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STUDIES ON THE ULTRAVIOLET-SPECTRAL DISPLACEMENTS OF CATTLE RHODOPSIN

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SUMMARY

1. The displacements of the C-band ($\lambda_{max.} = 278$ mμ) and D-band ($\lambda_{max.} = 231$ mμ) in the absorption spectrum of cattle rhodopsin are probably concerned with some changes in the protein moiety of rhodopsin on illumination.

2. When a rhodopsin solution is illuminated, the absorption maxima at 231, 278, 286.5 and 292 mμ shift towards shorter wavelength (blue-shift) by approx. 1 mμ, resulting in a difference spectrum characterized by a prominent negative peak at 234 mμ ($\Delta\varepsilon = -37000$), two negative ones at 279 and 287 mμ ($\Delta\varepsilon_{287\,m\mu} = -2400$) and a positive one at 291 mμ ($\Delta\varepsilon = 1000$).

3. The displacements are not influenced by pH (4.7–9.7), ionic strength ($I = 0$–5), digitonin concentration (0.1–2 %), ageing or glycerol (up to 80 %, w/v), but they are reduced to 70 % in concentrated sucrose solution and increased to 160 % in concentrated urea.

4. Rhodopsin in rod outer segments shows similar spectral changes as in solution.

5. From the experimental results on low temperature illumination, etc., the displacements are considered to take place in the course from meta-rhodopsin to indicator yellow but not in the earlier processes of the rhodopsin cycle.

INTRODUCTION

Ultraviolet spectral displacements are considered to indicate changes in the configuration of proteins, as discussed in general reviews by LEACH AND SCHERAGA[1] and SCHERAGA[2]. We have found that rhodopsin, when illuminated, shows displacements both in the C- and the D-band regions[3] (250–300 mμ and 220–250 mμ). The present paper reports a detailed spectroscopic study for the purpose of elucidating configurational changes in the protein moiety of rhodopsin under illumination.

MATERIAL AND METHODS

Preparation

Rhodopsin solution was prepared after COLLINS *et al.*[4] (optical purity: $A_{500\,m\mu}/A_{520\,m\mu}$, 0.25–0.30; $A_{278\,m\mu}/A_{500\,m\mu}$, 2.4–3.5); opsin solution was similarly prepared under bright light instead of dim red light. pH was adjusted with 0.022 M phosphate

or borate buffer. For preparations of alkaline solutions above pH 9, NaOH was used without buffer. The measurement of pH was carried out at 25° with a glass electrode (Toa Dempa Kogyo, Model HM-5). NaCl was added to adjust the ionic strength ($I = 0.5$).

Dry rhodopsin preparation

A piece of filter paper soaked in rhodopsin solution was dried in a desiccator over P_2O_5 and then soaked in liquid paraffin for spectroscopic measurement. Another preparation was obtained by drying rhodopsin solution in the desiccator to produce a transparent film, which was then broken into pieces in liquid paraffin and applied to a piece of filter paper*. All procedures were carried out under dim red light.

Rod outer segments

The segments were separated from retinal homogenate in 36 % sucrose solution–0.06 M phosphate buffer (pH 6.5) by centrifugation for 20 min at 4500 rev./min, followed by washing with 0.06 M phosphate buffers (pH 4.7 and pH 6.5). The outer segments were suspended in 36 % sucrose solution (pH 6.5) for spectrophotometry.

Illumination

Samples were ordinarily illuminated at room temperature for 2 min with white light of 6000 lux through a 3-cm water layer.

Intense illumination was carried out likewise through a water layer with a flash bulb (22000 lumen second in total light output, 7 msec in duration) placed in a box with a window (5 × 7 cm) provided with a shutter in order to shut off the after-glow of the bulb. Light intensity was adjusted by varying the distance from the bulb; it could be calculated roughly from the distance, the total light output and the duration of the flash.

Illumination through interference filters ($\lambda_{max.}$, 442 mμ; half width, 25.6 mμ; $\lambda_{max.}$, 575 mμ; half width, 12.5 mμ) was carried out for 15 min at −78° (dry ice–acetone) and at −195° (liquid nitrogen) after KITO *et al.*[5].

Spectroscopic measurement

Rhodopsin-solution measurement: this was carried out at room temperature with either a Cary automatic recording spectrophotometer, Model 14, or Hitachi spectrophotometer, EPU-2A using 10-mm or 2-mm quartz cells which had been previously calibrated. The latter instrument was operated at monochromatic slit widths of 0.68–0.12 mm (200–300 mμ) thus defining the wavelengths with an accuracy of ± 0.5 mμ.

Absorption of suspension of rod outer segments: this was measured with the Hitachi spectrophotometer with 2-mm quartz cells mounted in a holder, the back of which was covered with a piece of filter paper soaked in liquid paraffin, according to SHIBATA *et al.*[6]. The wavelengths were specified with an accuracy of ± 1 mμ. 36 % sucrose solution–0.06 M phosphate buffer (pH 6.5) was used as blank.

Measurement of dry rhodopsin: this was performed against a piece of filter paper soaked in liquid paraffin with an accuracy of wavelength of ± 1 mμ

* Both preparations gave similar results.

