Photoreduction of Methyl Red and Tetrazolium Blue by Spinach Chloroplasts and Chromatophores of *Rhodospirillum rubrum* *

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1. Chloroplasts prepared from spinach leaves have been shown to catalyze a photoreduction of both methyl red and tetrazolium blue in a regular Hill reaction. Addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) *** at 10^{-5} M inhibited both reactions, but the further addition of ascorbate and 2,6-dichlorophenolindophenol (DPIP) restored the activity of the chloroplasts in the photoreductions.

2. Chromatophores of *Rhodospirillum rubrum* also catalyzed a photoreduction of both dyes in the presence of DPIP and ascorbate. Addition of quinacrine markedly stimulated both reactions, with half-maximal stimulation occurring at 20 μ M quinacrine. Experiments performed in red light showed the quinacrine did not increase the reaction rate by itself acting as a photocatalyst in the reactions. Addition of quinacrine inhibited the succinate-supported photoreduction of DPN by the chromatophores.

3. The respiratory inhibitors antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide both failed to stimulate the photoreduction of tetrazolium blue and methyl red by *R. rubrum* chromatophores, but instead were slightly inhibitory. These inhibitors strongly inhibited the succinate-supported photoreduction of DPN. Thus in terms of inhibition characteristics with chromatophores, quinacrine did not resemble antimycin A and HQNO. The photoreductions catalyzed by chloroplasts were not affected by added quinacrine, which is a distinguishing feature of the two photosynthetic systems.

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*** The abbreviations used are: DPIP, 2,6-dichlorophenolindophenol; Tris, tris hydroxymethylaminomethane; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FMN, flavin mononucleotide, and HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide.

Experiments performed with both plant chloroplasts and chromatophores of *Rhodospirillum rubrum* have shown that with both photosynthetically active systems a photoreduction of an added electron acceptor can be coupled to a photooxidation of ascorbate¹⁻⁴. The photooxidation of ascorbate, furthermore, was to a large degree dependent upon the presence of DPIP². This oxidation-reduction dye, which is reduced directly by ascorbic acid, appears to serve as an intermediate electron carrier between the chlorophyll system and ascorbate. An extensive series of investigations by Krasnovsky and coworkers⁵ has shown that ascorbate can participate in photoreduction reactions catalyzed by isolated chlorophylls and porphyrins, in which the final electron acceptors may be oxidation-reduction dyes such as methyl red or safranin T^{6,7}, pyridine nucleotides⁸, or the chlorophyll or porphyrin itself⁹. A study of the photoreduction of selected reagents by chloroplasts, chromatophores and isolated chlorophylls is desirable, since it will allow a comparison of the various systems and may indicate to what extent the photo-reactions catalyzed by isolated chlorophylls are representative of the photo-reactions taking place in the organized biological systems. For purposes of comparison, the photoreductions catalyzed by the isolated chlorophylls and porphyrins should be studied in aqueous media. The present paper describes the photoreduction of two such compounds, methyl red and tetrazolium blue, by both chloroplasts and chromatophores, while the following paper describes the photoreduction of these compounds by chlorophylls and porphyrins. In the case of the chloroplasts, both dyes are photoreduced in a regular Hill reaction, in which water is oxidized to produce oxygen. The dyes are also photoreduced by either chloroplasts or chromatophores when ascorbate is the electron donor, but the presence of DPIP is also required.

