Chlorophyll-Sensitized Oxidation–Reduction Reactions of Hemin in Pyridine*

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ABSTRACT: In one of the photochemical reaction systems of photosynthesis (system I), the primary process is believed to involve an electron transfer between cytochrome and chlorophyll. As an *in vitro* model for this system, a study has been made of the photochemical reactions between chlorophyll a and hemin, in pyridine solution. A slow dark reduction, undergone by hemin in pyridine, is accelerated by the presence of chlorophyll, and both red and green light further increase the rate of the reaction. A mechanism proposed for this reaction is electron transfer from chlorophyll to hemin, with recovery of oxidized chlorophyll at the expense of solvent. Chlorophyll also sensitizes the photooxidation of reduced hemin in air.

The kinetics of this reaction are similar to those of other sensitized photooxidations, indicating a similar mechanism. Quenching of chlorophyll fluorescence by low concentrations of hemin or reduced hemin suggest the existence of a complex between chlorophyll and hemin.

While the exact nature of the participation of *c*-type cytochromes in photosynthesis is still in question, results obtained by many workers have been interpreted as evidence for their involvement in primary photochemical events (for reviews, see Clayton, 1965; Vernon and Ke, 1966). A current formulation of the role of cytochromes *in vivo* (see, for example, Clayton, 1965, p 50) is summarized below.

There are two distinct photochemical reaction systems in photosynthesis, referred to as I and II. Excitation of system II results in a sequence of electron transport reactions associated with the liberation of oxygen from water and the reduction of a *c*-type cytochrome. Excitation of system I brings about oxidation of a special form of chlorophyll a, P 700 (so called because of its absorption maximum near 700 nm). This initiates a series of oxidation–reduction reactions which result finally in the formation of reduced pyridine nucleotide. The *c*-type cytochrome is believed to couple systems I and II; in its reduced form, it transfers an electron to oxidized P 700, returning this special chlorophyll to its original state. Evidence for the existence of a cytochrome–P 700 complex has been presented (Chance and Devault, 1964).

Since cytochromes are proteins, they are insoluble in organic media in which chlorophyll is soluble. While it has, therefore, not been possible to study interactions between cytochromes and chlorophyll in true solution, some *in vitro* studies have been made with colloidal suspensions. Vernon and Shaw (1965) and Ke *et al.* (1965) have studied the photoreduction of cytochromes catalyzed by chlorophyll a. They worked in 95% ethanol or in aqueous systems containing Triton X-100, with added reduced trimethyl-*p*-benzoquinone ($TMQH_2$).[1] Krasnovsky (1955) and Vernon (1961) have also observed the chlorophyll-sensitized photooxidation of reduced cytochrome *c*, in air. In the same work, Vernon noted a photoreduction of cytochrome *c*, in the presence of chlorophyll, at the expense of an unknown electron donor.

As a model system for cytochrome–chlorophyll interactions in photosynthesis, we have studied solutions containing chlorophyll a and hemin in pyridine. The prosthetic group of cytochrome *c* is a derivative of hemin in which the vinyls are reduced because of cysteinyl substituents (Caughey, 1966). A dark reaction was found, in which chlorophyll promotes the reduction of hemin. Light absorbed by either pigment increases the rate of this reaction. It was also found that chlorophyll sensitizes the photooxidation of reduced hemin in air. Such reactions, involving chlorophyll and another porphyrin in true solution without added reagents, do not seem to have been reported previously.

Experimental Section

Materials. Crystalline chlorophyll a was prepared by a combination of methods (Anderson and Calvin, 1963; Jacobs *et al.*, 1954) described previously (Broyde and Brody, 1966). It was free of chlorophyll b and yellow pigments, as judged by its visible absorption spectrum. Hemin, twice recrystallized, was obtained from Sigma

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: $TMQH_2$ and TMQ, reduced and oxidized trimethyl-*p*-benzoquinone.

Biochemicals, St. Louis, Mo., and was stored at 0°. β-Carotene was obtained from Nutritional Biochemicals Co., Cleveland, Ohio. Spectroquality pyridine obtained from Matheson Coleman and Bell, East Rutherford, N. J., was employed without further purification. Prepurified nitrogen (99.997%) and prepurified hydrogen (99.95% pure) and oxygen–nitrogen mixtures (1, 5, and 10% oxygen) were also purchased from Matheson.

For experiments involving hemin, pyridine solutions of the pigment were freshly prepared from the commercial reagent, as needed. Reduced hemin in pyridine solution was prepared in the dark by hydrogenation with palladium as a catalyst. It was maintained in the reduced state under hydrogen atmosphere in the presence of palladium. Under these conditions the reduced hemin was stable for several hours. Our criterion of stability was the maintenance of constant spectral properties, in particular, an unchanging ratio of absorbance (2.0) of the α and β peaks of reduced hemin at 558 and 526 nm, respectively. Exposure of this stock solution to air was kept to the minimum involved in the transfer of an aliquot to the reaction vessel by means of a microliter syringe. Treatment of hemin with hydrogen did not reduce the vinyl groups, since the α and β bands of the mesoporphyrin are blue shifted to 546 and 516 nm (Alben *et al.*, 1968).

Procedures. The source for illumination was a slide projector equipped with a 500-W tungsten lamp. The sample was placed 25 cm from the projector lens. For studies involving white light, a Corning 3-73 glass filter (Corning Glass Co., Corning, N. Y.) was employed to eliminate ultraviolet light of wavelengths less than 400 nm. In addition, a heat filter, consisting of 5 cm of 0.1 M $CuSO_4$ solution, was placed before the sample. The energy incident on the sample was 7×10^5 ergs/cm^2 per sec as determined with a thermopile (Eppley Laboratories, Newport, R. I.) calibrated by the National Bureau of Standards. For measurements of the quantum yield of the photochemical reduction of hemin, nonmonochromatic bands of red or green light were employed (because monochromatic light, isolated by interference filters, was found to be too feeble). These bands were obtained by using Corning glass filters 2-60, and 4-71 plus 3-71, respectively. The wavelengths used for calculations of quantum yields were 671 nm for red light and 530 nm for green light, since these are the respective absorption maxima of chlorophyll a and hemin in pyridine. Based on these wavelengths, the energies incident on the sample were found to be 3.9×10^{-6} einstein/cm^2 per sec for red light and 1.7×10^{-6} einstein/cm^2 per sec for green light.

