Photoreduction of Methyl Red and Tetrazolium Blue by Ascorbate: Catalysis by Chlorophylls and Porphyrins in Aqueous Media *

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1. A photoreduction of methyl red and tetrazolium blue by ascorbate has been observed in the presence of pure chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b*, protoporphyrin IX, hematoporphyrin IX and coproporphyrin III in aqueous solutions under anaerobic conditions. The chlorophylls and pheophytins, which were added to the aqueous reaction system absorbed on sucrose, gave "solutions" which were visually clear, but were apparently suspensions of small aggregates of the pigment molecules.

2. The photoreductions of both methyl red and tetrazolium blue were stimulated (with chlorophylls or porphyrins as catalysts) if 2,6-dichlorophenolindophenol (DPIP)*** was also present. This indicates a role of intermediate electron carrier for DPIP, serving to increase the flow of electrons from ascorbate to the photocatalyst under the influence of light. Addition of quinacrine had no effect upon tetrazolium blue photoreduction, but showed slight inhibition in the case of methyl red photoreduction.

3. A comparison of a number of porphyrins and porphyrin derivatives as catalysts for these reactions has been made. Activity was observed for the chlorophylls, pheophytins and porphyrins. Iron porphyrins, hemoproteins and FMN were inactive.

4. During photoreduction of methyl red a simultaneous oxidation of ascorbate was observed. Glutathione could substitute for ascorbate but gave a slower rate of reaction.

5. In the presence of air, the chlorophylls and protoporphyrin catalyzed a photooxidation of DPNH, reduced cytochrome *c*, reduced DPIP and ascorbate. Comparative rates are given.

6. These results are discussed in relation to the same photoreduction reactions catalyzed by plant chloroplasts and bacterial chromatophores.

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*** The abbreviations used are: DPIP, 2,6-dichlorophenolindophenol; Tris, tris(hydroxymethyl)-aminomethane; FMN, flavin mononucleotide, and DPNH, reduced diphosphopyridine nucleotide.

The ability of isolated chlorophylls, pheophytins and porphyrins to catalyze photochemical oxidation-reduction reactions has been shown by the extensive investigations of Krasnovsky and his coworkers¹. Following the original paper of Krasnovsky which reported a photochemical reduction of chlorophyll by ascorbic acid in pyridine solution², it was shown that chlorophylls in light could transfer electrons from ascorbate to a wide variety of compounds including oxidation-reduction dyes, riboflavin, quinones, hematin, dehydroascorbic acid and pyridine nucleotides^{3,5}. A chlorophyll-catalyzed photoreduction of methyl red by phenylhydrazine in methanol was demonstrated by the earlier experiments of Livingston and Pariser⁶. The photoreduction of the chlorophylls and pheophytins proceeds rapidly in the presence of pyridine, piperidine, nicotine and quinoline⁷, and most of the investigations by the Krasnovsky group have involved such non-aqueous media. That some of the reactions can also proceed in aqueous media was shown by the experiments of Krasnovsky and Umrikhina⁸, in which ascorbic acid, ferrous ion and ferrocyanide served as electron donors in a photochemical transfer of electrons to methyl red in the presence of chlorophyll, hematoporphyrin and protoporphyrin.

The present investigation is concerned with the photoreduction of two dyes, methyl red and tetrazolium blue, which have been shown to be readily photoreduced by plant chloroplasts and by chromatophores of *Rhodospirillum rubrum*⁹. With chromatophores, the photoreduction reactions require the presence of both ascorbate and DPIP. Accordingly, the photoreduction of these two dyes by ascorbate using chlorophylls, porphyrins and their derivatives as the photocatalyst was examined, with particular emphasis upon the role of DPIP in such photoreductions. The reactions were all performed in aqueous media, in order that a direct comparison could be made with the data obtained from the experiments with chloroplasts and chromatophores. The chlorophylls were added to the aqueous medium adsorbed on powdered sucrose, and as the sucrose dissolved, the chlorophyll remained in a fine colloidal suspension. The results obtained for methyl red photoreduction are in agreement with the previous data reported by the Krasnovsky group⁸, but the presence of DPIP in the reaction system was found to stimulate the reactions.

