THE ROLE OF SULFHYDRYL GROUPS IN THE BLEACHING AND SYNTHESIS OF RHODOPSIN

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A mixture of the carotenoid retinene$_1$ and the protein opsin, placed in the dark, reacts spontaneously to form the light-sensitive pigment of rod vision, rhodopsin (Wald and Brown, 1950). The nature of the binding between retinene$_1$ and opsin governs the conditions under which this synthesis occurs. Conversely the opening of the linkage between opsin and its chromophore as rhodopsin bleaches may have some fundamental connection with the excitation process in rod vision.

The demonstration by Morton and his colleagues that retinene$_1$ is vitamin A_1 aldehyde aroused interest in the general array of aldehyde reactions in which retinene$_1$ takes part. Attention centered originally on the condensation of retinene$_1$ with amino groups in a variety of organic amines, amino acids, and proteins. Such complexes vary in spectrum with pH, suggesting a relationship to the product of bleaching rhodopsin ("indicator yellow"). Some of them also are brightly colored in acid solution (0.1 N HCl), suggesting some connection with the rhodopsin chromophore (Ball, Collins, Morton, and Stubbs, 1948; Ball, Collins, Dalvi, and Morton, 1949; Collins and Morton, 1950).

We shall present evidence here, however, that sulfhydryl groups of opsin play a central part in the synthesis and bleaching of rhodopsin. The optimal conditions for rhodopsin synthesis favor the condensation of retinene$_1$ with sulfhydryl rather than with amino groups. The synthesis is blocked completely by low concentrations of the sulfhydryl reagent, *p*-chloromercuribenzoate; and this inhibition is reversed by the addition of glutathione. Furthermore the bleaching of rhodopsin exposes new sulfhydryl groups.

In a subsequent paper we will show that retinene$_1$ condenses spontaneously in solution with the sulfhydryl groups of cysteine and glutathione (Wald and Brown, 1952–53). Such complexes arise under the same conditions as are optimal for the synthesis of rhodopsin; and like rhodopsin, they do not vary in spectrum with pH.

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These observations have important consequences for the structure of rhodopsin, and its role in visual excitation. They emphasize also an important and largely neglected aspect of aldehyde biochemistry. Certain aldehydes are very effective sulfhydryl reagents; and with the growing recognition of the importance of sulfhydryl groups in the reactions of enzymes and other proteins, this property should become increasingly significant. It is hoped that our experiments, beyond their special province, will contribute to this development.

I

General Procedures

Rhodopsin.—The essential elements of this procedure are the isolation of the outer segments of the rods from the remaining retinal tissues; tanning with alum to make other proteins insoluble; leaching with water and buffer solutions to remove other water-soluble substances; extraction with petroleum ether to remove lipids; and finally the extraction of rhodopsin with the aid of the detergent, digitonin.

Twenty five to fifty cattle eyes are obtained fresh from the slaughter house, having been placed in the dark as soon as possible after slaughtering. The retinas are dissected out under dim red light, and are usually kept frozen at about −15°C. until needed. They are thawed, ground thoroughly in a mortar, and the disintegrated tissue is taken up in neutral M/15 phosphate buffer. Centrifuging at about 1200 R.P.M. brings down the larger bits of tissue, leaving the outer segments of the rods in suspension. This suspension is decanted, and is recentrifuged at about 5000 R.P.M. to sediment the rods.

The supernatant is discarded, and the rod residue stirred into 45 per cent sucrose in phosphate buffer. The suspension is centrifuged at about 1800 R.P.M. for 5 to 10 minutes to bring down bits of pigment epithelium and other heavy tissues; the rods are less dense, and remain in suspension. This suspension is decanted, diluted with 4 to 5 volumes of buffer solution, and is recentrifuged at about 5000 R.P.M. The supernatant is discarded, and the rod residue stirred up in 4 per cent potassium alum solution, and left to tan for several hours. It is centrifuged out, and washed twice in distilled water and once in neutral phosphate buffer in the centrifuge. The residue of these washings is frozen-dried, and the dry material extracted twice by stirring with petroleum ether to remove lipids. The final residue of all these treatments is leached for several hours with 2 per cent aqueous digitonin solution to extract rhodopsin. The rhodopsin solutions so prepared have ratios of extinction at 400 and 500 mμ (400/500 ratios) of 0.25 to 0.35.

Frog rhodopsin was prepared in the same way, using whole retinas rather than rod suspensions. Squid rhodopsin was prepared from rod suspensions, separated from the remainder of the retinas by differential centrifugation, washed with water and alkaline buffer to remove screening pigment, and extracted directly with digitonin solution.

Opsin.—This is prepared from cattle retinas by the same procedure as used for rhodopsin, except that the retinas are bleached to colorlessness by exposure to light after removal from the eye. We have found also that it is advisable to omit here the

freeze-drying and extraction with petroleum ether, for these procedures tend to yield opsin preparations of relatively low activity.

Retinene$_1$.—This is prepared by the chromatographic oxidation of vitamin A_1 alcohol on solid manganese dioxide (Wald, 1947–48). The retinene$_1$ is purified by adsorption out of petroleum ether solution on a column of calcium carbonate, on which it forms a diffuse band which travels down the column and eventually is collected in the filtrate. For reactions in water, the retinene$_1$ is taken up in 2 per cent digitonin solution.

Regeneration of Rhodopsin.—Rhodopsin solutions bleached in the presence of added retinene$_1$ regenerate a large proportion of their rhodopsin in the dark (Wald and Brown, 1950). In the present experiments, 0.5 ml. of a rhodopsin solution is mixed in the dark with 0.25 ml. of a concentrated solution of retinene$_1$ in digitonin, and with 0.25 ml. of buffer or another reagent. Changes in rhodopsin concentration are measured by following the extinction at 500 mμ—the rhodopsin maximum—with the Beckman spectrophotometer, using quartz microcells 1 cm. in depth and 3 mm. wide (Pyrocell, New York). The mixture is bleached for 15 minutes in the concentrated light of a 160 watt tungsten lamp, passing through a Jena GG3 filter glass to remove ultraviolet radiation, and a Corning 3962 glass to remove heat. The mixture is then allowed to regenerate in the dark for 2 to 2.5 hours at 24–27°C. Then 0.1 ml. of a 1 M solution of freshly neutralized hydroxylamine is added, to prevent further regeneration and to remove fortuitous retinene$_1$ complexes. The absorption spectrum is measured in the dark, and again after bleaching completely in the light. The difference in absorption spectrum before and after bleaching is the "difference spectrum" of the regenerated rhodopsin. The difference in extinction at 500 mμ measures the amount of rhodopsin regenerated. A control bleaching of another sample of the same preparation of rhodopsin, brought to the same dilution and mixed with hydroxylamine, yields a measure of the rhodopsin present initially. The one quantity divided by the other is the percentage regeneration.

II

Sulfhydryl Groups and Rhodopsin Synthesis

The synthesis of rhodopsin from retinene$_1$ and opsin is inhibited completely by 0.1 M hydroxylamine or by 0.7 M formaldehyde (Wald and Brown, 1950). Hydroxylamine blocks this synthesis by condensing with retinene$_1$ to form retinene$_1$ oxime; in a sense it competes with opsin for retinene$_1$. In the same sense formaldehyde competes with retinene$_1$ for certain groups on opsin.

Initially we thought that these were probably amino groups. Aldehydes, however, react not only with amino groups—a reaction made familiar by Sørensen's formol titration—but with sulfhydryl groups (Jowett and Quastel, 1933; Schubert, 1936; Ratner and Clarke, 1937). Which of these alternative reactions provides the basis for rhodopsin synthesis?

Both types of reaction seem to involve the amino and sulfhydryl groups in their uncharged states. Below pH 6–7, amino groups exist primarily as ammonium ions ($—NH_3^+$), and combine only slightly even with formalde-

hyde, probably the most active of the aldehydes. Sulfhydryl groups, however, remain largely un-ionized up to pH 8–9, and at lower pH's condense readily with formaldehyde and other aldehydes.

Thus Ball *et al.* (1949) have reported that retinene$_1$ reacts with aliphatic amines and with amino acids only in alkaline solution, and then only slowly unless the amino compound is present in large excess. Even in 0.033 M sodium hydroxide, retinene$_1$ does not react appreciably with β-alanine when mixed in equimolar proportions. At this alkalinity, it does react with excess β-alanine; but in neutral solution with excess β-alanine very little reaction can be observed even after 48 hours.

