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Rhodopsin. Purification and Recombination with Phospholipids Assayed by the Metarhodopsin I → Metarhodopsin II Transition†

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ABSTRACT: Studies of the nature of interaction between the visual protein rhodopsin and the rod outer segment (ROS) membrane phospholipid components have been initiated. To assay this interaction, a flash photolysis instrument has been built with microsecond resolution allowing the kinetic observation of the spectroscopic intermediates metarhodopsin $I_{480\,nm}$ → metarhodopsin $II_{380\,nm}$ in the bleaching process of rhodopsin. A single first-order rate has been established for the kinetic appearance of metarhodopsin $II_{380\,nm}$ in preparations of rhodopsin in its native disc membrane environment (ROS membranes) and for dodecyldimethylamine oxide (DDAO) detergent solubilized rhodopsin. A purification procedure has been developed for the preparation of rhodopsin free of phospholipid and detergent and the isolated protein can be recombined with phospholipids to obtain a "reconstituted" lipid–protein species of defined composition. The spectroscopic assay is a sensitive indication of the protein–lipid interaction. The following lifetimes for metarhodopsin I → metarhodopsin II were observed at 20°: sonicated ROS membranes, 20 msec; purified lipid-free rhodopsin in DDAO, 0.08 msec; rhodopsin reassembled with egg phosphatidylcholine, 9 msec (70% component), 2 msec (30% component). The transition is blocked for rhodopsin free of detergent and lipid but can be restored by addition of detergent.

The molecular photoreceptor in both vertebrate and invertebrate rod cells of the retina has been shown to be the chromoprotein rhodopsin. This membrane protein contains the tightly coupled chromophore retinal which isomerizes from the 11-cis to the all-trans form upon absorption of light (Wald, 1968). The ultimate consequence of this absorption is the triggering of a bioelectrical activity in the cell which can be transmitted to higher order neurons (Sillman *et al.*, 1969; Hagins *et al.*, 1970, Hagins, 1972).

Following absorption of a photon a series of intermediate steps have been spectrally defined for this chromoprotein. The

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initial intermediates, which have characteristic absorption maxima and lifetimes at 20°, are

rhodopsin
↓ $\tau > 6 \times 10^{-12}$ sec
prelumirhodopsin (545 nm)
↓ $\tau \simeq 50 \times 10^{-9}$ sec
lumirhodopsin (497 nm)
↓ $\tau \simeq 50 \times 10^{-6}$ sec
metarhodopsin I (480 nm)
↓ $\tau = 18 \times 10^{-3}$ sec
metarhodopsin II (380 nm)

(Yoshizawa and Wald, 1963; Matthews *et al.*, 1963; Ostroy *et al.*, 1966; Busch *et al.*, 1972; Cone, 1972). The interconversion from meta I to meta II[1] is the last step which is fast enough to be involved in the triggering mechanism. It seems likely that these spectrochemical changes of the chromophore must be tightly coupled to protein conformational changes, one or more of which remotely triggers the change in potential of the outer plasma membrane of the rod outer segment. To describe the trigger mechanism as a molecular event, or events, requires a greater understanding of the tightly coupled interaction between the chromophore retinal and the protein moiety opsin as well as greater understanding of the interactions between the protein and lipids of its native membrane environment.

This paper reports the development of a method for the study of the functional interaction between rhodopsin and components of its membrane environment as "assayed" by observing the rate of interconversion of meta I → meta II.

Experimental Section

Materials. Dark adapted bovine retinae were purchased from George Hormel Company, Austin, Minn., and stored at <−60°. Sucrose "ultra pure" was obtained from Schwarz/Mann. Dithiothreitol (Cleland's Reagent) came from Cal Biochem. Imidazole (Im) from Sigma was recrystallized from benzene before use. Hydroxylapatite and Bio-Gel P-100 are products of Bio-Rad; Sephadex G-100 is from Pharmacia. *all-trans*-Retinal and hydroxylamine were from Eastman. Ammonyx-LO is a product of Onyx Chemical Co., Hoboken, N. J.,[2] *N,N*-dimethyltetradecylamine was obtained from K and K Labs and *N,N*-dimethyldodecylamine from Eastman Organic. Phosphatidylethanolamine and phosphatidylserine were obtained from Supelco. Egg lecithin (phosphatidylcholine) came from Sylvania, Millburn, N. J. All other chemicals were reagent grade. Collodion bags came from Schleicher and Schüll, W. Germany.

Dodecyldimethylamine Oxide. N,N-Dimethyldodecylamine was distilled under vacuum and then fractionally distilled on a spinning band column to obtain the dodecylamine, bp 76–77° (1 mm). Analysis by gas chromatography showed the fraction to be 99% dimethyldodecylamine.[3]

Synthesis of the *N,N*-dimethyldodecylamine oxide was carried out by a modification of the procedure described by Sheng and Zajacek (1970). All filtrations and transfers were carried out in a drybox flushed in N_2. Fractionally distilled *N,N*-dimethyldodecylamine (40 g) was heated to 55°; 30% aqueous hydrogen peroxide (22.4 g) was slowly added over a period of 1 hr followed by 60 ml of *tert*-butyl alcohol. The reaction was stirred at 55° for 6 hr. The solvent was removed on a rotatory evaporator. The slightly wet material was redissolved in absolute ethanol, molecular sieves were added, and the solution was allowed to stand overnight. The solution was filtered, the solvent was removed, and the paste was redissolved in warm toluene at a temperature under 75°. The compound crystallized as white needles upon cooling. The crystals were washed with cold anhydrous ether (0–5°) and dried under vacuum for several days to remove traces of toluene; mp 132–133°; yield recrystallized product, 25–30 g. The material was checked by thin-layer chromatography (Pelka and Metcalfe, 1965) and had no visible contaminants. A 0.5 M solution had no absorption above 230 nm. Herrmann (1962) reported the critical micelle concentration as 2.84 mM at 1° and 2.10 mM at 27° with a minimum molecular weight of 17,700 and 17,300, respectively, and an approximate p*K* of the amine oxide of 5.0; thus above pH 7 the material is virtually nonionic.

[^{14}C]Dimethyldodecylamine Oxide. ^{14}C-labeled dimethyldodecylamine oxide was synthesized from lauric acid labeled at the α carbon. The acyl chloride was prepared with $SOCl_2$, converted to the dimethylamide, and reduced with $LiAlH_4$. The oxide was prepared from this labeled dimethylamine by addition of aqueous hydrogen peroxide. The final product was extracted twice with ether, dried, and washed twice more with ether. The sample was then exhaustively dried and weighed to obtain a specific activity. The material was shown by thin-layer chromatography (Pelka and Metcalfe, 1965) to have greater than 95% of the ^{14}C radioactivity in the DDAO band. This material was used to quantitate the amount of detergent present in the following preparative steps. All samples counted for radioactivity were dissolved in a dioxane base counting solution.

Isolation of ROS. All operations were carried out under dim red light in an ice bath or in a cold room at 4° unless otherwise noted; 200–250 bovine retinae were thawed, then ground with mortar and pestle to the consistency of a smooth thick paste. The retinae were diluted with 200 ml of cold 0.1 M KCl-0.01 M Im (pH 7) and homogenized by 15–20 passes with a Potter-Elvehjem homogenizer. The homogenized retinae were centrifuged at 1000 rpm, 15 min (100*g*). The thick ROS containing supernatant was then recentrifuged at 15,000 rpm for 20 min (27,000*g*) to collect the ROS pellet.

