SQUID RETINOCHROME

Configurational Changes of the Retinal Chromophore

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ABSTRACT The configurations of the retinal chromophore in light and dark reactions of squid retinochrome were investigated by means of high-performance liquid chromatography. Orange light isomerized the chromophore of retinochrome, all-*trans*-retinal, mainly to the 11-*cis* configuration in metaretinochrome. Irradiation with shorter-wavelength lights not only accelerates the photoreversal of metaretinochrome to retinochrome but also leads to a slight production of isoretinochrome (13-*cis*-retinochrome), yielding a photoequilibrium mixture of three kinds of retinochrome. 13-*cis*- and 9-*cis*-retinochromes are photosensitive, and are converted into metaretinochrome upon irradiation with orange light. When steadily exposed to orange light in the presence of a trace of retinochrome-protein, all of the all-*trans*-, 13-*cis*-, and 9-*cis*-retinals are catalytically isomerized only to the 11-*cis* form, although the reaction rate is reduced in the order of the retinals listed above. In the dark, 9-*cis*-retinochrome, like retinochrome, remains unchanged, but both meta- and 13-*cis*-retinochromes slowly change to retinochrome. The chromophore of 13-*cis*-retinochrome changes directly to the all-*trans* form, whereas the 11-*cis* chromophore of metaretinochrome goes to all-*trans* mainly through the 13-*cis* form. The direct isomerization from 11-*cis* to all-*trans* hardly occurs at temperatures as low as 20°C, and shows high values of the activation enthalpy and entropy changes. Based upon these findings, the role of retinochrome in the photoreception of the visual cells is discussed.

INTRODUCTION

The visual cells of cephalopods contain retinochrome in addition to rhodopsin. Retinochrome differs from rhodopsin in many respects, as has been revealed by accumulated studies of absorption characteristics, photochemical behavior, biochemical properties (cf. Hara and Hara, 1972, 1973*a*, 1982), and location in the visual cells (cf. Hara and Hara, 1976, 1982). When irradiated with green light at liquid-nitrogen temperature, *Todarodes* retinochrome (λ_{max} = 496 nm, −190°C) is converted mainly to an intermediate lumiretinochrome with a slightly lower absorbance at λ_{max} 475 nm, its retinal chromophore being changed from the all-*trans* to the 11-*cis* form. Unlike rhodopsin, retinochrome irradiated with blue light at −190°C does not produce a batho-type intermediate with a peak at a wavelength longer than its own λ_{max}. On warming to above −20°C in the dark, lumiretinochrome is further converted into metaretinochrome with a λ_{max} of 470 nm (Hara et al., 1981). Metaretinochrome has been shown to be a photoproduct of retinochrome at room temperature. Either of the photoproducts, lumi- or metaretinochrome, is partially but rapidly regenerated back to retinochrome by reirradiation (photoreversal), reaching a photoequilibrium mixture whose component amounts depend on the wavelength of the irradiating light (cf. Hara and Hara, 1972). When left alone in the dark, metaretinochrome also returns slowly to retinochrome (spontaneous regeneration), as observed by Hara and Hara (1969) and Sperling and Hubbard (1975). Such a regeneration without light does not take place in the rhodopsin system. When incubated in the dark with all-*trans*-retinal, metaretinochrome is changed quickly to retinochrome (dark regeneration), as demonstrated by Hara and Hara (1968). Furthermore, the free protein moiety of retinochrome, aporetinochrome, has the capacity of coupling with various geometrical isomers of retinal (13-*cis*, 11-*cis*, 9-*cis* and all-*trans*) to synthesize particular pigments (dark synthesis), each of which shows highly individual absorption in the visible range (Hara and Hara, 1973*a*, *b*).

The properties of retinochrome described above have been found mainly by means of spectrophotometry. The isomerization behavior of the retinal chromophore has been studied qualitatively and quantitatively using the coupling capacity of cattle opsin for the 11-*cis*- and 9-*cis*-retinals and by the coupling capacity of aporetinochrome for the all-*trans* and 13-*cis* isomers. Recently, it has been shown that the stereoisomeric composition of retinal can be determined by high-performance liquid

chromatography. In the present study,[1] new findings on the light and dark reactions of retinochrome and metaretinochrome (for example, photoreversal and regeneration) are added to our earlier investigations. A main purpose of this paper is to elucidate the property of photopigments synthesized with 13-*cis*- and 9-*cis*-retinal and to describe the behavior of the retinal chromophore during the spontaneous regeneration characteristic of the retinochrome system.

METHODS

Preparation of Retinochrome

The squids, *Todarodes pacificus*, were collected during the night from the southwestern waters of the Japan Sea. The method for extracting retinochrome is the same as described previously (Ozaki et al., 1982; Hara and Hara, 1982). All operations were carried out at ~4°C under dim red light. The bisected posterior half of the dark-adapted eye was shaken in 67 mM phosphate buffer, pH 6.5, to detach the outer segments of the visual cells. The remaining retina, consisting mainly of the inner segments, was lifted out, homogenized with the same buffer, and centrifuged. The pellet was then suspended in a 43% (wt/vol) sucrose solution at pH 6.5 and centrifuged for 15 min at 12,000 rpm to float retinochrome-rich cell fragments. They were washed successively with sodium carbonate, water, Weber-Edsall's solution (0.6 M KCl, 40 mM $NaHCO_3$, and 10 mM Na_2CO_3) and petroleum ether, and finally extracted with a 2% aqueous solution of digitonin. The pH of the retinochrome extract was between 5 and 6. For experiments, the extract was diluted with 67 mM phosphate buffer to adjust it to the desired concentration and pH.

Preparation of Aporetinochrome

The protein moiety of retinochrome, aporetinochrome, was prepared by a modification of the method described by Hara and Hara (1973*b*). Retinochrome extract at pH 6.5 was irradiated at 0°C alternatively with orange light (10 min) and with intense near-ultraviolet light from a 500 W Xenon lamp passed through a Toshiba UV-D2 black glass (1–2 h), until the solution became colorless. These irradiations gradually destroyed the retinal chromophore, but scarcely damaged aporetinochrome. This technique is not only convenient but is useful in assessing the density and purity of retinochrome and also of colorless aporetinochrome.

Irradiation and Absorption Measurements

For irradiation of the experimental samples, a 100 W tungsten filament projector lamp shielded by a 8-cm water cell was used for a light source. Orange light exceeding 560 or 530 nm in wavelength was selected by either a Toshiba V-O 56 or V-O 53 cutoff glass; the former was used unless otherwise specified. Monochromatic lights of 480 and 420 nm were isolated by the combination of an interference and a glass filter (Toshiba KL-48 with Toshiba V-Y 46 and Toshiba KL-42 with Hoya L-40, respectively). Near-ultraviolet light (maximally 360 nm) was obtained from the same light source shielded by a Toshiba UV-D2 black glass. Absorption measurements were performed with a Hitachi model 323 (Hitachi Corp., Tokyo) or a Shimazu model UV-202 (Shimazu Corp., Kyoto) recording spectrophotometer.