On the other hand we have found that retinene$_1$ reacts rapidly with the sulfhydryl group of cysteine, when the latter is in excess, at pH 6.5. The reaction goes to completion within about 5 hours at 23°C. (Wald and Brown, 1952–53).

One has therefore a reasonably clear distinction in the pH range in which retinene$_1$ reacts with amino and with sulfhydryl groups. The optimal pH for the reaction of retinene$_1$ with opsin to form rhodopsin should indicate to a degree which type of group is involved.

The pH-activity curve for the regeneration of rhodopsin from retinene$_1$ and opsin is shown in Fig. 1. The optimum pH is about 6; the synthesis proceeds almost as well at pH 5, but falls to low values at pH 7 and above. That is, the synthesis goes best at pH's at which amino groups are primarily ionic, while —SH groups remain uncharged.[1]

One can conclude from these measurements alone that the synthesis of rhodopsin is optimal at pH's which are disadvantageous for the condensation of retinene$_1$ with amino groups, yet favor its condensation with sulfhydryl groups.

Experiment.—Three rhodopsin solutions, prepared as described above, were used in these experiments. In each series of measurements, aliquot samples of a single preparation were examined under identical conditions except for pH. The method of measuring the percentage regeneration also has been described. At pH 4–5, citrate or acetate buffers were used; at pH 6–8, sodium-potassium phosphate mixtures; and at pH 9 a borate buffer.

We have examined the effect upon rhodopsin synthesis of monoiodoacetic acid and monoiodoacetamide, reagents which remove sulfhydryl groups irreversibly by alkylation (*cf.* Barron, 1951). These substances had no ap-

[1] Several years ago Chase and Smith (1939–40) reported measurements of the effect of pH upon the regeneration of rhodopsin. They found a pH optimum at about 6.7, the regeneration falling to low values at pH 5 and 9. These measurements cannot be compared readily with our own. Apart from various differences in procedure, they involve frog rather than cattle rhodopsin; solutions not supplemented with retinene$_1$ as in the present experiments; and very small regenerations, the largest of which represented a change in extinction at 560 mμ of 0.034.

preciable effect upon either the rate or the extent of rhodopsin regeneration, even in a concentration of 0.03 M, some ten times the concentration that inhibits a number of sulfhydryl enzymes.

It is known, however, that in the hierarchy of sulfhydryl poisons, iodoacetic acid and its amide stand low in both specificity and potency (Barron and Singer, 1945; Singer and Barron, 1945). In both respects they are sur-

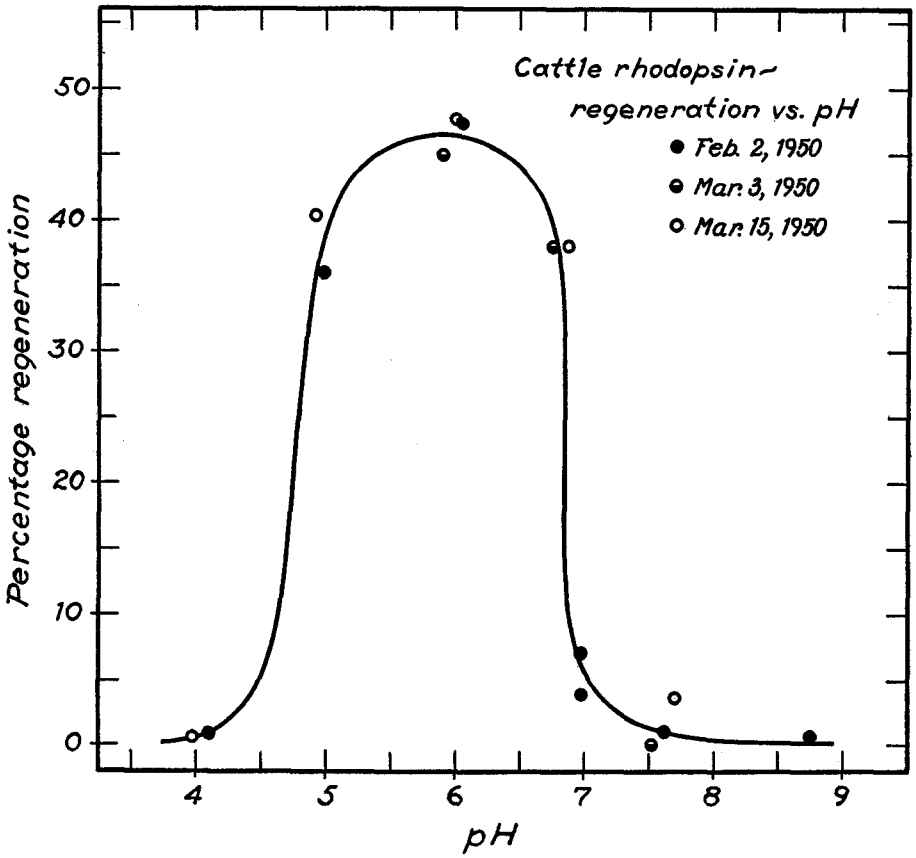


FIG. 1. The regeneration of cattle rhodopsin at various pH's. Data from three experiments, each with a different preparation. At each pH the rhodopsin, supplemented with retinene$_1$, was bleached completely and allowed to regenerate at 24–27°C. for 2 to 2.5 hours.

passed by *p*-chloromercuribenzoate, PCMB (Hellerman, Chinard, and Ramsdell, 1941). A variety of enzymes that are unaffected by iodoacetate are strongly inhibited by PCMB.[2]

[2] We are indebted to Dr. H. Stanley Bennett of the University of Washington School of Medicine, and to Dr. L. Hellerman of the Johns Hopkins School of Medicine, for initial gifts of *p*-chloromercuribenzoate.

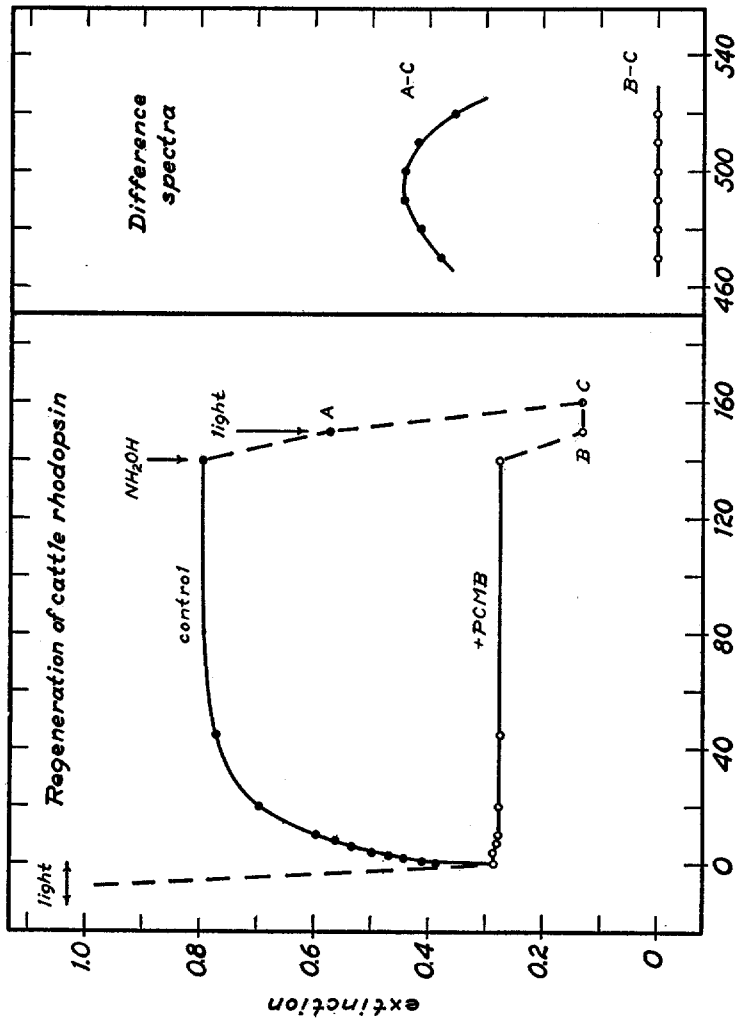


FIG. 2. The inhibition of rhodopsin synthesis with *p*-chloromercuribenzoate (PCMB). The left-hand portion of the figure follows the extinction at 500 mμ of a rhodopsin preparation, supplemented with retinene$_1$, to half of which PCMB had been added (7×10^{-4} M), the other half being held as control. Both solutions were bleached completely, and replaced in the dark at time 0. The control undergoes a rapid regeneration of rhodopsin, the poisoned solution shows no appreciable change. At the end of this period, both solutions are reilluminated in the presence of hydroxylamine. The control mixture bleaches from A to C; the difference spectrum of this change, shown on the right, has the maximum at about 495 mμ characteristic of regenerated rhodopsin. The poisoned solution undergoes no further bleaching (B to C).

