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ASSESSMENT OF PHYSIOLOGICAL INTEGRITY OF SONICATED RETINAL ROD MEMBRANES

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As the size of rod membrane fragments was reduced by sonication or by addition of the detergent Emulphogene, the intensity of the circular dichroism (CD) bands (210 and 221 nm) increased progressively with a blue shift in position. The intensity of the visible CD bands (340 and 495 nm) was also increased by sonication. Since the intensity increase of the CD bands was related to a reduction in turbidity, the anomalous CD features of intact membranes could be attributed to optical artifacts caused by the particulate nature of the material.

Because the magnitude of the CD bands at 221 nm and 340 nm was essentially identical for the sonic suspension and detergent-clarified solution, the adequacy of sonic suspensions can be assured by checking whether detergent affects the intensity of these bands.

Suspensions of sonicated rod membranes, purified on Agarose, contained vesicles of 112 nm in average diameter. The morphology and size of the vesicles did not change upon photobleaching of rhodopsin. The vesicles retained such rod membrane properties as conformational insensitivity to photobleaching of the retinal chromophore, thermal stability, and pigment regenerability. Thus, the physiological integrity of rod membranes was maintained by the sonicated vesicles.

From the most reliable estimate of the molecular ellipticity at 221 nm, the helical content of membrane-bound rhodopsin was determined to be approximately 47%.

INTRODUCTION

For conformational analysis of biological molecules in solution, the technique of circular dichroism (CD) has been used with considerable success and the method has recently been applied to the study of various membrane systems (1–4). The CD spectra of membrane suspensions show generally similar anomalies which have been attributed by recent studies to optical artifacts (5–7). Although theoretical and experimental attempts have been made to correct CD spectra for the artificial effect of light scattering (7–9), satisfactory solutions applicable to different membrane systems are yet to be found. If membranes can be disintegrated to such an extent that spectral distortions due to light scattering are made negligible without destroying biological properties of the whole

membrane, such preparations would not only permit reliable CD measurements but also studies of membrane functions under more simple conditions.

In the retinal rod outer segment membrane of bovine (10) and frog (11) retinas, the visual pigment rhodopsin reportedly accounts for 80–90% of the total protein. Because of the unusually high concentration of a single protein in the photoreceptor membrane, the membrane has great potential as a simple model for studies on other biological membrane systems. This paper reports that fragmentation of retinal rod membranes by ultrasonic irradiation produces clarified suspensions comparable in optical properties to those produced by detergent and that the membrane vesicles thus prepared still retain virtually all of the properties of the unsonicated rod membranes. It is also reported that the adequacy of preparations for CD measurements can be critically assessed on the basis of a detergent effect on the CD spectra of membrane suspensions.

MATERIALS AND METHODS

Sonication of Bovine Retinal Rod Outer Segment

Retinas were collected from fresh dark-adapted bovine eyes (50 eyes) and rod outer segments (ROS) were prepared by flotation in surcorse as previously described (12). The possibility of contamination of ROS by mitochondria was checked by following the A_{550} of reduced cytochrome c and no cytochrome c oxidase activity could be detected in the purified preparation. ROS were suspended in about 5 ml of deionized water containing 10^{-2} M ethyleneglycol-bis (β-amino ethyl) N, N′ -tetracetic acid (EGTA, Sigma, St. Louis) at pH 7.0 and sonicated at 20 KC at 0–3°C with a Sonifier Model 140D (Heat System Co., New York) with an output control setting of 5. In some experiments ROS were sonicated in deionized water or in 0.067 M potassium phosphate buffer, pH 6.5. ROS were sonicated for 120 sec and centrifuged at 80,000 × g for 60 min. The supernatant (ca. 3 ml, A_{498} = 0.2) was placed on a 1.8 × 57 cm column of 200–400 mesh Agarose (Bio-Gel A-5 m; Bio-Rad Lab., Richmond, Calif.) and eluted with sonication medium at a rate of 10 ml/hr at 3°C. Approximately 2 ml was collected for each fraction; those containing rhodopsin were pooled and designated Ps. Purified rhodopsin was prepared by chromatography on calcium phosphate as described previously (12) using Emulphogene BC 720 (GAF, New York) as extractant.

