THE STABILITY OF RHODOPSIN AND OPSIN

Effects of pH and Aging

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In the course of a study of acid-base changes on bleaching rhodopsin, we found that at pH more acid than 5.5 or more alkaline than 7 the exposure of rhodopsin to light is followed by the denaturation of its protein moiety, opsin (Radding and Wald, 1955–56). In the present paper we examine the stability of rhodopsin and opsin as functions of age and pH.

We have used two criteria of stability: maintenance of the absorption spectrum of rhodopsin or of its maximal extinction at 500 mμ; and retention of its ability to regenerate after exposure to light.

Aging of Rhodopsin

Rhodopsin solutions can be kept at 3°C. for as long as 6 months with no appreciable change in absorption spectrum in the visible region (the α-band).[1] The capacity to regenerate after exposure to light, however, declines throughout this period.

To measure the regenerability, an aliquot of cattle rhodopsin solution is diluted with 2 to 3 volumes of phosphate buffer, pH 6.4. It is exposed to the short intense light of a photoflash bulb, and neo-*b* retinene in digitonin solution in excess is added immediately. This mixture is incubated in the dark at 25–27°C. for 2.5 to 3 hours. The amount of rhodopsin regenerated is estimated by adding hydroxylamine (0.17 M), and measuring the extinction at 500 mμ before and after bleaching (*cf.* Hubbard and Wald, 1952–53; Wald and Brown, 1953–54).

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[1] Though the α-band of rhodopsin, with $\lambda_{max.}$ 500 mμ, remains intact on aging, the absorption spectrum in the near ultraviolet undergoes changes. Thus one of our best preparations had a ratio of extinctions at 400 and 500 mμ (400/500 ratio) of 0.21, a 280/500 ratio of 2.4, and a distinct β-band at 350 mμ. Several days later the β-band had become a minor inflection on a general absorption rising into the ultraviolet, and the 280/500 ratio had risen. The 400/500 ratio, however, had remained at 0.22.

Fig. 1 and Table I show the stability of 5 different preparations of cattle rhodopsin, measured by both criteria. The extinction at 500 mμ (K_{500}) remains constant in all these preparations throughout the period of storage. The regenerability, however, declines in all of them, and at very different rates.