This is true also of opsin (Fig. 2). Rhodopsin supplemented with retinene$_1$ regenerates in large part after bleaching by light. In the presence of 7×10^{-4} M PCMB, no regeneration occurs. Alternatively, opsin alone, on mixing with retinene$_1$ in digitonin solution, forms rhodopsin in the dark. If the opsin is treated previously with PCMB (7×10^{-4} M), no rhodopsin is formed.

Experiment.—Two 0.5 ml. portions of a solution of cattle rhodopsin were mixed with 0.25 ml. of concentrated retinene$_1$ in digitonin. To one (*a*) was added 0.25 ml. of neutral phosphate buffer; to the other (*b*) the same volume of a neutral solution of PCMB to make a final concentration of 7×10^{-4} M. The final pH in (*a*) was 6.82, in (*b*) 6.72. The extinction of rhodopsin in each mixture, measured at 500 mμ in a 10 mm. layer, was 1.385.

Both solutions were bleached together for 15 minutes, then left in the dark to regenerate at about 25°C. for 140 minutes. The regeneration was followed with periodic measurements of the extinction at 500 mμ. At the end of this interval, hydroxylamine was added, and the percentage regeneration and the difference spectra of the regenerated pigment were determined as described above. The difference spectra are shown at the right in Fig. 2; the changes in extinction at 500 mμ are shown at the left. In the presence of PCMB no rhodopsin had regenerated; in its absence, about 42 per cent regeneration had occurred.

In another experiment for which no figure is shown, opsin, prepared as described above, was mixed with retinene$_1$, and with either neutral phosphate buffer, or with PCMB in a final concentration of 7×10^{-4} M. Both mixtures were incubated in the dark for 120 minutes, and were then examined as above. The control mixture had synthesized rhodopsin in a yield corresponding to an extinction at 500 mμ of 0.123. The mixture containing PCMB yielded no measurable rhodopsin.

The initial action of PCMB on proteins is reversible. The heavy metal replaces hydrogen in the —SH group, forming a mercaptide. This is readily dissociated by introducing some other sulfhydryl compound for which PCMB has a higher affinity (Barron and Singer, 1945).

In this way the inhibition of rhodopsin synthesis by PCMB is reversed in part by the addition of glutathione (Fig. 3). As usual in such reactions, the reversal of inhibition is not complete, and is more effective the earlier the competing mercaptan is added. Apparently the removal of free —SH groups by PCMB is followed in opsin, as in other proteins, by slower, irreversible changes, probably structural rearrangements having the character of a denaturation.

Experiment.—In the experiment of Fig. 3, two 0.25 ml. portions of a cattle rhodopsin solution were mixed with 0.125 ml. of concentrated retinene$_1$, and with 0.125 ml. of neutral PCMB. The final concentration of PCMB was 7×10^{-5} M.

Both solutions were illuminated for 15 minutes. Then 1 mg. of powdered glutathione was added to one solution, and both were placed in the dark for $2\frac{3}{4}$ hours at room temperature. Then they were tested for the presence of regenerated rhodopsin by measuring the difference spectra as described above. These are shown in Fig. 3.

The solution treated with PCMB alone had not regenerated any rhodopsin. That which had received glutathione after 15 minutes' action of PCMB had produced rhodopsin, corresponding in amount to a 30 per cent reversal of the PCMB inhibition.

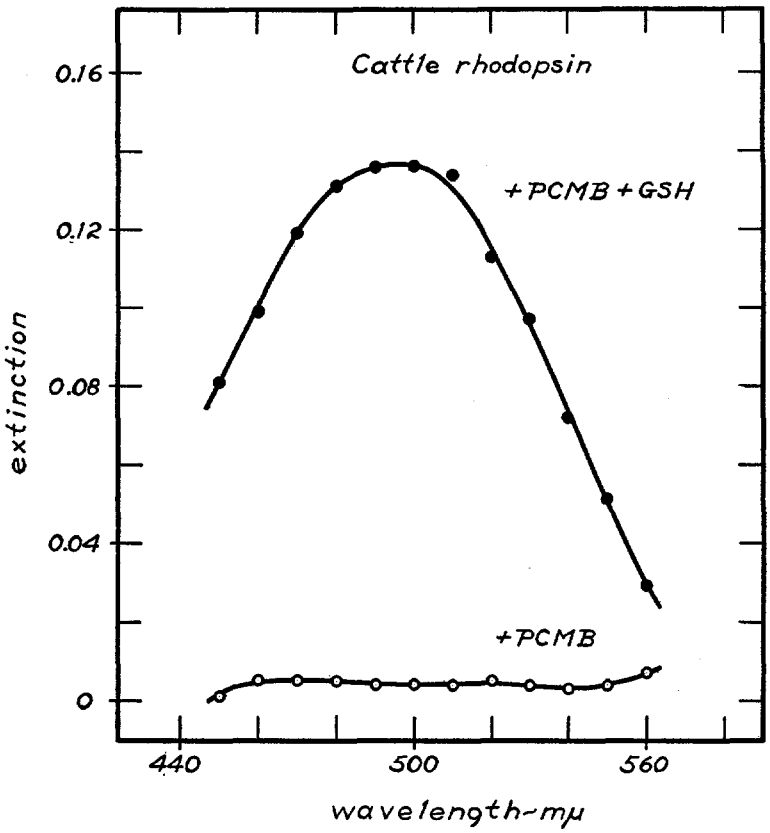


FIG. 3. Reversal of the PCMB-inhibition of rhodopsin synthesis with glutathione (GSH). Two portions of a rhodopsin preparation, both poisoned with 7×10^{-5} M PCMB, were bleached in white light for 15 minutes. Then glutathione was added to one solution, and both were incubated in the dark. They were then tested for regenerated rhodopsin by measuring the absorption spectra before and after reexposure to light. The difference spectra in the figure show that PCMB had completely prevented the regeneration of rhodopsin; but that the later addition of glutathione had reversed this inhibition about 30 per cent.

Rhodopsin is not attacked by PCMB in the concentrations which poison its synthesis. To test this we mixed a cattle rhodopsin solution with neutral buffer and added PCMB in a final concentration of 7×10^{-4} M. This solution had an initial extinction at 500 mμ of 1.525. After standing for 3 days at

about 25°C., its extinction had fallen only 5.6 per cent. On irradiation, this solution bleached normally, the extinction at 500 mμ falling to 0.435.

We have confirmed also the *a priori* supposition that retinene$_1$ does not react with PCMB. A solution containing 1.34×10^{-5} M retinene$_1$ was mixed with 100 times this concentration of PCMB. The absorption spectrum, measured at once and again 2.5 hours later, showed no appreciable difference from that of retinene$_1$ alone.

The inhibition of rhodopsin synthesis by PCMB depends therefore upon the reaction of this substance with opsin. It involves certain —SH groups of opsin which are not available to PCMB in rhodopsin.

In the experiments described above we have used PCMB in concentrations sufficient to block completely the synthesis of rhodopsin. The effects upon this synthesis of graded concentrations of PCMB are shown in Fig. 4.

Rhodopsin regeneration is inhibited with the first addition of PCMB, and decreases linearly as the concentration of the reagent rises. An appreciable inhibition is evident at a PCMB concentration of 7×10^{-6} M. At 8×10^{-5} M PCMB, the inhibition is complete.

In the experiments shown in Fig. 4 the rhodopsin concentration was equivalent to 1.5×10^{-5} M retinene$_1$; *i.e.*, bleaching the rhodopsin completely yielded this concentration of retinene$_1$.[3] Therefore to inhibit completely the regeneration of rhodopsin in this experiment required 5 to 6 times as many moles of PCMB as the rhodopsin contained retinene$_1$. It is perhaps more significant to state this relation in terms, not of the retinene$_1$ originally bound in rhodopsin, but of the retinene$_1$ it is capable of recombining in regenerating. With no PCMB present, this rhodopsin preparation regenerated 50 per cent after bleaching (*cf.* Fig. 4). Therefore one can say in this instance that to prevent retinene$_1$ from condensing with opsin to form rhodopsin required the addition of 10 to 12 mole equivalents of PCMB. It would seem from this that our preparation contained many more —SH groups titratable with PCMB than participated directly in rhodopsin synthesis. The preparation was not altogether pure, and some of the —SH groups may have been on other molecules than opsin itself.