Spectral Measurements

Absorption spectra were recorded at 20°C with a Cary 14 spectrophotometer. CD spectra were recorded at 27°C using a Cary 61 spectropolarimeter which had been previously calibrated at 290 nm with d-10-camphosulfonic acid as described in the Cary 61 manual. The scanning speed was 30 nm/min in the visible wavelength region. At this speed, bleaching of rhodopsin after each run was less than 3%. For recordings in the far UV, the scanning speed was set at 7.5 nm/min. The molecular ellipticity θ was calculated from $\theta = 100\ \psi / l\ M$ where ψ is observed ellipticity; M is the concentration (moles/liter); l is light path (in cm). The molecular weight of rhodopsin was assumed to be 30,000 and a mean residue molecular weight of 128 was used (12).

Protein and Lipid Analyses

Protein was determined with Folin reagent (13). Lipids were removed from the lyophilized material by two extractions with 10 volumes of chloroform-methanol (2:1, v/v). After evaporation of the solvent, the phospholipid content of the residue was determined by phosphorus analysis (14). Phosphatidyl-L-serine and phosphatidylethanolamine were purchased from Applied Science (State College, Pa.).

Thermal Bleaching of Rhodopsin

Aliquots (2 ml) of sample (A_{498} = 0.6–0.8) were subjected to thermal treatment at a given temperature for a specific time, cooled rapidly to room temperature and mixed with 0.1 ml of 0.5 M NH_2OH (pH 6.0). Rhodopsin concentrations before and after heat treatment were determined from A_{498} using $\epsilon M_{,\,498} = 40 \times 10^3$. The first order velocity constant (k) for thermal bleaching reaction was caluclated from the equation:

$$k = \frac{2.303}{t_2\ t_1} \log \frac{\triangle A_1}{\triangle A_2}$$ in which $\triangle A_1$ and $\triangle A_2$ are optical absorbances at 498 nm at times t_1 and t_2.

Synthesis of Rhodopsin and Isorhodopsin

ROS (from 50 retinas) suspended in 20 ml of 0.067 M potassium phosphate, pH 6.5 were mixed with 10 ml of 0.5 M NH_2OH (pH 6.5) in an ice bath and allowed to bleach under room light (irradiation with six 40-watt cool lamps from a distance of 60 cm) for 60 min and centrifuged. After washing, the final pellet was subjected to sonic disruption to prepare retinal-free Ps. To synthesize isorhodopsin, retinal-free Ps were incubated with 9-cis retinal (o.1 mmole retinal/mg protein; Sigma) in the dark for 1 hr. Rhodopsin was regenerated by mixing with all-trans retinal in ethanol (0.1 mmole retinal/mg protein; Sigma), subsequently exposing the mixture to room light for 1 hr and incubating it in the dark for 1 hr. Pigment formation was stopped by shaking the mixture with a buffered solution of NH_2OH (1 mmole per 1 μmole rhodopsin).

Electron Microscopy

The sonicated retinal disc membranes were negatively stained in 2% sodium phosphotungstate, pH 6.4, and examined and photographed in a Siemens 101 electron microscope using 80 KV accelerating voltage and a 50 μm objective aperture.

RESULTS

Sonic Disruption of Disc Membranes

The concentration of rod particles nonsedimentable at 80,000 X g reaches a maximum

