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PHOTOOXIDATIONS CATALYZED BY PLANT AND BACTERIAL EXTRACTS AND BY RIBOFLAVIN-5'-PHOSPHATE

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The first simplification of the process of photosynthesis was accomplished by HILL[1] when he successfully reacted certain oxidants with the photochemical reducing system generated by chloroplasts (photochemical [H]*). These oxidants, called Hill reagents, were reduced photochemically, with the simultaneous evolution of oxygen by intact chloroplasts. Thus, since oxygen was evolved, the chloroplasts effected a net reduction in the system under the influence of light. Recently, chloroplasts have been shown to catalyze the photooxidation of several compounds, *i.e.*, reduced glutathione[2], the dye Janus Green B[3], ascorbic acid[4,5], manganous ion[6] and TPNH[7]. Also, illumination of chloroplasts in the absence of added Hill reagent results in an uptake of oxygen[8]. GOOD AND HILL[9] ascribe this to the reduction of some autooxidizable substance (flavin nucleotide) by photochemical [H], followed by an oxidation of the reduced compound by oxygen to form hydrogen peroxide. Thus, it is another manifestation of the Hill reaction, with oxygen serving as the oxidant. The hydrogen peroxide produced in the reaction can serve in further oxidation reactions, and many of the photooxidations catalyzed by chloroplasts can be explained in this manner[4,5,6]. In some cases, however, the photochemical [OH] system may be involved[5].

The addition of ascorbic acid and DPIP to plant leaf homogenates or chloroplasts results in a rapid oxidation of ascorbate by oxygen in the light[5]. Balance studies indicate the oxidation of ascorbate proceeds via two pathways, one resulting from

* Photochemical [H] system refers to the reducing power generated by illuminated chloroplasts during photosynthesis, with no attempt to describe the components of the system. Likewise, photochemical [OH] system refers to the system which liberates oxygen during normal photosynthesis. In addition the following abbreviations will be used: DPIP, 2,6-dichlorophenolindophenol (2,6-dichlorobenzenoneindophenol); $DPIPH_2$, reduced 2,6-dichlorophenolindophenol; TPNH, reduced triphosphopyridine nucleotide, IC, indigo carmine; chph, chlorophyll, FMN, riboflavin-5'-phosphate.

References p. 123.

the peroxide generated by the reaction of oxygen with the photochemical [H] system, and the other involving the photochemical [OH] system directly, as shown in the following scheme, which is essentially that of VERNON AND KAMEN[5]:

$$1.\quad 4\,H_2O \xrightarrow[\text{chloroplast}]{h\nu} 4\,[H] + 4\,[OH]$$

$$2.\ (a)\ 4\,[H] + 2\ \text{flavin} \longrightarrow 2\ \text{flavin}\,H_2$$

$$(b)\ 2\ \text{flavin}\,H_2 + 2\,O_2 \longrightarrow 2\ \text{flavin} + 2\,H_2O_2$$

$$(c)\ 2\,H_2O_2 + 2\,DPIPH_2 \longrightarrow 4\,H_2O + 2\,DPIP$$

$$3.\quad 4\,[OH] + 2\,DPIPH_2 \longrightarrow 4\,H_2O + 2\,DPIP$$

$$4.\quad 4\,DPIP + 4\ \text{ascorbate} \longrightarrow 4\,DPIPH_2 + 4\ \text{dehydroascorbate}$$

$$4\ \text{ascorbate} + 2O_2 \longrightarrow 4\ \text{dehydroascorbate} + 4\,H_2O.$$

Reaction 3 of this scheme represents an interception of the photochemical [OH] system with $DPIPH_2$, and would be an oxidative counterpart of the Hill reaction.

