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# Chlorophyll-photosensitized Reduction of Triphenyltetrazolium Chloride by Hydrazine Hydrate

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The fluorescence of chlorophyll a and b in methanol is gradually activated in the presence of hydrazine hydrate. The absorption spectrum of this reaction product between chlorophyll and hydrazine shifts to shorter wave lengths than that of chlorophyll itself. On the other hand, the fluorescence of chlorophyll and chlorophyll–hydrazine is efficiently quenched by triphenyltetrazolium chloride. Thus, chlorophyll can photosensitize the reduction of triphenyltetrazolium chloride by hydrazine, which occurs slowly even in the dark. This mechanism is discussed in connection with the primary quantum conversion of photosynthesis.

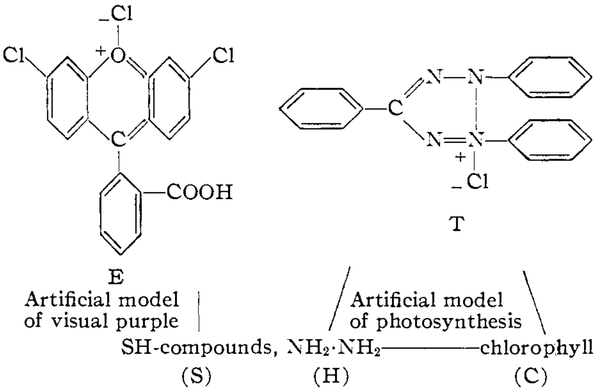
## Introduction

The main chlorophyll-photosensitized reaction, which has been studied,[1] is the autoxidation of benzidine, isoamylamine, allylthiourea, etc. Since the discovery of the Hill reaction of oxygen evolution caused by light in the presence of chloroplast and some suitable oxidizing agent, it has been supposed that in the chlorophyll-photosensitized decomposition of water *in vivo* hydrogen will be caught by some oxidizing agent, probably by TPN,[2] or more possibly by lipoic acid (6,8-thioctic acid),[3] with the production of oxygen, so that the hydrogen can be utilized for the reduction of fixed carbon dioxide. Therefore, studies on the chlorophyll-sensitized oxidation–reduction reaction involving some oxidizing agent other than oxygen seem necessary for the understanding of the primary photochemical reaction in photosynthesis.

The reaction between methyl red and phenylhydrazine studied by Ghosh and Sen-Gupta[4] and further developed by Livingston and co-workers,[5] and the reaction between riboflavin or DPN and phenylhydrazine or ascorbic acid studied by Krasnovskii and co-workers[6] belong to such a category. Unfortunately their conclusions are rather varied and the mechanism of the participation of chlorophyll in photosensitization still remains vague.

During the course of investigations on complicated organic fluorescent and photosensitive compounds, the author noted that in suitable complexes between a fluorescent molecule and certain other molecules, the specific transfer of excitation energy may introduce photosensitivity into the complex similar to that which occurs in vision and in photosynthesis.

An example is the artificial model of visual purple: the photosensitive complex between a fluorescent pigment (E) and SH-compound (S).[7] A further example, the artificial model of photosynthesis, is described here. This is the chlorophyll-(C)-photosensitized reduction of triphenyltetrazolium chloride (T) by hydrazine hydrate (H).



## Experimental Methods and Results

**Reagents.**—A mixture of chlorophyll a and b from potato leaves was extracted and purified according to the method of Zscheile and Comar.[8] Hydrazine hydrate was quantitatively determined by the iodine method.[9] Bayer reagent grade triphenyltetrazolium chloride was used.

**Measurements.**—The fluorescence intensity of chlorophyll solution excited by mercury lines at 3650 Å. was measured by the R.C.A. 931A photomultiplier through a Mazda V-R1 red glass filter. Absorption spectra were measured by the Beckman spectrophotometer. For the photosensitized reaction, the red light of a 200-watt tungsten lamp filtered through the Mazda V-R1 glass filter was allowed to fall upon the reaction cell of 2.5 × 2.5 × 2.5 cm. at a distance of 20 cm. from the light source. The appearance of red color from reduced triphenyltetrazolium chloride was photoelectrically measured by the use of a 6-volt head-light lamp with a Mazda V-G1 filter as a light source, at right angles to the irradiation source.

(1) **Activation of Chlorophyll Fluorescence by Hydrazine Hydrate.**—It was found that the fluorescence of chlorophyll is gradually elevated by

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(1) G. O. Schenck, *Angew. Chem.*, **64**, 2 (1952); *Z. Elektrochem.*, **56**, 855 (1952); *Naturwiss.*, **40**, 205, 229 (1953).

