ACID-BASE PROPERTIES OF RHODOPSIN AND OPSIN

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(Received for publication, December 9, 1955)

When rhodopsin is bleached by light, its prosthetic group, retinene, is broken away from its original sites of attachment on the protein, opsin. This process can be expected to uncover new groups on opsin, at the least those groups which had been involved in holding retinene, and perhaps others, associated with some general loosening of structure of the protein. Indeed it has been recognized that the bleaching of rhodopsin possesses some of the characteristics of a protein denaturation (Wald, 1935–36; Mirsky, 1936; Wald and Brown, 1951–52).

Only two changes in the protein have heretofore been measured. Broda and Victor (1940) reported that on bleaching, the isoelectric point of frog rhodopsin shifts from 4.47 to 4.57. Wald and Brown (1951–52) found that the bleaching of rhodopsin (frog, cattle, squid) exposes 2 to 3 sulfhydryl groups for each retinene molecule liberated.

This last observation implied that at sufficiently alkaline pH to ionize sulfhydryl groups, the bleaching of rhodopsin should liberate hydrogen ions. This is indeed the case. If a slightly alkaline rhodopsin solution (pH 9–10) is brought into balance in a pH meter, exposure of the solution to bright white light causes an immediate deflection of the needle in the acid direction.

There is no *a priori* reason to believe that the changes in rhodopsin on bleaching are confined to sulfhydryl groups or alkaline pH. We undertook therefore the titration of rhodopsin over its entire stability range, and a study of the acid-base changes associated with bleaching.

General Procedure

The titration curves of rhodopsin and bleached rhodopsin are shown in Figs. 1 and 2. Fig. 1 shows the pH to which a rhodopsin solution is brought by the addition of measured quantities of acid or base, before and after bleaching; and as control, the pH to which the solvent, 2 per cent aqueous digitonin, is brought under the same circumstances. Fig. 2 shows the corrected titration curves, in terms of moles of acid and base bound per mole rhodopsin or bleached rhodopsin. Bleached rhodopsin here is equivalent to opsin, since the other product of bleaching, retinene, does not contribute to the titration.

* This investigation was supported in part by grants from The Rockefeller Foundation and the Office of Naval Research.

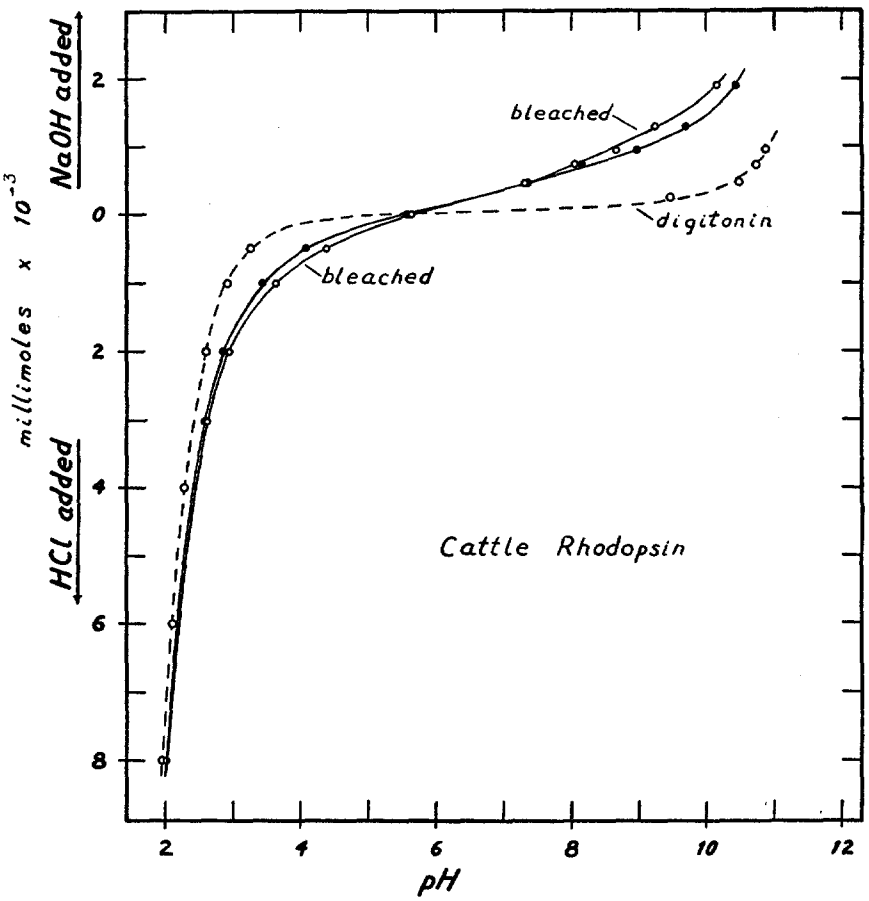

FIG. 1. The titration of cattle rhodopsin, of the product of its bleaching (opsin), and the control titration of the solvent 2 per cent digitonin in water. Measurements at 20°C. with a single preparation having the following characteristics: age, 5 to 7 days; K_{500}, 3.46; 400/500 ratio, 0.26; 280/500 ratio, 2.3; 4.26×10^{-8} moles in 0.5 ml.

