THE EFFECT OF ILLUMINATION ON THE ELECTRICAL CONDUCTANCE OF RHODOPSIN SOLUTIONS*

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ABSTRACT

An apparatus was constructed in order to record continuously and simultaneously changes in extinction and electrical conductance of rhodopsin solutions. With this apparatus, changes in electrical conductance on exposing rhodopsin to light were investigated. On illumination solutions of rhodopsin revealed a conductance change so long as they preserved their photosensitivity. The conductance change begins almost immediately upon illumination and is almost proportional to the amount of rhodopsin decomposed, continuing until rhodopsin is converted to indicator yellow. Near pH 7 the conductance is apt to increase slightly, while it decreases considerably outside the range of pH 6–9, being accompanied by a pH change towards neutrality. The conductance change is regarded as an essential property of rhodopsin, because it occurs in aqueous suspension as well as in digitonin solution; it may be caused by hydrogen or hydroxyl ions and some other conductive substances. It is also noteworthy that the petroleum ether–soluble component of the rod outer segments—presumably the lipide—tends to increase the conductance change. In suspensions of rod outer segments and retinal homogenates, the conductance increases on illumination irrespective of pH: this may be due to secondary reactions following the photic reaction of rhodopsin. We shall discuss the significance of the conductance change in relation to the initiation of visual excitation.

Recent progress in the biochemistry of rhodopsin has been achieved mainly by Wald and other workers at Harvard University, as well as by Morton, Collins, and others of the University of Liverpool (*cf.* Wald, 1953, and Collins, 1954). They have even carried out enzymological studies on the "rhodopsin cycle" in recent years. Nowadays one may be sure that incident light activates rhodopsin molecules and leads eventually to the stimulation of rod vision through a series of physical and chemical events. As the process related to completion of vision must certainly involve quick reactions, a chemical study in this field is attended with many difficulties. Thus the analysis of the elementary process contributing to the formation of retinal impulses

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has been reasonably allotted to the electrophysiologists, such as Granit, Hartline, and others (*cf.* Granit, 1947).

The writer is of the opinion that light may cause an ionization or photoelectric effect in rhodopsin molecules and that, if so, the activation of rhodopsin must be accompanied by a change in the electrical properties of rhodopsin solutions. In the present study we shall demonstrate a change in the electrical conductance of rhodopsin solutions on exposure to light, in order to establish the physicochemical basis of an initial phenomenon in the excitation of vision.

Experimental Procedures

Preparation of Rhodopsin Solution.—Cattle rhodopsin was prepared by a modification of the methods described by Collins *et al.* (1952 *a*) and Wald *et al.* (1951–52). Under a dim red light, thirty to fifty retinas are dissected from fresh eyes derived from the slaughter house and kept in the dark at 0°C. They are vigorously shaken with about half their volume of physiological saline (0.9 per cent) in order to detach the rod outer segments from the remaining retinal tissues. The thoroughly stirred mixture is filtered through a 60 mesh brass gauze shaped in the form of a hollow, whereupon the retinal debris is transferred into a glass tube to be shaken again with saline. This procedure is repeated three times, until a crude rod suspension containing melanin, blood, and retinal tissue fragments is obtained. This is transferred into a centrifuge tube, and saturated sucrose solution is carefully poured down the side of the tube so as to form a lower sugar layer of about half the volume of the saline suspension. With a flat-ended glass rod, the saline-sugar interface is stirred so that a gradient of concentration of sucrose may be produced between the top and the bottom. The tube is then centrifuged at 4,000 R.P.M. for 10 minutes, and now all the components of the suspension are found to be accumulated in their own pycnotic levels. As this centrifugation brings down blood, melanin, and other tissue fragments, the rod outer segments are separated as a deep scarlet-colored layer, which can be easily removed from the tube with a pipette. The rod outer segments are centrifuged once more with saturated sugar solution. They are then washed with about 50 ml. of saline and centrifuged at 4,000 R.P.M. for about 10 minutes. The washing is repeated more than three times until the supernatant becomes clearly colorless. The residue—the rod outer segments—is stirred in 4 per cent potassium aluminum sulfate solution, left to harden for 1 hour at 5°C., and washed three times with ion-free water. The final material is stored frozen at −10°C., and, when needed to extract rhodopsin, it is treated with 1 per cent aqueous solution of digitonin for about 1 hour at room temperature.

In order to remove as much of the lipides as possible, the following treatment has been adopted. The rod outer segments are dried with a vacuum pump, ground in a mortar with about five times their volume of anhydrous sodium sulfate, and extracted two or three times by vigorous shaking with petroleum ether. The dried material is then washed with ion-free water in the centrifuge more than three times until it becomes free of sodium sulfate. The procedure for extracting rhodopsin is the same as previously described. Rhodopsin solutions so prepared will be provisionally called "lipide-free."

In the course of preparation we use no buffer, because the rhodopsin solution should be as free from ions as possible for reliable measurements of the conductance change. The pH of the rhodopsin solution is usually 4.4–5.2, probably being affected by the isoelectric point of the protein. Before each experiment, the absorption spectrum of the preparation is measured with a Beckman spectrophotometer between 320 and

