Changes in Electrical Conductance of Rhodopsin on Photolysis

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ABSTRACT The change in electrical conductance of rhodopsin solutions was studied with flash-photolysis techniques. The whole pattern of the conductance change on illumination consists of three different processes: (I) the initial decrease, (II) the increase, and (III) the slow decrease, which are in decreasing order of reaction rate. The processes I, II, and III can be most distinctly recognized on flash illumination of acid, slightly acid, and alkaline rhodopsins, respectively. On the other hand, the bleaching of rhodopsin also shows at least three successive phases of different rates, but none of them corresponds in reaction rate to any of the processes of the conductance change. The conductance change may be related to conformational changes of opsin following photoisomerization of retinene, being due to hydrogen or hydroxyl ions and some other inorganic electrolytes. The amount of the change, especially the initial decrease, is proportional to the amount of rhodopsin bleached, even when the photochemical back reaction towards rhodopsin and isorhodopsin occurs in the chromophore depending on the intensity of illumination. Of the three processes, the slow decrease is most severely affected by aging, but the initial decrease and increase are slightly affected. These two processes promptly caused by illumination are connected closely to the conformational changes during the conversion of rhodopsin to metarhodopsin, and perhaps to the initial stage of excitation of rod cells.

One may be sure that incident light activates rhodopsin molecules contained in rod cells and eventually leads to the initiation of rod vision. The rhodopsin molecule consists of two parts; *i.e.*, retinene as prosthetic group and opsin as protein moiety. In the light, a cis isomer of retinene is converted to the all-trans configuration, and is finally removed from its original site of attachment to opsin. This decomposition process as demonstrated by bleaching may be accompanied by the uncovering of some chemical groups in the protein moiety. In fact, Wald and Brown (20) and Radding and Wald (15) observed liberation of chemical groups such as sulfhydryl groups on illumina-

tion of rhodopsin solutions. This fact suggests that incident light finally produces a certain loosening in protein structure or denaturation of rhodopsin. Recently Hara (6) examined the effect of illumination on changes in transmission and electrical conductance of rhodopsin solutions, and found that on illumination solutions of rhodopsin revealed a conductance change depending on pH, as long as they preserved their photosensitivity. He was of the opinion that the conductance change during illumination should correspond to weak denaturation of rhodopsin, which might contribute to the excitation of rod cells.

Since the visual excitation must be triggered by photoexcitation of retinene, we intended to study subsequent changes in protein characteristics of the rhodopsin molecule following illumination. For the purpose of examining these changes exactly, it would be desirable to expose rhodopsin solutions to a short, intense flash of light, instead of to a continuous light. In the present study, we shall analyze the change in electrical conductance of rhodopsin solutions on illumination by means of the flash-photolysis technique, and above all try to elucidate the following problems: What kinds of elementary processes are participating in the over-all change in conductance, and how far are these processes related to photoexcitation of rhodopsin molecules?

EXPERIMENTAL PROCEDURES

Preparation of Rhodopsin Solutions

Rhodopsin solutions were prepared by almost the same method as described by Hara (6). Under dim red light, the retinas dissected from fresh cattle eyes are vigorously shaken with physiological saline and filtered through a brass gauze. Upon the crude rod suspension thus obtained, saturated sucrose solution is poured so as to form a lower sugar layer, and a gradient of sucrose concentration is brought about in the tube by stirring the saline-sugar interface. After centrifugation a scarlet-colored layer containing rod outer segments is separated from other retinal fragments. After washing with three successive portions of saline, the rod outer segments are suspended for hardening in potassium aluminum sulfate solution for 30 minutes. They are then dehydrated with anhydrous sodium sulfate, treated with petroleum ether three times to remove as much lipid as possible, and washed with ion-free water in the centrifuge more than three times. Finally rhodopsin solutions are prepared by extraction of this material with 2 per cent aqueous digitonin for about 1 hour at room temperature.

The rhodopsin preparation was usually in the range of pH 4.6–5.2. It was kept between −5 and 0°C in a Dewar vessel until needed and used for all the experiments—except those on the effect of aging—within 3 days after preparation. Before each experiment, the absorption spectrum of the preparation was measured with a Beckman spectrophotometer; the optical density at 500 mμ (D) and the optical purity (P, a ratio of extinction at 400 to 500 mμ) were determined. Aliquots of the preparation were used as the experimental samples without adjustment of pH, but, if needed, the desired pH was obtained by the addition of a trace of sodium hydroxide

or hydrochloric acid. The pH measurement was carried out with a glass electrode pH meter.

Construction of Apparatus

For the purpose of short period illumination of high intensity, an apparatus was constructed, as illustrated in Fig. 1, which enables us to record continuously not only

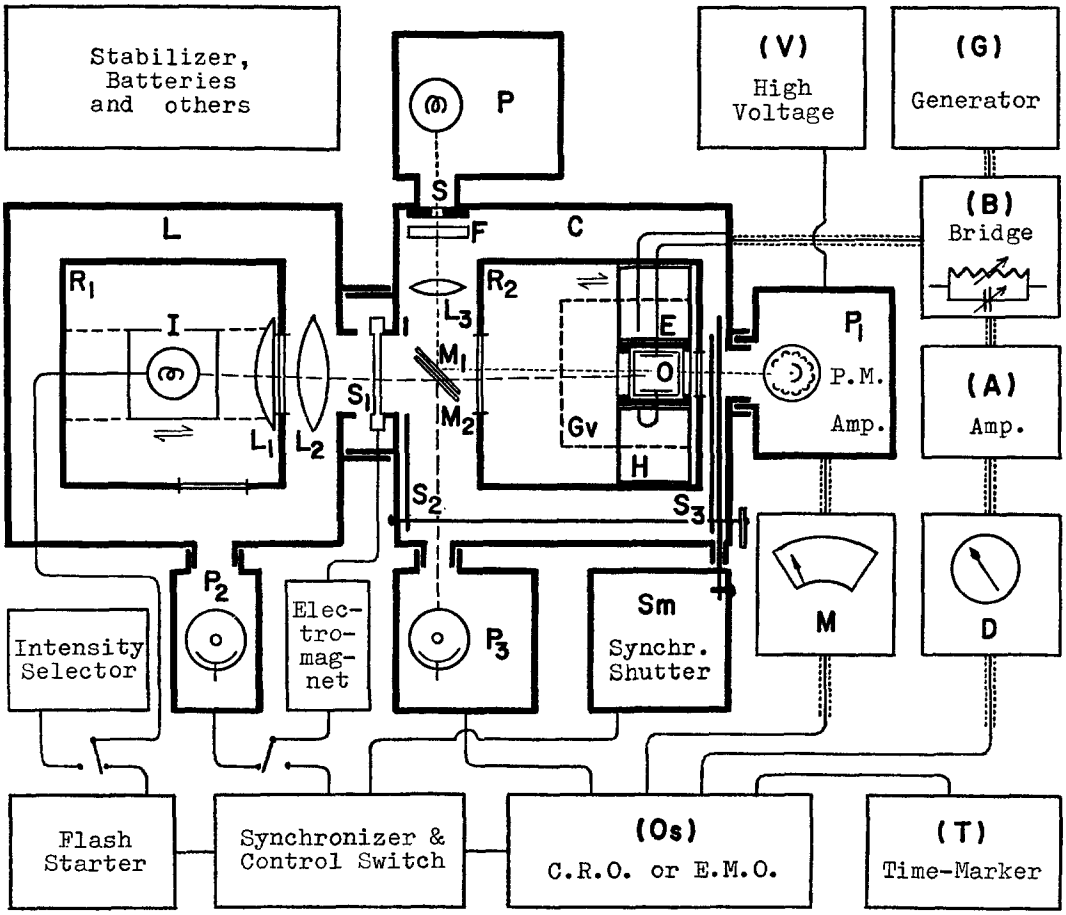

FIGURE 1. Diagram of the equipment used for measurement of transmission and electrical conductance of rhodopsin solution on illumination. See text for further details.

the electrical conductance but also the transmission of rhodopsin solutions on illumination.