(2) W. Vishniac and S. Ochoa, *J. Biol. Chem.*, **195**, 75 (1952); **198**, 501 (1952).

(3) M. Calvin and J. A. Barltrop, THIS JOURNAL, **74**, 6153 (1952); M. Calvin, *Chem. Eng. News*, **31**, 1735, 3550 (1953).

(4) J. C. Ghosh and J. B. Sen-Gupta, *J. Indian Chem. Soc.*, **11**, 65 (1934).

(5) R. Livingston, D. Sickle and A. Uchiyama, *J. Phys. Colloid. Chem.*, **51**, 775 (1947); R. Livingston and R. Pariser, THIS JOURNAL, **70**, 1510 (1948).

(6) A. A. Krasnovskii, *Doklady Akad. Nauk.*, *S.S.S.R.*, **61**, 91 (1948); A. A. Krasnovskii and G. P. Brin, *ibid.*, **67**, 325 (1949); **73**, 1239 (1950); A. A. Krasnovskii and V. A. Gavrilova, *ibid.*, **81**, 1105 (1951); A. A. Krasnovskii and K. K. Voinovskaya, *ibid.*, **87**, 109 (1952).

(7) E. Fujimori, *Bull. Chem. Soc. Japan*, **28**, 334 (1955).

(8) F. P. Zscheile and C. L. Comar, *Botan. Gaz.*, **102**, 463 (1941).

(9) I. M. Kolthoff, THIS JOURNAL, **46**, 2009 (1924); W. C. Bray and E. J. Cuy, *ibid.*, **46**, 858 (1924).

the reducing agent, hydrazine hydrate, at room temperature. Figure 1 shows the experimental results obtained in a methanol solution of a mixture of chlorophyll a and b in the presence of hydrazine.

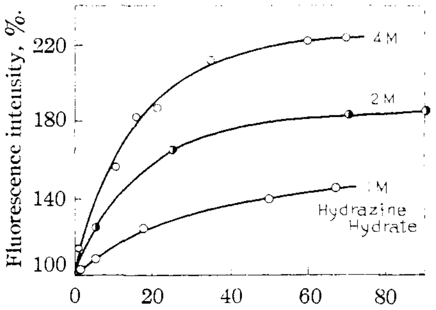

Fig. 1.—Activation of chlorophyll fluorescence by hydrazine hydrate as a function of time: $10^{-4}$ $M$ chlorophyll a and b methanol solution in the presence of various concentrations of hydrazine hydrate (20°).

The fact that the fluorescence increases rapidly in the dark with the increase of hydrazine concentration indicates the dark activation of fluorescence by a chemical reaction between chlorophyll and hydrazine. Since the concentration (H) of hydrazine is much higher than that of chlorophyll, this reaction is of the first order for chlorophyll. Assuming that the reaction responsible for the activation of fluorescence is bimolecular, the intensity of fluorescence as a function of time is represented by the equation

$$(\phi_{\max} - \phi) = (\phi_{\max} - \phi_0) \exp{[-k(\mathrm{H})t]} \qquad (1)$$

where $\phi_0$ is the initial intensity of fluorescence, $\phi_{\max}$ the final one, and $k$ the reaction rate constant. The experimental results are in considerable agreement with this equation. During the preparation of this paper, Weller and Livingston[10] published a study of the reaction of chlorophyll in the presence of amines, the results of which are in part comparable with the results of the present reaction. However, hydrazine hydrate will react not only with ring-V of chlorophyll, as Weller and Livingston described, but also with the phytol and methyl ester to produce a hydrazide and further with the formyl group, and with the magnesium atom to form a reversible complex, as suggested by Krasnovskii and Brin.[11] Actually, this chlorophyll hydrazine product was obtained as a water-soluble compound and studies on its purification and photochemical properties are in progress.