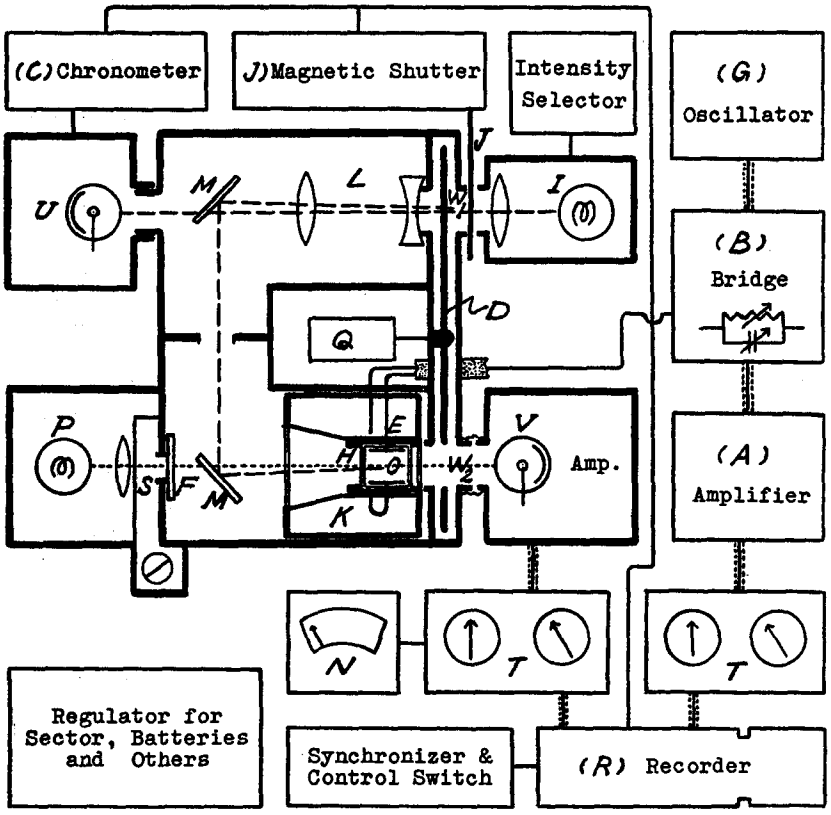

FIG. 1. Diagram of the equipment used for measurement of transmission and electrical conductance of rhodopsin solution on illumination. See text for further details in the figure.

700 mμ, and the optical density (D) at 500 mμ and the optical purity (P), *i.e.* the ratio of extinction at the minimum to the maximum, is thus determined.

Construction of Apparatus.—As every rhodopsin solution varies in respect to such properties as density, purity, and pH, it cannot always show a similar process of bleaching, even if illuminated with a constant light. Therefore, we must record continuously and simultaneously the transmission and the electrical conductance during illumination, in order to examine exactly the amounts of electrical change at the time when a given amount of rhodopsin has bleached. For this purpose, the following apparatus has been constructed (Fig. 1).

A 40 c.p. tungsten point light (*I*), run off a constant voltage supply, is used as the source of illumination. After passing through a sector (*D*) rotated by an electrical motor (*Q*), the light is formed into a parallel beam by a condensing lens system (*L*). Reflecting at a pair of mirrors (*M*), it finally falls on the front face of the optical cell (*O*, internal dimensions, 1.0 × 1.0 × 3.5 cm.) containing the rhodopsin solution. The maximum illumination measured at the optical cell is about 3,000 or 12,000 lux, depending on whether the sector is rotated or stopped.

On the other hand, an 8 c.p. light (*P*) supplied by a battery is used as the source for electrophotometry. After passing through a lens, a variable slit (*S*), and a glass filter (*F*, Mazda V-G 1 with main wavelength, 518 mμ, or Hitachi's interference filter with main wavelength, 503 mμ), the light is projected on the optical cell containing the sample solution, and hereupon, it falls through the rotatory sector on a photoelectric tube (*V*, Mazda PS-50-V : Sb−Cs), housed in an earthed container settled on the vibration-proof table. Since the transmitted light becomes intermittent on account of rotation of the sector, the photoelectric current is enlarged by a three stage A.C. amplifier at about 600 to 1,000 C.P.S. After rectification and filtration, the current goes to a detection box (*T*) consisting of a crude and a fine meter, and gives certain deflections which are recorded by the meters, corresponding to the transmission of rhodopsin solution. As the rectified current is also led to an electromagnetic recorder (*R*) by metal-covered wire, the bleaching process of rhodopsin can be recorded on a photographic paper.

The rotatory sector (*D*) plays an important role in this apparatus. The sector is a thin circular disc (30 cm. in diameter), on which 20 slots of 1 cm. square are arranged in a circle with intervals of 3 cm. It rotates at 30 to 50 R.P.S. in a narrow compartment, each wall of which is endowed with a pair of windows 1 cm. square (W_1 and W_2). On the rotation of the sector, the slots and the windows are synchronized as follows. When the monochromatic beam reaches the photoelectric tube through the sample, the illumination light (*I*) has been completely screened at the window W_1, while the light (*I*) can reach the sample only when W_2 is closed. The sample solution bleaches in an instant, and its transmission is measured at the following one, and thus the bleaching process of rhodopsin can be exactly recorded.

In order to keep the temperature of the sample practically constant during illumination (a few minutes), a water reservoir (*K*, made of brass, 8 × 8 × 10 cm.) is prepared. It has a front and a rear window both with plane parallel faces, allowing the light to pass through the optical cell towards the tube. A cell holder (*H*), immersed in the reservoir, supports not only the optical cell but also a pair of platinized platinum electrodes (*E*), fitted exactly into the cell (cell constant, 0.564). For the measurement of electrical conductance, the electrode leads are connected to the unknown arm of a Wheatstone bridge (*B*), into which a variable condenser is added in parallel with the variable arm. The alternating current sent to a diagonal of the bridge is generated in a CR oscillator (*G*), usually 1,000 C.P.S. and less than 1.0 volt. To detect the breaking of balance in the bridge and to record the conductance change, the slight potential difference between both ends of a high resistance placed in another diagonal is enlarged by a three stage A.C. amplifier (*A*), and the resulting rectified voltage is sent to another detection box and finally to the recorder, where the magnitude of conductance change on illumination can be recorded continuously on the microampere scale.

An electromagnetic shutter (*J*) is placed between the illumination source and the sector. The window opens completely in less than 1 millisecond. A part of the illumination light is not reflected at the first mirror, but goes on to another photoelectric tube (*U*) which signals the illumination period on the photographic paper. On the way to the recorder, the leads sending the signal are provided with a chronometer (*C*) for marking the time.

All the equipment except the motor driving the sector is served by batteries in order to avoid various troubles resulting from fluctuation of voltage during the experiment. As any slight change in the rotation rate of the sector exerts little influence on the experimental results, the stability of the apparatus can be regarded as sufficient to perform the recordings. The sensitivity of the apparatus can be varied by adjusting the gain of the circuit with the attenuator. At its greatest sensitivity, a 0.01 per cent increase or decrease in the resistance of the variable arm corresponds to a 30 mm. deflection on the photographic paper; that is, we can easily detect a 10^{-5} change in the electrical resistance of the sample, while a 1 per cent change of transmission indicated by an accessory fine meter (*N*) corresponds to a 10 mm. deflection.