An initial sucrose flotation was carried out by homogenizing the ROS pellet in 200 ml of 40% sucrose (w/w)-0.01 M Im (pH 7). The suspension was centrifuged at 15,000 rpm for 20 min. The floating material was loosened from the sides of the tube with a flexible rod and the upper half of supernatant collected. The ROS were obtained from this supernatant by diluting the suspension 1:4 with 0.1 M KCl-0.01 M Im (pH 7), and collected as a pellet by centrifugation at 15,000 rpm for 15 min. The segments were then isolated as suggested by McConnell (1965). Gradients were prepared at 4° with 13 ml of 20% sucrose (w/w), 0.01 M Im (pH 7) (d = 1.085), and 13 ml of 40% sucrose (w/w), 0.01 M Im (pH 7) (d = 1.182). The ROS were layered upon six 26-ml gradients and centrifuged in Spinco Model L in an SW 25.1 head at 22,500 rpm (51,505*g*) for 2 hr at 5°. The ROS band was located near the center of the gradient and was collected by a syringe with a long needle. These ROS may be used immediately or may be frozen in liquid N_2 and stored at <−60° for at least 4–6 months with no noticeable effects.

Solubilization of ROS Membranes. The ROS were thawed, diluted 1:4 with 0.1 M KCl-0.01 M Im (pH 7), and collected

[1] Abbreviations used are: DDAO, dodecyldimethylamine oxide (also known as LDAO); ROS, rod outer segments; meta I, metarhodopsin I; meta II, metarhodopsin II; Im, imidazole; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

[2] This detergent is received as a 30% aqueous detergent solution and is composed of 65% dodecyldimethylamine oxide and 35% tetradecyldimethylamine oxide (R. Sorentino, personal communication, Onyx Chemical Co., Jersey City, N. J. 07302).

[3] Conditions: gas chromatography was performed on a 10 ft × 0.25 in. stainless steel column prepared by Analabs, New Haven, Conn., from 10% Apeizon L (KOH treated) on Chrom NAW 80–100 mesh; column temperature, 180°

by centrifugation. The pellet was washed twice with distilled water and extracted at 4° by gently stirring or mixing on a slowly rotating mixer for 4 hr to overnight; 100 retinae could be completely extracted with 5 ml of 0.1 M DDAO in buffer.[4] Following extraction, the solution was centrifuged and the supernatant collected to give a clear crude extract. The solubilized protein is stable in the detergent for a year or more at 4° and for hours at room temperature as judged by the lack of alteration of the chromoprotein absorption spectrum and the meta I → meta II transition kinetics.

Chromatography on Hydroxylapatite. Bio-Rad hydroxylapatite was extensively washed to remove fines and suspended in 1% Ammonyx-LO, 0.01 M Im (pH 7). Columns of 1.1 × 25 cm were poured and washed with 100 ml (5 void volumes) of 0.05 M DDAO, 0.01 M Im (pH 7), and 1 mM dithiothreitol. The 0.1 M DDAO extract of ROS was applied (samples contained about 25–35 $OD_{500\ nm}$ units per column) to the column which was eluted with a 140 ml phosphate gradient from 5 to 150 mM P_i in 0.05 M DDAO in buffer. Fractions with $OD_{500\ nm}$ greater than 0.2 were collected and pooled (Figure 2).

Chromatography on G-100. Sephadex G-100 columns of 2.5 × 80 cm were poured and equilibrated with 1 mM DDAO in buffer. Rhodopsin which had been chromatographed on hydroxylapatite was concentrated by vacuum dialysis in collodion bags, applied to the column, and eluted with the above buffer. Rhodopsin was eluted just beyond the void volume of the column (Figure 3). This protein is called phospholipid free and has less than 0.5 mol of phospholipid/mol of rhodopsin as assayed by phosphate analysis. When the detergent concentration is increased to reduce aggregation, the average absorbance ratios are 400/500 = 0.18 and 280/500 = 1.8.

Removal of Detergent. Pooled fractions of rhodopsin were dialyzed (1–200 ml of dialysate) for 2 days with at least six changes of dialysate buffer. The detergent-free material existed as a suspension in the absence of detergent and could be collected by centrifugation as a brilliantly red gelatinous material. The presence of detergent was assayed by its radioactivity.

Recombination with Phospholipid. Rhodopsin was recombined with lipid to restore a near normal rate for appearance of meta II under the following conditions: solvent was removed from phospholipid with a stream of nitrogen; the lipid was then suspended in aqueous buffer at a concentration of about 7.5 mM by vortexing the solution. This solution was made 100 mM in DDAO to completely solubilize the lipid. The phospholipid was added to phospholipid free rhodopsin to give the following incubation conditions: 1×10^{-5} M rhodopsin, 1×10^{-3} M phospholipid, 50 mM [^{14}C]DDAO, 1 mM dithiothreitol, and 0.01 M Im (pH 7). The sample was dialyzed against pH 6 buffer with multiple changes for 4–6 days and finally twice against pH 7 buffer. The harvested recombinant was assayed for radioactivity and shown to have less than 0.5 mol of DDAO/mol of rhodopsin. Before use, the recombinant was sonicated to give an optically clear solution.

Phospholipid Analysis. Phospholipid was analyzed by inorganic phosphate determination following ashing of the entire sample according to the procedure of Ames (1966).

Molar Absorption Coefficient at 500 nm. The molar absorption coefficient at 500 nm has been measured for both crude rhodopsin and purified material. The method of Wald and Brown (1953–1954) was used to determine the amount of *all-trans*-retinal oxime present before and after illumination of the rhodopsin. The molar absorption coefficient at 365 nm of *all-trans*-retinal oxime in 1% Ammonyx-LO, 0.01 M Im (pH 7), and 0.1 M hydroxylamine (or 0.05 M hydroxylamine) was determined to be 5.28×10^4 M^{-1} cm^{-1}. The 500-nm absorption coefficient for the retinal chromophore of rhodopsin was determined from the ΔOD at 500 nm before and after illumination of the chromophore and OD at 365 nm after illumination and formation of retinal oxime. The base line for the later measurement was that of the solvent; no correction was necessary for rhodopsin plus hydroxylamine before illumination. The molar absorption coefficient determined and used throughout this work is 40,000 ± 2000 M^{-1} cm^{-1}.

Gel Electrophoresis. Protein solutions were incubated in 5% sodium dodecyl sulfate, 1% β-mercaptoethanol, 0.02 M P_i buffer (pH 7) for 2 hr at 37°. Electrophoresis was then carried out according to Weber and Osborn (1969). Gels were stained with Coomassie Brilliant Blue and destained by diffusion. Gels were stained for carbohydrate according to Segrest and Jackson (1972) and stained for lipid according to Chiffelle and Putt (1951).

Spectra. All spectra were taken with a Cary 14 or Cary 17. Low-temperature spectra were taken with the Cary 14 fitted with a flow-through dewar for passing a controlled mixture of cold and warm nitrogen past the cell. Temperatures were recorded at the cell surface with a copper *vs.* constantan thermocouple. For illuminating the solutions, the cell was turned 90° and bleached with light from a xenon lamp focused on the cell. The beam was prefiltered by a cell of flowing water and Corning filter 4-65. Glasses were prepared from equal volumes of sample solution and glycerol or ethylene glycol.

Flash Photolysis. A high-sensitive single-flash apparatus was assembled to observe the transient rise of the meta II species. The instrument was similar to that described by Rüppel and Witt (1969). The monitoring source intensity was sufficiently low so that no bleaching of the sample was observed over a period of minutes. The flash lamp (Xenon, 200J, S340) had a risetime of less than 4 μsec and a decay time of about 12 μsec; thus the photolyzing pulse was of shorter duration than the risetime of the meta II intermediate. In this first generation flash apparatus not all of the trigger pickup from the sparkgap discharge was eliminated and the initial 25 μsec of data were flawed with pickup noise as can be observed in Figure 6. Thus for data collected at 0.5 μsec/channel, the first 25 μsec of data were not used for data processing. The photolysis flash was filtered through two interference filters (Optic Technology) with a band pass of 75 nm centered at 510 nm. The sample cell (Scientific Cell Co. 431F) was jacketed for temperature control and the temperature was measured within the cell with a thermocouple. The reaction was monitored at 380 nm by a 1P-28A photomultiplier and recorded by a Biomation transient recorder (Model 802). The latter possessed 8 bit data (256 data units) resolution and 10 bit time (1024 channels) resolution. The kinetic record, digitized and stored by the transient recorder, was transferred to permanent storage on magnetic tape cassettes. For this purpose, the data were first processed for sequential transmission *via* a parallel to serial data converter and the signals were modulated to give proper frequencies for recording on a Lafayette tape recorder.[5] The data were later demodulated and transmitted *via* an Execuport (Computer Transceiver Systems, Paramus, N. J.) onto file in the house computer (GE-355) where they could be processed. Processing consisted of a program to plot the data, to convert the voltage output signals to changes in absorption (a), to compute the ln

[4] Buffer throughout this work refers to 0.01 M Im–0.1 mM dithiothreitol (pH 7).

