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Circular Dichroism of Native and Illuminated Bovine Visual Pigment$_{500}$ at 77°K in the 620- to 320-nm Region*

Joseph Horwitz† and Joram Heller

ABSTRACT: Absorption and circular dichroism spectra of bovine digitonin- and cetyltrimethylammonium bromide (CTAB)–visual pigment$_{500}$ complexes were measured at 77°K in glycerol–water mixtures in the 620- to 320-nm range. Upon cooling from 298°K, the absorption maxima shifted to the red and increased by a factor of 1.15, while the area under the absorption peak increased by 16% in the digitonin and by 5% in the CTAB–pigment. The small change in absorption was attributed mostly to the effect of volume changes upon cooling. Cooling of native CTAB–visual pigment from 298 to 77°K resulted in the disappearance of the circular dichroism positive peak at 490 nm. The positive peak at 340 nm was unchanged. Due to poor glass and accompanying uncertainty in the base line of digitonin–visual pigment at 77°K it was impossible to ascertain whether the circular dichroism band at 490 nm disappeared upon cooling.

Illumination at 500 nm of either CTAB–or digitonin–visual pigment at 77°K produced a new circular dichroism spectrum with a positive peak at approximately 560 nm and a negative peak at approximately 480 nm. The circular dichroic band at 340 nm was only minimally affected by illumination. This new spectrum, which had very large circular dichroism signals compared to the native pigment signals, was called state I. Continuous illumination of state I progressively increased the circular dichroism signals to a maximum after which the spectrum changed. When the illuminated visual pigment was kept in the dark the circular dichroism spectrum of state I was unchanged after 30 min. The circular dichroism spectrum of state I could be produced, at different rates, by illumination at any wavelength between approximately 400 and 560 nm. State I was always the first circular dichroic spectrum produced by illuminating visual pigment at 77°K. Illumination of state I at 560 nm or longer illumination at 500 nm produced a different circular dichroism spectrum. This spectrum had a negative peak at approximately 540 nm and a positive peak at approximately 460 nm. The peak at 340 nm was only minimally changed. This circular dichroism spectrum was called state II. When the illuminated visual pigment was kept in the dark at 77°K the spectrum of state II was unchanged after 30 min. The circular dichroism spectra of states I and II could be reversibly transformed, one into the other, by proper illumination. States I and II had an isosbestic point at approximately 512 to 518 nm, depending on the concentration of glycerol in the medium. The circular dichroism spectrum of native digitonin–or CTAB–visual pigment did not pass through this isosbestic point, and once illuminated, the circular dichroism spectra of both states I and II were always different from that of native pigment. No illumination conditions were found that could produce the native circular dichroism spectrum from either states I or II. From these experiments it was concluded that illumination of native visual pigment at 77°K produces two new states which exhibit very large circular dichroism signals. These states are always different from the native visual pigment, are interconvertible by illumination, and cannot be made to regenerate native visual pigment by illumination.

When native bovine visual pigment$_{500}$ is illuminated it undergoes a series of transformations which result, ultimately, in the dissociation of the chromophore, as *all-trans*-retinal, from the apoprotein. Several spectroscopically defined intermediates in this process have been recognized (for reviews, see Wald, 1968, Abrahamson and Ostroy, 1967, and Morton and Pitt, 1969). These intermediates of illuminated visual pigment, which are unstable at room temperature, were all found by low-temperature absorption spectroscopy. Despite a great amount of research in this field, little information is available about the structure of these intermediates. For instance, it is not known whether the various spectroscopic intermediates reflect changes in the structure or conformation of the chromophore, of the apoprotein, or both. This paucity of structural information stems from the difficulty of relating absorption spectroscopy data to molecular architecture in the absence of a model system for correlating the visual pigment spectrum with its structure.

Since circular dichroism is an expression of molecular geometry, it can yield additional structural information not obtainable from absorption spectroscopy. Several studies on the circular dichroism spectra of visual pigment$_{500}$ have been reported (Crescitelli *et al.*, 1966; Takezaki and Kito, 1967; Kito *et al.*, 1968; Shichi *et al.*, 1969; Shichi, 1970). These studies show that native bovine, frog, and squid visual pigments, when measured at room temperature,

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have circular dichroic bands with peaks at approximately 490 and 340 nm. Illumination at room temperature results in the disappearance of the circular dichroism bands. The development of instruments capable of recording circular dichroism spectra at low temperatures (Horwitz *et al.*, 1969; Strickland *et al.*, 1969) coupled with the computer-assisted analysis and recording of the spectra (Horwitz *et al.*, 1968) led us to investigate the circular dichroism spectra of native and illuminated visual pigment$_{500}$ at 77°K. Previously reported results on the absorption spectra of visual pigment$_{500}$ at 77°K were obtained exclusively with the digitonin–visual pigment complex (Yoshizawa and Wald, 1963). To facilitate comparisons between our circular dichroism data and the previous work on the absorption spectra, we report results obtained with digitonin–visual pigment in addition to results obtained with purified CTAB[1]–visual pigment (Heller, 1968).

