Phototropy of New Photosensitive Complexes between a Fluorescent Pigment and SH-Compounds

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Introduction

Recently it has been recognized that the SH-group plays an important role not only in coenzyme-A and many enzymes, but also in the synthesis of the hormone and its activity, the coagulation of blood, the penetration of cellular membrane, the contraction of muscles, and cellular mitosis. It would be specially significant also in the reversibility of light energy conversion in vision and photosynthesis. On the other hand, there are many biological fluorescent pigments in the organism, such as porphyrin, carotenoids, flavin, and pteridine, which exhibit their physiological functions in close association with other biological substances such as protein and suitable oxidizing or reducing agents.

During the course of the investigation on the synthesis and preparation of organic fluorescent and photosensitive substances, their spectral structures, their abilities of emission, and their photosensitized and other photochemical reactions, the author noticed that the combination and interaction of these fluorescent pigments with some foreign substances possessing the SH-group responsible for the migration of excitation energy by molecular complex formation and electron transfer might be significant for their photosensitivity as light-sensitive substances. In confirmation of this point of view, it has been found in this laboratory that the complexes between a fluorescent pigment and SH-compounds selected as the reaction partner for the above mentioned reasons exhibit a specific photosensitivity.

It is well known that there are several photosensitive pigment-protein complexes in the retina of the eye[1]. One of them, visual purple or rhodopsin has the light absorption maximum at about 500 mμ. The absorption of light by this rhodopsin activates the pigment molecules and stimulates rod vision. Rhodopsin is bleached by light to retinene$_1$ and protein opsin, and is then regenerated from them in the dark. Recently, the liberation of SH-groups in the photobleaching of rhodopsin has been discovered by Wald and Brown[2], who ascertained the important role of SH-groups in the synthesis and bleaching of rhodopsin. As the author succeeded in fitting a fluorescent pigment into the SH-group of SH-protein or other SH-compounds

1) G. Wald, *J. Opt. Soc. Am.*, **41**, 949 (1951); *Federation Proc.*, **12**, 606 (1953); *Ann. Rev. Biochem.*, **22** 497 (1953).

2) G. Wald and P. K. Brown, *J. Gen. Physiol.*, **35**, 797 (1952); **37**, 189 (1953).

and since these pigment-SH compound complexes showed a phototropy analogous to this rhodopsin, the experimental results are described in this paper.

Experimental Methods

Materials.—The fluorescent pigment (I) used in this experiment was derived from fluorescein by the same method as described in the previous paper[3]. This yellow pigment showed a stable yellow fluorescence in a neutral aqueous solution. Hoffmann-La Roche reagent grade glutathione and Wako reagent grade egg albumin, cysteine, and thioglycolic acid were used.

On addition of this pigment solution to the alkaline solution of SH-compounds such as egg albumin, glutathione, cysteine, and thioglycolic acid, the yellow color disappeared with a slow appearance of an absorption in the longer wave length range, and its yellow fluorescence was partly quenched at the moment of the addition and then gradually deactivated as the reaction proceeded. These pigment-SH compound complexes were bleached by light and regenerated thermally in the dark. Since the experimental results were different in each complex, each case will be described separately below.

Measurements.—For the photobleaching reaction, the white light of a 1-KW projection lamp was allowed to fall upon a reaction cell of 2.5 cm. $\times$2.5 cm. $\times$3 cm. at a distance of 20 cm. from the light source. The variation of green light absorption during the formation reaction and regeneration reaction of the complexes was photoelectrically measured by the use of a 6-volt headlight lamp filtered through the Mazda glass filter V-G1 (maximum transmission at about 520 mμ) as a light source, at right angles to the irradiation source for photobleaching, and would be represented by the ratio of the initial value at the beginning of the reaction, I_i, to the respective measured value, I. The absorption spectra were measured by the Beckman spectrophotometer.