For quantum yield measurements of the photooxidation of reduced (hydrogenated) hemin, an interference filter (Farrand Optical Co., New York, N. Y.) with maximum transmission at 660 nm was employed. With this filter, the energy incident on the sample was 2.0×10^{-8} einstein/cm^2 per sec, assuming the weighted transmission of band pass to be at 660 nm.

The sample vessel was a rectangular silica absorption cuvet (Pyrocell Manufacturing Co., Westwood, N. J.) of 1-cm path length, with an extended neck. When the reaction was to be carried out in the presence of a gas mixture other than air, a set of hypodermic needles equipped with valves was used. The needles were inserted into a rubber stopper which fitted snugly in the ground-glass neck of the cuvet. Gas was bubbled through the reaction mixture, in the dark, for 15 min and then the valves were closed.

To analyze for reaction, absorption spectra were measured on a Cary Model 14R recording spectrophotometer. Concentrations of hemin [H] and reduced hemin [RH] were calculated using the following equations $OD_{(558)} = \epsilon_{(558)\mathrm{H}}[\mathrm{H}] + \epsilon_{(558)\mathrm{RH}}[\mathrm{RH}]$ and $OD_{(526)} = \epsilon_{(526)\mathrm{H}}[\mathrm{H}] + \epsilon_{(526)\mathrm{RH}}[\mathrm{RH}]$, where $\epsilon(\lambda)_{\mathrm{H}}$ and $\epsilon(\lambda)_{\mathrm{RH}}$ are the molar absorption coefficients of hemin and reduced hemin, respectively, at wavelength, λ, in nanometers. The values of ϵ shown below, and used in the above equations are based on ones given by Lemberg and Legge (1949): λ, 558 and 526; ϵ_{H}, 8.5 and 10.2×10^3 l./mole cm; and ϵ_{RH}, 32.4×10^3 and 16.2 l./mole cm. When chlorophyll was present, its contribution to optical density at 558 and 526 nm was subtracted from the measured values.

Chlorophyll concentration was determined from the optical density at 671 nm, using an extinction coefficient of 7.69×10^4 l./mole cm. Unless otherwise specified, photochemical reactions were excited by white light for 10 min and absorption spectra were measured before and after irradiation. Concurrent control experiments were made. For the study of the "reduction" of hemin in pyridine (see Discussion), concentrations of $3\text{–}4 \times 10^{-5}$ mole/l. were used for hemin and 1.8×10^{-5} mole/l. for chlorophyll, when present. For other types of experiments, concentrations of reagents are given in the Results section.

Fluorescence intensities and spectra were measured with a photofluorometric apparatus described elsewhere in detail (Brody *et al.*, 1965). Fluorescence emission was excited with the 436-nm line isolated from a low-pressure mercury arc lamp (General Electric H100-A4) by means of Corning glass filters 3-73 and 5-60. Fluorescence was both excited and examined from the front surface of the sample. The emitted light was passed through a monochromator to a Dumont type 6911 multiplier phototube. For quenching studies 20 ml of chlorophyll a solution was placed in a dewar which had an optically flat bottom; this served as sample holder. The optical path length through the solution was 1 cm. Stock solutions of concentrated hemin or reduced hemin were added in 10-μl aliquots. After each addition, the solutions were quickly stirred and fluorescence intensity was measured at 676 nm (the emission maximum of chlorophyll a in pyridine). Light was incident on the sample for only those few seconds during which intensity of fluorescence was being monitored. The volume added was, in total, less than 0.2 ml and one such experiment took 10–15 min. A correction was applied to the measured fluorescence intensities to take into account the decrease in fluorescence which arises from competitive absorption at 436 nm by hemin or reduced hemin. The correction is made with the equation (see, for example, Förster, 1951)

$$F = \frac{1 - 10^{-(\mathrm{OD_C}+\mathrm{OD_H})}}{1 - 10^{-\mathrm{OD_C}}} \frac{OD_{\mathrm{C}}}{OD_{\mathrm{C}} + OD_{\mathrm{H}}}$$

F represents the fraction of the original value to which the fluorescence intensity has been decreased through competitive absorption by hemin (or reduced hemin), and OD_C and OD_H are the optical densities of chlorophyll and hemin, respectively, at 436 nm. The optical densities of hemin or reduced hemin were calculated from the dilution factor of the stock solution, whose optical density had been determined.

For the fluorescence quenching study at 0°, the chlorophyll solution was placed in a small vial, to a depth of 1 cm. The vial was surrounded by coils, through which a liquid refrigerant at 0° was circulated by a Forma-Temp (Forma Scientific, Marietta, Ohio), Model 2095, constant-temperature bath. The vial was then placed in the dewar, and fluorescence intensities were measured, as at room temperature.

Results

Reduction of Hemin in Pyridine

Without Added Reagents. IN THE DARK. In pyridine (even in the absence of added reducing agent) there is a slow change in absorption in the green region of the spectrum, as has been noted previously (Rackow *et al.*, 1957). The diffuse absorption band of hemin is converted into two sharp bands with maxima at 526 and 558 nm; these strongly resemble the hemochromogen bands characteristic of chemically reduced hemin.

In Figure 1 may be seen plots of ln ($[H]_t/[H]_0$) as a function of time, in air (curve D), and under nitrogen (curve C), where $[H]_0$ and $[H]_t$ are hemin concentrations at times 0 and t. (In those cases where the systems were deoxygenated with nitrogen, timing begins after completion of bubbling.) Although, at first, both these curves are linear and rates of reaction are about the same, the rate soon decreases in air. The linearity of curve C indicates that the reaction is first order with respect to hemin concentration, with a first-order rate constant of 1.1×10^{-5}/sec.

UPON ILLUMINATION. It was observed that illumination with white light increases the rate of reaction under nitrogen notably. For example, in a 3.3×10^{-5} M hemin solution, 0.04×10^{-5} mole/l. of hemin was reduced in the dark in 20 min, while in light 0.24×10^{-5} mole/l. was reduced.

