Structure of Visual Pigments. I. Purification, Molecular Weight, and Composition of Bovine Visual Pigment$_{500}$*

Joram Heller

ABSTRACT: Native bovine visual pigment$_{500}$ has been extracted from isolated rod outer segments with the cationic detergent cetyltrimethylammonium bromide and purified by gel filtration chromatography on Sephadex G-200. The protein is homogeneous as judged by rechromatography, spectral data, and polyacrylamide gel disc electrophoresis.

The molecular weight estimated by gel filtration chromatography on a calibrated column of agarose is 27,000–30,000. The minimal molecular weight of the apoprotein found by amino acid analysis is 26,400, and the calculated molecular weight of the native pigmnet is 27,707. Of 235 amino acid residues present, 118 have nonpolar side chains. Bovine visual pigment is a glycoprotein containing three glucosamine, two mannose, and one galactose residues per molecule. Purified native visual pigment contains little or no phospholipids (<2%). No free amino- or carboxyl-terminal residues were found by any of the various methods used. The spectrum of purified native pigment has no β peak. Native visual pigment (as a complex with cetyltrimethylammonium bromide) has an ϵ_{500} of 23,100 ± 800, ϵ_{280} of 37,000 ± 1000, and an A_{280}/A_{500} ratio of 1.6 ± 0.08.

Visual pigments obtained from vertebrate and invertebrate eyes are conjugated proteins with either retinal or 3-dehydroretinal as a prosthetic group (for recent reviews, see Pitt (1964), Hubbard *et al.* (1965), and Bridges (1967)). In all the visual pigments examined, these were the only prosthetic groups found. The variety of visual pigments with absorption maxima which cover practically the whole visible spectrum, and which are quite different from the absorption maxima of free retinal and 3-dehydroretinal, must therefore be generated by a specific interaction of the prosthetic groups with a series of unique apoproteins. Although the nature and transformations of the prosthetic group of visual pigments are well understood, mainly through the elegant studies of Wald and his colleagues (Wald *et al.*, 1963), very little is known about the apoprotein. This lack of knowledge is primarily due to the problems involved in studying a highly insoluble protein that can be brought into aqueous solutions only with the aid of detergents, thus making the determination of its homogeneity and physicochemical properties a difficult task.

The present paper reports the molecular weight, composition, and some structural aspects of purified bovine visual pigment$_{500}$. An accompanying paper describes studies on the nature of the bond between retinal and the protein and on the conformational changes in visual pigment on exposure to light (Heller, 1968).

Experimental Section

Purification of Visual Pigment. Bovine retinas, dissected under dim red light, were obtained frozen from G. Hormel Co., Austin, Minn., and stored at −20°. There was no loss of visual pigment when retinas were stored for 3–4 months. All operations were performed at 3–4°, either in total darkness in a specially fitted, light-sealed cold room, or under dim red light obtained from a 40-W white lamp through a Wratten Safelight series 2 filter (Eastman Kodak, Rochester, N. Y.). Rod outer segments were prepared by adding 100 ml of 0.066 M sodium phosphate buffer (pH 7.0) to *ca.* 100 thawing retinas and homogenizing in a Waring Blendor for 10 sec. The homogenate was centrifuged for 30 min at 27,000*g*. The cloudy red supernatant was discarded (the red color is due to hemoglobin). The sediment containing the rod outer segments was dispersed in 100 ml of 1.02 M sucrose in 0.066 M sodium phosphate (pH 7.0, density 1.139 at 4°) and centrifuged for 45 min at 27,000*g*. The rod outer segments floated to the top of the tube. The density of sucrose was most critical; a too low density resulted in sedimenting the rod outer segments, whereas if the density was too high, mitochondrial contamination increased.

After loosening the part adhering to the upper tube wall, the rod outer segments plus the upper two-thirds of the supernatant were collected by decantation, and the heavy black precipitate was discarded. The supernatant was diluted by adding three volumes of 0.066 M sodium phosphate buffer (pH 7.0), and the rod outer segments were sedimented by centrifuging for 20 min at 27,000*g*. The clear supernatant was discarded, and the sedimented rod outer segments were suspended in 70 ml of 1.02 M sucrose in 0.066 M sodium phosphate (pH 7.0). The flotation in sucrose was repeated two more times, cen-

* From the Jules Stein Eye Institute, University of California School of Medicine, Los Angeles, California 90024. *Received April 16, 1968.* This investigation has been supported by Research Grant NB-06592 from the National Institutes of Health, U. S. Public Health Service, and Research Grant G-358 from the National Council to Combat Blindness, Inc.

trifuging each time for 1 hr at 39,000*g*. The floated rod outer segments were collected by diluting the sucrose solution with buffer and centrifuging for 20 min at 27,000*g*. The insoluble rod outer segments preparation was washed 20 times in 0.066 M sodium phosphate (pH 7.0) by repeated centrifugation for 10 min at 12,000*g*, thus removing soluble protein impurities.

The visual pigment was extracted from washed rod outer segments with 5 ml of the cationic detergent cetyltrimethylammonium bromide (Eastman, technical) (0.04 M) in 0.066 M sodium phosphate (pH 7.1). The rod outer segments were dispersed in the detergent solution by pipeting rapidly back and forth for several minutes with a Pasteur pipet and then by incubating for about 4 hr. Longer extraction times did not improve the yield. The solubilized visual pigment was separated from the insoluble residue by centrifugation at 12,000*g* for 10 min. The precipitate was reextracted with 5 ml of detergent solution as above, and the two extracts were combined. After the second extraction, the precipitate was either colorless or had a very faint red color. After the first 24 hr, a slight turbidity occasionally developed in the extract, and a colorless precipitate was removed by centrifugation at 48,000*g* for 30 min. Thereafter, the extract stayed optically clear and was stable for at least 1 month, when kept in the dark at 4°.