The difference spectrum: this was obtained by the method of "differential spectrophotometry"[7]. The concentrations of rhodopsin and rod outer segments were selected so as to give readings within the ranges of the instruments, obeying Beer's law. The first and the second derivative curves were constructed from the original spectrum with a view to detecting any low intensity bands obscured by those of high intensity[8].

RESULTS

Influences of pH, ionic strength, digitonin concentration and ageing

Fig. 1A shows the absorption spectra of rhodopsin solutions at pH 9.0 (25°) before and after illumination. The non-illuminated solution clearly shows four absorption bands, *i.e.* A-band[9,10] ($\lambda_{max.}$, 500 mμ); B-band ($\lambda_{max.}$, approx. 350 mμ); C-band[4] ($\lambda_{max.}$, 278 mμ) and a shoulder in the 230-mμ regio.. (D-band), the latter two of which are considered to be due to aromatic groups in the protein moiety of rhodopsin[4,11].

In Fig. 1B, the first derivative curve for a non-illuminated solution is seen to have a large negative peak at 234 mμ, and to intersect the base line at 278 mμ indicating that $\lambda_{max.}$ of the C-band is at this wavelength. The second derivative curve (Fig. 1C) reveals that the C-band includes two other components with maxima at 286.5 mμ and 292 mμ in addition to that at 278 mμ, and that the D-band has its maximum at 231 mμ, since a negative peak in the second derivative curve must correspond with $\lambda_{max.}$ in the original spectrum[8]. Thus, rhodopsin has ultraviolet absorption bands with maxima at 231 mμ, 278 mμ, 286.5 mμ and 292 mμ, besides two bands in the visible region. Rhodopsin solutions at pH 7.0 and pH 4.7 show no essential differences in absorption spectra from the solution at pH 9.0.

After illumination (pH 9.0), the A-band has disappeared and a band for alkaline indicator yellow ($\lambda_{max.}$, 365 mμ) has appeared, as expected[12]; moreover, the C-band and D-band have been slightly displaced (Fig. 1A). The first and the second derivative curves (Fig. 1B, C) give more detailed information about the ultraviolet-spectral displacements. In the C-band region, the intersection of the first derivative curve with the base line, *i.e.* $\lambda_{max.}$ of the original spectrum, has shifted from 278 mμ to 277 mμ, and two negative peaks at 286.5 mμ and 292 mμ in the second derivative curve have also shifted to shorter wavelengths by approx. 1 mμ. In the D-band region, the negative peak at 231 mμ in the second derivative curve has been similarly displaced. Thus, all the ultraviolet absorption maxima shift towards shorter wavelengths by approx. 1 mμ when rhodopsin is illuminated.

The spectral displacements on illumination result in a difference spectrum (pH 9.0) (Fig. 1D) which shows a prominent negative peak at 234 mμ ($\Delta\varepsilon = -3.7 \pm 0.1 \cdot 10^{4*}$), identical with the $\lambda_{max.}$ of the negative peak in the first derivative curve, two negative peaks at 279 mμ ($\Delta\varepsilon = -2.0 \pm 0.1 \cdot 10^{3}$) and at 287 mμ ($\Delta\varepsilon = -2.4 \pm 0.2 \cdot 10^{3}$) and a positive peak at 291 mμ ($\Delta\varepsilon = 1.0 \pm 0.1 \cdot 10^{3}$) in the ultraviolet region. In the visible region, there is a positive peak at 370 mμ and a negative peak at 500 mμ suggesting that rhodopsin has been converted to alkaline indicator yellow.

The difference spectra at pH 4.7 and 7.0 were found to be almost the same in the

* This and the subsequent values are calculated from $\Delta\varepsilon_{500\,m\mu} = 40\,600$, as determined by WALD *et al.*[9] and HUBBARD[10].