Operation of Apparatus.—The apparatus is ready for use about 20 minutes after all the parts have been supplied with electrical sources. 1.5 ml. of rhodopsin solution is introduced into the optical cell, a pair of electrodes is connected to the cable lead to the bridge, and then the balance in the bridge is examined. After the sensitivity of each circuit has been so adjusted as to complete the records of transmission and resistance, we reset each deflection of the meters just at the zero point with the compensator prepared in the detection box. Now, if one presses a remote control switch, the recording starts and soon the illumination begins by means of the electromagnetic shutter. At this time the deflections of the meters increase corresponding to the changes in transmission and in electrical resistance of the sample, and the course of the changes is automatically recorded on photographic paper in the recorder. When a shutter switch is turned off after a certain illumination period (usually within 1 minute), the recording soon finishes. If a scale-out should take place on account of the extraordinary magnitude of resistance change during the recording, reading of the meter would have to be carried out at short intervals as far as possible.

The unit of change is marked on the records as follows. As for photometry, the values of transmission before and after the recording can be determined from the accessory fine meter (*N*). In this case, transmissions are, of course, proportional to deflections of the vibrator in the recorder. As for measurement of resistance change, when the apparatus is just ready for recording and the bridge is balanced, the resistance of its variable arm is altered by a unit ohm and every reading in microamperes is noted down, corresponding to this breaking of the balance. The unit of change can be deduced from these readings and inserted into the records (*cf.* Fig. 2). For better understanding, the bleaching of rhodopsin is rewritten in terms of its extinction instead of transmission, while the course of resistance change is denoted in terms of the percentage change of resistance (the amount of change in ohm/the initial resistance of the solution; $\Delta R/R$). Hereafter we shall name the former REC (rhodopsin extinction curve) and the latter RRC (rhodopsin resistance curve).[1]

[1] Let ΔC be the amount of conductance change, and C the initial conductance of

In general, electrical conductance is very susceptible to temperature. In our experiments, however, heat emitted by the illumination source is almost completely absorbed by the water reservoir, so that the temperature of the sample is kept practically constant during illumination. As rhodopsin is very labile in relation to various physical and chemical factors, it is also necessary to check the effect of alternating current. For instance, after 10 or 20 ma. A.C. had been sent for 10 minutes to the cell containing rhodopsin solution, the absorption spectrum was examined in the range of 300 to 700 mμ. The extinction at 500 mμ of the control sample decreased from 0.236 to 0.225 or 0.219 after exposure to 10 or 20 ma. respectively. The slight bleaching seems to be due to a small amount of heat produced by the alternating current. However, since the current applied in the experiments is less than 100 microamperes, rhodopsin can scarcely be bleached in the course of the experiments.

RESULTS

1. Bleaching and Resistance Change of Rhodopsin Solutions.—An example of the records revealing an intimate relation between bleaching and resistance change on exposure of rhodopsin to light is shown in Fig. 2. When a rhodopsin solution is exposed to light, the resistance increases, and when screened, it no longer shows any remarkable change. The process of resistance increase fully corresponds to that of bleaching, both accompanied by an after-effect of illumination. When most of rhodopsin has bleached into indicator yellow a few minutes after illumination, further illumination does not cause any remarkable resistance change. It is therefore clear that the conductance change in question takes place while rhodopsin undergoes the bleaching into indicator yellow.

As long as the solution shows a red color, its ability to exhibit this resistance change remains virtually unaffected, even after the solution has been stored for several weeks. Consequently, the resistance change is related to the photosensitivity of rhodopsin. In other words, the rhodopsin molecule may have in itself a capacity for electrical response to the light. The resistance change is also observed in rhodopsin solutions prepared from the retinas of frogs (*Rana nigromaculata*). Therefore, such an electrical response may be regarded as one of the general characteristics of rhodopsin solution, irrespective of animal species.

2. Resistance Change in Relation to Rhodopsin Density.—If the resistance change is associated with the rhodopsin molecule, the amount of change must be dependent on the concentration of rhodopsin. Another example revealing the relation between REC and RRC is shown in Fig. 3. The change in ex-

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the solution, then $\frac{\Delta C}{C}$ will be equal to $\frac{R}{R+\Delta R} - 1$ or $-\frac{\Delta R}{R+\Delta R}$. As $R \gg \Delta R$ in our case, we also have $\left|\frac{\Delta C}{C}\right| = \left|\frac{\Delta R}{R}\right|$. Therefore, we can regard RRC as the process of percentage change of conductance.

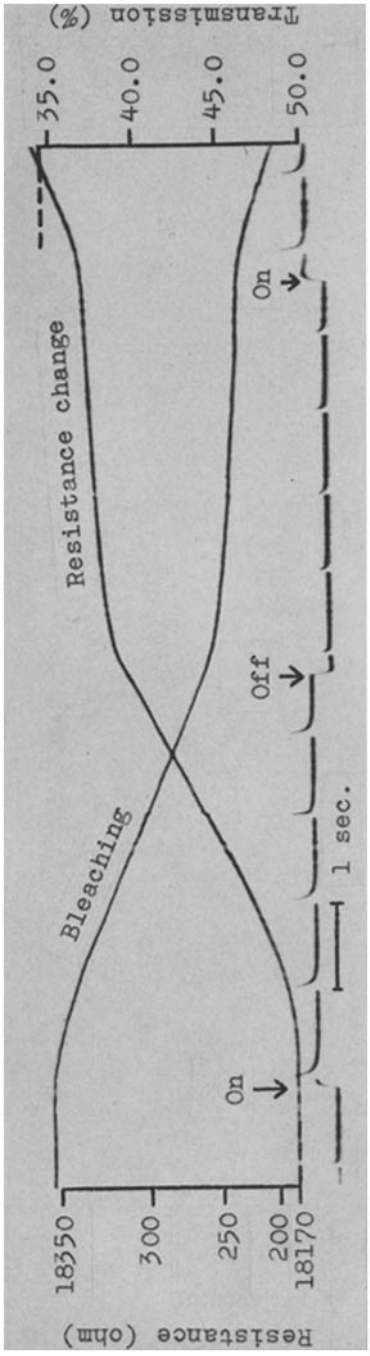

FIG. 2. A record of bleaching and resistance change of rhodopsin solution. Illumination, 3,000 lux. Sample, 0.459 in optical density at 500 mμ, 0.50 in optical purity, and pH 5. Transmission maximum of interference filter, 503 mμ.

tinction (REC) is clearly exponential with illumination time at least within 20 seconds after illumination, indicating that it involves a reaction of the first order. We find also that the resistance change is almost proportional to the amount of rhodopsin bleached during every illumination period; hence RRC must also be exponential. The results of two further experiments made under the same conditions were identical with those shown in Fig. 3.