The visual pigment was further purified by gel filtration. Sephadex G-200 (fine, Pharmacia) was swollen in deionized water at 80° for 10 hr and cooled to room temperature; the fines were removed by at least 20 cycles of water washing. Two columns of 3.2 × 97 and of 3.2 × 60 cm were packed and connected in series through minimal-length Teflon tubing (0.7-mm i.d.). Bed volume was 1256 ml with an external dead volume of *ca.* 4 ml. The column was equilibrated with 0.04 M CTAB[1] in 0.066 M sodium phosphate (pH 7.1) and operated with an upward flow using a metering pump with a constant flow rate of 25 ml/hr. Fractions of 10 ml were collected.

Visual pigment extract up to 20 ml (1.6% of bed volume) was applied to the column through the buffer line and eluted with the equilibrating buffer. Absorbencies and spectra of appropriate fractions were further analyzed with a recording spectrophotometer. The ratio A_{280}/A_{500} was used in this investigation as an assay for the degree of purity of visual pigment. In the initial phases of this work, ϵ_{280}, ϵ_{500}, and the "true" ratio A_{280}/A_{500} for bovine visual pigment were unknown, and the purification procedure was based on obtaining the lowest possible ratio. The values of A_{280} reported here are corrected for nonspecific absorption by linear extrapolation of the absorption between 310 and 340 mμ into the 280-mμ range.

Dilute solutions of native visual pigment were concentrated in the dark at 4° by pressure dialysis, using the Diaflo Model 50 ultrafiltration cell (Amicon, Cambridge, Mass.) equipped with a UM-1 membrane. The A_{280}/A_{500} ratios were higher after pressure dialysis due to concentration of CTAB which passes only incompletely through the membrane. Consequently the A_{280}/A_{500} ratio can not be used to assess purity after pressure dialysis without another cycle of gel filtration.

Removal of Detergent. Attempts to remove CTAB from bovine visual pigment by extensive dialysis or by gel filtration were unsuccessful. The detergent could be completely removed by incubating CTAB–visual pigment either in 80% (v/v) ethanol or 90% (v/v) acetone at 20° for 6–8 hr. The denatured protein precipitated and was collected by centrifugation.

Spectra. All spectra were recorded and absorption was measured with the Coleman-Hitachi Model EPS-3T double-beam, ratio-recording spectrophotometer in two matched 1-cm light-path cells at 20°.

Polyacrylamide Gel Disc Electrophoresis. The system of Davis (1964) was modified by omitting the "spacer" and "sample" gels. Runs were made in 7.5% gel at pH 8.9, 3.5 mA/tube at room temperature for 1 hr. Since visual pigment cannot be electrophoresed as the CTAB complex, the detergent was removed either by alcohol (80%) or by acetone (90%) precipitation. The precipitated protein was only sparingly soluble at pH 8.5 in 0.05 M Tris, even in the presence of 8 M urea; consequently only limited amounts of protein could be applied to the gel. Some of the analytical runs were made with visual pigment purified on Sephadex G-200 in 0.03 M SDS–0.05 M (pH 8.5). A number of the electrophoretic runs were made with gels containing 8 M urea, and the visual pigment, either ethanol or acetone precipitated, or as the SDS complex (see Results) was applied in 8 M urea.

Molecular Weight Determination. Gel filtration chromatography was performed according to Andrews (1964, 1965) with the following modifications. A column (1.95 × 150 cm) of agarose (*ca.* 8% gel, Bio-Gel A, 1.5-m, 100–200 mesh, Bio-Rad, Richmond, Calif.) was equilibrated with 0.04 M CTAB in 0.066 M sodium phosphate (pH 7.1) at 4°. Bed volume was 447 ml with an external dead volume of less than 1 ml. The column was operated with downward flow at a constant flow rate of 17.8 ml/hr and with a head pressure of 100 cm of water. The effluent transmittance at 280 mμ was monitored with the Uvicord II Model 8300A (LKB) using a fluorescent rod, a 280-mμ interference filter, and a flow cell with a 3-mm light path. This detection system, which has an extremely low radiation in the ultraviolet region and none in the visible region, caused no measurable loss of visual pigment. Samples (dissolved in the equilibrating buffer) were applied in 3 ml (0.7% of bed volume), and fractions of either 5 or 3 ml were collected from the time the sample touched the gel surface. The protein concentrations in the applied samples were 0.1–0.5%. The column was eluted with the equilibrating buffer. The effluent volume corresponding to maximum concentration of the test material (V_e, elution volume) was measured directly with a measuring cylinder accurate to 0.5 ml. The column was calibrated with known proteins and glycoprotein. Each standard was chromatographed at least twice. The elution volumes, V_e, of the calibrating proteins were plotted as a function of the logarithm of their molecular weight (Andrews, 1965).

Amino Acid Analysis. Samples of protein or peptide

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate.

(0.01–0.1 μmole) were hydrolyzed in 6 N HCl in sealed, evacuated tubes at 110° for various times. The content of the tubes was evaporated to dryness in a desiccator over NaOH pellets. Amino acids were analyzed with the Beckman Model 120C analyzer according to Spackman *et al.* (1958) as modified for accelerated analysis on spherical resins (Benson and Patterson, 1965). The columns used were 0.9 × 12.5 and 0.9 × 55 cm.