MATERIALS AND METHODS

Chloroplasts were prepared from spinach as previously described³. The growth of *R. rubrum* cells and the preparation of chromatophores from the cells of this bacterium have been reported previously⁴. The experiments were performed in a Thunberg tube which was adapted in such a manner that two regular 1 cm Pyrex absorption cells were joined to the main part of the tube through a T joint. The two absorption cells were spaced so that they would fit into positions 1 and 4 of the cell holder of a Beckman DU spectrophotometer. A special black cardboard cover was constructed to cover the adapted Thunberg tube which extended above the cell compartment of the spectrophotometer. The reagents required for a particular experiment were added to the reaction vessel, after which anaerobicity was obtained by means of three one-minute evacuations with a vacuum pump interspaced with the admission of nitrogen gas and mixing of the nitrogen gas with the contents of the vessel. Thus, the oxygen was flushed out and replaced with nitrogen. The contents of the reaction vessel were initially at 0°C. After the evacuation procedure the contents of the reaction vessel were distributed in approximately equal amounts in the two absorption cells, the reaction vessel was placed for four minutes in a glass chamber which was maintained at the desired temperature with an immersion heater, cooling coil and thermoregulator, following which the illumination period was initiated. One of the arms of the adapted Thunberg tube was covered with a cap of black cloth, and the contents of this absorption cell served as a dark control system. Absorbancy readings were made at 5 min intervals against a blank of appropriate absorbancy so that the observed readings were on the most sensitive part of the absorbancy scale. Illumination was performed with a 150 W tungsten reflector flood lamp, which gave a light intensity of approximately 1 700 foot candles in the reaction vessel. The photoreduction of methyl

Fig. 1. Photoreduction of tetrazolium blue by spinach chloroplasts and chromatophores of *R. rubrum*. The experimental conditions were those of Tables 1 and 2, with the chlorophyll concentrations being 4.8 and 5.5 μ M for the experiments with chloroplasts and chromatophores respectively. Anaerobic conditions were employed. A, chloroplasts in light; B, chromatophores in light; C, chromatophores in the dark, and D, chloroplasts in dark

Fig. 2. Photoreduction of methyl red by spinach chloroplasts and *R. rubrum* chromatophores under anaerobic conditions. The experimental conditions were the same as given in Tables 1 and 2, including the concentrations of chlorophyll. A, chromatophores in the light; B, chloroplasts in the light; C, chromatophores in the dark; D, chloroplasts in the dark.

red was followed at 440 $m\mu$, while tetrazolium blue was determined at 580 $m\mu$. The reactions were run at a temperature of 30°C.

Chlorophyll was determined by the method of Vernon¹⁰ and bacteriochlorophyll was determined by the method of van Niel and Arnold¹¹. Tris and FMN were purchased from Sigma Chemical Co. of St. Louis, ascorbic acid from the Nutritional Biochemical Corp., DPIP from Eastman Organic Chemical Co., methyl red from Merck and Co., tetrazolium blue from Mann Research Laboratories, Inc., DCMU was a gift from Dr. N. Bishop, and HQNO was a gift from Dr. J. W. Lightbrown.

RESULTS

Photoreductions catalyzed by spinach chloroplasts. The photoreduction of tetrazolium blue and methyl red by spinach chloroplasts in a regular Hill reaction is shown in Figs. 1 and 2, respectively. For these reactions there was no added electron donor, and the photoreduction of the dye was presumably accompanied by an evolution of oxygen. In the case of tetrazolium blue there was no observable reduction of the dye in the dark, while a slow but detectable reduction of methyl red did take place in the dark. Apparently the chloroplast preparation contained sufficient endogenous electron donors to catalyze the slow dark reaction.

The photoreaction with methyl red was linear with time, but this was not the case with tetrazolium blue. It is difficult to make a quantitative measurement of tetrazolium blue reduction in an aqueous solution, due to the formation of the insoluble formazan. Lester and Smith¹² have solubilized the formazan by adding a detergent to the reaction mixture in their experiments. It was not possible to do so in the present case, however, due to the difficulty of removing

Table 1. Photoreduction of tetrazolium blue and methyl red by spinach chloroplasts under anaerobic conditions in the presence and absence of ascorbate and DPIP. The reaction mixture contained 83 mM Tris buffer pH 7.0, 120 μ M methyl red or 67 μ M tetrazolium blue and 4.8 μ M chlorophyll which was contained in spinach chloroplasts. Where indicated there was also present 0.67 mM ascorbate, 34 μ M DPIP and 10 μ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The final volume was 6.0 ml and the final pH of the reaction system was 6.9. The control system contained only the dye, chloroplasts, and buffer. A photoreduction is indicated by an increase in absorbancy at 580 $m\mu$ in the case of tetrazolium blue (formation of blue color), and in the case of methyl red a decrease in absorbancy at 440 $m\mu$ indicated a photoreduction (loss of yellow color). Illumination was carried out with a 150 W tungsten reflector flood lamp.