In the Presence of Chlorophyll a. IN THE DARK. The presence of chlorophyll a causes an increase in the rate of reduction of hemin. From Figure 1 it may be seen that the rate is still first order with respect to hemin concentration, with rate constants that are between three- and fourfold higher than in the absence of chlorophyll. The slopes are 3.7×10^{-5}/sec in air (curve B) and 4.1×10^{-5} per sec under nitrogen (curve A).

A study was also made of the rate of reaction under nitrogen, as a function of chlorophyll concentration. For these experiments an initial hemin concentration of 4.9×10^{-5} mole/l. was used. Observations made after 3 hr revealed that, at most, 20% of the original hemin had been converted. The data are given in Table I.

In the absence of chlorophyll a, a rate of 0.14×10^{-9} mole/l. per sec was found, and the rates given in Table I have been adjusted by subtracting this value. Since the rate varies linearly with increasing chlorophyll concentration, the reaction is also first order with respect to chlorophyll concentration. A value of 3.7×10^{-5}/sec was obtained for the slope of this line.

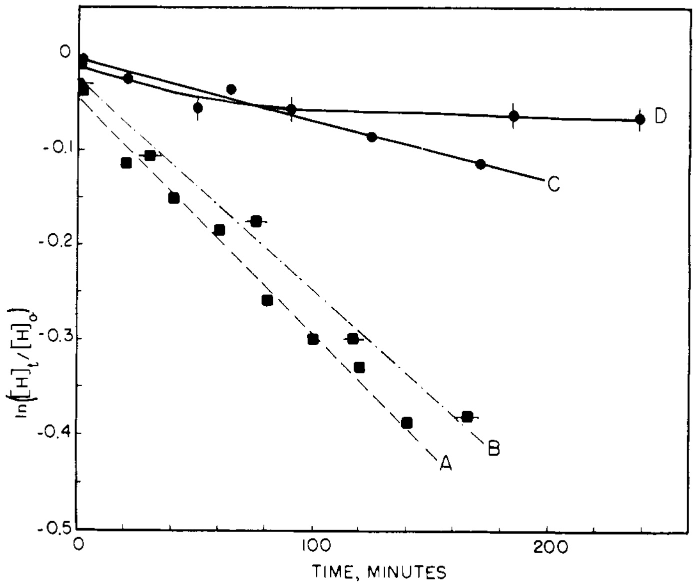


FIGURE 1: Rate of reduction of hemin in pyridine solution, in the dark. $[H]_0$ is the initial hemin concentration and $[H]_t$ is the hemin concentration at a specific elapsed time. Curve A: in the presence of 1.8×10^{-5} mole/l. of chlorophyll a, under nitrogen atmosphere; $[H]_0$ 3.3×10^{-5} mole/l. Curve B: in the presence of 1.8×10^{-5} mole/l. of chlorophyll a, in air; $[H]_0$ 3.0×10^{-5} mole/l. Curve C: under nitrogen atmosphere; $[H]_0$ 3.9×10^{-5} mole/l. Curve D: in air; $[H]_0$ 4.0×10^{-5} mole/l.

The time-dependent transformations in the absorption spectrum of hemin, which occur in the presence of chlorophyll a, may be seen in Figure 2. An isosbestic point occurs at 570 nm. It should also be noted that absorption by chlorophyll a remains constant.

UPON ILLUMINATION. The reduction of hemin in the presence of chlorophyll a is further accelerated upon irradiation. Under nitrogen, the rate in white light was five times greater than in the dark. In order to determine whether chlorophyll or hemin, or both are photochemically active in this reduction, quantum yields were measured in red and in green light. Samples were irradiated

TABLE I: Rate of Hemin Reduction as a Function of Chlorophyll Concentration.

Concn of Chl $\times 10^6$ (moles/l.)	Concn of Hemin Converted/sec, $\times 10^9$ (moles/l.)
2.0	0.04
6.4	0.25
7.4	0.22
15.6	0.52
18.1	0.56
25.0	0.93

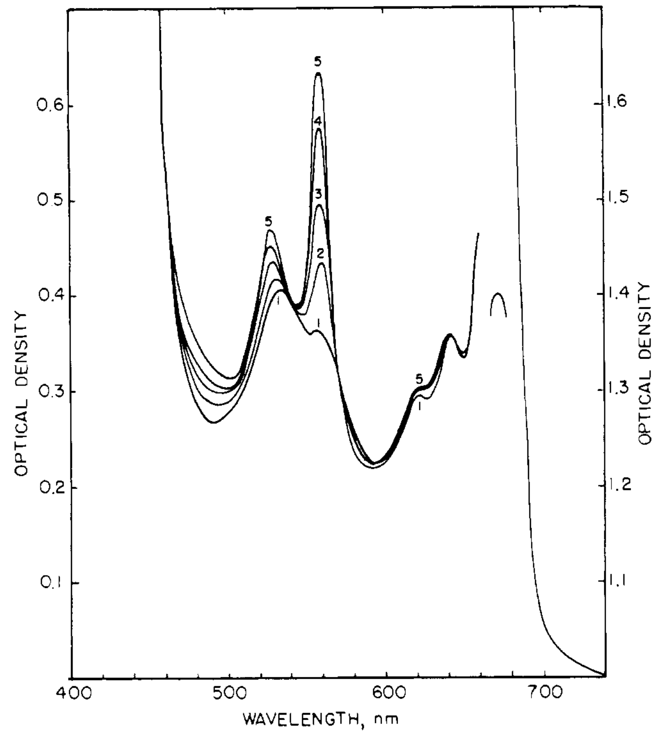


FIGURE 2: Spectral changes during reduction of hemin in pyridine in the presence of 1.8×10^{-5} mole/l. of chlorophyll a. Initial concentration of hemin, 3.3×10^{-5} mole/l. Curves 1–5 are, respectively, immediately after preparation, after bubbling 15 min with nitrogen, on standing in the dark for additional 20, 60, and 120 min. Right-hand ordinate applies to optical density at 671 nm (red absorption maximum of chlorophyll a).

for 30 min, under nitrogen atmosphere. Net quantum yields (light minus dark) for this reaction were found to be $3.1 \times 10^{-4} \pm 0.5$, for both red and green light. During the course of the reaction, the absorption spectrum of chlorophyll a remains virtually unchanged, both in the light and dark; see Figure 3 (curve D), points given by crosses (red light) and rectangles (dark). In the absence of hemin, chlorophyll in pyridine is also stable in the dark, especially under nitrogen (see curve C). However, in the absence of hemin and under irradiation with red light, chlorophyll fades under nitrogen (curve B), and even more rapidly in air (curve A).