[1]Preliminary accounts have been presented elsewhere (Ozaki et al., 1978), and discussed at the International Symposia on Visual Pigment and Purple Membrane held in Kyoto in September, 1978 and on Transduction Mechanism in Visual Cells held in Ohtsu in November, 1979, and at the fifth ICER (International Congress of Eye Research) held in Veldhoven in October, 1982.

Preparation of Retinal Isomers

All-*trans*-retinal was made from all-*trans*-retinol by our routine method (Hara and Hara, 1968). The retinol was oxidized to retinal by pouring it onto a column of active manganese dioxide, and all-*trans*-retinal was crystallized several times from its concentrated solutions in petroleum ether at about −70°C. Crystalline 13-*cis*- and 9-*cis*-retinals were purchased from the Sigma Chemical Co. (St. Louis, MO). Every crystal was dissolved in *n*-hexane, purified by high-performance liquid chromatography (HPLC), transferred into ethanol, and stored under nitrogen gas at −20°C. The purity of each retinal isomer was >99%. Before use, the stock solution was properly diluted with ethanol so that the concentration of ethanol might be <1.0% in the experimental samples.

Analysis of Retinal Isomers

The analysis of retinal isomers in *n*-hexane was performed with a Hitachi model 635 HPLC system (Hitachi Corp., Tokyo) equipped with a column (250 mm × 4 mm) of silica gel (Lichrosorb SI-60, 5 μm, Merck Chemical Div., Merk & Co., Inc., Rahway, NJ). The adsorbent in the column was preequilibrated with *n*-hexane containing 10% diethyl ether, and the sample was eluted at a constant flow rate of 1.2 ml/min with the same solvent. Five retinal isomers were detected by the absorption at 360 nm with a spectrophotometric detector (Hitachi Corp., Model 100-50). The proportion of the isomers was printed out as a molar ratio by a digital integrator (Takeda-Riken Industry Co. Ltd., Tokyo, Model TR-2200). Extinction coefficients at 360 nm required for computation were found from the absorption spectra measured by Kropf and Hubbard (1970) for 13-*cis*, 11-*cis*, 9-*cis*, and all-*trans* and by DeGrip et al. (1976) for 7-*cis*.

Extraction of the Retinal Chromophore

To study the configuration of the chromophore in various products formed by light and dark reactions of retinochrome with the aid of HPLC, we first examined treatments necessary for releasing the chromophore from retinochrome. The use of dichloromethane (CH_2Cl_2) has been recommended for detaching the 11-*cis* chromophore from rhodopsin without thermal isomerization (Pilkiewicz et al., 1977). Modified chemical treatments were therefore tested and compared with thermal treatment. 1 ml cold CH_2Cl_2 (0°C) was added to 1 ml of retinochrome extract at pH 6.5 (absorbance at λ_{max} 495 nm = 1.4), emulsified by a syringe in ice, and centrifuged to yield a lower layer of CH_2Cl_2 that included retinal. This layer was withdrawn, dried over anhydrous sodium sulfate (Na_2SO_4), and evaporated completely with a gentle stream of N_2 gas. The residual retinal was dissolved in 1.5 ml cold *n*-hexane, concentrated down to ~40 μl, and injected onto the silica gel column of the HPLC system. Methanol (CH_3OH) and sodium dodecyl sulfate (SDS) were also used; 1.5 ml cold CH_3OH or 0.1 ml of a 22% aqueous solution of SDS was mixed with 1 ml of the same retinochrome, emulsified in 1.5 ml cold *n*-hexane by vigorous shaking, and centrifuged to obtain a hexane extract of retinal. The extract was similarly dried, concentrated, and analyzed by HPLC. In the thermal treatment, the sample protein was kept in a water bath at 75°C for 45 s, chilled at once in ice, shaken vigorously for 3 min with 1.5 ml cold *n*-hexane, and centrifuged to separate the hexane extract. As shown in Table IA, when retinochrome extract was treated by heat, the percentage of all-*trans*-retinal amounted to a high value of 94%. However, when retinochrome was denatured with CH_2Cl_2, CH_3OH, and SDS, the percentage of all-*trans*-retinal was distinctly reduced along with a corresponding increase of 13-*cis*-retinal.

To make sure of this point, a separate study was carried out with synthesized pigments, as shown in Table IB. An artificial mixture of all-*trans*, 13-*cis*, and 9-*cis* retinal isomers was added to aporetinochrome to produce a sample consisting of various synthesized retinal pigments. The retinal chromophores were then released from the sample by heat or 60% CH_3OH treatment. As a result, the isomeric composition in the initial mixture of retinal isomers was well preserved even after warming, but the CH_3OH treatment caused a marked decrease of the all-*trans* and an increase of the 13-*cis* form. In the absence of protein, all-*trans*- and

TABLE IA
ISOMER ANALYSIS OF THE RETINAL CHROMOPHORE IN RETINOCHROME EXTRACT SUBJECTED TO THERMAL AND CHEMICAL TREATMENTS AT pH 6.5

Isomer	Warmed at 75°C, 45 s	Treated with		
		CH_2Cl_2	60% CH_3OH	2% SDS
	%	%	%	%
13-*cis*	6	19	23	34
11-*cis*	0	0	0	1
9-*cis*	0	0	0	2
7-*cis*	0	0	0	1
all-*trans*	94	81	77	62

13-*cis*-retinals were not affected by the addition of CH_2Cl_2, CH_3OH, and SDS, and successive extracts of retinals with *n*-hexane from the denatured sample gave a constant ratio of retinal isomers. We think, therefore, that the thermal isomerization from the all-*trans* to the 13-*cis* form takes place during the protein denaturation in the presence of chemical denaturants. In any case, the chemical procedures were not suitable for the isomer analysis in the retinochrome system, so the thermal method was used in the present studies.

The effects of the period of warming and the presence of digitonin were further examined as follows. A single preparation of retinochrome ($A_{495\,nm}$ = 1.4) was divided into two portions, and one was changed to metaretinochrome by exposure to orange light for 10 min. Each portion was further divided into 0.5-ml aliquots, and each aliquot was diluted with 0.5 ml of 4% digitonin. These samples were warmed for different times, cooled in ice, extracted with *n*-hexane, and centrifuged to effect phase separation. The hexane layer was dried, concentrated, and injected onto the HPLC column. The analytical results of all the samples are summarized in Table II. In the case of retinochrome, the high proportion of the all-*trans* isomer after warming for 30 s, 96%, was decreased for longer warming, while the 13-*cis* isomer increased. In the case of metaretinochrome, the high proportion of the 11-*cis* isomer at 30 s, 84%, was decreased as the warming proceeded, while the all-*trans* and 13-*cis* forms increased. These alternations in the isomeric composition indicated that the warming procedure was not completely free of possible thermal isomerization of retinal. The concentration of detergent in the samples was an important factor, and the presence of ~2% digitonin was effective in suppressing the undesired isomerization. When the digitonin concentration was as low as 0.5%, all the results were distorted, as seen in the percentages shown in parentheses in Table II. In particular, the 11-*cis* chromophore of metaretinochrome did not remain stable with low concentrations of digitonin.