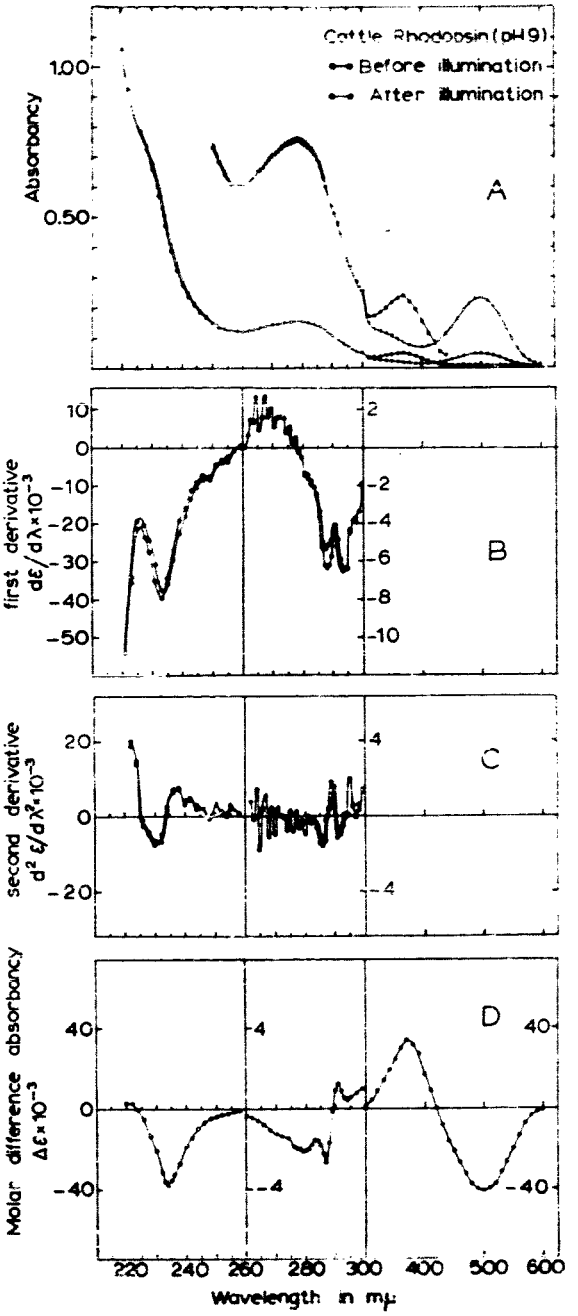

Fig. 1. (A) Spectra of rhodopsin solution (pH 9.0) before and after illumination; sample b, upper curves, is 5 times more concentrated than sample a, lower curves. (B) First derivative curves plotted from Fig. 1A. (C) Second derivative curves. (D) Difference spectrum before and after illumination

ultraviolet region as at pH 9.0. However, in the visible region, the difference spectrum depended greatly on the pH as is well-known[12].

In Fig. 2, the values of $\Delta\varepsilon_{234\,m\mu}$ and $\Delta\varepsilon_{287\,m\mu}$ are plotted against pH. They are seen to be independent of pH in the range pH 4.7 to 9.7. This corresponds to the pH range in which rhodopsin is regarded as stable in the dark[13]. The values of $\Delta\varepsilon$ at 29° are somewhat higher than those at 10°.

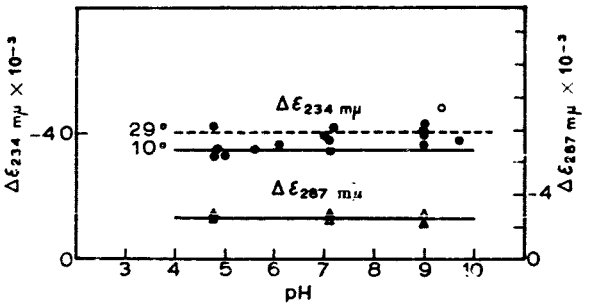

Fig. 2. Difference absorbancies at 234 mμ (upper two) and 287 mμ (lower one) of rhodopsin solution before and after illumination.

The absorbancies of the illuminated sample mostly showed no further change for 60 min or more after cessation of illumination (*cf.* Fig. 5), but sometimes, especially in the acid samples at 29°, they increased in the whole ultraviolet range, resulting in a difference spectrum quite similar to that between the fresh and the aged rhodopsin solution[11].

No influence of ionic strength (I = 0–5), digitonin concentration (0.1–2 %), rhodopsin concentration (1–3 folds) and ageing (storage for more than two weeks in an ice box) could be observed on the spectrum of rhodopsin or on the spectral change on illumination. When opsin solution was illuminated, there was no change in the spectrum.

Influence of urea, sucrose and glycerol

After addition of urea, etc. and buffers, rhodopsin solution was kept at 25° for more than 3 h before the illumination experiment.

Urea is able to bleach rhodopsin (approx. 10^{-6} M) even in the dark and there are ultraviolet-spectral displacements ($\Delta\varepsilon_{234\,m\mu} = -7\cdot10^4$) after incubation at 30° for 3 h; 1.6 M urea bleached 25 % of the rhodopsin; 2.4 M, 50 %; 3.2 M, 80 % and 4.8 M, 100 %*.

The effect of illumination on rhodopsin remaining unbleached even after urea treatment was studied. The values of $\Delta\varepsilon_{234\,m\mu}$ on illumination were plotted against the concentration of urea as shown in Fig. 3, from which it can be seen that urea has magnified the spectral change up to 1.6 fold. The difference absorbancies of the illuminated — non-illuminated sample did not alter after cessation of illumination (Fig. 5).

* Similar spectral displacements could also be observed in heat bleaching. Rhodopsin solution of pH 7 was incubated at 90° for 10 min in the dark and, after cooling, the difference spectrum was examined. This showed 100 % bleaching of rhodopsin with $\Delta\varepsilon_{234\,m\mu} = -7\cdot10^4$.

When opsin solution was subjected to the same treatment, it resulted in similar ultraviolet displacements with $\Delta\varepsilon_{233\,m\mu} = -7\cdot10^4$, while no change could be observed in the visible range.

Concentrated sucrose solution suppressed the spectral displacements of the D-band on illumination, but did not affect the A-band. The values of $\Delta\varepsilon_{234\,m\mu}$ were plotted against the sucrose concentration (Fig. 4): maximum suppression (30 %) occurred above a sucrose concentration of 40 % (w/v).