Materials and Methods

Bovine rod outer segments were isolated by flotation in 1.02 M sucrose as previously described (Heller, 1968). The purified rod outer segments were kept frozen until used. Rod outer segments were solubilized with various concentrations of digitonin or CTAB in 0.066 M sodium phosphate buffer (pH 7.0). CTAB–visual pigment was also purified by gel filtration chromatography as previously described (Heller, 1968). Results obtained with CTAB–visual pigment extracts and with pigment purified by gel filtration were identical. Digitonin–visual pigment solutions were obtained by solubilizing rod outer segments in 2% digitonin in 0.066 M sodium phosphate buffer (pH 7.0). The digitonin–visual pigment complex was concentrated by centrifugation in swinging-bucket rotor-type SW50L, in the Beckman L2-65 centrifuge at 40,000 rpm (175,000*g* maximum) for 12 hr at 4°. The digitonin–visual pigment obtained as a precipitate was dissolved in a small volume of buffer. The visual pigment preparations used in this work were mostly freshly prepared and in no case older than 1 week.

Absorption spectra were measured with the Cary 15. In some cases the position of the absorption maximum of the illuminated visual pigment was determined by monitoring the dynode voltage of the circular dichroism spectrophotometer simultaneously with the recording of the circular dichroism spectrum. This was achieved by using half of the computer memory to record the circular dichroism spectrum, leaving the other half for a record of the dynode voltage. Although absorption is proportional to the logarithm of the dynode voltage (Hariharan and Bhalla, 1956) it was possible to determine the position of the absorption maximum to within ±3 nm when checked with a Hg source by a direct measurement of the voltage.

Circular dichroism spectra were recorded on a modified Beckman circular dichroism spectrophotometer (Horwitz *et al.*, 1969; Strickland *et al.*, 1969) using a computer of average transients (Horwitz *et al.*, 1968). The circular dichroism spectrophotometer used a 75-W xenon lamp. Spectra were scanned at 3 nm/sec using a 0.1-sec time constant.

Samples were illuminated at a particular wavelength for various times in the circular dichroism spectrophotometer. The illuminating light was the circular polarized light used for normal circular dichroism measurements. Control experiments showed that linearly polarized light and unpolarized light gave the same results. This system of illumination enabled us to follow directly the kinetics of changes in the circular dichroism signal during illumination.

Sample Preparation. The nature of glasses produced by freezing mixtures of glycerol–visual pigment at 77°K had a profound influence on the various spectroscopic measurements. This is especially true for circular dichroism spectra where poor glasses result in drastic changes in the base line and great modification of the signal (Horwitz *et al.*, 1969; Strickland *et al.*, 1969). Glasses produced by freezing mixtures of glycerol–digitonin–visual pigment (1:1) showed multiple cracks. Raising the glycerol concentration to 66% somewhat improved the quality of the glass. Due to the multiple cracks in the glass, the light path used with digitonin samples was limited to 0.5 mm or less where the cracks were parallel to the light path and had no effect on the circular dichroism (Horwitz *et al.*, 1969; Strickland *et al.*, 1969). CTAB–visual pigment, on the other hand, yielded excellent glasses, showing one or very few cracks which were parallel to the light path. Moreover, we used mostly CTAB glasses that were completely free of any cracks in the light path. The glycerol concentration could be reduced to 40% and the light path increased to 1 mm. In the present study we have used only glasses (digitonin or CTAB) that produced no or only a very small offset in the base line in either the absorption or in the circular dichroism spectra when cooled from room temperature to 77°K (Strickland *et al.*, 1969). Samples, mixed with various proportions of glycerol, were cooled from 298 to 77°K within approximately 20 sec by plunging the cell and cell holder into liquid nitrogen held in the spectroscopic dewar (Horwitz *et al.*, 1969; Strickland *et al.*, 1969).

Because the circular dichroism spectrophotometer employs relatively strong illumination for its normal scanning, several precautions were taken to ensure that the scanning beam did not affect the photosensitive pigment. A relatively fast scanning speed was employed (3 nm/sec) and the slit was made as narrow as possible. Moreover, we have checked the effect of successive scanning on the circular dichroism spectrum. Some states of illuminated visual pigment were observed to be more sensitive to repetitive scanning than others. While it was possible to scan some states more than ten times without noticeable change, in other cases even one or two scans were enough to modify the circular dichroism spectrum.

The products formed by illuminating visual pigment at 77°K were found to be sensitive to the dim red light used in visual pigment work (Kodak, Wratten series 2 filter). As a result, once the sample was illuminated at 77°K, it was not exposed to the red light.

Results

Absorption Spectra. When solutions of digitonin–visual pigment with glycerol were cooled from 298 to 77°K, the absorption band was sharpened and the peak position shifted from approximately 500 nm to approximately 505 nm (Figure 1). These results were similar to the previously published data by Yoshizawa and Wald (1963). On the other hand, the peak height at 77°K (505 nm) was only 1.15 times the peak height at 298°K (500 nm). The area under the absorption band (620–400 nm) at 77°K was 1.16 times the area at 298°K. Yoshizawa and Wald (1963) found that the rise in the absorption peak upon cooling was 1.7 ± 0.11 or 1.58 when corrected for the volume contraction. In more recent measurements however, Yoshizawa (T. Yoshizawa and G. Wald, personal communication, 1970) has found

[1] Abbreviation used is: CTAB, cetyltrimethylammonium bromide.

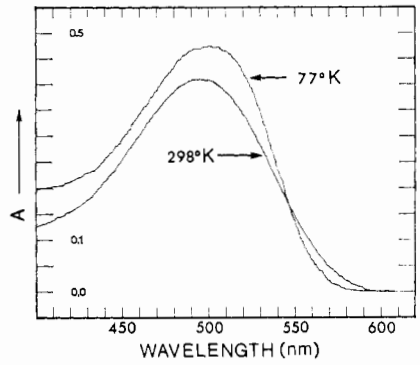

FIGURE 1: Absorption spectra of digitonin–bovine visual pigment$_{500}$. Visual pigment in 2% digitonin–0.066 M sodium phosphate buffer (pH 7.0) was adjusted to 55% glycerol and the spectrum recorded. The cuvet was then plunged into liquid nitrogen and the spectrum recorded again. Light path was 0.5 mm.