According to spectrophotometric inspection, metaretinochrome was entirely bleached 30 s after warming at 75°C: retinochrome was a little more resistant to heat but was bleached almost completely (>95%) after a 45-s warming. For the isomer analysis in the retinochrome system, the procedure of warming for 45 s with 2% digitonin was therefore considered to be the best for denaturing all the pigments and products in the mixture. In any case, the retinochrome extract contains a majority of the all-*trans* isomer (>93%), whereas its irradiated product (metaretinochrome fraction) is rich in the 11-*cis* isomer (>79%). In the text, we shall call the component with the all-*trans* chromophore "retinochrome," and that with the 11-*cis* chromophore at room temperatures "metaretinochrome."

TABLE IB
ISOMER ANALYSIS OF THE RETINAL CHROMOPHORE IN SYNTHESIZED PIGMENTS SUBJECTED TO THERMAL AND CHEMICAL TREATMENTS AT pH 6.5

Isomer	Mixture of retinals	Synthesized pigments (Retinals + aporetinochrome)	
		Warmed at 75°C, 45 s	Treated with 60% CH_3OH
	%	%	%
13-*cis*	43	42	63
11-*cis*	0	1	2
9-*cis*	3	3	5
7-*cis*	0	0	0
all-*trans*	54	54	30

The prior treatment for releasing the retinal chromophore from the samples (except a retinal mixture) was carried out in the presence of ~2% digitonin.

TABLE II
ISOMERIC COMPOSITION OF THE RETINAL CHROMOPHORE IN PREPARATIONS OF RETINOCHROME AND METARETINOCHROME AFTER WARMING FOR DIFFERENT TIMES

Sample (pH 6.5)	Isomer	Warmed at 75°C for			
		30 s	45 s	60 s	75 s
		%	%	%	%
Retinochrome	13-*cis*	4 (8)	7 (10)	11 (14)	13 (16)
	11-*cis*	0 (0)	0 (0)	0 (0)	1 (0)
	9-*cis*	0 (0)	0 (0)	0 (0)	0 (1)
	7-*cis*	0 (0)	0 (0)	0 (0)	0 (0)
	all-*trans*	96 (92)	93 (90)	89 (86)	86 (83)
Metaretinochrome	13-*cis*	4 (10)	6 (12)	10 (15)	—
	11-*cis*	84 (59)	79 (49)	66 (40)	—
	9-*cis*	0 (0)	0 (1)	1 (1)	—
	7-*cis*	0 (0)	0 (0)	0 (0)	—
	all-*trans*	12 (31)	15 (38)	23 (44)	—

The warming of every sample was performed in the presence of ~2% digitonin. For comparison, the results obtained in the presence of ~0.5% digitonin are shown in parentheses.

RESULTS

Photoreaction of Retinochrome and Metaretinochrome

Based upon the spectral observations (Hara and Hara, 1972), it was suggested that metaretinochrome would be driven toward regeneration of retinochrome by short-wave irradiation, and consequently the irradiation of either retinochrome or metaretinochrome would yield an equilibrium mixture consisting of both of them. To examine this idea, the isomer analysis was performed for the photoproducts in the photoequilibrium mixture. When aliquots of retinochrome at pH 6.5 were exhaustively irradiated each with orange (>560 nm), blue (480 nm), violet (420 nm), and near-ultraviolet (~360 nm) light, the photoequilibrium spectra were arranged in an increasing order of the wavelength of irradiating light. However, no distinct isosbestic point was observed among the spectra. The corresponding data for the isomeric composition are pre-

sented in Table III. While the all-*trans* isomer was dominant before irradiation (retinochrome, 94%), the 11-*cis* isomer dominated after irradiation with orange light (metaretinochrome, 79%). The other photoproducts on irradiation with short-wavelength lights contained less 11-*cis* and more all-*trans* isomer. The irradiation at shorter wavelengths promotes an increase of the all-*trans* and a decrease of the 11-*cis* isomer, presenting direct evidence that retinochrome has been photoreversed from metaretinochrome. 13-*cis*-retinal tends to increase gradually as the wavelength of the irradiating light is reduced, indicating that 13-*cis*-retinochrome (previously called isoretinochrome) has been newly produced in the photoequilibrium mixture by irradiation with short-wavelength light. This may be one of the reasons that an isosbestic point did not appear in spectra on irradiation of retinochrome with the varied light.

When all-*trans*-retinal is mixed with a small amount of retinochrome and steadily irradiated with an orange light that is not absorbed by retinal, the λ_{max} of the mixture, which is near 390 nm, is shifted toward 380 nm, accompanied by a marked decrease in absorbance. This phenomenon is closely associated with the capacity of retinochrome-protein to catalyze the photoisomerization of all-*trans*-retinal to the 11-*cis* form (Hara and Hara, 1968, 1973*a*, *b*). In fact, all-*trans*-retinal in 0.5% digitonin was not isomerized at all in the control mixture kept in the dark, whereas it was almost entirely converted to the 11-*cis* form (98%) in the irradiated mixture. When an artificial mixture consisting of 13-*cis*, 9-*cis*, and all-*trans* isomers ($A_{384\,nm} = 0.61$) was irradiated with orange light for 3 h in the presence of retinochrome ($A_{495\,nm} = 0.05$), its initial proportions of 34:38:28 were decreased to 4:22:0, respectively, and the percentage of 11-*cis* isomer produced was as high as 74%, indicating that all-*trans*- and 13-*cis*-retinals were isomerized to the 11-*cis* form rapidly, but to 9-*cis*-retinal slowly. The relatively slow isomerization suggested that 9-*cis*-retinal cannot quickly replace the 11-*cis* chromophore of metaretinochrome. Actually, 9-*cis*-retinal combines with aporetinochrome slowly, as seen in the following section.

TABLE III
ISOMERIC COMPOSITION OF THE RETINAL CHROMOPHORE IN RETINOCHROME EXTRACT BEFORE AND AFTER IRRADIATION WITH DIFFERENT SPECTRAL LIGHTS

Isomer	Before irradiation	After irradiation with light of			
		>560 nm	480 nm	420 nm	NUV
	%	%	%	%	%
13-*cis*	6	6	9	10	11
11-*cis*	0	79	65	50	42
9-*cis*	0	0	2	2	1
7-*cis*	0	0	0	0	0
all-*trans*	94	15	24	38	46

NUV, near-ultraviolet.