WESSEL[10] in his investigation of the photooxidation described above has advanced the following as an alternate hypothesis to explain the results:

$$\text{chph} \xrightarrow{h\nu} \text{chph}^* \tag{5}$$

$$\text{chph}^* + O_2 \longrightarrow \text{chph}^* \ldots O_2 \tag{6}$$

$$\text{chph}^* \ldots O_2 + DPIPH_2 \longrightarrow \text{chph} + DPIP + H_2O_2 \tag{7}$$

$$DPIP + \text{ascorbate} \longrightarrow DPIPH_2 + \text{dehydroascorbate} \tag{8}$$

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}\,O_2. \tag{9}$$

This scheme differs from the former in that it requires no organization of chlorophyll in the chloroplasts, does not involve the photolysis of water, and thus may reflect a property of molecular chlorophyll which is not involved in photosynthesis *per se*. The supporting evidence for WESSEL's scheme was the ability of chlorophyll *a* in alcohol solution to catalyze the photo-oxidation of ascorbate in the presence of DPIP, resulting in an accumulation of H_2O_2. The present investigation was undertaken to examine the reaction further in view of the two reaction mechanisms advance. In the course of the experimentation, it was found that FMN would catalyze the oxidation of $DPIPH_2$ and reduced cytochrome *c*, being better in this respect than either chloroplasts or chlorophyll *a*.

METHODS

Leaf homogenates and chloroplasts were prepared from spinach and sugar beet leaves as reported previously[5]. The preparation of sonic extracts of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* has been previously described[11]. Chlorophyll content of leaf homogenates was determined by the method of ARNON[12]. Bacterial chlorophyll content of bacterial extracts was determined by the method described by VAN NIEL AND ARNOLD[13]. All experiments were carried out with conventional manometric equipment and a Beckman DU spectrophotometer. For manometric experiments involving DPIP and ascorbic acid, the experimental vessels were maintained at 30° C, and contained the appropriate reagents in the main compartment with KOH in the center well. Total volume was 3 ml. Dark controls were included in each experiment by enclosing a vessel of similar composition with aluminium foil. For further details see ref.[5]. Light was supplied by a bank of tungsten bulbs suspended above the manometers, which resulted in a light intensity of approximately 200 foot-candles at the level of the reaction flasks.

For the experiments in which the rate of photo-oxidation of exactly reduced DPIP was determined spectrophotometrically, the 3.0 ml of test system was contained in test tubes which were exposed at 20 cm by a 200 watt tungsten bulb backed by a reflector. The light was filtered through 5 cm of water to prevent heating of the test system. Light intensity was approximately

References p. 123.

800 foot-candles. For each experiment an identical system covered by aluminium foil constituted the dark control. Immediately following the illumination period, both tubes were plunged into an ice bath to decrease oxidation of the $DPIPH_2$ between illumination and determination of optical density at 590 mμ. The values reported are the optical density differences between the light and dark tubes. For experiments on cytochrome *c*, the same procedure was used, except that the optical density determinations were made at 550 mμ.

For experiments involving exactly reduced DPIP, the reduced dye was prepared by exact titration with ascorbic acid. Cytochrome *c* was reduced by the addition of 1.1 equivalents of ascorbic acid. After 30 minutes at room temperature the solution was gently aerated to remove excess ascorbic acid. Chlorophyll *a* was prepared by chromatography from a dried petroleum ether solution of crude chlorophyll, using 20% diethyl ether in petroleum ether for development of the powdered sugar column.

The DPIP used in this investigation was obtained from Eastman Organic Chemical Co., Rochester, N.Y., riboflavin-5′-phosphate and cytochrome *c* from Sigma Chemical Co., St. Louis, Mo., and catalase, peroxidase, and ascorbic acid from Nutritional Biochemical Corp., Cleveland, Ohio. Other chemicals were reagent grade compounds commercially available.

RESULTS

In the presence of excess ascorbate, any $DPIPH_2$ photooxidized was immediately rereduced by the ascorbate, effectively presenting the system with completely reduced DPIP at all times. This system, called the DPIP-ascorbate couple, behaved differently from the system containing exactly reduced DPIP. Consequently the results obtained from photooxidation experiments on these two systems will be considered separately.

Photooxidation of DPIP-ascorbate couple

In their investigation of the mechanism of oxygen uptake by illuminated chloroplasts devoid of any Hill reagent, GOOD AND HILL[9] were able to show that several redox

TABLE I

STIMULATION OF DPIP-ASCORBATE PHOTO-OXIDATION

Manometric vessels contained, in a total volume of 3 ml, 50 μmoles phosphate buffer pH 7.0, 1 μmole DPIP, 20 μmoles ascorbic acid, sugar beet leaf homogenate equivalent to 110 μg of chlorophyll, and 0.1 μmole of the designated addenda. See METHODS for operational procedure.

