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Formation of 9-*cis*- and 11-*cis*-Retinal Pigments from Bacteriorhodopsin by Irradiating Purple Membrane in Acid†

Akio Maeda,* Tatsuo Iwasa,‡ and Tôru Yoshizawa

ABSTRACT: Both light-adapted and dark-adapted forms of bacteriorhodopsin in purple membrane in 67% glycerol solution were allowed to stand in acidic conditions by the addition of HCl to final concentrations from 4×10^{-4} to 2×10^{-2} M for 24 h at 3 °C. Over this concentration range, the acid-induced products from both species showed a maximum absorbance around 600 nm and high-performance liquid chromatography of extracted retinal isomers revealed that the acid-induced form of bacteriorhodopsin has 13-*cis*- and *all-trans*-retinals in a molar ratio of 4:6, which is intermediate between those of the dark-adapted and the light-adapted forms at neutral pH values. Exposure of the acid-induced form of bacteriorhodopsin to light at wavelengths longer than 670 nm at 3 °C caused a decrease of the absorbance around 600 nm with a concomitant rise of the absorbance around 500 nm. The extract from the irradiated products of bacteriorhodopsin in acid contained 9-*cis*- and 11-*cis*-retinals in addition to 13-*cis*- and *all-trans*-retinals. The absorbance maximum estimated from the analysis of the absorption spectra and the composition of the isomers was found at 495 nm for the 9-*cis*-retinal pigment and around 560 nm for the 11-*cis*-retinal pigment. On irradiation with 438-nm light, the 9-*cis*-retinal pigment disappeared with a concomitant increase of both the 13-*cis*- and *all-trans*-retinal pigments as judged by chromophore analysis and the absorption spectrum. The 9-*cis*-retinal pigment brought to pH 9 exhibited a maximum absorbance at 450 nm; this could be decomposed by the action of hydroxylamine or converted to a form resembling normal bacteriorhodopsin by 438-nm irradiation.

It was previously shown (Maeda et al., 1979) that the chromophore retinal in squid rhodopsin produces all kinds of mono-cis isomers including 7-*cis*- and 13-*cis*-retinals under appropriately selected conditions of irradiation. A further examination of this process revealed that the 7-cis chromophore was produced from lumirhodopsin, one of the light-induced intermediates, but not directly from bathorhodopsin. This implies that some protein factors may determine the direction for the light-induced isomerization of the chromophore retinals. The 7-*cis*-retinal pigment was also produced in cattle rhodopsin (Maeda et al., 1978) under the same experimental conditions as those employed for squid rhodopsin.

Bacteriorhodopsin (bR)[1] is another pigment which has retinal as a chromophore [see a recent review by Stoeckenius et al. (1979)]. It has been well established that the chromophore retinal extracted from the light-adapted form of bR (bR^L) is *all-trans*-retinal and that from the dark-adapted form of bR (bR^D) is an equimolar mixture of both 13-*cis*- and *all-trans*-retinals (Pettei et al., 1977; Maeda et al., 1977). So far, no other isomers have been found from irradiated bR.

It is known that bR undergoes a reversible structural alteration in acidic solution accompanied by a shift of the absorption spectrum to longer wavelength (Oesterhelt & Stoeckenius, 1971). It is expected that the protein environment of bR molecules in acid will affect the chromophore differently on irradiation from that of bR under neutral conditions. From flash photolytic experiments, Lozier et al. (1978) observed that acidic bR undergoes a unique photochemical reaction, forming short-lived species with the wavelength of maximum absorbance (λ_{max}) at shorter wavelengths.

Under continuous irradiation of bR in acid at 3 °C, we observed the formation of 9-*cis*- and 11-*cis*-retinal pigments, which have not been observed by irradiating bR at neutral pH

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[1] Abbreviations used: bR, bacteriorhodopsin; bR^L, light-adapted form of bacteriorhodopsin; bR^D, dark-adapted form of bacteriorhodopsin; λ_{max}, the wavelength of maximum absorbance; CTAB, cetyltrimethylammonium bromide; LC, high-performance liquid chromatography.

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values. We further studied several spectral properties of these pigments.

Materials and Methods

Preparation of Purple Membrane. Halobacterium halobium strain R_1, kindly provided by Professor W. Stoeckenius, was used. Purple membrane prepared as described by Oesterhelt & Stoeckenius (1974) was finally suspended in the unbuffered, distilled water and dialyzed against 100 volumes of distilled water. External fluid was replaced 3 times during dialysis for 24 h. The dialyzed preparation was then mixed with 2 volumes of glycerol and served for experiments at a concentration of A_{560nm} = 0.6–0.8 cm^{-1} by diluting with 67% glycerol. bR^D was obtained by incubation of the sample at 4 °C in the dark for at least 3 days before use. bR^L was prepared by irradiating bR^D with 530-nm light (a Toshiba interference filter, KL 53) for 20 min in an ice bath until no further spectral change was noticed.

Spectroscopy. The absorption spectrum was measured at 3 °C in a Hitachi recording spectrophotometer, Type 124, equipped with a cuvette holder in which ice-cold water was circulated. Condensation of moisture on the cuvette was prevented by purging the sample compartment with dry nitrogen gas.

The absorption spectrum of the sample bleached by the addition of cetyltrimethylammonium bromide (CTAB) (Danon & Stoeckenius, 1974) was measured at room temperature for the purpose of avoiding the precipitation of CTAB at 3 °C. The difference spectrum was calculated by subtracting the spectrum of the CTAB-bleached sample from that of the corresponding sample.