Methionine and the sum of cysteine and cystine were determined after oxidation with performic acid (Moore, 1963). Recoveries were calculated assuming a yield of 100% for methionine sulfone and 94% for cysteic acid (Moore, 1963).

Tryptophan was determined by the spectrophotometric method of Bencze and Schmid (1957). The value for tyrosine, obtained by direct amino acid analysis, was used to calculate the amount of tryptophan from the molar ratio of tyrosine to tryptophan. Tryptophan was also determined by spectrophotometric titration with *N*-bromosuccinimide at pH 4.0 according to Patchornik *et al.* (1958) using visual pigment apoprotein. Essentially the same results were obtained when retinal was not removed prior to titration. In some cases, the titration was performed in the presence of 8 M urea, again without affecting the end results. The uptake of *N*-bromosuccinimide was 9.5 moles/mole of tryptophan titrated.

Amino Sugars. Samples of visual pigment (0.01–0.1 μmole) were hydrolyzed in 4 N HCl in sealed, evacuated tubes at 100° for 3 and 12 hr. The hydrolysate was evaporated to dryness in a desiccator over NaOH pellets. Hexosamines were determined on the 0.9 × 55 cm column of the amino acid analyzer.

Neutral Sugars. The phenol–sulfuric acid method of Dubois *et al.* (1956) was used with a mixture of D-mannose and D-galactose (2:1) as standard. An amount of CTAB–buffer equal to that used in the sample was added to the blanks and standards. Identical results were obtained when the phenol–sulfuric acid method was used to assay either native or apo visual pigment.

Neutral sugars were identified qualitatively after hydrolysis in 2 N HCl at 100° for 8 hr in sealed, evacuated tubes. Acid, salts, and peptides were removed by passing the hydrolysate through a 0.9 × 18 cm column of mixed-bed ion-exchange resin eluted with deionized water. The effluent containing the neutral sugar was evaporated to dryness in a rotary evaporator at 30°. The residue was dissolved in water and chromatographed (descending) on Whatman No. 1 paper at 20° for 18 hr in ethyl acetate–pyridine–water–glacial acetic acid (5:5:3:1) (Fischer and Nebel, 1955). Sugars were located on the chromatogram by the silver nitrate method (Trevelyan *et al.*, 1950) and the silver oxide background was removed with Kodak liquid X-ray fixer (Benson *et al.*, 1952). The relative amounts of sugars present were estimated by serial dilution. The unknown sugars were identified by cochromatography with known sugars.

Sialic Acids. The method of Svennerholm (1957) was used to determine sialic acids. The amount of visual pigment taken (up to 0.04 μmole) was enough to determine with certainty one residue of *N*-acetylneuraminic acid per molecule. CTAB did not interfere with the reaction.

Phosphorus Determination. Rod outer segments purified by sucrose flotation as described above were washed six times in 0.02 M Tris (pH 7.1). Visual pigment was purified on a column (2.5 × 92 cm) of Sephadex G-200 (fine, Pharmacia) equilibrated with 0.04 M CTAB in 0.02 M Tris (pH 7.1). The purified native visual pigment (0.54 μmole) (63 ml) was extracted with 200 ml of ethanol–ether (3:1, v/v) at room temperature. Phosphorus was determined as described by Chen *et al.* (1956).

Studies of N- and C-Terminal Residues. Attempt to determine the N-terminal residue were made qualitatively using the dinitrophenylation method according to Fraenkel-Conrat *et al.* (1955) and the Edman degradation as described by Margoliash (1962) and Königsberg and Hill (1962). The cyanate method was performed quantitatively exactly as described by Stark and Smyth (1963). Digestions with carboxypeptidases were performed as described by Ambler (1967) and hydrazinolysis was performed as described by Fraenkel-Conrat and Tsung (1967).

Nomenclature. The suggestion of Dartnall (1952) was followed in naming visual pigments according to their absorption maximum in the visible (in millimicrons). This form is referred to as native visual pigment. Visual pigment that has been exposed to light and which shows an absorption maximum different from that of the native form, but with the retinal prosthetic group still covalently bound to the protein, is called light-exposed visual pigment. If a particular form of light-exposed visual pigment is to be specified, the position of its peak (in millimicrons) follows the term "light-exposed visual pigment." The term "bleached," commonly used for light-exposed visual pigment, was avoided because of its ambiguity in chemical terms and because it does not tell whether the retinal is still attached to the protein. After the retinal is hydrolyzed, the remaining molecule is referred to as visual pigment apoprotein.

Results

Attempts to purify native visual pigment as the digitonin or sodium cholate complex by gel filtration on Sephadex G-200 were unsuccessful. Visual pigment eluted mostly in the void volume with some trailing throughout the column elution volume. Gel filtration of light exposed, sodium borohydride reduced visual pigment in 8 M urea or 6 M guanidine hydrochloride at pH 8.5 or 2.0 yielded material which eluted in the void volume.

The chromatographic separation of native visual pigment (as the CTAB complex) is shown in Figure 1. Visual pigment appears as a symmetrical peak completely separated from a colorless high molecular weight protein contaminant. The yield of visual pigment, calculated on the basis of A_{500} is 85–90% of the amount applied to the column. The final purified visual pigment represents 70–80% of the amount present in the starting material (homogenized retinas extracted with CTAB) and 100 bovine retinas yield 0.5–0.7 μmole of pure pigment.

The chromatographic run supplied information on the initial state of the visual pigment applied to the column.