The titration was performed in dim red light at 20°C., with a glass electrode pH meter (Cambridge, Model R). In this instrument the sample to be measured is introduced into a narrow, tubular glass electrode, enclosed in a glass jacket.

To a small sample of rhodopsin solution, (in Fig. 1, 0.5 ml., 8.5×10^{-5} molar) a measured amount of acid or alkali, 0.05 to 0.1 ml., was added. This mixture was introduced into the tubular glass electrode. The pH was measured in dim red light until constant. Then the solution was bleached in place, by exposure to the brilliant "white" light from a magnesium flash bulb,[1] two such exposures in rapid succession.

[1] The light from a No. 5 G.E. photoflash bulb was passed through a combination of the Jena BG 18 and 19 and GG5 filters. These transmit a wide band of light be-

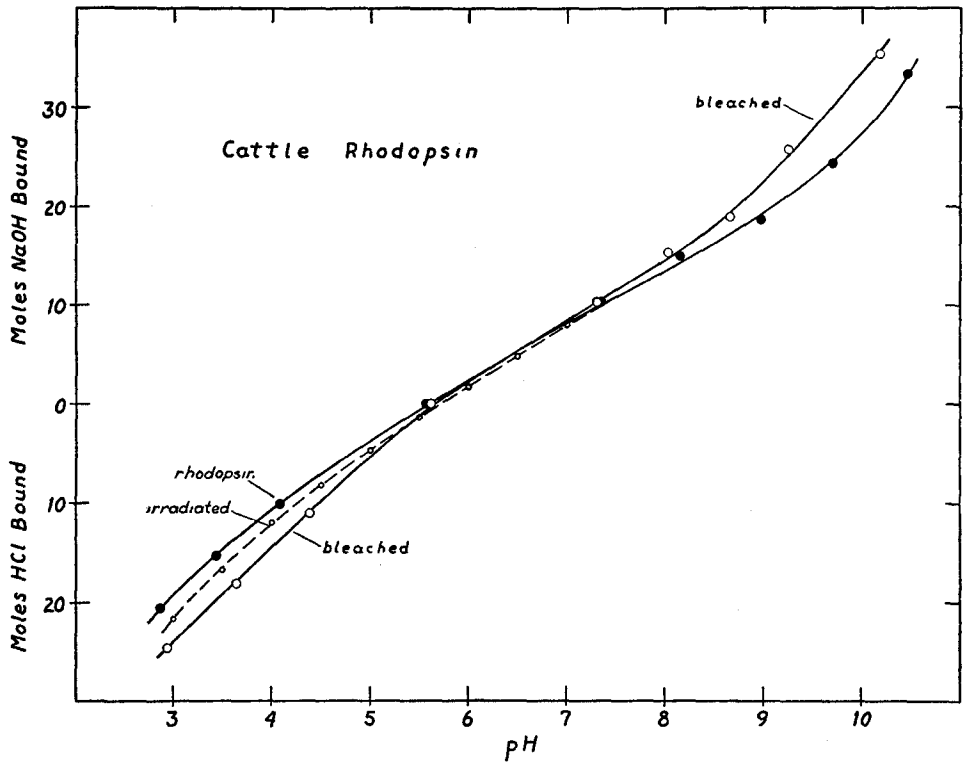

FIG. 2. Molar titration curves of rhodopsin and of the products of its exposure to light at various pH. Moles acid and base bound per mole rhodopsin. The broken curve ("irradiated") represents the product measured 30 seconds after exposure to light. The "bleached" curve represents the final product, after all changes of pH are completed (opsin).

The pH readings were immediately resumed in red light, and continued at intervals of 1 minute until virtually constant. At some pH's this took 30 to 40 minutes. The solution was exposed to a final flash of white light, and the pH again recorded; usually it had changed very slightly or not at all. Each sample of rhodopsin yielded in this way a point on the titration curve of both the unbleached and the bleached pigment.

Rhodopsin

Purity.—Our methods of preparing rhodopsin depend upon the removal of as many potential impurities as possible before bringing the pigment itself into solution. The essential procedures have been described by Wald and Brown (1951–52). The outer segments of cattle rods are separated from the remaining

tween 440 and 600 mμ, greenish in appearance, and containing almost all visible wave lengths absorbed by rhodopsin.

retinal tissues by differential centrifugation. They are tanned with alum to render other proteins insoluble; leached with buffers to remove water-soluble substances, and with distilled water to remove the buffer; and extracted with petroleum ether to remove lipids. Finally the rhodopsin is brought into solution with the aid of the solubilizer, digitonin. For the present experiments, the rhodopsin was in addition dialyzed overnight against 7 to 8 times its volume of 2 per cent digitonin solution in distilled water.