Irradiation in Acid. A sample (A_{595nm} = 0.5–0.7 cm^{-1}) was put in a quartz cuvette of 1 cm in optical path and 1 cm in width, cooled in an ice bath, and irradiated with deep red light or blue light from a slide projector with a 1-kW tungsten lamp. The whole surface of the cuvette was exposed to light. Deep red light was obtained by a cutoff filter (VR 69, Toshiba), which transmits light at wavelengths longer than 670 nm. Blue light (438 nm) was isolated by a combination of an interference filter (KL 44, Toshiba) and a cutoff filter (VO 42, Toshiba). Light intensities measured by a thermopile (Kipp and Zonen, Type CA1) at the place of the cuvette were 2.6×10^5 erg cm^{-2} s^{-1} for >670-nm light and 9.4×10^2 erg cm^{-2} s^{-1} for 438-nm light, respectively.

pH Measurement and Adjustment. pH was measured in a Radiometer pH meter, Type 26, with a glass electrode (G2222C) together with a calomel electrode (K4112). The pH value of the purple membrane suspension before addition of HCl was ~6.

One-fiftieth volume of HCl of a known concentration was mixed rapidly with purple membrane suspension. The pH was decreased to 3.2, 2.9, 2.4, and 1.5 at 20 min after addition of 0.02, 0.04, 0.1, and 1 M HCl, respectively. Since the pH changed gradually with time, acid conditions were expressed by the concentration of HCl added.

The sample in acid solution was brought to pH 9.0 ± 0.2 by adding 0.04 volume of 0.25 M sodium phosphate buffer, pH 9.0, containing NaOH, the amount of which was previously determined to just neutralize the HCl contained in the acidic suspension.

Analysis of Retinal Isomer Composition. The method for extraction of chromophore retinal and its isomer composition analysis by means of high-performance liquid chromatography (LC) were based on the procedures described by Pettei et al. (1977) and by Maeda et al. (1979). The total volume of the sample was adjusted to 1 mL by adding distilled water. The acidic sample was brought to pH 9 as described under pH Measurement and Adjustment, and then 1 mL of dichloromethane (Merck, spectral grade) was added. The whole content in the tube was mixed vigorously 3 times with a syringe. bR underwent denaturation by this procedure. Then 0.1 mL of 10% acetic acid was added and the whole content was further mixed repeatedly. Three milliliters of petroleum ether was added, and the retinal was extracted by vigorous mixing with a Pasteur pipet. This step was at 10 min after addition of dichloromethane. The same extraction procedures were repeated 3 times by adding 1 mL of dichloromethane, mixing, and extracting with petroleum ether. We observed some isomerization of 13-*cis*- to *all-trans*-retinal in the sample of the fifth or latter cycles of extraction. Therefore, the extraction was stopped at 40 min (the fourth cycle) from the beginning. The procedures after extraction and LC analysis were the same as those described previously (Maeda et al., 1979). All the procedures were carried out under dim red light with the sample on ice. The results from the four extractions were averaged and expressed with the standard deviation. Because the ratios for the successive extracts coincided well with one another, the averaged values were regarded as representative of the isomer composition in spite of the low yield (several percent for each extract).

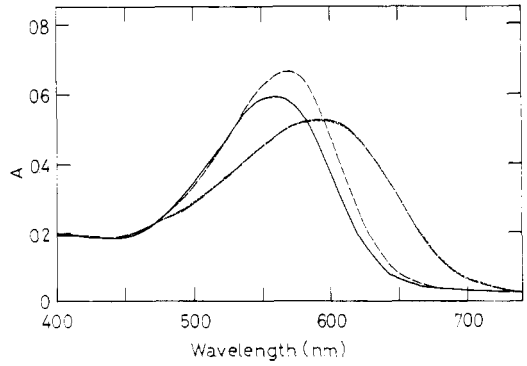

FIGURE 1: The absorption spectra of (—) bR^D, (– – –) bR^L, and the acidified forms derived from (–·–) bR^D and (···) bR^L in 67% glycerol at 3 °C. Acidification was done by allowing bR^D or bR^L to stand in 8×10^{-4} M HCl for 24 h at 3 °C.

Preparation of Artificial Pigments with Cattle Opsin. 11-*cis*- and 9-*cis*-retinal fractions from LC were collected. Solvent was evaporated by a stream of dry nitrogen gas. Cattle opsin, kindly provided by Dr. Y. Shichida in our laboratory, was mixed with the dried retinal fraction, and the mixture was incubated for 90 min in the dark. Then hydroxylamine was added to a final concentration of 0.05 M in the dark. Spectra were measured at 25 °C before and after light irradiation, and the difference spectrum was obtained by calculation from the absolute spectra.

Results

Purple Membrane in Acid. bR^D in 67% glycerol was allowed to stand in 8×10^{-4} M HCl for 24 h at 3 °C, and the spectrum was measured at 3 °C (Figure 1). λ_{max} was found at 595 nm. bR^L gave the same spectrum under these conditions. The acid-induced products from bR^D in 2×10^{-3} M HCl and 4×10^{-4} M HCl passed the same isosbestic point, and the extent of the transformation determined from the difference of A_{630nm} was ~95 and ~90%, respectively. The spectrum in 2×10^{-2} M HCl did not cross with the isosbestic point described above, and the difference of A_{630nm} lowered to 80%.