Syntheses and Photoreactions of 13-*cis*- and 9-*cis*-Retinochrome

When all-*trans*-retinal is added to about a two times molar excess of aporetinochrome, most of the retinal is quickly used up, combining with the protein to yield retinochrome (Hara and Hara, 1973*b*). We similarly tried adding 13-*cis*- and 9-*cis*-retinal drop by drop to aporetinochrome in the dark at pH 6.5, 20°C. The absorption spectrum of each mixture was measured after pigment formation was finished: 3 min following each addition of 13-*cis*-retinal and 10 min following that of 9-*cis*-retinal. With the successive addition of 13-*cis*-retinal, the visible absorption first increased around 475 nm, indicative of the formation of 13-*cis*-retinochrome. When the amount of added retinal was greater than ~35% of aporetinochrome, a secondary peak began to appear in the near-ultraviolet, probably due to unreacted retinal. The addition of 9-*cis*-retinal, however, gave a 9-*cis* product with an absorbance low in the visible and relatively high in the near-ultraviolet, even though the amount of retinal was not greater than ~15% of the protein. Both the peaks became higher and higher simultaneously with the increase of 9-*cis*-retinal. For preparing 13-*cis* and 9-*cis* pigments in the subsequent experiments, the amount of each isomeric retinal used was limited to ~30% of the aporetinochrome, using the molar extinction coefficients (ϵ_{max}) in ethanol, 35,600 $cm^{-1}M^{-1}$ for 13-*cis*-retinal and 36,100 $cm^{-1}M^{-1}$ for 9-*cis*-retinal (cf. Robeson et al., 1955).

In Fig. 1 *A* are shown the synthesis and photoreaction of 13-*cis*-retinochrome. The pigment synthesized (spectrum 2) by coupling 13-*cis*-retinal with aporetinochrome (spectrum 1) was finally bleached by irradiation with orange light (spectrum 3). Spectra 4 and 5 were obtained from spectra 2 and 3, respectively, after correction for excess aporetinochrome. The λ_{max} of 13-*cis*-retinochrome is at 475 nm, and its ϵ_{max} was calculated to be about 41,000 $cm^{-1}M^{-1}$ from the peak height of spectrum 4 and the amount of retinal added to aporetinochrome, assuming all the retinal reacted. This value was fairly small compared with the ϵ_{max} of 60,800 for retinochrome (Hara and Hara, 1982). Upon exposure to orange light 13-*cis*-retinochrome, like retinochrome, is readily bleached and its λ_{max} shifts to 470 nm. The absorption spectrum of this photoproduct (spectrum 5) agrees well with that of metaretinochrome at the same concentration (broken-line spectrum). In the difference spectrum before and after photobleaching of 13-*cis*-retinochrome, the λ_{max} and λ_{min} were at 485 and 380 nm, respectively. The isomeric composition of the retinal chromophore in the preparation of 13-*cis*-retinochrome and its photoproduct is shown in Table IV. It is clear that, on irradiation, the chromophore of 13-*cis*-retinochrome is

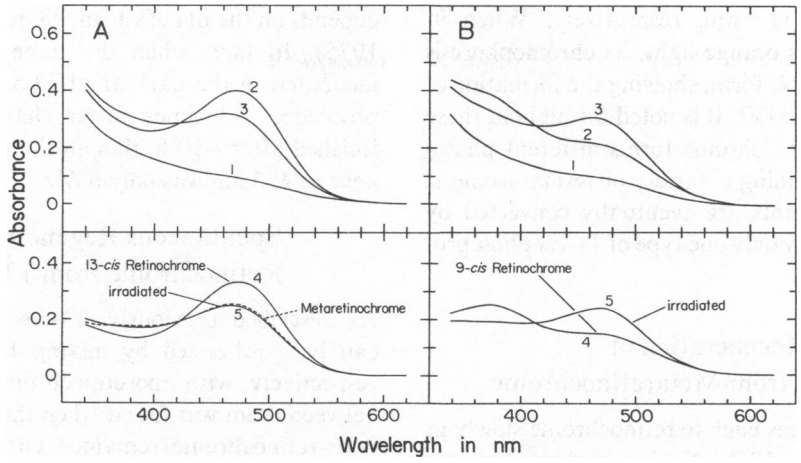

FIGURE 1 Syntheses and photochemical reactions of 13-*cis*- and 9-*cis*-retinochrome at pH 6.5, 20°C. (*A*) To 27.9 nmol of aporetinochrome (spectrum *1*), 8.1 nmol of 13-*cis*-retinal was added, and the spectrum was measured 3 min later (spectrum *2*). The product was irradiated for 10 min with orange light of >530 nm, and the spectrum was measured (spectrum *3*). The absorption characteristics of 13-*cis*-retinochrome and its photoproduct are shown by spectra *4* and *5*, which have been corrected by subtracting the absorption of excess aporetinochrome from spectra *2* and *3*, respectively. On irradiation, 13-*cis*-retinochrome (spectrum *4*) was bleached to the photoproduct (spectrum *5*) whose absorption spectrum coincided with that of metaretinochrome (broken-line spectrum). (*B*) To 28.6 nmol of aporetinochrome (spectrum *1*), 8.3 nmol of 9-*cis*-retinal was added and the spectrum was measured 10 min later (spectrum *2*). The product was irradiated for 20 min with orange light of >560 nm and the spectrum was then recorded (spectrum *3*). Spectra *4* and *5* were respectively derived from spectra *2* and *3* after correction for the remaining aporetinochrome. Unlike 13-*cis*-retinochrome, 9-*cis*-retinochrome did not show any bleaching on irradiation, but its absorption increased in the visible and decreased in the near-ultraviolet range. Spectrum *5* also fit well to the absorption spectrum of metaretinochrome. See Table IV for the isomeric form of the retinal chromophore in the compounds represented by the spectra in *A* and *B*.

isomerized mainly to the 11-*cis* form. The composition of the photoproduct is consistent with that of metaretinochrome, which has been shown in Tables II and III. We concluded that 13-*cis*-retinochrome is converted into metaretinochrome by exposure to orange light.

As seen in Fig. 1 *B*, 9-*cis*-retinochrome was also synthesized (spectrum 2) by incubating 9-*cis*-retinal with aporetinochrome. Unlike 13-*cis*-retinochrome, the absorbance increased in the visible and decreased in the near-ultraviolet (spectrum 3), when this pigment was irradiated with orange light. The corrected spectrum for the photoproduct (spectrum 5) resembled the spectrum of metaretinochrome in shape. In the difference spectrum before and after irradiation of 9-*cis*-retinochrome, the λ_{min} and λ_{max}

TABLE IV
CONFIGURATIONAL CHANGE OF THE RETINAL CHROMOPHORE ON IRRADIATION OF 13-*cis*- AND 9-*cis*-RETINOCHROMES, pH 6.5, 20°C

Isomer	13-*cis*-retinochrome		9-*cis*-retinochrome	
	Kept dark	Irradiated >530 nm, 10 min	Kept dark	Irradiated >560 nm, 20 min
	%	%	%	%
13-*cis*	81	10	6	7
11-*cis*	2	78	6	80
9-*cis*	1	0	81	2
7-*cis*	0	0	0	0
all-*trans*	16	12	7	11

The absorption spectra of the samples are shown in Fig. 1.