Conditions	Change in absorbancy after 10 min (Light minus dark)	
	Methyl red 440 $m\mu$	Tetrazolium blue 580 $m\mu$
Control	-0.202	0.575
+ ascorbate	-0.497	0.621
+ ascorbate, DPIP	-1.23	0.701
+ DCMU	+ 0.021	0.051
+ DCMU, ascorbate, and DPIP	-0.817	0.481
+ ascorbate, DPIP, boiled chloroplasts	-0.016	0.006
Control, chloroplasts aged 100 h, 0°C.	+ 0.012	0.002
+ ascorbate, DPIP	-0.622	0.051

aliquots from the anaerobic system and maintaining the anaerobicity required. Accordingly, the data recorded for tetrazolium blue were those obtained directly, and as the reaction proceeded there was formation of the insoluble product. However, for the purposes of these experiments the reproducibility of the reaction for the short times investigated was sufficient to show the qualitative aspects of the reaction. It should be mentioned that while there was visible precipitation of the reduced dye in the experiments involving chloroplasts (and microscopically it was shown to collect on the chloroplasts), when chromatophores were used a much clearer "solution" was obtained. This was probably due to the smaller particles involved in the photoreaction in the case of the chromatophores.

The ability of chloroplasts to utilize ascorbate (in the presence of DPIP) as the electron donor for the photoreduction of both tetrazolium blue and methyl red is shown in Table 1. The addition of DCMU, a potent inhibitor of the oxygen evolving system¹³, completely inhibited methyl red photoreduction in the absence of ascorbate and decreased the photoreduction of tetrazolium blue to less than 10 % of the original activity. This demonstrates that the photoreduction of the dyes in the control system was coupled to the evolution of oxygen. Addition of ascorbate increased the rates of the reactions, and further addition of DPIP gave the maximal rates. When DCMU was present in the system, ascorbate and DPIP were able to overcome the inhibitory effect of this compound, which is readily explained in terms of the ascorbate and DPIP couple serving as the electron donor for the photoreduction of the dyes.

Table 2. Photoreduction of methyl red and tetrazolium blue by chromatophores of *Rhodospirillum rubrum*. The reaction system contained 33 mM Tris buffer pH 7.2, 0.67 mM ascorbate, 34 μ M DPIP, 11 μ M bacteriochlorophyll (in the chromatophores) and either 44 μ M tetrazolium blue or 120 μ M methyl red in a final volume of 6.0 ml. Anaerobic conditions were employed. Illumination was performed with a 150 W tungsten reflector flood lamp.

Conditions	Absorbancy change in 10 min Light minus dark	
	Methyl red 440 m μ	Tetrazolium blue 580 m μ
Complete system	-0.439	0.539
— DPIP	+ 0.025	-0.011
— ascorbate, DPIP	+ 0.012	-0.009
— chromatophores	0	0
Chromatophores heated at 95°C for 10 min	+ 0.008	0.039

The behavior of the chloroplasts in these reactions is the same as was previously observed³ for the photoreduction of TPN, DPN, indigo carmine, FMN and vitamin K₃. Thus, the chloroplast appears to be employing its usual reaction mechanisms in the present photoreductions.

As shown in Table 1, boiling the chloroplasts destroyed their ability to catalyze the photoreduction reactions in the presence of ascorbate and DPIP. The accompanying paper in this series¹⁴ shows that isolated chlorophylls are capable of catalyzing a photoreduction of both dyes in the presence of ascorbate and DPIP. Since the isolated chlorophylls can catalyze the reactions, the boiling process apparently either causes a change in the chlorophyll molecules, or more likely, through some physical process makes the chlorophyll unavailable to the reagents in solution. The latter could be due to a structural change in the chloroplasts or a covering of some specific reaction sites by chloroplast material released by the boiling process.