Each acid-induced sample was then brought to pH 9 at 3 °C as described under Materials and Methods. Since the

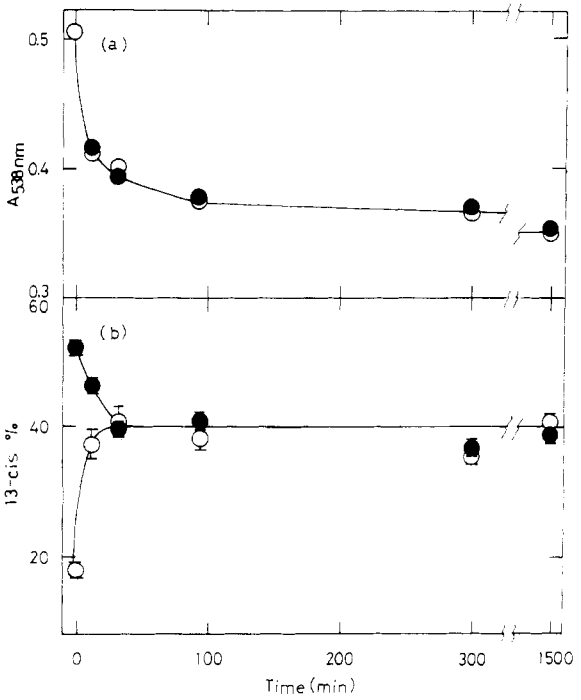

FIGURE 2: The courses of the transformation of (●) bR^D and (○) bR^L in 8×10^{-4} M HCl. (a) A_{538nm} and (b) percentage of 13-*cis*-retinal content are plotted against time. The standard deviation is shown by bars.

spectrum of bR^L remained unchanged within 60 min under these conditions, no thermal isomerization was expected to occur over a period for the subsequent spectral measurements and the extraction of retinals. The spectrum thus obtained gave λ_{max} at a slightly longer wavelength as compared with that of bR^D along with some rise of the absorbance in a shorter wavelength region. On incubation for 24 h at 20 °C, the samples acidified over a range of HCl concentration from 4 $\times 10^{-4}$ to 2×10^{-3} M returned nearly completely to bR^D, but the extent of recovery was ~95% when bR was treated in 2 $\times 10^{-2}$ M HCl.

LC analysis on the retinal extracted from acid-treated bR, either derived from bR^D or bR^L, gave 13-*cis*- and *all-trans*-retinals in a molar ratio of 40:60 (±1%), whereas the molar ratio observed for bR^D was 57:43 (±1%) and that for bR^L was 15:85 (±3%). The results on acid-treated bR were obtained with the retinals extracted from the sample adjusted to pH 9. The same ratio of the isomers was obtained when the retinals were extracted directly from the purple membrane suspended in acid. In this case, however, trace amounts of 11-*cis*- and 9-*cis*-retinals were found probably due to acid-catalyzed isomerization. The nearly same results on the isomer ratio were obtained for purple membrane over a range of HCl concentration from 4×10^{-4} to 2×10^{-2} M.

Figure 2 shows the kinetics on the acid-induced transformation of bR^D and bR^L in 8×10^{-4} M HCl. A_{538nm}, the absorbance at an isosbestic point for bR^D–bR^L conversion, decreased with the transformation of bR to its acid form (see Figure 1). No striking difference was noticed between the rate starting from bR^D and that from bR^L. The reaction proceeded very rapidly within 10 min and then proceeded slowly. A similar kinetic process was reported by Druckmann et al. (1979). Overall rates increased with increasing the concentration of HCl to 2×10^{-2} M and became very small at 4×10^{-4} M HCl. Isomer composition analyzed by LC almost attained a final constant level (approximate ratio of 13-cis and all-trans = 4:6) within the first 30 min in 8×10^{-4} M HCl

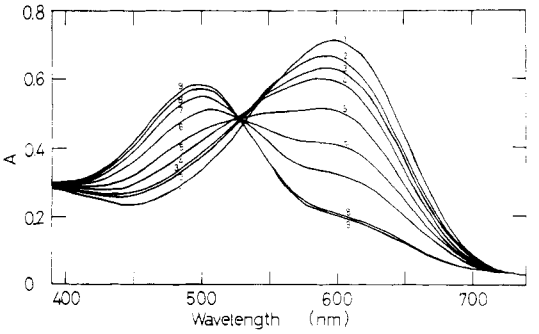

FIGURE 3: Spectra of bR in 67% glycerol in the course of light-induced transformation at 3 °C. (Curve 1) 24 h after addition of 2×10^{-3} M HCl. After irradiation with >670-nm light for (curve 2) 10, (curve 3) 20, (curve 4) 40, (curve 5) 80, (curve 6) 160, (curve 7) 320, (curve 8) 640, and (curve 9) 960 min.

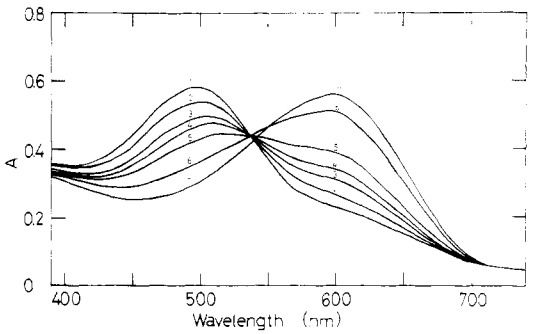

FIGURE 4: Spectra in the course of the light-induced backward reaction from the blue-shifted product. bR treated in 2×10^{-3} M HCl was first irradiated with >670-nm light for 960 min (curve 9 in Figure 3). Curve 1 in this figure: An aliquot of this sample was transferred another cuvette of 2 mm in width. After irradiation with 438-nm light for (curve 2) 10, (curve 3) 20, (curve 4) 40, (curve 5) 80, (curve 6) 160, and (curve 7) 320 min.

on bR^D and also on bR^L. On the other hand, even after 30 min, the decrease of A_{538nm} continued and λ_{max} of the difference spectrum before and after addition of HCl moved slightly to shorter wavelength around 635 nm.