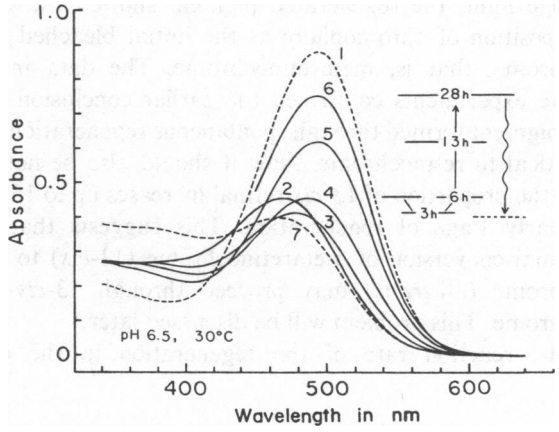

FIGURE 2 Spontaneous change in absorption of metaretinochrome in the dark. Retinochrome at pH 6.5 (spectrum *1*) was converted to metaretinochrome (spectrum *2*) by a 10-min irradiation with orange light, and incubated in the dark at 30°C. Transient spectra were recorded 3, 6, 13, and 28 h after incubation (spectra *3*, *4*, *5*, and *6*), and finally the sample was reirradiated for 10 min with the same orange light (spectrum *7*). During dark incubation, the absorption of metaretinochrome decreased slightly in the early stage (cf. spectrum *3*) and then gradually increased with time, the λ_{max} shifting toward 495 nm. The absorbance at λ_{max} after 28 h amounted to ~85% of the original retinochrome shown by spectrum *1*. The inset on the right side briefly shows the transition of absorbance at the spectral peak. In this experiment, six 0.5-ml portions of the sample were withdrawn to examine the configurational change of the retinal chromophore. Those results are shown in Table V.

appeared at 470 and 375 nm, respectively. When 9-*cis*-retinochrome absorbs orange light, its chromophore is also converted to the 11-*cis* form, showing the formation of metaretinochrome (Table IV). It is noted throughout these experiments that aporetinochrome forms different photosensitive pigments containing a variety of retinal isomers, but that all those pigments are eventually converted by orange light to principally only one type of 11-*cis* photoproduct, metaretinochrome.

Spontaneous Regeneration of Retinochrome from Metaretinochrome

Metaretinochrome changes back to retinochrome slowly in the dark (Hara and Hara, 1969). Such spontaneous regeneration has never been observed in the rhodopsin system. In the present experiment, metaretinochrome was prepared by irradiation of retinochrome with orange light, and incubated in the dark at pH 6.5, 30°C. In Fig. 2 we show the spectral changes during the incubation of metaretinochrome. During the initial period of incubation, the 470-nm absorption peak of metaretinochrome was lowered and shifted toward longer wavelengths. Later on, the absorbance increased gradually with time. The corresponding change in the configuration of the retinal chromophore is shown in Table V. During dark incubation, the amounts of 11-*cis*-retinal decreased and all-*trans* increased, and after 28 h the proportion of all-*trans*-retinal was 86% of the total chromophore. When irradiated with orange light, the regenerated pigment showed the same composition of chromophore as the initial bleached retinochrome, that is, metaretinochrome. The data in the above experiments confirmed the earlier conclusion that the pigment formed through spontaneous regeneration was identical to retinochrome. Now it should also be noticed that the proportion of 13-*cis*-retinal increases up to 15% at an early stage of regeneration. This suggests that the thermal conversion of metaretinochrome (11-*cis*) to retinochrome (all-*trans*) may proceed through 13-*cis*-retinochrome. This problem will be discussed later.

The reaction rate of the regeneration in the dark depends on the pH of the medium (Sperling and Hubbard, 1975). In fact, when the same metaretinochrome was incubated in the dark at pH 7.5, 30°C, the regeneration proceeded 2.3 times faster than that at pH 6.5, and finished after ~10 h, though the regenerability in absorbance at 495 nm was only 66%.

Spontaneous Regeneration of Retinochrome from 13-*cis*-Retinochrome

As described previously, 13-*cis*- and 9-*cis*-retinochrome can be synthesized by mixing 13-*cis*- and 9-*cis*-retinal, respectively, with aporetinochrome. A marked difference between them was found when they were kept in the dark; 9-*cis*-retinochrome remained virtually unchanged in the dark, whereas 13-*cis*-retinochrome behaved like metaretinochrome. Fig. 3 shows the spectral change of 13-*cis*-retinochrome at pH 6.5 during incubation in the dark at 30°C, where every spectrum has been corrected for excess aporetinochrome. The chromophore change in this experiment is shown in Table VI. The chromophore of 13-*cis*-retinochrome was gradually converted into the all-*trans* form, and after 12 h the chromophore composition (all-*trans*, 94%) reached that of retinochrome (cf. Table V). When irradiated with orange light, this all-*trans* pigment was converted to the same 11-*cis* product as metaretinochrome. Consequently, it was clear that 13-*cis*-retinochrome kept in the dark could be changed completely into retinochrome, which shows a 1.5 times higher extinction coefficient. Furthermore, 13-*cis*-retinochrome regenerates retinochrome more rapidly than metaretinochrome, and the dark process in the former is associated with no isomer other than 13-*cis* and all-*trans* (cf. Table VI).

Mechanism of Spontaneous Regeneration of Retinochrome

As demonstrated in Figs. 2 and 3, both meta- and 13-*cis*-retinochromes are spontaneously converted to retinochrome in the dark. To clarify the behavior of the retinal chromophore in these conversions, the following experiments were carried out. As soon as meta- and 13-*cis*-

TABLE V
CONFIGURATIONAL CHANGE OF THE RETINAL CHROMOPHORE DURING THE INCUBATION OF METARETINOCHROME IN THE DARK

Isomer	Retinochrome	Metaretinochrome	Kept dark for			Irradiated >560 nm, 10 min
			6 h	13 h	28 h	
	%	%	%	%	%	%
13-*cis*	4	5	15	11	6	5
11-*cis*	0	80	40	16	6	80
9-*cis*	0	0	1	1	2	1
7-*cis*	0	0	0	0	0	0
all-*trans*	96	15	44	72	86	14

Retinochrome at pH 6.5 was converted to metaretinochrome by a 10-min exposure to orange light (>560 nm), kept dark at 30°C for many hours, and finally irradiated with the same light. The spectral changes are presented in Fig. 2.