The dependence of rhodopsin synthesis upon free sulfhydryl groups introduces a further consideration. Such groups are highly vulnerable under ordinary conditions of experimentation to autoxidation and attack by heavy

[3] The retinene$_1$ equivalent of rhodopsin is evaluated on the following basis. Retinene$_1$ dissolved in 2 per cent aqueous digitonin, and measured in a 10 mm. layer, has an extinction at 385 mμ of 1, when its absolute concentration is 8.8 μg. per ml. or 3.1×10^{-5} M. Rhodopsin and the retinene$_1$ derived from it by bleaching have very nearly the same extinctions at their respective absorption maxima. Therefore a rhodopsin solution with an extinction at 500 mμ of 1 contains the equivalent of 3.1×10^{-5} M retinene$_1$.

metal ions. For this reason we have tested the possibility that the addition of cysteine or glutathione might protect opsin from both sources of deterioration, and so might aid the regeneration of rhodopsin.

Such an experiment is shown in Fig. 5. A solution of cattle rhodopsin supplemented with concentrated retinene$_1$ was divided into two portions. To one portion, neutral phosphate buffer was added; to the other, the same

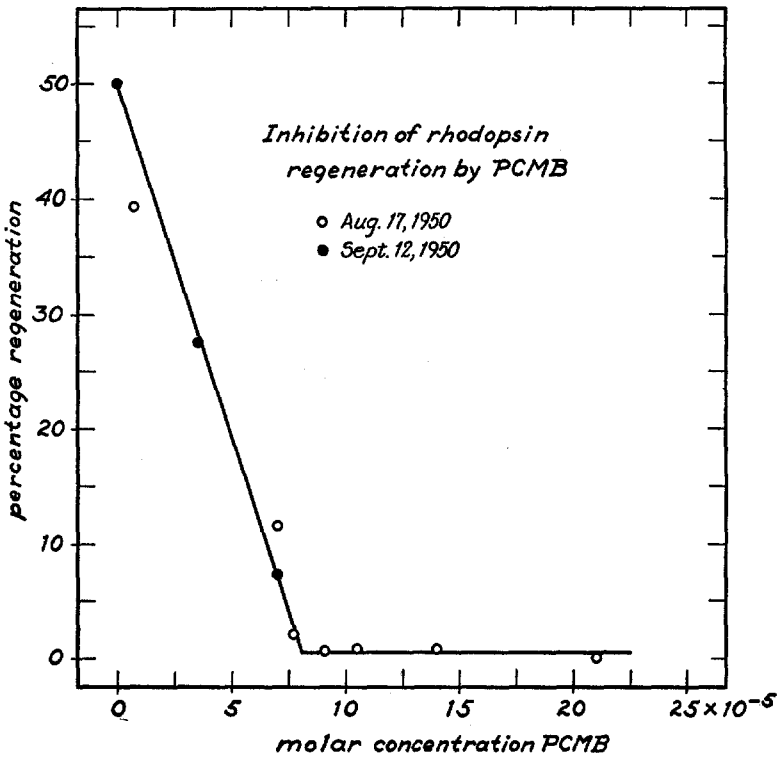


FIG. 4. The regeneration of rhodopsin in the presence of graded concentrations of *p*-chloromercuribenzoate. Data from two experiments. The regeneration falls linearly as the PCMB concentration is increased, until at 8×10^{-5} M PCMB the inhibition is complete.

volume of neutral glutathione solution. The final concentration of glutathione was 0.71 mg. per ml. Both solutions were bleached by irradiating for 15 minutes, and then were allowed to regenerate in the dark for 3.5 hours at room temperature. The control mixture regenerated 62 per cent, that containing glutathione regenerated 71 per cent. This difference appears to be significant, particularly since the solution containing glutathione was at a slightly less advantageous pH (6.85) than the control (6.65).

It seems therefore that the addition of low concentrations of free sulfhydryl can aid rhodopsin synthesis, apparently by helping to maintain intact the sulfhydryl groups of opsin. It should be noted that higher concentrations of

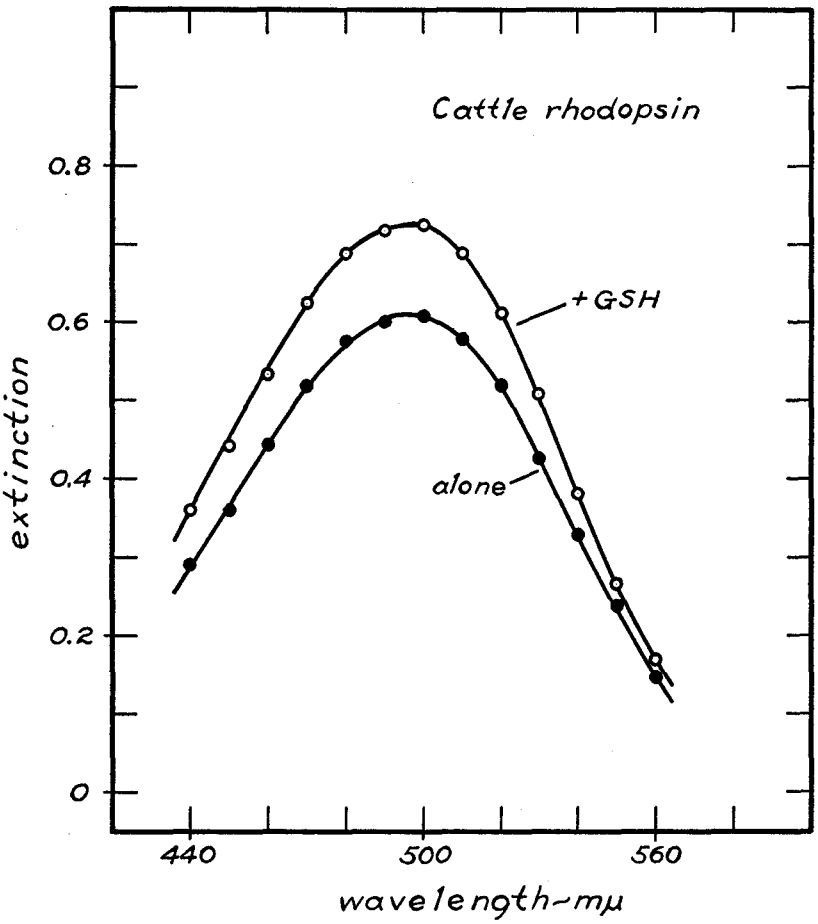


FIG. 5. The effect of added glutathione upon rhodopsin synthesis. Two portions of a preparation of rhodopsin, to one of which glutathione was added (0.71 mg. per ml.), were bleached and allowed to regenerate. The difference spectra of regenerated rhodopsin are shown. The mixture containing glutathione regenerated 71 per cent, the unsupplemented mixture 62 per cent.

foreign sulfhydryl groups, particularly in such highly reactive molecules as cysteine, might inhibit the regeneration of rhodopsin by competing with opsin for retinene$_1$ (Wald and Brown, 1952–53).

To summarize these observations, it has been shown that the condensation

of retinene$_1$ with opsin to form rhodopsin is optimal at pH's which favor the reaction of retinene$_1$ with —SH rather than with $-NH_2$ groups. The synthesis of rhodopsin, though unaffected by the less powerful sulfhydryl reagents, monoiodoacetic acid and its amide, is inhibited completely by *p*-chloromercuribenzoate. This inhibition is reversed in part by the addition of other sulfhydryl compounds. PCMB does not attack rhodopsin or retinene$_1$. Its action in this system appears to be confined to the —SH groups of opsin. The synthesis of rhodopsin can on occasion be aided by the addition of such a sulfhydryl compound as glutathione, which helps to keep the sulfhydryl groups of opsin in the free and reduced condition.

It may be concluded that certain sulfhydryl groups of opsin play a central part in the synthesis of rhodopsin. In opsin these groups are available to PCMB; in rhodopsin they are not available. As will appear below, neither are they available in rhodopsin for reaction with silver ions. These groups are not only required for rhodopsin synthesis, but are removed or bound in the course of this process, and are formed or released as rhodopsin bleaches. The latter phenomenon is demonstrated directly in the experiments which follow.