The bulb as the source of illumination (I) is immersed in a water reservoir (R_1, made of brass, 15 × 15 × 18 cm) with windows allowing the light to pass. The reservoir itself is placed in a lamp housing (L, 25 × 25 × 20 cm), the inside of which is entirely covered with aluminum sheets to avoid electrical noise and thermal dis-

turbance caused by lighting the bulb. After passing through a condenser system (L_1 and L_2) and two shutters (S_1 and S_2), the light is projected on the front face of an optical cell (O, 1.0 cm thick) containing the rhodopsin solution.

A tungsten point lamp (P) supplied by a battery is used as the source for electrophotometry. After passing through a variable slit (S), an interference filter (F, Hitachi No. 50, main wavelength: 503 mμ), and a lens (L_3), the monochromatic light reflected at a metallic mirror (M_1) falls on the optical cell. When a shutter (S_3) is open, the transmitted light finally reaches a photomultiplier tube (P_1, RCA 1P28) served by a high voltage supply (V). The shutter S_3 is driven either by a hand lever or by a synchronized shutter mechanism (Sm) operating simultaneously with another shutter S_2, by which the illuminating light is completely screened while the transmitted light is projected on the photomultiplier tube. The photoelectric current amplified gives a deflection on a meter (M), corresponding to the transmission of the sample. When the output from the amplifier is also led to a multiple channel cathode ray oscilloscope (Os, Iwasaki DS-5155), the apparatus can exactly follow transmission changes occurring in time as short as 1 microsecond.

For shielding the sample from electrical disturbance and stray light, a grounded container (C, the same size as L) is prepared. In this container another water reservoir (R_2, made of brass, the same size as R_1) is placed in order to keep the sample at constant temperature during illumination. A cell holder (H) supports not only the optical cell but also a pair of platinized platinum electrodes (E) fitted exactly into the cell (cell constant: 0.565). For the measurement of electrical conductance, a Wheatstone bridge (B) is used, into which the alternating current less than 1.0 volt of 1 kc is supplied by a sine-wave generator (G). To detect unbalance in the bridge or a change in the electrical conductance of the sample, the potential difference appearing at the diagonal of the bridge is amplified by an AC amplifier with narrow band pass (A, Yokogawa DV-CR-201). The resulting voltage is rectified by a half-wave detector (D) and sent to the *C.R.O.* directly or to an electromagnetic oscillograph (Os, Yokogawa N-3-D) after filtration. The final time constant of the apparatus is 8 milliseconds in the former and 40 milliseconds in the latter.

The start of illumination is controlled by a voltage generated synchronously with the recording. When a tungsten point lamp is used, the synchronizer output directly drives the electromagnet to open the photographic shutter S_1. In the case of a photoflash lamp, when the output stimulates the bulb to fire and its flash intensity reaches a constant level, a thyratron excited by a photoelectric tube (P_2) triggers the electromagnet to open the shutter. This mechanism serves not only to expose the sample to the light of constant period around the peak of flashing, but also to cut off fore- and after-glow of the flash. In the case of a xenon-filled discharge tube, this is mounted in its own lamp housing instead of L, and is fired by the synchronizer output. At this time, immediately after the shutter S_3 has been closed, the sample is exposed to a flash through the shutter S_1, and afterwards, when the shutter S_1 screens the sample from the intense after-glow of the flash, S_3 is reopened for the measurement of transmission. After being reflected at a mirror (M_2), a small fraction of the illuminating light falls upon another photoelectric tube (P_3) to signal the illumination period on the record. The leads sending the signal are provided with a time-marker (T).

The electrical conductance is susceptible to temperature. In the present equipment, however, heat produced by the illumination source is completely removed by two water reservoirs, so that the temperature of the sample is never influenced by illumination. For experiments at low temperature, a glass vessel (*Gv*, 12 × 8 × 12 cm) filled with a freezing mixture is immersed into the reservoir R_2, and the sample is illuminated through a narrow channel left in the mixture. In this way, the front and rear windows of the reservoir R_2 are prevented from frosting during the experiments.

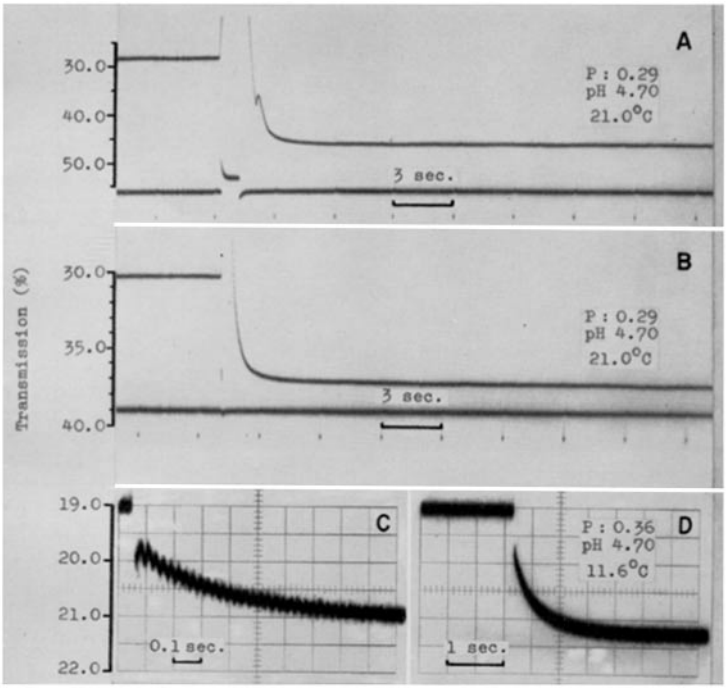

FIGURE 2. Records of transmission change of rhodopsin solution on illumination with (A) tungsten point lamp, (B) photoflash lamp, and (C, D) xenon-filled discharge tube. Transmission maximum of interference filter, 503 mμ. *P*, optical purity ($E_{400m\mu}/E_{500m\mu}$). Other explanation, see text.

Measurements

Into the optical cell 1.5 ml of rhodopsin solution is introduced, and the attenuators of the amplifiers are so adjusted as to complete the records of transmission and resistance. After original values of transmission and electrical resistance of the sample have been noted, all the operations are initiated by a remote control switch. After recording, the transmission and pH of the sample illuminated are checked. The unknown arm of the bridge is connected to a variable resistance which has been set equal to the original resistance of the sample. After the balance has been reestablished, the resistance of the variable arm is altered 1 ohm at a time, and every reading in microamperes corresponding to the unbalance is also recorded. Then the unit of

change deduced from these readings is expressed in terms of the change in specific conductance, and is marked on the experimental record. As for photometry, the values of transmission before and after the recording are written on the ordinate of the record. In the text, the bleaching of rhodopsin is denoted in terms of optical density instead of transmission.