MATERIALS AND METHODS

A description of the reaction vessels used in this experimental work has been given in the previous paper⁹. Anaerobic conditions were obtained in the manner reported, with the exception that argon gas was used for the intermediate flushings in the present investigation. Illumination was performed with either a 150 or 500 W tungsten reflector flood lamp which gave a light intensity at the reaction site of approximately 1500 or 4500 footcandles, respectively. The reactions were run in a water bath with a controlled temperature of 26°C, and the reaction vessels were illuminated through the transparent walls of the plastic container. Absorbancy readings were made on the illuminated and non-illuminated systems at 5 min intervals in the case of the tetrazolium blue experiments and at 2 min intervals for the methyl red experiments. A Beckman DU spectrophotometer was used for all measurements.

Chlorophyll *a* and chlorophyll *b* were prepared from spinach leaves by means of chromatography on a powdered sucrose column as previously reported¹⁰. Following the final separation of the chlorophylls on the column, the sucrose adsorbing the pure chlorophylls was dug out of the column, air dried in the dark, and stored at -20°C in the dark in a drying chamber. For the experiments involving chlorophyll *a* and chlorophyll *b*, the

Fig. 1. Absorption spectra of pure chlorophyll *a* and chlorophyll *b* dissolved in Tris buffer solution after adsorption on sucrose. In addition to the chlorophyll the solution contained only 10 mM Tris buffer pH 8.0 and the sucrose which had adsorbed the pigment. The final sucrose concentration was approximately 0.5 M. The spectra were obtained with a Beckman DK2 automatic recording spectrophotometer.

chlorophylls were added to the reaction system on the sucrose. The sucrose quickly dissolved as the liquid was swirled around, and the chlorophyll was thus dispersed through the medium. Although not a true solution of chlorophyll, the reaction mixture containing the chlorophyll was visually clear and there was no tendency for the chlorophylls to precipitate out of solution unless it was allowed to stand for several hours. The chlorophyll adsorbed on sucrose could not be stored for extended periods without losing activity, and the experiments described in this investigation were performed with freshly prepared chlorophylls. The absorption spectra of "solutions" of chlorophyll *a* and chlorophyll *b* prepared in this manner are given in Fig. 1. The maxima of the main absorption bands of chlorophyll *a* are at 669–671 and 418–422 $m\mu$, while for chlorophyll *b* they occur at 655–658 and 464–468 $m\mu$. Although no experiments have been performed to determine the state of the chlorophyll in such experiments, the absorption bands of both pigments are shifted to longer wavelengths and are flattened when compared to the corresponding spectra in aqueous 80 % acetone¹⁰, in a manner similar to the chlorophylls present in the intact leaf¹.

Pheophytins *a* and *b* were prepared from the purified chlorophylls by adding a drop of concentrated HCl to the chlorophylls in petroleum ether, allowing the solutions to stand for 5 min, following which the acid was washed out with 8 successive washings with water, and the final solution rechromatographed on a powdered sucrose column. The pheophytins were added to the reaction system in the same manner as the chlorophylls, adsorbed on powdered sucrose. The absorption maxima of pheophytin *a* under the same conditions as given in Fig. 1 were at 681–684 and 400–405 $m\mu$ for the two main bands, which were less sharp than the corresponding bands for the pigment dissolved in acetone or ether. The absorption maxima for pheophytin *b* were observed at 667–669 and 423–427 $m\mu$. Again these bands were not sharp.

For some experiments a non-purified preparation of the chlorophylls was used. This was prepared by extracting spinach leaves with acetone, transferring the extracted pigments into petroleum ether, and adsorbing the pigments on powdered sucrose. No chromatography was performed. Consequently, this preparation contained both chlorophylls and other acetone-soluble compounds, including the carotenoids. Since the composition of such a preparation was not controlled, a variation would be expected. Whereas with some preparations an almost clear "solution" was obtained upon dissolving it into water,

with other preparations there was a tendency for the "solution" to become cloudy, and in this case the chlorophylls would precipitate. This could be avoided by adding small quantities of sodium deoxycholate to the system, and accordingly this was routinely done when using the non-purified chlorophyll preparation.