Concentrated glycerol solution revealed no remarkable effect on the spectral displacements of either the D- (Fig. 4) or A-bands.

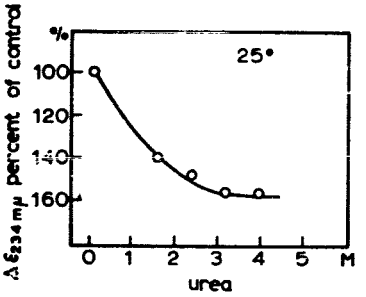

Fig. 3. Difference absorbancies at 234 mμ of rhodopsin–urea solution before and after illumination.

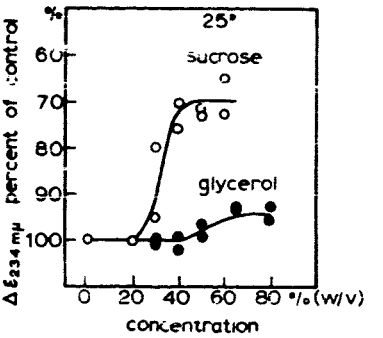

Fig. 4. Difference absorbancies at 234 mμ of rhodopsin–sucrose and rhodopsin–glycerol solution before and after illumination.

Rod outer segments

Fig. 6A shows two examples of the absorption spectra of a suspension of rod outer segments in a concentration range where Beer's law holds. The first and the second derivative curves were examined; the former has a large negative peak at 237 mμ and intersects the base line at 278 mμ, while the latter has a large negative peak at 231 mμ. We can conclude that a suspension of rod outer segments has an A-band with maximum at 500 mμ, a C-band at 278 mμ and a D-band at 231 mμ. All the characteristics, which remain unchanged for at least two days, are the same as those of rhodopsin in solution. The intensities of the bands are affected by the flattening effect[14] which must be taken into consideration when dealing with pigments concentrated in cell structures.

Illumination resulted in a spectral change similar to that of rhodopsin in solution (Fig. 6B). The A-band has disappeared. The significance of the difference spectrum in the C-band region is doubtful. In the difference spectrum in the D-band region a negative peak is found at 238 mμ, which is close to the $\lambda_{max.}$ of the negative peak in the first derivative curve (237 mμ).

Illumination at low temperature and high intensity

In Fig. 7, Curve a shows the difference spectrum at 25° before and after illumination at — 195° with 575-mμ light. According to KITO *et al.*[5], this treatment converts rhodopsin to iso-rhodopsin. There is no difference in the ultraviolet region but certain characteristic differences in the visible region, probably due to the production of iso-rhodopsin ($\lambda_{max.}$, 487 mμ).

We found no change in the ultraviolet difference spectrum (Fig. 7, Curve b) obtained at 25° before and after re-illumination of the sample at — 195° with 575-mμ

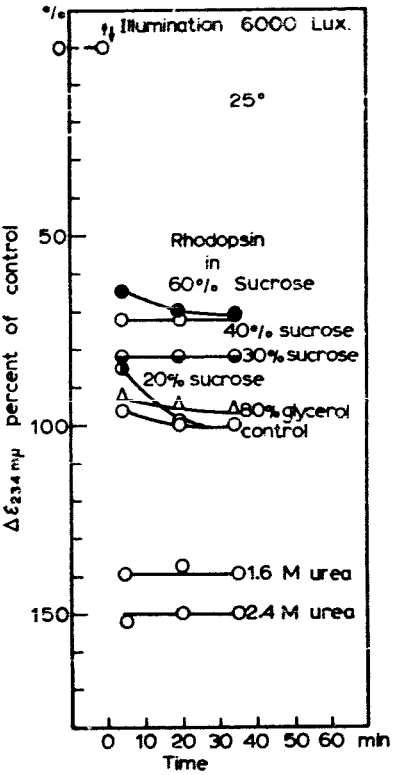

Fig. 5. Time course of difference absorbancies at 234 mμ (illuminated – non-illuminated rhodopsin solutions).

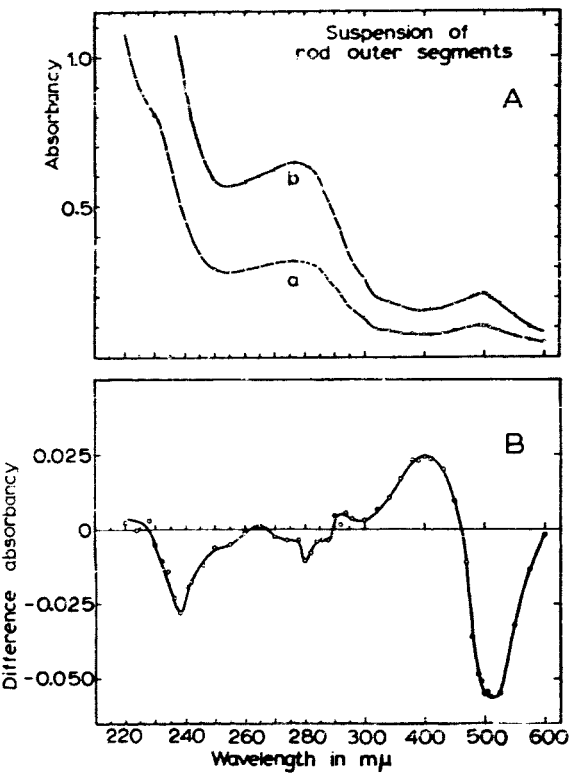

Fig. 6. (A) Spectra of rod outer segments suspended in 36% (w/v) sucrose solution–0.06 M phosphate buffer (pH 6.5) determined according to SHIBATA (see text). (B) Difference spectrum before and after illumination of Sample a.

light, which had been previously illuminated at —195° with 442-mμ light. After this treatment, rhodopsin should be converted to iso-rhodopsin through labile fraction[5].