(2) **Change of Absorption Spectrum by the Reaction of Chlorophyll with Hydrazine Hydrate.**—This activation of fluorescence was accompanied by the change of absorption spectrum to shorter wave lengths than that of chlorophyll itself (Fig. 2). The absorption maxima of this fluorescence-activated complex were at 415 and 636 mμ in the case of chlorophyll a and b mixture. The spectral shift of red absorption maximum was larger than that of blue-violet maximum, but the change of absorption intensity was inverse. This shift of absorption maxima was analogous to that of the reaction products from amines and chlorophyll a, in which case the principal maxima were at 411 and 640 mμ in ether solution.[10]

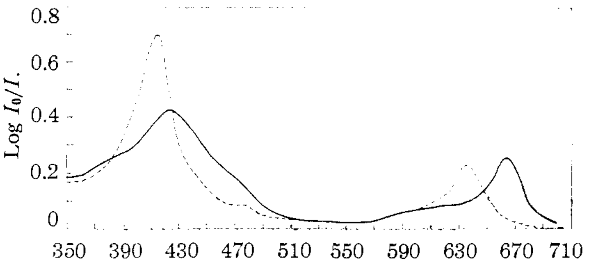

Fig. 2.—Absorption spectra of chlorophyll in the presence and absence of hydrazine hydrate: ——, $10^{-5}$ $M$ chlorophyll a and b mixture methanol solution in the absence of hydrazine; ------, in the presence of 0.5 $M$ hydrazine.

(3) **Quenching of Chlorophyll Fluorescence by Triphenyltetrazolium Chloride.**—The fluorescence of chlorophyll was found to be quenched efficiently by an oxidizing agent such as triphenyltetrazolium chloride (T) (Fig. 3) or an oxonium type of fluorescein derivative (E). In this case, however, the change of absorption spectrum was not observed. This suggests that the quenching of chlorophyll fluorescence by oxidizing agents having an oxonium chloride or ammonium chloride bond can be ascribed to the interaction only in the excited state of chlorophyll. This effect has been observed previously in a reducing fluorescent molecule, hydroquinone-2,5-disulfonic acid, and an oxidizing agent, nitrate ion.[12]

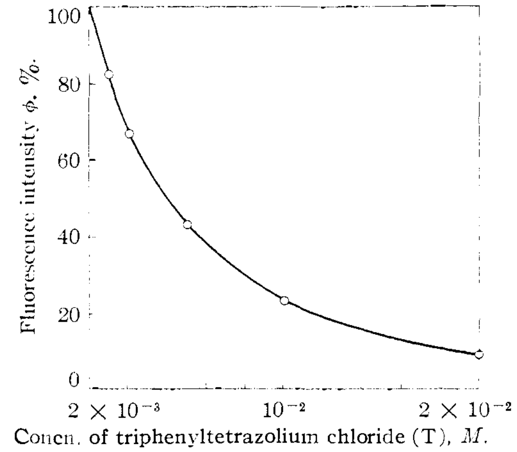

Fig. 3.—Quenching of chlorophyll fluorescence by triphenyltetrazolium chloride: $10^{-4}$ $M$ chlorophyll a and b methanol solution in the presence of various concentrations of triphenyltetrazolium chloride (20°).

The variation of the intensity, $\phi$, of the fluorescence as a function of the concentration (T) of triphenyltetrazolium chloride showed a positive deviation from the Stern–Volmer equation (Fig. 4)

$$\phi_0/\phi = 1 + k(\mathrm{T}) \qquad (2)$$

where $\phi_0$ is the intensity of fluorescence in the absence of the quencher and $k$ is called the quenching

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[10] A. Weller and R. Livingston, THIS JOURNAL, **76**, 1575 (1954).

[11] A. A. Krasnovskii and G. P. Brin, *Doklady Akad. Nauk., S.S.S.R.*, **89**, 527 (1952).

[12] E. Fujimori, *J. Chem. Soc. Japan, Pure Chem. Sect.*, **72**, 358 (1951).

constant. For such positive deviations the modified Stern–Volmer equation was offered by Rollefson and Boas[13] and used by Livingston and Ke[14];

$$\phi_0/\phi = 1 + k_1(\mathrm{T}) + k_2(\mathrm{T})^2 \qquad (3)$$

The plot of $[(\phi_0/\phi) - 1]/(\mathrm{T})$ against (T) showed a linear relation (Fig. 4). This quenching effect of triphenyltetrazolium chloride was more efficient than other quenchers studied by Krasnovskii, *et al.*,[15] or Livingston and Ke. Furthermore, the fluorescence of the more fluorescent complex between chlorophyll and hydrazine was also quenched by triphenyltetrazolium chloride.