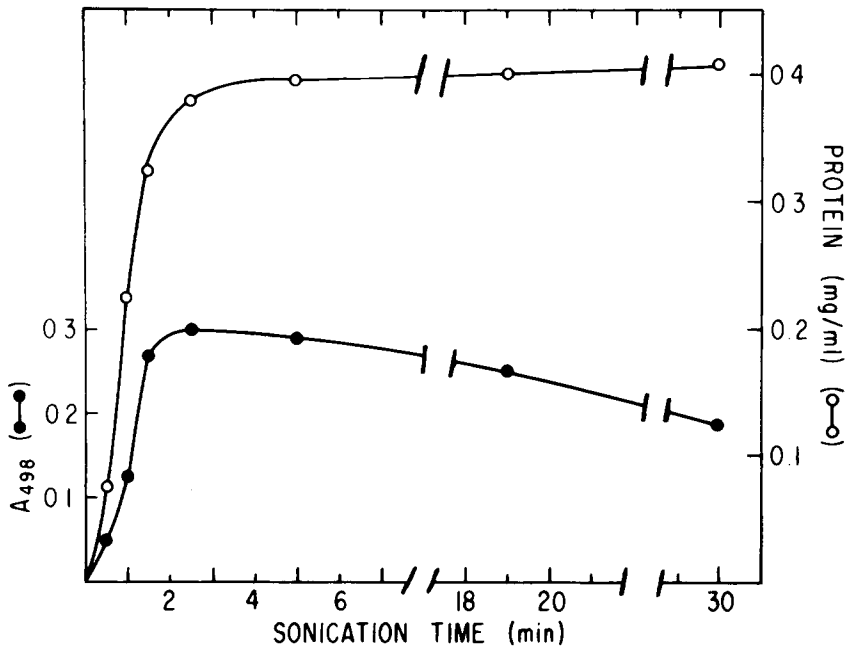

Fig. 1. Dependence of the formation of rhodopsin-containing particles on sonication time. After sonication at 20 KC for given times, the suspension was centrifuged at 80,000 × g for 60 min and the A_{498} and protein concentration of the supernatant were determined.

at 2–3 min of sonication. After this time the chromophore of rhodopsin decomposes gradually (Fig. 1). The effects of sonication on CD properties of rod membranes are illustrated in Fig. 2. Unsonicated membrane fragments show negative CD bands at around 212 nm and 225 nm which remain unchanged upon bleaching of the pigment by light. Sonic disruption of the membrane is accompanied by an increase in ellipticity of the CD bands and a shift in the CD maxima to 209 nm and 221 nm, respectively. The increase of ellipticity with sonication is paralleled by a decrease in light scattering as determined by measuring the absorbance at 620 nm (Table I). Similar results are obtained when the detergent Emulphogene is added to the initial unsonicated suspension. The magnitude of the 221 nm CD band obtained after extensive sonication is essentially identical with that observed after clarification of the suspension with Emulphogene. Thus, the sonically dispersed suspension is comparable in optical transparency to the detergent-solubilized preparations but different from the latter in its insensitivity to light-induced conformational change (Fig. 2). It is noted that, compared to the 221 band (CD change on sonication: $-0.078°$ to $-0.120°$), the intensity increase of the 209 nm band concurrent with sonication is less marked (CD change on sonication: $-0.050°$ to $-0.068°$). Therefore, the 221 nm band should be more reliable than the 209 nm band for estimation of helical content of opsin associated with Ps. Similar observations were made on these bands of other membrane systems (15). As shown in Fig. 3, the CD bands at 340 and 495 nm of unsonciated suspensions are less intense than those of sonicated suspensions. Since rhodopsin concentrations in these preparations, as measured from difference (unbleached vs. bleached) spectra in Emulphogene, are identical, the low band intensities must be related to light scattering or turbidity of the solution. In nonsonicated suspensions, the 340 nm band has a higher intensity than the 495 nm band. With a reduction in particle size,

TABLE I. The Effect of Light Scattering on the Circular Dichroism Bands of Various Rhodopsin Preparations

	Light scattering[a] (%)	θ_{221}	θ_{340}	θ_{495}	$\theta_{495/340}$
Not sonicated	100	−7,300	35,500	33,300	0.938
Sonicated in water	60	−10,500	47,400	51,700	1.090
Sonicated and centrifuged (15,000 × g, 10 min)	43	−12,000	52,800	63,100	1.195
Sonicated and centrifuged (50,000 × g, 40 min)	12	−13,800	55,200	65,400	1.185
Ps; sonicated, centrifuged (80,000 × g, 60 min) and purified on Agarose	0	−14,000	57,200	66,700	1.166
Rhodopsin solubilized in 2% digitonin (pH 6.5)	0	−14,200	62,000	42,000	0.677
Rhodopsin purified in 1% Emulphogene (pH 6.5)	0	−14,300	61,000	26,500	0.434

[a]Measured from the OD at 620 nm. Light scattering of unsonicated suspension taken as 100%. Light scattering referred to solutions with the same amount of rhodopsin.

the relationship is reversed (Fig. 3 and Table I). The intensity difference of the 340 nm band is small between Ps and purified rhodopsin in Emulphogene, while the intensity of 495 nm band is much higher in rhodopsin associated with Ps. Since light scattering is not prominent in these preparations, the intensity loss of 495 nm band is probably attributable to the direct effect of detergent on the chromophore.