The photoreduction of tetrazolium blue was followed at 580 $m\mu$ and no attempt was made to solubilize the formazan formed in the reaction. Accordingly, the results reported for tetrazolium blue cannot be translated into quantitative terms, but are intended only to show the qualitative aspects of the reaction. The photoreduction of methyl red was followed at 440 $m\mu$. For converting the absorbancy readings into definite quantities of methyl red, a molar extinction coefficient of 17 000 was used. This value was determined for the pH used in this investigation.

The tetrazolium blue was purchased from Mann Research Laboratories, New York, methyl red from E. Merck and Co., Darmstadt, ascorbic acid from Hoffman La Roche and Co., Basle, DPIP from Eastman Chemical Co., Rochester, glutathione from Nutritional Biochemicals Corp., Cleveland, cytochrome c, FMN, catalase and peroxidase from Sigma Chemical Co., St. Louis, and protoporphyrin IX, hematoporphyrin IX and hemin from California Corp. for Biochemical Research, Los Angeles. The protoporphyrin was substantially purified before use in these experiments. The coproporphyrin and hemato-heme were obtained from Dr. B. Burnham.

Quantitative determination of photocatalysts used. The concentrations of chlorophylls *a* and *b* as well as the concentrations of pheophytins *a* and *b* were determined from absorbancy measurements made at the appropriate maxima in the red spectral region. These readings were related to concentrations by use of the specific absorption coefficients for these compounds given for aqueous 80 % acetone¹⁰. The concentration of chlorophylls in the nonpurified preparation was determined from the absorption maximum in the red, using the specific absorption coefficients for the chlorophylls as above and assuming a ratio of chlorophyll *a* to *b* of 2.5¹⁰. The concentrations of protoporphyrin and coproporphyrin were determined from absorbancy readings, using extinction values of $E_{1\text{ cm}}^{1\%} = 4900$ in dilute HCl at the Soret band maximum for protoporphyrin¹¹ and a molar extinction coefficient of 47 000 at 402 $m\mu$ in 1 M HCl for coproporphyrin¹². The molar extinction coefficient given for coproporphyrin was also used for determining hematoporphyrin concentration, utilizing the Soret maximum of hematoporphyrin for the measurement. The concentrations of hemin and hematoheme were determined using the spectroscopic data given by Paul¹³ for these compounds. Catalase and peroxidase concentrations were calculated by utilizing the molecular weights of the proteins, and cytochrome c and FMN concentrations were determined from absorbancy values at 550 (in the reduced form) and 450 $m\mu$, respectively^{14,15}.

RESULTS

The photoreduction of tetrazolium blue by chlorophyll *a*, chlorophyll *b* and protoporphyrin IX is shown in Table 1. The activity with all three photocatalysts was maximal in the presence of both ascorbate and DPIP. As in the previous experiments there was excess ascorbate present, so that the DPIP was maintained in the reduced state, and the stimulation by the added DPIP is best explained in terms of its acting as an intermediate electron carrier between the ascorbate and photocatalyst, thereby increasing the rate of photoreduction of the photocatalyst and increasing the rate of the overall reaction. In the present case, a good rate of tetrazolium blue photoreduction was observed in the absence of DPIP, which is in contrast to the reaction catalyzed with *R. rubrum* chromatophores⁹. At the pH of the reaction there was no observable reduction of tetrazolium blue in the dark. At a pH of 10 or above, a dark reduction of tetrazolium blue was observed, pointing out the fact that the photoreduction of this dye by ascorbate is a thermodynamically favorable one, and in this case there is no storage of energy as a consequence of the photochemical