Three kinds of illumination source[1] are used depending on the purpose of the analytical experiments: (1) a tungsten point lamp, (2) a photoflash lamp, and (3) a xenon-filled discharge tube, in ascending order to the illumination intensity and in descending order of the exposure period. (1) and (3) always give constant amounts of light, whereas (2) presumably does not. Examples of the bleaching of rhodopsin following exposure to bulbs (1), (2), and (3) are presented in Fig. 2. The decrease in optical density at 503 mμ in A, B, and D is calculated as 0.271, 0.096, and 0.049 respectively at the end of recording. The figures show that the decrease in optical density is essentially complete within a few seconds after the end of illumination. Especially, C and D clearly indicate that the optical density decreases significantly even in darkness.

RESULTS

1. *Conductance Change of Rhodopsin Solution*

According to Hara (6), continuous illumination with 3,000 lux evoked a gradual change in electrical conductance of rhodopsin solutions at any pH, as the bleaching proceeded. Both processes were exponential with the illumination period for a time, indicating that the conductance change was proportional to the amount of bleached rhodopsin at least within 20 seconds. When the light was turned off, the changes almost ceased, although both showed slight after-effects of illumination. In the present experiments with techniques of flash-photolysis, however, we have observed patterns in the conductance change different from the above-mentioned result with continuous illumination of low intensity.

Fig. 3 gives two examples of the conductance changes in acid and alkaline samples derived from a single preparation of rhodopsin on exposure to a photoflash lamp. In the acid sample (A), the conductance considerably decreases at first, slightly increases, and soon attains a constant level. At a little lower pH, the phase of initial decrease was not always followed by that of slight increase. In the alkaline sample (B), the conductance rapidly decreases at first, and then slowly further for a fairly long time after the bleaching of rhodopsin stops (*cf.* Fig. 2B). The alkaline sample is apparently dif-

[1] (1), 15 volts, 200 watts. Illumination, about 60,000 lux at the sample. Exposure period, 1 second. Distance between lamp and sample, 36 cm. (2), West 6A or FP-6. Total light output, 33,000 visual lumen seconds. Flash duration at ½ peak, 40 milliseconds. Exposure period, 40 milliseconds controlled by the shutter. Distance, 20 cm. (3), Ushio UF-880-B. Light intensity when operated at 4,500 volts, approximately 2×10^8 candles. Flash duration at ½ peak, about 200 microseconds. Distance, 20 cm.

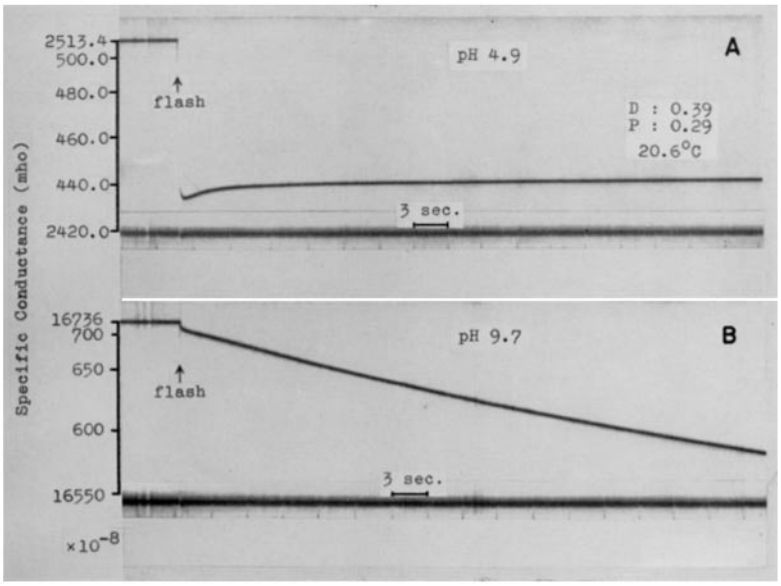

FIGURE 3. Records of conductance change of (A) acid and (B) alkaline solutions of rhodopsin on exposure to photoflash lamp. *D*, optical density at 500 mμ; *P*, optical purity.

ferent from the acid one in the pattern of the conductance change after the flash. In Fig. 3, the initial decrease is evaluated as 80×10^{-8} mho in A and 25×10^{-8} mho even in B. As the decrease in optical density was 0.193 in A and 0.220 in B, the initial decrease per unit density decrease can be calculated as 415×10^{-8} mho and 114×10^{-8} mho in acid and alkaline solutions respectively.

2. *Dependence of Conductance Change on pH*

In order to examine dependence of the conductance change on pH, a single preparation of rhodopsin was divided into many aliquots of different pH and their conductance changes were recorded after illumination with a tungsten point lamp and a photoflash lamp. As 1 second illumination with the tungsten lamp was more suitable for delivering a constant amount of light and far easier for observing the pattern of the changes revealed during illumination, a series of results concerning pH dependence obtained by this illumination method is represented in Fig. 4. The decrease in optical density of each aliquot after illumination was in the range of 0.27–0.31, depending somewhat on pH. Near neutrality (pH 6.0–7.0) the initial decrease in conductance becomes extremely small, but one can clearly recognize the transitions in the whole pattern of the conductance change according to the shift

of pH.[2] The initial decrease in conductance amounts to 76.8 × 10^{-8} mho at pH 5.1 and 18.7 × 10^{-8} mho at pH 5.4. It is therefore markedly depressed by the slight shift of pH towards neutrality. On the contrary, the conductance increase after the initial decrease is apparently enhanced as the result of the same slight shift of pH.

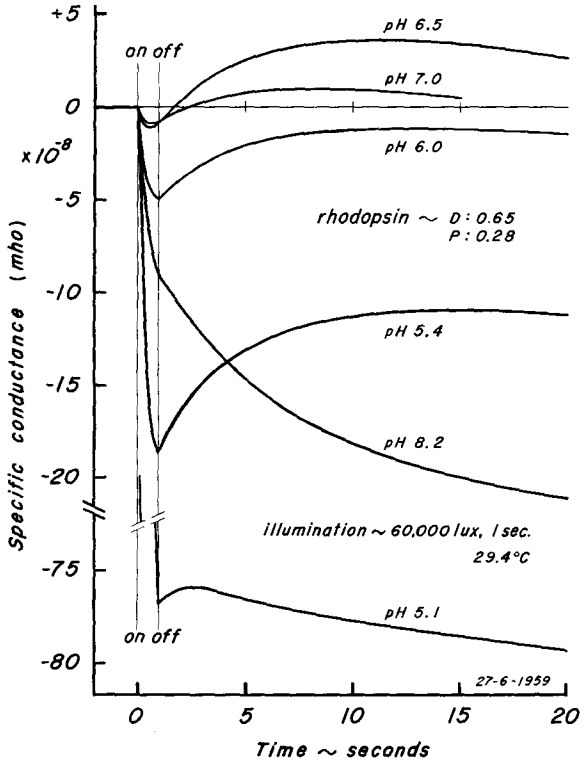

FIGURE 4. Conductance change of rhodopsin solutions of various pH following 1 second illumination with 60,000 lux.

Now, it may be suggested that the whole pattern of the conductance change consists of three different types of change: (I) the initial decrease, (II) the increase, and lastly (III) the slow decrease. The changes (I), (II), and (III) could be typically recognized in acid, slightly acid, and alkaline rhodopsins, respectively, upon illumination with a flash of light. They will be distinctly defined by the value of the reaction rate, as discussed later. Hereafter we shall provisionally call them processes I, II, and III.