III

The Liberation of Sulfhydryl Groups in the Bleaching of Rhodopsin

We have sought to complete the argument developed in the preceding section by measuring directly the sulfhydryl groups in preparations of rhodopsin, and the effects of bleaching upon them.

In these experiments we used a modification of the amperometric silver titration of Kolthoff and Harris (1946), which has been applied extensively by Benesch and Benesch in estimating the sulfhydryl groups of proteins (1948) and glutathione (1950). The method is highly specific and is not interfered with by any of the common amino acids, purines, pyrimidines, cozymase, or ascorbic acid. This procedure has a particular significance for rhodopsin and its role in visual excitation. We shall therefore describe it in some detail.

Fig. 6 shows a diagram of the arrangements. A standard mercury-mercuric iodide half-cell (I) is connected with a salt bridge to another half-cell (II) in which a rotating platinum electrode is in contact with an ammoniacal solution containing a mercaptan. The latter solution is titrated with silver nitrate. The sulfhydryl groups remove the silver ions, keeping the current close to zero, until no free sulfhydryl remains. With the addition of further silver ions, the current rises proportionately with silver ion concentration, marking the end of the titration.

The mercury-mercuric iodide reference cell (I) was assembled just as described by Kolthoff and Harris. The composition of the titration cell (II) was adapted in various ways to meet our special needs.

Vessel I contains in solution over a pool of mercury, 4.2 gm. of potassium iodide and 1.3 gm. of mercuric iodide in 100 ml. of saturated potassium chloride. The salt bridge is formed of a rubber tube filled with saturated KCl, connected with a glass tube containing 30 per cent KCl in 3 per cent agar gel, and closed by a sintered glass membrane. This in turn connects with a second such tube, filled in part with 30 per

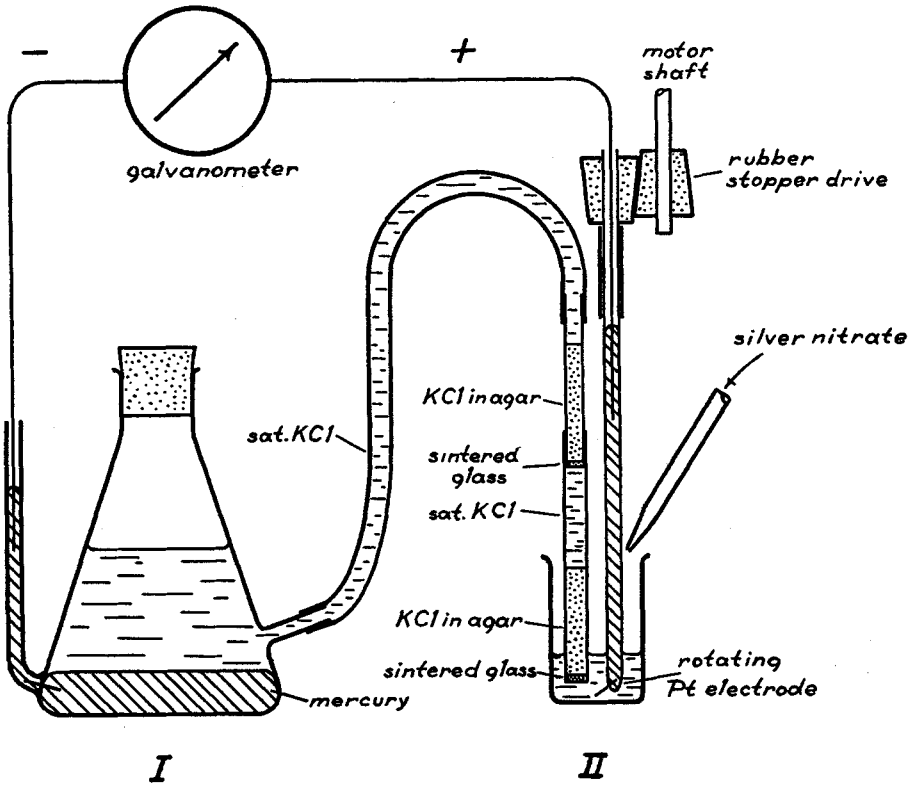


FIG. 6. Assembly for the amperometric silver titration of sulfhydryl groups, modified from Kolthoff and Harris (1946). Vessel I is the reference mercury-mercuric iodide half-cell. This is connected through a salt bridge with the titration vessel (II), in which a rotating platinum electrode is in contact with an ammoniacal solution of a mercaptan. The latter is titrated with silver nitrate. When all —SH groups have been removed by combination with silver ions, the addition of further silver nitrate causes a flow of current proportional in strength to the silver ion concentration.

cent KCl, and ending in a second plug of KCl in agar and a sintered glass membrane. The point of this construction is that the final section of the bridge can be refilled from time to time without dismantling the rest of the assembly.

In the original arrangement of Kolthoff and Harris, vessel II contains about 5 mg. of mercaptan sulfur in 100 ml. of an ethyl alcohol–water mixture, made about

0.25 M in ammonia, and 0.01 to 0.1 M in such a non-interfering electrolyte as ammonium nitrate. This is titrated with 0.005 M silver nitrate solution.

Our modifications of the procedure all involved vessel II. They included: (1) The titration was performed in a total volume of only 30 ml. (2) The silver nitrate soluused in the titration was 0.001 M. (3) In titrating rhodopsin, it was necessary to avoid the use of ethyl alcohol, which readily destroys this pigment. We found no difficulty in performing these titrations in aqueous solution. Benesch and Benesch (1948) also report the successful titration of a protein in water solution. (4) To keep rhodopsin or opsin in water solution we have had to use 1 to 2 per cent digitonin. Control measurements with cysteine and glutathione in digitonin solution showed that the presence of the detergent does not interfere with the titration.

In every instance our titrations were checked with control measurements on cysteine or glutathione. It was only after we were sure that the procedure yields accurate results with one of these molecules, that it was applied to rhodopsin or opsin; and check titrations with glutathione were carried out repeatedly in direct association with each of our experiments. Glutathione proved altogether to be a much more reliable check reagent than cysteine. With the latter, the end-point of the titration usually was not sharp, the base-amperage rising gradually throughout the titration, and lower —SH values than expected theoretically were usually obtained.

As an example of the working of this procedure, the amperometric titration of glutathione is shown in Fig. 7. In this case a solution of 0.002 M glutathione was prepared in M/15 neutral phosphate buffer. 0.5 ml. of this was mixed with 1.5 ml. of ammonium nitrate solution (1 M) and 0.5 ml. of ammonia solution (2.5 M), and brought with water to a volume of 30 ml. This mixture was titrated amperometrically with 0.001 N silver nitrate solution, added in portions of 0.1 ml. When 0.8 ml. of silver nitrate had been added, the current was still zero. At 0.9 ml. silver nitrate, the current rose somewhat, and on further addition of silver ions it increased linearly. The extrapolation of this straight line portion of the titration back upon the base line yields a value of 0.93 ml. silver nitrate which had reacted with glutathione —SH. A perfect titration would have yielded the value 1.0 ml.

When enough excess silver ions had been added to establish a reasonable flow of current, 0.1 ml. of 0.001 M glutathione was run into the titration vessel. The current fell as an equivalent amount of silver ion was removed from solution. The addition of 0.1 ml. of 0.001 N silver nitrate solution brought the current back to its former value. This was done again and again, each time with the same result. The horizontal distances between either the upper or the lower cusps in Fig. 7 measure the silver ion equivalent of the —SH added; always 0.1 ml. of 0.001 N silver nitrate solution compensated for each addition of 0.1 ml. of 0.001 M glutathione.