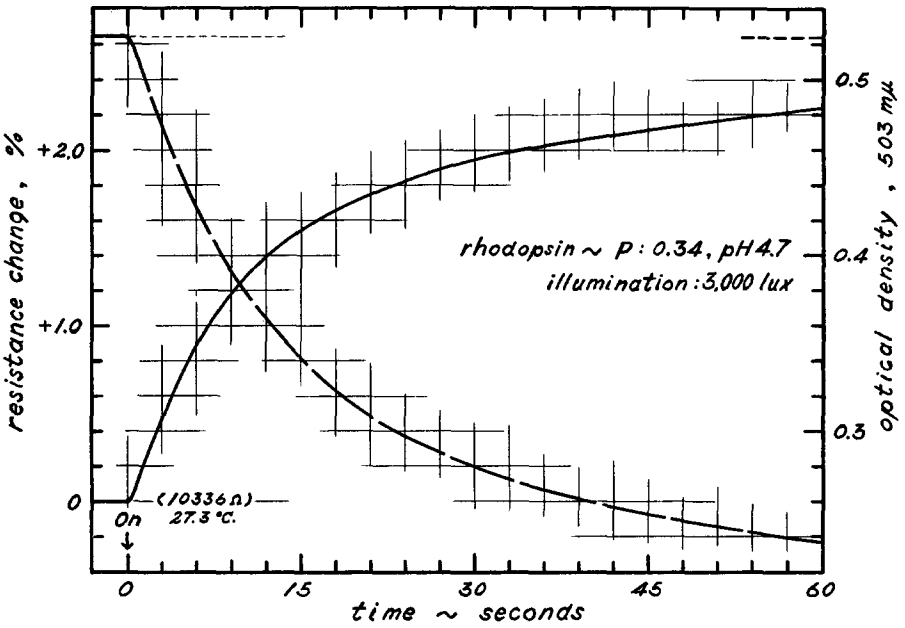

FIG. 3. An example of RRC and REC. Solid line, RRC; broken line, REC. A value in parentheses denotes the initial resistance.

Let us now calculate the actual conductance change in this instance. The optical density of rhodopsin decreases from 0.524 to 0.392 during the initial 9 seconds, while the resistance change amounts to 1.185 per cent. Therefore the actual conductance change[2] is calculated as 0.115×10^{-5} mho per 0.132 of optical density, or 0.87×10^{-5} mho per unit optical density. This means that the change in a solution with a $E_{503m\mu}$ of 1.0 amounts to 0.49×10^{-5} mho in specific conductance at pH 4.7.

[2] As described in the preceeding footnote, $\left|\frac{\Delta R}{R}\right|$ is equal to $\left|\frac{\Delta C}{C}\right|$, so that the actual conductance change ΔC is obtained from $\left|\frac{\Delta C}{C}\right| \times C$ or $\left|\frac{\Delta R}{R}\right| \times \left|\frac{1}{R}\right|$. In order to show the change in specific conductance, ΔC must be multiplied by the cell constant 0.564.

3. Initiation of Resistance Change.—The initial phase of the process of resistance change goes on as shown in Figs. 4 and 5. The record shown in Fig. 4 was taken on illumination with 12,000 lux, while that in Fig. 5 was obtained on exposure to a photoflash lamp. The former record clearly indicates that the resistance increase begins within 20 milliseconds after the beginning of illumination. From the latter record which is taken with the apparatus adjusted for high sensitivity, we remark that the change begins at the very moment when the light reaches the sample. We must here give attention to the following points. First, a resistance increase apparently occurs—in spite

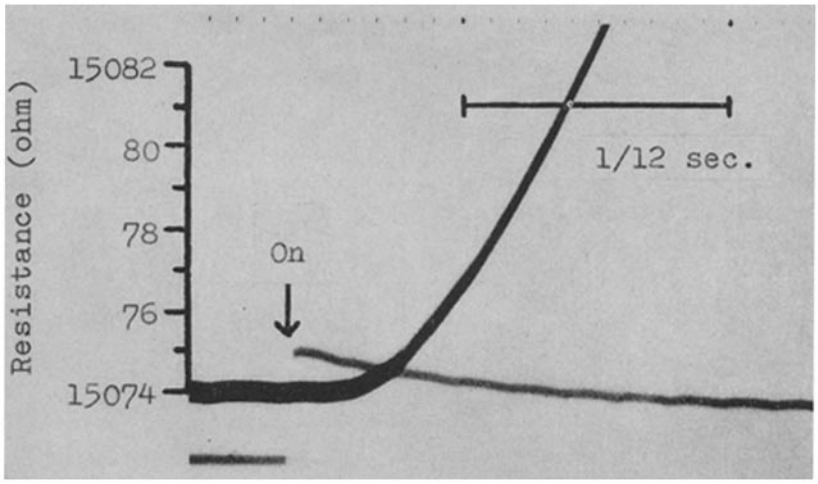

FIG. 4. A record of resistance change on illumination with 12,000 lux. Sample, 0.484 in D, 0.35 in P, and pH 4.5.

of a resistance-decreasing tendency owing to a rise in temperature resulting from intense illumination; and second, the electrical response takes place quite rapidly in the rhodopsin solution, which is still more remarkable when we take into consideration the delayed movement of the vibrator.

4. The Dependence of Resistance Change on pH.—As shown in the above experiments, rhodopsin solutions at about pH 5 always show an increase in the electrical resistance, *i.e.* a decrease in the conductance, on exposure to light. However, it seemed possible that the resistance change would depend on the pH of the solution. A single preparation of rhodopsin was therefore divided into aliquots which were brought to various pH by addition of a small quantity of hydrochloric acid or sodium hydroxide.[3] Because of these addi-

[3] pH must be varied within the range in which rhodopsin undergoes no bleaching. In fact, a sample irradiated at pH 2 showed no more resistance change.