[5] The data processing instrumentation used was designed and constructed by D. Z.

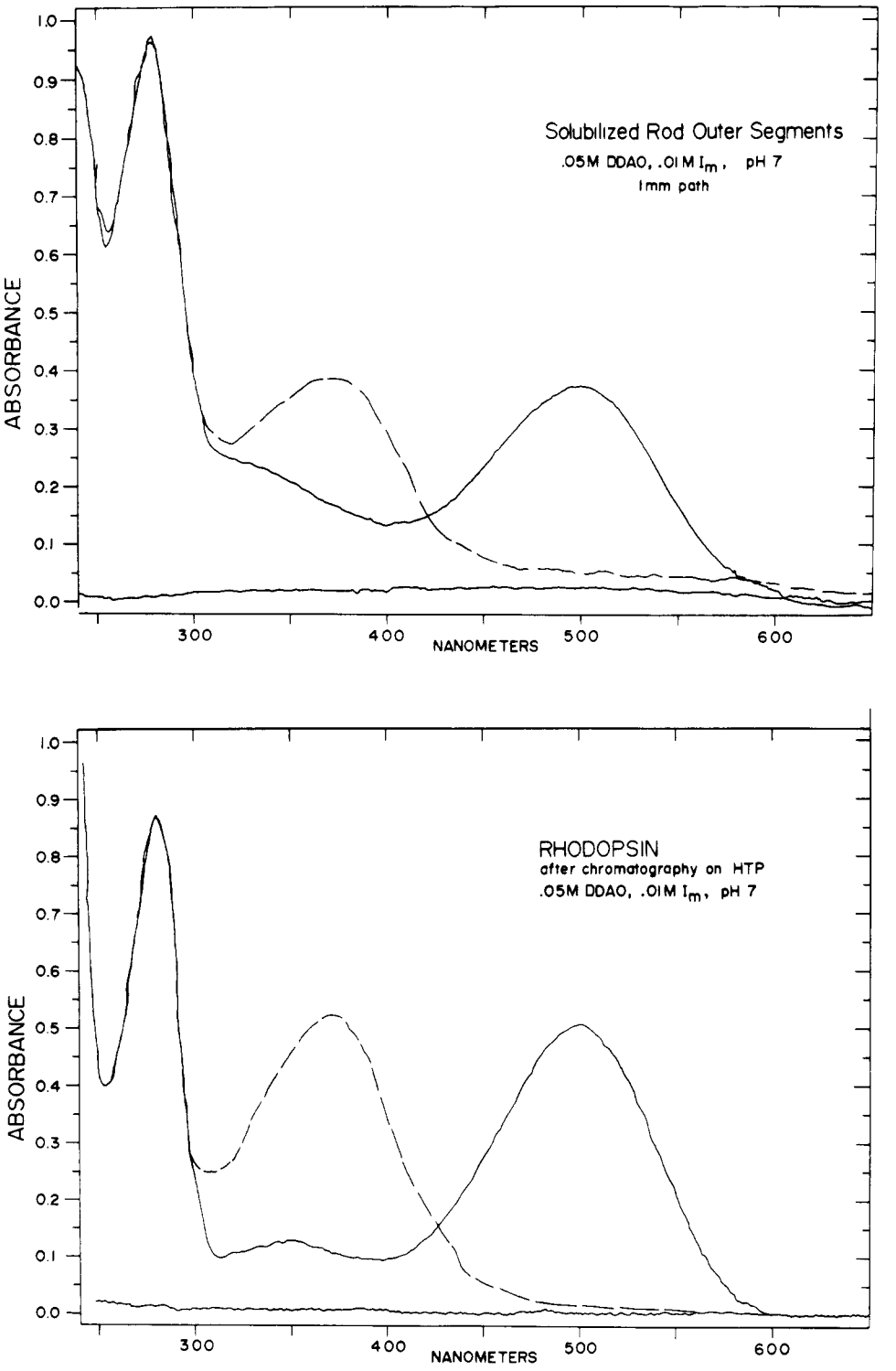

FIGURE 1: Absorption spectrum of bovine rhodopsin. The tracings are taken directly from the Cary 17: (——) no illumination, (- -) post illumination. Upper figure: Isolated ROS solubilized with 0.05 M DDAO, buffer,[4] 1-mm path length. Lower figure: Rhodopsin after purification on hydroxylapatite in presence of 0.05 M DDAO in buffer, 1-cm path length. The base line in both figures is recorded with 0.05 M DDAO–0.01 M Im (pH 7).

($a_\infty - a_t$) as a function of time, and to calculate a least-squares slope of selected points and the rate constant. All graphic output were produced on a Hewlett Packard recorder which was interfaced with the house computer. Typical records are given in Figures 6 and 7.

A second generation instrument has been built in Göttingen. It utilizes a tuneable dye laser flash and a DEC PDP 11 computer for data acquisition. Its properties and use will be reported in a forthcoming publication. This instrument was used in the study of the recombinant lipid–rhodopsin species. The data acquired for ROS and solubilized rhodopsin have been reproduced with this instrument. It has a very low signal to noise ratio and the data acquired reconfirm the single first-order nature of the rise of meta II with no appearance of faster initial processes.

Results

Isolation and Solubilization of ROS Membranes. A large batch of ROS from 250 retinae may be prepared in 1 day and stored at −60° for up to a year with no noticeable effects on

TABLE I: Purification and Characterization of Rhodopsin.

Sample	Absorbance Ratio 400/500	Absorbance Ratio 280/500	mol of Phospholipid / mol of Rhodopsin[a]	mol of DDAO / mol of Rhodopsin[a]
ROS (sonic.)	0.60	5.0 ± 1.0	110 ± 20	
Solubilized ROS	0.30 ± 0.01	3.0 ± 0.5	80 ± 10	900
Hydroxylapatite–rhodopsin	0.19 ± 0.01	1.8 ± 0.2	5 ± 5	200
G-100 rhodopsin[b]	0.23 ± 0.04	2.2 ± 0.1	1 ± 1	40
Detergent-free rhodopsin (sonic.)[b]	0.61	3.0	<1	<0.2
Rhodopsin + egg PC[b]	0.9	5.0	100	<0.1

[a] Molarity of rhodopsin based on a_m (500 nm) = 4.0×10^4 M^{-1} cm^{-1}. [b] Absorbance ratios are high due to scatter but are reduced by addition of DDAO.

the rate of meta II production in sonicated ROS or in solubilized rhodopsin. The use of Ammonyx-LO (a mixture of dodecyl- and tetradecyldimethylamine oxide) as a solubilizing agent has been previously described by Ebrey (1971). DDAO alone is equally efficient in solubilizing the ROS membranes. Use of 0.1 M DDAO allows total solubilization of the ROS membranes in a very small volume. The yield of solubilized rhodopsin from ROS membranes varies from batch to batch, but it is quite high, 3–9 nmol/retina. Up to 0.7 M DDAO has been added to these membranes without affecting the 500-nm chromophore absorption or kinetic parameters of meta II as measured in detergent. Spectra of solubilized ROS membranes show the characteristic absorption at 500 nm of the retinal chromophore of unbleached rhodopsin, a defined β band at about 340 nm, and the 280-nm protein absorbance (Figure 1). The ratio of protein absorbance (280 nm) to chromophore absorbance (500 nm), however, is relatively high at this point (Table I). Multiple bands are also observed upon gel electrophoresis (Figure 4).