In spite of all these measures, we cannot be sure that the rhodopsin was free from contaminating substances which might have entered the titration. Its absorption spectrum offered some assurance that colored contaminants and irrelevant proteins were low in amount. Colored impurities raise the extinction at 400 mμ relative to that at the maximum 500 mμ (400/500 ratio); similarly, irrelevant proteins raise the extinction in the region of the protein band, 280 mμ, causing an abnormally high 280/500 ratio. The preparations which yielded Figs. 1 and 2 possessed a 400/500 ratio of 0.26; the purest samples of cattle rhodopsin have the ratio 0.22–0.24. This preparation also had a 280/500 ratio of 2.3; the lowest such ratio yet found is 2.1. It is significant also that the titration curves of several such preparations were in good agreement. Furthermore, careful control measurements yielded no evidence that the presence of digitonin affects the titration.

All these considerations still do not ensure that the titration curves obtained with such preparations of rhodopsin represent the pure substance.

Stability.—Judging by its absorption spectrum, rhodopsin undergoes no change whatever on being kept for 1 hour at 27°C. at any pH between 4 and 10. At more acid and alkaline pH, the extinction falls. Less than 20 per cent is lost in 1 hour at pH 3.5 and 10.5 (27°); at the temperature of our titrations the loss is considerably smaller. We attempted to carry our titrations as far as pH 2. At this acidity a considerable portion of the rhodopsin bleaches in the course of the measurements; this accounts in part for the fact that the titration curves before and after bleaching come together in this region.

Control Titration.—To ascertain the amounts of acid and base bound by rhodopsin, it is necessary to determine as control the pH of solutions in which the same amounts of acid and base were added to aqueous digitonin. Such a control titration is shown as a broken line in Fig. 1. It differs little from the titration curve of water. At any pH, the amount of acid or base added to the digitonin control, subtracted from that added to the rhodopsin solution, yields the amount bound by rhodopsin. In this way the data in Fig. 1 yielded the corrected curves shown in Fig. 2.

Acid and Base Bound.—Fig. 2 shows that our rhodopsin preparations possess about 54 titratable groups per mole rhodopsin within the pH stability range: about 34 base-binding and 20 acid-binding groups. This result is more or less typical for the class of "neutral" soluble proteins, "neutral" because they have

comparable numbers of acidic and basic groups, and soluble because both types of group are plentiful. It is not clear why rhodopsin, if it possesses so many reactive groups, requires a solubilizer to bring it into solution. Our reservations with regard to purity are important in this respect.

The point at which the titration curve of rhodopsin crosses the zero ordinate, *i.e.* no acid or base bound, is a rough indication of the isoelectric or isoionic point. In three preparations this comes out to be 5.2, 5.43, and 5.57. It appears from this that cattle rhodopsin is more alkaline than frog rhodopsin, which has been found electrophoretically to have an isoelectric point at pH about 4.5 (Wald and Raymont, cited in Wald, 1938–39; Broda and Victor, 1940).

The Over-All Effect of Bleaching: Opsin

The bleaching of rhodopsin introduces marked changes in both the acid and alkaline titration (Fig. 2). These, when completed, lie always in the direction of neutrality: acid solutions become more alkaline on bleaching, alkaline solutions, more acid. At pH 6–7 there is almost no change.

That is, the titration curve of opsin is steeper than that of rhodopsin. Opsin is the better buffer.

Fig. 2 shows that the point at which no net acid or base is combined, the zero level on the ordinate, lies at a slightly higher pH for opsin than for rhodopsin. Bleaching apparently shifts the isoelectric point slightly, about 0.1 pH unit, toward higher pH; opsin is slightly more alkaline than rhodopsin. A comparable change has been observed electrophoretically on bleaching frog rhodopsin (Broda and Victor 1940).

Whatever reservations involve the titration curve of rhodopsin on the score of purity do not apply to the changes in acid and base bound on bleaching. These are accurate, whatever impurities may be present. The final changes are shown with a solid line in Fig. 3.

The bleaching of rhodopsin, when completed, induces virtually no net change in acid or base bound between pH 6 and 7. To both sides, the balance changes rapidly, to about 5 moles new acid bound per mole at pH 3.5, and to about 7 moles new base bound at pH 10. That is, in terms of our original rhodopsin preparation, we find about a 20 per cent increase in titratable groups on bleaching. It should be recalled that these changes accompany the release of a single mole of retinene.

The Time Course of Acid-Base Changes on Bleaching

Our titration curves were obtained by measuring the pH repeatedly, starting some time before each sample of rhodopsin was exposed to light, and ending as much as 30 to 40 minutes afterward. Fig. 4 shows representative examples of such measurements.