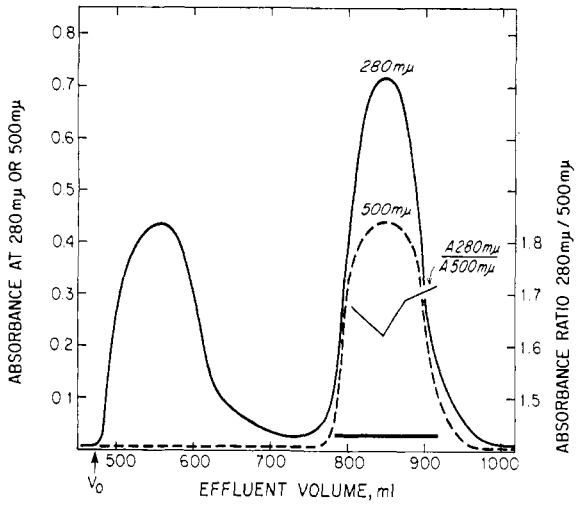

FIGURE 1: Chromatography of 3.9 μmoles of native bovine visual pigment on Sephadex G-200 (3.2 × 157 cm). Column conditions were as described in the Experimental Section. Bar represents pooled fractions.

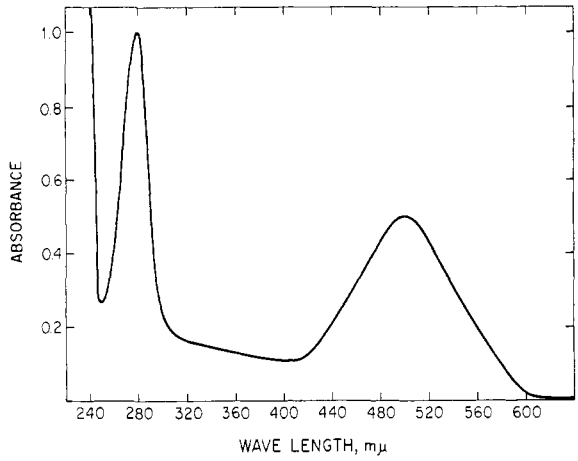

FIGURE 2: Spectrum of native bovine visual pigment$_{500}$ in 0.04 M CTAB–0.066 M sodium phosphate (pH 7.1) at 20°.

Since free retinal derived from light-exposed visual pigment eluted much later, and because it was completely separated from the native pigment and the apoprotein, the extent to which the original material was not all native could be estimated. Under optimal conditions, no yellow color appeared in the "small molecules" fraction.

By essentially the same procedure, visual pigment can be purified as the SDS complex by monitoring the specific 330-mμ peak of the borohydride-reduced form. SDS–visual pigment has the same elution volume from the Sephadex G-200 column and fulfills all criteria of purity as the CTAB complex except for the spectral ones which cannot be tested in this case. The elution volume of SDS–visual pigment complex is not changed by the addition of 8 M urea (pH 8.5) to the eluting solvent.

Criteria of Purity. RECHROMATOGRAPHY. When native visual pigment, purified once by chromatography on Sephadex G-200, was concentrated in the dark by pressure dialysis and rechromatographed under the same conditions, only one symmetrical peak was obtained. This peak appeared at the same elution volume as the visual pigment in the initial chromatography and had the same constant A_{280}/A_{500} ratio across the peak. If care is taken to avoid exposure to light, this cycle of chromatography and pressure dialysis can be repeated at least twice. In order to obtain these results, it should be stressed that visual pigment has to be in the native form. Light-exposed visual pigment has different chromatographic properties (Heller, 1968).

SPECTRAL DATA. The spectrum of purified native visual pigment (as CTAB complex) is shown in Figure 2. Visual pigment shows a peak at 500 mμ, a minimum at 410 mμ, and a sharp peak at 279–280 mμ. Purified visual pigment had a A_{280}/A_{500} ratio of 1.55–1.68 in different preparations. In individual runs, the ratio across the peak remained constant. The differences in the A_{280}/A_{500} ratio from run to run cannot be ascribed to partial light exposure as, under the experimental conditions employed, the native form is chromatographically distinguishable from the light-exposed form (Heller, 1968). The observed absorbance at 280 mμ, which was always corrected for nonspecific absorption (mainly light scattering), introduces some uncertainty as to the true A_{280} value and probably accounts for the variations in the ratio. Some of the best A_{280}/A_{500} ratios reported in the literature are 2.05 (Hubbard, 1954), 1.8–2.18 (Erhardt *et al.*, 1966), and 1.98 (Wolken, 1966). There is a question though, as to whether these reported values were corrected for nonspecific absorption, which can be considerable in detergent solutions.

Previously published spectra of visual pigment indicate a rather low broad peak with a maximum at about 350–360 mμ. This so-called β peak is presumed to be part of the spectrum of native visual pigment (for review, see Wald, 1953). The best preparations of purified visual pigment obtained in the present study had a continuously decreasing, flat absorption curve from about 320 mμ to about 400 mμ. Preparations that were partially light exposed had a clear broad peak with a maximum at *ca.* 350–360 mμ. Based upon these results and upon the known difficulty of obtaining preparations of visual pigments that are completely native, we conclude that the β peak is not part of the spectrum of native visual pigment but is due to free or bound retinal resulting from partial transformation to the light-exposed form of native visual pigment during the various isolation procedures.