Substance added to basic system	*Oxygen consumed in 45 min (light minus dark)* mm^3
None	61
FMN	142
1.2-Naphthoquinone-4-sulfonic acid	103
Cresyl violet	33
Indigo carmine	144
Toluyl blue	42
Methyl green	78
Benzyl violet	39
Methyl blue	10
Phenosafrinin	53
Gallocyanin	6
Brilliant cresyl blue	18
Indulin scarlet	32
Neutral red	39
Thionin	13
Litmus	62
Janus green B	22
Manganous chloride	65

References p. 123.

substances capable of being oxidized by molecular oxygen, particularly riboflavin and flavin nucleotides, stimulated oxygen consumption by illuminated chloroplasts. Accordingly, the effect of such additions upon the rate of oxygen uptake by illuminated leaf homogenate and the DPIP-ascorbate couple was investigated. Table I presents the results of a survey of many reagents in this regard. Stimulation was accomplished not only by FMN, as expected, but also by IC and 1,2-naphthoquinone-4-sulfonic acid, a vitamin K analog. The most efficient compounds were FMN and IC, both low potential compounds capable of reoxidation by oxygen. Presumably both compounds were reduced via the photochemical [H] system, followed by an oxidation by molecular oxygen. The compound or compounds responsible for the initial reduction of the FMN and IC are not known, but since the reaction was rapid they would have a potential at least as low as the dyes themselves.

The ability of FMN and IC to stimulate the photooxidation of the DPIP-ascorbate couple was examined for both leaf homogenate and *R. rubrum* extract. Table II shows that either of these compounds at a final concentration of 10^{-5} *M* stimulated the photooxidation catalyzed by leaf homogenate, but both were virtually inactive with the bacterial system. Higher concentrations of FMN gave an apparent stimulation in the case of *R. rubrum* extracts, but this was due to the ability of FMN itself at concentrations greater than 10^{-5} *M* to catalyze the reaction. At higher concentrations, IC inhibited the reaction for both plant and bacterial extracts.

TABLE II

STIMULATION OF DPIP-ASCORBATE PHOTO-OXIDATION BY FMN AND IC

Manometric vessels contained, in a total volume of 3 ml, 50 μmoles phosphate buffer, pH 7.0, 1 μmole DPIP, 20 μmoles ascorbic acid, and photosynthetic material as noted. When present, the final concentration of FMN or IC was 10^{-5} *M*. For the control experiments the leaf of bacterial extract was heated at 70° C for 15 minutes.

Photosynthetic material	*Addition*	*Oxygen consumed in 60 minutes* mm^3
Sugar beet leaf homogenate containing 0.047 mg chph	none	57
Sugar beet leaf homogenate containing 0.047 mg chph	FMN	162
Sugar beet leaf homogenate containing 0.047 mg chph	IC	122
Sugar beet leaf homogenate containing 0.047 mg chph (heated)	FMN	12
Sugar beet leaf homogenate containing 0.047 mg chph (heated)	IC	7
R. rubrum sonic extract containing 0.134 mg chph	none	137
R. rubrum sonic extract containing 0.134 mg chph	FMN	166
R. rubrum sonic extract containing 0.134 mg chph	IC	106
R. rubrum sonic extract containing 0.134 mg chph (heated)	FMN	11
R. rubrum sonic extract containing 0.134 mg chph (heated)	IC	—9
Chlorophyll *a*, 0.50 mg	none	9
Chlorophyll *a*, 0.50 mg	FMN	—11
Chlorophyll *a*, 0.50 mg	IC	9

References p. 123.