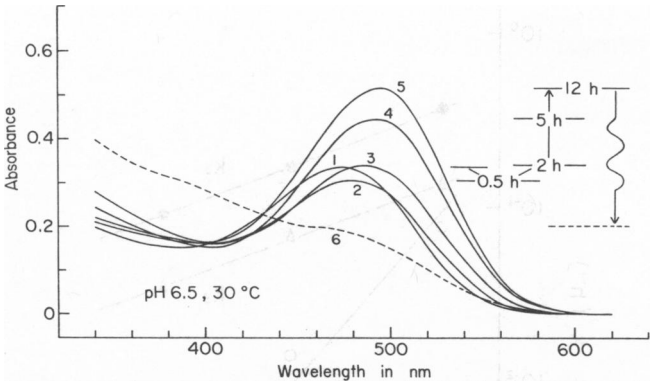

FIGURE 3 Spontaneous change in absorption of 13-*cis*-retinochrome in the dark. 13-*cis*-retinochrome was synthesized by mixing 16.2 nmol of 13-*cis*-retinal with 53.9 nmol of aporetinochrome at pH 6.5 (spectrum *1*), and incubated in the dark at 30°C. Spectra were measured after 0.5, 2, 5, and 12 h (spectra *2*, *3*, *4*, and *5*), and finally the sample was irradiated for 15 min with orange light of >530 nm (spectrum *6*). During the experiment, four 0.5-ml portions of the sample were withdrawn in order to examine the configurational change of the retinal chromophore. These data are presented in Table VI.

TABLE VI
CONFIGURATIONAL CHANGE OF THE RETINAL CHROMOPHORE DURING THE INCUBATION OF 13-*cis*-RETINOCHROME IN THE DARK

Isomer	13-*cis*-retinochrome	Kept dark for		Irradiated >530 nm, 15 min
		2 h	12 h	
	%	%	%	%
13-*cis*	82	50	5	7
11-*cis*	2	2	1	80
9-*cis*	0	0	0	1
7-*cis*	0	0	0	0
all-*trans*	16	48	94	12

13-*cis*-retinochrome at pH 6.5 was kept dark at 30°C for many hours, and finally irradiated with orange light (>530 nm). The spectral changes are presented in Fig. 3.

retinochromes were prepared at pH 6.5, each of them was incubated in the dark at 30°C. Small aliquots were frequently withdrawn from the sample to measure the isomeric composition of the retinal chromophore after various periods of incubation, and the molar percentages of 11-*cis*, 13-*cis*, and all-*trans* isomer to the total retinal were plotted against the incubation time, as shown in Fig. 4 *A*

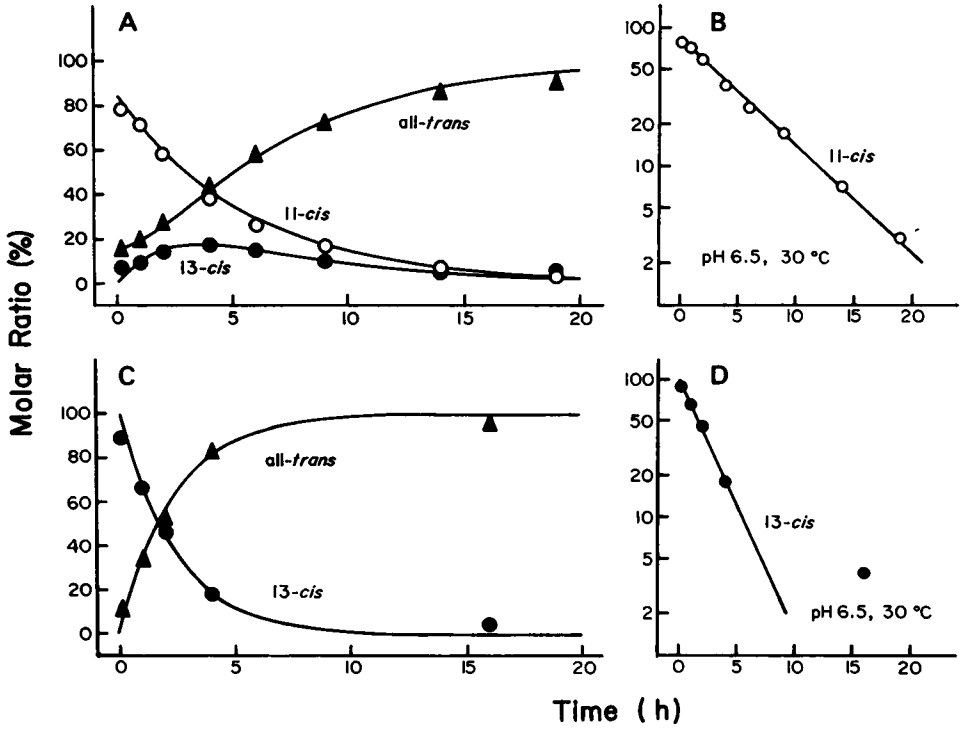

FIGURE 4 Changes in the isomeric form of the retinal chromophore during spontaneous regeneration of retinochrome from metaretinochrome (*A*) and 13-*cis*-retinochrome (*C*). Metaretinochrome and 13-*cis*-retinochrome at pH 6.5 were incubated in the dark at 30°C, and 0.5-ml portions were frequently withdrawn from each sample in order to analyze for the isomeric composition of the retinal chromophore. The molar percentages of 11-*cis* (○), 13-*cis* (●), and all-*trans* (▲) isomer to the total retinal are plotted against time. Solid lines (—) are obtained from the computation explained in the text. In *B* and *D*, the decrease of the 11-*cis* chromophore (○) of metaretinochrome and of the 13-*cis* chromophore (●) of 13-*cis*-retinochrome are replotted on a semilogarithmic scale, respectively, based upon the results shown in *A* and *C*. The slopes of the straight lines were used for determination of the rate constants for the dark isomerization of the retinal chromophore.

and *C*, for meta- and 13-*cis*-retinochrome, respectively. Based upon the facts that the all-*trans* chromophore of retinochrome remains unchanged in the dark and that no isomer other than all-*trans* and 13-*cis* appears during incubation of 13-*cis*-retinochrome, the experimental results were analyzed according to a kinetic model proposed in Fig. 5. This system consists of the 13-*cis*, 11-*cis*, and all-*trans* isomers, where the isomerizations from the all-*trans* to 11-*cis* and 13-*cis* and from the 13-*cis* to 11-*cis* are prohibited. In other words, the simple model in Fig. 5 contains the dark reactions of isomerization from 11-*cis* to all-*trans* (rate constant, k_1), from 11-*cis* to 13-*cis* (k_2), and from 13-*cis* to all-*trans* (k_3), and is useful enough to examine the mechanism of spontaneous regeneration of retinochrome. In Fig. 4 *B* and *D*, the changes in molar percentage of the 11-*cis* and 13-*cis* isomers are drawn on a semilogarithmic scale according to the plots shown in Fig. 4 *A* and *C*, respectively. The slopes of those straight lines correspond each to ($k_1 + k_2$) and k_3, as explained in the Appendix. As a result of the treatment described in the Appendix, the rate constants, k_1, k_2, and k_3 were determined to be 0.02, 0.16, and 0.42 h^{-1}, respectively. It is noted that k_1 (11-*cis* → all-*trans*) is markedly smaller than k_2 (11-*cis* → 13-*cis*). By using these rate constants and the initial concentrations of the 13-*cis*, 11-*cis*, and all-*trans* isomers, the time courses of the change of each retinal chromophore were obtained by a computor from Eqs. A4, A5, and A6 given in the Appendix, as drawn in Fig. 4 *A* and *C* (solid-line curves). Every curve fits all the observed plots, indicating that the present kinetic model and the rate constants are relevant to the processes of dark isomerization.