DISC ELECTROPHORESIS. The results obtained from polyacylamide gel disc electrophoresis of visual pigment are shown in Figure 3. Two bands were seen in crude extract of visual pigment, while the purified material shows a single narrow band moving toward the anode at pH 8.9. The same pattern is obtained in the presence of 8 M urea in the gel or when CTAB is removed by ethanol or acetone precipitation. All runs were made with light-exposed visual pigment, as the native form in CTAB cannot be electrophoresed owing to opposite mobilities at pH 8.9. A certain amount of caution should be exercised in interpreting electrophoretic patterns of a protein that is essentially denatured when applied to the gel. Because all the above experiments yielded the same result, it was concluded that the disc electrophoresis shows the presence of only one protein component in purified visual pigment.

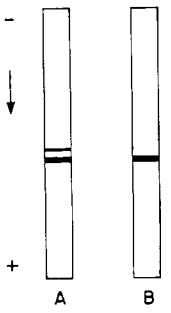

FIGURE 3: Polyacrylamide gel disc electrophoretic patterns of crude extract (A) and purified visual pigment (B). Ethanol-precipitated material dissolved in 8 M urea–0.05 M Tris (pH 8.5). The gel contained 8 M urea. For more details, see Experimental Section.

Molecular Weight. The results of gel filtration chromatography of native visual pigment on a calibrated column of agarose are shown in Figure 4. Bovine visual pigment chromatographs as if it were a globular protein of a molecular weight of 27,000–30,000. Although visual pigment is a glycoprotein with a carbohydrate content of *ca.* 4% its behavior is similar to that of ovalbumin, another glycoprotein with a low carbohydrate content, rather than that of ovomucoid or fetuin, both of which are glycoproteins with a high carbohydrate content (Andrews, 1965). The minimal calculated molecular weight of the apoprotein obtained by amino acid analysis is 26,397, while the calculated molecular weight of the native cycloprotein is 27,707.

Amino-Terminal Residue. Both dinitrophenylation and the Edman degradation, performed qualitatively, failed to demonstrate any free N-terminal residue. In the cyanate procedure, the protein remained soluble throughout, and amino acid analysis showed that 85% of the lysine residues was converted into homocitrulline, a figure to be expected from a fully carbamylated protein (Stark and Smyth, 1963). In several independent experiments, no more than 0.1 residue/molecule was found for any amino acid except lysine. Lysine was found in fractions "c_1" and "c_2" in amounts of 0.1–0.4 residue/molecule. This result is sometimes seen, and the lysine does not seem to come from the N-terminal position (see comments in Rombauts *et al.*, 1967). The combined results of these experiments can be accepted as tentative evidence of a blocked N-terminal residue.

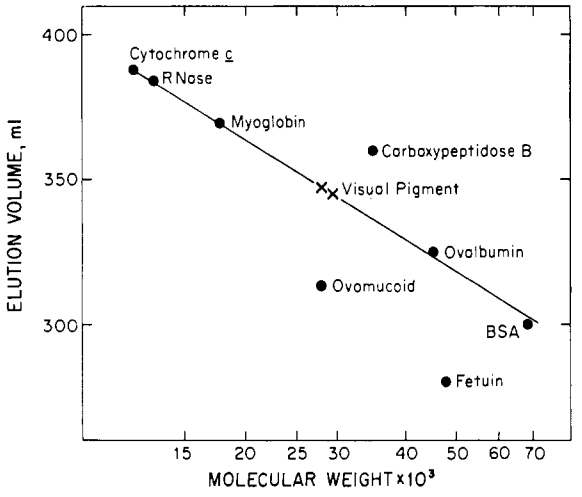

FIGURE 4: A plot of molecular weight as a function of the elution volume after gel filtration chromatography on a column of agarose of various calibrating proteins and native visual pigment. BSA is bovine serum albumin. For more details, see Experimental Section.

TABLE I: Composition of Bovine Visual Pigment.[a,b]

	6 N HCl, 110°			Nearest Integer
	24 hr	48 hr	72 hr	
Lys	10.0	9.8	9.8	10
His	3.9	3.9	4.0	4
Arg	5.9	5.9	5.9	6
$CySO_3H$	5.0	4.9		5
Asp	15.1	15.1	15.3	15
$MetSO_2$	8.0	8.3		8
Thr	16.0	14.7	15.7	17[c]
Ser	11.1	9.9	9.8	12[c]
Glu	21.1	20.7	21.0	21
Pro	12.9	12.8	13.3	13
Gly	16.2	15.9	16.1	16
Ala	19.9	19.7	19.9	20
Val	17.0	18.5	19.7	20[d]
Ile	11.6	12.9	13.0	13[d]
Leu	19.1	19.6	20.0	20[d]
Tyr	11.1	10.9	10.7	11
Phe	19.0	19.2	19.0	19
Trp				5[e]
Glucosamine				3[f]
Mannose				2[g]
Galactose				1[g]
Phospholipids[h]				0–2%

[a] Data are expressed as residues per molecule of protein. [b] Values are average of at least two determinations. [c] Extrapolated to zero time. [d] Extrapolated to infinite time. [e] Determined by titration with *N*-bromosuccinimide. [f] Determined after hydrolysis for 3 and 12 hr, 4 N HCl, 100°. [g] Determined by quantitative total neutral sugars and semiquantitative paper chromatography. [h] Determined as ethanol–ether-soluble P_i.

Carboxyl-Terminal Residue. Attempts to determine the carboxyl-terminal residue were unsuccessful. No amino acids were released from visual pigment by digestion with carboxypeptidase A, carboxypeptidase B, or with both enzymes together under any of the various digestion conditions employed. Hydrazinolysis did not reveal any carboxyl-terminal residue.