The stimulation by FMN and IC observed in the case of the leaf homogenate indicates some involvement of these compounds in the photooxidation of the DPIP-ascorbate couple, most probably by means of reaction 2 (b). The substitution of IC for FMN in this reaction was plausible, since IC is known to react with physiological reducing systems[14]. The failure or these compounds to stimulate the bacterial system was surprising, since in all other respects the bacterial extracts resembled the leaf homogenate in the photooxidation of the DPIP-ascorbate couple[5]. Sonic extracts of *Rsp. spheroides* also showed no stimulation by FMN or IC, and gave results identical to *R. rubrum* extracts. This behavior of the bacterial extracts indicates a difference in the photochemical [H] systems in plants and photosynthetic bacteria.

It was possible to substitute other dyes for DPIP in the above reaction, as shown in Table III. However, those compounds which substituted best are closely related structurally to DPIP. Since DPIP was one of the most active, it was used for all experiments, so that comparison could be made with previously reported data[5].

TABLE III

SUBSTITUTION OF OTHER DYES FOR DPIP

Manometric experiments were conducted using the same test system as for Table I, with 0.1 μmole FMN added. The compounds substituted were added in amounts equimolar to DPIP.

Substitute for DPIP	*Oxygen consumed in 40 min (light minus dark)* mm^3
None	153
Sodium benzenoneindo-2-methylphenol	84
Sodium benzenoneindo-2-methyl-5-*iso*propylphenol	160
Sodium benzenoneindo-3-sulfonaphthol	73
Sodium 2,6-dichlorobenzenoneindo-3-methylphenol	132
Sodium benzenoneindo-3-chlorophenol	95
Sodium benzenoneindo-3-methylphenol	114
Sodium benzenoneindophenol	141
Sodium 2,6-dibromobenzenoneindo-3-methoxyphenol	120
Sodium 2,6-dichlorobenzenoneindo-3-chlorophenol	152

Photooxidation of exactly reduced DPIP

Both reaction mechanisms proposed above to explain the photooxidation of ascorbate involve $DPIPH_2$ as the reagent directly photooxidized. Accordingly, the effect of chloroplasts, bacterial extracts and isolated chlorophyll on the photooxidation of exactly reduced DPIP was investigated. The chloroplasts used in these experiments were prepared from commercially available spinach and were not able to carry out the Hill reaction. Such chloroplasts were used because in such preparations the oxygen-evolving capacity would probably be impaired and the demonstration of the photochemical [OH] by a direct oxidation reaction would be more likely. Also, intact chloroplasts capable of performing the Hill reaction catalyze the reduction of DPIP, just the reverse of the reaction studied.

In the early experiments it was difficult to demonstrate any consistent photooxidation of the reduced DPIP with either chlorophyll or chloroplasts in an aqueous medium. Addition of FMN to the system caused a rapid photooxidation, but it was soon evident that the oxidation was almost exclusively due to the added FMN. Therefore, the ability of FMN alone to catalyze the reaction was studied, and Fig. 1

References p. 123.

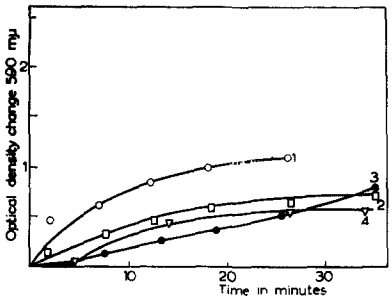

Fig. 1. Photo-oxidation of $DPIPH_2$ by various sensitizers. For each experiment there was an illuminated and non-illuminated tube containing, in 3 ml, 1 μmole exactly reduced DPIP, 30 μmoles phosphate buffer, pH 7.0 and 0.1 μmole of the designated sensitizer. In the case of chloroplasts, an amount equal to 0.1 μmole of chlorophyll was added. The values given are optical density differences between the exposed tube and the dark control. Tube 1, FMN; tube 2, chlorophyll *a*; tube 3, spinach chloroplasts; tube 4, no sensitizer added.