Process II usually appears after the cessation of illumination, so that process II and accordingly process III must be dark reactions. Although the

[2] The pattern of the changes recorded with a photoflash lamp was essentially similar to that shown in Fig. 4. Then the phase of increase began soon after the flash died out, as seen in Fig. 3A.

bleaching of rhodopsin may stop within 3 seconds after 1 second illumination with 60,000 lux (*cf.* Fig. 2A), process II can continue for about 10 seconds as shown in the case of pH 5.4 in Fig. 4. This fact means that processes II and III cannot coincide with the bleaching process. In the neutral sample, the conductance begins to increase within 1 second of illumination, so that process II must start soon after the beginning of illumination.

3. *Initiation of Conductance Change*

On 100 millisecond exposure of acid rhodopsin to a photoflash lamp, the initial decrease in conductance was observed only during illumination, suggesting an intimate connection with the photoreaction (9). However, the shorter the illumination period becomes, the greater is the part of the initial decrease that is shifted into the dark period. In fact, the record of illumination with the xenon-filled discharge tube clearly demonstrates that process I is a dark reaction lasting for about 150 milliseconds (*cf.* Fig. 6A).

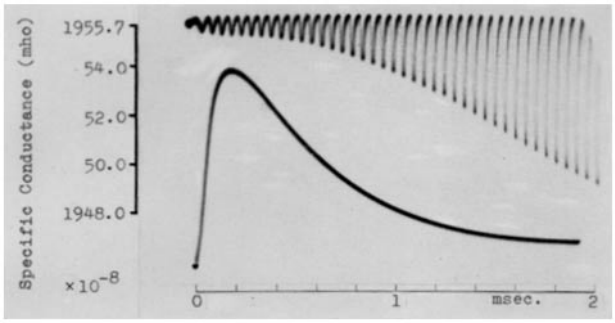

FIGURE 5. A record of the initial phase of conductance decrease on exposure to a flash of the xenon-filled discharge tube. Sample, 0.82 in *D*, 0.28 in *P*, and pH 4.8. 17.2°C. Lower trace indicates the duration of flash.

In order to examine the relation between the start of illumination and initiation of process I, the acid sample was exposed to a flash from the xenon-filled discharge tube. Exceptionally, alternating current of 20 kc was used for the conductance measurement, and the time constant of the apparatus was 300 microseconds. Fig. 5 represents a typical record of this experiment. In this figure, one can recognize the slight decrease in conductance at the peak of the flash and the remarkable decrease a little later. Anyhow, the electrical response in process I must take place immediately upon illumination, if we take into consideration the delayed diffusion of electroconductive substances in rhodopsin solutions.

Now we will examine how far process I is related to the bleaching of rhodopsin. A simultaneous record of bleaching and conductance change is

represented in Fig. 6A. This indicates that a rapid phase of bleaching had finished within the period[3] of 20 milliseconds after the beginning of illumination, though it could not be traced on the record, and that this rapid phase was followed by a slow phase. From Fig. 6B, the slow phase of bleaching in darkness continues for about half a second under the same conditions (18.9 C). On the other hand, the initial decrease in conductance is revealed as an exponential curve, corresponding neither to the rapid phase nor to the slow phase of bleaching.

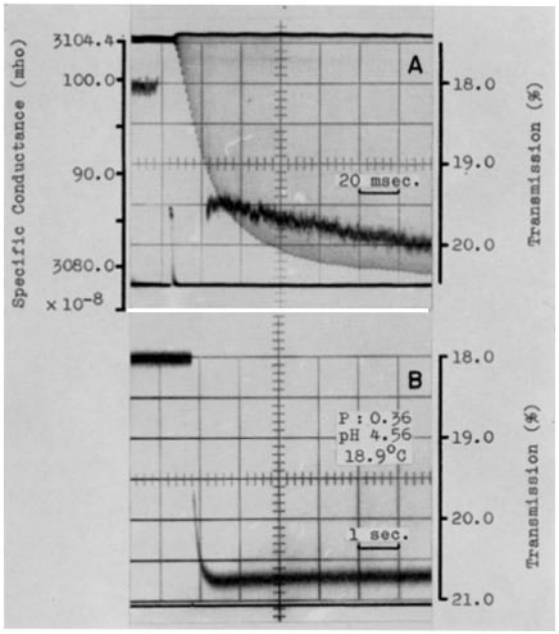

FIGURE 6. Relationship between changes in transmission (503 mμ) and in conductance on exposure of acid rhodopsin to a flash of the xenon-filled discharge tube. See text for further details.

None of the records in this series exhibited any symptom of the conductance increase of process II, because of the low pH of the sample and the short period of the recording. For initiation of process II, when slightly acid samples were similarly illuminated with the xenon-filled discharge tube, the conductance, for example, showed a minimum about 500 and 200 milliseconds after the beginning of illumination at 12.9 and 29.4°C, respectively. The photoflash lamp, though of a lower intensity, gave almost the same values as those in the above-mentioned experiments.

[3] Although this period before the shutter S_3 was reopened was shortened to 5 milliseconds, records similar to those of Fig. 6A were obtained.

4. *Quantitative Analysis of Conductance Change*

Although we find three different processes in the conductance change on illumination of rhodopsin, the quantitative analysis was carried out exclusively with regard to the initial decrease in conductance. The amount of the initial decrease was obtained from the maximum decrease revealed soon after illumination. The decrease in optical density at 503 mμ was also determined from the difference of transmissions measured before and about

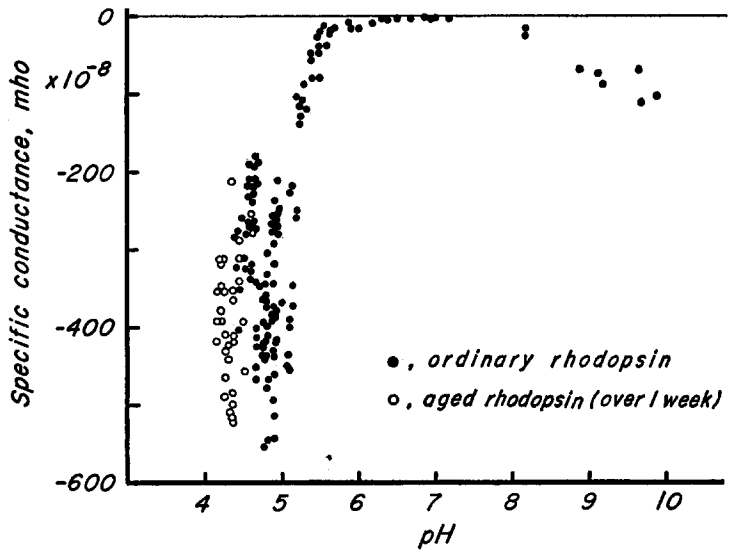

FIGURE 7. pH dependence of the initial decrease in conductance per unit decrease in optical density at 20°C.