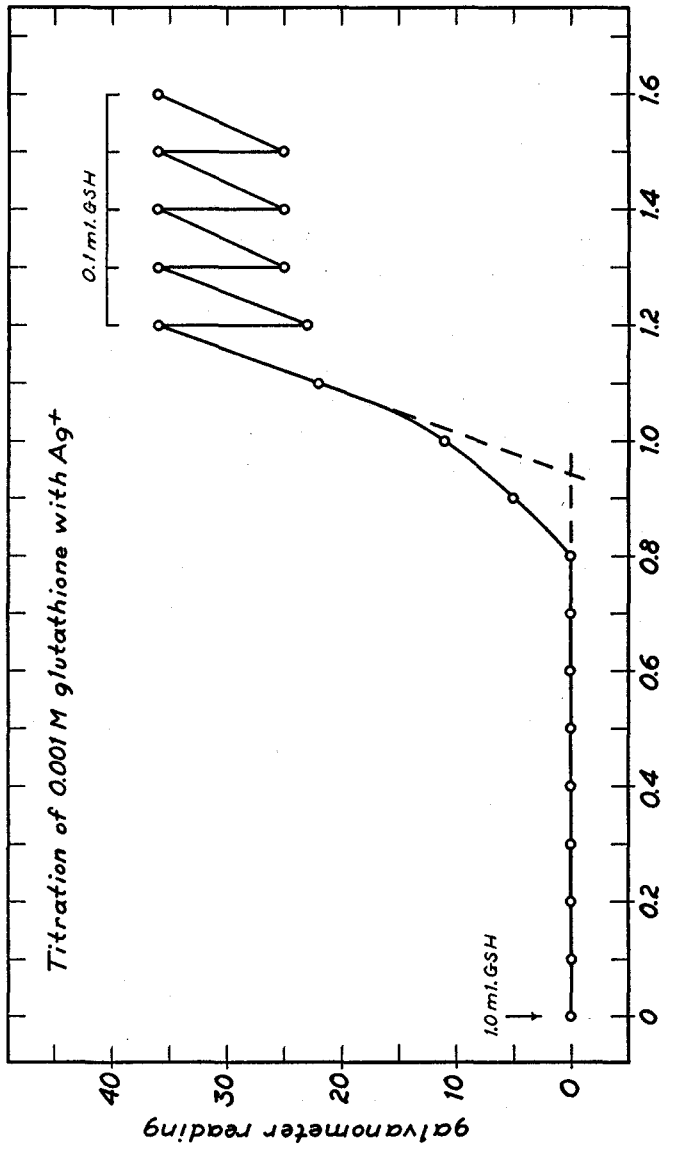


FIG. 7. Amperometric silver titration of glutathione. The titration was begun with 1 ml. of 10^{-3} M glutathione. This should have taken up 1 ml. of 10^{-3} M silver nitrate; the value obtained by extrapolating the straight-line portions of the titration is 0.93. After the initial titration was completed, 0.1 ml. portions of 10^{-3} M glutathione were added repeatedly. On each such addition the current fell, as the added —SH took up free silver ions; and each time it was brought back by the further addition of 0.1 ml. of 10^{-3} M silver nitrate.

As a further precaution, we have run check titrations with cystine in 1 per cent digitonin, and with retinene$_1$ in digitonin solution. In both cases blank titrations resulted; the current rose at once and linearly with the first addition of silver nitrate.

With these observations as background, we are prepared to understand the amperometric titration of rhodopsin itself. Such a titration, carried out with a preparation of cattle rhodopsin, is shown in Fig. 8.[4]

This preparation had an initial extinction at 500 mμ of 1.646. Two ml. of the rhodopsin solution were used in the titration, and the procedure was carried out in dim red light to avoid bleaching the pigment. Until 0.2 ml. of 0.001 N silver nitrate had been added, the current remained minimal. Then it rose linearly with further addition of silver ions; when 0.6 ml. of silver had been added the galvanometer read 42. At this point an intense white light was turned on the titration vessel. The rhodopsin was seen to bleach from pink to almost colorless, and as this happened the galvanometer reading dropped to 22. The bleaching of rhodopsin had liberated new sulfhydryl groups. The amount of —SH was equivalent to 0.19 ml. of the silver nitrate solution. When further silver nitrate was added, the amperage rose again, on a line parallel with that obtained previously. The horizontal distance between the cusp that marks the bleaching of rhodopsin and this new line again measures the —SH liberated on bleaching: 0.20 ml. -equivalent of silver nitrate solution.

The rhodopsin titrated in this instance was equivalent to 2 ml. of 5.1×10^{-5} M retinene$_1$ or 1.0×10^{-7} mols; *i.e.*, its bleaching yielded this amount of retinene$_1$. The sulfhydryl groups liberated in bleaching were equivalent to 0.2 ml. of 0.001 M sulfhydryl, or 2×10^{-7} mols of sulfhydryl. Therefore the bleaching of this preparation of rhodopsin yielded retinene$_1$ and —SH groups in the proportion 1:2.

We have obtained much the same result with a number of rhodopsin preparations from cattle, frogs, and squid. A representative series of such experiments with all three animals is shown in Fig. 9. In each case the titration of the rhodopsin solution was begun in red light. At the point indicated in the figure, the rhodopsin was bleached in bright white light. Invariably this was accompanied by a fall in current. The further addition of silver nitrate brought the current back to its former value and beyond.

The bleaching of rhodopsin under these circumstances is irreversible. The removal of the sulfhydryl groups of opsin by the formation of silver mercaptide poisons the resynthesis of rhodopsin just as does their removal by mercaptide formation with PCMB.

[4] In all such titrations, the rhodopsin was mixed with 1.5 ml. of ammonium nitrate solution (1 M) and 0.5 ml. of 2.5 N ammonium hydroxide made up to a total volume of 30 ml. with water. The final pH of the mixture was about 9.

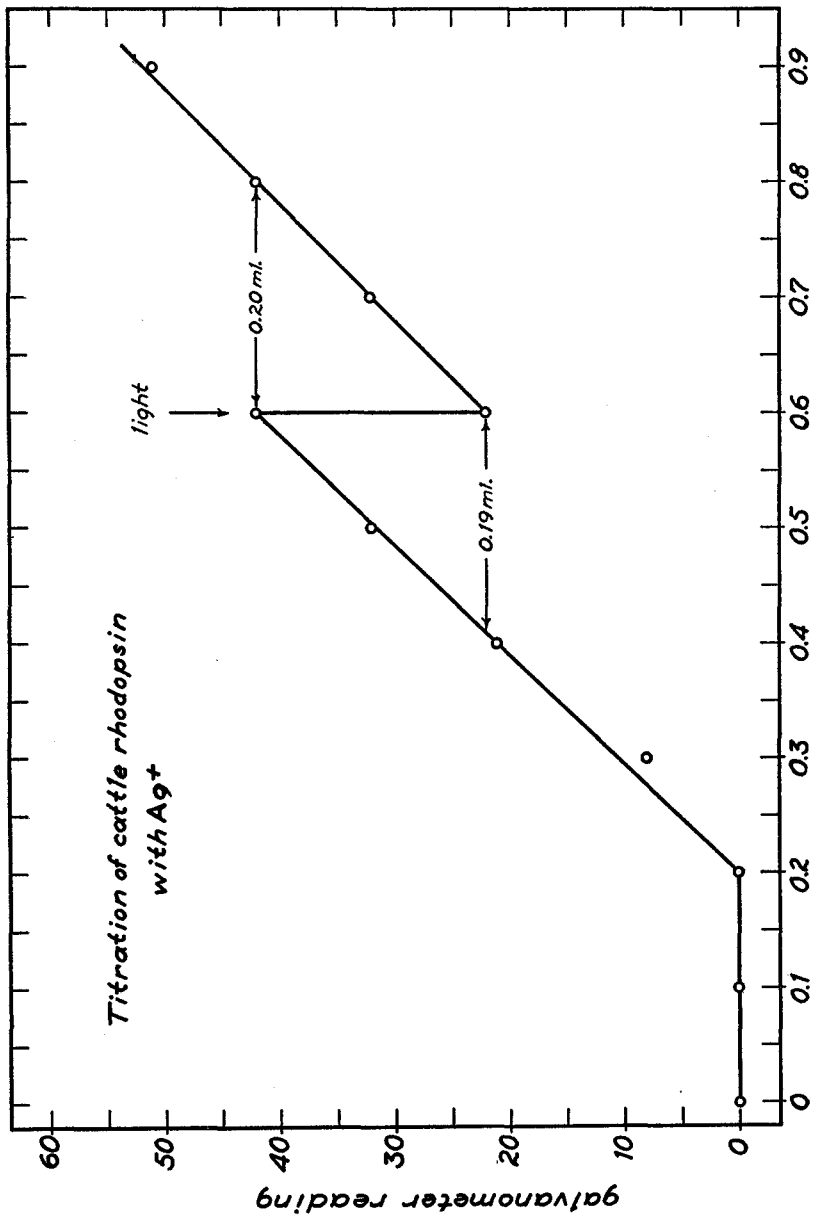


FIG. 8. Amperometric silver titration of rhodopsin. The titration was begun in dim red light to prevent the bleaching of the pigment. No current flowed until after 0.2 ml. of silver nitrate had been added; this equivalent of free sulfhydryl groups was present therefore initially. After a further 0.4 ml. of $AgNO_3$ had been added, producing a considerable flow of current, the rhodopsin was bleached in white light. The current immediately fell, as new —SH groups were exposed. Addition of more silver nitrate brought the current back to its former level and beyond. The horizontal distances marked on the figure measure the silver ion equivalent of the —SH liberated by bleaching. This corresponds to two —SH groups for each retinene$_1$ molecule that appears.