Formation of 9-cis- and 11-cis-Retinal bR. Purple membrane was allowed to stand in various concentrations of HCl for 24 h at 3 °C and then was irradiated with deep red light (>670 nm) at 3 °C. The spectral changes of the sample in 2×10^{-3} M HCl are shown in Figure 3. The absorption band at 595 nm decreased gradually with the concomitant appearance of a new band around 500 nm. An apparent isosbestic point was found at 528 nm (curves 5–9 in Figure 3), except that the spectral curves (curves 2–4 in Figure 3) crossed with the original one (curve 1 in Figure 3) above 530 nm at the initial step of photoconversion. The deviation of the crossing points at the initial step was more pronounced when the sample was irradiated in 2×10^{-2} M HCl and became less pronounced in 8×10^{-4} M HCl.

Irradiation of the blue-shifted product with blue light (438 nm) generated the species with λ_{max} at 595 nm (Figure 4). The spectral shape was similar to that of bR in acid before irradiation (curve 1 in Figure 3). However, the height of this band did not return to the original level and an apparent isosbestic point found at 535 nm (curves 1–6 in Figure 4) was somewhat different from that at 528 nm observed in the process of the forward reaction (Figure 3). These results as well as the deviation of the crossing point at the latest time in the course of the backward reaction (curve 7 in Figure 4) could be partly due to some destruction caused by acidic conditions, but this was not further pursued.

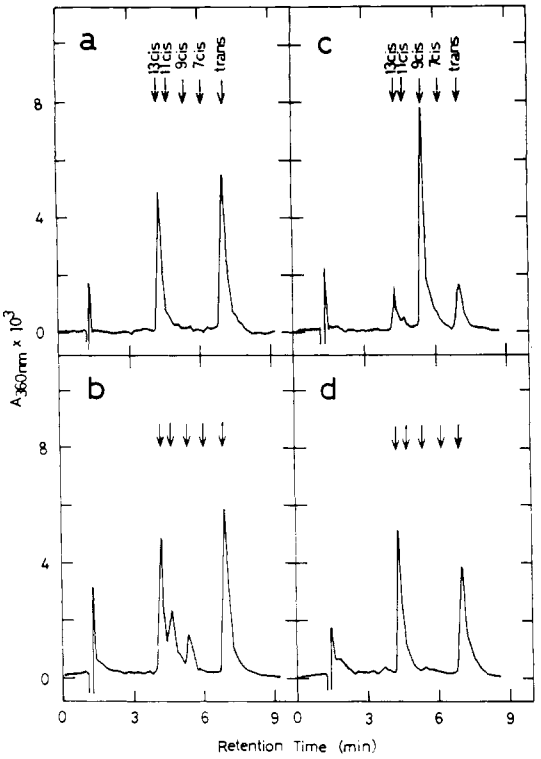

FIGURE 5: LC patterns of retinal isomers extracted from a portion of the following samples shown in Figures 3 and 4. (a) bR allowed to stand in 2×10^{-3} M HCl for 24 h at 3 °C (curve 1 in Figure 3). (b) The sample of (a) was irradiated with >670-nm light for 40 min (curve 4 in Figure 3). (c) The sample of (b) was irradiated further for a total of 960 min (curve 9 in Figure 3). (d) The sample of (c) was irradiated with 438-nm light for 320 min (curve 7 in Figure 4). Arrows indicate the retention times of respective isomers. The first spike in the chromatogram indicates a solvent front.

The retinal extract from unirradiated bR in 2×10^{-3} M HCl showed two peaks in the LC system (Figure 5a), which were identified to be 13-*cis*- and *all-trans*-retinals from their retention times, bR in acid was then irradiated with deep red light (>670 nm) for 960 min (Figure 5c). A large peak which was absent in the extract from the unirradiated sample (Figure 5a) was found. This material corresponds to 9-*cis*-retinal from its retention time. Another peak besides the 9-*cis*-retinal peak was found at an early time of irradiation (Figure 5b) and was assigned to be 11-*cis*-retinal. Each peak material was separately collected and mixed with cattle opsin to construct pigments. 11-*cis*- and 9-*cis*-retinal fractions extracted from purple membrane irradiated in 2×10^{-2} M HCl produced pigments having λ_{max} at 498 and 485 nm, respectively (data not shown). These values are just the same as those of cattle rhodopsin (498 nm) and isorhodopsin (485 nm), respectively (Yoshizawa, 1971). These results also exclude the possibility that the retinal with the retention time of 11-*cis*-retinal may be 9,13-di-*cis*-retinal, which was reported to have a retention time close to 11-*cis*-retinal but produces a pigment having λ_{max} at 483 nm (Crouch et al., 1975). Extensive irradiation of the 9-*cis*-retinal pigment of bR with 438-nm light caused the complete disappearance of the 9-*cis*-retinal peak (Figure 5d). This is consistent with the results obtained spectroscopically (see Figure 4).

