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Effects of Detergents and High Pressures upon the Metarhodopsin I ⇄ Metarhodopsin II Equilibrium†

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ABSTRACT: The effects of high pressure upon the equilibrium (3°) between metarhodopsin I ⇌ metarhodopsin II has been examined in several different preparations of bovine visual pigment. The equilibrium is shifted to the left for sonicated rod outer segments (ROS) (ΔV(1 atm) $\geq$ 60 cm^3/mol) and digitonin extracts of ROS ($\Delta V \simeq$ 10 cm^3/mol). Pressure has no effect on the equilibrium ($\Delta V \simeq 0$) for extracts of ROS made with the detergents emulphogene or lauryldimethylamine oxide. These observations support the proposal that in outer segment disk membranes rhodopsin–phospholipid interactions are coupled with the transformation metarhodopsin I → metarhodopsin II.

Functional interactions between the rhodopsin molecules and lipids in the outer segment membranes of vertebrate photoreceptor cells are implied in most models for signal transduction in photoreception (see Hagins, 1972; Blasie, 1972; Abrahamson and Wiesenfeld, 1972; Cone, 1972; Robinson and Weir, 1974). What is suggested in some models is that key structural changes in the disk membrane accompany one or more of the steps in the rhodopsin bleaching sequence. We have been seeking evidence for such rhodopsin–lipid coupling by measuring changes in the thermodynamics of bleaching which occur as a consequence of disrupting the lipid field surrounding the rhodopsin (*cf.* Applebury *et al.*, 1974).

It occurred to us that the molar free volume change (ΔV) might be a useful parameter to consider in this regard because large ΔV values are expected for processes in which the exposure of hydrophobic groupings to water is altered and because of the large compressibility of assemblies of hydrocarbon chains. Thus, we examined the pressure dependence of the equilibrium (3°) between the intermediates, metarhodopsin I (478 nm), and metarhodopsin II (380 nm) in the bleaching of bovine rhodopsin to obtain the ΔV for the transformation metarhodopsin I → metarhodopsin II. We found modest values of ΔV in sonicated rod outer segments (ROS)[1] and digitonin extracts of ROS but $\Delta V \simeq 0$ for extracts using detergents which displace nearly all the lipid surrounding the pigment protein. We describe these pressure experiments in this report.

Materials and Methods

Detergents, Buffers, and Reagents. Digitonin (Fisher Certified Reagent) and commercial lauryldimethylamine oxide (a 30% aqueous solution trade named Ammonyx LO was provided by the Onyx Chemical Co.) were used without further purification. Emulphogene BC720 (General Aniline and Film Corporation), a polyether detergent, was filtered through Celite. Imidazole (Calibiochem, A grade), recrystallized from ethanol, was neutralized with hydrochloric acid. Cacodylic acid (Fischer Reagent) was used as the sodium salt.

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[1] Abbreviation used is: ROS, rod outer segments.

A sample of 11-*cis*-retinal was kindly provided to us by the Hoffmann-La Roche Co. *n*-Butylamine (Eastman Organic Chemicals) was distilled just prior to use. Ethanol and *n*-hexane were of spectroquality grade.

Rhodopsin Preparations. Rod outer segments (ROS) were prepared from bovine retinas (G. Hormel Co.) using the procedure of Applebury (Busch *et al.*, 1972; Applebury *et al.*, 1974). The ROS were washed three times with distilled water before sonication or solubilization with detergent. All operations were carried out under dim red light.

Sonicates of ROS were prepared using a Branson Sonifier Cell Disrupter Model W185. ROS prepared from 25 retina in 3.5–5 ml of distilled water contained in a 25 × 50 mm tube cooled in an ice–water mixture were subjected to five 1-min bursts of ultrasound (output control setting 4, normal tip). The sample was allowed to cool for about 1 min between sonic bursts. After sonication, buffer was added and the sample was centrifuged at 100,000*g* for 30 min. The absorption spectrum of the clear supernate was recorded, the volume adjusted to suit the experiments to be performed, and the pH recorded (0°).

ROS (from 25–50 retina) were solubilized by agitation with buffered detergent solution (2–4 ml) using a slowly rotating mixer. The detergent solutions and extraction times were: 2% digitonin, 2–4 hr, room temperature; 1% emulphogene, 2–4 hr, room temperature; 2% Ammonyx LO containing 1 mM dithiothreitol, 12–16 hr, 4°. The mixtures were centrifuged at 100,000*g* for 30 min. The spectrum of the clear supernate was recorded, its volume adjusted, and the pH recorded (0°). The Ammonyx LO extracts were diluted (final Ammonyx LO concentration 0.1%) with 2% digitonin or 1% emulphogene for use in the high pressure experiments. The former will be referred to as Ammonyx LO–digitonin extract and the latter as Ammonyx LO–emulphogene extract. Experiments were performed within a few hours after sonication or detergent extraction of the ROS.

Pressure Generation Apparatus and Optical Bomb. The apparatus used for pressure generation and measurement and the optical bomb are described in detail elsewhere (Kliman, 1969; Zipp, 1973).

The high-pressure optical bomb, designed by Professor W. B. Daniels of the University of Delaware, was constructed from Vascomax 300 CVM maraging steel and rated to 10 kbars. The sealing capability of the high-pressure vessel was provided by unsupported area seals. To avoid contamination of the pressurizing fluid (spectroquality hexane), all packing material was metal. The high-pressure windows were of ultraviolet (uv) grade sapphire. The bomb fitted into the sample compartment of a Cary 14 recording spectrophotometer. The rhodopsin solutions were contained in an internal cell of quartz (3.4-cm path length and 1.0 cm o.d.) and were isolated from the pressure fluid by means of a flexible Teflon sleeve. This cell has been described by Kliman (1969).