In each case, as already described, rhodopsin, having been mixed with acid

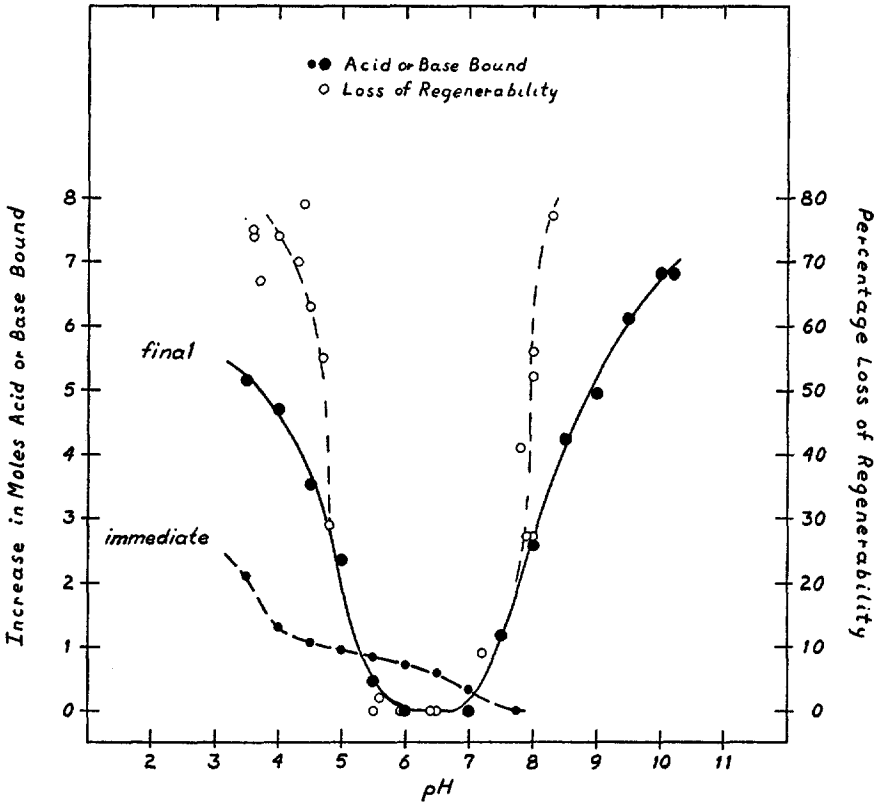

FIG. 3. Increases in acid or base bound as the result of exposure of rhodopsin to light at various pH. The immediate curve represents measurements made 30 seconds after the start of irradiation, the final curve after all pH changes were completed. Between pH 4.5 and 7.5 the immediate change involves the liberation of 1 mole of an acid-binding group with pK about 6.6. Below pH 4, further acid-binding groups are liberated, perhaps owing to denaturation. The final changes are associated with the irreversible acid and alkaline denaturation of opsin, as evidenced by the loss of capacity to regenerate rhodopsin (open circles, broken line).

or alkali, was allowed to equilibrate for a short time in the dark in the pH meter. Occasionally the pH drifted considerably during this interval (*cf.* Fig. 4, lower left). After settling down at a steady value, the sample was irradiated in place, and subsequent changes in pH were recorded.

In the acid region, at pH about 4, exposure to light induces an immediate leap of pH in the alkaline direction. This is followed by a slow drift in the same direction, which may go on for an hour (Fig. 4, upper left). At pH about 5.5 (upper right), on exposure to light there is again an immediate change toward