A further increase of HCl to 1 M reversed the spectral shift of unirradiated bR back to shorter wavelengths (λ_{max} at 556 nm), as was shown by Lozier et al. (1978). The acidification and the subsequent irradiation with >610-nm light caused decreases in the content of 13-*cis*-retinal to 23 ± 2% and 12 ± 2%, respectively, with corresponding increases in the content

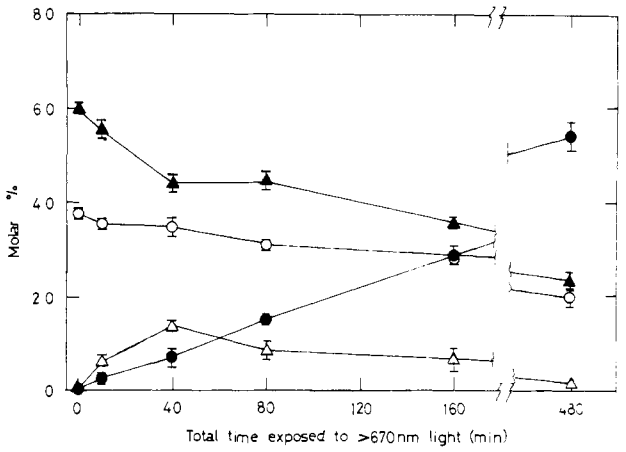

FIGURE 6: Changes of retinal isomer composition during irradiation of bR in 2×10^{-3} M HCl: (○) 13-*cis*-retinal; (△) 11-*cis*-retinal; (●) 9-*cis*-retinal; (▲)) *all-trans*-retinal. The standard deviation is shown by bars.

of *all-trans*-retinal. No 9-*cis*- and 11-*cis*-retinal formation was noticed, however, with either >610-nm light or 526-nm light. These isomers were also not found when bR without acid was irradiated.

As shown in Figure 6, the amount of 11-*cis*-retinal found at early times of irradiation in 2×10^{-3} M HCl decreased with further exposure to light and disappeared at the final step, while the amount of 9-*cis*-retinal increased steadily. The amount of 11-*cis*-retinal was greater than that of 9-*cis*-retinal within 40 min, and then the amount of 9-*cis*-retinal became greater. These results may explain the deviation of the crossing point during the first 40 min (curves 2–4 in Figure 3). The amount of 13-*cis*-retinal decreased in parallel with that of *all-trans*-retinal during irradiation.

The product formed on irradiation in 8×10^{-4} M HCl with deep red light for 960 min was a mixture composed of 10 ± 2% of 13-*cis*-retinal, 75 ± 3% of 9-*cis*-retinal, and 15 ± 2% of *all-trans*-retinal and contained less than 1% of 11-*cis*-retinal. The spectrum of the 9-*cis*-retinal pigment was then roughly estimated by subtracting 25% of the spectrum of unirradiated bR in acid from the spectrum of the overall mixture, with the assumption that the ratio of 13-cis to all-trans isomer was not grossly changed and that the spectra of these pigments were unchanged on irradiation. The first assumption seems to be valid (see Figure 6). The 9-*cis*-retinal pigment thus calculated has λ_{max} at 495 nm (Figure 7a). A similar analysis was applied to the spectrum of the mixture formed by irradiating bR in 2×10^{-2} M HCl for 80 min. This mixture contained 26 ± 2% of 13-*cis*-retinal, 17 ± 1% of 11-*cis*-retinal, 15 ± 2% of 9-*cis*-retinal, and 42 ± 3% of *all-trans*-retinal pigments. Although the calculated spectrum may involve some uncertainty mainly due to a small percentage of 11-*cis*-retinal content, λ_{max} of the 11-*cis*-retinal pigment could be estimated to lie in the region around 560 nm (Figure 8).

The 9-cis-Retinal Pigment at pH 9. The mixture containing 13-*cis*-, 9-*cis*-, and *all-trans*-retinal pigments prepared as described above and also the unirradiated purple membranes in acid were brought to pH 9. These spectra are shown in Figure 7b together with a spectrum of the 9-*cis*-retinal pigment at pH 9, which was calculated by subtracting the fraction contributed by the starting material consisting of 13-*cis*- and *all-trans*-retinal pigments in the same way as was done in Figure 7a. The resulting spectrum was very smooth in shape, having λ_{max} at 450 nm. Its molar absorbance was about half of that of the unirradiated sample.