Constant temperature was maintained by means of a copper–brass jacket which fitted snugly over the entire length of the high-pressure vessel. The temperature was maintained constant to ±0.05°.

Correction for Solvent Compression. The absorbance of any solution in an optical cell of fixed length will increase with increasing pressure due to compression. Before two-state equilibrium constants can be determined, the experimentally determined absorbances must be corrected for this effect. This correction can be achieved by multiplying the experimental absorbance values by the relative volume, $V(P,T)/V(1\text{ atm},T)$, of the solvent. The pressure dependence of the relative volume of water was taken from Bridgeman (1931) and Vedam and Holton (1968).

Effect of Pressure on the pH of Buffers. The pH values of solutions of the various commonly used buffers exhibit very different pressure dependences (Neuman *et al.*, 1973; Zipp and Kauzman, 1973). Since the metarhodopsin I ⇄ metarhodopsin II equilibrium is pH dependent it was necessary to choose a buffer with a negligibly small pressure coefficient.

The largest contribution to the molar free volume change upon dissociation of an acid in water is due to alterations in solvation associated with the change in the number of charged species. Only small values of ΔV(1 atm), $\sim+1$ cm³/mol, are observed for the dissociation reactions of conjugated acids of neutral bases such as amines (Hamann, 1965). For such buffers the pH increases by no more than 0.1 on increasing the pressure from 1 atm to 6000 kg/cm² (*cf.* phosphate buffer, ΔpH = ΔpH = -1.6 for ΔP = 6000 kg/cm²). On this basis, imidazole-HCl (usually 0.01 M, pH 7.0) was chosen as the buffer in most of the pressure experiments reported here. The pH of each sample was measured at 0° and 1 atm and was taken to be negligibly different at the pressures (up to 7000 kg/cm²) which were employed.

In some experiments 0.01 M cacodylate buffer was employed. This buffer has a ΔV(1 atm) of -13 cm³/mol. The ΔpH values are reported (Neuman *et al.*, 1973) to be -0.21, -0.39, -0.51, and -0.65 at pressures of 1000, 2000, 3000, and 4000 kg/cm², respectively.

Effect of Pressure on the Detergent Solutions. Aqueous emulphogene solutions (1%) contained in the optical bomb at 3° did not exhibit light scattering at pressures up to 7000 kg/cm². The same is true of 2% aqueous digitonin. However, the latter sometimes scattered light badly and precipitated when the pressure was changed rapidly, perhaps due to accompanying fast temperature changes. Consequently care was taken to change the pressure sufficiently slowly during experiments to avoid precipitation of the digitonin. The Ammonyx LO precipitated from aqueous solutions containing as little as 0.05% of the detergent when the pressure was raised above about 2000 kg/cm². On the other hand the mixed detergent systems 2% digitonin–0.1% Ammonyx LO and 1% emulphogene–0.1% Ammonyx LO showed no light scattering at pressures up to 7000 kg/cm². The optical densities at 400 nm of the detergent solutions employed in the experiments increased less than 0.01 unit when the pressure was increased from 1 atm to 3000 kg/cm².

High-Pressure Experiments. The procedures used in the high-pressure experiments were generally as follows. The rhodopsin preparations were centrifuged at 100,000*g* for 30 min within 2 hr before use and very often just prior to use. This was done to minimize light scattering due to aggregated material. The quartz optical cell was loaded with the sample ($A_{500\text{nm}} \simeq 0.2$ cm⁻¹) and placed in the pressure bomb. The bomb was inserted into its cooling jacket and the whole assembly placed in the sample compartment of a Cary M14 spectrometer. All these operations were carried out in dim red light. After cooling to 3° an absorption spectrum was recorded over the range 350–660 nm. If the sample was not to be irradiated, spectra were recorded at various pressures up to the maximum pressure employed and then at various pressures as the pressure was released and finally at atmospheric pressure. If the sample was to be irradiated the optical bomb in its cooling jacket was removed from the spectrometer after recording a spectrum at atmospheric pressure. The optical path of the pressure bomb was aligned with the beam of light from a focused incandescent lamp (Bausch & Lomb microscope

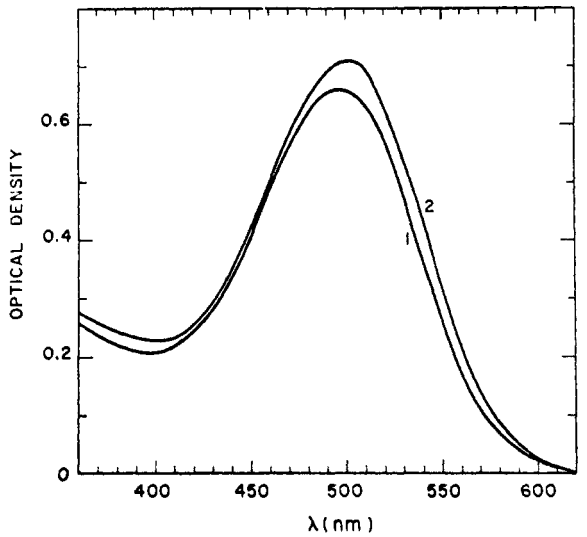

FIGURE 1: The absorption spectrum of a digitonin (2%) extract of ROS (0.01 M imidazole (pH 7), 3°) at atmospheric pressure (1 atm = 1.03 kg/cm²) (curve 1) and at 4000 kg/cm² (curve 2). The spectra are not corrected for scattering or contraction.