Purification of the Sonically Dispersed Membrane Particles

The Ps are stable for at least three days at 3°C in deionized water or 0.067 M potassium phosphate, pH 6.5, but they gradually aggregate and precipitate on prolonged standing at 3°C or freezing. While Na^+ and K^+ at 10^{-2} M show little effect on their dispersion, the addition of Mg^{++} (10^{-2} M) and Ca^{++} (10^{-3} M) results in aggregation and the CD spectrum of the aggregates shows a red shift and a decrease in band intensity at 222 nm. Since the sonicated particles are found to be stable in EGTA (10^{-2} M, pH 6.5), the rod outer segments are sonicated in aqueous EGTA and the Ps are eluted from the Bio-Gel column in the same solution. The rhodopsin containing fraction appears near the end of the void volume, followed by a colorless protein peak (Fig. 4). Similar elution patterns are obtained for Ps sonicated in deionized water or in 0.067 M phosphate buffer, and for Ps prepared from partially delipidated ROS. The second protein peak does not reconstitute rhodopsin or isorhodopsin when mixed with suitable retinal isomers and is not opsin.

The rhodopsin containing fraction is shown by electron microscopy to be composed entirely of smooth walled, apparently empty, vesicles (Fig. 5). The size of the vesicles ranges from 25–300 nm in diameter with an average diameter of approximately 112 nm. Prolonging the sonication time from 2 to 20 minutes does not appreciably increase the population of smaller particles. No morphologic difference is detected in vesicles prepared from unbleached and bleached outer segments.