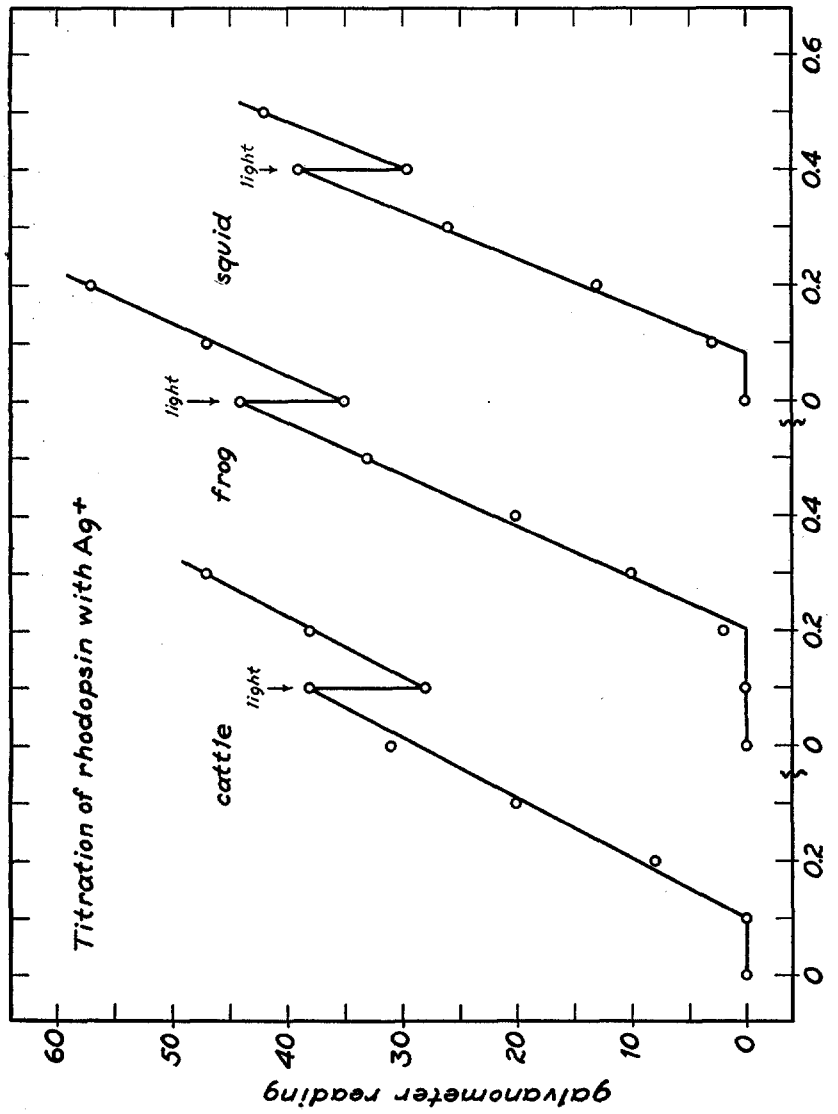


FIG. 9. The amperometric silver titration of rhodopsin preparations from cattle, frogs, and squid. The titrations were all begun in dim red light. In each case small amounts of silver ion were taken up by —SH groups present initially Then the current rose proportionately with the addition of further silver ions. In each case, bleaching of the rhodopsin with light caused a fall in the current, as new —SH groups appeared. On adding more silver nitrate, the current rose again. In all these preparations about two —SH groups were liberated for each retinene$_1$ molecule.

Each experiment of this kind yields an estimate of the number of sulfhydryl groups exposed per retinene$_1$ liberated in the bleaching of rhodopsin. All our measurements of this ratio are collected in Table I. In frog, cattle, and squid rhodopsin the result is essentially the same—*two* new —SH groups become titrable with silver for each molecule of retinene$_1$ that appears. The actual results vary between 1.8 and 2.4, this last figure obtained in one experiment with a squid preparation. The average for all the preparations is 2.1.[5]

TABLE I

The Liberation of Sulfhydryl Groups by the Bleaching of Rhodopsin

The retinene$_1$ content of the rhodopsin was estimated on the basis that a rhodopsin solution which in a 10 mm. layer has an extinction at 500 mμ ($K_{max.}$) of 1, has a potential retinene$_1$ content of 3.1×10^{-8} mols per ml. The —SH groups liberated by bleaching rhodopsin were estimated by the amperometric silver titration.

Source	Rhodopsin titrated	$K_{max.}$ of rhodopsin	Ratio, —SH: retinene$_1$	Date
	ml.			*1950*
Cattle (1)	1.0	1.646	2.0	Nov. 6
	0.5		1.8	
	0.5		1.8	
Cattle (2)	1.0	2.458	2.12	Dec. 6
Frog (1)	0.5	1.025	2.05	Nov. 15
	0.3		2.35	
Frog (2)	1.0	1.586	2.16	Nov. 22
	0.5		2.19	
Squid	1.0	0.940	2.4	Dec. 12
Average			2.1	

IV

Significance of the Observations

Bleaching and Denaturation.—Some years ago Mirsky (1936) suggested that the bleaching and resynthesis of rhodopsin might be regarded as a reversible denaturation. This seemed at the time a gratuitous view, unless the concept of denaturation were to embrace all reversible reactions of proteins; for none of the changes which regularly accompany denaturation had yet been shown to occur during the bleaching of rhodopsin (Wald, 1937–38).

[5] Bennett (1951) reports the histological demonstration of —SH groups in the outer segments of the rods of frogs, using a mercurial dye closely related to PCMB. It may prove possible with this reagent to show *in situ* that light-adapted rods contain a higher concentration of —SH groups than dark-adapted.

It is a curious fact nevertheless that the more we have come to know about the bleaching and resynthesis of rhodopsin, the more closely these phenomena have come to resemble protein denaturation and renaturation.[6] So, for example, the bleaching of rhodopsin by heat, in some ways a typical heat denaturation, has been shown to merge its properties imperceptibly with the bleaching induced by light (St. George, 1949; 1951–52). The bleaching of rhodopsin has been reported to shift the isoelectric point from 4.47 to 4.57 (Broda and Victor, 1940); such small displacements of isoelectric point in the alkaline direction occur commonly during protein denaturation. Rhodopsin, though it undergoes certain preliminary reactions when illuminated in the dry state, requires water to bleach (Wald, Durell, and St. George, 1950); the need for water in protein denaturation has been familiar since the work of Chick and Martin (1910). Rhodopsin regenerated *in solution* is usually slightly different in its properties from rhodopsin extracted from the retina (Collins and Morton, 1950; Wald, 1951); such differences are found regularly between native and renatured proteins. We have shown that the regeneration of rhodopsin from retinene$_1$ and opsin is a spontaneous, *i.e.* an energy-yielding reaction; it is the bleaching of rhodopsin that requires energy, usually furnished by light (Wald and Brown, 1950). Similar relations have recently been demonstrated in the reversible denaturation of a number of proteins (Anson and Mirsky, 1934; Kunitz, 1948–49; Eisenberg and Schwert, 1950–51). An equilibrium exists between native and denatured protein, which below a temperature specific for each protein, favors the native state. Below this critical temperature, denatured protein goes over spontaneously to the native condition, and conversely work must be done to denature the protein.

The present experiments add a significant element to this comparison, for such an increase in titrable sulfhydryl groups as accompanies the bleaching of rhodopsin is one of the most characteristic signs of protein denaturation.

The bleaching of rhodopsin therefore shares a number of important properties with denaturation, the synthesis of rhodopsin with renaturation. It can hardly be doubted that this is a meaningful relationship, and that some measure of clarification, both of the behavior of rhodopsin and of denaturation, may be derived from it.

Sulfhydryl Groups and the Structure of Rhodopsin.—The relationship just discussed draws attention to a curious issue. The —SH groups which appear in the course of protein denaturation are commonly believed to exist preformed in the native protein, though not accessible to reagents. One thinks of them as somehow folded into the protein structure, or perhaps engaged in weak types of linkage, *e.g.* hydrogen bonds—with other groups.