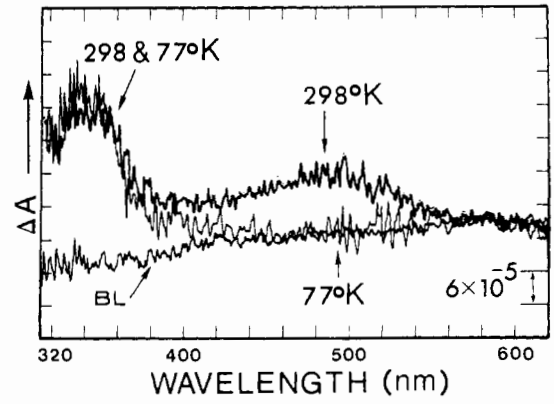

FIGURE 3: Circular dichroism spectra of native CTAB–visual pigment at 298 and 77°K. The circular dichroism spectrum of visual pigment in 0.07 M (final) CTAB–43% glycerol was recorded at 298°K after four scans. The mixture was then cooled to 77°K and recorded after four scans. Base line (BL) was recorded at 298 and 77°K with solvent only. Base lines at 298 and 77°K were identical. Light path, 1 mm. A_{500} (final), 0.575. Note the increase in noise over the absorption peaks.

that the increase in absorptivity upon cooling is a factor of only 1.14 (bovine visual pigment in 2:1 glycerol–digitonin mixtures). These investigators ascribe the previously high figure to multiple reflections in the glass caused by the many cracks which increase the light path.

The peak position at 298°K and at 77°K was dependent upon the relative amount of glycerol in the solution. Increasing the concentration of glycerol from 50 to 66% shifted the peak at 298°K from approximately 500 to 494 nm, while the peak at 77°K shifted from approximately 505 to 507 nm.

When CTAB–visual pigment was cooled from 298 to 77°K the change in the absorption spectrum was similar to those observed with digitonin–visual pigment (Figure 2). The peak at 298°K was at 500 nm, and it shifted to 505 nm at 77°K. The peak height increased by a factor of 1.15, while the area under the absorption band (620–400 nm) increased by a factor of 1.05.

Cooling the samples from 298 to 77°K causes a volume contraction which increases the concentration. Since neither the absolute nor relative magnitude of this contraction was known, it was difficult to judge the significance of the changes in peak height and area under the absorption band. It is probable that these changes are due to the effect of volume changes caused by the cooling and that cooling *per se* has no effect on digitonin and CTAB–visual pigment absorption spectra. The slight increase in absorption maximum and area under the absorption peak seen here was within the range observed with many proteins upon cooling (Strickland *et al.*, 1969; Horwitz *et al.*, 1970; E. H. Strickland and J. Horwitz, unpublished results).

Circular Dichroism Spectrum of CTAB–Visual Pigment at 298°K. At room temperature, native CTAB–visual pigment had two positive circular dichroism bands with peaks at approximately 490 and 340 nm (Figure 3). The peak height of the 340-nm band was about twice that at 490 nm. Illumination at 500 nm resulted in gradual decrease and ultimate disappearance of both peaks.

Circular Dichroism Spectra of Native and Illuminated CTAB–Visual Pigment at 77°K. Lowering the temperature of a solution of native CTAB–visual pigment from 298 to 77°K resulted in a marked change in the circular dichroism spectrum. The broad positive peak at 490 nm disappeared, the spectrum in this region having returned to base line (Figure 3). On the other hand, the positive peak at 340 nm did not seem to be affected by the cooling (Figure 3). The circular dichroism band at 490 nm reappeared when the sample was warmed from 77 to 298°K.

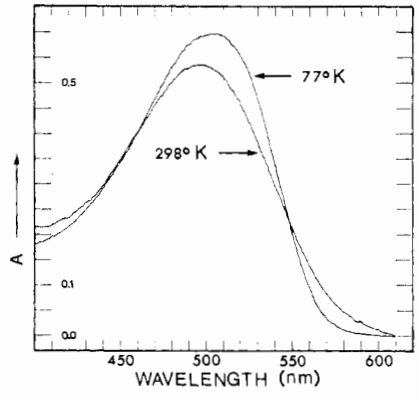

FIGURE 2: Absorption spectra of CTAB–bovine visual pigment$_{500}$. Visual pigment in 0.14 M CTAB–0.066 M sodium phosphate buffer (pH 7.0) was mixed with an equal volume of glycerol and the spectrum recorded. The cuvet was then plunged into liquid nitrogen and the spectrum recorded again. Light path was 1 mm.