If water is added to the reaction mixture so that the solvent is 20% aqueous pyridine, absorption by chlorophyll declines very rapidly upon illumination under nitrogen, reaching half its initial optical density in 20 min, even though hemin is present.

Photooxidation of Reduced Hemin in Air

Using Hemin Reduced in the Presence of Chlorophyll. If after reduction of hemin in the presence of chlorophyll, as described above, air is admitted and illumination with red or white light continues, the hemochromogen absorption bands decrease in intensity and there is a marked increase in absorption in the 620–640-nm region of the spectrum. These spectral changes are identical with the ones reported below and correspond to photooxidation of reduced hemin.

When a mixture of chlorophyll and hemin is irradiated in air, competition between reduction of hemin and oxidation of reduced hemin is observed. Under such conditions the hemochromogen bands begin to appear slowly, but after 25 min they decrease again, with attendant increased absorption near 640 nm, as described above. Under these conditions absorption by chlorophyll decreases to half its original value in 1 hr.

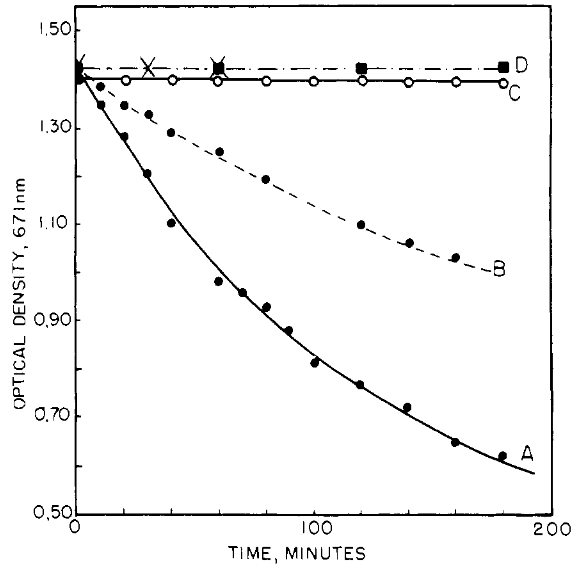


FIGURE 3: Optical density of chlorophyll a (1.8×10^{-5} mole/l.) in pyridine at 671 nm, as a function of time. Curves A and B: on irradiation with red light (660 nm) in air and under nitrogen, respectively. Curve C: in the dark, under nitrogen atmosphere. Curve D: in the presence of hemin, $[H]_0$ 3.0×10^{-5} mole/l. Rectangles: in the dark, under nitrogen atmosphere, crosses: on irradiation with red light, under nitrogen atmosphere.

Using Hemin Reduced by Hydrogenation. IN THE ABSENCE OF CHLOROPHYLL. Although reduced hemin, produced by hydrogenation, is unstable in air, the initial rate of oxidation is slow in the dark. For example, a 2.6×10^{-5} M solution of reduced hemin is stable in the first 10 min, and even after 30 min, only 8% oxidation was found. In the absence of chlorophyll as sensitizer, light does not accelerate the oxidation of reduced hemin.

IN THE PRESENCE OF CHLOROPHYLL. When chlorophyll is present, reduced hemin is even more stable. In comparison with the 8% reoxidation of reduced hemin cited above, a solution containing 1.6×10^{-5} mole/l. of chlorophyll a and 2.6×10^{-5} mole/l. of reduced hemin results in less than 0.5% reoxidation in the same 30-min interval.

Illumination with red or white light strongly accelerates the oxidation of reduced hemin, when chlorophyll is present. Green light was found to be relatively inactive in this reaction; its effectiveness was limited to the extent to which it was absorbed by chlorophyll.

In Figure 4 may be seen the time course of the spectral changes which occur in a solution containing 5.6×10^{-6} mole/l. of chlorophyll a and 1.7×10^{-5} mole/l. of reduced hemin, upon irradiation with white light, in air. This reaction is marked by a decrease in the sharp hemochromogen bands in the green region of the spectrum as well as an increase in absorption in the vicinity of 620–

640 nm. Absorption by chlorophyll remains unchanged. Again an isobestic point is seen to occur near 570 nm.

Kinetics of the chlorophyll-sensitized photooxidation of reduced hemin were studied as a function of various parameters. The relative quantum yield of this reaction, in air, as a function of reduced hemin concentration is given in Figure 5. The chlorophyll concentration was 6.0×10^{-6} mole/l. This curve is described by the empirical equation $\phi_1 = [RH]/(\beta + [RH])$. ϕ_1 is the relative quantum yield of the reaction, [RH] is the initial concentration of reduced hemin, and β, a constant chosen to give a good fit, is equal to 8.6×10^{-6} mole/l.

In Figure 6 is given the relative quantum yield of the reaction as a function of concentration of oxygen in solution. The initial concentrations of reduced hemin and chlorophyll were 1.7×10^{-5} and 4.4×10^{-6} mole per l., respectively. The solubility of oxygen in pyridine is approximately four times greater than that in water (Stephen and Stephen, 1963) or about 5×10^{-3} mole/l., at 298°K and 760 mm of oxygen. Oxygen concentration in solution was calculated by application of Henry's law. Measurements with more than 20% oxygen (air) was not possible, for under these conditions dark oxidation became significant. An empirical equation which describes the curve in Figure 6 is $\phi_2 = [O_2]/(\alpha + O_2)$, where $[O_2]$ is the oxygen concentration in solution and α is 3.5×10^{-5} mole/l. As can be seen from the figure, the rate of reaction is very low under nitrogen, and is sharply dependent upon oxygen concentration in the low concentration range. At 2.75×10^{-4} mole/l. of oxygen (obtained by flushing with 5% oxygen in nitrogen), the maximum rate is very nearly achieved.