Purification of Rhodopsin. Chromatography on hydroxylapatite in the presence of DDAO separates solubilized lipid from solubilized rhodopsin and in addition separates bleached rhodopsin from unbleached rhodopsin (Figure 2). Recovery of rhodopsin from the hydroxylapatite column is greater than 90%. After pooling the fractions of the major 500-nm absorbing peak, and exhaustively dialyzing to remove inorganic phosphate, assay for phospholipid shows that most of the lipid is separated from the rhodopsin (Table I). Remaining phospholipid content as determined by phosphate analysis may be tightly bound to rhodopsin, may be independently solubilized by DDAO but eluted in the same fractions, or may arise from failure to remove residual phosphate eluent. In the best preparations less than 0.5 mol of phospholipid/mol of rhodopsin is present.

The spectrum of rhodopsin eluted from this column (Figure 2) demonstrates a good spectral purity (Figure 1). The β peak is well-defined at 340 nm and the spectral ratios are low indicating absence of other contaminating species (Table I). Gel electrophoresis shows the presence of only two bands, a major

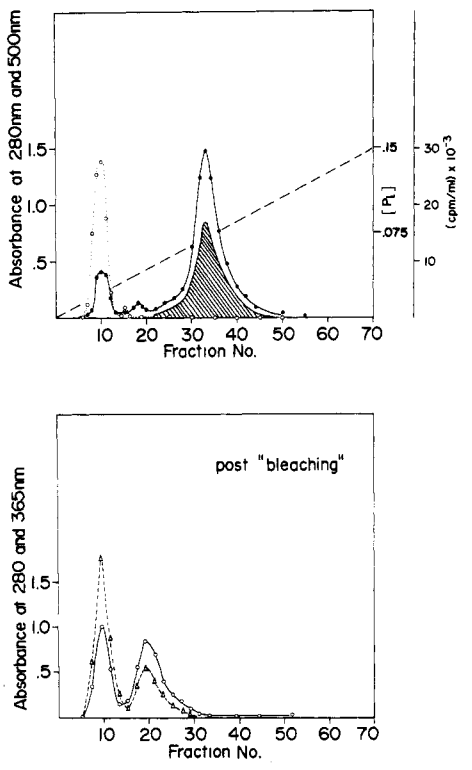

FIGURE 2: Chromatography of solubilized rhodopsin on hydroxylapatite; 1.1 × 25 cm columns initially equilibrated at 5 mM P_i, 0.05 M DDAO in buffer. Upper figure: Chromatography of isolated ROS solubilized with [^{14}C]DDAO. Applied 14 $OD_{500\ nm}$ units. Eluted by peristaltic pumping with a phosphate gradient from 5 to 150 mM P_i in cold 0.05 M DDAO buffer. Collected 15-min fractions (2 ml): (●—●) $OD_{280\ nm}$; (——) with slashed fill, $OD_{500\ nm}$; (O ···O) [^{14}C]DDAO cpm/ml. Lower figure: Chromatography of isolated ROS solubilized with DDAO but "bleached" before application. Conditions are same as for the upper figure except DDAO was not radioactive: (O——O) $OD_{280\ nm}$; (Δ - - - Δ) $OD_{365\ nm}$.

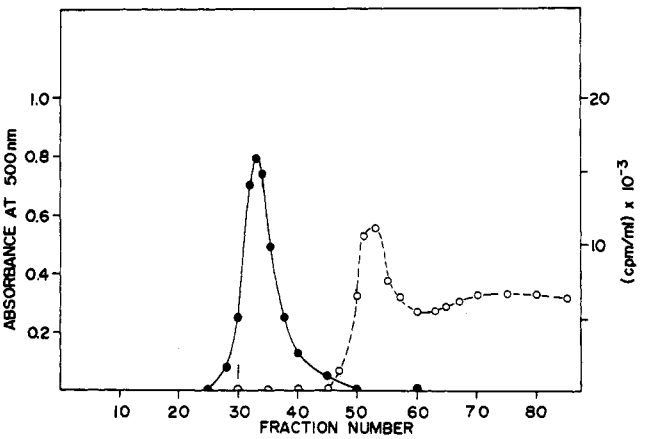

FIGURE 3: Chromatography of rhodopsin on Sephadex G-100; 2.5 × 82 cm column initially equilibrated with 1 mM DDAO, buffer pH 7. Applied 2.8 ml of 9×10^{-5} M rhodopsin in 0.5 M [^{14}C]DDAO in buffer. Eluted by peristaltic pumping with cold 1 mM DDAO in buffer; 4-ml fractions: (· — ·) $OD_{280\ nm}$; (O - - - O) [^{14}C]DDAO cpm/ml.

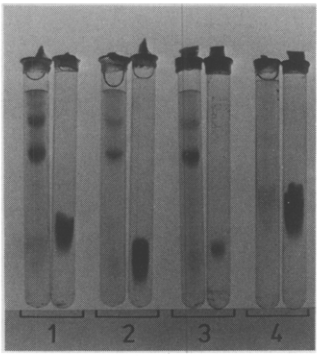

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis. Procedure carried out in accordance with Weber and Osborn (1969): (1) ROS solubilized in DDAO; (2) rhodopsin after chromatography on hydroxylapatite; (3) rhodopsin after chromatography on G-100; (4) DDAO as a reference. For each pair the right gel is stained with Coomassie Brilliant Blue; the left gel is stained with fuchsin for carbohydrate. Lipid stained very poorly and is not photogenic. The lipid stain was positive only in gel 1 and indicated lipid only in the faster moving band which also stains for carbohydrate. Note that the DDAO detergent itself stains as carbohydrate.

rhodopsin band and a minor second band which moves like a dimer form of rhodopsin (Figure 4).

To remove residual traces of lipid, contaminating phosphate from hydroxylapatite chromatography, and to remove excess detergent from rhodopsin, gel-filtration chromatography may be carried out after the suggestions of Helenius and Simons (1971). Rhodopsin is separated from the smaller detergent–lipid micelles (Figure 3). Some detergent must be present to elute the rhodopsin, therefore, the column is run in the presence of a DDAO concentration below its critical micelle concentration. The material eluted is nearly free of lipid and contains less than 0.5 mol of phospholipid/mol of retinal chromophore (Table I). The eluted protein, now in low amounts of detergent, aggregates upon standing which increases the absorption ratios due to scattering (Table I). The low spectral ratios are restored upon readdition of detergent. Heavily loaded gels show one major rhodopsin band and less than 10% of a lighter secondary band (Figure 4).

Characterization of the Thermal Intermediates of Solubilized Rhodopsin. Spectral bleaching studies carried out at low temperatures illustrate that the material purified in DDAO detergent undergoes the normal dark thermal intermediate transitions upon bleaching (Yoshizawa and Wald, 1963; Matthews *et al.*, 1963; Yoshizawa, 1972) (see Figure 5). Upon cooling rapidly from 27 to −196°, the main 498-nm rhodopsin peak shifts slightly to about 502 nm and increases in absorptivity by about 15%. Upon irradiation with light of wavelength greater than 440 nm, a new maximum appears at 525 nm (prelumirhodopsin). This transition to prelumirhodopsin is reversible since upon irradiation with light of wavelength greater than 580 nm a spectrum with a maximum at 502 nm can be reestablished. If the solution is irradiated at −196° to produce prelumirhodopsin and then slowly warmed, new species appear. At tem-

TABLE II: Kinetic Data For Meta I (480 nm) → Meta II (380 nm).