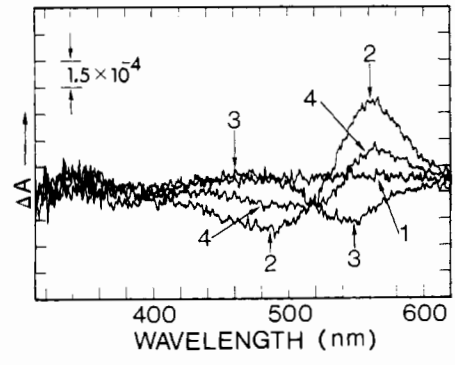

FIGURE 4: States I and II of CTAB–visual pigment. Visual pigment in 0.07 M (final) CTAB–50% glycerol at 77°K. Curve 1, native pigment; curve 2, after illumination at 500 nm (0.4-mm slit) for 30 sec; curve 3, after illumination at 560 nm (0.4-mm slit) for 100 sec; curve 4, after further illumination at 500 nm (0.4-mm slit) for 30 sec. Light path, 0.5 mm, A_{500} (final) 0.28.

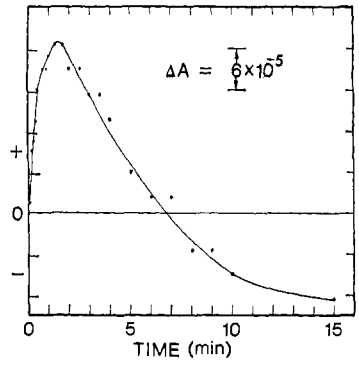

FIGURE 5: Kinetics of development and decay of the positive circular dichroism peak at 560 nm as a result of illuminating native visual pigment at 500 nm. Visual pigment in 0.04 M CTAB–0.066 M sodium phosphate buffer (pH 7.0), in 44% glycerol at 77°K was illuminated with 500-nm light (half-bandwidth approximately 4 mm) and the circular dichroism spectra were recorded at 560 nm. The cell had a 1-mm light path, A_{500} (final) 0.142. Results are expressed as change of circular dichroism signal at 560 nm relative to native visual pigment signal at this wavelength.

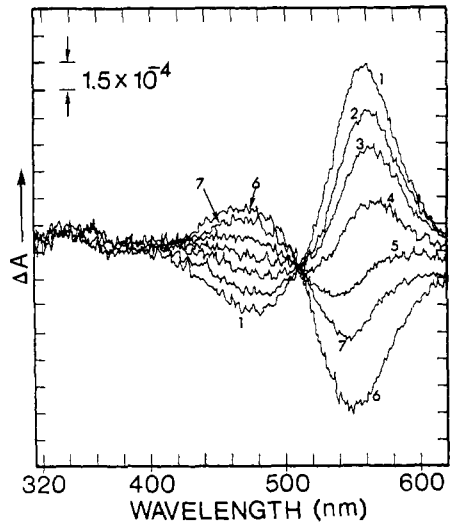

FIGURE 6: States I and II of CTAB–visual pigment. Visual pigment in 0.14 M CTAB (final)–50% glycerol at 77°K. Illumination was performed with 0.2-mm slit width and scanning with 0.05-mm slit width. Curve 1, after 34-min illumination at 400 nm; curve 2, additional 1.5-min illumination at 500 nm; curve 3, additional 1.5-min illumination at 500 nm; curve 4, additional 3-min illumination at 500 nm; curve 5, additional 3-min illumination at 500 nm; curve 6, additional 1.5-min illumination at 560 nm; curve 7, additional 10-min illumination at 560 nm. Light path, 1 mm; A_{500} (final), 0.22.

When native CTAB–visual pigment (at 77°K) was illuminated with monochromatic light at 500 nm (approximately 4-mm half-bandwidth), a greatly different circular dichroism spectrum was produced. The new circular dichroism spectrum had a large positive band with a peak at 560 nm and a smaller negative band peaking at 480 nm (Figure 4). The positive peak at 340 nm which was present in the native pigment was affected very little, if at all, by illumination. For descriptive purposes this circular dichroism spectrum will hence be referred to as illuminated visual pigment (77°K) state I, or state I for short. Continuing illumination at 500 nm led to the progressive increase of state I with both circular dichroism peaks always at the same wavelength (560 and 480 nm). The kinetics of development of state I as a function of illumination is shown in Figure 5. The 560 nm circular dichroism peak height of state I increased linearily with time of illumination up to 30 sec, when 73% of the maximal signal had developed, and then peaked at about 90 sec. Further illumination at 500 nm led to the progressive decay of state I. The kinetics of state I described here are only qualitative since the time course depends on factors such as the intensity and spectral purity of the illumination, the nature and concentration of the detergent, pH, and other factors.

After about 10–15-min illumination at 500 nm the circular dichroism spectrum of state I was replaced by a different spectrum. The positive peak at 560 nm and the negative peak at 480 nm of state I were replaced by a negative peak at 540 nm and a positive peak at approximately 460 nm (Figures 4 and 6). This circular dichroism spectrum will be referred to as illuminated visual pigment (77°K) state II, or state II for short. State I and state II had an isosbestic point at approximately 518 nm (Figure 4) or approximately 512 nm (Figure 6) showing that the circular dichroism spectra of state I and state II represent only two chemical species. The exact position of the isosbestic point was dependent on the percent glycerol in the medium. This is understandable in light of the fact that the position of the absorption maxima was also dependent on the per cent glycerol.