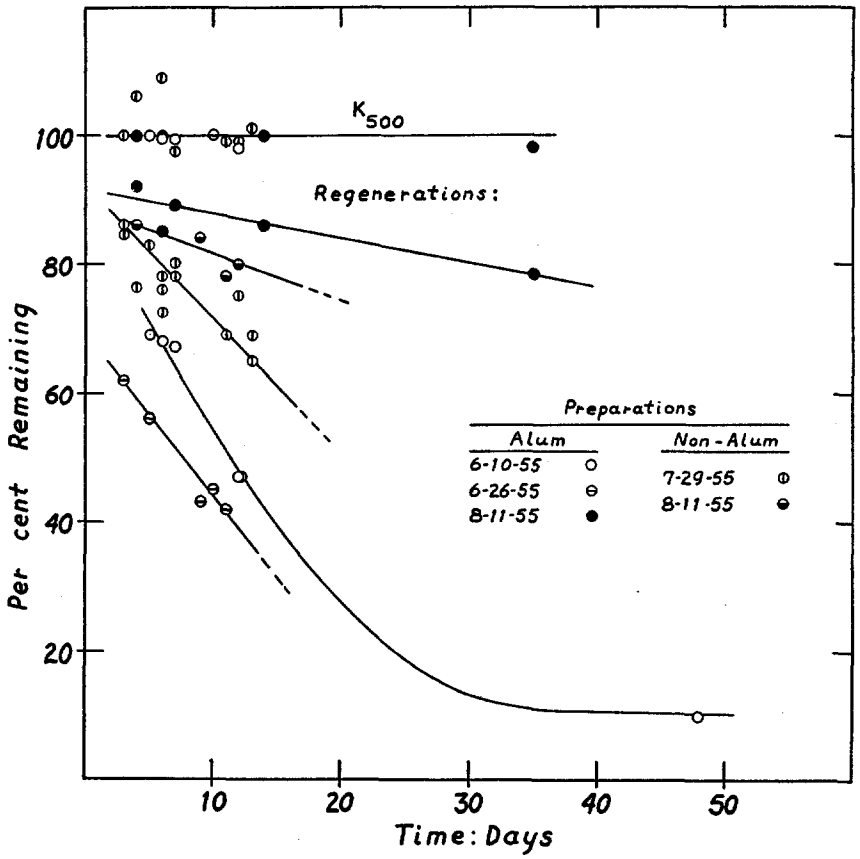

FIG. 1. The stability of rhodopsin on aging. Five rhodopsin solutions were stored at 3°C., and their stability measured in two ways: (*a*) maintenance of the extinction at 500 mμ; and (*b*) capacity to regenerate after exposure to light. By the first criterion the rhodopsin remains intact throughout the period of measurement; but its regenerability continuously declines, at a different rate in each preparation. The use of alum in preparing the rhodopsin, or its omission, seems to make no difference.

We have not yet been able to determine what causes the decay of regenerability. When rhodopsin is extracted in successive portions from a single preparation of rod tissue, all the extracts seem to lose regenerability at about

the same rate.[2] The use of alum in the preparation of rhodopsin, or the omission of this step, seems to make no difference, though its omission approximately doubles the relative amounts of impurities (proteins, etc.) absorbing at 278 mμ (Fig. 1, Table I). Dialysis against 2 per cent digitonin in water also seems to have no effect; though, in addition to removing some contaminants, dialysis shifts the pH from about 6.5–7.0 to 5.2–5.7. The addition of a chelating agent (EDTA, ethylenediaminetetracetic acid), glutathione, calcium or magnesium

TABLE I

Rate of Loss of Regenerability of Rhodopsin on Aging, Compared with Variations in Mode of Preparation, Purity, and Initial Capacity to Regenerate

The preparations were made from rods tanned with alum or in which this procedure was omitted; and involved first and second extractions of the rods with digitonin. The purity is measured by the ratios of extinctions at 280 and 400 mμ to the extinction at the $\lambda_{max.}$ 500 mμ (280/500 and 400/500 ratios). The lower these ratios, the purer the preparation.

Date	Alum treatment	Extraction No.	280/500 ratio	400/500 ratio	Initial percentage regeneration	Initial rate of loss of regenerability (percentage per day)
1955						
June 10	+	1	—	0.33	—	3.3
		2	—	0.26	69	3.3
June 26	+	2	—	0.26	62	2.6
July 29	−	1	4.9	0.34	85	1.9
		2	4.0	0.31	86	1.9
		3	—	0.28	—	1.9
Aug. 11	+	1	2.5	0.23	92	0.4
		2	2.5	0.27	—	0.4
Aug. 11	−	1	4.1	0.25	86	0.7

chloride, or serum albumin to aged rhodopsin solutions did not significantly improve their regenerability. (We have not, however, *stored* rhodopsin in the presence of any of these substances.) Rhodopsin preparations vary considerably in their initial ability to regenerate, and again this does not correlate with the rate at which regenerability is lost (Fig. 1, Table I).

It is plain that whatever the cause, the regenerability of rhodopsin may be

[2] The rod particles, after separation from the remaining retinal tissue by differential centrifugation, hardening with alum, freeze-drying, and extraction with petroleum ether, are in the form of a dry powder. The yield of rhodopsin is greatest if this is extracted in successive steps with small volumes of 2 per cent digitonin in water.

lost during storage at a neutral pH without any change in its absorption spectrum. The significance of this observation is discussed further below.

pH-Stability of Rhodopsin

Spectral Stability.—Chicken rhodopsin can be kept for an hour at 23°C. at any pH between 4 and 9 without loss of extinction (Wald, Brown, and Smith, 1954–55). In the present experiments cattle rhodopsin has been kept for 1 hour at 25–27°C. at various pH, and the extinction at 500 mμ measured. To set each pH, 0.1 ml. of rhodopsin was diluted with 0.4 ml. of buffer. The Michaelis acetate-veronal buffer was used from pH 2.5 to 8.4, glycine buffer from pH 9.6 to 11.4. The pH of the mixtures was measured at the beginning, and in questionable cases also at the end of the experiment.

The results are shown in Fig. 2, in terms of the extinction at 500 mμ remaining after 1 hour, as a percentage of the initial extinction. By this criterion the rhodopsin is stable between pH 3.9 and 9.6. To both sides of this range, the pigment bleaches, the extinction at 500 mμ falling to half its initial value in 1 hour at pH 3.3 and 10.5. The use of alum or its omission in the preparation of rhodopsin seems to make no difference.