	mol of Phospholipid / mol of Rhodopsin	k ($sec^{-1} \times 10^{-2}$)[a] (at 20°, pH 7)	
			E_A = 35 kcal/mol
ROS Membranes			
Sonicated ROS	110	0.55	ΔG^* = 14.5 kcal/mol (20°)
		24 (37°)[b]	ΔH^* = 35 kcal/mol
			ΔS^* = 69 eu
Rhodopsin Solubilized in 0.1 M DDAO			
ROS	100	120	E_A = 19 kcal/mol
Rhodopsin	5	110	ΔG^* = 11.5 kcal/mol (20°)
		900 (37°)[b]	ΔH^* = 19 kcal/mol
			ΔS^* = 28 eu
Rhodopsin	<1	120	
Rhodopsin + 0.1 M NaCl	<1	110	
Rhodopsin + 0.1 M KCl	<1	125	
Rhodopsin + 1 mM Mg^{2+}	<1	135	
Rhodopsin + 1 mM Ca^{2+}	<1	140	
Rhodopsin + 0.05 M NH_2OH	<1	130	
Detergent-Free Rhodopsin			
DF-Rhodopsin	<1	Blocked	
DF-Rhodopsin + 0.1 M DDAO	<1	140	
Rhodopsin Recombined with Phospholipid			
DF-Rhodopsin[c] + PC ± Mg^{2+}	100	Blocked	
DF-Rhodopsin + PE ± Mg^{2+}	100	Blocked	
DF-Rhodopsin + PC, inc. 37°	100	Blocked	
DF-Rhodopsin + PE, inc. 37°	100	Blocked	
Rhodopsin in DDAO + PC in DDAO (DDAO removed by dialysis)	100	1.2k_2 (70%)	
		5.0k_1 (30%)	

[a] The precision of the kinetic constants is about 20%. [b] Extrapolated to 37°. [c] Detergent-free (DF) rhodopsin used contained 1 mol of phospholipid/mol of rhodopsin.

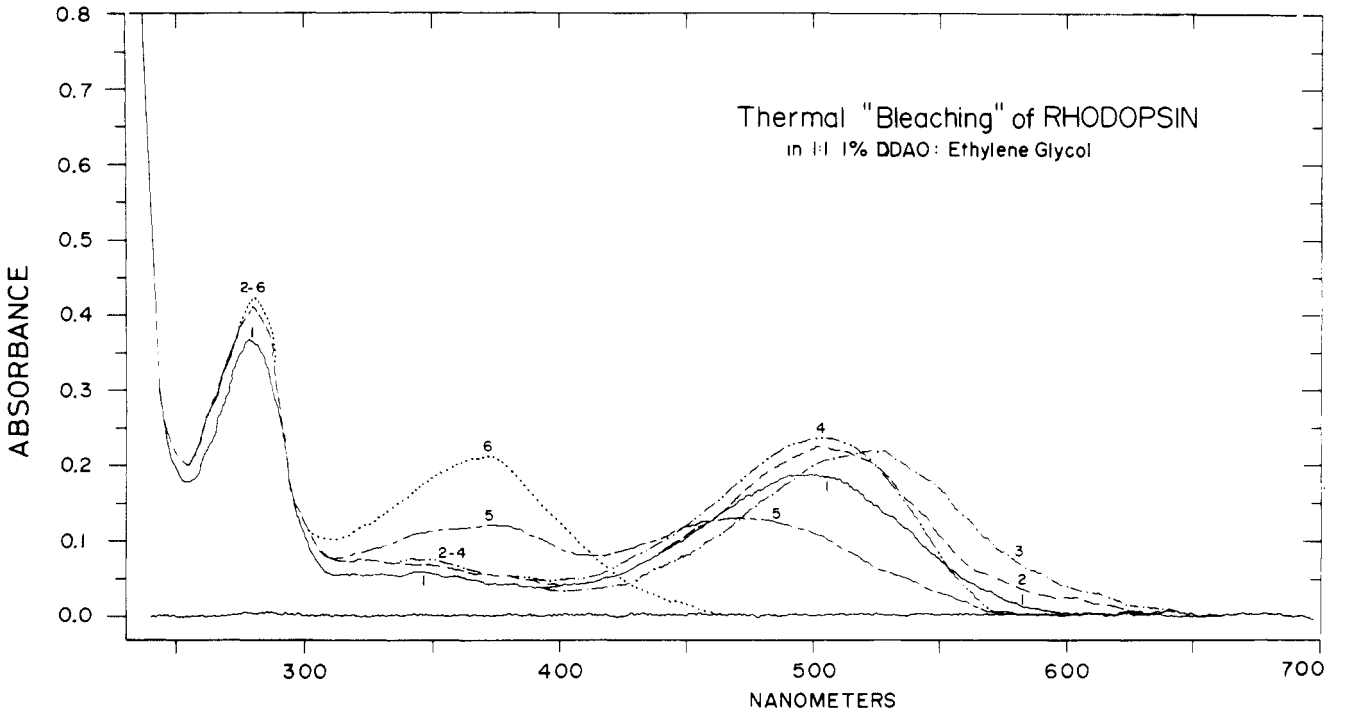

FIGURE 5: Spectral intermediates observed in the thermal bleaching of rhodopsin. Tracings are of spectra taken in the Cary 14 fitted with an optical dewar. Cell, 1 mm. Sample, 1:1 ethylene glycol–1.4 × 10^{-4} M rhodopsin in Ammonyx-LO in buffer: (1) 27°, rhodopsin, λ_{max} 498 nm; (2) −196°, rhodopsin, λ_{max} 502 nm; (3) −196°, irradiated 10 min at 440 nm, prelumirhodopsin, λ_{max} 525 nm; (4) −113°, lumirhodopsin, λ_{max} 500 nm; (5) −35°, mixture of meta I (480 nm) and meta II (380 nm) rhodopsin; (6) 27°, metarhodopsin II, λ_{max} 380 nm.

peratures above −150°, the spectral maximum shifts from 525 to 500 nm (lumirhodopsin). This species is stable until at least −113°. Unfortunately, the DDAO glass mixture becomes opaque between −90 and −35° so no changes can be observed in this region. At −35° a mixture of species absorbing at 480 and 380 nm is observed (metarhodopsin I and metarhodopsin II). As the solution is further warmed, the 480-nm species converts entirely to the 380-nm species (meta II). This conversion from meta I to meta II shows an isosbestic point at 415 nm as has been observed earlier (Matthews *et al.*, 1963). Thus rhodopsin devoid of lipid and solubilized in DDAO detergent demonstrates the normal spectral thermal intermediates. A notable difference, however, is the establishment of the meta I and meta II species at lower temperatures. In digitonin solutions, the thermal limit for conversion of meta I to meta II is >−15° (Matthews *et al.*, 1963); in DDAO it is below −35° (Figure 5).

Detergent-Free Rhodopsin and Rhodopsin–Lipid Recombinants. Rhodopsin can be completely freed of detergent by dialysis against imidazole buffer containing 10^{-4} M dithiothreitol (Table I). Such detergent-free material is not soluble but a suspension of detergent-free rhodopsin may be sonicated for use in spectral studies and shows the 500-nm absorbing chromophore in recorded spectra. The aggregated material is completely resolubilized by readdition of detergent.

Several methods for producing recombinants which would demonstrate a transient production of the meta II were attempted. Simple addition of phospholipid to detergent free rhodopsin does not produce a recombinant which shows the meta I to meta II reaction; this step is blocked as in the detergent-free rhodopsin species. Addition of Mg^{2+}, sonication, or incubation at 37° of the combined lipid and protein is also not successful in producing a functional species (Table II). If both the protein and the phospholipid are first dispersed in detergent and then allowed to slowly recombine while the detergent is removed by dialysis, a recombinant species capable of undergoing the meta I to meta II conversion is produced. Although a full analysis has not been carried out, the initial detergent concentration must be greater than 5 mM and need not be greater than 50 mM.

Kinetics of Metarhodopsin I → Metarhodopsin II. The method of collection of data for kinetic observation of the meta I to meta II reaction is illustrated for rhodopsin solubilized in 0.05 M DDAO in Figure 6. The reaction is rapid. The upper figure shows the raw data observed, *i.e.*, the transient change in transmission at 380 nm resulting from the absorption of photons from the flash at 500 nm. The real noise in the collected data is illustrated in the preflash and postflash records. The data as displayed have been smoothed by averaging values over 2.5 μsec (5 channels at 0.5 μsec/channel). The lower graph illustrates the first-order kinetic plot of the data. a_∞ is taken directly as the relative voltage at infinity, *i.e.*, at least tenfold the lifetime. The noisy data are real data; the smooth line is calculated from the least-squares slope which is directly proportional to the first-order rate constant.