Although illumination at 500 nm could produce both the circular dichroism spectra of state I and its transformation into state II, a much more efficient way of producing state II from state I was by illumination of state I at 560 nm. Although state II could be produced from state I by illuminating with light of longer wavelength (up to approximately 620 nm), this mode of generating state II was less efficient (slower) than illumination at 560 nm. The decay of the positive circular dichroism band at 560 nm (state I) upon illumination at 560 nm and the build-up of state II could be followed in the spectrophotometer (Figure 7). Thus illumination of native visual pigment (77°K) at 500 nm rapidly produced the circular dichroism spectrum of state I, and illumination of state I at 560 nm rapidly produced state II. The circular dichroism spectrum of state I could then be regenerated rapidly by illumination at approximately 440 nm. States I and II passed smoothly into each other with a clear isosbestic point (Figures 4 and 6). A mixture of states I and II can be produced (Figure 6) by a short illumination at either 560 or 440 nm (starting with state I or II, respectively). As can be seen from Figure 6 the mixture of states I and II have their respective positive and negative circular dichroism peaks

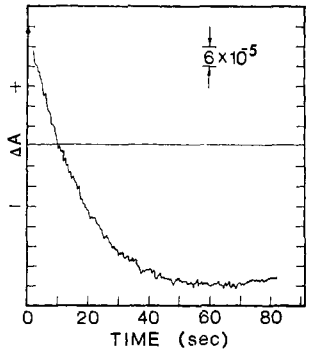

FIGURE 7: Instrument trace of the transformation of the positive circular dichroism peak at 560 nm of state I upon illumination at 560 nm. Visual pigment in 0.07 M CTAB (final)–50% glycerol, 1-mm light path, A_{500} (final), 0.31. State I was illuminated at 560 nm with a slit width of 0.5 mm (half-bandwidth 15 nm).

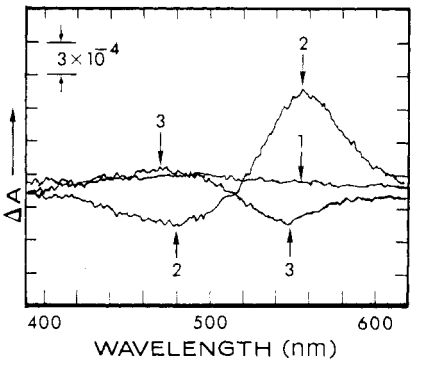

FIGURE 8: Circular dichroism spectra of digitonin–visual pigment. Visual pigment in 0.7% digitonin (final)–66% glycerol at 77°K. Curve 1, native pigment; curve 2, after 1-min illumination at 500 nm (0.4-mm slit width); curve 3, after additional 100-sec illumination at 560 nm (0.4-mm slit width). Light path 0.5 mm A_{500} (final) 0.345.

displaced—yet they always pass into each other through the same isosbestic point.

Under appropriate illumination conditions, states I and II could be produced in such a way that their spectra almost canceled each other, resulting in a flattened circular dichroism spectrum in some respects similar to the original native one. Yet, the original native circular dichroism spectrum was not produced again after the initial illumination (which produced state I), and the mixtures of states I and II were always different from the native spectrum. The native circular dichroism spectrum (at 77°K) did not pass through the isosbestic point of states I and II (Figure 4). Thus the flattened circular dichroism signal produced by mixtures of states I and II seemed to be different from the native spectrum. This particular experiment was repeated a number of times and in all cases the isosbestic point of states I and II was distinct from the native state.

This series of experiments showed that illumination of native CTAB–visual pigment produced two new chemical species. These chemical species (state I and II) were reversibly interconvertible by illumination at appropriate wavelengths. *The original native-state circular dichroism spectrum could not be regenerated by illumination at any wavelength.*

State I could be produced from native CTAB–visual pigment by illumination at wavelengths other than 500 nm. When native visual pigment (77°K) was illuminated at progressively shorter wavelengths starting at 620 nm, no change in the circular dichroism spectrum was observed until illuminated at approximately 560 nm. Short illumination at this wavelength produced state I which rapidly transformed into state II after further illumination at 560 nm. On the other hand, illuminating at 400 nm was quite efficient in producing state I from native CTAB–visual pigment. With illumination at this wavelength, state I was quite stable and was converted into state II only by illumination at longer wavelengths. *These experiments showed that illumination of native CTAB–visual pigment always resulted in the initial production of state I.* State II could then be generated from state I.

In transforming the native to illuminated visual pigment, the flat base-line spectrum between 620 and 400 nm (at 77°K) was replaced by the unique, large circular dichroism signals of states I and II. The positive circular dichroism peak at 340 nm, which was found in native visual pigment, seemed to have changed only minimally during these transformations (Figures 4 and 6). The circular dichroism band in the 340-nm region seems to be similar in the native and in the illuminated visual pigment states I and II. The positive circular dichroism band at 340 nm shown by native visual pigment at 298°K was thus only minimally affected by cooling to 77°K or by illuminating at 77°K.

Results obtained with extracts or with purified CTAB–visual pigment were identical. Thus, in this region of the spectrum which is dominated by the contribution of the chromophore alone, further purification, which removes other colorless proteins and lipids, does not seem to affect the circular dichroic spectra.

When the circular dichroism spectrum and the absorption maxima were recorded simultaneously, it was found that states I and II had absorption peaks around 515–520 and 500–505 nm, respectively. Thus these results correspond to the spectral shifts observed by Yoshizawa and Wald (1963).

Circular Dichroism Spectrum of Native and Illuminated Digitonin–Visual Pigment at 298°K. At room temperature, native digitonin–visual pigment had two positive circular dichroism bands with peaks at approximately 490 and 340 nm. The peak heights at 490 and 340 nm were in the ratio of 1:1.5. These data are similar to the results obtained by Crescitelli *et al.* (1966).