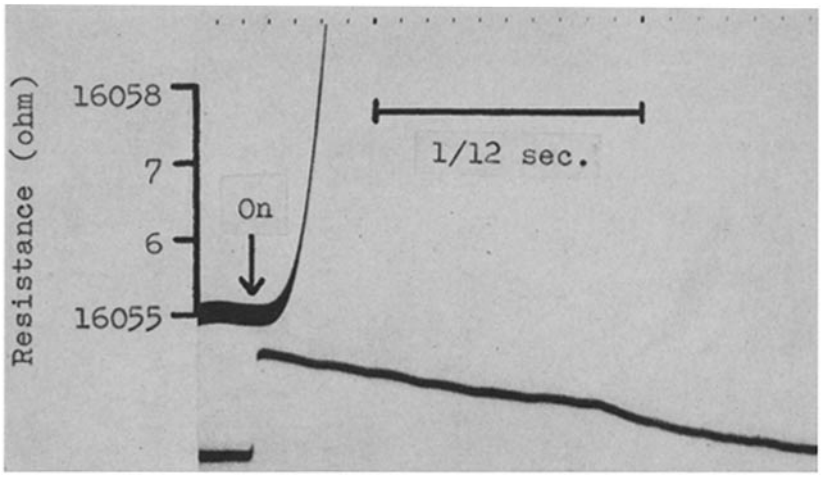

FIG. 5. A record of resistance change on exposure to a photoflash lamp (total light output, 65,000 lumen seconds), placed at a distance of 50 cm. from the sample. Sample, 0.484 in D, 0.35 in P, and pH 4.5.

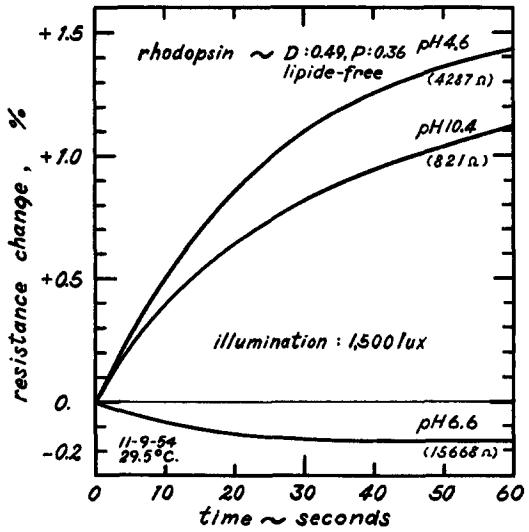

FIG. 6. RRC at various pH.

tions, every aliquot has, of course, a different initial resistance, but this fact does not interfere with the qualitative examination of the effect of pH. A group of RRC obtained from such a set of preparations is illustrated in Fig. 6. In both acid and alkaline samples, the resistance conspicuously increases on illumination, while it slightly decreases in an almost neutral sample. An

example of the experimental results obtained from another set of preparations is shown in Fig. 7, in which we have plotted the percentage change in RRC of every aliquot 30 seconds after illumination with 3,000 lux. It is clearly

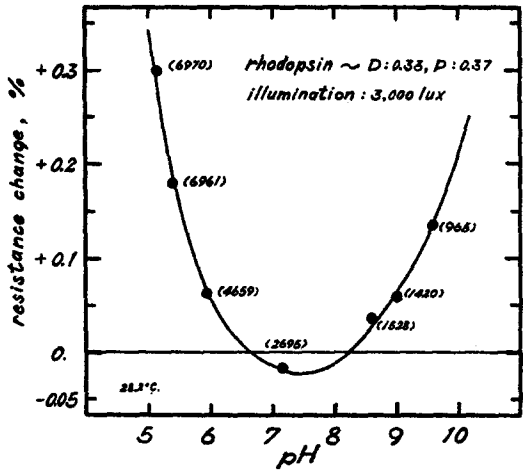

FIG. 7. pH dependence of the resistance change of lipide-free rhodopsin solution.

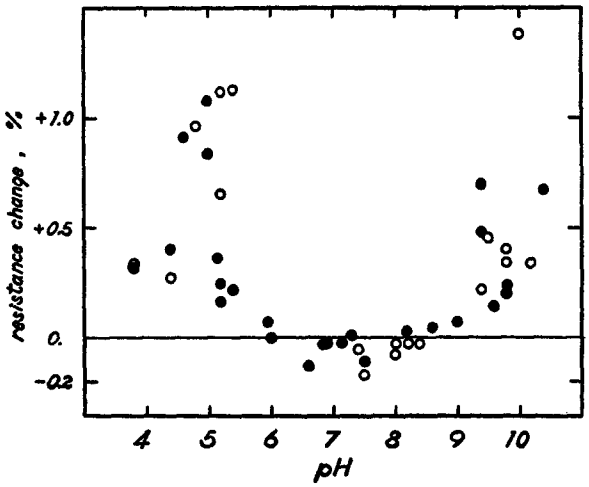

FIG. 8. Resistance change of rhodopsin solution at various pH. Open circles, ordinary solution; filled circles, lipide-free solution.

shown that the resistance increases progressively as the pH of the solution is shifted towards either extreme, while it decreases slightly in the pH range of 6–9.

A resistance-decreasing pH range determined on a set of preparations might not always hold on the other preparations with different optical purities. All the results obtained from several sets of preparations are brought together in Fig. 8. In the figure, there is plotted the percentage change in RRC 30 seconds after illumination, when the initial density of every sample is converted into 0.400. Although each experiment could not be made under the same conditions of optical purity and intensity of illumination, the figure seems to be sufficient to show the relation of pH to the increase and decrease of resistance. It must be remarked that the resistance-decreasing range is always between pH 6–9. It should be noted that in Fig. 7 the curve intersects the line for zero resistance change at two points. At these points there is, therefore, no resistance change in spite of the bleaching of rhodopsin. The fact that the resistance change is dependent on the pH of the rhodopsin solution may be due to the protein character of rhodopsin, since it is known that the dissociation or denaturation of proteins is related to pH.

5. pH Change on Illumination.—In order to understand the way in which resistance change depends on pH, one must know the change in pH which accompanies the bleaching of rhodopsin solutions, because the pH change may sometimes cause the resistance change of the solutions. With the glass electrode pH meter (one drop type), the pH change of the rhodopsin solution during bleaching was continuously measured. Table I is obtained from this experiment. Following illumination, the rhodopsin solution shows a slight change in pH; the initial pH shifts more or less towards the neutral.[4] As the pH meter cannot trace the pH change without lag, it is difficult to analyze the process of pH change of the solution during illumination, but we can roughly estimate that the rhodopsin solution (about 0.40 in D and P) reveals a pH change of as much as 0.01 unit 30 seconds after illumination with 3,000 lux.