5 minutes after illumination, in order to calculate the conductance decrease per unit decrease in optical density ($\Delta C/\Delta D$). To show clearly at first the pH dependence of the value of $\Delta C/\Delta D$, all the results from many different preparations used throughout the present studies are brought together in Fig. 7. When the pH of the acid sample is brought towards neutrality, the amount of the initial decrease steeply declines, approaching the line for zero change. As seen clearly in the range of pH 4–5, the values of $\Delta C/\Delta D$ tend to scatter widely at one pH. Referring to Hara (6), this fact would be due to variations in the character of lipoprotein according to preparation of rhodopsin. For all the following experiments, the acid aliquots obtained from single preparations were employed, usually without any pH adjustment of the extracted solution of rhodopsin. Although the minimum in conductance may be caused by a balance between processes I and II, the initial decrease

in these acid samples would be little influenced by process II, because process II hardly appears at such a low pH.

The relation between ΔC and ΔD after various periods of exposure was studied by means of illumination with 60,000 lux, as shown in Table I. Both the density decrease (ΔD) and conductance decrease (ΔC) become larger with longer exposure times. The value of $\Delta C/\Delta D$ is practically constant up to 1/5 second exposure. But this ratio tends to be slightly smaller in the cases of exposure of half a second and 1 second, probably because the conductance change is more or less suppressed by the conductance increase

TABLE I

RELATION BETWEEN INITIAL DECREASE IN CONDUCTANCE AND DECREASE IN OPTICAL DENSITY ON ILLUMINATION OF RHODOPSIN SOLUTION

Experimental conditions			Changes on illumination		
Sample	Illumination, 60,000 lux	Original conductance, $\times 10^{-8}$ mho	Initial decrease in conductance, $\times 10^{-8}$ mho, ΔC	Density decrease at 503 mμ, ΔD	Conductance change per unit of density decrease, $\Delta C/\Delta D$
	sec.				
D:0.42	1/25	1910.2	9.7	0.021	462
P:0.26	1/10	2010.0	22.6	0.048	471
pH 4.8	1/5	1937.3	45.2	0.097	465
24.1°C	1/2	1867.1	75.0	0.169	443
	1	1814.7	92.6	0.217	427

D, optical density at 500 mμ; *P*, optical purity.

due to process II, which is previously noted in section 3 to start about 200 milliseconds after illumination. If the exposure is not too long, the initial decrease in conductance is proportional to rhodopsin bleached, as indicated by constant value of $\Delta C/\Delta D$. By the way, it was considered that the short photoflash used in later experiments is more suitable than the illumination with 60,000 lux for determination of $\Delta C/\Delta D$ because of its capacity to cause more remarkable bleaching even during 40 milliseconds.

It is known that the rhodopsin bleaching with flashes of light is influenced by the photorecovery of rhodopsin from some intermediate product (1, 2, 9, 13, 23, 24). Then the effects of the photorecovery on the bleaching and conductance change of rhodopsin were examined with the photoflash lamp. As for the bleaching, some samples placed at various distances were illuminated at one time by the photoflash, and their variation in optical density at 500 mμ was traced for 1 hour with a Beckman spectrophotometer, as illustrated in Fig. 8. As the sample is placed nearer the light source, the optical density following illumination becomes lower, until it reaches a

minimum value at a certain illumination (15 cm away from the light). After this, the more intense illumination causes a rather smaller decrease in the optical density. It is also noticed that the optical density of every sample continues to decrease gradually over 1 hour in darkness, showing a far slower phase of bleaching than the two phases cited already in Fig. 6. In any case,

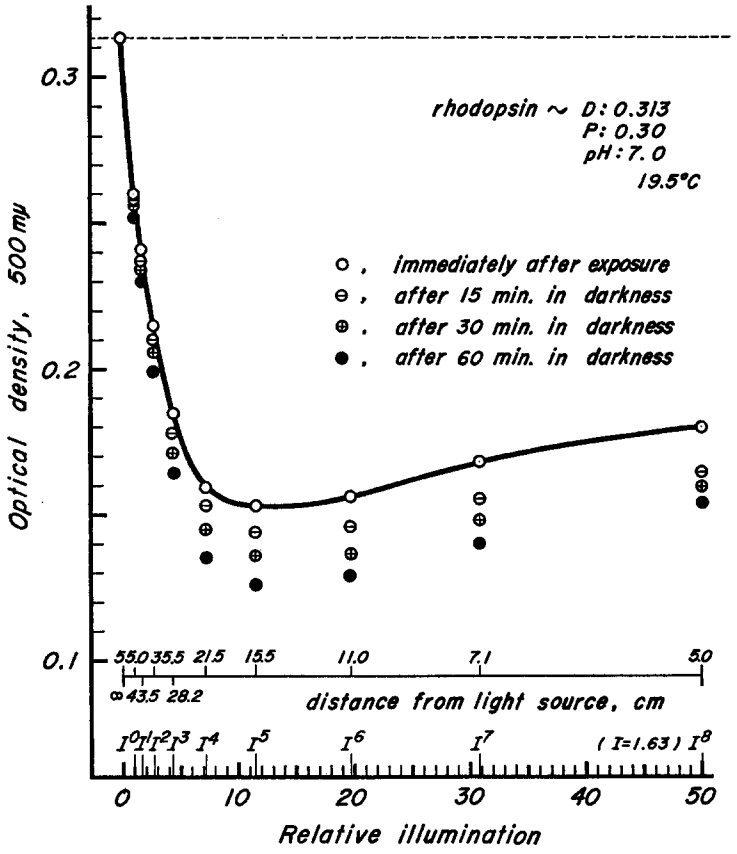

FIGURE 8. Effect of a flash of light on bleaching of rhodopsin solutions placed at various distances from the photoflash lamp.

however bright a flash may be, rhodopsin cannot be bleached more than about 70 per cent of the total amount.[4] Such a fact may suggest that the intense light is used not only for the bleaching of rhodopsin but also for the photorecovery even at room temperature. As for the conductance change,

[4] In Fig. 8, being illuminated with 10,000 lux for 15 minutes, every sample finally fell to 0.042 from 0.313 in optical density. As the sample bleached most by a flash of light showed 0.126 in optical density, the maximum amount of rhodopsin bleached was calculated as (0.313 − 0.126)/(0.313 − 0.042) or 0.69 of the total amount of rhodopsin.

TABLE II

INITIAL DECREASE IN CONDUCTANCE ON ILLUMINATION WITH FLASHES OF DIFFERENT INTENSITY

Experimental conditions			Changes on illumination		
Sample	Relative intensity of illumination, photoflash lamp	Original conductance, $\times 10^{-8}$ mho	Initial decrease in conductance, $\times 10^{-8}$ mho, ΔC	Density decrease at 503 mμ, ΔD	Conductance change per unit of density decrease, $\Delta C/\Delta D$
D:0.62	1	2172.3	16.0	0.109	147±4
P:0.33	4	2066.0	38.4	0.271	142±3
pH 5.3	12	2028.2	37.6	0.278	135±1
31.3°C					

Each value of the changes is derived from two experiments.