The 9-*cis*-retinal pigment brought to pH 9 was sensitive to

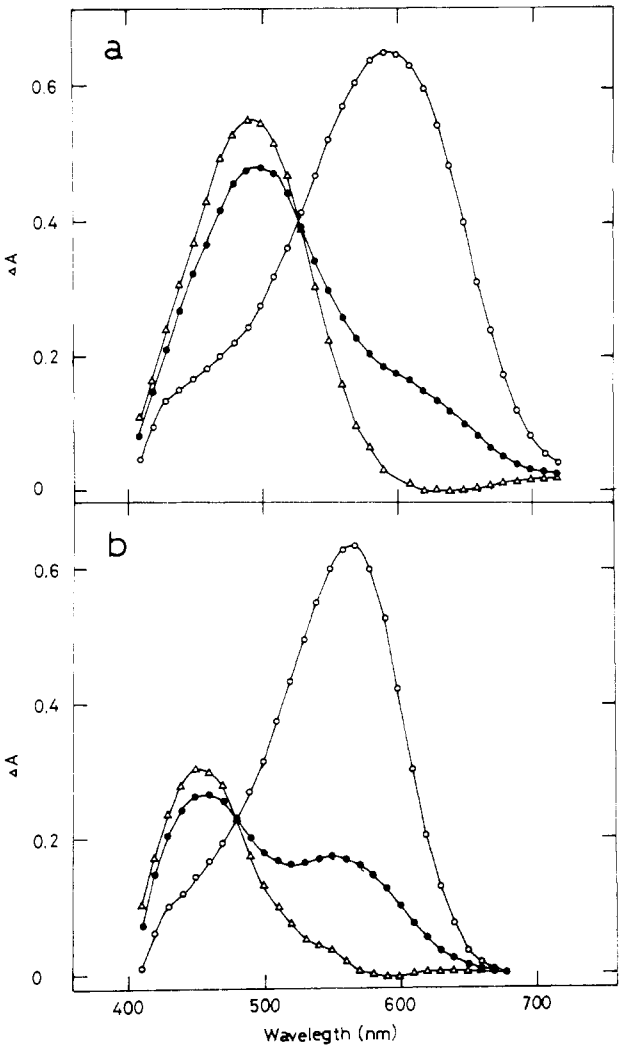

FIGURE 7: A calculated spectrum of the 9-*cis*-retinal pigment produced with light in acid. (a) The spectrum of (○) bR in 8×10^{-4} M HCl and (●) its irradiated product with >670-nm light. Both were depicted as the difference spectra to the CTAB-bleached sample in order to minimize errors due to light scattering and red membranes contained in the sample. (△) The spectrum of "pure" 9-*cis*-retinal pigment was calculated by subtracting 25% of (○) from (●) and then multiplying by 100/75 the spectrum after subtraction. (b) The spectra of bR samples observed by bringing the respective acid-treated samples in (a) to pH 9. The procedures for calculation and notations are same as those in (a).

438-nm light and generated a spectrum similar to that of bR^L (Figure 9). It also underwent dark adaptation after being kept at 37 °C. Both samples before and after irradiation with 438 nm light were kept in 0.05 M hydroxylamine in the dark, and the change of the absorbance at their respective λ_{max} was followed at 3 °C (Figure 10). The product generated by 438-nm light irradiation was completely resistant to the action of hydroxylamine, as was known for normal bR under neutral conditions. In contrast, the 450-nm peak of the 9-*cis*-retinal pigment decreased gradually with the concomitant appearance of the absorption band around 360 nm due to retinal oxime.

Discussion

Lozier et al. (1978) incorporated purple membranes into acrylamide gels for the purpose of avoiding aggregation in acid. Here, the spectral properties of bR in acid were satisfactorily studied by use of purple membranes in 67% glycerol at 3 °C without serious problems arising from precipitation. The λ_{max} of bR in 4×10^{-4} to 2×10^{-2} M HCl was found to be around

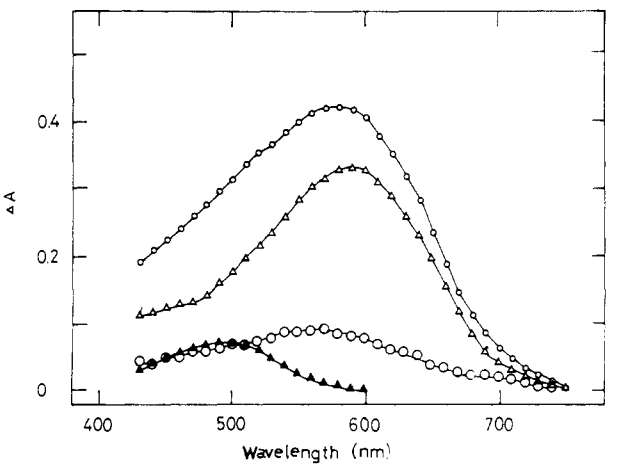

FIGURE 8: A calculated spectrum of the 11-*cis*-retinal pigment produced with light in acid. (○) The spectrum of the product by irradiating bR in 2×10^{-2} M HCl for 80 min with >670-nm light. (△) Spectral contribution from untransformed bR which comprises 68% (26% of 13-cis plus 42% of all-trans; see the text) of the sample is depicted as 68% of the starting material of acid-treated bR. (▲) Spectral contribution from the 9-*cis*-retinal pigment which comprises 15% of the product is depicted as 15% of the 9-*cis*-retinal pigment shown in Figure 7a. (◯) The spectrum of 11-*cis*-retinal pigment was then calculated by subtracting (△) and (▲) from (○). The curve was not normalized as was done for Figure 7.

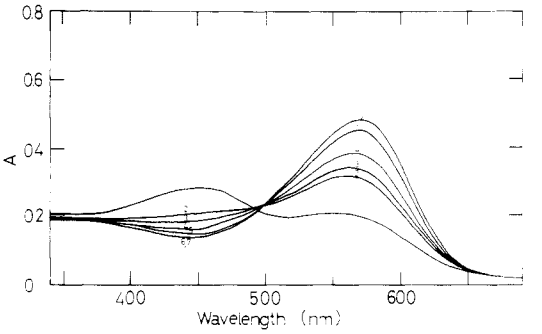

FIGURE 9: Spectra in the course of the light-induced generation of bR-like pigment from the 9-*cis*-retinal pigment at pH 9. bR in 8×10^{-4} M HCl was first irradiated with >670-nm light as described in Figure 3. The sample was brought to pH 9 (curve 1). This was irradiated with 438-nm light for (curve 2) 10, (curve 3) 20, (curve 4) 40, (curve 5) 80, (curve 6) 160, and (curve 7) 320 min.