Circular Dichroism of Native and Illuminated Digitonin–Visual Pigment at 77°K. Results obtained with digitonin–visual pigment were similar to the results obtained with CTAB–visual pigment. Due to the poor quality of the digitonin glasses and the resulting variation in the base line from experiment to experiment, some of the points could not be established as unequivocally as for CTAB–visual pigment. In particular, it was impossible to establish with assurance whether the positive circular dichroism peak at 490 nm, which is found in native digitonin–visual pigment at 298°K, disappears upon cooling at 77°K. As mentioned above, this band disappeared at low temperature in the CTAB–visual pigment. In all other respects the digitonin and CTAB–visual pigments were identical. Illumination at 500 nm always produced state I as the initial state. State I and state II had their positive and negative circular dichroism peaks at the same positions as the state I and II of CTAB–visual pigment (Figure 8). State I could be transformed into state II most efficiently by illumination at 560 nm while state II could best be transformed into state I by illumination at 440 nm. State I and II were transformed into each other through an isosbestic point at approximately 514 nm.

Discussion

Absorption Spectra. Jurkowitz (1959), Balke and Becker (1967), and Sperling and Rafferty (1969) have shown that the cooling of *all-trans*-retinal from room temperature to 77°K results in a red shift of approximately 14 nm of the absorption peak and an increase of approximately 10% in the peak height. On the other hand, when the same experiment was performed with 11-*cis*-retinal, the peak shifted approximately 16 nm to the red and the peak height increased by some 62%. This remarkable increase in absorption of 11-*cis*-retinal on cooling was ascribed by Jurkowitz (1959) and Wald (1959) to the release of the steric hindrance of 11-*cis*-retinal at 77°K. Yoshizawa and Wald (1963) found that the absorption peak of digitonin–visual pigment increases 1.7 ± 0.11-fold on cooling to 77°K. They ascribed this large increase in absorption to the same release of steric hindrance observed in the free chromophore 11-*cis*-retinal. In view of the small increase (1.15-fold) of the absorption maxima of both

digitonin- and CTAB-visual pigment observed in this paper and the more recent results of T. Yoshizawa and G. Wald (personal communication, 1970) it seems that this interpretation needs revision. Thus the analogy suggested by Yoshizawa and Wald (1963) between the rise in absorption of 11-*cis*-retinal and native visual pigment upon cooling does not hold. These authors did already realize that such a possibility existed, since the 9-cis pigment (isorhodopsin) which is relatively unhindered gave the same intensification upon cooling as did the 11-cis pigment (rhodopsin).

One of us (Heller, 1968) had claimed in the past, on the basis of absorption spectra of purified CTAB-visual pigment, that there is no obvious absorption band in the 340-nm region. On the basis of the results of Shichi (1970) and our current work, this claim seems to be incorrect. Although purified CTAB-visual pigment shows a continuous smooth descending absorption curve from 300 to 400 nm, the evidence of a clear circular dichroism peak at 340 nm indicates there must be an absorption band in this region, even if only a very small one which can be recognized only with difficulty by absorption spectroscopy.

Circular Dichroism. When native CTAB-visual pigment was cooled from 298 to 77°K the positive circular dichroism band at 490 nm disappeared, while the band at 340 nm appeared unchanged (Figure 3). This remarkable finding shows that upon cooling, there was either a change in the chromophore itself or in its environment, or both. Likewise, the power of the circular dichroism technique is shown by the fact that this change was not detected in the absorption spectrum. This change of the circular dichroism signal was reversible, the signal at 490 nm reappearing upon warming. This shows that the conformational changes caused by cooling are not irreversible.

It was surprising to find that when native visual pigment was illuminated at 77°K the resulting circular signal was much larger than the original signals either at 298 or 77°K (Figure 4, 6, and 8 compared to Figure 3). We have no explanation at this time for this unique increase in signal magnitude upon illumination, and further experimentation is necessary to interpret this observation.

The nature of the dissociating agent seems to have only a minimal effect on the circular dichroism spectra in the 620–320-nm region. The only difference observed in this study between the digitonin- and CTAB-visual pigments was the peak height ratio of the 490- and 340-nm bands. Another potential difference was the disappearance upon cooling of the 490-nm band in CTAB-visual pigment. This was a difficult point to establish in the digitonin-visual pigment samples due to poor glasses with the resulting shifts in base line. The cause of the different peak height ratio in digitonin and CTAB preparation is not clear.

The effects of light scattering from dispersed membranes, suspended mitochondria, and other systems of relatively large particles on circular dichroism spectra has recently come under intensive investigation (Urry and Ji, 1968; Urry *et al.*, 1970; Urry and Krivacic, 1970; Schneider *et al.*, 1970). These effects may result in decreasing the amplitude and slightly shifting the position of circular dichroism bands. Since visual pigments are solubilized in detergent micelles, some error due to scattering may be introduced. However, the exact effect to be expected from light scattering upon circular dichroism band and its importance is still an open question (Ottaway and Wetlaufer, 1970). The effects of light scattering, if any, on the circular dichroism spectra described in this paper are not significant because all measurements were done in frozen glasses. Light is not expected to change the physical character of the detergent–protein micelle in this rigid medium.