Experimental Results

(1) **Egg Albumin.**—When 2.5×10^{-4} M pigment (I) reacted with 1–7.5 % egg albumin in 0.1–3 N NaOH solution, its yellow color disappeared and a red color of the reaction product appeared gradually as shown in Fig. 1. Fig. 2 shows the absorption spectra of this pigment and the reaction complex. The absorption maximum of the yellow pigment at 453 mμ shifted to the longer wave length of 520 mμ, together with the deactivation of its fluorescence, which occurred instantaneously at the beginning of the reaction and continued to complete disappearance as the reaction proceeded.

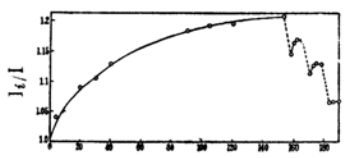


Fig. 1. Formation and phototropy of pigment-SH-protein complex. Dotted lines indicate bleaching during the illumination by light. 2.5×10^{-4} M pigment and 7.5 % egg albumin in 3 N NaOH.

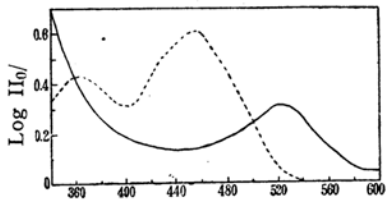


Fig. 2. Absorption spectra of pigment (I) and its complex with egg albumin:....., 10^{-4} M pigment in phosphate buffer solution at pH 7.0;—, 10^{-4} M pigment and 1 % egg albumin in 3 N NaOH.

This non-fluorescent red pigment-protein complex was bleached by light and regenerated rapidly within about two minutes at room temperature in darkness (Fig. 1). In this case, the more often the photobleaching was repeated, the lower the degree of regeneration became. The average initial regeneration degree was about 30, 15, and 8 %, respectively, to 7.5, 5 and 2.5 % of protein concentration; thus the regeneration degree was parallel to the protein concentration.

The addition of cysteine to this protein complex altered the color from red to orange. This fact seems to suggest the reversible formation of an orange pigment-cysteine complex; i, e., the exchange reaction of the SH-compound.

(2) **Glutathione.**—The absorption maximum of a complex produced by the reaction of glutathione with the pigment was at the same 520 mμ as in the csae of protein. In light this complex showed a deep color, in-

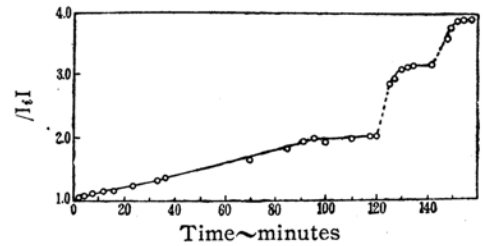


Fig. 3. Formation and phototropy of pigment-glutathione complex. 2.5×10^{-4} M pigment and 0.05 M glutathione in 3 N NaOH.

3) E. Fujimori, *J. Chem. Soc. Japan (Pure Chem. Sect.)*, 75, 24 (1954); *J. Am. Chem. Soc.*, in the press.

stead of bleaching, which changed towards regeneration in darkness (Fig. 3). This strange regeneration reaction was completed within about ten minutes at room temperature. Such a photochemical change to a deep color took place even in the case of cysteine and thioglycolic acid, but on account of its slowness the ordinary photobleaching and regeneration were observed on short irradiation, as will be mentioned below. The light absorption maximum before and after irradiation was at the same wave length, namely 520 mμ (Fig. 4).

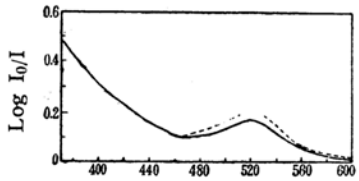


Fig. 4. Absorption spectra of pigment-glutathione complex:—, before irradiation of light;....., after irradiation and regeneration. 2.5×10^{-4} M pigment and 0.02 M glutathione in 1.2 N NaOH.

(3) **Cysteine.**—The reaction between the pigment and cysteine of high concentration in alkaline solution produced an orange complex whose absorption maximum was at 505 mμ (Fig. 6). It was found that the phototropy is repeated many times as shown in Fig. 5. As long as the irradiation time was short, the bleached product showed a pale yellow color which returned to the original orange color in the dark, but on repeating the illumination many times or irradiating it for a long time it changed to a pale red and in the dark converted into a deep red color whose absorption maximum gradually approached 520 mμ (Fig. 6). In both cases the regeneration degree was almost 100%. The initial orange complex fluoresced more weakly than the original pigment, while the red complex produced photochemically showed the almost complete disappearance of fluorescence as in protein complex. This color and phototropy remained unchanged by neutralization with hydrochloric acid.