Photoreductions catalyzed by R. rubrum chromatophores. The ability of *R. rubrum* chromatophores to catalyze a photoreduction of methyl red and tetrazolium blue is shown by the data presented in Figs. 1 and 2. In order for the photoreduction to proceed, the presence of ascorbate and DPIP was required, since unlike the plant chloroplast, the bacterial system cannot catalyze a photoreduction unless some electron donor is added to the reaction system. On the basis of the chlorophyll content of the chloroplasts and the chromatophores used in the experiments, it is apparent that the chloroplasts were more effective in both reactions. However, as shown below, the activity of the chromatophore system was greatly stimulated by the addition of quinacrine. The ability of the ascorbate and DPIP couple to donate electrons to the dyes used in the present experiments agrees with previously published data, in which the ascorbate-DPIP couple was shown to support the photoreduction of DPN by *R. rubrum* chromatophores⁴. The strict dependence of the photoreductions upon the presence of both DPIP and ascorbate is shown in Table 2.

Table 3. Stimulation by quinacrine of the anaerobic photoreduction reactions catalyzed by chromatophores of *R. rubrum*. The composition of the complete system was the same as given for Table 2, with 0.2 mM quinacrine also present. The reaction time was decreased to 5 min. For experiment 2 the chromatophores were heated at 95°C for 10 min.

Conditions	Absorbancy change in 5 min Light minus dark	
	Methyl red 440 m μ	Tetrazolium blue 580 m μ
1. Complete system	-1.42	0.902
— quinacrine	-0.230	0.296
— DPIP	+ 0.003	0.071
— ascorbate, DPIP	+ 0.008	0.008
2. Complete, chromatophores heated	-0.060	0.024
— quinacrine	+ 0.030	0.020

As was the case with chloroplasts, heating the chromatophores destroyed their ability to catalyze both reactions. The inability of ascorbate alone to support the photoreactions agrees with the previously advanced hypothesis that although the ascorbate serves as the ultimate electron donor, it is the reduced form of the DPIP which interacts with the chlorophyll system, and the presence of excess ascorbate insures that the DPIP remains in the reduced form and allows the reactions to proceed ^{3,4}.

The addition of quinacrine to chromatophores of *R. rubrum* produces a marked inhibition of photosynthetic phosphorylation ¹⁵. In this phosphorylation process ATP is formed by reactions coupled with the recombination of the electrons from the primary reducing site with the oxidized product formed *via* the primary photochemical act of the chromatophore. Since such a recombination would not favor the demonstration of a net photoreduction of tetrazolium blue or methyl red, it was thought that addition of this compound might serve to stimulate the photoreduction reactions. The data presented in Table 3 show that such was the case, and marked stimulation was observed if quinacrine was present in the reaction system. Furthermore, quinacrine was not effective in the absence of DPIP, indicating that the quinacrine did stimulate the reaction already taking place, and the increase in activity was not due to the introduction of a new type of reaction catalyzed by the quinacrine. Again, heating the chromatophores served to remove almost all of the activity.

The illumination for the preceding experiments was accomplished with white light. Since quinacrine is structurally somewhat similar to the flavins and the flavins are capable of catalyzing photoreduction reactions in white light ¹⁶, it was necessary to perform the same type of experiment using red light which was not absorbed by the quinacrine. For a comparison, the effect of FMN upon the reaction run in both white and red light was examined. The results of these experiments are shown in Table 4. Concerning the photoreduction of tetrazolium blue, the presence of either FMN or quinacrine resulted in an increased rate of photoreduction in white light. Performing the experiment in red light, however, showed that the stimulating effect of quinacrine was

Table 4. The effect of quinacrine and FMN on the anaerobic photoreduction reactions of *R. rubrum* chromatophores using red and white light. The composition of the experimental system was the same as given in Table 2, with the exception of the bacteriochlorophyll concentrations which were $3.7 \mu\text{M}$ for the experiments using tetrazolium blue and $3.0 \mu\text{M}$ for the experiments with methyl red. Also present were $83 \mu\text{M}$ FMN and $100 \mu\text{M}$ quinacrine where indicated.