Yoshizawa and Wald (1963) illuminated digitonin–bovine visual pigments$_{500}$ at 77°K and followed the reaction products by absorption spectroscopy. They found that when visual pigment was illuminated with monochromatic light at 440 nm, the absorption peak shifted from 505 to 517 nm. By the use of calculated difference spectra, they identified the product of illumination as a species absorbing maximally at 543 nm and called it prelumirhodopsin. They interpreted prelumirhodopsin to be the initial product of illumination where the native 11-*cis*-retinyl-apoprotein was isomerized to the *all-trans*-retinyl-apoprotein. An important consideration in this interpretation was the further observation by Yoshizawa and Wald (1963) that when the visual pigment derivative(s) produced by illumination at 440 nm and which has its peak at 517 nm, was illuminated at 600 nm the original spectrum (peak at 505 nm) was restored. The peaks with maxima at 517 and 505 nm were converted into each other with a sharp isosbestic point at 512.5 nm. This was interpreted to mean that only two compounds were present and that the only change caused in the 543-nm-absorbing species (prelumirhodopsin) was an isomerization of the retinyl group. Illumination at 600 nm reisomerized the *all-trans*-retinyl to 11-*cis*-retinyl, thereby regenerating the original native visual pigment (Yoshizawa and Wald, 1963; Wald, 1968). The explicit inference is that rhodopsin and prelumirhodopsin differ only in the state of isomerization of the prosthetic retinyl group. Moreover, rhodopsin and prelumirhodopsin are freely interconvertible as a result of an appropriate monochromatic illumination.

The circular dichroism studies reported in this paper show that the effects of illumination on visual pigment at 77°K are more complex than the absorption studies suggest. When native visual pigment (either the digitonin or CTAB complex) was illuminated, a unique circular dichroism spectrum was produced. The flat base-line spectrum between 620 and 400 nm was replaced by a much larger signal with a positive peak at 560 nm and a smaller negative peak at 480 nm (Figure 4). For descriptive purposes we called this spectrum state I. Both the positive and negative peaks of state I increased (up to a point) as linear functions of illumination. The circular dichroism spectrum of state I was always the first to be produced from native visual pigment upon illumination, irrespective of the wavelength of the illumination. As the visual pigment was progressively illuminated state I transformed into a different spectrum that was, in some senses, almost a mirror image of state I. This circular dichroism spectrum, called state II, had a negative peak at 540 nm and a positive peak at 460 nm. State I and state II could be produced most efficiently by illumination at 440 and 560 nm, respectively. The transformation of state I into state II and back could be repeated more than once. State I and II passed smoothly into each other through a sharp isosbestic point. We have never succeeded in regenerating the original native circular dichroism spectrum by any kind of illumination. Moreover, the circular dichroism spectrum of the native pigment does not pass through the isosbestic point of state I and II, and thus could not be derived from any combination of state I and II. These results show that the native visual pigment is different from its illuminated state and that illumination by itself cannot regenerate the native state. These results can be summarized as follows (Figure 9). Can the experimental observations of Yoshizawa and Wald

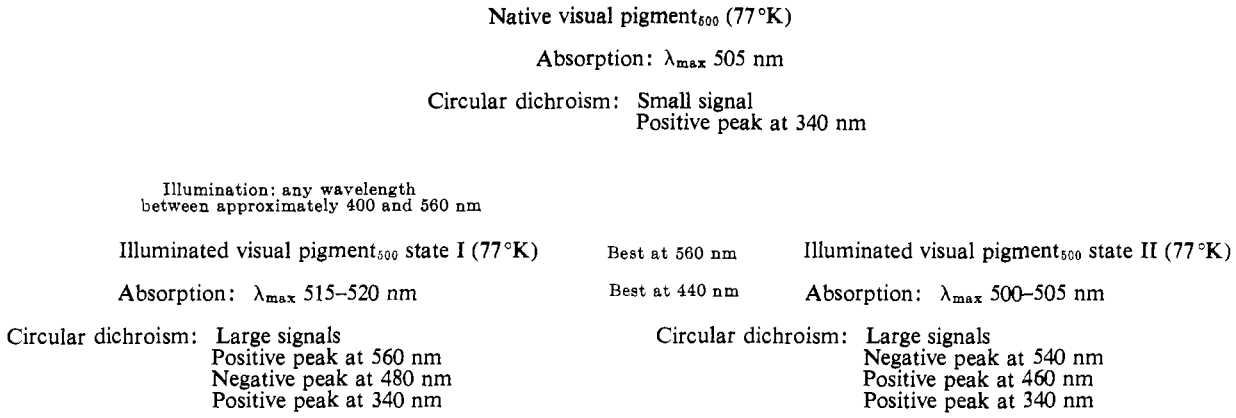

FIGURE 9: A schematic diagram of the interrelationship between native visual pigment and its illuminated derivatives as discussed in the text. The scheme applies equally to the digitonin–and CTAB–visual pigments except for the uncertainty concerning the 490-nm band in the native digitonin pigment.

(1963) be reconciled with the results obtained in this paper? As reported in Results, when native visual pigment was illuminated at 500 nm (at 77°K), its absorption maximum shifted from approximately 505–517 nm. These observations are identical with those of Yoshizawa and Wald. Under these conditions, a circular dichroism spectrum was produced with peaks at 560 and 480 nm. Yoshizawa and Wald calculated a spectrum for the derivative produced by illuminating visual pigment at 77°K and found it should have an absorption peak at 543 nm (prelumirhodopsin). This might be the species which has a circular dichroism signal of state I and shows peaks at 560 and 480 nm. Yoshizawa and Wald illuminated this product ("prelumirhodopsin") with light of 600-nm wavelength and found that the absorption spectrum returned to the original absorption of native visual pigment (rhodopsin). They arrived at the most logical conclusion that the native state was regenerated. Although the product of illuminated state I, which we call state II, does indeed show an absorption maximum at 505 nm similar to the native state, the circular dichroism measurements make it obvious that native visual pigment and state II are very different. Since the absorption spectra of native visual pigment and illuminated visual pigment state II are very similar, the spectra of prelumirhodopsin and the product of its illumination with light of 600 nm, which were observed by Yoshizawa and Wald, passed through an isosbestic point with the rhodopsin spectrum, thus seemingly showing that this procedure regenerated the native state (rhodopsin).