Table 1. Component study for tetrazolium blue photoreduction catalyzed by chlorophyll *a*, chlorophyll *b* and protoporphyrin IX. The complete reaction system contained 3.0 mM ascorbate, 30 μ M DPIP, 15 mM Tris buffer pH 7.5 and 0.1 mM tetrazolium blue in 5.5 ml of solution. When present, quinacrine and β -carotene were 36 μ M and sucrose was 0.5 M. The ascorbic acid was neutralized with NaOH just prior to addition, and the tetrazolium blue was added after the ascorbate had reduced the DPIP. Illumination was with a 150 W tungsten reflector flood lamp. Anaerobic conditions were employed. The chlorophylls were added to the reaction system adsorbed on sucrose, and consequently those reaction systems were also approximately 0.5 M in sucrose.

Conditions	Absorbancy increase at 580 m μ in 20 min.		
	Chphyll <i>a</i> 2.5 μ M	Chphyll <i>b</i> 3.4 μ M	Protoporphyrin 4.0 μ M
Complete system	0.196	0.155	0.168
— DPIP	0.156	0.061	0.094
— ascorbate, DPIP	0.001	0.001	0.006
— tetrazolium blue	0.002	0.002	0.001
+ quinacrine	0.210	0.155	0.167
+ β -carotene	0.169	0.134	0.161
+ sucrose	—	—	0.175
— photocatalyst	0.008	—	—

reaction. In view of the marked stimulating effect of quinacrine on the photoreduction of tetrazolium blue by bacterial chromatophores, this compound was tested with the isolated pigments. As shown, it had no significant effect upon the three photoreactions. Addition of β -carotene to the reaction resulted in a slight decrease in activity, which agrees with previously reported data on the inhibition of photoreactions of chlorophyll by this compound^{16,17}.

A similar series of experiments in which methyl red was the electron acceptor is reported in Table 2. Perusal of the table reveals that this reaction resembles

Table 2. Component study for methyl red photoreduction. The complete reaction system contained 6.0 mM ascorbate, 90 μ M DPIP, 15.6 mM phosphate buffer pH 6.7 and 0.13 mM methyl red in 5.5 ml of solution. When present, quinacrine and β -carotene were 36 μ M and sucrose was 0.5 M. The ascorbic acid was neutralized just prior to addition to the system, and the methyl red was added last. Illumination was with a 150 W tungsten reflector flood lamp. Anaerobic conditions were employed. The chlorophylls were added to the reaction system adsorbed on sucrose, and consequently those systems contained approximately 0.5 M sucrose.

Conditions	Absorbancy change at 440 m μ in 6 min.		
	Chphyll <i>a</i> 2.5 μ M	Chphyll <i>b</i> 3.4 μ M	Protoporphyrin 3.0 μ M
Complete system	−0.166	−0.057	−0.812
— DPIP	−0.059	+0.008	−0.132
— ascorbate, DPIP	+0.004	+0.020	+0.023
— methyl red	+0.001	+0.001	−0.005
+ quinacrine	−0.103	−0.045	−0.131
+ β -carotene	−0.181	−0.095	−0.795
+ sucrose	—	—	−0.820
— photocatalyst	+0.012	—	—

the photoreduction of tetrazolium blue in most of the aspects investigated. Thus, the maximal reaction rate was observed when DPIP was present in addition to the ascorbate. In fact, there was no activity observed with chlorophyll *b* unless the DPIP was present. No activity was observed in the absence of both compounds, and the presence of quinacrine served to inhibit the reaction, with the reaction catalyzed by protoporphyrin being particularly susceptible. In the present case, however, the presence of β -carotene caused a slight stimulation of the reactions catalyzed by the chlorophylls. The photoreduction of methyl red was observed to take place over a wide range of pH values. Experiments run at pH 4, in which case the dye was in the red form, showed good rates of photoreduction with protoporphyrin as the catalyst. The pH used in these experiments gave maximal activity, and at this pH the dye was primarily in the alkaline yellow form.