In conclusion, the 11-*cis* chromophore of metaretinochrome is changed to the all-*trans* form mainly through 13-*cis*-retinochrome, and the 13-*cis* chromophore is directly isomerized to the all-*trans* form to produce retinochrome.

To determine the activation parameters in the dark isomerization, similar experiments were repeated to obtain the rate constants, k_1, k_2, and k_3 at different temperatures (22.5°, 26.6°, and 31.7°C), and they were plotted on a semilogarithmic scale against the reciprocal of temperature (Arrhenius plot), as illustrated in Fig. 6. The decrease

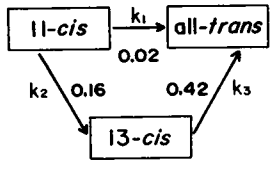

FIGURE 5 A kinetic model for the spontaneous regeneration of retinochrome in the dark. Based on the data shown in Fig. 4, the rate constants of isomerization, k_1, k_2, and k_3 at 30°C, pH 6.5, are noted in the figure. Most of the 11-*cis* chromophore of metaretinochrome is isomerized indirectly to the all-*trans* form through 13-*cis*, because k_2 is much larger than k_1.

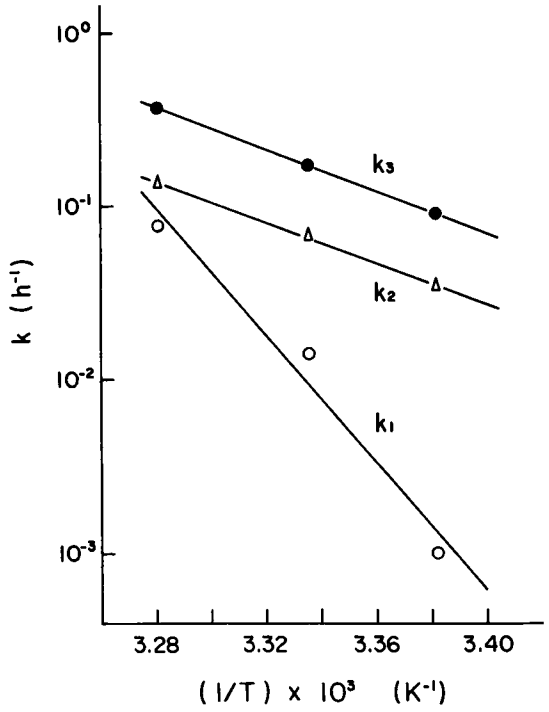

FIGURE 6 Arrhenius plot of the rate constants, k_1, k_2, and k_3. Three sets of the preparations of meta- and 13-*cis*-retinochrome, pH 6.5, were incubated each at 22.5°, 26.6°, and 31.7°C in the dark. 0.5-ml portions were withdrawn from each set after various periods of incubation, and analyzed with HPLC to find the rate constants at different temperatures. The slope of the straight line gave the activation energy change of each process of isomerization. See text for further details.

of k_1 with the fall of temperature was far more marked than that of k_2 or k_3. At 22.5°C, k_1 (0.001 h^{-1}) was negligible compared with k_2 (0.034 h^{-1}) and k_3 (0.089 h^{-1}). The activation enthalpy change (ΔH^*) was found from the slope of the straight lines in Fig. 6, and the activation entropy change (ΔS^*) and activation free energy change (ΔG^*) were computed according to the theory of absolute reaction rates. Those results are summarized in Table VII. It is worth noting that both ΔH^* and ΔS^* for the direct process from 11-*cis* to the all-*trans* form were much larger than the corresponding quantities for the two-step reaction passing through the 13-*cis* form.

TABLE VII
ACTIVATION PARAMETERS FOR THE DARK ISOMERIZATION OF THE RETINAL CHROMOPHORE IN THE RETINOCHROME SYSTEM

	ΔH^*	ΔS^*	ΔG^*
	kcal/mol	*cal/deg/mol*	*kcal/mol*
11-*cis* → all-*trrans*	82.9	189	27.0
11-*cis* → 13-*cis*	27.5	8.45	25.0
13-*cis* → all-*trans*	28.2	12.8	24.4

ΔH^*, activation enthalpy change; ΔS^*, activation entropy change; ΔG^*, activation free energy change (at 22.5°C).

DISCUSSION

Dark Isomerization of the Retinal Chromophore

As seen from Table VII, the isomerization of the 11-*cis* chromophore directly to the all-*trans* form in metaretinochrome is greatly inhibited by a high energy barrier, $\Delta H^* = 82.9$ kcal/mol, which is about twice as large as the value calculated for the isomerization around the 11–12 double bond in the protonated retinal Schiff-base (PRSB) (Suzuki et al., 1974; Kakitani and Kakitani, 1975). Furthermore, for the direct isomerization to retinochrome, a large conformational change of the protein moiety is indicated by the high value of ΔS^*, 189 cal/deg/mol. These values of ΔS^* and ΔH^* in the present reaction are about two times larger than those measured for the transition of metarhodopsin I to II during the photobleaching of cattle rhodopsin (cf. Ostroy, 1977). On the other hand, for each of the isomerization processes from the 11-*cis* to the 13-*cis* form and from the 13-*cis* to the all-*trans* form within the protein molecule, ΔH^* is small compared with the energy barrier calculated for the isomerization in the PRSB. The value of ΔS^* is also small, suggesting that the activation is accompanied by no large conformational change of the protein molecule. This means that the interaction between the chromophore and the protein makes the direct isomerization from the 11-*cis* to the all-*trans* form difficult, but the overall conversion can be eventually facilitated by a chain reaction; the 11-*cis* to the 13-*cis* and then to the all-*trans* form.

A *cis* → *cis* isomerization of the retinal chromophore as found in the dark during spontaneous regeneration from metaretinochrome (11-*cis* → 13-*cis*) has never been observed in any light reactions of retinochrome or rhodopsin, although a possibility of *cis* → *cis* isomerization was theoretically proposed in the bicycle-pedal model (Warshel, 1976). The dark isomerization from the 13-*cis* to the all-*trans* form was reported in bacteriorhodopsin (Maeda et al., 1977), where $\Delta H^* = 24$ kcal/mol (Tokunaga and Ebrey, 1978), as low as the value in retinochrome. In a previous report (Ozaki et al., 1982), we pointed out that retinochrome and bacteriorhodopsin have common characteristics in the effects of salts and pH on the absorption spectrum. It is interesting that those two photopigments are also similar to each other in the isomerization of the retinal chromophore.