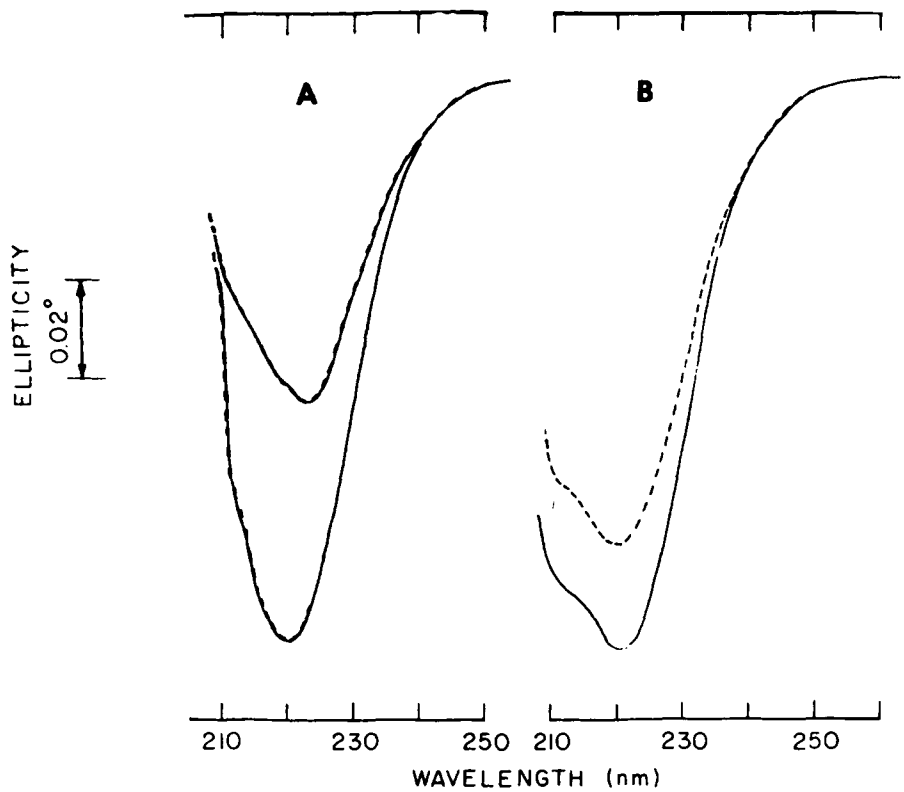

Fig. 2. Effect of sonication on the UV circular dichroism spectra of rod membranes. The upper curves in (A) are for unsonicated ROS; unbleached (——) and bleached (- - - - -) ROS. In the course of sonication, the band intensity increases progressively to a constant level (lower curves). The solid and dotted lines are for unbleached and bleached particles, respectively. The solid line in (B) was obtained after addition of Emulphogene to the unsonicated membrane suspension. After bleaching, the dotted curve was recorded. Rhodopsin concentration = 3.3×10^{-5} M. $l = 0.2$ cm.

Comparison of Ps with Unsonicated Membrane Fragments

The thermal stabilities and the ratios of phospholipid to rhodopsin of the Ps and ROS membranes are very similar but purified rhodopsin, with only 15 moles of phospholipid per mole of rhodopsin, is much less stable to heat than the membrane-bound rhodopsin (Table II). The molecular ellipticity of the Ps measured at 221 nm is found to be $-(1.44 \pm 0.04) \times 10^4$. The helical content of the Ps protein (largely rhodopsin) is estimated as 47%, with the assumption that $\theta_{221} = -3.04 \times 10^4$ for a perfect helix (16). Provided that sonication does not have a direct effect upon opsin conformation, this value may be taken as an approximate estimate of the helical content of rhodopsin associated with intact rod membranes. Purified rhodopsin was previously reported (12) to have a somewhat higher helical content (50–60%). In preliminary experiments, the infrared spectra of Ps in a dry film show a shoulder at 1630 cm^{-1} (amide I band), suggesting the presence of β-structure in the Ps protein.

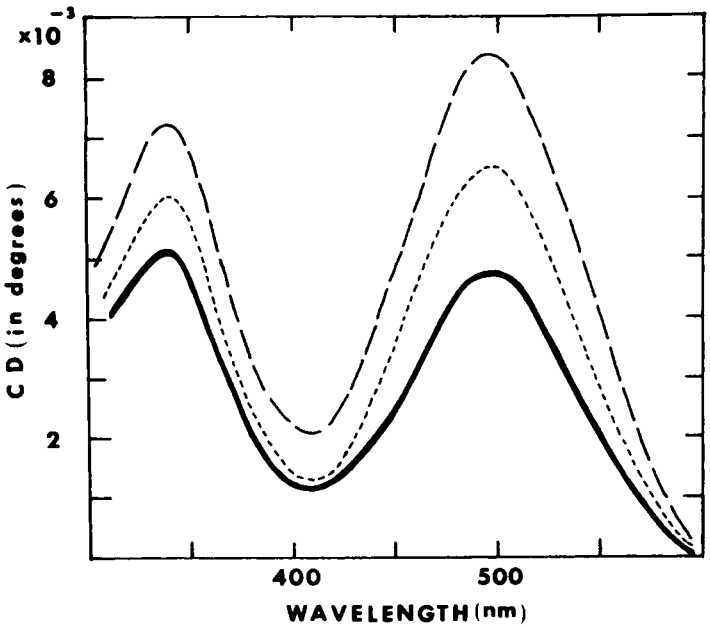

Fig. 3. Effect of sonication on the visible circular dichroism spectra of rod membranes. Suspension prepared by gentle homogenization of rods (——); suspension sonicated at 20 KC for 120 sec (– – – – –); sonicated suspension centrifuged at 80,000 × g for 60 min and purified on Agarose (– – –). Rhodopsin concentration = 6.3×10^{-5} M. l = 0.2 cm.