Amino Acid Composition. The results of several analyses of bovine visual pigment are presented in Table I. The spectrophotometric method of Bencze and Schmid (1957) showed 6 residues tryptophan/molecule when the value of 11 residues of tyrosine/molecule, obtained by amino acid analysis, was used as the basis of calculation (Tyr/Trp = 1.86). Titration with *N*-bromosuccinimide

showed only 5 residues of tryptophan/molecule. The latter value was considered more accurate because of the spectrophotometric methods, which involve a certain amount of extrapolation, are known to give results that tend to be too high (Noltman *et al.*, 1962). A tryptophan content of 5 residues/molecule is also in better agreement with ϵ_{280} value for this protein.

Carbohydrate Content. The carbohydrate composition of bovine visual pigment is shown in Table I. Evidence that the carbohydrate is an integral part of the molecule is shown by the fact that it is found in the preparations purified by gel filtration chromatography, and that repeated precipitation of purified visual pigment from an 80% ethanolic solution did not change it. There are 3 residues of glucosamine and 3.1–3.3 residues of neutral sugar. The neutral sugar fraction is composed of 2 residues of mannose and 1 residue of galactose per molecule. No galactosamine, glucose, xylose, fucose, or sialic acids were found on repeated examination of different preparations in tests designed to detect a single residue per molecule.

Phospholipid Content. The amount of phospholipid present in purified visual pigment was estimated by measuring the ethanol–ether-soluble P_i. No phosphorus was found in 0.54 μmole of purified native visual pigment. Assuming an average of 4% P_i in phospholipids, native visual pigment (as CTAB complex) contains at most 2% of phospholipids.

Molar Absorptivity. Samples of purified native visual pigment of known A_{500}, A_{280}, and an A_{280}/A_{500} ratio of 1.6 were subjected to amino acid analysis (24- and 72-hr hydrolysis at 110°, 6 N HCl). The calculated amounts of protein (assuming a molecular weight of 26,400) was used to calculate ϵ_{280} and ϵ_{500}. The recovery from the analyzer was taken as 100% (Spackman, 1967). The average of four analyses was ϵ_{280} 37,000 ± 1000 and ϵ_{500} 23,100 ± 800. The reported value of ϵ_{500} 40,600 (Wald and Brown, 1953) is probably too high due to the indirect method of measurement and the assumptions involved.

Discussion

Purification. The only parameter which can be used as an "assay" during purification of visual pigments is their characteristic spectral properties. This dependence upon a unique spectrum imposes some rather strict limitations on the experimental approach to any purification procedure. On the one hand, visual pigments being extremely photosensitive it is imperative that all operations be performed in the dark or under dim red light. On the other hand, it is possible to develop a method of purifying some unique derivative of visual pigment that has characteristic spectral properties, stable to ordinary illumination and to various denaturants. Such a derivative is light-exposed, sodium borohydride reduced visual pigment (Bownds and Wald, 1965; Akhtar *et al.*, 1965). Using both approaches to the purification procedure, namely, purification of native visual pigment in the dark and isolation of a recognizable stable derivative, a further major methodological consideration was realized in the early part of this investigation. Visual pigment being a membrane structural protein has a marked tendency to aggregate. Several solubilizing agents such as 2% aqueous digitonin, 8 M urea, 6 M guanidine hydrochloride, or 2% sodium cholate, that to all intents and purposes form true "solutions" of visual pigment, break the polymeric membrane structure only partially and then only to oligomeric forms of various sizes, as judged by gel filtration chromatography on Sephadex G-200. The only agents found to dissociate the structure to its "monomeric" units were SDS and CTAB. SDS bleaches the visual pigment (Smith, 1941), it gels at low temperatures, and is almost impossible to remove from the protein. The agent of choice and the one finally adopted throughout the purification procedure was the quarternary ammonium salt, CTAB, a cationic detergent (Bridges, 1957; Snodderly, 1967). CTAB was found to dissociate visual pigment into its "monomeric" form without causing any change in its spectral properties; it is a most effective extracting agent (much more so than digitonin or cholate) and it forms stable, clear solutions of visual pigment. CTAB is very soluble in water; it does not gel at 3–4°; is itself colorless; and it has only a minimal, flat, absorption curve down to 230 mμ, which makes it possible to follow the ultraviolet absorption of the protein. CTAB can be removed quite easily from the protein by precipitation of the protein from an 80% ethanolic solution. And finally, although it is not possible to use the Folin reagent to estimate protein (Lowry *et al.*, 1951), both the ninhydrin procedure for proteins and peptides (Hirs *et al.*, 1956) and the phenol–sulfuric acid procedure for sugars (Dubois *et al.*, 1956) are compatible with the presence of CTAB.