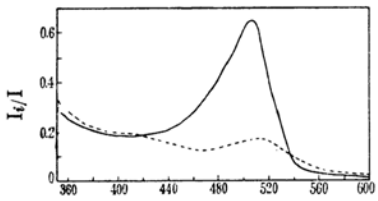


Fig. 6. Absorption spectra of pigment-cysteine complex:—, before irradiation of light;....., after irradiation and regeneration. 10^{-4} M pigment and 0.4 M cysteine-HCl in 1.2 N NaOH.

(4) **Thioglycolic Acid.**—Thioglycolic acid behaved in a manner analogous to that of

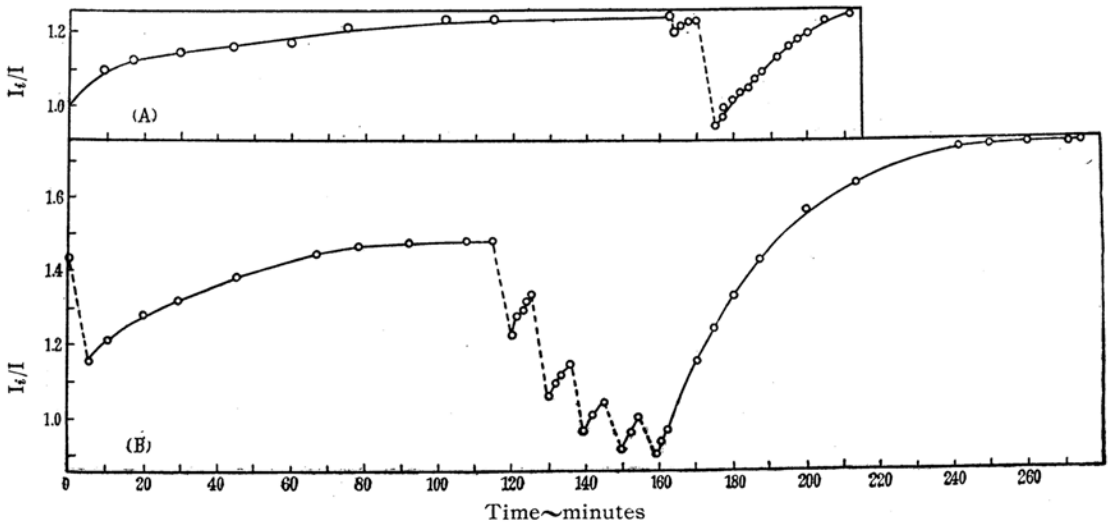


Fig. 5 (A) Formation and phototropy of pigment-cysteine complex. 2.5×10^{-4} M pigment and 1 M cysteine-HCl in 3 N NaOH.
(B) Phototropy followed after standing for about 15 hours from the terminal of Fig. 5A.