The relative efficiency of various porphyrins and porphyrin derivatives in catalyzing the two photoreactions is shown in Table 3. It is seen that both the chlorophylls and pheophytins were active in the reactions, with chlorophyll *a* being better than chlorophyll *b*, and the chlorophylls being superior to the pheophytins. All the porphyrins investigated were active, with protoporphyrin being superior in both reactions. The presence of iron in the porphyrin caused a loss of photochemical activity. This is in accord with data reported by Krasnovsky¹⁸. As expected, the heme proteins examined were inactive in the reactions, as was FMN. The FMN result was also expected, since the presence of ascorbate serves to inhibit the direct photoreduction of FMN itself¹⁹. In comparing the two photoreductions, chlorophyll *a* was the most efficient of the

Table 3. Comparison of chlorophylls, porphyrins and porphyrin derivatives in the photoreduction reactions. The anaerobic experimental conditions utilized were the same as given for Tables 1 and 2. The observed rates have been corrected for a 4.0 μ M concentration of photocatalyst. In the actual experiments the concentrations of photocatalyst were close to 4 μ M with the exception of the iron porphyrins (10 μ M), the heme proteins (24 μ M) and FMN (166 μ M).

Photocatalyst	Absorbancy change, light minus dark	
	Methyl red 6 min 440 m μ	Tetrazolium blue 20 min 580 m μ
Chlorophyll <i>a</i>	-0.265	0.312
Chlorophyll <i>b</i>	-0.135	0.168
Pheophytin <i>a</i>	-0.174	0.097
Pheophytin <i>b</i>	-0.102	0.029
Protoporphyrin	-1.09	0.171
Hematoporphyrin	-0.568	0.026
Coproporphyrin	—	0.019
Hemin	-0.002	0.006
Hematochrome	-0.009	0.005
Cytochrome <i>c</i>	-0.001	0.001
Catalase	-0.003	0.001
Peroxidase	-0.003	0.003
FMN	—	0.001

Table 4. Substitution of glutathione for ascorbate in the photoreduction of methyl red and tetrazolium blue. Conditions were the same as given in Tables 1 and 2 with the exception that illumination was with a 500 W tungsten reflector flood lamp and where indicated, equimolar quantities of glutathione were substituted for ascorbate.

Conditions	Absorbancy change, light minus dark	
	Methyl red 6 min 440 m μ	Tetrazolium blue 10 min 580 m μ
2.5 μ M chlorophyll <i>a</i> with ascorbate	−0.414	0.388
with glutathione	−0.286	0.208
with glutathione, minus DPIP	−0.198	0.161
3.0 μ M protoporphyrin with ascorbate	−2.76 ^a	0.200
with glutathione	−0.530	0.116
with glutathione, minus DPIP	−0.218	0.046

^a This value was calculated by extrapolation of the rate observed for a two minute illumination period.

compounds investigated for tetrazolium blue photoreduction, while for methyl red photoreduction, protoporphyrin was most active.

The function of ascorbate in the experiments described above is that of an electron donor, since Krasnovsky has repeatedly shown that ascorbate can directly photoreduce either chlorophylls, pheophytins or porphyrins¹. The possibility of using another compound for the electron donor was investigated. It was found that glutathione would support the photoreductions, but at a somewhat slower rate. Table 4 presents the data obtained with glutathione. For these experiments illumination at a higher light intensity was employed in order that the reactions would be in the range where they could be readily measured. As shown in the table, glutathione substituted for ascorbate in the reaction, giving about half the activity of ascorbate except in the case of methyl red photoreduction by protoporphyrin, where it was only about 1/5 as active. As was the case with ascorbate, a couple consisting of glutathione and DPIP gave more activity than the glutathione alone, indicating that here also the DPIP could serve as an intermediate electron carrier between the glutathione and photocatalyst. This is in agreement with the activity observed with glutathione in the early photooxidation experiments of Vernon and Kamen¹⁰.