The plot is linear over at least 3 lifetimes which indicates that the detergent-solubilized rhodopsin has a single first-order rate for the appearance of the meta II species (Figure 6, lower half). The rate constants observed are the same for ROS membranes solubilized in high concentrations of DDAO and for solubilized rhodopsin that has been separated from all phospholipid (Table II). Hydroxylamine, NaCl, KCl, Mg^{2+}, and Ca^{2+} have no effect on this rate (Table II).

Figure 7 illustrates the use of the same techniques for measuring the lifetime of meta II in sonicated ROS. For freshly prepared ROS or ROS stored below −60° the data demonstrate a single first-order process for the rise of meta II. Data have been accumulated over a variety of temperatures and recorded as an Arrhenius plot in Figure 8. Sonicated ROS membranes have a linear inverse temperature dependence over the range of 10–35°. The experimental data of Rapp (1970) (see also Abrahamson and Wiesenfeld, 1972) have also been plotted and show good agreement with the data obtained here. The data when extrapolated to 37° give a rate constant of 2.4 × 10^3 sec^{-1} which is in the same range for those observed for *in vitro*

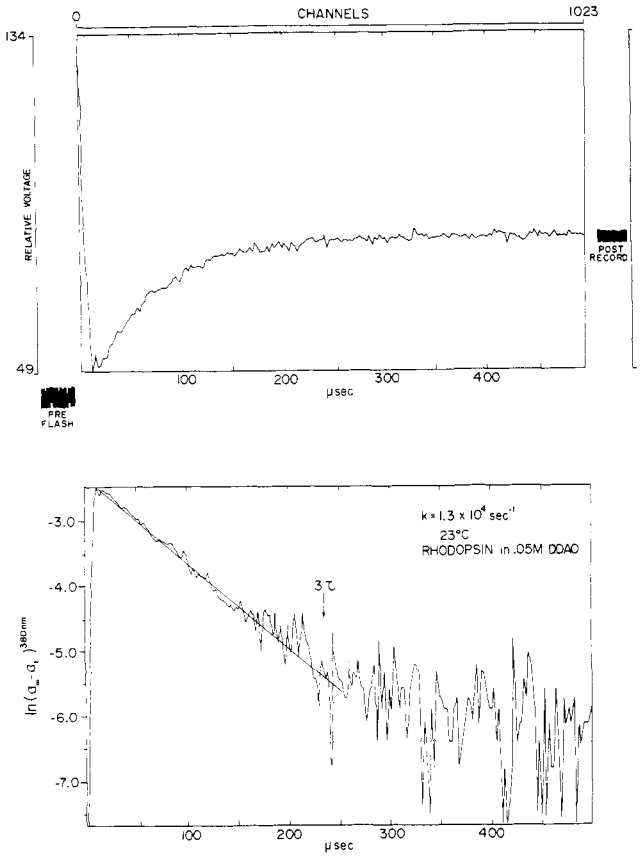

FIGURE 6: Kinetic data showing the rise of meta II in DDAO solubilized rhodopsin: 1.8×10^{-5} M rhodopsin, 0.05 M Ammonyx-LO, in buffer after purification on hydroxylapatite and 5 mol of phospholipid/mol of rhodopsin. Upper figure: Analog reconstruction of raw data observed. Vertical axis: 5 V full scale = 256 units. Horizontal axis: 0.488 μsec/channel. The vertical axis from bottom to top decreases in per cent transmission, increases in absorbance. Lower figure: First-order kinetic plots of raw data. Noisy data are real processed data. Smooth line is calculated least-squares slope. $k = 1.3 \times 10^4$ sec^{-1}, 23°. 3τ (3 lifetimes) equals 95% of the reaction.

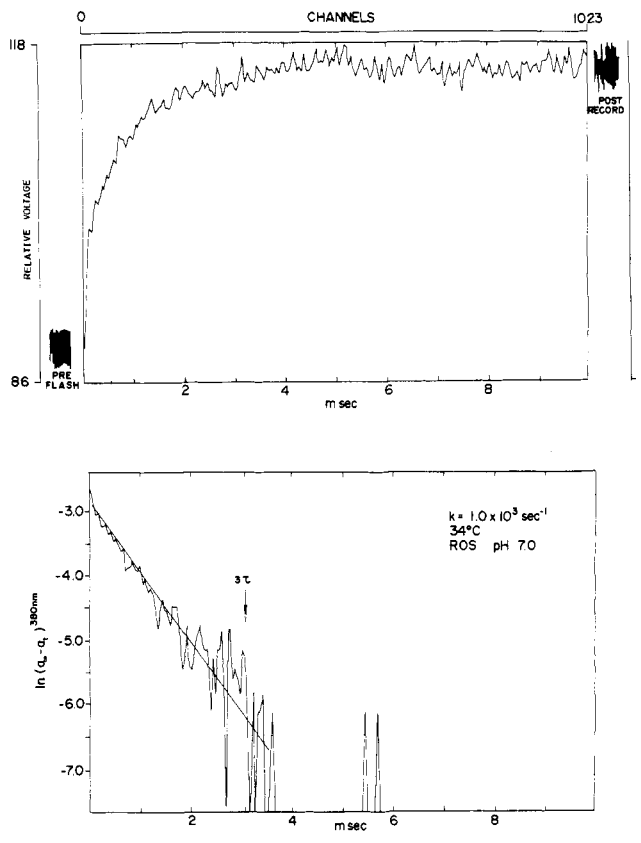

FIGURE 7: Kinetic data showing the rise of meta II in sonicated ROS: 1.45×10^{-5} M rhodopsin, 0.01 M Im (pH 7) and 93 μmol of phospholipid/mol of rhodopsin. Upper figure: Analog reconstruction of raw data observed for ROS. Vertical axis: 1 V full scale = 256 units. Horizontal axis: 0.975 msec/channel. Lower figure: First-order kinetic plot of raw data. Noisy data are real processed data. Smooth line is calculated least-squares slope. $k = 1.0 \times 10^3$ sec^{-1}, 34°. 3τ (3 lifetimes) equals 95% of the reaction.

studies (Hagins, 1972; Penn and Hagins, 1973). The energy of activation found is 35 kcal/mol and one may calculate the activation parameters accordingly as given in Table II.

When detergent is completely removed from rhodopsin by dialysis an aggregated material is obtained which can be sonicated to disperse the rhodopsin such that spectral studies may be carried out. No transient spectral change at 380 nm is observed over a time range from 10 μsec to 2 min (Figure 9). The appearance of meta II appears to be effectively blocked. Upon resolubilizing the detergent-free material with DDAO, the reaction is completely restored (Table II). Spectra taken with the Cary 17 of the detergent-free rhodopsin before and after flash photolysis indicate that rhodopsin does undergo some photochemical reaction. The OD at 500 nm is decreased and the maximum is shifted from 500 nm toward 480 nm. This might suggest that rhodopsin undergoes transitions up to the meta I intermediate but the meta I to meta II conversion is blocked. Upon addition of detergent to the photolyzed detergent-free material the spectrum shifts from the blocked intermediate to the 380-nm absorbing meta II.