Regenerability.—We have made a few measurements at acid and alkaline pH to determine whether rhodopsin, while maintaining its absorption spectrum, loses its regenerability. Such measurements at pH 4.5 and 7.9 are shown in Fig. 3.

A sample of rhodopsin was titrated to pH 4.5. An aliquot was immediately removed and 3 volumes phosphate buffer added to bring to pH 6.2. This portion was exposed to a short flash of bright light, excess neo-*b* retinene added immediately, and the mixture incubated in the dark for 3 hours. At the end of incubation, the rhodopsin regenerated was measured by the difference in extinction at 500 mμ before and after bleaching in the presence of hydroxylamine. This procedure yielded the first point in Fig. 3.

The remainder of this rhodopsin solution was held in the dark at pH 4.5 for an hour. Then a second aliquot was withdrawn and treated just as before. This procedure yielded the second point in Fig. 3. Exactly parallel measurements were made at pH 7.9. It is clear that within this range of pH, rhodopsin maintains its regenerability for at least an hour at 25–27°C.

pH-Stability of Opsin

Opsin is very much less stable to acids and bases than rhodopsin. This difference is illustrated by continuing the experiment just described (Fig. 3).

Following an hour in the dark at pH 4.5 or 7.9, the rhodopsin was exposed to a short flash of intense light. A first sample was withdrawn at once, brought to pH 6.2, and incubated in the dark with excess neo-*b* retinene for 3 hours. Thereafter samples were withdrawn at various intervals and treated similarly. Thus a series of samples was obtained of opsin which had been exposed to acid or

alkaline pH for various lengths of time before bringing to an optimal pH for regeneration. The amount of rhodopsin regenerated in each sample was estimated by the loss of extinction at 500 mμ on bleaching in the presence of hydroxylamine.