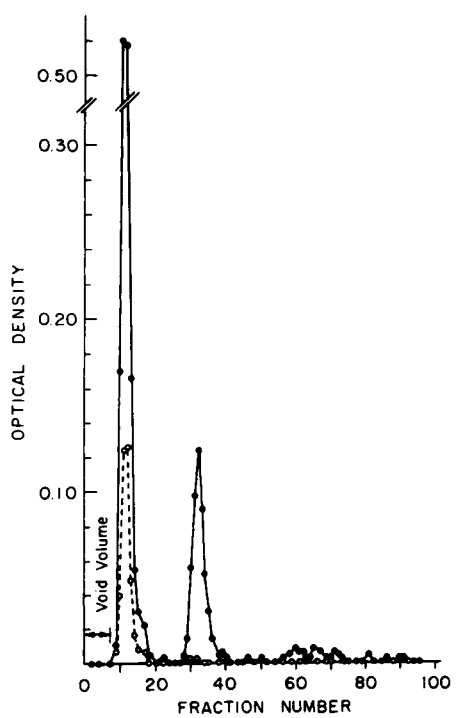

Fig. 4. Purification of sonicated particles on Agarose. The solid and dotted lines are the absorbances at 280 nm and 498 nm, respectively.

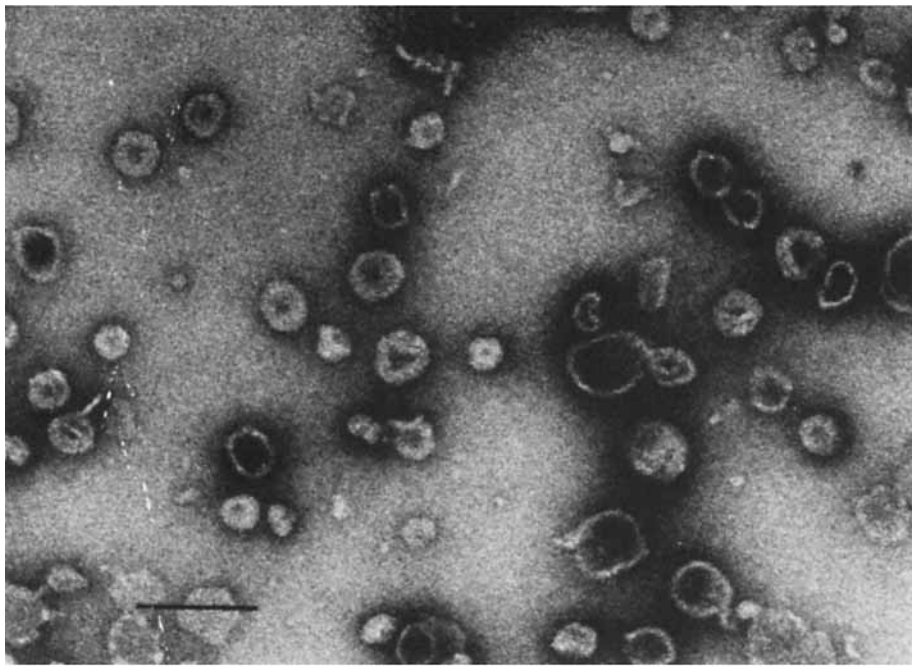

Fig. 5. Electron micrograph of particles resulting from the sonic disruption of retinal rod outer segment. The particles were eluted from Agarose column and suspended in 10^{-2} M EGTA. Droplets were fixed on copper grid in osmium tetroxide vapor for 3 min and negatively stained in 2% sodium phosphotungstate. 160,000 ×. Bar length is 100 nm.

TABLE II. Thermal Bleaching and Phospholipid Content

	Molar ratio of phospholipid to rhodopsin (mean ± s. d.)	First-order rate constant of bleaching reaction (sec^{-1})
ROS membrane (in water)	110 ± 10	k (at 62°) = 8.3×10^{-4}
Sonic particles (in water)	120 ± 10	k (at 62°) = 10.4×10^{-4}
Sonic particles from partially delipidated ROS (in water)[a]	80 ± 10	k (at 62°) = 23.7×10^{-4}
Purified rhodopsin (in water)	15 ± 5	k (at 47°) = 16.2×10^{-4}

[a]Lyophilized ROS which had been extracted twice with n-hexane were sonicated in water.

To test whether Ps can bind retinal, bleached Ps are incubated with 9-cis retinal, or irradiated in the presence of all-trans retinal and incubated. In each experiment, isorhodopsin or rhodopsin is formed, respectively (Fig. 6). The result indicates that Ps, like intact membranes, are capable of binding and photoisomerizing retinal for rhodopsin synthesis.