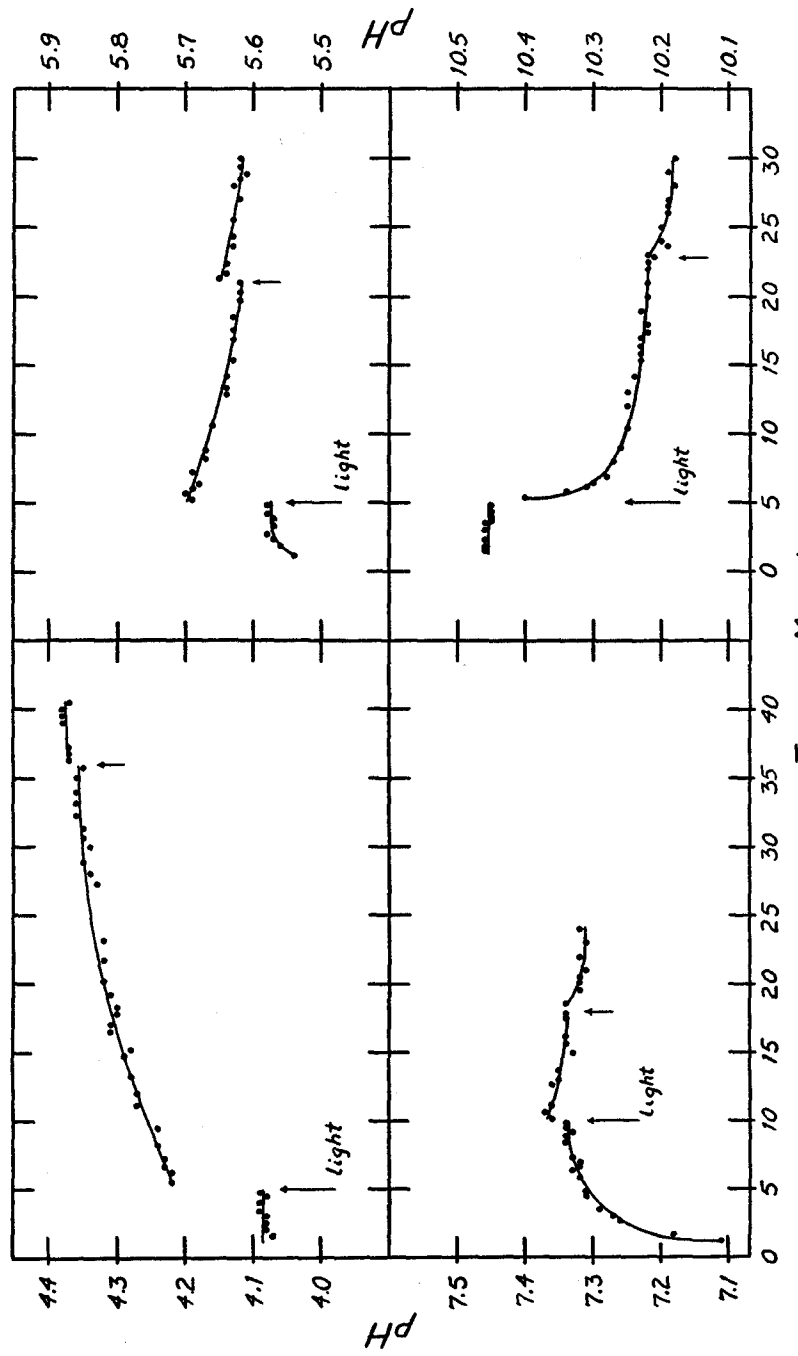

FIG. 4. The change of pH of rhodopsin in solution following exposure to flashes of intense light at various pH (20°C.). The pH of the rhodopsin solutions was measured in dim red light until stable. The solutions were then exposed to two flashes of light a few seconds apart (arrow), and pH measurements resumed in the dark until again stable. A second exposure to light (arrow) induced little further change.

higher pH, followed by a slow drift in the *reverse* direction. Near neutrality, pH 6.5 to 7.5, bleaching involves only very small changes, a small immediate rise of pH followed by a slow return to the original level (lower left). In the alkaline region, there is a rapid fall of pH on exposure to light, not obviously discontinuous as in the acid range, but seeming usually to extrapolate back to the instant at which irradiation was begun (lower right).

It is important to note that bleaching itself has a characteristic time course. The exposure of rhodopsin to light yields as the immediate photochemical product lumi-rhodopsin, which is then converted by ordinary dark reactions to meta-rhodopsin, and finally to a mixture of retinene and opsin. The loss of color goes largely with the last step (Wald, Durell, and St. George, 1950).

At pH 10 and above, bleaching is completed within a few seconds; the pH continues to change however for 10 to 15 minutes. At pH about 7, bleaching goes on for about an hour; whereas the pH changes only very slightly, during the first few minutes. At pH about 4, large spectral changes continue for several hours following irradiation, whereas the pH becomes virtually constant within an hour (*cf.* Wald, 1937–38). There is little obvious relation therefore between the pH changes observed and bleaching. The spectral changes involve principally the prosthetic group of rhodopsin, the pH changes principally opsin.

Our pH changes are of two distinct types, originating, as we shall see, in two different types of chemical process: (*a*) immediate changes, apparently connected more or less with the light reaction. These are observed unequivocally only at neutral and acid pH, where they precede the bulk of bleaching; and (*b*) relatively slow changes, which seem to pursue a course independent of changes in the absorption spectrum at both acid and alkaline pH. The curves for bleached rhodopsin in Fig. 1 to 3 represent the final resultant of both types of change.

Immediate Change

The immediate rise of pH observed in neutral and acid solutions is shown in Fig. 5. This figure brings together measurements with three different preparations of very nearly the same concentration (4.26×10^{-8} moles rhodopsin in 0.5 ml). All yield substantially the same result.

Below pH 3, rhodopsin is highly unstable, bleaching rapidly in the dark (Radding and Wald, 1955–56). In this region therefore the data are not reliable. At pH above 3.5 both rhodopsin and opsin are stable within the short times involved in these measurements (*cf.* Fig. 6). The increase in pH on irradiation rises to a maximum value of 0.135 at about pH 5 (Fig. 5). It falls off nearly symmetrically to both sides of this maximum, reaching very low values at pH 3 and 7.5.

From the data of Fig. 5 the moles acid bound by this "immediate" product

of irradiation can be computed. This is entered as a broken line in Fig. 2. Presumably it represents primarily the titration curve of lumi- and meta-rhodopsin (the "transient orange" of Lythgoe).