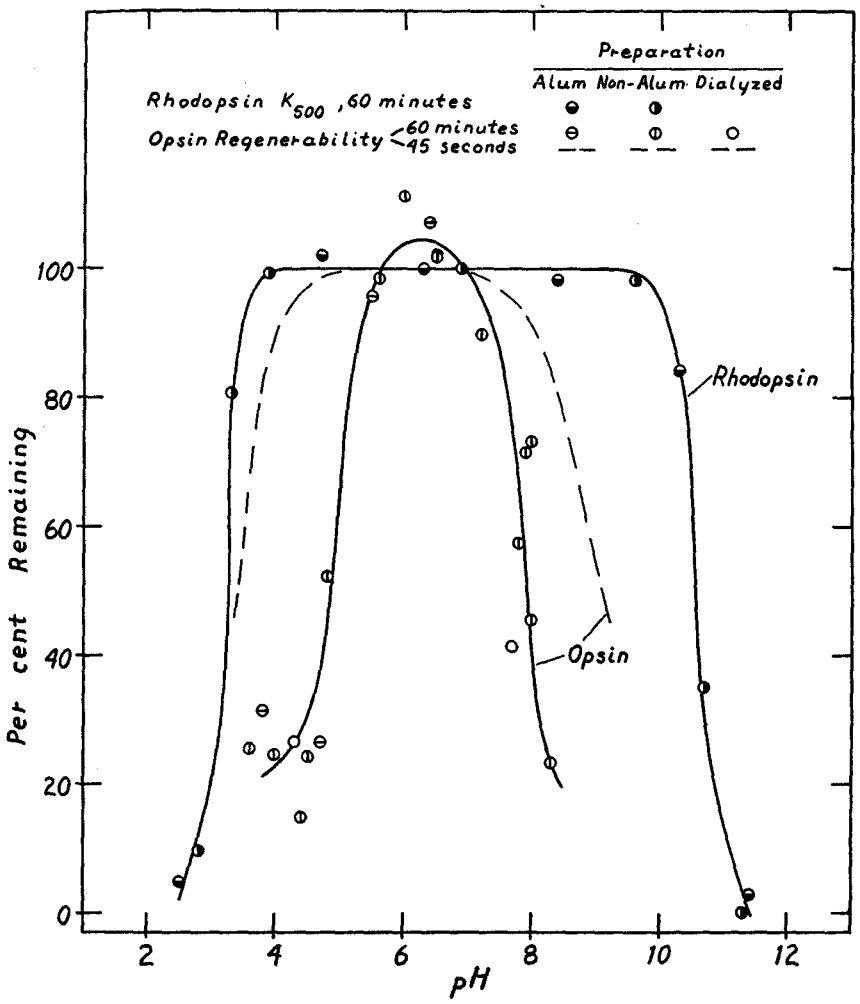

FIG. 2. Stability of rhodopsin and opsin as a function of pH. The curve for rhodopsin shows the maintenance of the extinction at 500 mμ following incubation for 1 hour at 25–27°C. The capacity of opsin to regenerate rhodopsin was measured 45 seconds (broken line) or 1 hour after the exposure of rhodopsin to light. To measure regenerability, each sample of opsin was brought to pH 6.4 ± 0.2, and incubated with excess neo-*b* retinene. Though rhodopsin is stable for at least 1 hour at pH 3.9–9.6, opsin is stable only between pH 5.5 and 7.

Fig. 3 shows that the stability which rhodopsin displays at pH 4.5 and 7.9 is lost at once on exposure to light. In the hour following irradiation, the opsin formed had lost 25 (pH 7.9) and 75 (pH 4.5) per cent of its regenerability. Light in splitting off the prosthetic group exposes new sites on opsin, and introduces

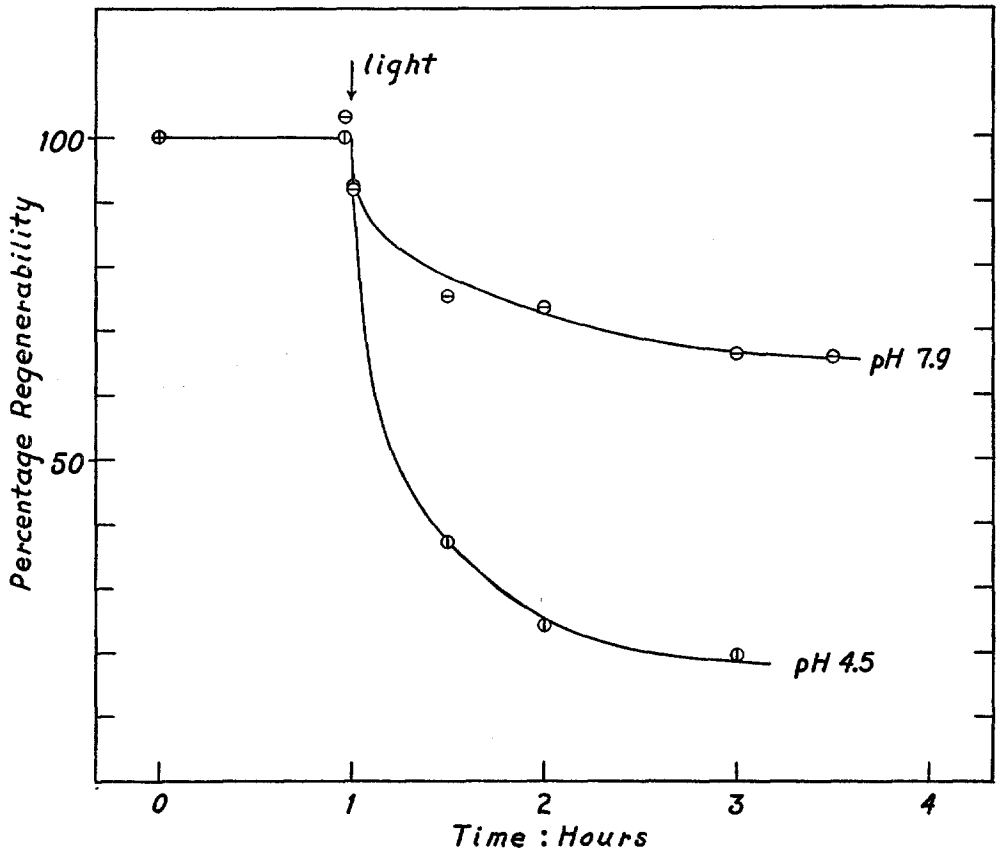

FIG. 3. Stability of rhodopsin before and after exposure to light at pH 4.5 and 7.9. In the hour before exposure to light, rhodopsin maintains its full regenerability; but the regenerability begins to decay immediately on irradiation. Light in splitting the prosthetic group from rhodopsin, leaves its protein residue, opsin, much more vulnerable to acids and bases.

a greatly increased vulnerability to acids and bases (*cf.* Wald and Brown, 1951–52; Radding and Wald, 1955–56).