The purified visual pigment is judged to be *native*, mainly on the strength of its spectral characteristics. The term "native" is somewhat ambiguous and ill defined. It is used generally in the sense that, as far as can be determined experimentally, the protein under consideration has the same conformation and the same properties it had *in situ*. However, if a rigorous proof is asked for the native state for proteins that are part of an assembled complex, it turns out in many cases that this is more of an assumption than of a proven fact. This is mostly so because of the inherent difficulty in determining the *true* state of a protein in its *original* site (see, for instance, discussion about cytochrome *c*, a component protein derived from the respiratory chain; Margoliash and Schejter, 1966). Color is still the only criterion we have by which to define the native state of visual pigments, and until other criteria become available, this problem cannot be answered conclusively. There is always the possibility that the detergent causes some changes in the structure of the protein that are not accompanied by spectral changes. Snodderly (1967) has reported that in the presence of CTAB visual pigment does not re-form from the aproprotein and 11-*cis*-retinal. Preliminary experiments in this laboratory show this is due to the effects of the detergent on the pigment *after* it had first been exposed to light. The fact that a strong cationic detergent like CTAB is able to dissociate the membrane structure completely and yet to preserve the native state is rather remarkable. This becomes even more so in view of the fact that the anionic detergent, SDS, which dis-

sociates the aggregate visual pigment to the same degree as CTAB, nevertheless discharges the color. Even more remarkable is the fact that such strong dissociating agents as 6 M guanidine hydrochloride or 8 M urea discharge the color readily but do not disaggregate the protein. This is an interesting reminder that dissociation of an aggregate protein and conformational changes are not necessarily the same process (Tanford, 1964). It is possible to have conformational changes without dissociation (urea and guanidine hydrochloride), dissociation without conformational changes (CTAB), or both dissociation and conformational changes (SDS).

The relative ease with which visual pigment can be purified is probably due to two factors. On the one hand, the sucrose flotations and extensive washings remove most, if not all, of the soluble protein contaminants from the insoluble rod outer segments. On the other hand the solubilizing action of CTAB must be quite selective, and the only other protein contaminant present happens to have a much higher molecular weight and thus is easily removed by gel filtration.

The molecular weight of native visual pigment in CTAB, as estimated by gel filtration chromatography on calibrated columns of agarose, is 27,000–30,000. Gel filtration chromatography was the method of choice in determining the molecular weight of native visual pigment; this is the only method that can be used rather simply in the dark. Moreover, the position of the peak in gel filtration is independent of the concentration of the applied protein; and yet aggregation, when it occurs, can be readily identified. In using the gel filtration method several assumptions were made and tested. It was assumed that the calibrating proteins and visual pigment bind CTAB proportionately to their molecular weight and that the calibration curve thus obtained can be used to determine the molecular weight of an unknown, *e.g.*, visual pigment. As can be seen from Figure 4, the straight line obtained with calibrating proteins is consistent within itself and can be used to check proteins of known molecular weight. Even more so, fetuin and ovomucoid, two glycoproteins that have smaller elution volume relative to their molecular weight (Andrews, 1965), appear at their expected elution volume in presence of CTAB. Assuming that visual pigment adheres to the normal relationship shown by globular proteins, its molecular weight is 27,000–30,000. Visual pigment is a glycoprotein with a relatively low carbohydrate content (*ca.* 4%) and thus seems similar to ovalbumin, another glycoprotein of low sugar content that chromatographs as a normal globular protein. Actually, if it is assumed that visual pigment behaves similarly to fetuin or ovomucoid, its molecular weight would be much smaller than 27,000, probably in the order of 12,000–15,000. All other supporting evidence, amino acid composition, number of sulfhydryl per molecule (Heller, 1968), number of prosthetic groups per molecule (Heller, 1968) and number of sugar residues per molecule, indicates that the molecular weight of bovine visual pigment glycopoprotein is about 27,000.

A blocked N terminal is probably the reason no amino-terminal residue was found. It is more difficult to to see why no carboxyl-terminal residue was found. An ultimate or penultimate proline residue would be expected to prevent both carboxypeptidases A and B from releasing any amino acid but this should have been demonstrated by hydrazinolysis. The experimental difficulties and ambiguity in demonstrating any C-terminal residue are known (see recent papers by Rombauts *et al.*, 1967; Englund *et al.*, 1968).

Amino Acid and Carbohydrate Composition. The mole (per cent) content of nonpolar residues (Pro, Ala, Cys, Val, Met, Ile, Leu, and Phe) in visual pigment, which is 50.2%, is one of the highest reported and is characteristic of highly insoluble, membraneous-structural proteins (Margolis and Langdon, 1966). The mole (per cent) content of nonpolar residues is 31.7% for bovine and horse heart cytochrome *c* (Nakashima *et al.*, 1966; Margoliash *et al.*, 1961), 36.3% for bovine ribonuclease (Smyth *et al.*, 1963), 41.2% for bovine chymotrypsinogen A (Brown and Hartley, 1966), 41.8% for TMV (Anderer *et al.*, 1965), 43% for beef heart mitochondrial structural protein (Criddle *et al.*, 1962), and up to 43.7% for some chloroplast lamellar proteins (Lockshin and Burris, 1966).

This high content of nonpolar residues in visual pigment is not unexpected. For soluble globular proteins, the division of polar–nonpolar residues can be taken as an approximation of the number of outside–inside residues (Kendrew, 1962). Visual pigment is an aggregated, insoluble membrane component, and it is to be expected that some of the oustide residues are actually nonpolar. This will facilitate the building of a membrane structure by favoring protein–protein interactions over protein–solvent interactions (Tanford, 1964). In this highly hydrophobic, insoluble matrix, the covalently attached carbohydrate moiety probably serves as an orientation factor in directing the visual pigment molecule into a particular steric position in the membrane by carbohydrate (hydrophilic)–solvent interactions.