Table II gives the results obtained from the experiments of illuminating the acid samples placed 35, 15, and 6 cm from the photoflash lamp, where relative intensity of illumination is respectively estimated as about 1, 4, and 12 from Fig. 8. The decrease in optical density of the sample subjected

TABLE III

EFFECT OF IONS ON INITIAL DECREASE IN CONDUCTANCE ON ILLUMINATION OF RHODOPSIN SOLUTIONS

Experiment	Experimental conditions: Sample			Original conductance, $\times 10^{-8}$ mho	Changes on illumination: Initial decrease in conductance, $\times 10^{-8}$ mho ΔC	Density decrease at 503 mμ, ΔD	Conductance change per unit of density decrease, $\Delta C/\Delta D$	No. of measurements
	D:0.40	in H_2O	pH 4.4	4352.1	58.0	0.149	389±13	2
I*	*P*:0.55	in NaCl (0.01 M)	pH 4.5	97,892.6	68.0	0.179	380±18	2
	27.2°C	in KCl (0.01 M)	pH 4.5	111,638.9	66.5	0.169	393±29	2
	D:0.39	in H_2O	pH 4.5	3126.6	43.5	0.132	330±7	2
II*	*P*:0.47	in $MgCl_2$ (0.002 M)	pH 4.6	46,816.7	39.1	0.133	294±20	2
	28.3°C	in $CaCl_2$ (0.005 M)	pH 4.5	131,929.8	40.8	0.138	296±10	3
	D:0.59	in H_2O	pH 5.2	2876.3	83.3	0.260	320±18	3
III	*P*:0.26	in $AlCl_3$ (0.005 M)	pH 5.2	148,903.7	66.3	0.254	261±11	4
	16.7°C							

* Preparation without petroleum ether treatment of rods.

to the most intense illumination is as relatively slight as expected, probably because of the photorecovery mentioned above. On the other hand, the conductance decrease is likewise small, so that the value of $\Delta C/\Delta D$ differs little from those of the other two cases. This fact shows that the initial decrease in conductance can be regarded as proportional to the amount of rhodopsin converted to final products, even when the photorecovery of rhodopsin is evoked.

According to Hara (7), none of the ions such as Na^+, K^+, Mg^{++}, and Ca^{++} is able to give any marked effect on the initial phase of bleaching of rhodopsin solutions. Although the effect of these ions on the initial decrease in conductance was at first examined, no marked influence on the value of $\Delta C/\Delta D$ could be found as shown by Experiments I and II in Table III. However, the ion of Al^{+++} tended to decrease the value of $\Delta C/\Delta D$ at pH 5.2, as indicated by Experiment III. This was also the case at pH 4.1. The higher the valency of the ion captured by rhodopsin becomes, the more effectively the ion would be able to depress the initial dissociation of rhodopsin on illumination. When we also tested the conductance changes of slightly acid samples on illumination with monochromatic lights of 460, 500, 540, and 600 mμ, all the records obtained were similar to those in Fig. 4 and there was no appreciable difference in the value of $\Delta C/\Delta D$.

5. *Effect of Temperature on Conductance Change*

Previous experiments with continuous illumination (11) showed that the whole pattern of conductance change was little influenced by low temperatures near 0°C, and especially, that the conductance decrease in acid solution could still be recognized even at as low a temperature as -12°C. To observe the effect of temperature on the conductance change and the bleaching following illumination, rhodopsin solutions were illuminated with the tungsten lamp for 1 second at room temperature and near 0°C. For ease in observing the general trend, a preparation was previously adjusted to about pH 5.5 from the original pH 4.7. A set of results is presented in Fig. 9. The conductance changes in processes I and II proceed more slowly at low temperature than at room temperature, and the amounts of the initial decrease and the increase may decline as the temperature goes down. On the other hand, the bleaching almost stops 2 seconds after illumination at room temperature, but proceeds gradually for about 10 seconds at low temperature. The final level of transmission may be a little higher at the higher temperature.

The temperature dependence of the amount of the initial decrease was investigated with aliquots of a rhodopsin preparation without any adjustment of pH. The experimental results are summarized in Table IV. Although the minimum conductance is reached immediately after illumination at

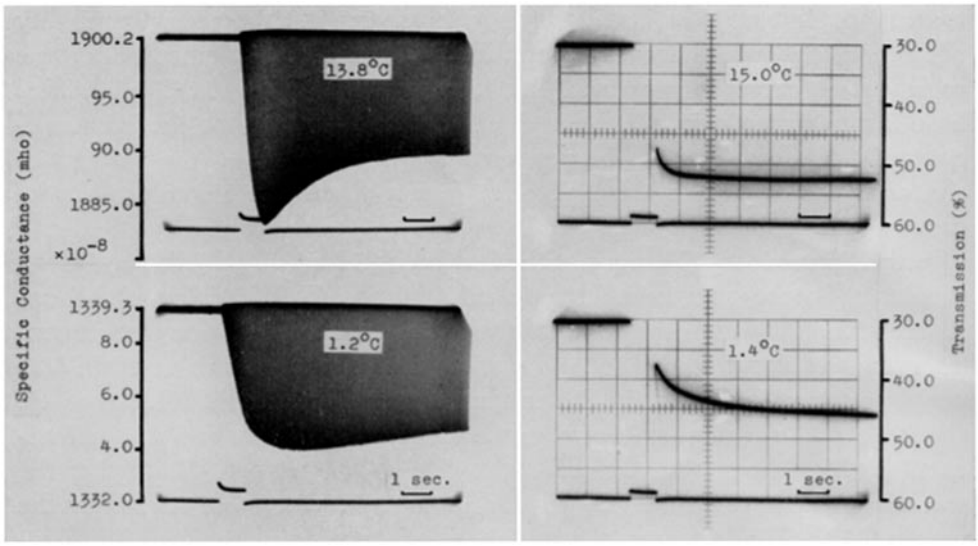

FIGURE 9. Records of changes in transmission (503 mμ) and in conductance on illumination of slightly acid rhodopsin at different temperatures. Sample, 0.54 in *D*, 0.36 in *P*, and pH about 5.5.

TABLE IV

INITIAL DECREASE IN CONDUCTANCE AND DECREASE IN OPTICAL DENSITY ON ILLUMINATION OF ACID RHODOPSIN AT DIFFERENT TEMPERATURES

	Experimental conditions			Changes on illumination			
Experiment	Sample	Temperature	Original conductance, $\times 10^{-8}$ mho	Initial decrease in conductance, $\times 10^{-8}$ mho, ΔC	Density decrease at 503 mμ, ΔD	Conductance change per unit of density decrease, $\Delta C/\Delta D$	No. of measurements
		°C					
I	*D*:0.46 *P*:0.29 pH 5.1	23.0	1706.5	104.2	0.237	440±2	2
		3.5	1084.2	42.1	0.134	314±7	2
II	*D*:0.57 *P*:0.37 pH 4.9	17.5	1219.3	30.0	0.121	248±21	4
		3.3	815.3	15.3	0.081	189±11	4
III*	*D*:0.58 *P*:0.47 pH 4.9	18.2	3643.5	43.3	0.112	387±15	5
		3.0	2483.2	23.0	0.079	291±19	5

* Omission of petroleum ether treatment of rods.

room temperature but some seconds later at low temperature, the initial decrease in conductance (ΔC) is expressed by the maximum decrease in conductance following illumination in both cases. The decrease in optical density at 503 mμ (ΔD) was measured about 5 minutes after illumination as the amount of rhodopsin bleached. Each value of pH was determined at room temperature. The samples were illuminated with flashes of light for 40 milliseconds in Experiments II and III, and with 12,000 lux for 60 seconds in Experiment I. Through all the experiments both ΔC and ΔD become smaller at low temperature. However, the conductance decrease per unit of density decrease, $\Delta C/\Delta D$, is clearly smaller at low temperature than at high temperature. The ratio of $\Delta C/\Delta D$ at high temperature to $\Delta C/\Delta D$ at low temperature may be calculated as 1.401, 1.312, and 1.330 in Experiments I, II, and III, respectively. Accordingly, the temperature coefficient of the conductance change; *i.e.*, the percentage increase in $\Delta C/\Delta D$ per unit degree of temperature, is 2.1, 2.2 and 2.2 per cent/°C in Experiments I, II, and III respectively, estimated on the average as 2.2 per cent/°C. Clearly, these values lie in the range of the coefficients for the conductance of dilute solutions of inorganic electrolytes, especially between those of ordinary salts (2.2 to 2.7 per cent/°C) and those of acids or bases (1.6 to 2.0 per cent/°C). Therefore, the difference in $\Delta C/\Delta D$ according to temperatures may be fully explained as the temperature dependence of mobility of any inorganic substance reacting on illumination. If so, the amount of the conductive substance which contributes to the conductance change can be regarded as decided by the amount of rhodopsin bleached, irrespective of temperature. In other words, the amount of change in the protein moiety induced by light must be intimately linked only to the amount of rhodopsin bleached.