In Figure 7 is given the actual quantum yield of the reaction in red light (660 nm), as a function of chlorophyll concentration. The initial concentration of reduced hemin was 3.0×10^{-5} mole/l. For our lowest chlorophyll concentration (1.9×10^{-6} mole/l.), a yield of 1.1×10^{-2} was obtained; the yield declines rapidly with chlorophyll concentration and approaches a value of 1.8×10^{-3}. The data are approximated by the empirical equation $\Phi = \gamma/(1 + \delta\,[\mathrm{Chl}])$ with γ equal to 2.5×10^{-2} and δ equal to 7.3×10^{5} l. per mole.

The relative rates of reaction were measured as a function of concentration of β-carotene, a known quencher of the chlorophyll triplet state (Fujimori and Livingston, 1957). Concentrations of chlorophyll and reduced hemin were 4.4×10^{-6} and 1.7×10^{-5} mole per l., respectively. For this study, red light of wavelengths greater than 600 nm (Corning glass filter 2-63) was used for excitation, in order to avoid the problem of competitive absorption by carotene. As may be seen in Figure 8, retardation of the reaction follows the Stern-Volmer equation (Förster, p 197, 1951) and the rate is reduced to half its initial value in the presence of 2.5×10^{-5} mole/l. of β-carotene. Because absorption by carotene obscures the 526-nm hemochromogen band, relative concentrations of hemin were determined from increase in absorption at 640 nm (see Figure 4).

Quenching of Chlorophyll a Fluorescence by Hemin and by Reduced Hemin.

The fluorescence of chlorophyll a in pyridine, in air, is quenched by both hemin and reduced hemin. Quenching follows the Stern-Volmer equation, as shown in Figure 9, in which F_0 and F_Q are, respectively, fluorescence intensities in the absence of quencher, and in its pres-

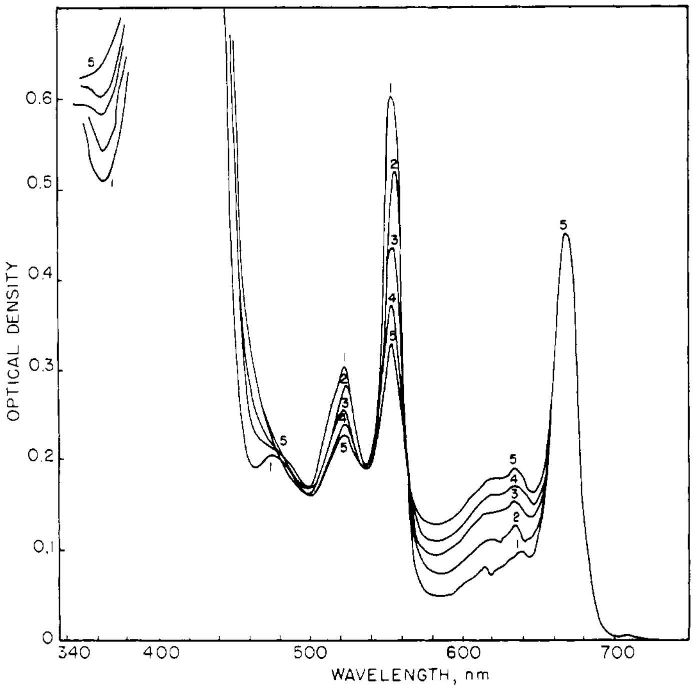


FIGURE 4: Spectral changes during chlorophyll-sensitized photooxidation of reduced hemin. Chlorophyll concentration 5.6×10^{-6} mole/l. Initial concentration of reduced hemin 1.7×10^{-5} mole/l. Curves 1-5 are, respectively, after 0-, 2-, 5.- 7-, and 10-min illumination with white light, in air.

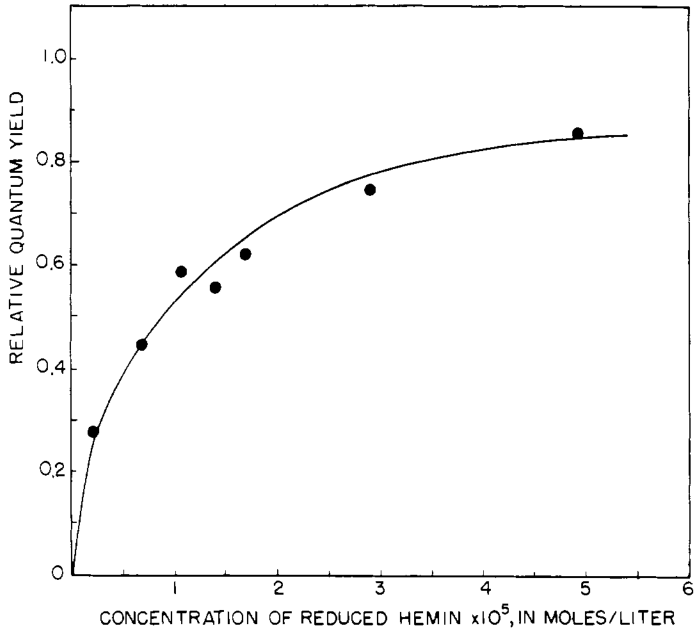


FIGURE 5: Relative quantum yield of chlorophyll-sensitized photooxidation of reduced hemin, in air, as a function of initial concentration of reduced hemin. Chlorophyll concentration 6.0×10^{-6} mole/l. Curve is plot of empirical equation given in text.