illuminator 31-33-53) filtered through an Optics Technology Spectracoat "600 nm" cut-off filter and a Corning C.S. #2-63 color glass filter. This filter combination has an absorbance greater than two at wavelengths shorter than 575 nm. The filters and lamp were placed as close as possible to the window of the bomb. Irradiation of the sample for 10 min at the maximum setting of the lamp was sufficient to achieve a photostationary absorption spectrum. The bomb was replaced in the spectrometer and a spectrum recorded at atmospheric pressure. Spectra were recorded at one or more high pressures and then the pressure released and a spectrum recorded at atmospheric pressure to check the reversibility of the pressure-induced spectral changes. Spectra were recorded at other high pressures as long as the spectrum at atmospheric pressure remained unchanged. In several experiments the sample was irradiated while under high pressure. This required to differences in procedures except added safety precautions.

Calculations of the Ratio [Metarhodopsin II]/[Metarhodopsin I] from Absorption Spectra. In order to calculate the ratio [metarhodopsin II]/[metarhodopsin I] from the absorption spectrum of an irradiated sample of a rhodopsin preparation recorded at high pressure several manipulations must be made. One must correct for sample compression and scattering, subtract the absorption due to residual rhodopsin and isorhodopsin, and subtract the absorption due and other materials present in the preparation. In addition one must correct for the presence of subsequent species in the bleaching scheme such as metarhodopsin$_{465}$, and correct for pressure-induced changes in the spectrum of the various species. It was found (*vide infra*) that the absorption spectra of rhodopsin and metarhodopsin I exhibit only very small shifts and virtually no change in extinction coefficient at pressures as high as 7000 kg/cm². The criterion of reversibility was employed with respect to the question of other species such as metarhodopsin$_{465}$. In experiments in which the presence of metarhodopsin$_{465}$ was suspected, checks based on the observations of Ostroy *et al.* (1966) regarding the kinetics of the thermal decay of the metarhodopsins were employed. The small amount of rhodopsin and isorhodopsin remaining after exhaustive irradiation of the various preparations with the filtered light source was determined in separate experiments carried out at atmospheric pressure and 3° in a jacketed 1-cm quartz cell using the procedure described by Matthews *et al.* (1963). The relative extinction coefficients at the maximum ϵ_{max} of the main absorption band of rhodopsin, metarhodopsin I, and metarhodopsin II were taken to be 1.00, 1.06, and 1.00, respectively (Matthews *et al.*, 1963).

Ratios of [metarhodopsin II]/[metarhodopsin I] = K were determined from the absorption spectra in two ways. The spectral corrections and subtractions described above were applied and the resulting difference spectrum resolved into contributions from the two metarhodopsins by employing the spectrum of metarhodopsin I reported by Kropf and Hubbard (1958). Values of K were then calculated from the ratio of the intensities of the two absorption curves and the ratio of ϵ_{max}. The sum [metarhodopsin I] + [metarhodopsin II] calculated from these resolved curves was 10–20% higher than [rhodopsin] photolyzed. That this was due to an overestimate of the amount of metarhodopsin II is indicated by the fact that the amount of metarhodopsin I determined from spectra of irradiated digitonin extracts of ROS recorded at 7000 kg/cm² where virtually no metarhodopsin II is found (*vide infra*) agreed very well (2–4% discrepancy) with the amount of rhodopsin photolyzed. Greater error is expected in determining metarhodopsin II compared to metarhodopsin I because the spectra of the intermediates are not as well determined below about 400 nm as they are above this wavelength, there is more interference at wavelengths below 400 nm from other absorbing species in the preparations, and the light scattering increases greatly toward the lower wavelengths. It was decided to take [rhodopsin]$_{photo}$ − [metarhodopsin I] as the amount of [metarhodopsin II] in the sample after making sure that contributions from other species, such as metarhodopsin$_{465}$, were negligible. The values of K reported here were determined in this way. Despite the fact that the K values determined in the two ways differed, plots of ln $1/K$ *vs.* pressure had very similar slopes indicating that the error in determining values of ΔV was smaller than the error in determining the K values.

Results

Effects of High Pressure upon Rhodopsin. Absorption spectra (3°) of digitonin extracts of ROS (pH 5.5–7.4) and of an Ammonyx LO-digitonin extract of ROS (pH 7.0) were recorded at various pressures from 1 atm to 7000 kg/cm²; spectra of a sample of sonicated ROS (pH 6.7) were measured at pressures up to 4000 kg/cm². The spectral changes were similar for all these samples, namely, the "500-nm" absorption band increased in intensity and shifted slightly to higher wavelengths under pressure. These changes were completely reversible upon release of the pressure even when the samples were kept under pressure for more than 1 hr. An example is given in Figure 1 which shows the absorption spectrum (660–350 nm) of a digitonin extract of ROS (0.01 M imidazole, pH 7.0, 3°) recorded at 1 atm and at 4000 kg/cm². The contraction of the sample under pressure, expected to be 12% at 4000 kg/cm², accounts for the increase in absorptivity, about 10%, measured at the maximum of the "500-nm" band. The maximum of the band shifted from 498 nm at 1 atm to 502 nm at 4000 kg/cm². Upon release of the pressure the spectrum was virtually identical with the spectrum recorded at atmospheric pressure before any pressure was applied. Spectral data obtained at various pressures are given in Table I. Because of the large path length of the absorption cell many of the samples scattered light badly in the ultraviolet range. Because of this