We have made extensive measurements of the regenerability of opsin following incubation at various pH. The results are shown in Fig. 4.

In each experiment a sample of rhodopsin was titrated to the desired pH. An

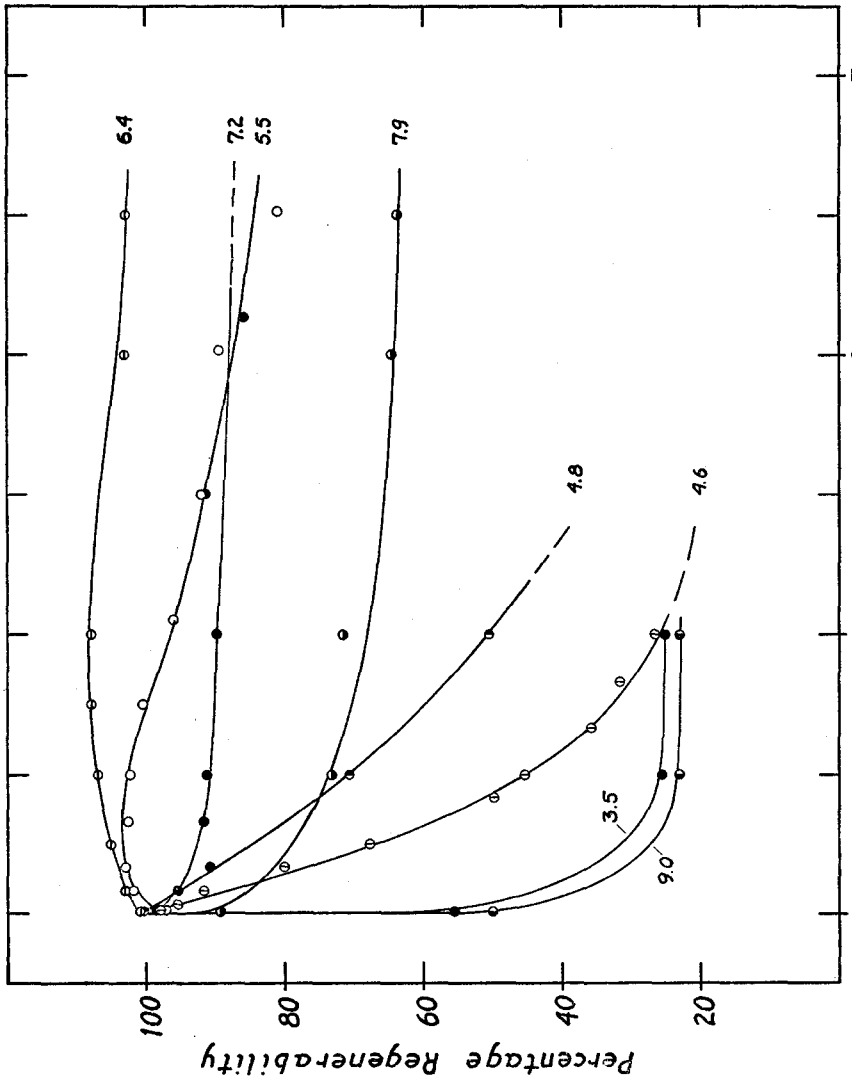

FIG. 4. The regenerability of opsin held at various pH. Following exposure of rhodopsin solutions to light, the opsin which results was held in the dark at the pH shown, at 25–27°C. Periodically samples were withdrawn, brought to pH 6.4 ± 0.2, and their regenerability tested. This is expressed as percentage of the control regenerability, measured on the same preparation.

aliquot was removed immediately, brought to pH 6.4 ± 0.2, exposed to a short flash of bright light, and neo-*b* retinene in excess added at once. After incubating for 2.5 hours, the amount of regenerated rhodopsin was estimated by measuring the fall in extinction at 500 mμ on bleaching in the presence of hydroxylamine. The result represented the maximum regenerability of the preparation, given always the value 100 per cent in Fig. 4.

The remainder of the rhodopsin at each pH was exposed to a flash of light, then left in the dark at 25–27°C. Aliquots were withdrawn at various intervals, brought to pH 6.4 ± 0.2, and tested as above for regenerability. The first aliquot was started incubating with neo-*b* retinene at 45 seconds following exposure to light, the last from 1 to 2.5 hours later.