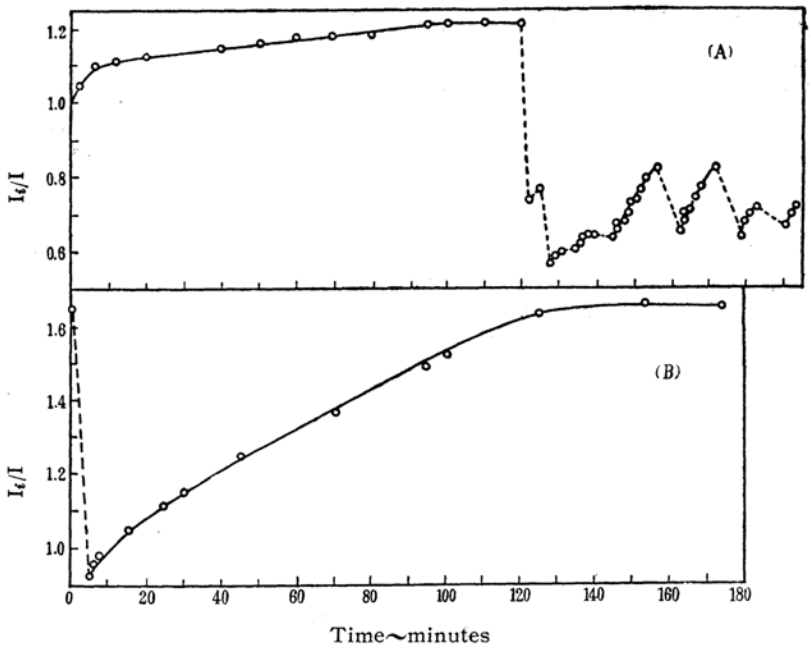


Fig. 7 (A) Formation and phototropy of pigment-thioglycollic acid complex. 2.5×10^{-4} M pigment and 1.5 M thioglycolic acid in 3 N NaOH.
(B) Phototropy followed after standing for about 15 hours from the terminal of Fig. 7A.

cysteine. The absorption maxima of pigment-thioglycollic acid complex were initially at 470 and 495 mμ, but later changed to 520 mμ on illumination (Fig. 8). Since this spectral shift to 520 mμ due to some photochemical reaction took place more easily than that of cysteine-complex, the same change to a deep color as in the case of glutathione was observed in the third irradiation of Fig. 7A. The phototropy was observed in both complexes of different spectra. The regeneration degree was 100 % and the regeneration time about two hours at room temperature, which was longer by about one hour than in the case of cysteine.

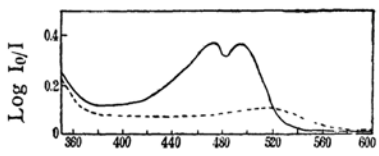


Fig. 8. Absorption spectra of pigment-thioglycolic acid complex:—, before irradiation of light;....., after irradiation and regeneration. 10^{-4} M pigment and 0.6 M thioglycolic acid in 1.2 N NaOH.

It was found, moreover, that this dark regeneration is generally a thermal reaction accelerated by heat. The relation between temperature and regeneration time in the case of thioglycolic acid is shown in Table I. Since the initial absorption maximum, the degree of regeneration, and the time required for regeneration are very different with each SH-compound, the summary of these experimental results is shown in Table II.

TABLE I
RELATION BETWEEN TEMPERATURE AND REGENERATION TIME AFTER IRRADIATION FOR FIVE MINUTES

Temp. (°C)	Reg. time (min.)
15	120
30	85
50	60

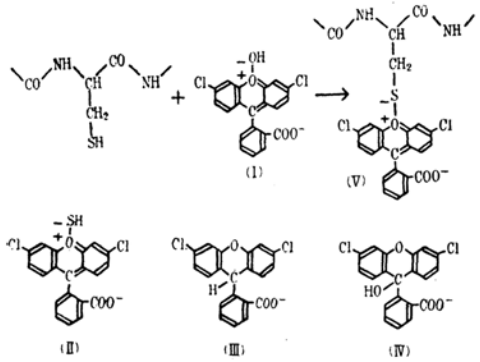
TABLE II
VARIOUS PROPERTIES OF PIGMENT-SH COMPOUND COMPLEXES

SH-compound	Absorp. Max. (mμ)	Reg. Deg. (%)	Reg. time* (min.)
Egg albumin	520	30–8	about 2
Glutathione	520	—	10
Cysteine	505→520	100	60
Thioglycolic acid	470. 495→520	100	120

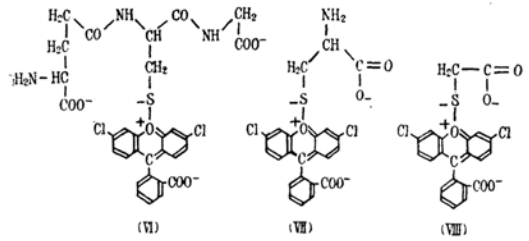
* Regeneration time at room temperature after irradiation for fiver minutes.