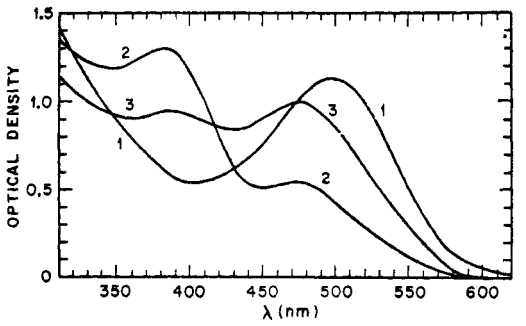

FIGURE 2: The absorption spectrum of sonicated ROS (0.01 M imidazole (pH 6.7), 3°) before irradiation (curve 1), at atmospheric pressure after irradiation with light of $\lambda > 580$ nm (curve 2), and after pressurizing the irradiated sample to 1100 kg/cm² (curve 3). The spectra are not corrected for scattering or contraction.

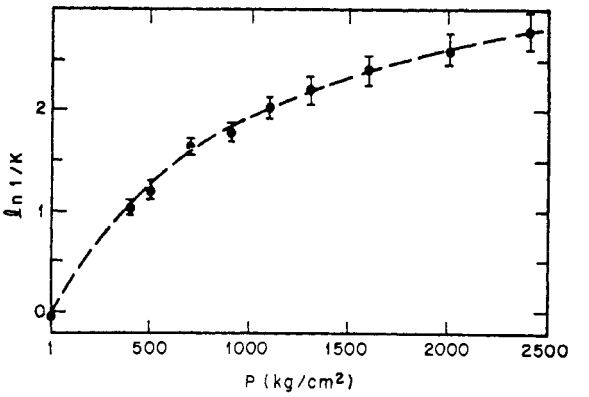

FIGURE 3: A plot of ln $1/K$ *vs.* pressure (P) for the sample of sonicated ROS from which the spectra of Figure 2 were obtained.

TABLE I: Wavelength λ_{max} and Relative Intensity $\epsilon(P)/\epsilon(1\text{ atm})$ of the Maximum of the Main Absorption Band of a Digitonin Extract of ROS (pH 7, 3°) at Different Pressures.[a]

P (kg/cm²)	λ_{max} (nm)	$\epsilon_{max}(P)/\epsilon_{max}(1\text{ atm})$	$V(1\text{ atm})/V(P)$
1 atm	498	(1.00)	(1.00)
1000	500	1.03 ± 0.03	1.04
2000	501	1.05	1.09
4000	502	1.09	1.12
7000	503	1.12	1.16

[a] Compression data for water are listed as $V(1\text{ atm})/V(P)$ and are taken from Bridgeman (1931) and Vedam and Holton (1968).

and absorption due to the buffer no attempt was made to monitor pressure effects upon the "280-mn" absorption band.

Effects of Detergents and High Pressures on the Metarhodopsin I ⇄ Metarhodopsin II Equilibrium. The absorption spectrum at 1 atm of a sample of sonicated ROS (0.01 M imidazole, pH 6.7) contained in the optical pressure bomb and kept at 3° was recorded (Figure 2, curve 1). The bomb was removed from the spectrometer and the sample was irradiated as described until a "photostationary" absorption spectrum was obtained (10–15 min). This spectrum (curve 2 in Figure 2) exhibits two bands in the near-ultraviolet and visible regions with maxima close to 380 and 480 nm, respectively. The spectrum is similar to those described by Matthews *et al.* (1963) and Ostroy *et al.* (1966) for digitonin extracts of rhodopsin treated in a similar way. Both groups have shown that the bands at "480 nm" and "380 nm" are associated with two species, called metarhodopsin I and metarhodopsin II, respectively, which are in equilibrium with each other during the slow transformation to other species which occurs under these experimental conditions. We found (in a separate experiment) that under the conditions of pH and temperature employed there was no change in the spectrum of the irradiated sample left in the dark for 1 hr.

When high pressure was applied to the irradiated sample the "480-nm" absorption band increased in intensity while the "380-nm" band decreased in intensity. The spectrum recorded at 1100 kg/cm² is shown as curve 3 in Figure 2. The intensity changes were accompanied by small shifts in the maxima of the bands; the long-wavelength band maximum shifted from 477 nm at 1 atm to 479 nm at 2400 kg/cm². Spectra recorded after the release of the pressure were generally identical with the original spectrum recorded postirradiation at 1 atm. Irreversible changes were observed to occur only if the sample was kept at high pressures for times on the order of 1 hr. The high pressures appear to encourage the formation of a species which absorbs maximally near 460 nm, presumably the species described by Ostroy *et al.* (1966) called metarhodopsin$_{465}$.

Values for the equilibrium constant K = [metarhodopsin II]/[metarhodopsin I] were calculated from the absorption spectra as described in the section on methods. Values of K for high pressures were determined from only those spectra which showed reversibility upon release of the pressure.