In the difference spectrum (Fig. 7, Curve c) determined at 25° before and after illumination at — 195° with 442-mμ light, the characteristic spectral changes in the D-band region corresponded to the amount of bleached rhodopsin as revealed by the spectral change in the A-band region. This treatment is said to convert one half of the rhodopsin into a mixture of indicator yellow and retinene + opsin through labile fraction.

As illustrated in Fig. 8 (Curve b) the difference spectrum before and after re-illumination of the sample at —195° with 575-mμ light, which had been warmed to —78° after pre-illumination at —195° with 442-mμ light, gives an ultraviolet change corresponding to amount of bleaching. After this treatment, one half of the rhodopsin should remain unchanged, while the other half might be partly (one quarter) converted to iso-rhodopsin through labile fraction and meta-rhodopsin, and partly (the other quarter) to indicator yellow, etc. that has been derived from the component of meta-rhodopsin incapable of being photorecovered.

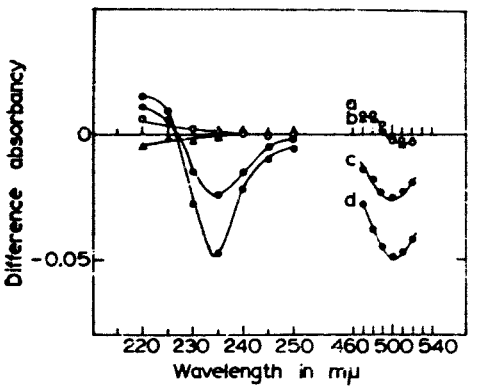

Fig. 7. Difference spectra of rhodopsin solution before and after illumination, (a) at −195° with 575-mμ light (O—O); (b) at −195° with 442-mμ followed by 575-mμ light (▲—▲); (c) at −195° with 442-mμ light (●—●) and (d) difference spectrum of iso-rhodopsin solution (produced by illumination at −195° with 575-mμ light) before and after illumination with 575-mμ light at 25° (Ⓘ—Ⓘ). Duration of illumination, 20 min.

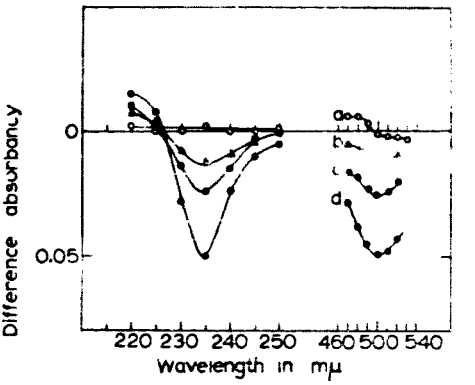

Fig. 8. Difference spectra of rhodopsin solution before and after the following treatments: (a) incubation at −195° and −78° (dry ice–acetone) in dark, thenillumination at −195° with 575-mμ light (O—O); (b) illumination at −195° with 442-mμ light and incubation at −78°, then incubation at −195° with 575-mμ light (△—△); (c) illumination at −195° with 442-mμ light and incubation at −78°, then −195° in dark (●—●) and (d) illumination at 25° with 442-mμ light (⊖—⊖).

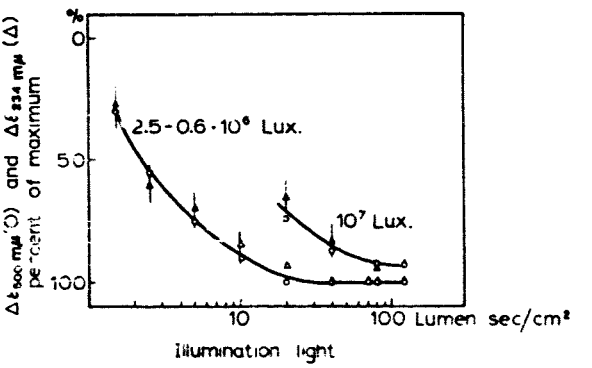

Fig. 9. Difference absorbancies at 500 mμ and 234 mμ before and after flash illumination of rhodopsin solution.

In Fig. 9, the difference absorbancies at 234 mμ and 500 mμ are plotted against the illumination at various light intensities. It is obvious from this figure that illumination with 10^7 lux can bleach a rather smaller amount of rhodopsin than illumination with $2.5–0.6 \cdot 10^6$ lux, and furthermore, that the values of $\Delta\varepsilon_{234\,m\mu}$ and $\Delta\varepsilon_{500\,m\mu}$ show almost the same curves. The smaller amount of bleached rhodopsin under intense light may be ascribed to photorecovery from the labile fraction to rhodopsin or iso-rhodopsin[16]. No ultraviolet spectral change can be detected in rhodopsin and iso-rhodopsin which have been photoreversed through the labile fraction and even through meta-rhodopsin.