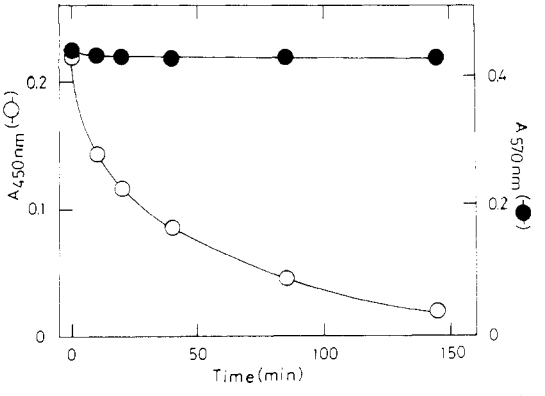

FIGURE 10: The course of the reaction with 0.05 M hydroxylamine at pH 9. (○) The change of the absorbance at 450 nm, the λ_{max} of the 9-*cis*-retinal pigment at pH 9, with the sample containing the 9-*cis*-retinal pigment. (●) The change of the absorbance at 570 nm, the λ_{max} of the light-induced pigment, with the light-generated product.

600 nm. In accordance with the previous results by other authors (Oesterhelt & Stoeckenius, 1971; Moore et al., 1978; Lozier et al., 1978), an acid-induced shift of the spectrum to

longer wavelength was observed. The amount of untransformed bR in acid could be estimated from the amount of the light-induced intermediates formed therefrom. In our preliminary experiments, batho-bR produced from bR in acid at −190 °C was at most 5% of that found at neutral pH values. Furthermore, an almost identical final spectrum was observed over the wide range of HCl concentrations used. It is therefore likely that nearly complete transformation to the acidic species was attained in the present experiments.

Although bR^D and bR^L gave the same final spectrum in acid, the spectral change at very early times after addition of HCl was complex due to overlapping of the blue shift caused by isomerization from *all-trans*- to 13-*cis*-retinal and the red-shift caused by transformation to the acidic form of bR. Some differences between the spectrum of the acidified sample derived from bR^D and that from bR^L observed by Lozier et al. (1978) could be due to that their spectral measurements were done before the transformation was complete.

Under acidic conditions, rapid isomerization of retinals occurred and a unique equilibrium state in the dark was established with a smaller content of 13-*cis*-retinal than that of bR^D under neutral pH values. This is consistent with a recent result by Mowery et al. (1979). A small amount of 13-*cis*-retinal in bR^L, which was not found in the previous experiments (Maeda et al., 1977), was detected. Since the present experiments were done in unbuffered 67% glycerol of pH 6, the rate for the dark adaptation may be somewhat greater than that in 10 mM phosphate buffer of pH 7 (Ohno et al., 1977), in which the previous experiments were conducted. A small amount of 13-*cis*-retinal was, however, also found in bR^L at pH 7 by Mowery et al. (1979). The content of 13-*cis*-retinal in bR^D was slightly greater than that observed previously (Maeda et al., 1977). Since the samples were left in CTAB before addition of dichloromethane in our previous experiments, a slight difference in the state of the sample before the extraction of retinals might partly affect these results. These discrepancies, however, are presently unsolved.

Any way, both bR^D and bR^L converged rapidly into the same product in view of the spectral data and the retinal isomer composition. Because of a rapid isomerization rate in acid (Ohno et al., 1977), it is quite difficult to prepare samples containing only *all-trans*-retinal from bR^L. Therefore, bR^D was chosen as a starting material for experimental convenience.

On irradiation of acidified bR with deep red light under acidic conditions, 9-*cis*- and 11-*cis*-retinal pigments, hitherto undetected pigments from native bR, were found to be produced. These pigments were still photosensitive and were transformed to other pigments including the 13-*cis*- and *all-trans*-retinal pigments on irradiation with appropriately selected light.

Schreckenbach et al. (1977) observed the transient formation of a 430-/460-nm pigment from the reaction of *all-trans*-retinal with apopurple membranes. This pigment has nearly the same λ_{max} as that of the protonated Schiff base, implying few interactions of retinal moiety with protein. The 9-*cis*-retinal pigment described here was susceptible to hydroxylamine at pH 9. It will be supposed that the Schiff base portion was exposed to the environmental aqueous phase when the pigment was brought to pH 9. The 9-*cis*-retinal pigment at pH 9 had a smooth spectrum in contrast to that described by Schreckenbach et al. (1977), which had shoulders at 400, 430, and 460 nm. The 9-*cis*-retinal pigment here described was sensitive to light and returned to the normal 13-*cis*- and *all-trans*-retinal pigments under acidic and presumably weakly alkaline conditions. The 11-*cis*-retinal–apopurple membrane complexes constructed from apomembranes and 11-*cis*-retinal also form normal bR on irradiation (Oesterhelt & Schuhmann, 1974).

Similar blue-shift transient species at low pH values have been observed in flash photolytic experiments by Lozier et al. (1978). They observed the changes of crossover points in the difference spectrum from 540 to 520 nm between 80 μs and 10 ms after the flash. This correlates with our experiments in which at least two different pigments were detected. In contrast to these transients, however, our 9-*cis*-retinal and 11-*cis*-retinal pigments are quite stable products. Relations between these spectrally similar products shound be explored in further studies. Quantum efficiencies for the formation of our 9-*cis*- and 11-*cis*-retinal pigments have not accurately been measured but definitely are very low. It is likely that the transient species described by Lozier et al. (1978) may have some structural resemblance to the 9-*cis*- and 11-*cis*-retinal pigments and these pigments may be formed in a low probability from the transients.