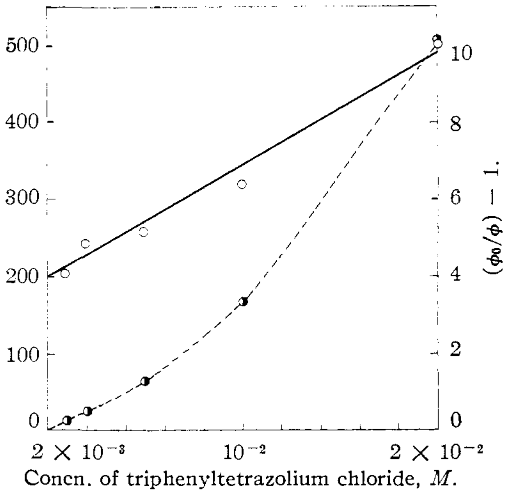

Fig. 4.—Plots of $[(\phi_0/\phi) - 1]/(\mathrm{T})$ (——) and $(\phi_0/\phi) - 1$ (------) *vs.* (T) for the quenching of chlorophyll fluorescence by triphenyltetrazolium chloride.

**(5) Chlorophyll-photosensitized Reduction of Triphenyltetrazolium Chloride by Hydrazine Hydrate.**—The foregoing experimental results indicate that these three compounds, *i.e.*, a photosensitizer chlorophyll, a reducing agent hydrazine, and an oxidizing agent T or E, are in close enough relation to interact with each other. It was also found that T can be slowly reduced by a large amount of hydrazine even in the dark. However, when the ternary system of T, hydrazine and chlorophyll was irradiated with orange-red light, the reaction between T and hydrazine was highly accelerated and showed a noticeable photosensitized reduction. In these experiments in air-saturated solutions, there was no observable induction period of the oxygen-inhibition originating in the chlorophyll-photosensitized autoxidation of hydrazine. This experimental result is shown in Fig. 5. Since the dark bleaching reaction with hydrazine occurred comparatively rapidly in the case of E, it was difficult to recognize this photosensitized reaction.

The water-soluble product, described in section (1), of the reaction between chlorophyll and hydrazine, the fluorescence of which was quenched by triphenyltetrazolium chloride, also could exhibit a similar remarkable photo-reducing power in the presence of hydrazine.

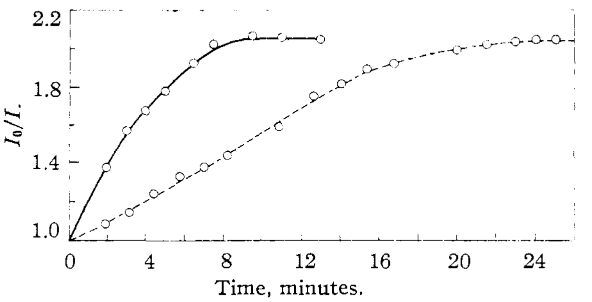

Fig. 5.—Chlorophyll-photosensitized reduction of triphenyltetrazolium chloride by hydrazine hydrate, followed by the variation of green light absorption during the reaction: $10^{-5}$ *M* chlorophyll a and b methanol solution in the presence of $10^{-4}$ *M* triphenyltetrazolium chloride and 3 *M* hydrazine hydrate (20°): ------, in darkness; ——, in light.

## Discussion

The foregoing experiments are still too preliminary to allow discussion of a detailed mechanism of this chlorophyll-photosensitized reaction but consideration of a possible mechanism would be of significance. The mechanism may be represented by the reactions

$$\mathrm{F} + \mathrm{N_2H_4} \longrightarrow \mathrm{F{-}N_2H_4} \qquad (4)$$

$$\mathrm{F}^* + \mathrm{T} \longrightarrow (\cdot\mathrm{F}\cdot + \mathrm{T}) \longrightarrow {}^{+}\mathrm{F}\cdot + \cdot\mathrm{T}^- \qquad (5)$$

$$^{+}\mathrm{F}\cdot{-}\mathrm{N_2H_4} \longrightarrow \mathrm{F}{-}\cdot\mathrm{N_2H_3} + \mathrm{H}^+ \qquad (6)$$

where F is chlorophyll; $N_2H_4$, hydrazine; T, triphenyltetrazolium chloride; $F{-}N_2H_4$, a chlorophyll–hydrazine complex; F*, an excited chlorophyll with an activated electron; ·F·, a diradical; $^{+}F\cdot$, a semiquinone radical with a missing electron; and $\cdot T^-$, a semiquinone radical with an accepted electron. Although the reaction (4) indicates a dark interaction between chlorophyll and hydrazine accompanying the activation of fluorescence and the shift of absorption spectrum, the intimate contact important for the photosensitized reaction will also be possible. According to the reaction (5), the light reaction between excited chlorophyll and triphenyltetrazolium chloride which is the cause of the quenching of chlorophyll fluorescence, the chlorophyll molecule absorbs one quantum, resulting in the activation of an electron to such a high energy level that it is easily extracted by a suitable oxidizing agent intimately related to the chlorophyll molecule. In such a case, there is the possibility that electron transfer would take place through the diradical ·F· induced from the normal excited state F* of chlorophyll. The photochemical formation of these diradical and semiquinone radicals was recently shown by Livingston,[16] *et al.*, in the phototropy of chlorophyll in fluid solutions and by Linschitz and Rennert[17] in the photochemical reaction between chlorophyll and quinone. On the other hand, such a quenching of chlorophyll fluorescence by oxidizing agents, such as benzoquinone and azo dyes, was also studied by Livingston and