Dry rhodopsin

In Fig. 10A, the absorption spectra of dry rhodopsin are represented for two different concentrations. It will be observed that at higher concentration the maximum of the A-band is located at 500 mμ and that of the C-band at 278 mμ. As regards the maximum of the D-band, the second derivative curves were plotted for several samples of different concentrations; the negative peak was at 231 mμ, shifting from longer wavelengths according to the decrement in the concentration. Considering stray radiation[16], 231 mμ should be regarded as $\lambda_{max.}$ of the D-band.

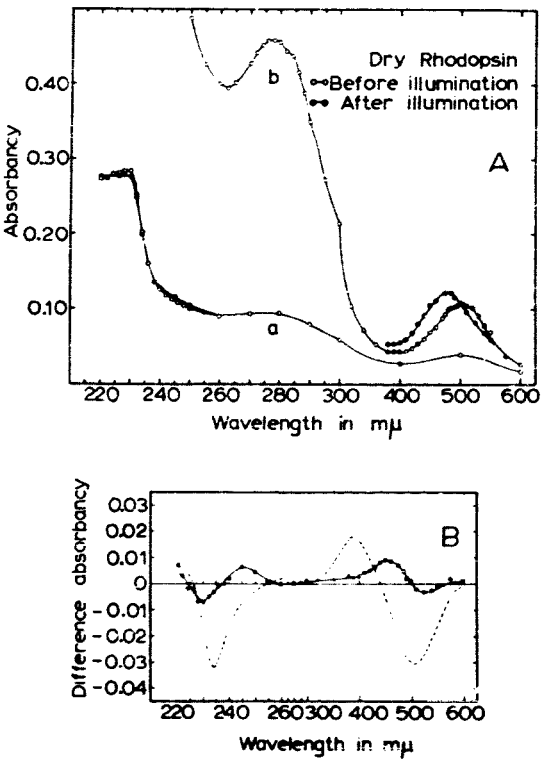

Fig. 10. (A) Spectra of dry rhodopsin. (B) Difference spectrum before and after illumination of Sample a at 25°. For reference, the broken line shows a difference spectrum of rhodopsin solution.

When illuminated by white light of 60000 lux for 1 min at 25°, there occurs a shift of the A-band maximum from 500 mμ to near 480 mμ ($\lambda_{max.}$ of meta-rhodopsin) and a slight broadening of the D-band; no change can be detected in the C-band. The difference spectrum of dry rhodopsin before and after illumination is shown in Fig. 10B. In the D-band region, there is a negative peak at 230 mμ ($\Delta\varepsilon = -9000$) and a positive peak at 245 mμ, both of which have never been observed in the other cases. In the visible region, a positive peak is found at 450 mμ and a negative one at 525 mμ.

DISCUSSION

Ultraviolet absorption bands of rhodopsin

The first and the second derivative curves of the ultraviolet absorption spectrum of cattle-rhodopsin solution show that the $\lambda_{max.}$ of the D-band is 231 mμ at pH 7.0, moreover, that the C-band consists of three bands with maxima at 278 mμ, 286.5 mμ and 292 mμ in the pH range 4.7–9.0. Of these four bands, 278 mμ was ascribed to tyrosine and tryptophan residues in the protein moiety of rhodopsin molecule by COLLINS *et al.*[4]. The others have not been discussed hitherto.

Considering that aromatic amino acids in a protein molecule show $\lambda_{max.}$ shifted to a longer wavelength by 1–6 mμ compared with free amino acids[1], it would be supposed that incorporation of tryptophan into the protein moiety of rhodopsin would shift the maximum at 288 mμ (see ref. 17) (at neutral pH) to 292 mμ, and the maximum at 279 mμ (see ref. 17) probably to 286.5 mμ. Similarly, tyrosine maxima at 274.5 mμ and 280 mμ (see ref. 17) might be shifted to 278 mμ and 286.5 mμ respectively. Lastly, the maximum at 231 mμ was considered in the previous experiments* to be due to tyrosine residues, assuming that the maximum at 223 mμ (see ref. 19) (at neutral pH) might be shifted towards longer wavelength by 8 mμ by incorporation into the protein molecule.

Ultraviolet spectral changes on illumination

When rhodopsin is illuminated, all the ultraviolet absorption maxima mentioned above are shifted towards shorter wavelength (blue-shift) by approx. 1 mμ, resulting in the difference spectrum which is characterized by three negative peaks at 234 mμ, 279 mμ and 287 mμ and a positive peak at 291 mμ (Fig. 1). The similar blue-shift of ultraviolet absorption bands was observed on denaturation of ribonuclease, insulin, lysozyme, pepsin, etc.,[20–25] where the peaks at 278–279 mμ and 287 mμ in the difference spectra were ascribed to the perturbation of the tyrosine band and those at 291–294 mμ to that of the tryptophan band. Therefore, we can also consider that the peaks at 279 mμ and 287 mμ in the difference spectrum of rhodopsin might be due to tyrosine residues and the one at 291 mμ to tryptophan residues.