This subtracted from the titration curve for rhodopsin yields the immediate change in moles acid bound per mole rhodopsin, shown in Fig. 3. The latter curve has a complex form. Between pH 7.5 and 4.5, it has the conventional shape of a simple titration curve, as though marking the liberation of one

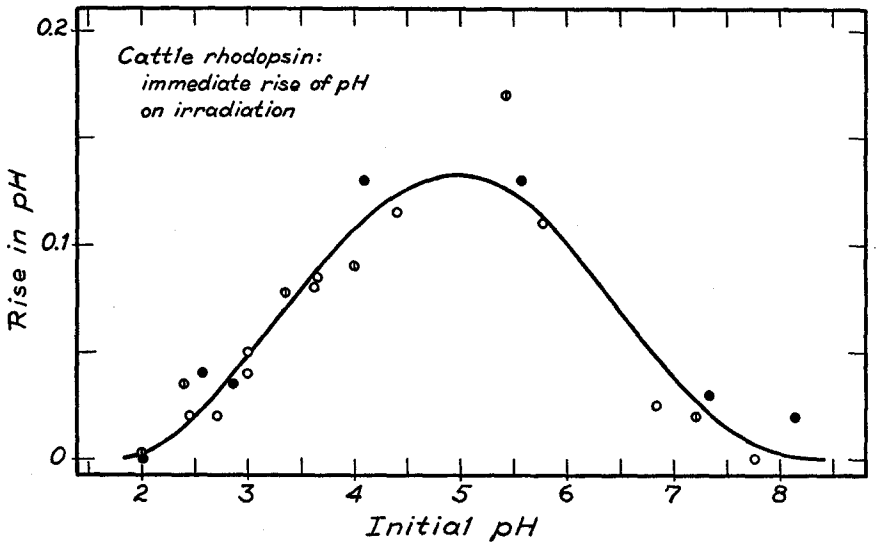

FIG. 5. Immediate rise of pH on exposure of rhodopsin to light at various pH. Measurements made 30 seconds after irradiation, with three different rhodopsin preparations containing 4.21, 4.26, and 4.30 $\times$ 10^{-8} moles rhodopsin in 0.5 ml. solution.

equivalent of an acid-binding group per mole of rhodopsin. The apparent pK is about 6.6, close therefore to the pK of the imidazole group of histidine. Below pH 4, the acid bound rises again, perhaps owing to the denaturation of opsin (*cf.* Fig. 6).

Subsequent Changes: Acid and Alkaline Denaturation

It seemed possible that the later changes in pH which follow the irradiation of rhodopsin might involve the denaturation of opsin. This was tested by measuring the capacity of opsin to regenerate rhodopsin, after various times spent at various pH's (*cf.* Radding and Wald, 1955–56).

The regeneration experiments were carried out in dim red light at 25–27°C. A solution of rhodopsin was titrated to the desired acid or alkaline pH. A small