Although not systematically examined, it was observed that phenylhydrazine could also serve as an electron donor in these reactions, but it was not very effective. It should also be mentioned that in experiments designed to see if mammalian cytochrome *c* could serve as an electron donor in reactions of this type, there was no oxidation of the reduced cytochrome *c* added. However, if the oxidized form of the cytochrome was added to the chlorophylls with no other additions, a slow but consistent photoreduction of the cytochrome was

Table 5. Stoichiometry of anaerobic photoreduction involving methyl red, with ascorbate as the electron donor. The reaction system contained 1.8 mM ascorbate, 55 μ M DPIP, 9.1 mM phosphate buffer pH 7.4, methyl red as indicated in the table and either 4.0 μ M protoporphyrin or chlorophyll *a* in 5.5 ml of solution. The reaction was allowed to proceed until all the methyl red was reduced as shown by visual inspection, following which the ascorbate remaining in the illuminated system was determined by titration with a standard solution of DPIP. A comparison was then made with a control system which was identical in composition with the exception that it contained no methyl red. The control system was also illuminated, and the difference in ascorbate between the two systems corresponded to that which entered into the photochemical reaction with methyl red. The standard DPIP solution was prepared by standardization against a solution of ascorbic acid whose concentration was determined by the method of Hewitt and Dickes ²².

Photocatalyst	μ moles ascorbate oxidized	μ moles methyl red reduced
Protoporphyrin	1.87	1.64
Chlorophyll <i>a</i>	1.72	1.55

observed. This again agrees with previous experiments performed by Krasnovsky ²¹, with the reduction coming at the expense of some unknown hydrogen donor in the medium, probably protein.

In the experiments reported to date it has been assumed that the ascorbate was functioning as a hydrogen donor, and was consumed in the photochemical reaction. To show that this actually was the case, the stoichiometry of the reaction was determined, with the results of the experiments shown in Table 5. The photoreduction of methyl red was chosen, both because of the rapidity of the reaction and because of the fact that the final solution was colorless and allowed a titration of the ascorbate remaining in the medium. With both chlorophyll *a* and protoporphyrin as photocatalyst, the values obtained were close to the expected 1 to 1 ratio. In both cases there was slightly more ascorbate disappearing than would be expected, and the cause for this is not known. The experiments do show the fundamental aspects of the photoreduction, that ascorbate is oxidized in the reaction as methyl red is reduced.

Krasnovsky *et al.* have shown that it is possible to photoreduce chlorophylls, pheophytins and porphyrins in organic solvents such as pyridine or piperidine if no electron acceptor is added ^{1,2,23,24}. Similar experiments were performed in the present investigation, to see if the photoreduced forms of the chlorophylls and porphyrins could be demonstrated in an aqueous medium. It was possible to show changes in the absorption spectrum of hematoporphyrin in the presence of ascorbic acid. Illumination caused a visible bleaching of the hematoporphyrin, with a decrease in the absorption of the Soret band and an increase in absorption at 500 $m\mu$. These changes were more pronounced under acid conditions. A measure of the reaction is the change in absorbancy in the vicinity of the Soret peak, and the data presented in Fig. 2 represent such changes. A rapid decrease in the absorbancy was observed in the light, and a slower reverse reaction was seen in the dark. Addition of methyl red to the system prevented the absorbancy changes related to this reaction until the methyl red was reduced, at which time photoreduction of the porphyrin commenced (however at a slower rate). In the absence of ascorbate no reaction was

Fig. 2. Direct photoreduction of hemato- porphyrin by ascorbate in the absence of other electron acceptors. The reaction mixture contained 1.8 mM ascorbate, 50 μ M hematoporphyrin and 10 mM acetate buffer pH 4.6. A, Composition as given above; B, 0.2 mg of methyl red was also present; C, same as A except ascorbate was not present.

observed. Similar experiments performed with chlorophyll *a* revealed that changes in the absorbancy of the chlorophyll at its absorption maxima could be observed, but the rates were very slow, and it is questionable if they were reversible. Since protoporphyrin is not appreciably soluble at the pH utilized, it was difficult to perform experiments with this compound. However, the same general type of reaction as shown in Fig. 2 was observed with protoporphyrin, with the rate of reaction being much slower due to the low concentration of the porphyrin.