One may ask whether the —SH groups which emerge when rhodopsin

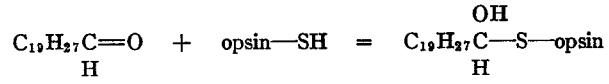
[6] All aspects of the chemistry of protein denaturation have lately been reviewed by Neurath, Greenstein, Putnam, and Erickson (1944) and by Anson (1945).

bleaches also are present in the intact pigment, and are merely "uncovered" by the dissociation of the prosthetic group. This seems to us improbable. If sulfhydryl groups played a passive role in rhodopsin, if they were only covered in its synthesis and uncovered in its bleaching, it is difficult to understand why the poisoning of —SH groups with PCMB or silver ions should block rhodopsin synthesis. Our observation that retinene$_1$ condenses readily with the —SH groups of cysteine and glutathione encourages the notion that such linkages are involved directly in binding retinene$_1$ to opsin (Wald and Brown, 1952–53). In all likelihood, sulfhydryl groups are literally removed in the synthesis of rhodopsin, and are regenerated when this pigment bleaches.[7]

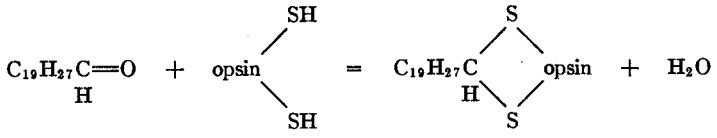
What then is their place in the structure of rhodopsin? We do not wish to deal with this problem here beyond the confines of a preliminary statement.

Sulfhydryl groups may take part in rhodopsin synthesis in either of two ways. They could be engaged directly in binding the prosthetic group to opsin; or alternatively they could yield hydrogen atoms for some reductive transformation of retinene$_1$ into the prosthetic group, simultaneously forming a disulfide (—S—S—) linkage. One could imagine also a combination of both types of reaction.

The simplest reaction which retinene$_1$ can undergo with the sulfhydryl groups of opsin is direct addition to form a hemithioacetal:



This product might condense with a second sulfhydryl group to yield a thioacetal:



[7] *Note Added in Proof.*—An added evidence that —SH groups of opsin are involved directly in binding the carotenoid prosthetic group, and are not merely "masked" by it, may be found in the fact that free silver ions in low concentration attack rhodopsin. We have noted in the text that concentrations of PCMB which poison the synthesis of rhodopsin do not attack the pigment once it is formed. We have since found that concentrations of free silver ion just sufficient to cause a flow of current in the amperometric titration (*i.e.*, higher than 0.5×10^{-5} M) slowly destroy rhodopsin. With rise in silver ion concentration, the attack is accelerated. At a concentration of free silver ions of 3×10^{-5} M, and at pH 9, about 40 per cent of the rhodopsin is destroyed in an hour at 25°C. This effect is too slow to interfere with the amperometric titration of sulfhydryl groups. The slowness of this reaction, however, together with its disruption of rhodopsin seems to us to argue that in this process silver ions attack linkages between certain sulfhydryl groups of opsin and the carotenoid chromophore.

The initial attachment of retinene$_1$ to opsin probably involves reactions of this nature. Something more than this, however, is required to yield the rhodopsin chromophore. Both the above formulations involve, without any compensatory change, the loss of the conjugated carbonyl group of retinene$_1$; and so produce, as we have shown with cysteine and glutathione, a shift of spectrum toward shorter wave lengths, just opposite in direction from the shift that accompanies rhodopsin synthesis.

The further developments which lead to the formation of the rhodopsin chromophore are still obscure. There is no want of theoretical possibilities, but one lacks as yet adequate information to choose among them.

Rhodopsin and Visual Excitation.—The bleaching of rhodopsin is a complex process. It is initiated by a light reaction, followed by dark changes, which bring it by way of the intermediate lumi- and meta-rhodopsin to a final mixture of retinene$_1$ and opsin (Wald, Durell, and St. George, 1950). Somehow this process, probably in its early stages, excites the rods; and this excitation, transmitted centrally from neurone to neurone, ends in the production of visual sensations.

At all levels of the optic pathways the nervous response is made manifest by electrical changes—action currents in the retina, optic nerve, and brain. Each of these structures in turn presents the fundamental problem of biological excitation—how a stimulus evokes the electrical changes which constitute the response.

When we set out to measure sulfhydryl groups in the present experiments, we chose the amperometric titration simply because it offered the best available procedure. There was no thought that the procedure itself would have an intrinsic interest for our problem. This method, however, exposes a phenomenon that is probably more significant than the measurements. As shown in Figs. 8 and 9, under the conditions of the amperometric titration, the bleaching of rhodopsin yields directly an electrical variation. In doing so it comes close, at least in a formal sense, to mimicking the excitation process.

The essence of this effect lies, not in the specific arrangements of the titration procedure, but in the liberation of sulfhydryl groups. The sulfhydryl group is the most reactive in biochemistry. In properly designed physical arrangements, all of its principal modes of reaction can be made to yield electrical effects. The —SH group is mildly acidic, and hence can affect the hydrogen ion potential; it is a strong reducing agent, and hence can change the oxidation-reduction potential; probably most important of all, it strongly binds certain metal ions, the property upon which our own measurements were based.

The liberation of these highly reactive groups by the stimulus, light, acting upon the receptor substance of rod vision, rhodopsin, is the crux of our observations. The means used in the present instance to turn this event into an

electrical effect is a purely arbitrary matter of physical organization. As already said, there exists a wide choice of such artificial arrangements, others of which we have already begun to explore.

What is needed now is to come closer to the particular physical arrangement within the rods that responds to the bleaching of rhodopsin with retinal potentials. This is a chemoanatomical problem, one that must fit rhodopsin in a meaningful way into the microstructure of the rods. If it is true that the liberation of sulfhydryl groups is the source of the retinal potential, the property of such groups that merits first consideration is probably their capacity to bind ions. Our own experiments rest upon this property; but from a physiological point of view we have worked with the wrong ion. Surely silver is not normally available in the rods, and in its presence rhodopsin cannot be resynthesized. One of our first tasks is to construct a system in which rhodopsin can bleach reversibly, and can give rise to reversible changes in the distribution of physiological ions.

Beyond this generalization of our immediate problem lies a further generalization. In principle the rhodopsin system differs from other excitation processes as encountered in other sense organs, nerves, and muscles, only in its sensitivity to light. No adequate theory of excitation exists as yet for any of these situations, yet certain ways of thinking about them have become habitual: ideas involving membranes, changes in permeability, and consequent upon these the transfer of ions into or out of the cell.

The main inference to be drawn from our observations is that without reference to membranes or permeability changes, the exposure of reactive groups in a receptor molecule by the action of the stimulus can itself evoke the redistribution of ions that generates the electrical response. In this sense our experiments with rhodopsin may provide a model for the excitation process in general.

SUMMARY

The condensation of retinene$_1$ with opsin to form rhodopsin is optimal at pH about 6, a pH which favors the condensation of retinene$_1$ with sulfhydryl rather than with amino groups. The synthesis of rhodopsin, though unaffected by the less powerful sulfhydryl reagents, monoiodoacetic acid and its amide, is inhibited completely by *p*-chloromercuribenzoate (PCMB). This inhibition is reversed in part by the addition of glutathione. PCMB does not attack rhodopsin itself, nor does it react with retinene$_1$. Its action in this system is confined to the —SH groups of opsin. Under some conditions the synthesis of rhodopsin is aided by the presence of such a sulfhydryl compound as glutathione, which helps to keep the —SH groups of opsin free and reduced.

By means of the amperometric silver titration of Kolthoff and Harris, it is shown that sulfhydryl groups are liberated in the bleaching of rhodopsin,

two such groups for each retinene$_1$ molecule that appears. This is true equally of rhodopsin from the retinas of cattle, frogs, and squid.

The exposure of new sulfhydryl groups adds an important element to the growing evidence that relates the bleaching of rhodopsin to protein denaturation. The place of sulfhydryl groups in the structure of rhodopsin is still uncertain. They may be concerned directly in binding the chromophore to opsin; or alternatively they may furnish hydrogen atoms for some reductive change by which the chromophore is formed from retinene$_1$.

In the amperometric silver titration, the bleaching of rhodopsin yields directly an electrical variation. This phenomenon may have some fundamental connection with the role of rhodopsin in visual excitation, and may provide a model of the excitation process in general.

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