Figure 3 is a plot of ln $1/K$ *vs.* pressure for the sample of sonicated ROS referred to in Figure 2. The effect of pressure (P) upon the equilibrium constant at constant temperature (T) is related to the molar free volume change (ΔV) for the reaction by

$$(\partial \ln K/\partial P)_T = -(\Delta V/RT)$$

where R is the gas constant. It can be seen from Figure 3 that ΔV is positive for the reaction metarhodopsin I → metarhodopsin II for sonicated ROS. The ΔV is not constant over the pressure range; ΔV is much larger at the lower pressures (<700 kg/cm²) than at the higher pressures (>1300 kg/cm²). A lower limit (63 cm³/mol) for the value of the molar free volume change at one atmosphere, $\Delta V(1\text{ atm})$, can be determined from the data. Another experiment with sonicated ROS at the slightly higher pH of 7.0 yielded similar results and $\Delta V(1\text{ atm}) > 48$ cm³/mol.

Several experiments were performed in the same way with extracts of ROS in 2% digitonin at various pH values. Values of K at 1 atm were found to be in reasonable agreement with those reported in the literature (Matthews *et al.*, 1963; Ostroy *et al.*, 1966). Some of the data are listed in Table II.

The pressure dependence of K for a digitonin extract of ROS at pH 5.5 (1 atm) (0.01 M cacodylate) is shown in Figure 4 as a plot of ln $1/K$ *vs.* P. The lowering (by not more than 0.5 pH unit) of the pH of the sample under pressure was ignored in the calculation of the K since only a small dependence of K on pH is expected in this pH range (Matthews *et al.*, 1963). From the plot one can see that ΔV is positive and, in contrast to the results obtained for sonicated ROS, ΔV is constant over the pressure range examined. The $\Delta V(1\text{ atm})$ of 10 cm³/mol is much smaller than that found for sonicated ROS. Similar re-

TABLE II: Equilibrium Constant K and Molar Free Volume Change at Atmospheric Pressure ΔV(1 atm) for the Reaction Metarhodopsin I $\rightleftarrows$ Metarhodopsin II in Various Preparations from Bovine Rod Outer Segments.

Preparation	pH[a] (0°)	K(1 atm, 3°)[c]	ΔV(1 atm, 3°) (cm³/mol)
Sonicated ROS	6.7	1.0	>63
	7.0	0.7	>48
Digitonin (2%) extract of ROS	5.5[b]	1.0	10 ± 2
	6.7	0.7	14 ± 3
	7.4	0.3	13 ± 4
Emulphogene (1%) extract of ROS	6.7	3.5	~0
	7.0	3.5	~0
Ammonyx LO extract of ROS diluted with digitonin solution (0.1% Ammonyx LO–2% digitonin)	7.0	5.2	~0
Ammonyx LO extract of ROS diluted with emulphogene (0.1% Ammonyx LO–1% emulphogene)	7.0	5.0	~0

[a] Buffer was 0.01 M imidazole-HCl except where noted. [b] Buffer was 0.01 M cacodylate. [c] Calculation of K based upon the metarhodopsin I absorption band intensity compared with the loss of Rhodopsin absorption upon photolysis.

sults were obtained for digitonin extracts in imidazole buffer at pH 7.0 and 7.4 (see Table II).

It was interesting to find that digitonin extracts of ROS could be irradiated under pressure (4000 and 7000 kg/cm²) to give spectra virtually identical with those obtained by applying pressure to samples which were irradiated at atmospheric pressure.

Experiments could not be performed with extracts of ROS containing Ammonyx LO as the only detergent since the latter precipitated at high pressures above about 2000 kg/cm². However, Ammomyx LO extracts diluted with 1% emulphogene or 2% digitonin so that the final Ammonyx LO concentration was 0.2% or lower did not precipitate or exhibit increased light scattering even at 8000 kg/cm².

Experiments performed with emulphogene (1%) extracts of ROS or with samples extracted using Ammonyx LO and then diluted with 1% emulphogene or 2% digitonin gave

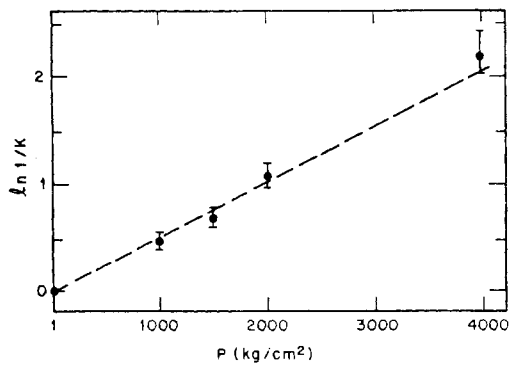

FIGURE 4: A plot of ln $1/K$ *vs.* pressure (P) for a digitonin extract of ROS at pH 5.5 (1 atm) (0.01 M cacodylate) and 3°.

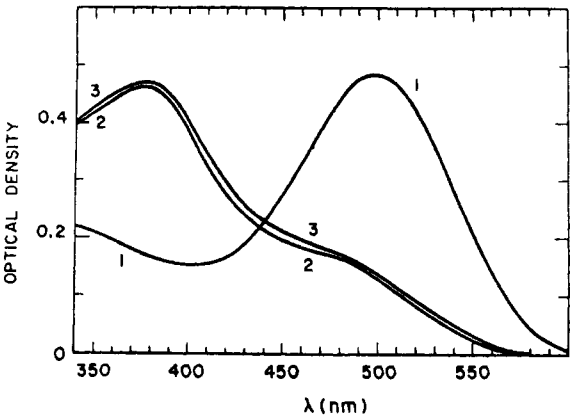

FIGURE 5: The absorption spectrum of an emulphogene (1%) extract of ROS (0.01 M imidazole (pH 7.0), 3°) before irradiation (curve 1), at atmospheric pressure after irradiation (curve 2), and after pressurizing the irradiated sample to 3000 kg/cm² (curve 3). The spectra are not corrected for scattering or contraction.

similar results (see Table II). Irradiation of each of these extracts at 3° in the usual way afforded a ratio of metarhodopsins more heavily weighted toward metarhodopisn II (K = 3.5–5) than that obtained for sonicated ROS and digitonin extracts.