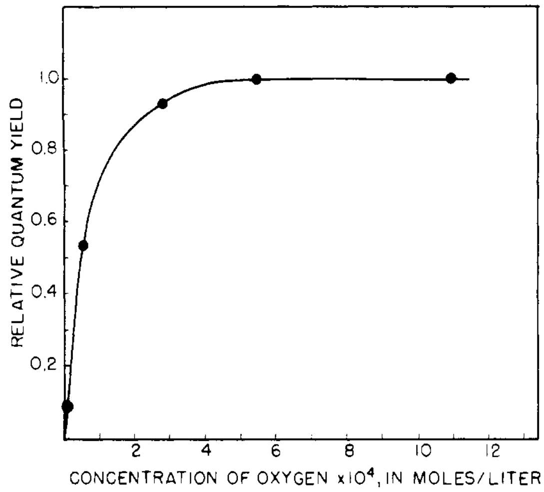


FIGURE 6: Relative quantum yield of photooxidation of reduced hemin as a function of concentration of oxygen in solution. Chlorophyll concentration 4.4 $\times$ 10^{-6} mole/l. Initial concentration of reduced hemin 1.7 $\times$ 10^{-5} mole/l. Curve is plot of empirical equation given in text.

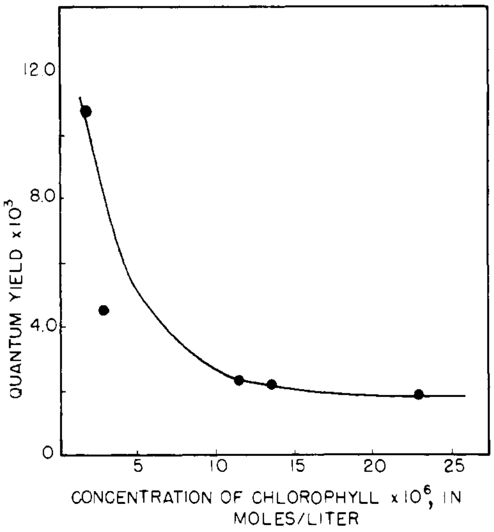


FIGURE 7: Quantum yield of chlorophyll-sensitized photooxidation of reduced hemin as a function of chlorophyll concentration, in air. Initial concentration of reduced hemin 3.0 $\times$ 10^{-5} mole/l. Curve is plot of empirical equation given in text.

ence at the concentration given on the abscissa. The quenching constant (the inverse of the concentration of quencher needed to reduce fluorescence intensity to one-half its original value) is 2.1 $\times$ 10^4 l./mole for hemin at both 298 and 273°K (curve A), and 1.9 $\times$ 10^4 l./mole for reduced hemin at 298°K (curve B). The difference between these two values may not be significant; within the range of experimental variation a single line could have been drawn.

The fluorescence of chlorophyll a in pyridine, in air, is undiminished upon standing in the dark, for the period of time required to carry out the fluorescence quenching measurements. In a control experiment, it was noted that following the immediate change in fluorescence produced by the addition of hemin or reduced hemin, no further changes in fluorescence intensity occurred, at least for the 30-min period of observation in this experiment. Hemin and reduced hemin do not fluoresce.

Because the observed quenching constants were so high, additional quenching experiments were made, in which techniques of measurement were varied, in order to determine if an artifact had caused chlorophyll fluorescence to appear diminished. Some measurements were made in which detection was at right angles to excitation. Others were made with detection in the same direction. In addition, systems with very low optical densities were examined (total optical density at 436 nm less than 0.1). In all cases, quenching was noted and the quenching constants were in reasonably good agreement with the value given above.

Quenching of chlorophyll fluorescence by hemin or reduced hemin is not accompanied by changes in the fluorescence spectrum of chlorophyll, or in the absorption spectra of either pigment in the region from 350 to 1000 nm. Even difference spectra (in which mixtures of chlorophyll a and hemin served as sample, and unmixed solutions in separate cuvets placed one behind the other, served as reference) revealed no absorption change.

Discussion

Formation of a Complex between Chlorophyll and Hemin. The fluorescence of chlorophyll a is very efficiently quenched by both hemin and reduced hemin. Because there are no time-dependent changes in intensity after the instantaneous quenching, it would appear that reaction between the pigments is not responsible the effect. Since the lifetime of chlorophyll fluorescence is about 10^{-8} sec (Brody and Rabinowitch, 1957) and encounter frequencies are of the order of 10^{10} for a 1 M solution at room temperature (Oster and Adelman, 1956), the observed high quenching constants (about two orders of magnitude larger than 10^2 l./mole) indicate that kinetic encounters alone are not responsible for the decreased fluorescence (Förster, p 209, 1951). On the other hand, the formation of a complex between chlorophyll and hemin is consistent with our observed high quenching constants. Chance and Devault (1964) have proposed the existence of a chlorophyll–cytochrome charge transfer complex *in vivo*.

The apparent absence of absorption changes and the absence of a temperature dependence for quenching in the 0–25° range indicate that the coupling between chlorophyll and hemin is weak.

Reduction of Hemin in Pyridine. SOLVENT AS REDUCING AGENT. The nature of the slow reaction which hemin undergoes in pyridine solution in the absence of added material is still a matter of controversy. The observed spectral changes are very much like those which accompany reduction of hemin by hydrogen, or other chemical means. The reaction has been interpreted as a reduction (Rackow *et al.*, 1957) either by solvent or by reducing impurity. If an impurity brought in with hemin were the reducing agent, the extent of reaction would be

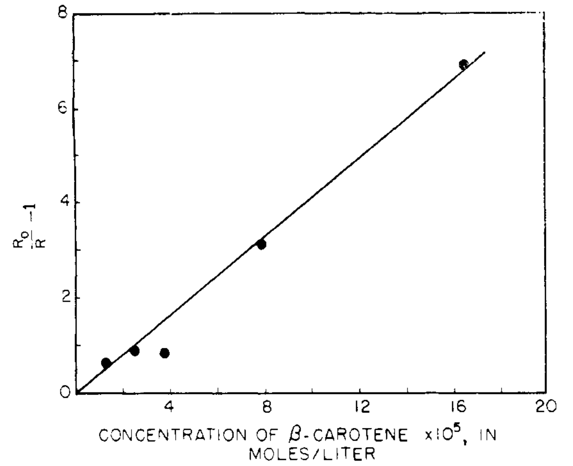


FIGURE 8: Relative rate of chlorophyll-sensitized photooxidation of reduced hemin as a function of concentration of β-carotene. Chlorophyll concentration 4.4×10^{-6} mole/l., initial concentration of reduced hemin 1.7×10^{-5} mole/l. R and R_0 are, respectively, the relative rates with and without β-carotene.