Yoshizawa and Wald have also observed that illumination of prelumirhodopsin with light of 600 nm, or especially 546 nm, produces a shift of λ_{max} to wavelength shorter than the original native rhodopsin. They interpreted this observation as showing that illumination of prelumirhodopsin produces both native 11-cis visual pigment and 9-cis visual pigment (isorhodopsin, λ_{max}, 485 nm). Could the circular dichroism spectrum of state II be due to photogeneration of 9-cis visual pigment? We have prepared the native 9-cis visual pigment and examined its circular dichroism spectrum at 77°K (J. Horwitz and J. Heller, unpublished results). The native 9-cis pigment shows a base line similar to the native 11-cis pigment in digitonin. Illumination of the 9-cis pigment produces initially a large circular dichroism signal similar to state I of the 11-cis pigment, the only difference being a shift of the position of the peaks. Illumination of this state I of the 9-cis pigment produces a state qualitatively similar to state II of the 11-cis pigment. It is clear from these experiments that state II of the 11-cis pigment can not be due to 9-cis visual pigment (isorhodopsin) since the prepared 9-cis pigment does not show the circular dichroism spectrum of state II.

What are the causes of the very large circular dichroism signals observed in visual pigment upon illumination? And what is the nature of the chemical substance(s) produced by illumination at 77°K? On the basis of our experiments, we think the large circular dichroism signals which were observed, and their behavior upon illumination as described above, are incompatible with a simple reversible cis–trans isomerization of the retinyl chromophore at 77°K. We feel, however, that too little information is as yet available to warrant the suggestion of any mechanism. We are currently working on the chemical identification of these low-temperature intermediates of illuminated visual pigment.

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Effect of Histidine on the Enzyme Which Catalyzes the First Step of Histidine Biosynthesis in *Salmonella typhimurium**

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ABSTRACT: The enzyme, phosphoribosyltransferase, which catalyzes the first step of the pathway for histidine biosynthesis in *Salmonella typhimurium*, is inhibited by the end product of the pathway, histidine. In this report we present studies on the mechanism of the inhibition of phosphoribosyltransferase by histidine. These studies bring to light the following findings. (1) In the presence of histidine the enzyme displays a positive ultraviolet difference spectrum. Analysis of this effect by the solvent perturbation method indicates a burying of chromophores upon addition of histidine. Spectrophotometric titration of the ionization of tyrosyl hydroxyl groups discloses that histidine prevents ionization of 12 tyrosyl residues in the enzyme. (2) Addition of histidine to phosphoribosyltransferase causes a shift in the fluorescence emission spectrum of the enzyme. The extent of the shift is dependent upon histidine concentration. Titration of the shift with histidine reveals a nonlinear behavior, suggesting a cooperativity in this effect of histidine. (3) Addition of histidine to phosphoribosyltransferase does not significantly alter the circular dichroic spectrum of the enzyme. This finding indicates that histidine does not cause a significant change in the secondary structure of the enzyme. (4) Histidine-insensitive phosphoribosyltransferase, isolated from a feedback-resistant mutant, does not show the change in fluorescence which is produced by histidine in the wild-type enzyme. The mutant enzyme does, however, bind histidine, judging from the fact that it, like the wild-type enzyme, is protected by histidine against denaturation by urea. We conclude that inhibition of phosphoribosyltransferase by histidine is brought about by a change in the conformation of the enzyme. Although phosphoribosyltransferase from a feedback-resistant mutant is able to bind histidine, it is unable to undergo the conformational change characteristic of the wild-type enzyme. Thus, the failure of the mutant enzyme to be inhibited by histidine is probably due to an alteration of its structure which does not permit a conformational change to occur when it binds histidine.

The biosynthesis of histine is carried out in *Salmonella typhimurium* through a series of ten steps, each catalyzed by a specific enzyme. The information for the structures of these enzymes is encoded in a small segment of the *Salmonella* chromosome, the histidine operon (for the most recent review, see Brenner and Ames, 1970). Regulation of histidine biosynthesis is accomplished through two different mechanisms. One mechanism, feedback inhibition, acts at the level of enzyme activity; the end product of the pathway, histidine, inhibits the first enzyme of the pathway, *N*-1-(5′-phosphoribosyl)adenosine triphosphate:pyrophosphate phosphoribosyltransferase (EC 4.2.1c) or, more simply, phosphoribosyltransferase, thereby shutting off the whole pathway (Ames *et al.*, 1961; Martin, 1963; Klungsoyr *et al.*, 1968). The second mechansim, *repression*, acts at the level of enzyme synthesis; the rate of synthesis of the histidine enzymes is increased under conditions of histidine limitation and is decreased under conditions of histidine excess (Ames *et al.*, 1960; Ames and Hartman, 1963). It appears, however, that His-tRNA, rather than histidine itself, is the mediator of this effect (Schlesinger and Magasanik, 1964; Silbert *et al.*, 1966; Roth *et al.*, 1966). The mechanism by which His-tRNA affects the synthesis of the histidine enzymes is not known.

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