Conditions	Absorbancy change, light minus dark	
	Methyl red 440 m μ 5 min	Tetrazolium blue 580 m μ 10 min
<i>White light</i>		
Control	-0.163	0.156
+ quinacrine	-1.16	0.693
+ FMN	-0.167	0.674
+ quinacrine, FMN	-1.04	0.745
<i>Red light</i>		
Control	-0.140	0.287
+ quinacrine	-1.05	0.924
+ FMN	-0.030	0.255
+ quinacrine, FMN	-0.176	0.992
+ quinacrine and $166 \mu\text{M}$ FMN	-0.340	—

retained while FMN was inactive. Thus, the stimulation by FMN in white light could be ascribed to the FMN itself acting as the photocatalyst for the reaction, while quinacrine stimulated the photoreaction catalyzed by the chlorophyll of the chromatophore. When the experiments utilizing methyl red as electron acceptor are considered, it is seen that whereas the quinacrine behaved in the same fashion as with tetrazolium blue, FMN failed to stimulate the reaction in white light, and in red light it inhibited the reaction. Furthermore, addition of both quinacrine and FMN to the system revealed that the FMN acted as an antagonist to the quinacrine and decreased the usual activation observed with quinacrine. The reason for the inhibitory effect of FMN in the case of methyl red photoreduction is not known. FMN may be acting in a competitive manner with quinacrine in the activated methyl red photoreduction, but the necessary experiments required to support this conclusion have not yet been performed. As shown in the subsequent paper of this series¹⁴, quinacrine does not stimulate the photoreduction of either tetrazolium blue or methyl red when the photocatalyst is isolated chlorophyll or porphyrin. Thus, an explanation of its effect must be found in terms of the organized electron transport system found in the chromatophore.

The effect of quinacrine concentration upon both tetrazolium blue and methyl red photoreduction is shown in Fig. 3. Both reactions respond in a similar manner to changes in quinacrine concentration, indicating a common point of action for the quinacrine in both photoreactions. The concentrations of quinacrine active in these photoreactions are close to those which are effective in inhibiting photosynthetic phosphorylation in chromatophores of the same organism¹⁵.

Fig. 3. The effect of quinacrine concentration upon the photoreduction reactions. The composition of the reaction systems was the same as given in Table 2, except that for the experiment with methyl red the final concentration of bacteriochlorophyll was $3.0 \mu\text{M}$

Aside from quinacrine, other compounds are known to inhibit the photosynthetic phosphorylation process in bacterial chromatophores, notably antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO)^{4,17}. Using the same reasoning advanced above, addition of these compounds would be expected to increase the rate of the photoreduction reactions. As shown in Table 5, such was not the case, and the presence of these compounds served to slightly inhibit the photoreductions. For comparison, the effect of these compounds upon the photoreduction of DPN is included. In the case of DPN photoreduction the electron donor was succinate, which couples with the chromatophores *via* succinic dehydrogenase¹⁸. Of the three inhibitors, quinacrine was unique, since it inhibited only DPN photoreduction, while stimulating the other reactions. Both antimycin A and HQNO inhibited all three reactions, but only DPN photoreduction was strongly inhibited. There is not enough evidence available at present to explain these differences in terms of the electron transfer

Table 5. Effect of respiratory inhibitors upon the photoreductions catalyzed by *R. rubrum* chromatophores. The composition of the control systems was the same as given for Table 2 for methyl red and tetrazolium blue photoreduction. For the experiments on DPN photoreduction, the ascorbate and DPIP were replaced with 5 mM succinate. The inhibitor solutions added to the reaction system were all 5 mg per ml, and the antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) were dissolved in ethanol. Anaerobic conditions were employed.