Ordinarily the solutions of rhodopsin used for these experiments had an ionic strength at the time of bleaching not greater than 0.01; and the addition of buffer prior to regeneration raised the ionic strength to about 0.07 to 0.08. Instances, however, in which bleaching occurred at ionic strength as high as 0.2 and regeneration at ionic strength as high as 0.5 involved no changes in regenerability.

Fig. 4 displays several interesting features. At high alkalinities or acidities the regenerability falls to very low values in 30 minutes (pH 3.5, 9.0) or 1 to 2 hours (pH 4.6, 4.8).[3]

At alkaline pH nearer neutrality (7.2, 7.9), the regenerability still declines in a simple fashion, but to a relatively steady value which is still quite high. At pH 7.2 this is about 85 per cent of the initial regenerability, at pH 7.9 about 60 per cent. What does this end-point mean? It cannot represent an equilibrium between native and denatured forms of opsin, for during the 3 hour incubation at pH 6.4 with neo-*b* retinene any reversibly denatured opsin has plenty of time to renature. The fraction of regenerability lost at these pH's represents an irreversible change; then why does it not come to involve all the opsin present? The result observed seems to imply some heterogeneity in the preparation. A fraction of the opsin produced by bleaching apparently is unstable at pH 7.2, a still larger fraction at pH 7.9. These unstable portions lose their regenerability, while the rest of the opsin remains intact.

The same problem arises at slightly acid pH, but to it is added another curious phenomenon: a rise of regenerability reaching a peak 30 minutes (pH 5.5) to an hour (pH 6.4) after the rhodopsin was exposed to light. There seems little doubt that this effect is genuine, but its cause is not known. The bleaching of

[3] In Figs. 2 to 4, the regenerability never falls to zero. The reason for this is that our initial exposures of rhodopsin to light did not produce complete bleaching, and left residues which added to our determinations of the rhodopsin regenerated. That is, our regenerability measurements have a "blank" of 20 to 30 per cent, for which we have not corrected. When therefore the apparent regenerability has fallen to 20 to 30 per cent, this is equivalent to complete failure to regenerate.

rhodopsin proceeds in stages. Our short intense illumination converts rhodopsin to lumi-rhodopsin, which goes on in the dark for an hour or longer at this temperature and pH to form a mixture of all-*trans* retinene and opsin (Wald, Durell, and St. George, 1951). The obvious thought that regeneration can proceed best after these processes are completed cannot apply here, for the subsequent 3 hour incubation at pH 6.4 with neo-*b* retinene gives more than enough time for all the dark phases of bleaching to be completed, however early the incubation is begun. The experiment at pH 6.4 is particularly revealing, for in this instance no adjustment of pH is needed—neo-*b* retinene is merely added to the irradiated rhodopsin at various times after exposure to light. Under these circumstances up to an hour of delay in adding neo-*b* retinene seems to aid the subsequent regeneration. Apparently some change occurs *before* neo-*b* retinene is added, not in its presence, that favors regeneration. We have as yet no idea what this change can be.

From the data of Fig. 4 and other comparable measurements the curves for regenerability of opsin are plotted in Fig. 2. The broken line shows the regenerability 45 seconds after the exposure of rhodopsin to a flash of light, the solid line and open circles the regenerability after an hour. The regenerability remains intact for 45 seconds at pH 5–7, falling steeply to both sides, and reaching half its maximum value at pH 3.4 and 9.1. At 60 minutes after exposure of rhodopsin to light, the stability range of opsin is very narrow. The regenerability falls sharply below pH 5.5 and above pH 7.0, reaching half the maximum at about pH 5 and 8. The regenerability seems to rise a little above the 45 second value in the neighborhood of pH 6.4, as already noted in the discussion of Fig. 4. The use or omission of alum in preparing the rhodopsin apparently makes no difference in any of these measurements, nor does dialysis or its omission (*cf.* Fig. 2).