If one considers the resistance change with reference to the pH change on illumination, it will be noted that the pH change is always accompanied to some extent by an increase in resistance of rhodopsin solution. Near pH 4 or 10, the resistance increase as the result of exposure of rhodopsin to light may be mainly dependent on the pH change of the rhodopsin solution, since the initial conductance of the sample is principally due to hydrogen or hydroxyl ion. In these cases, a pH change of 0.01 unit may correspond to as much as a 2 per cent increase in resistance. Near neutrality, even if there were a slight change of pH, it could not exert a remarkable influence on the resistance change, because the initial conductance of solution must be mainly ascribed to conductive substances other than hydrogen or hydroxyl ion.

[4] The results generally coincide with those of Radding and Wald (1955–56 *a*, *b*), who recently reported on the change of pH in rhodopsin solution following exposure to flashes at various pH.

Anyhow, the resistance decrease observed in the range of pH 6–9 is likely to have an essential and important significance with regard to the photochemical response of rhodopsin. Outside the neutral range, we must suppose that the resistance decrease is cancelled out by the pH change which is responsible for the increase in resistance.

Radding and Wald (1955–56 *a*) found two distinct types of pH changes of rhodopsin in solution following exposure to flashes at various pH: (1) immediate changes, apparently connected more or less with the light reaction and unequivocally observed only at neutral and acid pH, and (2) relatively slow changes, independent of the changes in absorption spectrum at both acid and

TABLE I

pH Change of the Rhodospin Solution on Illumination

Conditions	pH before illumination	pH shift after illumination
D:0.42	4.52	+0.04
P:0.37	5.37	+0.05
lipide-free	5.99	+0.12
1 min. after	6.26	+0.12
exposure to 10,000 lux	9.51	−0.05
D:0.17	4.92	+0.06
P:0.39	4.95	+0.06
lipide-free	7.26	−0.02
2 min. after	9.75	−0.03
exposure to 10,000 lux	10.43	−0.06

D, optical density; P, optical purity.

alkaline pH. As the conductance change which we observe at acid and alkaline pH may depend mainly on the pH changes following illumination, our results suggest that an immediate change in pH takes place during bleaching of rhodopsin at alkaline as well as acid pH.[5]

6. Removal of Petroleum Ether-Soluble Component.—According to Collins and his coworkers (1952 *b*), the rod outer segment contains a large amount of phospholipide—about 30 per cent of the dry weight. In order to examine the influence of lipide on the resistance change of rhodopsin solutions, we have prepared two types of rhodopsin solution, depending on whether the rod outer segments have been previously extracted with petroleum ether or not. As the lipide-free solutions are usually of far greater purity than the ordinary ones, they can be compared only by means of simultaneous measurements of bleaching and resistance change.

[5] Recently we recorded an immediate increase in resistance during exposure of rhodopsin solution to flashes of intense light at alkaline pH as well as acid pH.

An example of the experiments is illustrated in Fig. 9. In this example, the decrease of 0.06 in the optical density—at 518 mμ—corresponds to the resistance increase of 0.33 or 0.49 per cent according to whether the rods were treated with petroleum ether or not. In general, the percentage change of resistance for bleaching a given amount of rhodopsin is clearly larger in ordinary solutions than in lipide-free solutions. This is also the case at other pH's, as shown in Table II. Thus the lipide raises the percentage change of resistance in rhodopsin solutions irrespective of pH. Moreover, as the ordinary solution is usually lower in the initial resistance than the lipide-free

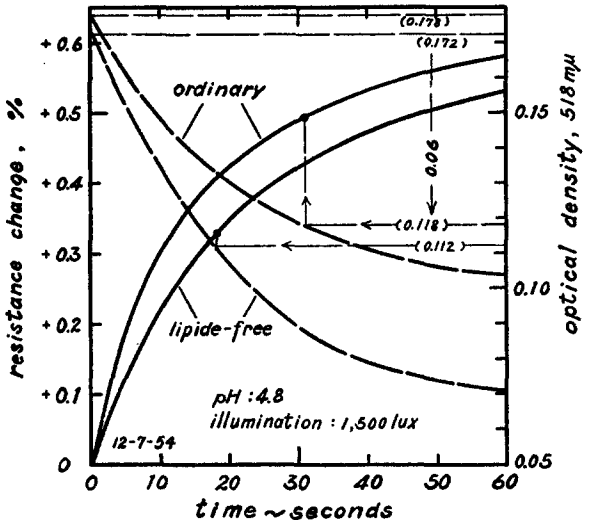

FIG. 9. Effect of lipide on resistance change of rhodopsin solution. Solid line, RRC; broken line, REC.

one, it is obvious that the former must show a larger change of actual conductance as well as a larger percentage change of resistance on illumination.

Strictly speaking, such a simple representation cannot always hold good for samples near both ends of the resistance-decreasing pH range. In fact, the reversal of resistance change near the end of this range (*cf.* the case of pH 8.2 in Table II) is related to whether the solution contains lipide or not. Considering the results in Fig. 8, the lipide also seems to make wider the resistance-decreasing pH range, or to shift it slightly towards the more alkaline side. Though the resistance change in this range is so slight and moreover so complicated that the effect of lipide cannot easily be determined, it cannot be denied that the lipide modifies the resistance change, even near pH 7.

7. *Influence of Digitonin.*—Rhodopsin can be brought into aqueous solu-

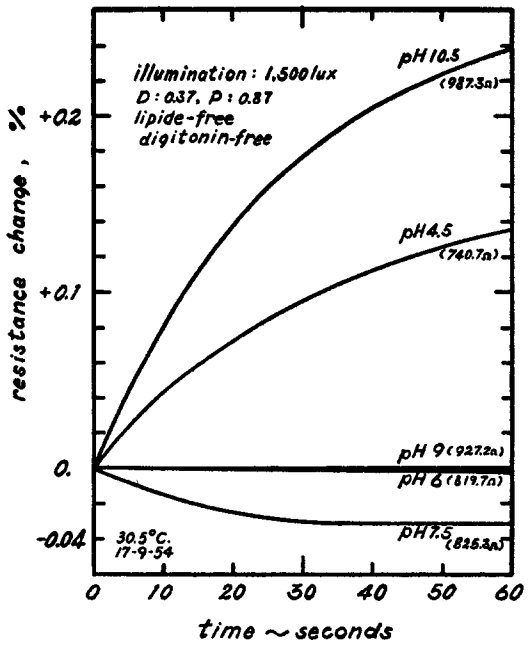

FIG. 10. Resistance change of digitonin-free rhodopsin at various pH.