DISCUSSION

Rod outer segment membranes show the same anomalous CD features which have been demonstrated in other membrane systems (1, 2, 5–7, 17). The CD distortions are

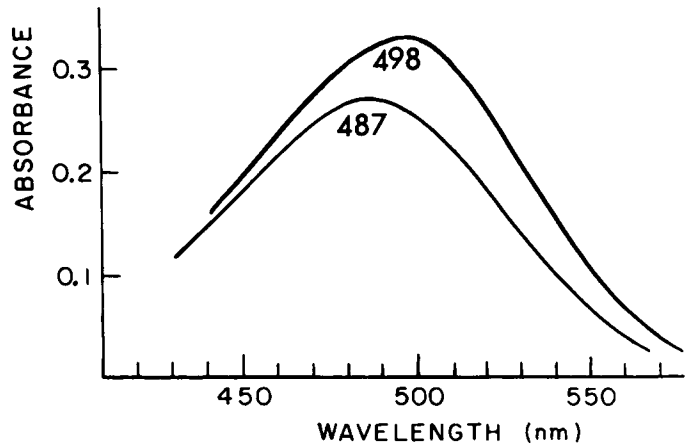

Fig 6. Absorption spectra of reconstituted pigments. Difference (unbleached + NH_2 OH vs. bleached + NH_2OH) spectra were recorded at 20°. The upper curve (λ_{max} =498 nm) is rhodopsin and the lower curve (λ_{max} = 487 nm) isorhodopsin. The molar extinction coefficients for reconstituted pigments were 40.3 X 10^3 for rhodopsin (at 498 nm) and 42.5 X 10^3 for isorhodopsin (at 487 nm).

observed not only in the UV but also in the visible wavelength region. Whether it is the negative ellipticity in the UV or the positive ellipticity in the visible wavelength region, the major anomaly in the CD spectrum of a turbid ROS suspension is a low band intensity. A decrease in the turbidity of ROS suspensions after sonication results in a progressive increase in the intensity of the CD band, thus the increase of the molecular ellipticities at 340 and 495 nm is directly related to a reduction in particle size. From these results, we conclude that the CD distortions in the UV as well as visible wavelength region can be attributed to optical artifacts caused by light scattering.

In order to avoid the optical artifacts, it is important to test the feasibility of using membrane suspensions for accurate measurements. The present results show that this can be done by comparing the magnitude of molecular ellipticities at 221 nm and 340 nm before and after the addition of detergent to the suspensions. Smaller changes in the molecular ellipticity at these wavelengths indicate better quality samples for CD measurements. The detergent effect on the 221 nm band may be useful for assessment of the adequacy of other membrane suspensions for CD measurements. It is noted that membrane-bound rhodopsin shows a higher value of θ_{495} than detergent-solubilized rhodopsin, as observed previously (3). Since the magnitude of θ_{340} remains virtually constant between the preparations, this anomaly cannot be accounted for by light scattering. It is more likely to be due to the specific effect of detergent on the structural asymmetry of the chromophore. The magnitude of θ_{495} is affected even by such mild detergent as digitonin. This cautions us not to neglect the detergent effect in estimating such parameters as the rotatory strength from the CD spectra of detergent extracts.

Phospholipid vesicles, liposomes, have been prepared by ultrasonic treatment or swelling of phospholipid or a mixture of phospholipids and used as lipid bilayer models for biological membranes (18, 19, 20). These vesicles are useful models in that the phospholipids and the additional components to be incorporated into the vesicles can be clearly defined. On the other hand, a question can be raised as to whether the artificial vesicles accommodate every component required for characteristic membrane functions.

In contrast, membrane vesicles derived from biological membranes may not be well defined chemically but are likely to contain known, as well as unknown, components which are necessary for functions of intact membranes. Indeed, despite the drastic conditions of membrane disruption, rod particles still retain the physiological integrity of rod membranes and exhibit such properties as conformational stability of opsin to photobleaching of the chromophore, thermal stability, and rhodopsin regenerability. For these reasons, we believe that the membrane vesicles prepared by sonic disruption could serve as useful material for studies on structure–function aspects of rod membranes which involve various optical measurements.

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