Several methods of reconstitution have been tried. Only when both the phospholipid and the protein are dispersed in detergent and the detergent is slowly removed by dialysis is the transition from meta I to meta II restored (Figure 10). The recombination of egg phosphatidylcholine with rhodopsin served to restore a rate of the same order of magnitude as observed for ROS. Although the recombinant does not display a single first-

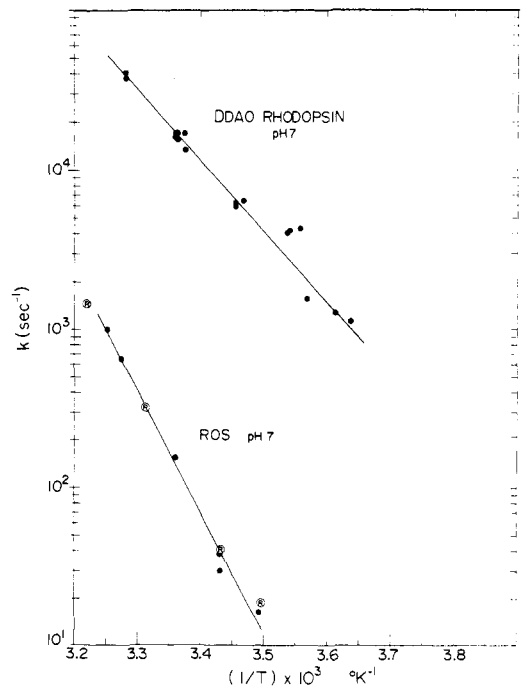

FIGURE 8: Arrhenius plot of kinetic data for the risetime of meta II. The data in the upper half of the figure are for DDAO solubilized rhodopsin. Data are equivalent for solubilized rhodopsin before and after purification to remove lipid and other non-$OD_{500\,nm}$ absorbing species, pH 7. The data in the lower half of the figure are for sonicated ROS, pH 7. Ⓡ refers to the data of Rapp (1970).

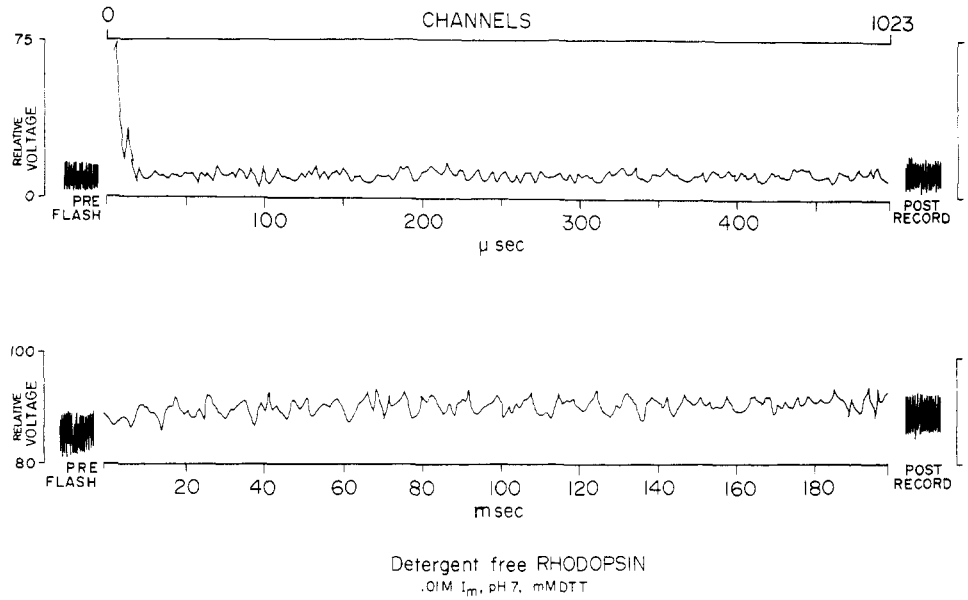

FIGURE 9: Kinetic record of detergent-free rhodopsin observed at 380 nm. Detergent-free rhodopsin 1×10^{-5} M, 0.2 mol of DDAO/mol of rhodopsin, 0.01 M Im (pH 7), 1 mM dithiothreitol, sonicated, 20°. Upper figure: Vertical axis, 200 mV full scale = 256 units; horizontal axis, 0.488 μsec/channel. Lower figure: Vertical axis, 200 mV full scale = 256 units; horizontal axis, 0.195 msec/channel.

order rate constant, it can be interpreted as a combination of two first-order rates. A rough estimate of the amplitudes of these two rates indicates that 70% of the reaction proceeds with a time constant (τ) of 9 msec and 30% proceeds with a time constant (τ) of 2 msec at 20°. In comparison ROS membranes have a time constant (τ) of 20 msec.

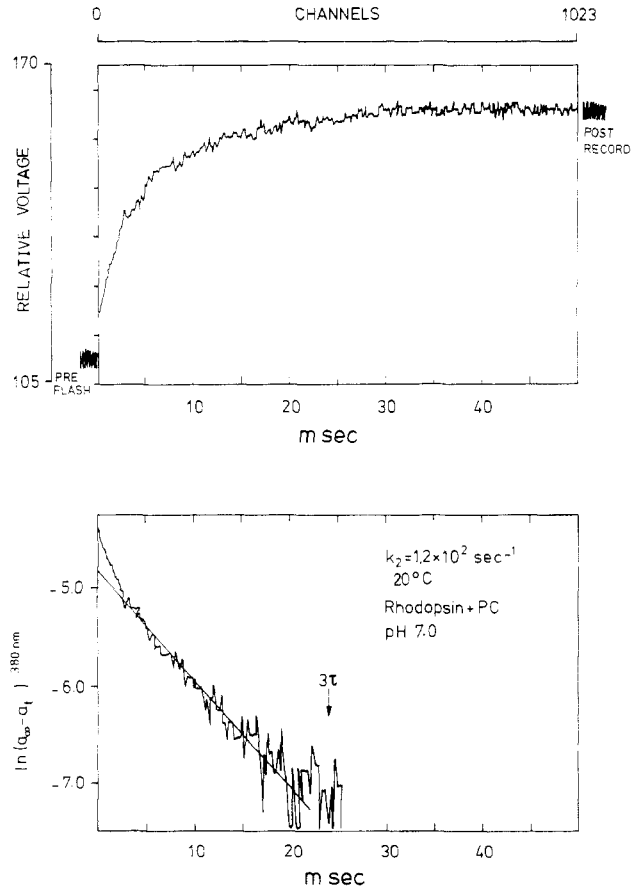

FIGURE 10: Kinetic data showing the rise of meta II in recombined rhodopsin-phosphatidylcholine, 1.1×10^{-5} M rhodopsin, 1.2×10^{-3} M phosphatidylcholine, 0.1 mM dithiothreitol, 0.01 M Im (pH 7). Upper figure: Analog reconstruction of raw data observed for protein-lipid recombinant. Vertical axis: 0.39 mV/data unit. Horizontal axis: 49.9 μsec/channel. Lower figure: First-order kinetic plot of raw data. Noisy data are real processed data. Smooth line is calculated least-squares slope with $k_2 = 1.2 \times 10^2$ sec^{-1}. $k_1 = 5 \times 10^2$ sec^{-1}, 20°.

Discussion

Reconstitution of biological membranes from specified component parts provides a feasible method for elucidating the molecular organization of membranes and understanding the specific interactions between components of that membrane (Razin, 1972). For informative studies it is important to obtain membrane protein components free of native lipid and to be able to recombine the protein with specified membrane lipid components to recreate a species which is functionally active. Detergents or other solubilizing agents used to disrupt the native membrane, to purify its component parts, and to recombine specific constituents should not irreversibly destroy the protein function and should be completely removed from the final recombinant species. With these constraints in mind the methods reported here were developed for the investigation of functional and structural interactions of rhodopsin with specific membrane components.

In order to characterize rhodopsin by methods now established for soluble proteins a variety of detergents has been employed for solubilization and purification of ROS membranes, *e.g.*, digitonin (Wald and Brown, 1951–1952), Emulphogene (Shichi *et al.*, 1969), cetyltrimethylammonium bromide (CTAB) (Heller, 1968), dodecyltrimethylammonium bromide (Hong and Hubbell, 1972), and Ammonyx-LO (Ebrey, 1971). Only with dodecyltrimethylammonium bromide has it been shown that all phospholipid from the native membrane can be removed from rhodopsin (Hong and Hubbell, 1972). Use of digitonin or the ionic detergents is less desirable since upon storage rhodopsin is unstable in their presence. Moreover, once rhodopsin is solubilized with these detergents they are very difficult to remove. The binding of detergents to proteins has been shown to depend upon the hydrocarbon chain length (Reynolds *et al.*, 1967). Thus to remove detergent from protein it is advantageous to use a detergent with a shorter length hydrocarbon tail. The detergent DDAO, the shorter chain component of Ammonyx-LO, seems to avoid the above problems.