The ratio of metarhodopsin I to metarhodopsin II in emulphogene or Ammonyx LO extracts at pressures as high as 3000 kg/cm² was found to be unchanged from the ratio of atmospheric pressure; that is, ΔV(1 atm) $\simeq$ 0. An example is shown in Figure 5 in which curve 1 is the absorption spectrum of an emulphogene (1%) extract of ROS (0.01 M imidazole (pH 7.0)), curve 2 is the absorption spectrum recorded immediately after irradiation in the usual way, and curve 3 is the absorption spectrum recorded at 3000 kg/cm². The increased intensity of the high-pressure spectrum was due to sample contraction. The spectrum recorded after the pressure was released was virtually identical with curve 2. In accord with the observations of Ostroy *et al.* (1966) production of metarhodopsin$_{465}$ during the course of the pressure experiments was accelerated in these samples containing a high proportion of metarhodopsin II. Thus, pressurization, spectrum recording, and depressurization were performed as quickly as possible. That no significant amount of metarhodopsin$_{465}$ was formed during an experiment was checked by quickly heating the sample to 25° after recording the final spectrum at atmospheric pressure and noting an appropriate loss of absorption attributable to metarhodopsin II and concomitant increase in absorption attributable to metarhodopsin I.

Effects of Pressure upon the Absorption Spectrum of 11-cis-Retinylidene-n-butylimine. 11-*cis*-Retinylidene-*n*-butylimine was prepared by stirring 11-*cis*-retinal with a large excess of *n*-butylamine for 2 hr at room temperature. The excess *n*-butylamine was evaporated in a stream of dry nitrogen and the residual product was taken up in solvent to give a 5 μM solution. Absorption spectra of the solution were recorded at various pressures at 3°. Two solvents were employed: *n*-hexane and ethanol–1 mM acetic acid in which the Schiff base is protonated.

The wavelengths of the maxima and the relative intensities the long-wavelength band extracted from the spectra are listed in Table III.

Discussion

Metarhodopsin I $\rightleftarrows$ Metarhodopsin II Equilibrium and Detergents. The equilibrium constant K = [metarhodopsin

TABLE III: Wavelength λ_{max} and Relative Intensity $\epsilon(P)/\epsilon(1\text{ atm})$ of the Main Absorption Band of *n*-Butyl-11-*cis*-retinylidenimine in *n*-Hexane and in Ethanol–1 mM Acetic Acid as a Function of Pressure.[a]

P (kg/cm²)	λ_{max} (nm)	$\epsilon_{max}(P)/\epsilon_{max}(1\text{ atm})$	$V(1\text{ atm})/V(P)$
	In EtOH–10^{-3} M HOAc		
1 atm	447	(1.00)	(1.00)
1000	452	1.10 ± 0.03	1.07
2000	455	1.14	1.12
4000	459	1.19	1.17
6000	462.5	1.24	1.22
	In *n*-Hexane		
1 atm	367	(1.00)	(1.00)
1000	371	1.14 ± 0.03	1.09
3000	376	1.28	1.18
5000	379	1.32	1.24

[a] Compression data for the solvents are listed as $V(1\text{ atm})/V(P)$ and were taken from Bridgeman (1931) and Brazier and Freeman (1969).

II]/[metarhodopsin I] at 3° at neutral pH (1 atm) is quite similar (~0.5) for sonicated bovine ROS and 2% digitonin extracts of ROS (Table II). Values of K at the same pH for ROS extracted with 1% emulphogene or with 2% Ammonyx LO are much larger, in the range 3.5–5 (Table II). A similar high value is found for the mixed detergent system 2% digitonin–0.1% Ammonyx LO.

A simple correlation between the position of the equilibrium and the rate of the metarhodopsin I → metarhodopsin II transformation is not to be expected (Abrahamson and Wiesenfeld, 1972). However, the much larger values of K for emulphogene and Ammonyx LO extracts compared to digitonin extracts and sonicated ROS are undoubtedly connected with the much faster formation of metarhodopsin II observed for extracts of ROS in "strong" detergents compared to sonicated ROS (Abrahamson and Wiesenfeld, 1972; Applebury *et al.*, 1974) and fresh digitonin extracts (Sengbusch and Stieve, 1971a,b). For example, Williams and Breil (1968) reported data showing that the rate of formation of metarhodopsin II at room temperature is at least ten times faster in extracts of ROS in 1% hexadecyltrimethylammonium bromide than it is in 2% digitonin (also see Williams and Baker, 1972). Applebury *et al.* (1974) have obtained flash kinetic data which show that at 3° the rate of formation of metarhodopsin II in Ammonyx LO extracts is more than 100 times greater than the rate in sonicated ROS.