TABLE II

Effect of Lipide on the Resistance Change of the Rhodopsin Solution on Illumination

Date	Condition		Percentage change of solution		Fall of density at 518 mμ in REC
	Initial density at 500 mμ	Initial pH	Ordinary	Lipide-free	
1954					
July 12	0.20	4.8	+0.49	+0.33	0.060
		5.0	+0.48	+0.30	
July 15	0.14	8.2	−0.02	+0.01	0.030
		9.8	+0.17	+0.05	
		3.8	+0.12	+0.07	
July 30	0.13	4.4	+0.09	+0.07	0.013
		7.5	−0.06	−0.02	

tion only with the aid of such solubilizers as bile salts or digitonin. Such rhodopsin solutions do not contain free rhodopsin molecules but micelles of rhodopsin in combination with the solubilizer (*cf.* Wald, 1944; Hubbard, 1954). One must, therefore, inquire whether the digitonin affects the resistance

change on illumination. After treatment with petroleum ether, the rod outer segments were ground with glass powder, suspended in a small amount of ion-free water, and centrifuged at 3,000 R.P.M. for several minutes. The supernatant contains a fine suspension of rhodopsin (pH 10.5), and is slightly more opalescent than the digitonin solution. A series of aliquots at various pH's is prepared by addition of hydrochloric acid to such a suspension, and similar experiments are performed.

A typical result is shown in Fig. 10. The general characteristics of the RRC and its pH dependence are the same as in digitonin solution. It may therefore be concluded that the resistance change on illumination is a specific property of rhodopsin and has nothing to do with the digitonin. Though the solubilizer may influence the dispersed state of rhodopsin molecules, the latter can still display the capacity for resistance change on illumination.

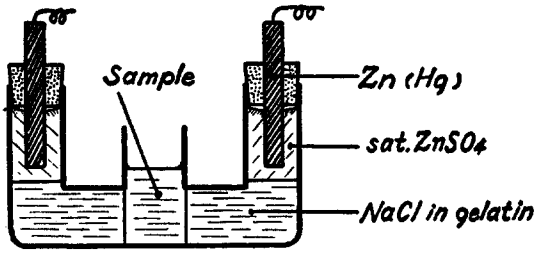

FIG. 11. Diagram of the cell used for measurement of the conductance of r.o.s. suspension and retinal homogenate.

In these experiments, the initial resistance of every aliquot is lower than in the previous experiments, because of inevitable contamination with salts in the course of preparation. Under such conditions, the component which mainly determines the initial resistance value is surely some other substance than hydrogen or hydroxyl ion. The percentage change in acid or alkaline aliquots is clearly lower than that found in the previous experiments. Therefore, it must be concluded that the resistance change in these experiments is markedly suppressed because the pH change is masked by a large amount of other conductive substances.

8. Resistance Change in Rod Outer Segment Suspension and Retinal Homogenate.—The change in conductivity of rhodopsin solutions upon illumination shows that such a change appears even in a medium containing fragments of visual cells. For the experiments with suspensions of rod outer segments and retinal homogenate, a pair of non-polarizable electrodes consisting of zinc-zinc sulfate and gelatin is used instead of platinum electrodes (Fig. 11). Into a cell made of glass tubing (diameter, 13 mm.), a suitable amount of warm physiological saline containing 10 per cent gelatin is poured, and after hardening, the gelatin is removed from the center of the tube, into which the

sample may then be introduced. Since the internal resistance of the cell is about ten times as high as that of the sample, the sensitivity is only about one-tenth that obtained with rhodopsin solutions. Moreover, local migration of ions between the electrodes and the sample is inevitable, so that the resistance changes slightly at a constant rate, at least during the short period of these experiments. The initial resistance of the sample is obtained from the difference between resistances measured before and after mercury (regarded as zero ohm) has been put in place of the sample.

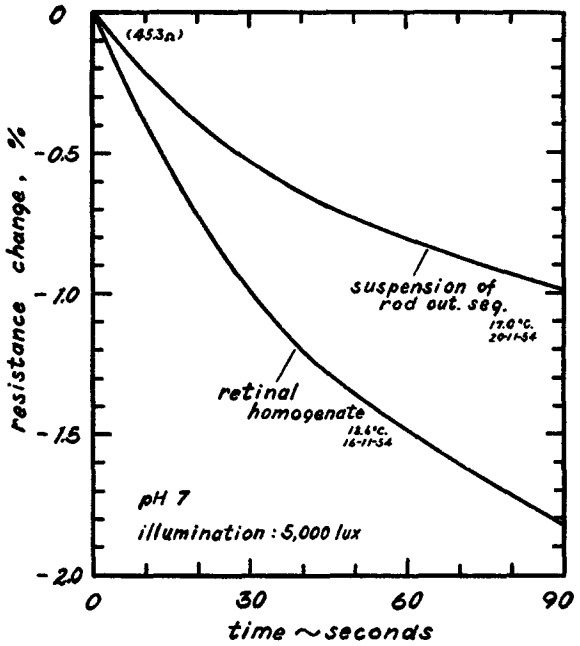

FIG. 12. Resistance change in saline suspension of the rod outer segments and of retinal homogenate.

The rod outer segments isolated from 45 fresh retinas are suspended in 10 ml. of saline, omitting extraction with petroleum ether, and 2 ml. of the suspension are used for each experiment. The conductivity cell containing the sample is immersed in a water bath, and illuminated through 20 cm. of water. One of the results—an average of six curves obtained at pH 7—is shown in Fig. 12. The conductance clearly increases with time after the beginning of illumination with 5,000 lux. The change is rather linear in spite of the relatively high illumination and hence somewhat different from that found with rhodopsin solutions. The resistance change is larger than that of the rhodopsin solutions, in spite of higher conductivity of 0.9 per cent saline (45.3 ohm), so that the cause of change may lie not only in the bleaching of rhodopsin,

but also in any other secondary reactions in cell fragments. Furthermore, as the current of frequency of 1 kc. may flow more through the medium than across the cell fragments, the resistance change is probably due to some conductive components diffused into the medium.

The pH dependence of conductance change in the rod outer segment (r. o. s.) suspension is indicated in Table III. Over the entire pH range, the conductance always increases on illumination, clearly indicating a different situation from that of the rhodopsin solution. The rod outer segments must liberate considerable amounts of conductive substances irrespective of the pH of the medium.

