more than one product as the bleaching continues since the solution remained clear.

When using phospholipase C on rhodopsin solubilized

in Emulphogene, Daemen and Bonting reported no loss of absorption at 498 nm. In fact, they used the enzyme to rid their Emulphogene preparation of phospholipids. Recent work by Abrahamson (2) has also shown that phospholipids are not essential to the stability of rhodopsin. There appears to be a flaw in the current reasoning about phospholipids, however. The fact that a stable rhodopsin can be prepared without phospholipids does not imply that phospholipids are not important to the functioning of rhodopsin in vivo. More important, just because Daemen and Bonting have shown phospholipids to be inessential in an Emulphogene-rhodopsin system, this does not imply that phospholipids are inessential in a CTAB-rhodopsin system.

Thin Layer Chromatography

It was possible to separate three isomers of retinal by thin layer chromatography: 9-cis, 11-cis, and all-trans. This work has since been duplicated by Daemen and Bonting (25). Direct application of light-bleached and urea-bleached rhodopsin samples to TLC plates showed qualitatively that the light-bleached sample contained primarily all-trans retinal, while the dark-urea bleached sample contained equal amounts of 11-cis and all-trans retinal.

When methanol extracts were taken of urea-bleached and light-bleached samples there was found to be one marked difference when their complement of phospholipids was separated.

In the light-bleached sample, one spot, R_f equals 0.19, appeared which gave a phosphorus positive reaction with molybdic acid, fluoresced with the same color as retinal under UV light, and moved with an R_f similar to that of PE. The urea-bleached sample produced two such spots, R_f's equal to 0.17 and 0.19, with the above characteristics. The two spots had a ΔR_f the same as that shown by 11-cis retinal and all-trans retinal. It is proposed that these two spots are the 11-cis retinylidene PE and the all-trans retinylidena PE from rhodopsin, transiminated during isolation. Figure 32 illustrates the sulfuric acid char of the plate.

General Conclusions

The processes of light-bleaching, dark urea-bleaching, and thermal bleaching, although having features in common were shown not to be identical. Moreover, evidence from the dark urea-bleaching and thermal bleaching studies has indicated that rhodopsin may have two discrete chromophores. Whether these are on the same molecule, different molecules, or subunits remains to be studied. These two chromophores may be spectrally identical but show different sensitivities towards the bleaching process being examined. Only the all-trans isomer seems to be generated when rhodopsin is bleached by light or by heat in the dark, as evidenced by thin layer chromatography and spectral analyses. Yet equal quantities of the 11-cis and all-trans isomers of retinal appear to be released when rhodopsin is bleached by urea in the dark.

Phospholipids appear necessary for the integrity of rhodopsin in CTAB, since treatment with phospholipase C destroys alpha band absorption. This may relate to the stability of the rhodopsin in CTAB, having a micellar weight of 310,000 Daltons, having a hydrated density of .9699 ml/g, and being spherical in nature. Since rhodopsin has recently been shown to be a freely rotating molecule within a liquid-like environment by Cone (20), this study in which rhodopsin molecules are packaged homogeneously within CTAB micelles may come close to approximating the behavior of rhodopsin <u>in vivo</u>.

CHAPTER V

SUMMARY

Vertebrate photoreceptors contain ordered layers of light-sensitive visual pigments. These pigments consist of a glycolipoprotein termed opsin, and an attached chromophore of 11-cis retinal. On exposure to light, an isomerization of the retinal takes place, with its concomitant release from opsin as the all-trans isomer.

A method was developed for the efficient extraction and solubilization of this visual pigment by the ionic detergent CTAB. Further purification using columns, ammonium sulfate fractionation, and organic solvent washes produced a pure visual pigment preparation, with $OD_{280\ nm}/OD_{498\ nm}$ equal to 6.0 and $OD_{400\ nm}/OD_{498\ nm}$ equal to 0.29. The material exhibited a single band on disc gel electrophoresis. The amino acid analysis of the visual pigment compared favorably with that of the literature. Using ultracentrifugation, the hydrated density of micelles was found to be 0.9699 ml/g and their weight to be 310,000 Daltons.

When solutions of the purified rhodopsin were mixed with urea in absolute darkness, a bleaching process was observed,

not unlike that due to light. The kinetics of this dark urea-induced bleaching was followed. Pseudo first order rate constants and half-lives were determined for several molarities of urea. When the rate of bleaching was equated to urea concentration, the concentration raised to the power of 2.5 fit the data. This low order of magnitude indicates that a simple chemical process, and not denaturation, was responsible for the observed bleaching. When light was admitted to the cell compartment, however, a different bleaching process was observed to occur. In these experiments, the dependence on urea concentration was to the power of 7.8.

It was also noted that at lower concentrations of urea in the dark, a solution of rhodopsin would only bleach to a limiting value of OD equal to $OD_o/2$. In addition, by dialyzing away the urea in the dark, these solutions of rhodopsin could be partially regenerated.

The heat inactivation of native rhodopsin in CTAB was found to occur at a temperature of 42°C. In another temperature study, the bleaching kinetics of rhodopsin in 1, 3, and 5 M urea was determined at 10°, 20°, 30°, and 40°C. Using the data, an Arrhenius plot was constructed, and E_a found to be approximately 20 Kcal/mole.

Thin layer chromatography was used to differentiate between the retinals and phospholipids extracted from light-bleached and urea-bleached rhodopsin. The light-bleached sample gave all-trans retinal, while the urea-bleached gave

equal quantities of 11-cis and all-trans retinal. The phospholipids from rhodopsin were easily isolated and identified with suitable standards. In the light-bleached sample a spot with R_f similar to PE, giving a phosphate-positive color with molybdic acid spray, and fluorescing under UV light in a manner characteristic of retinal was tentatively identified as N-retinylidine PE. In the dark urea-bleached extract, however, two spots were obtained with the above properties. These were postulated to be the 11-cis and all-trans N-retinylidine PE's.

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