The negative peak at 234 mμ in rhodopsin might be, at least in part, attributed to the blue-shift of the D-band, because that wavelength (234 mμ) coincides with that of the negative peak due to the D-band in the first derivative curve as shown in Fig. 1B**. Another factor responsible for this negative peak in the difference spectrum would be the decrease in the intensity of the D-band, and, according to GLAZER AND SMITH[27], the change in the absorption of peptide bonds when they are transformed from the helical structure to the random coiled one.

Any contribution of retinene to the ultraviolet-spectral change of rhodopsin is ruled out by the fact that the spectral changes in the ultraviolet region are independent of pH, while those in the visible region are considerably affected by pH of the medium[12].

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* In the previous experiments with rhodopsin, opsin, ribonuclease, ovalbumin, lysozyme, tyrosine and tryptophan, we suggested that a maximum in the range 228–231 mμ might be ascribed to tyrosine residues[19].

** If $\Delta\lambda$ and the change in intensity are small, $\Delta\varepsilon$ has the form of the negative of the first derivative of the original spectrum as pointed out by CHERVENKA[26] and SCHERAGA[3].

The spectral displacements in the tyrosine and tryptophan bands of ribonuclease, lysozyme, etc. on denaturation are considered to be ascribable to (a) the disruption of tyrosyl hydrogen bonds, (b) the exposure of these residues out of the protein molecule, (c) the receding of some polar groups from the residues; *i.e.*, some configurational deformation of the protein[1,2].

The same reasons can be advanced for the spectral displacements of rhodopsin on illumination. If configurational deformations really take place when rhodopsin is bleached, it must be expected that a denaturant (*e.g.* urea) will magnify the deformation and accordingly give larger ultraviolet-spectral displacements, while an anti-denaturant (sucrose[28,29]) may suppress the deformation, resulting in a smaller spectral change. Furthermore, if any tyrosine (and tryptophan) residues are newly exposed on illumination, solvents of high refractive index (red-shifting solvents) such as sucrose and glycerol should reduce the blue-shifts of the D-band[30].

The fact that urea magnified the spectral change (Fig. 4) seems to support this concept. Though two mechanisms can be suggested for the suppressive effect of sucrose, namely (a) inhibition of the denaturation, and (b) perturbation of the electrostatic field around the newly exposed chromophores, the contribution of the latter may be considered to be small. If this mechanism is operative, a concentrated glycerol solution should also suppress the spectral displacement; in fact, we found no effect of glycerol on the spectral change. Thus, it can be inferred that a concentrated sucrose solution probably acted as an anti-denaturant, protecting rhodopsin from denaturation on illumination.

We relate the ultraviolet-spectral changes to some slight configurational looseness in the protein moiety of rhodopsin on illumination. On the other hand, no essential difference in the ultraviolet spectrum can be observed between non-illuminated rhodopsin in rod outer segments, in aqueous solution of different pH and even in the dry state so that the protein moiety of rhodopsin seems to show no configurational difference such as to produce some changes in the electrostatic field around the tyrosine and tryptophan residues between those states.

pH independence

Although pH change[31] and conductance change[32] caused by the denaturation of rhodopsin on illumination are found to be affected considerably by the pH of the medium, pH independence of the configurational deformation of rhodopsin would be expected from pH independence of the ultraviolet-spectral changes. This can probably be explained as follows. After the pH-independent changes in the configuration of rhodopsin, the masked dissociable groups would become ionized, depending on pH of the medium, though these ionizations could not perturb the electrostatic field of the tyrosine or tryptophan groups so as to result in any spectral displacements.

In the section *Rod outer segments* it has been found that rhodopsin in rod outer segments undergoes the same ultraviolet-spectral change as in solution. Though $\lambda_{max.}$ of the negative peak (238 mμ) is longer than in solution (234 mμ), the former can also be related to the blue-shift of the D-band, since this is very close to the $\lambda_{max.}$ of the negative peak in the first derivative curve of the spectrum of rhodopsin in rod outer segments (237 mμ). Thus we can imagine that rhodopsin packed in the disks of rod outer segments might be configurationally deformed when illuminated

Rhodopsin cycle and denaturation

In which process of the rhodopsin cycle does the denaturation, as demonstrated by the ultraviolet-spectral change, take place? This question will be answered in part by the illumination experiments at low temperature. The fact that no ultraviolet change can be detected for the photoreversed rhodopsin or iso-rhodopsin, mentioned in the section *Illumination at low temperature and high intensity*, indicates that, at any step in the following cycle of rhodopsin, denaturation of the protein moiety does not occur, or even if it does, it will be reversed. The broadening of the D-band through conversion of rhodopsin to meta-rhodopsin (dry), as described in the section *Dry rhodopsin*, probably means that the protein moiety of rhodopsin becomes somewhat unstable, permitting greater intramolecular vibrations. This instability of the protein moiety of meta-rhodopsin may render it to some extent incapable of photorecovery mentioned above.