It is useful to compare the observations concerning the metarhodopsin I → metarhodopsin II transformation with those concerning the effects of detergents and phospholipids on the regenerability of bleached pigment. While bleached sonicated ROS and fresh digitonin extracts are highly regenerable (see Shichi, 1971a), bleached extracts in 1% emulphogene (Shichi, 1971a), 2% Triton X-100 (Johnson and Williams, 1970), and 2% Ammonyx LO (Applebury *et al.*, 1973; Ebrey, 1971) are not at all regenerable. Loss of regenerability of digitonin extracts can be effected by aging, petroleum ether extraction, or treatment with phospholipase A (Shichi, 1971a). Regenerability can be restored by addition of phospholipid to delipidated extracts (Shichi, 1971a; Zorn and Futterman, 1971; Hong and Hubbell, 1972). These and other observations have led to the suggestion that the phospholipid influences the strucure of the pigment protein and maintains a conformational state of opsin which is essential for regeneration (Zorn and Futterman, 1971; Shichi, 1971a,b). The phospholipid requirement is specific in that detergents even with long hydrocarbon chains cannot replace phospholipid with respect to this function. On the other hand phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine all of various fatty acid compositions appear to be equally effective in restoring regenerability to delipidated ROS extracts (Shichi, 1971a; Hong and Hubbell, 1972). Thus, the nature of the phospholipid–protein interactions connected with the function of phospholipid in pigment regeneration is obscure. Furthermore, the apparent requirement of phospholipid in regeneration does not necessarily relate to any function of phospholipid in signal transduction in photoreception.

What does seem clear from the regeneration studies is that extraction of ROS with detergent solutions such as 1% emulphogene or 2% Ammonyx LO severely disrupts the phospholipid milieu of the pigment protein while extraction with 2% digitonin is not so disruptive. Differentiation between sonicated ROS and digitonin extracts on the one hand and emulphogene and Ammonyx LO extracts on the other hand is also found for the rate of the metarhodopsin I → metarhodopsin II transformation and for the position of the metarhodopsin I ⇄ metarhodopsin II equilibrium. We take the attitude that these detergent-dependent properties reflect the state of the phospholipid surrounding the pigment protein and not the presence of the detergent *per se*. That the detergent Ammonyx LO does not significantly perturb the pigment protein at least in the vicinity of the chromophore is strongly suggested by the observations of Applebury *et al.* (1974) that the sequence of spectral changes (intermediates) observed upon photolysis of Ammonyx LO extracts of ROS is the same as observed in digitonin extracts of ROS.

Effect of High Pressure on the Metarhodopsin I ⇄ Metarhodopsin II Equilibrium. It appears that bovine rhodopsin is not susceptible to pressure denaturation under the conditions of our experiments. This conclusion is based on the insensitivity of the "500-nm" absorption band of extracts of ROS to pressures up to 7000 kg/cm², and the fact that irradiation of digitonin extracts at 7000 kg/cm² followed by release of pressure gives virtually the same spectrum as that obtained from an identical sample irradiated at atmospheric pressure. The insensitivities of the long-wavelength absorption bands of metarhodopsin I and metarhodopsin II to high pressures and the reversibility of the pressure-induced changes in their ratio indicate that these species intermediates are also not easily susceptible to pressure denaturation. Therefore, we take the view that the chromoproteins of metarhodopsin I and metarhodopsin II are not significantly perturbed from their native forms at 3° in the pressure range up to 3000 kg/cm² in which values of K were determined.

The effect of pressure on the metarhodopsin I ⇄ metarhodopsin II equilibrium differentiates between sonicated ROS and digitonin extracts which exhibit $\Delta V(1\text{ atm}) > 10$ cm³/mol and emulphogene and Ammonyx LO extracts of ROS which give $\Delta V \simeq 0$. We believe this difference reflects the state of the phospholipid associated with the pigment protein rather than effects of the detergents *per se* (emulphogene, Ammonyx LO–emulphogene, and Ammonyx LO–digitonin give the same results). Furthermore, we propose on the basis of these observations that protein–phospholipid interactions are coupled with the transformation metarhodopsin I →

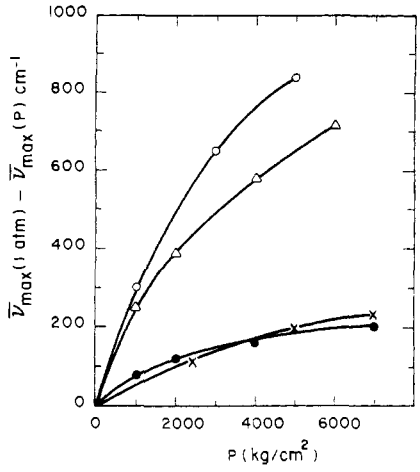

FIGURE 6: The shift in the frequency of the long-wavelength absorption maximum *vs.* pressure for rhodopsin (digitonin extract of ROS) (●); metarhodopsin I (irradiated digitonin extract of ROS) (X); 11-*cis*-retinylidene-*n*-butylimine in ethanol–1 mM acetic acid (△); 11-*cis*-retinylidene-*n*-butylimine in hexane (○). The shift $\bar{\nu}_{max}$(1 atm) − $\bar{\nu}_{max}(P)$ is in cm^{-1}.

metarhodopsin II. Arguments in support of this proposition follow.