DDAO solubilizes ROS membranes well giving a high yield of rhodopsin. This nonionic detergent is very soluble at cold-room temperatures (1.5 M or greater), has no adverse effect

upon either ion exchange or gel filtration chromatography, and has no absorption above 230 nm. It can be removed from rhodopsin to less than 1 mol/mol of rhodopsin by dialysis even in the presence of phospholipid. The following characteristics demonstrate that rhodopsin solubilized with DDAO and purified in its presence maintains its native structural integrity at least in the protein region surrounding the chromophore. (a) Rhodopsin shows no loss of its characteristic chromophore absorption in presence of DDAO, even at room temperature, high concentration (1 M or greater), and over long periods of time. (b) The chromophoric and protein spectral transitions are well-defined indicating that the rhodopsin preparation is free of other absorbing contaminants (Figure 1, Table I). (c) The molar absorbance at 500 nm is 40,000 M^{-1} cm^{-1} in agreement with that earlier reported (Wald and Brown, 1953–1954; Shichi, 1970). (d) Low-temperature spectral studies demonstrate the presence of the known dark reaction intermediates following the absorption of light, although there is a difference in the thermal lability of the meta I intermediate (Figure 5). (e) Sonication does not harm the environment of the chromophore, even in lipid-free, detergent-free preparations of rhodopsin. (f) Upon removal of detergent or recombination with phospholipid and detergent removal 85–100% of the protein as measured by $OD_{500\ nm}$ is recoverable.

To investigate the nature of the rhodopsin–lipid interactions in ROS membranes some indicative functional assay must be used. Although it is unknown how rhodopsin is involved in the visual transduction process, a primary sequence of events following photon absorption has been established and studied both *in vitro* (Yoshizawa and Wald, 1963; Matthews *et al.*, 1963; Abrahamson and Wiesenfeld, 1972; Yoshizawa, 1972) and *in vivo* (Hagins, 1956, 1972; Cone and Cobbs, 1969; Penn and Hagins, 1973). This sequence consisting of spectral intermediates is defined as follows: rhodopsin (500 nm) → prelumirhodopsin (545 nm) → lumirhodopsin (497 nm) → meta I (480 nm) → meta II (380 nm). Even though little is known about the nature of these intermediates they are considered essential to the transduction process and they may be used phenomenologically as a known "activity" of rhodopsin. Such changes must reflect subtle or dramatic structural changes in the lipoprotein around the chromophore region. Technically, the transition from meta I to meta II is the easiest to study and initial studies have been addressed to this step. This transition is the last one that is fast enough to be involved in the transduction process (Wald, 1968; Hagins, 1972). The step has a high entropy of activation (Table II) and has been interpreted as a dramatic unfolding of protein structure (Abrahamson *et al.*, 1960; Matthews *et al.*, 1963; Ostroy *et al.*, 1966).

In earlier *in vitro* kinetic studies the interpretation of this step was complicated by the observation of multiple first-order rates (Ostroy *et al.*, 1966; Pratt *et al.*, 1964; Abrahamson and Wiesenfeld, 1972). Both the work of Sengbusch (1970) and our data indicate that care must be taken in interpreting results obtained for membranes in the presence of detergent. Solubilization of membranes with digitonin leads to a decrease in the rate of reaction and it has been demonstrated that digitonin apparently breaks the membrane into nonhomogeneous pieces (Sengbusch, 1970; Sengbusch and Stieve, 1971). The appearance of multiple first-order rates for the intermediates of bleaching apparently arises from nonuniform dissolution of the membrane. Cetyltrimethylammonium bromide is known to effect an increase in the rate of conversion of meta I to meta II but it has been less systematically studied (Sengbusch, 1970; Williams and Breil, 1968).

Sonicated rod outer segment membranes for which the ratio of phospholipid to protein is 100:1 show a single first-order rate of appearance of meta II (Figure 7). A single first-order process for ROS with similar kinetic parameters have also been observed by Rapp (1970). Fresh preparations of ROS membranes or those stored at −60° or lower must be used. The rates observed are of the same order as those observed *in vivo*. At 37°, the kinetic rate of transition of meta I to meta II is 3×10^3 sec^{-1} for rat, 0.9×10^3 sec^{-1} for rabbit (Hagins, 1972; Penn and Hagins, 1973), and for isolated bovine ROS it is 2.4×10^3 sec^{-1} (Table II).

When ROS membranes are solubilized with DDAO they still show a single first-order rate of appearance of meta II, but the rate is dramatically increased (Figures 6 and 8). Whether this effect can be attributed to the influence of DDAO upon rhodopsin or to removal of phospholipid from rhodopsin is not distinguishable. Both whole solubilized membranes and solubilized rhodopsin from which all phospholipid has been removed show the same rate (Table II). When detergent is completely removed from lipid-free rhodopsin, the meta I to meta II conversion is effectively blocked. A similar effect is observed in dried ROS and has been attributed to the prevention of a hydrolytic step at this point (Wald *et al.*, 1950; Kimbel *et al.*, 1970). Results of this work suggest that aggregation could also prevent this reaction from occurring. The effect is reversible and readdition of detergent disperses the aggregates and restores the reaction (Table II). In contrast, addition of phospholipid to detergent-free rhodopsin without first solubilizing both phospholipid and rhodopsin does not restore this step (Table II).

The effect of DDAO upon rhodopsin is reversible. Reassociation of lipid-free rhodopsin with egg phosphatidylcholine brought about by slowly removing the dispersing detergent produces a recombinant species which undergoes the reaction from meta I to meta II. The rate of this reaction is of the same order of magnitude as observed for ROS membranes themselves. The reaction is not, however, a single first-order process. When the data are treated as two first-order rate processes at least 70% of the recombinant species have a lifetime of 9 msec. ROS have a lifetime of 20 msec in comparison. It is possible that this recombinant consists of fragments nonhomogeneous in the ratio of phospholipid to protein or in the degree of unsaturation in the fatty acid chains in the local rhodopsin environment. Still, it is possible to recreate an artificial lipid environment for rhodopsin in which it can behave functionally in a similar fashion as it behaves in its native membrane environment.

Phosphatidylcholine makes up only 35% of the lipids in ROS; the other native components are 35% phosphatidylethanolamine, 10% phosphatidylserine and a variety of less common lipids (Kimbel *et al.*, 1970, Borggreven *et al.*, 1970). To define the nature of rhodopsin–lipid interactions and possibly to define the molecular organization of the ROS membrane it is important to ask what effects these other species of lipid have upon the rate of conversion of meta I to meta II. In addition, does the length or degree of unsaturation of the lipid hydrocarbon chain have an effect on the function of rhodopsin? Is the fluidity of the membrane important for the photon induced spectral transitions of rhodopsin?

Other studies have reported the effect of lipids upon the regenerability of the rhodopsin chromophore (Zorn and Futterman, 1971; Shichi, 1971; Hong and Hubbell, 1972, 1973). This rather slow process, which takes place after the bleaching of rhodopsin and long after visual transduction is completed, requires the presence of lipid and addition of retinal *in vitro*. Hong and Hubbell (1973) have suggested that structural aspects of lipid–protein interactions rather than specific chemical

requirements are important for regeneration of the chromophore. It has not been proven, however, that this *in vitro* regeneration is analogous to the regeneration process which takes place *in vivo* (Cone and Brown, 1969).

Preparation and characterization of homogeneous recombinants can now be carried out for a more detailed study of these lipid-protein interactions. Certainly much further study is necessary to elucidate the structural and functional lipid requirements for the photon-induced spectrochemical transitions of rhodopsin which lead to visual transduction.

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