Temperature dependence of the electrical conductance in non-illuminated rhodopsin solutions was also examined. Although our rhodopsin preparations differed widely in the values of D, P, and pH, the temperature coefficient was calculated as 3.3, 3.5, and 3.1 per cent/°C in Experiments I, II, and III in Table IV respectively. Considered together with many other cases, it was distributed between 3.0 to 3.8 per cent/°C, the average being 3.4 ± 0.3 per cent/°C. This value is much higher than the temperature coefficient for the inorganic conductive substance participating in the conductance change of illuminated rhodopsin.

6. *Effect of Aging on Conductance Change*

The response to photoflashes of light of 40 milliseconds was examined with rhodopsin samples stored for many days. The whole pattern of the conductance change changed more or less with time after preparation. In general, as the aging proceeded, process II became obscure and process III more and more distinct. In acid solution, aged rhodopsin tended to reveal a pattern

of conductance change similar to that of fresh rhodopsin in alkaline solution (*cf.* Fig. 3B). The period of storage required for recognizing a constant effect of aging differed widely among preparations. The conspicuous effect was connected closely to the remarkable rise in optical purity during the storage of rhodopsin preparations. Considered together with many experiments, it seemed rather that the solution abundant in lipid showed resistance to aging.

Two representative series of experiments on the effect of aging on the

TABLE V

INITIAL DECREASE IN CONDUCTANCE AND DECREASE IN OPTICAL DENSITY ON ILLUMINATION OF AGED ACID RHODOPSIN

	Experimental conditions						Changes on illumination		Conductance change per unit of density decrease, $\Delta C/\Delta D$ at 20°C
		Sample							
Experiment	Aging period	D	P	pH	Temperature	Original conductance, $\times 10^{-8}$ mho	Initial decrease in conductance, $\times 10^{-8}$ mho ΔC	Density decrease at 503 mμ, ΔD	
	days				°C				
	0	0.30	0.31	4.8	17.6	1129.3	34.5	0.101	360
	6	0.27	0.37	4.8	18.6	1993.5	37.1	0.086	440
I	14	0.21	0.54	4.3	21.3	3330.7	16.2	0.034	460
	23	0.15	0.73	4.3	19.9	3266.1	17.7	0.039	450
	34	0.12	0.86	4.3	21.3	3487.3	9.2	0.024	370
	0	0.53	0.45	4.6	17.6	3968.6	27.9	0.150	200
	6	0.43	0.34	4.6	18.6	4418.7	32.2	0.145	240
II*	14	0.42	0.34	4.6	21.3	5118.1	27.6	0.100	270
	23	0.40	0.41	4.4	19.9	4981.8	32.4	0.104	310
	34	0.35	0.49	4.2	21.3	5692.5	30.4	0.098	300

* Preparation without petroleum ether treatment of rods.

amount of the initial decrease in conductance are shown in Table V, based on average values through four analyses repeated in order to cancel the irregularities due to photoflash lamps. ΔC corresponds to the amount of the initial decrease at the first second after illumination, and ΔD to the density decrease measured 5 minutes later. Optical density (D) and optical purity (P) gradually alter with age, and both ΔC and ΔD gradually decline. The value of $\Delta C/\Delta D$ becomes slightly large at one time during the progress of aging, though this tendency cannot be explained easily by the pH dependence of ΔC and ΔD. The more marked rise in optical purity with age in Experiment I than in Experiment II seems to exert no essential influence on the value of $\Delta C/\Delta D$.

Acid solutions of rhodopsin did not usually reveal any symptom of process

II about 1 week after preparation. For further examination of process II, several aliquots of aged rhodopsin were brought towards neutrality and illuminated with the tungsten lamp for 1 second. A series of results obtained after aging for 24 days is illustrated in Fig. 10. The decrease in optical density after illumination was 0.16 to 0.17. In this figure, process II is distinctly observed owing to the adjustment of pH, and accordingly aged rhodopsin never loses its capacity to exhibit process II. In contrast with the results in

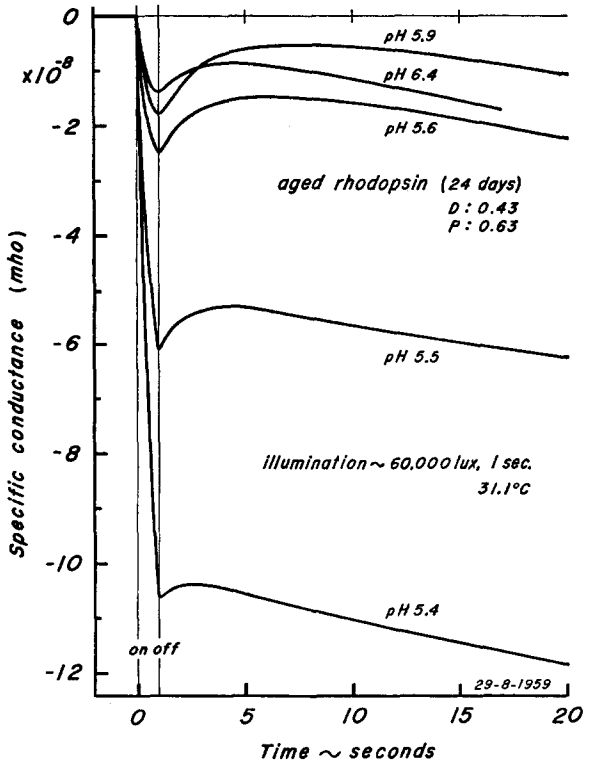

FIGURE 10. pH dependence of conductance change of aged rhodopsin.

Fig. 4, we do not observe that process II begins within 1 second of illumination, nor that the conductance continues to increase beyond the original level. One can consider that the disappearance of process II in acid solution of aged rhodopsin may result mainly from process III which becomes prominent in aged rhodopsin.

DISCUSSION

Elementary Processes of Conductance Change

Previously Hara (6) pointed out that, on continuous illumination, the electrical conductance of rhodopsin solutions changes almost exponentially for a

while with illumination time, decreasing markedly at acid and alkaline pH, but increasing slightly near neutrality. Now such patterns in the conductance change depending on pH may be easily understood as the result of combination of three different elementary processes; *i.e.*, process I (initial decrease), II (increase), and III (slow decrease).