In visual pigments the chromophore retinal isomerized to the 7-*cis*-retinal isomer in an environment which allowed the formation of lumirhodopsin (Maeda et al., 1979) but not in an environment which allowed only bathorhodopsin formation. These facts imply a strongly unique protein conformation in bathorhodopsin. It has been argued that bR in acid is a form of batho-bR from spectral resemblance (Lewis et al., 1978; Mowery et al., 1979). It is interesting that the formation of the 9-*cis*- and 11-*cis*-retinal pigments was only noticeable under acidic conditions where some negatively charged residues in purple membrane are blocked or positively charged residues lose their countercharges. The residues which are responsible for the low pH effect could be carboxyl groups in bR. The unique isomerization pattern in acid may be a consequence of such changes in the electrical environment of the retinal chromophore. In this respect it is worth noting that the light-induced formation of 7-*cis*-, 9-*cis*-, and 11-*cis*-retinals from *all-trans*-retinal is much greater in polar organic solvents than in apolar ones (Denny & Liu, 1977). Alternatively, the changes in charge distribution may result in a conformation change in the vicinity, of the chromophore and may make the space which allows accommodation of the 9-*cis*- or 11-*cis*-retinal isomer on irradiation. These alternatives should be explored further.

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Human Fibroblastoid Interferon: Immunosorbent Column Chromatography and N-Terminal Amino Acid Sequence†

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ABSTRACT: Three mice and one rabbit were inoculated with purified human fibroblastoid interferon. Neutralizing activity to human fibroblastoid interferon was observed in the serum of these animals with the rabbit showing the highest anti-interferon titers (10^6 neutralizing units/mL). Rabbit antiserum was coupled to cyanogen bromide activated Sepharose, and the resulting material was tested for use in the purification of human fibroblastoid interferon. Pure interferon obtained by this procedure was analyzed, and we report the sequence of the first 13 N-terminal amino acid residues of this protein.

In order to determine the primary structure of a scarce protein like human interferon, it is necessary to fulfil a few requirements: to maximize the cellular production of the protein (Tan et al., 1970; Ho et al., 1973; Zoon et al., 1980; Cantell & Hirvonen, 1977), to increase the sensitivity of amino acid sequence methodologies (Reinbolt et al., 1977; Hunkapiller & Hood, 1978, 1980; Wittmann-Liebold et al., 1977), and to increase the efficiency of the purification of the protein (Tan et al., 1979, 1980; Berthold et al., 1978; Knight, 1976; Knight et al., 1980; Edy et al., 1976; Davey et al., 1974; Len et al., 1978; Zoon et al., 1979; Rubinstein et al., 1978, 1979). Much effort has been made in the cellular production of interferon, and rapid advances have been made in the area of microsequencing proteins at the subnanomole to nanomole range (Hunkapiller & Hood, 1980). However, the available procedures for isolating pure interferon from serum containing a crude interferon preparation provide a yield of 1–10% of the pure protein. In this paper we report an efficient method using an immunosorbent column for the purification of human fibroblastoid interferon which provides a recovery of 30% or more pure interferon from a serum-containing preparation and 70% when the interferon is prepared in serum-free medium. The new procedure has produced preparations (400–1000 pmol) of pure interferon for its amino acid sequence analysis.

Experimental Procedures

Interferon Production and Assay. Interferon was produced and concentrated from a C-10 cell line as previously described in serum-containing or serum-free medium (Tan et al., 1979) and assayed on human fibroblasts according to a semimicro-method (Tan, 1975). Titers were determined by visual assessment of the viral cytopathic effect. A human fibroblast preparation referenced to 69/19 MRC human leukocyte interferon was used as the interferon standard. This standard was kindly tested and supplied by Dr. W. Merk and Dr. G. Bodo.

Protein for Inoculation. Pure interferon for inoculation was prepared by the method of Tan et al. (1979) and Berthold et al. (1978). The purity of interferon was verified by analytical slab polyacrylamide gel electrophoresis (Figure 1).

Generation of Antibodies. Three BALB/c mice were each inoculated with 10–20 μg of the purified interferon preparation weekly for 3 months. The purified antigen was mixed 1:1 v/v with Freund's adjuvant and injected intraperitoneally. One New Zealand White rabbit was inoculated with the same amount of antigen on a regimen similar to that for the mouse, except that in the first eight inoculations Freund's adjuvant was omitted and the routes of inoculation were different as indicated in Fugure 2. Test sera were obtained from the animals once weekly thereafter.

For determination of interferon neutralizing activity, an aliquot of the test serum diluted 100-fold was incubated with an equal volume of human interferon. After the incubation, the mixture was assayed for antiviral activity as described above. The neutralizing titer was obtained by first finding the well in which half the assay cells were protected; the value of the original interferon activity in that well was then multiplied by 200, the serum dilution factor. For example, if 50% protection occurred in a well containing 1×10^3 units of antiviral activity (prior to incubation), then the neutralizing titer of that serum would be 2×10^5 units/mL. Antiviral units were standardized as described above.

Preparation of Immunosorbent Column. Rabbit antiserum was treated with 40% ammonium sulfate. The precipitated immunoglobulin fraction was sedimented by centrifugation at

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