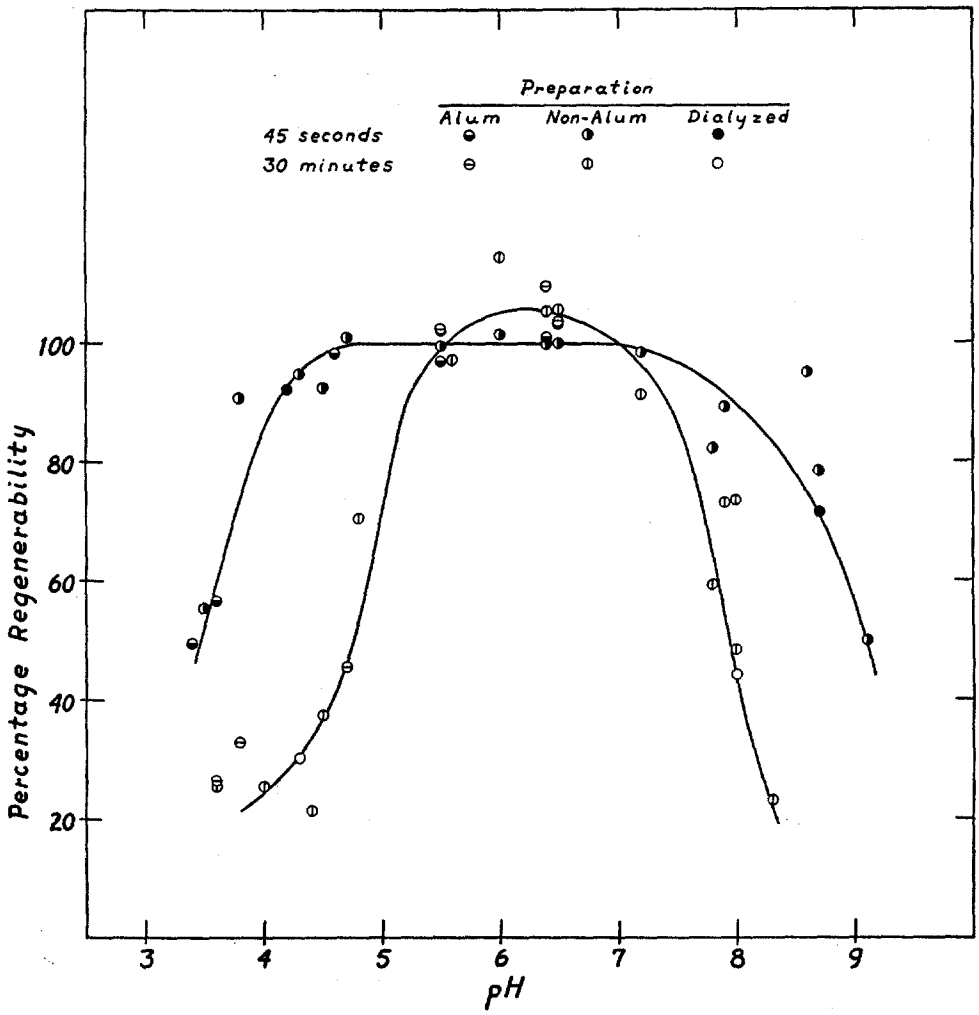

FIG. 6. The capacity to regenerate rhodopsin, measured 45 seconds and 30 minutes after the exposure of rhodopsin to light at various pH. The regenerability was measured by bringing the product of irradiation to pH 6.4, and incubating 2.5 to 3 hours in the dark with neo-*b* retinene. This incubation period is long enough to permit any renaturation to occur that is possible. The loss of regenerability therefore measures the degree of *irreversible* denaturation. At pH 4.5 and 8 half the product of bleaching (opsin) is denatured within 30 minutes; while at pH 3.5 and 9, half is denatured within 45 seconds.

sample was withdrawn, brought to pH 6.4, and exposed to light. At 45 seconds afterward, neo-*b* retinene was added, and the mixture incubated in the dark for 2.5 to 3 hours (*cf.* Hubbard and Wald, 1952–53). This was the control experiment, to measure the maximum capacity of the rhodopsin to regenerate after bleaching, under the conditions employed.

The remainder of the acid or alkaline rhodopsin was then bleached. From time to time, beginning at 45 seconds, a small aliquot was withdrawn, neutralized to pH 6.4 ± 0.2, mixed with neo-*b* retinene, and incubated 2.5 to 3 hours in the dark. After each such incubation, the rhodopsin which had regenerated was estimated by measuring the difference spectrum or the difference in extinction at 500 mμ before and after bleaching in the presence of 0.2 M hydroxylamine (*cf.* Wald and Brown, 1953–54).

Losses in the capacity to regenerate, compared with the control, can be regarded as an aspect of *irreversible* denaturation. The 2.5 to 3 hours' incubation in the dark after adding retinene should have been sufficient for any reversal of denaturation that could have occurred.

Fig. 6 shows the degree to which opsin retains its capacity to regenerate rhodopsin after being kept for 45 seconds or 30 minutes at various pH's. Several types of preparation were tested in these experiments: dialyzed preparations in which the retinal tissue had been hardened in alum, or in which this step was omitted; and undialyzed preparations. All yielded similar results.[2]

Opsin loses little of its regenerability after 45 seconds at pH 4.5–7.5. To both sides of this range, the regenerability declines measurably even within this short interval. It falls to about half within 45 seconds at pH 3.5 and 9.

Measured 30 minutes after exposure to light, the stability range is much narrower. The regenerability remains maximal between pH about 5.5 and 7; indeed it seems to rise a little above the 45 second value toward the middle of this range. To both sides it declines rapidly, falling to half at pH about 5 and 8.

In Fig. 3 the loss of regenerability 30 minutes after exposure to light is plotted as a broken line. It bears a close relation to the increase under the same circumstances of the acid or base bound. Between pH 6 and 7 the bleaching of rhodopsin neither changes the final pH nor induces denaturation. To both sides of this range, both types of effect rise together.

It is apparent that the slow changes of pH which follow the irradiation of rhodopsin are mainly the result of acid and alkaline denaturation of opsin. All but the changes (immediate and slow) observed at pH 5.5–7 may be accounted

[2] Wald and Brown (1951–52) have measured the percentage regeneration of cattle rhodopsin as a function of pH. This is maximal at pH 5–6.8, falling steeply to both sides of this range. The difference between this curve and those of Fig. 6 in the present paper is that whereas the earlier experiments measured regeneration at various pH, the present experiments measure regeneration at a single, optimal pH (6.4), following incubation at various pH (*i.e.*, regenerability).

for in this way. By the same token the final form of the titration curve of bleached rhodopsin (Fig. 2) represents native opsin only over the middle range (pH 5.5–7). Beyond this it is distorted by the effects of denaturation.

Changes in Base-Binding Capacity on Heat and Alkaline Denaturation of Rhodopsin

It seemed of interest to inquire whether the exposure of rhodopsin to frank denaturation procedures in the dark might induce acid-base changes comparable with those that occur on bleaching with light.

At pH above 10.5, rhodopsin rapidly denatures, as judged by the loss of its characteristic absorption spectrum. We find that as this change progresses, the pH of the solution becomes more acid by 1–1.5 pH units.

In another experiment, rhodopsin at pH 9.5 was divided into two portions. One was set aside as control; the other was denatured by heating at 86°C. for 1.5 minutes. The latter sample was found to have become more acid than the control by 0.5 pH unit.

It appears therefore that the changes in pH which follow the bleaching of rhodopsin by light at acid and alkaline pH's are mimicked by other procedures which denature this molecule.