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[13] G. K. Rollefson and H. Boas, *J. Phys. Colloid. Chem.*, **52**, 518 (1948); H. Boas and G. K. Rollefson, THIS JOURNAL, **72**, 3435 (1950).

[14] R. Livingston and Chun-Lin Ke, *ibid.*, **72**, 909 (1950).

[15] V. B. Evstigneev and A. A. Krasnovskii, *Doklady Akad. Nauk., S.S.S.R.*, **60**, 623 (1948); V. B. Evstigneev, V. A. Gavrilova and A. A. Krasnovskii, *ibid.*, **74**, 315 (1950).

[16] R. Livingston and V. A. Ryan, THIS JOURNAL, **75**, 2176 (1953); R. Livingston, G. Porter and M. Windsor, *Nature*, **173**, 485 (1954).

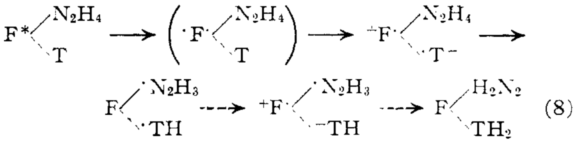
[17] H. Linschitz and T. Rennert, *ibid.*, **169**, 193 (1952).

Ke,[14] who presented a general quenching mechanism originating in electron transfer. This mechanism was supported by the author[12] in the combination of other reducing fluorescent molecules and oxidizing agents. Since this fluorescence quenching by T is observed even in the more fluorescent product between chlorophyll and hydrazine, the reaction (5) is modified as

$$F^{*}\text{—}N_2H_4 + T \longrightarrow (\cdot F^{\cdot}\text{—}N_2H_4 + T) \longrightarrow {}^{+}F^{\cdot}\text{—}N_2H_4 + \cdot T^{-} \quad (7)$$

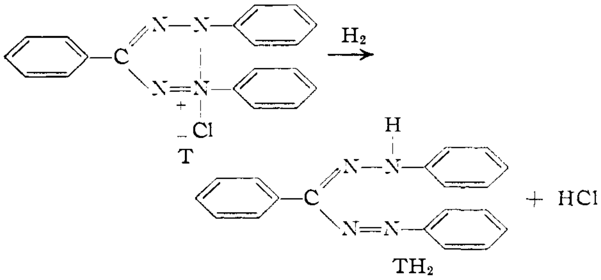
The reaction (6) means that chlorophyll, whose electron has been transferred to T, extracts an electron from a hydrazine molecule intimately attached to it. Whether or not chlorophyll exists intermediately as $^{+}F^{\cdot}$ in this scheme depends only on the time lag between the initial loss of an electron by the chlorophyll molecule and the subsequent recapture of an electron from a hydrazine molecule. If the time lag is so small that the new electron is acquired to replace the one removed from its normal energy level simultaneously with, or even before, the loss of the activated electron, the oxidative ionization of chlorophyll will not take place, or, inversely, the reductive ionization will take place. This seems to show the strange function of chlorophyll, which can act both as a reducing agent and an oxidizing agent. However, despite the evidence of Krasnovskii and co-workers[18] that chlorophyll is photoreduced by ascorbic acid and other reducing agents, chlorophyll is considered to be photochemically attacked more easily by oxidizing agents than by reducing agents from the point of view of fluorescence quenching.