Rhodopsin	Iso-rhodopsin
⇵	⇵
Labile fraction	(−195°)
⇵	
Meta-rhodopsin	(−78°)

Thus, the ultraviolet-spectral displacements can be considered to take place in the change from meta-rhodopsin to indicator yellow, rather than in the earlier processes. Therefore, we suppose that the denaturation would take place only in free water, because, neither in frozen water (at low temperature) nor in bound water (in dry state), could the ultraviolet-spectral displacements be detected.

If there is no water available, rhodopsin cannot be bleached and denatured, no matter how retinene may be isomerized to the all-*trans* form and the protein moiety may become unstable as described above. Therefore, the penetration of water molecules into the rhodopsin molecule is supposed to be the necessary condition for bleaching and denaturation. Water absorption of the rhodopsin molecule seems of interest in relation to the hydration and dehydration theory of nerve excitation postulated by TOBIAS[33]. The steadiness of denaturation represented by the independence of the ultraviolet absorption change of pH, ionic strength, etc. is considered to be one of the important qualities of rhodopsin as a sender of information in the photoreceptor.

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REFERENCES

1 S. J. LEACH AND H. A. SCHERAGA, *J. Biol. Chem.*, 235 (1960) 2827.
2 H. A. SCHERAGA, *Protein Structure*, Academic Press, New York, 1961, p. 217.
3 M. TAKAGI AND Y. SEKOGUTI, *Biochim. Biophys. Acta*, 49 (1961) 589.
4 F. D. COLLINS, R. M. LOVE AND R. A. MORTON, *Biochem. J.*, 51 (1952) 292.

[5] Y. KITO, M. ISHIGAMI AND T. YOSHIZAWA, *Biochim. Biophys. Acta*, 48 (1961) 287.
[6] K. SHIBATA, A. A. BENSON AND M. CALVIN, *Biochim. Biophys. Acta*, 15 (1954) 461.
[7] R. BASTIAN, R. WEBERLING AND F. PALLILA, *Anal. Chem.*, 22 (1950) 160.
[8] A. E. MARTIN, *Nature*, 180 (1957) 231.
[9] G. WALD AND P. K. BROWN, *J. Gen. Physiol.*, 37 (1954) 189.
[10] R. HUBBARD, *J. Gen. Physiol.*, 37 (1954) 381.
[11] M. TAKAGI, *Zool. Mag. (Tokyo)*, 69 (1960) 269.
[12] H. J. A. DARTNALL, *The Visual Pigments*, Methuen, London, 1957.
[13] C. M. RADDING AND G. WALD, *J. Gen. Physiol.*, 39 (1956) 923.
[14] L. N. M. DUYSENS, *Biochim. Biophys. Acta*, 19 (1956) 1.
[15] C. D. B. BRIDGES, *Biochem. J.*, 79 (1961) 135.
[16] I. FRIDOVICH, W. FARKAS, G. W. SCHWERT, JR. AND P. HANDLER, *Science*, 125 (1957) 1141.
[17] C. FROMAGEOT AND G. SCHNEK, *Biochim. Biophys. Acta*, 0 (1960) 113.
[18] M. TAKAGI, in preparation.
[19] L. J. SAIDEL, A. R. GOLDFARE AND S. WALDMAN, *J. Biol. Chem.*, 197 (1952) 285.
[20] M. LASKOWSKI, JR., J. WIDOM, M. MCFADDEN AND H. A. SCHERAGA, *Biochim Biophys. Acta*, 19 (1956) 581.
[21] H. A. SCHERAGA, *Biochim. Biophys. Acta*, 23 (1957) 196.
[22] M. LASKOWSKI, JR., S. J. LEACH AND H. A. SCHERAGA, *J. Am. Chem. Soc.*, 82 (1960) 571.
[23] O. O. BLUMENFELD AND G. E. PERLMANN, *J. Gen. Physiol.*, 42 (1959) 563.
[24] J. W. DONOVAN, M. LASKOWSKI, JR. AND H. A. SCHERAGA, *Biochim. Biophys. Acta*, 29 (1958) 455.
[25] J. HERMANS, JR. AND H. A. SCHERAGA, *J. Am. Chem. Soc.*, 83 (1961) 3283.
[26] C. H. CHERVENKA, *Biochim. Biophys. Acta*, 31 (1959) 85.
[27] A. N. GLAZER AND E. L. SMITH, *J. Biol. Chem.*, 236 (1961) 2942.
[28] A. BEILINSSON, *Biochem. Z.*, 213 (1929) 399.
[29] C. R. HARDT, I. F. HUDDLESON AND C. D. BALL, *J. Biol. Chem.*, 163 (1946) 211.
[30] C. C. BIGELOW AND I. I. GESCHWIND, *Compt. Rend. Trav. Lab. Carlsberg*, 31 (1960) 283.
[31] C. M. RADDING AND G. WALD, *J. Gen. Physiol.*, 39 (1956) 909.
[32] T. HARA, *J. Gen. Physiol.*, 41 (1958) 857.
[33] J. M. TOBIAS, *J. Cellular Comp. Physiol.*, 37 (1951) 91.