DISCUSSION

The exposure of rhodopsin to frank denaturing procedures—heat, mineral acids or alkalies, polar organic solvents, heavy metal ions—bleaches the molecule. Does bleaching by light also imply denaturation? A number of parallels may be drawn between the properties of bleaching by light in neutral solution and a protein denaturation (Wald and Brown, 1951–52); yet none of them is compelling. Some points of correspondence with denaturation derive simply from the fact that bleaching involves the partial or complete severance of retinene, the prosthetic group of rhodopsin, from the protein opsin. This necessarily exposes new groupings, with consequent effects on acid-base balance. We cannot yet make a clear argument that the bleaching of rhodopsin by light in neutral solution (pH 6–7) is significantly related to denaturation.[4]

[4] Obviously this discussion is based on a particular view of the meaning of denaturation. In our view this term always implies some unfolding of protein structure,

In mildly acid or alkaline solutions, however, in which rhodopsin itself is stable, exposure to light induces a rapid denaturation of opsin. This involves both loss of regenerability, and the exposure of numbers of new acid- and base-binding groups.

It is clear that the attachment of the prosthetic group to opsin exercises a marked protective effect. It seems to hold the protein together. Exposure to light introduces an immediate vulnerability: outside the narrow range of pH 5.5–7, the disruption by light of the retinene-protein linkage is followed by an apparently irreversible unravelling of the opsin structure (Radding and Wald, 1955–56).

We have described a further phenomenon, the significance of which is more ambiguous. This is the loss of regenerability of rhodopsin on aging, without change in its absorption spectrum. The maintenance of absorption spectrum implies that at least the portion of the protein bound to retinene is "native." Does the loss of regenerability imply that at other sites it has denatured? If so, these sites presumably lie close to the retinene-protein bond, for both effects involve this bond, the one its maintenance, the other its restitution. Again we seem to find a condition in which the attachment of the prosthetic group to opsin maintains an apparent *status quo* which, once interrupted, cannot be fully restored. If the loss of regenerability on aging is to be regarded as a partial denaturation, we have demonstrated independent, though interacting denaturations involving separate sites on the same protein molecule—a matter of considerable interest for general protein chemistry.

One cannot be sure, however, that the effect of aging has the character of denaturation. It would, for example, be possible to mimic this effect by adding to a rhodopsin solution small amounts of the sulfhydryl poison, *p*-chloromercuribenzoate. This would not disturb the absorption spectrum, yet would inhibit regeneration proportionately to the concentration (Wald and Brown, 1951–52). Could it be that some heavy metal ion leached from the glassware during storage has a similar effect on our rhodopsin solutions? Such effects are not unknown (*cf.* Pappenheimer and Johnson, 1936); and this kind of fortuitous contamination might explain the great variability in rate of loss of regenerability that we have observed. Against this specific notion, however, is our observation that addition of a chelating agent or of glutathione, reagents which would be expected to remove heavy metal ions, did not improve the regenerability.

Until the mechanism by which regenerability is lost on aging is determined,

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and some consequent degree of disorder as evidenced, for example, by failure to crystallize. We do not think the term is applied usefully to such specific reactions as the removal of a non-peptidic prosthetic group, or even a fragment of the protein itself; or the addition of reagents which combine, reversibly or irreversibly, with specific groups of the protein (—NH_2, —SH); though any such reaction may be a prelude to denaturation.

these considerations must remain open. We are left with the realization that the rhodopsin molecule is held intact to a degree by the attachment of its prosthetic group to the protein. The disruption of this attachment by light, though not itself certainly causing a structural rearrangement of the protein, opens the latter to a variety of attacks from which it had earlier been immune: denaturation at pH outside the range 5.5–7, and the progressive inhibition of regeneration within this range as rhodopsin ages.

SUMMARY

The stability of cattle rhodopsin and of its protein moiety opsin toward acids and alkalies and on aging was determined by two criteria: maintenance of absorption spectrum, and capacity to regenerate after exposure to light.

On storage at 3°C. at pH near neutrality, the absorption spectrum in the visible region may remain unchanged for as long as 6 months; but the regenerability progressively declines, at very different rates in different preparations. The cause of this decline has not been determined. It may involve denaturation at sites other than the retinene-protein bond, which by the evidence of the absorption spectrum remains intact.

Cattle rhodopsin maintains its absorption spectrum at any pH from 3.9–9.6 for at least an hour at 25–27°C. To both sides of this pH range the pigment bleaches, the extinction falling to half in 1 hour at pH 3.3 and 10.5.

The exposure of rhodopsin to light greatly increases the vulnerability of the product (opsin) to acids and bases. Opsin rapidly loses its capacity to regenerate rhodopsin to both sides of the range of pH 5.5–7.0. Half the regenerability is lost within 45 seconds at pH 3.4 and 9.1; and within 1 hour at pH 5 and 8.

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