TABLE III

Resistance Decrease of r.o.s. Suspension and Retinal Homogenate 60 Seconds after Illumination

Date	Sample	Illumination	Initial pH					
			4	5	7	8	9	10
1954			*per cent*					
Nov. 19	Suspension of rod outer segments	2,500 lux	0.58	—	0.19	0.20	0.53	0.46
Nov. 16	Retinal homogenate	5,000 lux	1.41	1.31	1.48	—	1.51	1.34

In the experiments with retinal homogenates (12 retinas per 10 ml.), the change is not very different from that found with the r. o. s. suspension (Fig. 11), and the increase in conductance on illumination is about the same over the whole pH range (Table III). Of course, one cannot be sure that changing the pH of the medium alters appreciably the pH inside the r. o. s. in suspensions or homogenates. It would appear from the results in Table III that the pH of the medium, however, exerts some influence on the r. o. s. in suspension, as exhibited by the conductance change. Although the pH change on illumination is observed with r. o. s. suspensions and retinal homogenates, hydrogen or hydroxyl ions are not likely to make a large contribution to the increase in resistance, because of the extremely low resistance of the saline solution; the liberation of other ions or radicals must, therefore, be mainly responsible for the increase in conductance. We must therefore conclude that the principal cause of the conductance change is some secondary process following the photoreaction of rhodopsin in the rod fragments.

DISCUSSION

1. Conductance Change and the Lipide.—The electron microscope studies of Sjöstrand (1949, 1953) revealed the submicroscopic structure of rods and cones in guinea pig and perch. The rod outer segment appears to consist of

a pile of regularly spaced membranes, which are associated in pairs and primarily composed of protein. Though the location of the lipide has not yet been determined, it seems justified to assume tentatively that the double layers of lipide molecules are restricted within the double membrane disc. For the rhodopsin, there remains little place other than in the membranes, and hence Sjöstrand's analysis implies that the lipide is located near the rhodopsin. Such a structure must provide a chance for the lipide to act closely upon the rhodopsin.

In our experiments, the influence of lipide on the change in conductivity upon illumination has already been pointed out, and as a consequence, we should like to propose the following consideration. In order that the rhodopsin play a sufficient role in a physiological sense through its conductance change, the lipide must be closely connected to the rhodopsin so as to participate in the excitation process. The rhodopsin is regarded chemically as a chromoprotein composed of retinene and opsin, but, when it reveals any physiological function, it must act as a "rhodopsin complex" constituted by retinene, opsin, and lipide. The chemical composition of the complex and the mode of its association with other components to form a rod disc, are important problems which must be investigated in the future.

In the experiments on the pH dependence of the conductance change, we noted that there is a pH at which the rhodopsin solution does not reveal any conductance change on illumination while it successively bleaches, and moreover that such a pH shifts, being affected by the lipide content of the solution. This may be important for the mechanism of photoreception in the rods. The lipide should not be regarded merely as an impurity in rhodopsin solutions; it must play an important role in the photoreceptive process of rhodopsin.

2. Denaturation of Rhodopsin.—Mirsky (1936) suggested that the bleaching and resynthesis of rhodopsin might be regarded as a reversible denaturation of its protein moiety. The shift of the isoelectric point (Broda and Victor, 1940) and the change in the rotatory polarization (Takamatsu, 1933) of rhodopsin solutions on illumination suggest that a denaturation of the protein is associated with the bleaching of visual pigments. Many other experiments appear to support a concept of rhodopsin denaturation on illumination.

Recently Wald and Brown (1951–52) showed by means of the amperometric silver titration that the bleaching of rhodopsin is accompanied by the liberation of sulfhydryl groups, a change which is known to accompany denaturation. Though the final pH of the mixture containing rhodopsin was about 9 in their experiments, the results have offered concrete evidence that the rhodopsin may be denatured in the course of bleaching. They pointed out that, in the silver titration, the bleaching of rhodopsin yields directly an electrical variation, and moreover suggested that this phenomenon may pro-

vide a model of the excitation process in general. The sulfhydryl groups may be expected to affect the hydrogen ion concentration because of their mild acidity, or to change the oxidation-reduction potential of rhodopsin due to their strong reducing power. In fact, the pH change was observed on illumination of rhodopsin solution (Radding and Wald, 1955–56 *a*), and we know that a change in the oxidation-reduction potential accompanies illumination (unpublished observation). At any rate, it is very interesting that denaturation may have some fundamental connection with the physiological role of rhodopsin.

According to Radding and Wald (1955–56 *a*, *b*), slow changes of pH following the illumination of rhodopsin are associated with the reversible denaturation of opsin in acid and alkaline solution, as evidenced by loss of its capacity to regenerate rhodopsin. Comparing our results with theirs, it is clear that the conductance changes do not correspond to the slow changes, but to the immediate changes in pH. We therefore believe that the conductance change caused by illumination corresponds to a change prior to the denaturation of opsin. The conductance change which depends on pH may be an expression of the weak denaturation of rhodopsin as a whole, which contributes to visual excitation.

3. Generation of Nerve Impulses.—The creation of nerve impulses may be considered as an event in which some electrical change is produced by means of reactions derived from photoactivation of rhodopsin. As mentioned previously, the changes in the electrical conductance of the rhodopsin complex imply the movement of some electrically conductive substances. When the charged substance migrates or is localized somewhere immediately after illumination, it must, even in the rod, be accompanied by a change in potential difference, which may contribute to the generation of nerve impulses. In a physiological framework, the phenomenon of conductance change may therefore be associated with visual excitation. However, in order to understand the significance of the conductance change, we must elucidate the following points: (1) What factors participate in the conductance change, and (2) When does the rhodopsin molecule initiate the conductance change?

The first question cannot be answered exactly. The electrical conductance of the solutions may be due to many kinds of conductive ion or radical, among which hydrogen and hydroxyl ions are registered as pH. As stated previously, the pH of rhodopsin solutions changes slightly during bleaching and shifts more or less toward neutrality. On the contrary, the pH remains virtually unchanged in neutral solutions. It is therefore probable that the pH change influences the conductance change in acid or alkaline solution, while it cannot be an important factor in the conductance change near neutrality. There must therefore be some other ions or radicals associated closely with rhodopsin besides hydrogen or hydroxyl ions.

As for the second problem, it has become clear in these experiments that the conductance change occurs while rhodopsin changes to indicator yellow. As the conductance change shows little lag after illumination, we assume that it is initiated during the early stages of bleaching. However, we hope to answer this more decisively in the near future.

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