The experiments described above were all performed under anaerobic conditions, so that nothing would impede the flow of electrons to the final electron acceptor. It is well known that the chlorophylls and porphyrins are active in photooxidations in air²⁵, and accordingly experiments were performed to compare some of the previously investigated photocatalysts in an aerobic

Table 6. Photooxidation of various substances by chlorophylls and protoporphyrin in the presence of air. The reactions were all performed in 5.5 ml of solution which contained 10 mM phosphate buffer pH 7.4, approximately 5 μ M photocatalyst and 0.1 mM DPNH, reduced cytochrome *c* or reduced DPIP. For the experiment with ascorbate it was present with an 1.8 mM concentration. The results have been adjusted to give the rate for 5.0 μ M photocatalyst. Cytochrome *c* was reduced with hydrosulfite and DPIP was reduced with an equivalent amount of ascorbic acid. The experiment with DPIP was only run for 2 min, since there was an oxidation of the dye in the dark. The rate so obtained was projected for the 10 min period. The rates were obtained from absorbancy differences between the illuminated and non-illuminated compartments of the adapted Thunberg tubes, except that ascorbate was determined by titration with a standard solution of DPIP. Illumination was obtained with a 500 watt tungsten reflector flood lamp.

Photocatalyst	μ Moles oxidized in 10 min for 5.0 μ M photocatalyst			
	DPNH	Cytochrome <i>c</i>	DPIP _H	Ascorbate
Protoporphyrin	0.159	0.170	0.465	0.38
Hemin	0.009	0.004	0.001	0
Chlorophyll <i>a</i>	0.038	0.135	0.180	0.22
Chlorophyll <i>b</i>	0.027	0.052	0.075	0.15

photooxidation of ascorbate and other reducing agents. As shown in Table 6, the same pattern of relative reactivity was shown for protoporphyrin, chlorophyll *a* and chlorophyll *b*, with activity decreasing in that order. An active photooxidation was observed for all four substrates examined, reduced cytochrome *c*, reduced DPIP, DPNH, and ascorbate. The oxidation of ascorbate was expected, as was the reaction with DPIP, since it stimulated the photoreductions reported above. Indeed, the demonstration of the photooxidation of reduced DPIP strengthens the concept that DPIP does function in the manner suggested in the photoreduction experiments. The photooxidation of cytochrome *c* has been previously described by Krasnovsky²¹ in the case of chlorophyll *a* and *b*, by Vernon and Kamen²⁶ for chromatophores of *R. rubrum* and by Nieman *et al.*²⁷ for a digitonin extract of chloroplasts. In the case of the photooxidations catalyzed by both chromatophores and the digitonin preparation from chloroplasts, it was supposed that the cytochrome was oxidized directly by the chlorophyll system. The data of Krasnovsky²¹ and those shown in Table 6 show that this is the case. The photooxidation of DPNH, though not as rapid as the other reactions, shows that this reaction can be catalyzed by the chlorophylls, and can explain the photooxidation of pyridine nucleotides by chloroplasts observed earlier²⁸.

A comparison of the rates of ascorbate oxidation using either oxygen or methyl red as the electron acceptor can be made by converting the rates for methyl red photoreduction given in Table 4, using a value of 17 000 for the molecular extinction coefficient for methyl red at this wave length. The comparable rate for methyl red photoreduction using protoporphyrin as the photocatalyst is 2.6 μ moles/10 min/5 μ M photocatalyst, while the rate for chlorophyll *a* is 0.47. So in both cases, the transfer of electrons was faster with methyl red. However, in the aerobic experiments there were no special provisions made for shaking the reaction system to insure ready solution of oxygen from the air, and accordingly the rates given for the aerobic reaction are probably not maximal.

DISCUSSION

The photoreductions studied in this investigation are the same ones which have been shown to take place with both plant chloroplasts and *R. rubrum* chromatophores⁹. Although the photoreactions do not involve the storage of energy such as does the photoreduction of pyridine nucleotides, and although the compounds photoreduced are not physiological compounds, a comparison of the reactions does allow some conclusions concerning the nature of the photochemical act in the chloroplast and chromatophore. The main feature is that the organized pigment systems found in the chloroplast and chromatophore can catalyze the same relatively simple reactions as do the isolated chlorophylls. Also, in both cases, the presence of DPIP stimulates the reaction.