Additions	Absorbance change, light minus dark		
	DPN 340 mμ, 10 min	Methyl red 440 mμ, 5 min	Tetrazolium blue 580 mμ, 5 min
None	0.194	-0.251	0.254
antimycin A, 0.5 mg	-0.009	-0.211	0.156
HQNO, 0.25 mg	0.025	-0.215	0.243
quinacrine, 0.5 mg	0.043	-1.08	0.777
antimycin A and quinacrine	—	-0.819	0.368
HQNO and quinacrine	—	-0.998	0.463

components of the chromatophore. If quinacrine stimulation of tetrazolium blue and methyl red photoreduction is due to its ability to prevent the cyclization of the electrons in the chromatophore, it is difficult to see why antimycin A and HQNO do not give the same response.

In comparing the photoreduction of DPN with the other two photoreactions, the differences in the two reaction systems employed became apparent. The succinate-supported photoreduction of DPN was much more sensitive to the respiratory inhibitors. Also, in comparing the abilities of both succinate and the ascorbate-DPIP couple to support DPN photoreduction, succinate was observed to give the better reaction. Furthermore, succinate could not support a photoreduction of methyl red and produced only a slow photoreduction of tetrazolium blue. Accordingly, there are unknown aspects of both systems which must first be clarified before the mode of action of these inhibitors can be ascertained.

Preliminary experiments have been performed to see if quinacrine was capable of stimulating the photoreductions catalyzed by chloroplasts. Although a few cases were observed in which there was slight activation, the general case was that quinacrine had no effect on the photoreductions. Accordingly, one significant difference between the chloroplasts and chromatophores is their response to quinacrine.

DISCUSSION

The photoreduction of methyl red and tetrazolium blue by spinach chloroplasts in the absence of any added electron donor shows the characteristics of a regular Hill reaction. However, a simpler reaction is taking place in the presence of ascorbate and DPIP. The information presented in the following paper¹⁴ shows that these dyes can be photoreduced by the ascorbate-DPIP couple in the presence of isolated chlorophylls or porphyrins, and there is no reason in the present case to rule out a direct photoreduction of the dyes by the chlorophyll system in the chloroplast. There is also no reason to rule out a direct reaction of the DPIP-ascorbate couple with the chlorophyll system. Indeed, in view of the data on the inability of DCMU to prevent the photoreduction of the dyes in the presence of the ascorbate-DPIP couple, a direct reaction of the couple with the chlorophyll seems most logical. Therefore, it would appear that the only part of the chloroplast utilized in these reactions is the photoactive chlorophyll system, making this reaction one of the most simple of the many photochemical reactions catalyzed by plant chloroplasts.

The ability of *R. rubrum* chromatophores to catalyze the same reactions as the plant chloroplasts again points up the basic similarity of the plant and bacterial systems. It has earlier been shown that with both chloroplasts and chromatophores a photooxidation of ascorbate and a corresponding photoreduction of DPN was possible³. This agrees with the concept that the bacterial chromatophores practice a more primitive photosynthetic process, and under certain conditions the plant chloroplast can be forced into the same pattern^{3,19}.

One difference in the two systems investigated is the manner in which quinacrine affects the photoreductions catalyzed by chloroplasts and chromatophores. From the information presented in the following paper¹⁴ it is

seen that quinacrine does not stimulate the corresponding photoreductions catalyzed by isolated chlorophylls, and therefore it does not affect the chromatophore by influencing the processes associated with the activated chlorophyll system. Therefore, it is most likely active by virtue of its ability to prevent the cyclization of electrons *via* the electron transport components of the chromatophore. However, further experimentation is required, since the differences found in the response of chromatophores to quinacrine and the respiratory inhibitors antimycin A and HQNO still cannot be explained in a satisfactory manner.

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