Discussion

The evidence that oxonium hydroxide of the pigment (I) reacts with SH-group in these reactions is obvious from the experimental facts that sodium sulfide also reacted to produce the non-fluorescent red compound (II) with the absorption maximum at 520 mμ and that such a reaction could not occur at all in the reduction product (III) and OH-transition product (IV) of the pigment (I). It is certain, therefore, that the reaction with protein should be represented by the following schema:



The reaction products with glutathione, cysteine, and thioglycolic acid would be (VI), (VII), and (VIII), respectively.



In egg albumin and glutathione, both of which are polypeptides, the absorption maximum is at 520 mμ from the beginning, while in cysteine and thioglycolic acid with free carboxyl group adjacent to the SH-group it is at a shorter wave length than 520 mμ and shifts to 520 mμ by light absorption. This fact seems to suggest some photochemical change of intramolecular orientation, namely photochemical isomerization, in these complexes and some effect of the adjacent free carboxyl ion upon this.

Since these red complicated complexes show the same absorption maximum at 520 mμ and non-fluorescence as in the more simple SH-pigment (II), the discussion described in the previous paper[3] will be true for the present complexes between a fluorescent pigment and complicated SH-compounds; thus the ionic combination of oxonium oxygen of the pigment (I) with SH-group, resulting in the formation of $-S^{-}-{}^{+}O=$ bond, would bring the remarkable deactivation of fluorescence and the shift of absorption spectrum into a longer wave length range. Moreover, the mechanism of this remarkable deactivation of fluorescence in these complexes will be analogous to that, in the SH-pigment (II), considered in connection with the instantaneous quenching of OH-pigment (I) fluorescence by SH-compounds; i. e., it may probably be due to the electron-transfer based on the reducing power of SH-group. Such a remarkable deactivation of fluorescence and a shift of absorption spectrum is likely to be observed in a static molecular complex formed in the ground state. In such a case, the transfer of excitation energy may sometimes bring about a specific photosensitivity. The present series of complexes seems to belong to this category.

Although (II) shows neither fluorescence nor photochemical change under irradiation, the pigment-SH compound complex is bleached in light, stays in some bleaching metastable state with the lifetime made longer by the specific effect of combined $^{-}$S-R, and is regenerated slowly to the original state in the dark as shown in the experiments. As such a bleaching state, a diradical and especially a semiquinone radical with an ·S-R free thiol radical may be of significance. If so, this phototropy of oxidizing oxonium pigment-reducing SH-compound complexes will be regarded as a sort of photo-reversible oxidation-reduction reaction where intramolecular electron transfer takes place through the diradical and semiquinone radical with the formation of ·S-R radical by light absorption.

The dark and light reactions of the pigment ant pigment-SH-compound complexes discussed above, including the previous results on the bleaching reaction of the pigment itself, will presumably be summarized by the following various model equations according to the above assumption:

1	$D—OH+h\nu_a \rightarrow D^*—OH$	Light absorption of the pigment
2	$D^*—OH \rightarrow D—OH+h\nu_f$	Fluorescence of the pigment
3	$D—OH \rightarrow HO—D$	Dark bleaching reaction of the pigment
4	$D^*—OH \rightarrow HO—D$	Light bleaching reaction of the pigment
5	$D^*—OH+{}^-S-R \rightarrow (\cdot D \cdot —OH+{}^-S-R) \rightarrow \cdot D^-—OH+\cdot S-R$	Quenching of the fluorescence
6	$\cdot D^-—OH+\cdot S-R \rightarrow D—OH+{}^-S-R$	Reverse reaction
7	$D—OH+{}^-S-R \rightarrow D—S—R_I+OH^-$	Formation of the complex
8	$D—S—R_I+h\nu_a' \rightarrow D^*—S—R_I$	Light absorption of the complex
9	$D^*—S—R_I \rightarrow \cdot D \cdot —S—R_I \rightarrow \cdot D^- \cdot S—R_I$	Bleaching reaction of the complex
10	$\cdot D^- \cdot S—R_I \rightarrow \cdot D \cdot —S—R_I \rightarrow D—S—R_I$	Regeneration reaction of the complex
11	$D^*—S—R_I \rightarrow D—S—R_{II}$	Photo-isomerization
12	$D—S—R_{II}+h\nu_a'' \rightarrow D^*—S—R_{II}$	Light absorption of the isomer
13	$D^*—S—R_{II} \rightarrow \cdot D \cdot —S—R_{II} \rightarrow \cdot D^- \cdot S—R_{II}$	Bleaching reaction of the isomer
14	$\cdot D^- \cdot S—R_{II} \rightarrow \cdot D \cdot —S—R_{II} \rightarrow D—S—R_{II}$	Regeneration reaction of the isomer
15	$D—S—R_I+{}^-S—R_{III} \rightarrow D—S—R_{III}+{}^-S—R_I$	Exchange reaction of SH-compound