DISCUSSION

The action of light on rhodopsin in the rods results in a nervous excitation. It is difficult to ascribe this effect to the action of retinene *per se*. Like other carotenoids, retinene is a bland, relatively inert substance, hardly to be conceived of as an irritant. Furthermore, at physiological temperatures and pH it is released slowly, as the last step in a chain of reactions initiated by light; whereas the nervous response, even in a cold blooded animal, appears within a fraction of a second. Changes in the protein component of rhodopsin would seem *a priori* to offer richer possibilities; and among these we have primarily to consider the most rapid changes—the conversion of rhodopsin to lumi- or at most meta-rhodopsin.

From this point of view, among the changes we have measured, the rapid rise of pH on irradiation in neutral and mildly acid solution holds the greatest interest. This—followed, it will be recalled, at pH 5.5–7.5 by its relatively slow partial or complete reversal—is indeed the only change we find at physiological pH. It appears in all solutions more acid than pH 7.5; and cannot be confused with changes associated with the denaturation of opsin, since between pH 5.5 and 7.5 opsin is stable within the period of our measurements.

As already noted, this immediate change seems to consist in the liberation by light of one equivalent of an acid-binding group with pK about 6.6. This pK is close to that of the imidazole group of histidine (or perhaps the α-amino group of cystine) (Edsall, 1943); yet amino acid pK's are so greatly modified

by their associations in proteins that no reliable inference can be drawn from the pK alone.

At pH 5.5–7.5, this change is reversed wholly or in part by a slow subsequent fall of pH (Fig. 4). Either the group exposed immediately on irradiation later reacts with others so as to be covered again; or some other slow modification of opsin that increases its net acidity tends to balance out the initial rise of pH.

All the other changes we have measured following irradiation can be ascribed to the denaturation of opsin, as evidenced by irreversible losses of the capacity to regenerate rhodopsin. It is plain that opsin does not tolerate exposure to pH's somewhat removed in either direction from neutrality, and bleaching at these pH's entails the irreversible denaturation of the molecule.

What of the earlier demonstration that sulfhydryl groups are liberated on bleaching rhodopsin, about 2 such groups per molecule in the cattle pigment (Wald and Brown, 1951–52)? These probably account for 2 of the 7 equivalents of base bound on bleaching (Fig. 3). Is this liberation of —SH groups an aspect of the denaturation of opsin, rather than an integral part of bleaching?

Mirsky (1936) once suggested that the bleaching and resynthesis of rhodopsin in the retina might be regarded as a reversible denaturation; and we have discussed the liberation of —SH groups on bleaching from this point of view (Wald and Brown, 1951–52). The new point at issue is whether instead the —SH groups we observed were an aspect of the irreversible denaturation of opsin, as demonstrated in the present experiments.

The amperometric silver titration by which Wald and Brown measured —SH groups was performed at pH about 9, at which opsin loses half its capacity to regenerate rhodopsin within 45 seconds following the onset of irradiation (*cf.* Fig. 5). There is little doubt therefore that considerable denaturation of opsin could have occurred in the course of those measurements. We are now repeating them in neutral solution.[3] On the other hand, it should be remembered that we found also that such a sulfhydryl poison as *p*-chloromercuribenzoate reversibly inhibits rhodopsin synthesis in neutral solution. It is clear therefore that —SH groups have a role in rhodopsin formation, whether or not they can be shown to appear under non-denaturing conditions of bleaching.

SUMMARY

Purified preparations of cattle rhodopsin have been titrated to various pH, irradiated, and the pH changes followed thereafter until completed. In this

[3] *Note Added in Proof.*—Mr. Paul K. Brown has repeated these measurements with cattle rhodopsin in neutral tris buffer at pH 7 (*cf.* Benesch, R. E., Lardy, H. A., and Benesch, R., *J. Biol. Chem.*, 1955, **216,** 663). On bleaching the rhodopsin, 2.4 sulfhydryl groups appear per retinene liberated, *i.e.* per molecule rhodopsin, confirming our earlier measurements. This exposure of new —SH groups therefore appears intrinsic to the process of bleaching, and does not demand the denaturation of opsin.

way we have obtained the titration curves of rhodopsin, of the immediate product of irradiation, measured within 30 seconds; and of the final product of irradiation (opsin).

The rhodopsin preparations display about 54 titratable groups per mole of pigment: about 34 base-binding and 20 acid-binding groups. In default of an absolute purification, one cannot be sure that all of these go with rhodopsin itself.

Exposure to light induces an immediate rise of pH between pH 2 and 8, maximal at about pH 5. This—followed by its slow partial or complete reversal —is the only change of pH in the physiological range (6–7). It involves the exposure of 1 new acid-binding group per mole of rhodopsin with pK about 6.6, close therefore to that of the imidazole group of histidine.

At acid and alkaline pH this immediate change is followed by slower changes, occupying up to 40 minutes at 20°C. These changes are always in the direction of neutrality. They involve increases of 5 to 6 moles acid bound at acid pH, and 7 moles base bound at alkaline pH. They are associated with the irreversible denaturation of opsin in acid and alkaline solution, as evidenced by loss of its capacity to regenerate rhodopsin. Such frank denaturation procedures as the exposure of rhodopsin to alkali or heat in the dark result in comparable acid-base changes.

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