Physiological Function of the Retinochrome System

In the present study, we have reconfirmed with the aid of chromatography that the protein moiety of retinochrome, aporetinochrome, combines with 13-*cis*-, 9-*cis*-, and all-*trans*-retinals to yield their particular photosensitive pigments, all of which are converted into 11-*cis* metaretinochrome by irradiation. In visible light, retinochrome (or, more specifically, aporetinochome) therefore shows the capacity for changing a variety of retinal isomers to 11-*cis*, the form that is required by opsin to form rhodopsin in the retina. The visual cells, in fact, contain much retinochrome in addition to rhodopsin located on the rhabdomal membranes, and the amount of retinochrome increases markedly in the outer segments during light adaptation (Hara and Hara, 1976). Our recent studies have indicated that retinochrome is bound at least to the lamellated bundles of membranes (myeloid bodies) observed abundantly in the inner segments (Seki et al., 1980; Fukushima et al., 1980; Ozaki et al., 1982, 1983). Because the myeloid bodies are also distributed in the proximal regions of the outer segments together with a number of thin-layered myeloid bodies and minute vesicles that may have retinochrome or aporetinochrome (cf. Hara and Hara, 1976), the possibility always exists that the two membrane systems carrying retinochrome and rhodopsin interact with each other in the outer segments. Recently we prepared a sample of rhabdomal membranes containing active opsin, which is capable of forming rhodopsin through coupling with 11-*cis*-retinal (Seki et al., 1980). When these membranes are incubated in the dark with metaretinochrome-bearing membranes prepared from the cell fragments of the inner segments, the 11-*cis* chromophore of metaretinochrome can enter the rhabdomal membranes to reconstitute rhodopsin there (Seki et al., 1982). Based upon these findings, we believe that metaretinochrome may contribute to rhodopsin formation as an 11-*cis*-retinal supplier in vivo.

Another notable fact is that the photoproducts of retinochrome, metaretinochrome (11-*cis*), and isoretinochrome (13-*cis*), are changed finally into retinochrome (all-*trans*) when kept in the dark. Such a mechanism of dark isomerization in spontaneous regeneration can take part in the transformation of various kinds of *cis* isomers of retinal into the all-*trans* form alone, so that retinal may be reserved in the stable form of retinochrome until required for rhodopsin formation. According to Bridges (1976), in the frog retina, retinoids are stored as the retinyl ester in oil droplets of the pigment epithelial cells. In the squid retina, retinochrome-protein may serve as a storage place for retinal, since the retina contains about as much retinochrome as rhodopsin. In view of the formation of rhodopsin, if one regards metaretinochrome and retinochrome as an "active form" and a "stock form," respectively, the active form gives 11-*cis*-retinal to opsin, and the excess slowly turns to the stock form in the dark. In this way, both the light and dark reactions developing in the retinochrome system would play an important role in maintaining available visual pigment for photoreception, constantly providing only two forms of retinal, 11-*cis* and all-*trans*.

APPENDIX

In the reaction system of spontaneous regeneration proposed in Fig. 5, when the rate constants of retinal isomerization from 11-*cis* to all-*trans*, from 11-*cis* to 13-*cis*, and from 13-*cis* to the all-*trans* form are respec-

tively expressed as k_1, k_2, and k_3, the following equations should hold:

$$\frac{d[all]_t}{dt} = k_1[11]_t + k_3[13]_t \quad (A1)$$

$$\frac{d[11]_t}{dt} = -(k_1 + k_2)[11]_t \quad (A2)$$

$$\frac{d[13]_t}{dt} = k_2[11]_t - k_3[13]_t, \quad (A3)$$

where $[all]_t$, $[11]_t$, and $[13]_t$ are the respective concentration of all-*trans*-, 11-*cis*-, and 13-*cis*-retinal at time t after the start of dark incubation. By solving these equations, the concentration of each isomer at time t is given as

$$[all]_t = ([all]_0 + [11]_0 + [13]_0) - \frac{k_1 - k_3}{k_1 + k_2 - k_3}[11]_0 e^{-(k_1+k_2)t} - \left(\frac{k_2}{k_1 + k_2 - k_3}[11]_0 + [13]_0\right) e^{-k_3 t} \quad (A4)$$

$$[11]_t = [11]_0 e^{-(k_1+k_2)t} \quad (A5)$$

$$[13]_t = -\frac{k_2}{k_1 + k_2 - k_3}[11]_0 e^{-(k_1+k_2)t} + \left(\frac{k_2}{k_1 + k_2 - k_3}[11]_0 + [13]_0\right) e^{-k_3 t}, \quad (A6)$$

where $[all]_0$, $[11]_0$, and $[13]_0$ are the initial concentrations of each isomer. Because $[11]_0$ is to be zero in the experiment with 13-*cis*-retinochrome, Eq. A6 may be written simply as

$$\log[13]_t = \log[13]_0 - 2.30\, k_3 t. \quad (A7)$$

The actual values of $[13]_0$ and k_3 are readily determined respectively to be 1.0 (100%) and 0.42 h^{-1} from the intercept at 0 time and the slope of the straight line shown in Fig. 5 *D*, where log $[13]_t$ is plotted against time t. Eq. A5 can also be rearranged to

$$\log[11]_t = \log[11]_0 - 2.30\,(k_1 + k_2)t. \quad (A8)$$

In the experiment with metaretinochrome, $\log[11]_t$ is plotted against time in Fig. 5 *B*, and the values of $[11]_0$ and $(k_1 + k_2)$ are found to be 0.85 (85%) and 0.18 h^{-1}, respectively. Supposing that each of the initial concentrations of 11-*cis*, all-*trans*, and 13-*cis* chromophore in metaretinochrome solution was 85, 15, and 0% ($[11]_0 = 0.85$, $[all]_0 = 0.15$ and $[13]_0 = 0$), Eq. A6 will be expressed as

$$[13]_t = k_2 \frac{0.85}{k_1 + k_2 - k_3}[e^{-k_3 t} - e^{-(k_1 + k_2)t}], \quad (A9)$$

where $(k_1 + k_2)$ and k_3 are already known. On substituting 0.17 for $[13]_t$ and 4 for t, based on the data shown in Fig. 5 *D*, Eq. A9 gives 0.16 h^{-1} as the value of k_2. Because $(k_1 + k_2)$ is 0.18 h^{-1}, the value of k_1 is determined to be 0.02 h^{-1}. All the values of k_1, k_2, and k_3 thus obtained are presented in Fig. 5.

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