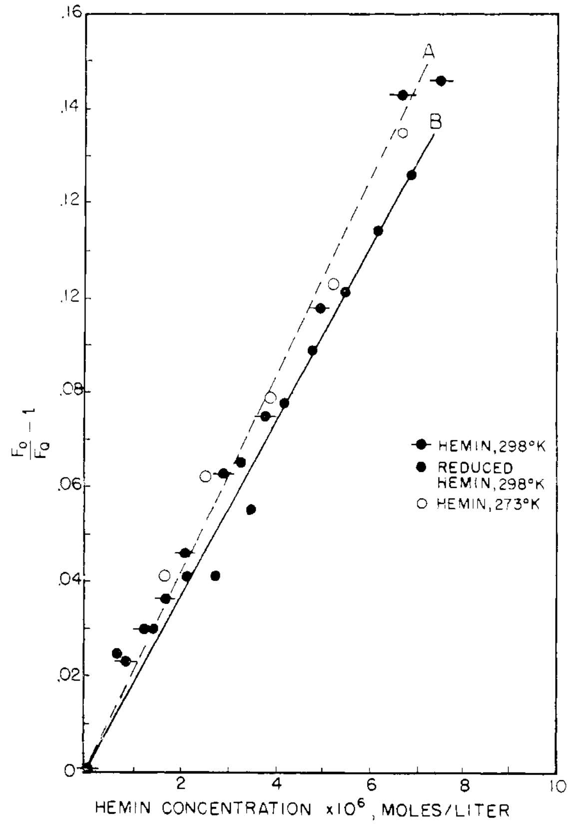


FIGURE 9: Stern–Volmer plot for fluorescence quenching of chlorophyll a by hemin (curve A at 298 and 273°K) and by reduced hemin (curve B, 298°K) in air. F_Q and F_0 are fluorescence intensities with and without quencher, respectively.

limited by impurity concentration which could constitute only a small fraction of that of the hemin. However, we have found the reaction can go to completion. Involvement of pyridine in the reaction is supported by the finding (W. Caughey and I. Cohen, personal communication) that 2,4-diacetyldeuterohemin dimethyl ester is not reduced in benzene, but it is in pyridine.

Our observation that this reaction is retarded in air and is no longer first order with respect to hemin concentration (see Figure 1, curve D) is also consistent with the interpretation that the reaction is a reduction, since accumulated reduced hemin is subject to reoxidation by air. For these reasons we have favored the reduction interpretation.

Light speeds up this reaction, even in the absence of chlorophyll, presumably by supplying activation energy.

NATURE OF THE PRODUCT WHEN CHLOROPHYLL IS PRESENT. The presence of chlorophyll a causes a large increase in rate of hemin conversion even in the dark. The product of this conversion is a single material, as revealed by the isosbestic point at 570 nm (Figure 2); it is also a reduced form of hemin, since its spectrum is again similar to the hemochromogens; in fact, the photooxidation of this material, in air, is sensitized by chlorophyll, and when the reduction of hemin is carried out in air and light, with chlorophyll present, photooxidation competes with reduction (Results).

ROLE OF CHLOROPHYLL. Besides the rate increase, there are other indications that chlorophyll participates in the reduction of hemin. For one, red light absorbed almost exclusively by chlorophyll, is as effective in promoting the reaction as green light. Secondly, both chlorophyll and reduced hemin are protected from oxidation, when in each other's presence. The enhanced stability of reduced hemin, in the dark, was shown by the first-order kinetics, even in air, when chlorophyll was present (see Figure 1; compare curves B and D); it was also noted directly (Results). Chlorophyll is stable in light, under nitrogen, when hemin is present, but it decomposes rapidly when hemin is absent. These stabilities of chlorophyll and hemin can be understood if chlorophyll is participating in reduction of hemin, thereby inhibiting competing oxidations (by air or by traces of oxygen in the nitrogen).

The reduction of hemin in pyridine, in the presence of chlorophyll, is analogous to a reaction reported by Ashkinazi *et al.* (1955, 1956) and Ashkinazi and Kryukov (1957) who found that ferric complexes of chlorophyll derivatives could be photoreduced by solvent, and that the rate of reaction was increased by reductants which formed complexes with the iron of the chlorophyll derivative. The mechanism here is believed to be electron transfer from reductant to iron. Subsequent oxidation of the solvent brings about recovery of the reductant. An analogous mechanism for our system would be electron transfer from chlorophyll to hemin in a complex, followed by recovery of the oxidized chlorophyll to its original state by reaction with pyridine. Such a mechanism would be consistent with the first-order kinetics in respect to both hemin and chlorophyll, as well as with the constant absorption properties of chlorophyll during the reaction. On the basis of potentiometric studies, Krasnovsky and Brin (1965) have also suggested that pyridine can act as a reductant.

Other mechanisms, consistent with the first-order rate dependences are also possible. One is electron transfer from chlorophyll to hemin, followed by reaction between two chlorophyll radicals, to produce one oxidized and one recovered chlorophyll. This explanation requires that the oxidized chlorophyll have the same absorption properties as chlorophyll itself. While such oxidation products are known, for example, one of the "allomerized" chlorophylls (Holt and Jacobs, 1954), oxidation usually leads to spectral changes.

For the chlorophyll-sensitized photoreduction of cytochromes in detergent-containing aqueous systems (Vernon and Shaw, 1965; Ke *et al.*, 1965), the mechanism is believed to be an initial electron transfer from the excited chlorophyll to TMQ (present as an impurity in the added $TMQH_2$ or formed during the reaction) to produce a semiquinone, which then reduces cytochrome. The oxidized chlorophyll regains its electron from $TMQH_2$. Since no added reductant is present in our system, an analogous mechanism would require pyridine to act in the role of TMQ and $TMQH_2$, as both the oxidant and reductant; this dual role seems less likely for pyridine.

EFFECT OF WATER. Water which inhibits the interaction between hemin and chlorophyll may do so by denying one pigment access to the other; although neither is water soluble, they both have hydrophyllic groups. In the presence of water the pigments are colloidally dispersed and apparently are shielded from each other.