The ΔV value for a reaction in solution is the sum of the volume change associated with the reactant molecule(s), and the volume change associated with the alterations in solvation which accompany the reaction. Several authors (Kauzmann, 1959; Brandts, 1968; Suzuki and Taniguchi, 1972) have reviewed the possible sources of ΔV for protein reactions in which the protein undergoes a structural change as, for example, in denaturation. While there exists no conclusive interpretation of the observed ΔV of denaturation of any protein, it appears clear from model studies that volume changes associated with solvation make significant contributions to the total ΔV and should dominate over other sources in many cases. For example, the solvation of hydrophobic side chains exposed during the denaturation of a water-soluble protein should lead to large decreases in partial molar volume. Kauzmann (1959) estimated that the volume should decrease by about 20 cm^3/mol at room temperature for each aliphatic side chain transferred from a hydrophobic environment to water. These volume changes are attributed to the formation of hydrophobic groups in water. On the other hand, volume changes associated with internal reorganization of the protein are expected to be very small.

In the absence of other information we interpret the $\Delta V \simeq 0$ value for the metarhodopsin I → metarhodopsin II transformation in emulphogene, Ammonyx LO–emulphogene, or Ammonyx LO–digitonin extracts to mean that little volume change is associated either with the chromoprotein or with its detergent–lipid surroundings. The alternative would be to invoke a coincidental cancellation of volume changes for the chromoprotein and each detergent system. Continuing with this line of reasoning, we attribute the positive ΔV values for the reaction in sonicated ROS and digitonin extracts to volume changes associated with the phospholipid. In other words the associated phospholipid is rearranged as a consequence of the metarhodopsin I → metarhodopsin II reaction. We further suggest that the much larger value of ΔV(1 atm) for the sonicated ROS compared to digitonin extracts reflects the perturbation of the phospholipid structure by the digitonin in the latter.

While the ΔV value for digitonin extracts of ROS was constant with pressure over the range examined, the ΔV for sonicated ROS was found to decrease substantially with increasing pressure: >50 cm^3/mol at 1 atm, ~10 cm^3/mol at 2000 kg/cm^2. This requires a greater compressibility to the metarhodopsin II state than to the metarhodopsin I state in sonicated ROS, but little compressibility difference between the corresponding species in digitonin extracts. If we associate the pressure effects with the phospholipid fraction, then we can further argue that there occurs a change in the packing of phospholipid moieties such that the lipid field of metarhodopsin II is more compressible than that of metarhodopsin I. We suggest that digitonin perturbs interactions among the phospholipid moieties and reduces the difference in compressibility between the two states.

The results of several kinds of experiments have evidenced rhodopsin–phospholipid interactions: rhodopsin becomes more labile to thermal bleaching upon solubilization with detergents (Hubbard, 1958; Johnson and Williams, 1970; Hong and Hubbell, 1972); delipidation of rhodopsin reduces its regenerability (Shichi, 1971a,b; Zorn and Futterman, 1971; Ebrey, 1971); incorporation of rhodopsin into egg lecithin vesicles inhibits the segmental motions of the fatty acid chains much the same as does cholesterol (Hong and Hubbell, 1972). A functional role for phospholipid in signal transduction in photoreception would seem to demand phospholipid–rhodopsin interactions which are coupled to some step in the bleaching of the pigment which occurs on a time scale of the metarhodopsin I → metarhodopin II step or shorter. We propose that the effects of detergents upon the rate of the metarhodopsin I → metarhodopsin II transformation (Applebury *et al.*, 1974; Williams and Breil, 1968) and the effects of detergents on the position of the metarhodopsin I ⇄ metarhodopsin II equilibrium reported here suggest that rhodopsin–phospholipid interactions are coupled with this step in the bleaching cycle. The effects of pressure upon the metarhodopsin I ⇄ metarhodopsin II equilibrium in various preparations lend excellent support for this notion.

Effects of High Pressures upon Pigment Spectra and Spectra of the Free Chromophore. It is on the basis of spectral criteria that we have concluded that the chromoproteins rhodopsin, metarhodopsin I, and metarhodopsin II are insensitvie to pressure denaturation. These criteria rely on the idea that the absorption spectrum of the chromoprotein is sensitive to changes in the structure of the protein at least in the vicinity of the retinyl chromophore. For the purpose of making comparisons it was of interest to determine the effects of pressure upon the absorption spectra of a model for the pigment chromophore, *n*-butyl-11-*cis*-retinylidenimine in hexane solution and in ethanol–1 mM acetic acid in which the Schiff base is protonated. The wavelength data of Tables I and III as well as data for metarhodopsin II (digitonin extract, pH 7.0) are shown in Figure 6 as a plot of the shift (in reciprocal centimeters) of the maximum of the main absorption band with pressure. It is most reasonable to compare the shifts for metarhodopsin I and the protonated model compound (in ethanol–acetic acid) since they have the most similar absorption spectra. The red shift accompanying pressurization of an absorbing solute in solution in the absence of any chemical change reflects primarily the increase in the dielectric constant and index of refraction of the medium due to the compression (Weigang and Robertson, 1963). We think that the much larger shift for the model chromophore in solution compared to that observed for metarhodopsin I indicates that the environs of the chromophore in the latter are not very compressi-

ble. This in turn suggests a picture for the chromoprotein in which the retinyl chromophore is well buried within a rigid protein structure.

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Added in Proof

Hong and Hubbell (1973) have recently reported that rhodopsin regeneration does not have an absolute requirement for phospholipid. Indeed they were able to regenerate delipidated rhodopsin in digitonin micelles. They suggest that the digitonin provides the adequately stable and rigid hydrophobic environment required for regeneration while detergents such as Ammonyx LO do not.

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