where D-OH is the pigment (I); D*-OH, the normal excited state (singlet); HO-D, the colorless OH-transition product (IV); $^-$S-R, the negative ion of SH-compound; D-S-R_I, the initial complex; D-S-R_{II}, an isomer produced by light; $\cdot D \cdot$, a diradical (tiplet state); $\cdot D^-$, a semiquinone radical; and ·S-R, also a free radical. The complex between $\cdot D^-$ and ·S-R, $\cdot D^- \cdot$S-R, will be regarded especially as a complex diradical responsible for the bleaching metastable state with a longer lifetime and seems to be closely related to the diradical, $\cdot D \cdot$-S-R, so as to stabilize it and the diradical by forming a resonance complex.

Thus this phototropy seems to be based on the photochemical formation of pigment free radicals and ·S-R free radical, especially $\cdot D^- \cdot$S-R complex through $\cdot D \cdot$-S-R. Although the formation of ·S-R free thiol-radical was assumed by Waters[4] or Barron[5] in the function of SH-enzymes, e.g., FAD- or DPN-SH protein enzyme, and ascertained by Crawshaw and Speakman[6] in the photolysis of disulfides, there are several studies on pigment free radicals in the field of photochemistry. In 1940, the phototropy due to the formation of a semiquinone radical was studied by Rabinowitch[7] in thionine-ferrous ion system. Schenck[8] suggested the possibility of a diradical in vision, and Livingston[9),10] showed that the formation of a diradical and a semiquinone free radical in the illuminated solution of chlorophyll is the cause of its phototropy.

4) W. A. Waters, "The chemistry of free radicals", Clarendon Press, Oxford, (1946), P. 257–283.
5) E. S. G. Barron, *Advances in Enzymol.*, **11**, 201. (1951).
6) G. H. Crawshaw and J. B. Speakman, *J. Soc. Dyers and Colourists*, **70**, 81 (1954).
7) E. Rabinowitch, *J. Chem. Phys.*, **8**, 551, 560 (1940).
8) G. O. Schenck, *Naturw.* **40**, 212 (1953); *Z. Elektrochem.*, **57**, 675 (1953).
9) R. Livingston and V. A. Ryan, *J. Am. Chem. Soc.*, **75**, 2176 (1953).
10) R. Livingston, G. Porter and M. Windsor, *Nature*, **173**, 485 (1954).

Summary

The absorption maximum of all pigment-SH compound complexes shifted to a longer wave length range than that of the pigment itself. Although in protein and glutathione the absorption maximum was at 520 mμ from the beginning, in cysteine and thioglycolic acid it was in a shorter wave length range than 520 mμ and shifted to 520 mμ after some photochemical reaction. These complexes with their absorption maxima in a shorter wave length range than 520 mμ fluoresced more weakly than the original pigment, while those at 520 mμ showed the almost complete disappearance of fluorescence. Before these SH-compounds combined with the pigment, its fluorescence was partly instantaneously quenched by them. Both complexes of different spectra showed a phototropy; i.e., bleaching in light and regeneration in darkness. The regeneration reaction was thermal: its velocity was parallel to the molecular weight of the SH-compound in these SH-compounds used in the present experiment. The degree of regeneration was low in protein but about 100% in cysteine and thioglycolic acid. The concentration of protein influenced the degree of regeneration but not the velocity of regeneration. This phototropy was discussed from the point of view of an ·S-R free radical and pigment radicals.

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