In the experimental records with the acid, slightly acid, and alkaline samples illuminated by flashes of light, these three processes were separated from each other almost perfectly, so that we could estimate the reaction rate of each process. The half-time of process I, as a thermal reaction of the first order, is approximately 15 milliseconds at 20°C (*e.g.* 16 milliseconds at 17.2°C and 15 milliseconds at 18.9°C) and the completion of process I requires about 150 milliseconds. Similarly, process II is also a thermal reaction of the first order with a half-time of about 2.3 seconds at 20°C (*e.g.* 1.7 seconds at 29.4°C and 2.7 seconds at 12.9°C) and requires as long as 10 seconds for its completion. Process III may be of far longer duration than processes I and II, with a half-time of more than several minutes. Thus, processes I, II, and III can be distinguished from each other by the difference in reaction rate.

Bleaching and Conductance Change

It is known from low temperature experiments (12, 13, 24) that illuminated rhodopsin passes at first into a labile fraction such as lumi- or metarhodopsin, which produces rhodopsin or isorhodopsin in the light and breaks down to final products in the dark. Since such a production of rhodopsin and isorhodopsin can be recognized on flash illumination of rhodopsin even at ordinary temperatures (1, 2, 22), it is also considered reasonable that the photoproduct produced by flash illumination of rhodopsin is a mixture consisting of one fraction that spontaneously decomposes in the dark (fraction 1) and another photosensitive fraction that is stable in the dark (fraction 2), and that the decrease in optical density of rhodopsin at 500 mμ after illumination is mainly due to the conversion of fraction 1 to final products, retinene and opsin. Wulff and his coworkers (22) have already pointed out that the photoproduct resembling metarhodopsin in absorption spectra decomposes in the dark through four different stages which require for completion (1) several hundred microseconds, (2) a few tens of milliseconds, (3) a few hundred milliseconds, and (4) 1 to 2 hours, but that (2) and (3) are rather difficult to separate from each other. Our experimental records in Fig. 6 suggest that there exists a phase of rapid decrease in optical density preceding another phase lasting for about 500 milliseconds after illumination. Besides these two phases of bleaching, an extremely slow phase continuing for more than 1 hour is recognized in Fig. 8. These results generally agree with their results, and now one may be sure that the decomposition of fraction 1 involves at

least three different phases of bleaching. On the other hand, each of three well defined processes of the conductance change is also a dark reaction initiated by light, and processes I, II, and III are in the decreasing order of reaction rate. There is no doubt, however, that in the reaction rate each process of the conductance change cannot correspond to any phase of bleaching.

According to Hubbard and her coworkers (12, 14), the transformation of rhodopsin to lumirhodopsin involves a configurational change of cis to all-trans retinene, so that most of the intermediates after this transformation may mainly have the same, all-trans configuration of chromophore. And consequently, the differences in color among some intermediates produced later during bleaching may be due to differences in the fit between the retinene and opsin surface. Such differences in the fit perhaps make a sequence of conformational changes of opsin. Since the conductance change does not proceed in parallel with the bleaching, all the processes of the conductance change might not be due to changes in the fit or in the conformation of opsin connected directly to the bleaching. Furthermore, considered in relation to the aging, which exerts somewhat different influences on the processes of the conductance change, each process seems to proceed at its own separate site in the protein moiety of the rhodopsin molecule.

The initial decrease in conductance is proportional to the amount of rhodopsin bleached, as shown by the constant value of $\Delta C/\Delta D$. This is also the case even when photosensitive fraction 2 is produced by the short intense flash illumination. These facts mean that the amount of the initial decrease is always proportional to the amount of fraction 1 which appears immediately after illumination and decomposes to final products. As to the increase and the slow decrease in conductance, we cannot easily make a clear argument with reference to the amount of rhodopsin bleached, but the ratio of the amount of the increase to that of the initial decrease seemed to be almost constant among samples of the same pH, even in different preparations. We believe, therefore, that the amount of the conductance increase is also proportional to the amount of rhodopsin bleached, which is related to the initial decrease.

Factors Contributing to Conductance Change

According to many workers, on illumination the pH of rhodopsin changes more or less towards neutrality, rapidly in an acid solution and slowly in an alkaline one; it is rather difficult to judge exactly the general trend of the pH change in almost neutral solution. Now it is clear that the acid sample, which undergoes the rapid rise in pH, mainly shows the conductance decrease of process I, while the alkaline sample, which undergoes the slow fall in pH, shows the slow conductance decrease of process III. Therefore

the pH change following illumination must contribute to the conductance decrease registered as processes I and III. According to Radding and Wald (15) and Fukami (4), a slow fall in pH sometimes follows the rapid rise in pH on exposure of weakly acid rhodopsin to a flash of light. Although process II, unlike the slow fall in pH, occurs without exception in the slightly acid samples and is of far shorter duration, it may be assumed that the conductance change in process II is also associated with such a fall in pH. Usually, on illumination with a flash of light, the samples of pH 5.0 and 6.0 may be respectively expected to show the rapid rise and the secondary fall in pH of about 0.05 unit per optical density decrease of 1.0. However, since it is considered that the actual amount of the initial decrease or the increase in conductance per unit decrease in optical density is rather too large to be explained only by the pH change of 0.05 (*cf.* Figs. 4 and 7), we cannot conclude that each process of the conductance change is wholly ascribable to the pH change mentioned above. It was suggested that the conductance change is partially due to the participation of some electrolytes such as alkali ions associated closely with rhodopsin molecules (8). On the other hand, Sekoguti (17, 18) ascertained the release of potassium ions from the retina with a change in ATPase activity of rod outer segments induced by illumination. Therefore, such phenomena might be connected to the processes of the conductance change on illumination, at least to process II.

Biological Meaning of Conductance Change

The photoisomerization of retinene may initiate various phenomena associated with conformational changes of the rhodopsin molecule. Indeed, many facts have been reported as follows: changes in isoelectric point (3), H ion concentration (4, 9, 15), electrophoretic characteristics (5), oxidation-reduction potential (10), and ultraviolet absorption spectra (19), as well as release of sulfhydryl groups (20, 21) and of acid- and base-binding titratable groups (15). In order to consider these phenomena as associated closely with the visual function, it seems, however, necessary to inquire whether these phenomena are sufficiently early and reversible for visual excitation. According to Radding and Wald (15, 16), the slow change in pH following illumination of rhodopsin is associated with the irreversible denaturation of opsin in acid or alkaline solution, as evidenced by loss of its capacity to regenerate rhodopsin. Referring to this opinion, it may be apparent that process III of the conductance change corresponding to the slow change in pH proceeds with the irreversible denaturation of opsin, while process I related to the rapid change in pH is associated with certain conformational changes revealed prior to the irreversible denaturation of opsin. As process II is a far earlier reaction than process III, processes I and II must arise from the reversible, weak denaturation of rhodopsin. Such an understanding would be

also supported by the following facts (unpublished experiments): the initial decrease and the increase in conductance appear during the production of metarhodopsin on illumination of squid rhodopsin at 5°C, and ultraviolet extinctions decrease instantly on flash illumination of rhodopsin. We believe that the conformational changes demonstrated by processes I and II bear a close relation to the initial stage of visual excitation, and that charged molecules and ions linked to those processes are produced within or around the disc structure of the rod outer segment to contribute to the generation of a potential for nerve impulses. It seems also of interest with regard to rod function that all the illuminated molecules of rhodopsin may not contribute to the excitation of the rod cell through the conductance change, because some molecules are converted not to fraction 1 but to fraction 2 including rhodopsin.

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