The summation of the reactions (4), (5) or (7) and (6) is given by



$$F^{*}\begin{smallmatrix}N_2H_4\\ T\end{smallmatrix} \longrightarrow \left(\cdot F^{\cdot}\begin{smallmatrix}N_2H_4\\ T\end{smallmatrix}\right) \longrightarrow {}^{+}F^{\cdot}\begin{smallmatrix}N_2H_4\\ \cdot T^{-}\end{smallmatrix} \longrightarrow$$
$$F\begin{smallmatrix}\cdot N_2H_3\\ \cdot TH\end{smallmatrix} \dashrightarrow {}^{+}F^{\cdot}\begin{smallmatrix}\cdot N_2H_3\\ {}^{-}TH\end{smallmatrix} \dashrightarrow F\begin{smallmatrix}H_2N_2\\ TH_2\end{smallmatrix} \quad (8)$$

where $\dashrightarrow$ indicates the secondary reduction to $TH_2$. The relation between T and $TH_2$ is



This entire process (8) between excited chlorophyll–hydrazine complex and T may be regarded as a flow of electrons activated by light or as a photoelectric current flowing from hydrazine through chlorophyll to T. On the basis of the view that hydrogen will be transferred from hydrazine to T through the diradical and semiquinone radical of chlorophyll, the excited chlorophyll seems to play the role of an oxidation–reduction enzyme.

Whether the excited chlorophyll molecule is utilized for the activation of a reducing agent or for that of an oxidizing agent in photosynthesis has been an open question until recently. In 1947, Livingston[5] assumed that in a chlorophyll-photosensitized reaction a reducing agent could reduce the metastable chlorophyll molecule, yielding the reduced form able to reduce an oxidizing agent. Although this assumption has been supported by Krasnovskii and co-workers[6] since 1948, Livingston corrected it in 1948 to the formation of a complex between metastable chlorophyll and an oxidizing agent[5] and, in 1953, suggested the production of a metastable diradical and of a semiquinone radical by reaction with an oxidizing agent in the photobleaching and regeneration of chlorophyll in solution.[16]

On the contrary, considering the observation that phenylhydrazine reacts with chlorophyll, Watson[19] emphasized in 1952 the significance of a combination between chlorophyll and a reducing agent. According to the studies of Watson[19] or Livingston[20] phenylhydrazine is known to react with chlorophyll to produce a non-fluorescent complex, while in this present experiment, hydrazine hydrate was found to produce a more fluorescent complex by a dark reaction with chlorophyll. This made it possible to verify the quenching effect of oxidizing agents on the fluorescence of a complex between chlorophyll and a reducing agent, as well as on the fluorescence of chlorophyll itself. Thus, the light-activated chlorophyll molecule will transfer an electron to an oxidizing agent and extract it from a reducing agent intimately associated with chlorophyll.

In the primary quantum conversion and the subsequent reaction of photosynthesis, it is a very important question whether water, which seems able to bind with the hydrophilic portion of chlorophyll according to the experiments *in vitro* by Livingston[21] or Krasnovskii,[22] could directly participate as a hydrogen donor,[23] following transfer of an electron from excited chlorophyll to TPN or thioctic acid, in a similar manner as in this chlorophyll-photosensitized reduction of triphenyltetrazolium chloride by hydrazine hydrate. It is well known that some photosynthetic bacteria with bacteriochlorophyll can use hydrogen sulfide as a reducing agent. In this connection, the significance of SH-protein in photosynthesis may suggest the contribution of a complex between hydrophilic protein and chlorophyll–water complex to the photo-decomposition of water. Accordingly, the study of fluorescent pigment-SH protein complexes seems to be of importance.

CHIBA, JAPAN

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[18] A. A. Krasnovskii, *Doklady Akad. Nauk., S.S.S.R.*, **60**, 421 (1948); A. A. Krasnovskii and G. P. Brin, *ibid.*, **63**, 163 (1948); A. A. Krasnovskii, G. P. Brin and K. K. Voinovskaya, *ibid.*, **69**, 393 (1949); A. A. Krasnovskii and K. K. Voinovskaya, *ibid.*, **81**, 879 (1951); A. A. Krasnovskii and V. A. Gavrilova, *ibid.*, **81**, 1105 (1951).

[19] W. F. Watson, *Trans. Faraday Soc.*, **48**, 526 (1952).

[20] R. Livingston, "Photochemistry of Chlorophyll" in "Photosynthesis in Plants," Iowa State College Press, Madison, Iowa, 1949, pp. 179, 196.

[21] R. Livingston, W. F. Watson and J. McArdle, THIS JOURNAL, **71**, 1543 (1949); R. Livingston and S. Weil, *Nature*, **170**, 750 (1952).

[22] V. B. Evstigneev, V. A. Gavrilova and A. A. Krasnovskii, *Doklady Akad. Nauk., S.S.S.R.* **66**, 1133 (1949); **70**, 261 (1950).

[23] L. S. Levitt, *Science*, **118**, 696 (1953).