The photooxidation experiments performed in the presence of air show that the activated chlorophylls are not specific for ascorbate as an electron donor, but can also react with glutathione, DPNH, reduced cytochrome *c* and reduced DPIP. Also, a wide variety of electron acceptors can be used, as shown by Krasnovsky and Brin⁵. Thus, a wide variety of reactions could theoretically be available to the chloroplast and chromatophore, and although

the intensive study now being made of the photosynthetic phosphorylation electron transport system shows that the chloroplasts can catalyze several photoreductions, it still seems that one of the remarkable things about the chloroplast is the selectivity they maintain in the photoreactions they perform. This may well be one of the prime advantages gained by the chloroplast and chromatophore from the organization present in the photosynthetic apparatus. The removal of carotenoids from chromatophores sufficiently upsets this control so that irreversible and lethal photooxidations take place^{29,30}. With the intact chloroplast there is also a high degree of selectivity concerning what can be photooxidized by the chloroplast, since only with aging or intentional damage to the oxygen evolving system can the chloroplast be made to substitute an oxidation of ascorbate in lieu of the natural reaction³¹.

In comparing the various photocatalysts that have been examined, it becomes apparent that the inclusion of iron in the porphyrin molecule causes a loss in activity. On the other hand, the presence of magnesium in the chlorophylls increases the activity over that of the pheophytins. This indicates that the metal ion is not necessary for the photoreactivity of the porphyrin, and one should consider the porphyrin system itself as the active agent in these reactions. The spectroscopic changes observed when the chlorophylls and porphyrins are photoreduced by ascorbate directly also indicate a change in the porphyrin system. One might wonder why the photosynthetic apparatus could not use porphyrins as well as the chlorophylls. It must be remembered, however, that although in the mechanistic sense the reactions described here may be representative of the ones taking place in the organized chloroplast or chromatophore, they are not the ones catalyzed by the organized pigment systems in nature. One cannot extrapolate from these data to the reactions taking place in the chloroplasts where water is oxidized to produce oxygen, or in the bacteria where an accessory hydrogen donor (not ascorbate) is oxidized in the normal photosynthetic process.

Although the photoreduction experiments described in the previous paper⁹ were performed in another laboratory, the conditions were approximately the same, and a general comparison of the rates of the photoreduction reactions catalyzed by the isolated photocatalysts and the chloroplasts and chromatophores can be made. Taking the data from Tables 1 and 4 of the previous paper and Table 3 of this paper, the rates for methyl red and tetrazolium blue photoreduction have been calculated and are shown in Table 7. The rates

Table 7. Comparison of photoreduction rates for the different photocatalysts.

Photocatalyst	Methyl red $\mu\text{moles/h}/\mu\text{mole}$ of photocatalyst	Tetrazolium blue Absorbancy change, 580 m μ per hour for μM photocatalyst
Chlorophyll <i>a</i>	51	0.27
Protoporphyrin	170	0.14
Chloroplasts	96	0.88
Chromatophores	24	0.36
Chromatophores plus quinacrine	145	1.11

obtained with the different photocatalysts are all in the same order of magnitude. The chloroplasts were more active than the chromatophores, but the addition of quinacrine stimulated the latter reaction to a rate above that for the chloroplasts. The quinacrine-stimulated chromatophores gave the highest rate for tetrazolium blue photoreduction, but protoporphyrin was the most active in methyl red photoreduction.

The activity of the porphyrins in the reactions described above may well have biological expression in the sensitization to light caused by high concentrations of porphyrins in the blood. The demonstrated ability of these compounds to photooxidize ascorbic acid, glutathione, reduced cytochrome c and DPNH suggests that their harmful effects in the case of porphyria may well be due to the photooxidation of some vital component of the cell or surrounding fluid thus upsetting the normal metabolic pattern.

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