Chlorophyll-Sensitized Photooxidation of Reduced Hemin. NATURE OF THE PRODUCT. Reduced hemin is oxidized to a product with a new absorption band in the vicinity of 640 nm (Figure 4). Such a band is characteristic of hemin and other iron(III) porphyrins under certain circumstances (Caughey, 1966; Havemann and Halberditzl, 1958; Braterman *et al.*, 1964; Mauzerall, 1965).

REACTION MECHANISMS. The kinetics of the chlorophyll-sensitized photooxidation of reduced hemin are similar to those of the sensitized photooxidation of other substrates by chlorophyll (for review, see Seeley, 1966) and by other dyes such as proflavin (Oster *et al.*, 1959). The empirical quantum yield of these reactions and of the one reported here depends upon reactant concentrations according to the equation

$$\Phi = \frac{[O_2]}{\alpha + [O_2]} \frac{[RH]}{\beta + [RH]} \frac{\gamma}{1 + \delta[Chl]}$$

where [RH] is the reduced hemin or other substrate and [Chl] is the chlorophyll or other sensitizer. Two mechanisms have been proposed which are consistent with this equation. One involves reaction between triplet excited sensitizer with oxygen, to form a complex called a moleoxide (Schenck and Ziegler, 1944) or photoperoxide (Oster *et al.*, 1959); this intermediate proceeds to oxidized the substrate and thereby reform the original sensitizer (mechanism A). The other mechanism (Kautsky *et al.*, 1933) requires energy transfer from triplet excited sensitizer to oxygen, to produce long-lived singlet excited oxygen, the actual oxidizing agent (mechanism B). Chl represents chlorophyll in the triplet state and $O_2(^3\Sigma_g)$ and $O_2(^1\Delta_g)$ are the ground triplet and excited singlet states of oxygen, respectively. A distinction between these mechanisms cannot be made on the basis of kinetic data. However, Porter (1965) has looked for the transient absorption spectrum of singlet oxygen, as an intermediate in the oxidation of naphthalene, and did not find it.

$$\left.\begin{array}{l} Chl' + O_2 \longrightarrow Chl \cdot O_2 \\ Chl \cdot O_2 + RH \longrightarrow RH \cdot O_2 + Chl \\ RH \cdot O_2 \longrightarrow H + O_2^- \end{array}\right\} \text{(mechanism A)}$$

$$\left.\begin{array}{l} Chl' + O_2(^3\Sigma_g) \longrightarrow Chl + O_2(^1\Delta_g) \\ O_2(^1\Delta_g) + RH \longrightarrow H + O_2^- \end{array}\right\} \text{(mechanism B)}$$

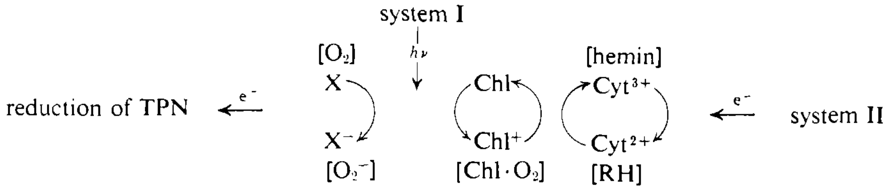
From our studies of the retardation of the reaction by minute amounts of β-carotene, we conclude that a long-lived state of chlorophyll is in fact the reactive one. We found that 2.5×10^{-5} mole/l. of carotene retarded the reaction to half the value in its absence. Taking the encounter frequency in pyridine at 25° as 6.8×10^9 mole/l. per sec (the product of the encounter frequency in water (Oster and Adelman, 1956) and the inverse ratio of the viscosities of the two solvents), the life-time for the photoreactive species in this system is 6×10^{-6} sec. Although β-carotene is itself oxidized in air in the presence of chlorophyll and red light, the rate of this reaction is slow compared with the sensitized photooxidation of reduced hemin. In the presence of 7×10^{-6} M chlorophyll a, only about 2% of a 4×10^{-5} M solution of carotene in benzene is oxidized in 0.5 hr under illumination comparable with ours (Claes, 1961). Therefore, the retardation noted by us is not due to this competing reaction.

One respect in which the sensitized photooxidation of reduced hemin differs from other chlorophyll-sensitized photooxidations is in its low quantum yield (of the order 10^{-2}); yields approaching unity have been obtained for other substrates, such as allylthiourea (Seeley, 1966). The low yield in the present system could be due to inactivity of the chlorophyll-reduced hemin complex as sensitizer. In the complex, access to the reactive site by oxygen may be restricted, and only the uncomplexed chlorophyll may promote the reaction.

ANALOGIES WITH PHOTOSYNTHESIS. The chlorophyll-sensitized photooxidation of reduced hemin is formally analogous to the photochemistry of system I in photosynthesis, as outlined earlier, if mechanism A obtains. In both cases chlorophyll is oxidized, in photosynthesis by an unknown electron acceptor, X, and in the *in vitro* system, by O_2. Chlorophyll is then regenerated, in photosynthesis by oxidation of reduced cytochrome *f*, in solution by reduced hemin. Scheme I summarizes this analogy, with the *in vitro* system given in brackets. On the other hand, the dark reaction between chlorophyll and hemin, which produces reduced hemin is analogous to a backup of photosynthesis, with electrons being transferred from chlorophyll to hemin *in vitro* and to cytochrome *in vivo*. Chance *et al.* (1963) previously proposed that such a dark reaction may occur *in vivo*.

It is of interest to compare quantitatively the reactions

SCHEME I



reported here with reactions *in vivo*. Chance and Devault (1964) obtained a quantum yield near unity for light-induced electron transfer from cytochrome to chlorophyll, in chromatium, while quantum yields approaching 10^{-2} were obtained by us for the chlorophyll-sensitized photooxidation of reduced hemin. However, quantum yields close to one have been obtained for these types of reactions *in vitro*, as noted above. Chance *et al.* (1963) also measured the rate of dark reduction of cytochrome in intact cells of chromatium and obtained a value of 0.01 μmole of iron converted/sec, at 300° K. By comparison, the dark reduction of hemin reported here is slow (see Table I).

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