Phospholipid Content of Visual Pigment. Previous reports on the composition of bovine visual pigment indicate a high content of *ca.* 45% phospholipid (Krinsky, 1958). This value was obtained from visual pigment preparations extracted from rod outer segments with digitonin. The experiments reported in the present paper show that purified native pigment contains less than 2% phospholipid if any is present. That the digitonin extract has such a high content of phospholipids is probably another manifestation of the incomplete disaggregation of the membrane structure brought about by digitonin. The digitonin particles actually represent a composite part of the membrane structure including phospholipids. In this sense, phospholipids are an integral part of the membrane as a whole but not of the visual pigment molecule. It is interesting to recall that digitonin particles from mitochondria, which contain 35–40% phospholipids, are apparently so complex they are capable of oxidative phosphorylation (Cooper *et al.*, 1955; Cooper and Lehninger, 1957).

The experiments described in the present report show that native visual pigment, a component of the membrane structure of the rod outer segment, can be isolated as a discrete molecule of relatively small size. This molecule, a conjugated glycoprotein, behaves in many re-

spects as a globular protein and contains little, if any, phospholipid. These observations support the idea that membrane proteins are not, of necessity, fundamentally different from ordinary globular proteins and that the unique membrane structure may be due primarily to strong protein–protein interactions and not to any peculiar structural features of the protein itself.

Acknowledgment

It is a pleasure to thank Professor E. L. Smith and Dr. F. S. Markland for helpful discussions and critical reading of the manuscript. I gratefully acknowledge the expert technical assistance of Mrs. Biruta R. Vellutini.

References

Akhtar, M., Blosse, P. T., and Dewhurst, P. B. (1965), *Life Sci. (Oxford) 4*, 1221.
Ambler, R. P. (1967), *Methods Enzymol. 11*, 155.
Anderer, F. W., Wittman-Liebold, B., and Wittman, H. G. (1965), *Z. Naturforsch. 20b*, 1203.
Andrews, P. (1964), *Biochem. J. 91*, 222.
Andrews, P. (1965), *Biochem. J. 96*, 595.
Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Benson, A. A., Bassham, J. A., Calvin, M., Hall, A. G., Hirsch, H. E., Kawaguchi, S., Lynch, V., and Tolbert, N. E. (1952), *J. Biol. Chem. 196*, 703.
Benson, J. V., Jr., and Patterson, J. A. (1965), *Anal. Chem. 37*, 1108.
Bownds, D., and Wald, G. (1965), *Nature 205*, 254.
Bridges, C. D. B. (1957), *Biochem. J. 66*, 375.
Bridges, C. D. B. (1967), *Comp. Biochem. 27*, 31.
Brown, J. R., and Hartley, B. S. (1966), *Biochem. J. 101*, 214.
Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.
Cooper, C., Devlin, T. M., and Lehninger, A. L. (1955), *Biochim. Biophys. Acta 18*, 159.
Cooper, C., and Lehninger, A. L. (1957), *J. Biol. Chem. 224*, 561.
Criddle, R. S., Bock, R. M., Green, D. E., and Tisdale, H. (1962), *Biochemistry 1*, 827.
Dartnall, H. J. A. (1952), *J. Physiol. 116*, 257.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.
Englund, P. T., King, T. P., Craig, L. C., and Walti, A. (1968), *Biochemistry 7*, 163.
Erhardt, F., Ostroy, S. E., and Abrahamson, E. W. (1966), *Biochim. Biophys. Acta 112*, 256.
Fischer, F. G., and Nebel, H. J. (1955), *Z. Physiol. Chem. 302*, 10.
Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.
Fraenkel-Conrat, H., and Tsung, C. M. (1967), *Methods Enzymol. 11*, 151.
Heller, J. (1968), *Biochemistry 7*, 2914 (this issue; following paper).
Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.
Hubbard, R. (1954), *J. Gen. Physiol. 37*, 381.
Hubbard, R., Bownds, D., and Yoshizawa, T. (1965), *Cold Spring Harbor Symp. Quant. Biol. 30*, 301.
Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15*, 216.
Königsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Krinsky, N. I. (1958), *Arch. Ophthalmol. 60*, 688.
Lockshin, A., Burris, R. H. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1564.
Lowry, O. H., Rosebraugh, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Margoliash, E. (1962), *J. Biol. Chem. 237*, 2161.
Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 114.
Margoliash, E., Smith, E. L., Kreil, G., and Tuppy, H. (1961), *Nature 192*, 1121.
Margolis, S., and Langdon, R. G. (1966), *J. Biol. Chem. 241*, 469.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Nakashima, T., Higa, H., Matsubara, H., Benson, A. M., and Yasunobu, K. T. (1966), *J. Biol. Chem. 241*, 1166.
Noltmann, E. A., Mahowald, T. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1146.
Patchornik, A., Lawson, W. B., and Witkop, B. (1958), *J. Am. Chem. Soc. 80*, 4747.
Pitt, G. A. J. (1964), *Exptl. Eye Res. 3*, 316.
Rombauts, W. A., Schroeder, W. A., and Morrison, M. (1967), *Biochemistry 6*, 2965.
Smith, E. L. (1941), *J. Gen. Physiol. 23*, 753.
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.
Snodderly, D. M., Jr. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1356.
Spackman, D. H. (1967), *Methods Enzymol. 11*, 3.
Spackman, D. B., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Svennerholm, L. (1957), *Biochim. Biophys. Acta 24*, 604.
Tanford, C. (1964), *Brookhaven Symp. Biol. 17*, 154.
Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.
Wald, G. (1953), *Ann. Rev. Biochem. 22*, 497.
Wald, G., and Brown, P. K. (1953), *J. Gen. Physiol. 37*, 189.
Wald, G., Brown, P. K., and Gibbons, I. R. (1963), *J. Opt. Soc. Am. 53*, 20.
Wolken, J. J. (1966), Vision, Springfield, Ill., Thomas, p 41.