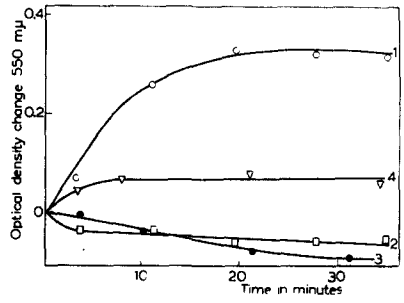

Fig. 2. Photooxidation of reduced cytochrome *c* by various sensitizers. The experimental conditions are the same as given for Fig. 1, with reduced DPIP being replaced by 0.15 μmoles of reduced cytochrome *c*. Tube 1, FMN; tube 2, chlorophyll *a*; tube 3, spinach chloroplasts; tube 4, no sensitizer added.

presents the activities of these various reagents in the reaction. There was some photooxidation by chlorophyll alone, but FMN was by far the most effective in this regard. Chloroplasts alone inhibited the self-photooxidation of $DPIPH_2$.

Since DPIP can substitute for cytochrome *c* in various enzymic reactions, the possibility that cytochrome *c* could be photooxidized by the above reagents was investigated. Fig. 2 presents the results so obtained. FMN was the only compound capable of photooxidizing cytochrome *c*. The apparent reduction caused by chlorophyll and chloroplasts was in reality an inhibition of the oxidation rate in the light when compared to the dark control.

Since the majority of known photooxidations in aqueous solution involve hydrogen peroxide, the effect of catalase and peroxidase upon the reaction was determined. Fig. 3 shows that a marked stimulation of $DPIPH_2$ photooxidation was caused by the addition of catalase to the FMN system. After an initial lag period, both chlorophyll and chloroplasts demonstrated a rate of photooxidation which was greater than the control. Peroxidase has also been tried in the same systems, and gave essentially the same result as catalase, causing a marked stimulation in only the FMN system. However, when catalase and peroxidase were added to the systems containing cytochrome *c* in lieu of DPIP, there was no significant difference when compared to the systems containing no catalase. This was not surprising, since reduced cytochrome *c* is not oxidized peroxidatively by peroxidase or catalase, but only by a specific enzyme, cytochrome *c* peroxidase. Thus, it would appear that the photooxidation of $DPIPH_2$ and cytochrome *c* is due to activated FMN, and the addition of catalase allows an additional means of oxidizing $DPIPH_2$ peroxidatively by the hydrogen peroxide accumulating.

From the above results, it is apparent that chlorophyll itself cannot catalyze the photooxidation of $DPIPH_2$ in aqueous solutions to any appreciable extent. Since WESSEL's experiments were conducted using ethanol as the solvent, the ability of chlorophyll *a* to catalyze the photo-oxidation at varying ethanol concentrations was determined. Fig. 4 shows that at high ethanol concentrations, chlorophyll *a*

References p. 123.

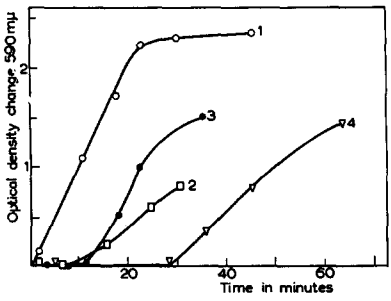

Fig. 3. Photo-oxidation of reduced DPIP by various sensitizers in the presence of catalase. The experimental conditions are the same as given for Fig. 1, with 3 mg catalase added to each tube. Tube 1, FMN; tube 2, chlorophyll *a*; tube 3, spinach chloroplasts; tube 4, no sensitizer added.

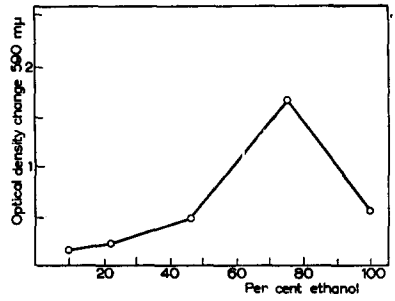

Fig. 4. Effect of ethanol concentration on the photo-oxidation of $DPIPH_2$ by chlorophyll *a*. The tubes contained, in 3 ml, 1 μmole $DPIPH_2$, 10 μmoles phosphate buffer pH 7.0, except in the case of 100% ethanol, and 0.2 mg chlorophyll *a*. A dark control was employed for each concentration of ethanol.

readily catalyzed the photo-oxidation of $DPIPH_2$, but this ability approached zero as the alcohol concentration approached zero. These data indicate that alcohol is intimately involved in the photo-oxidation as studied by WESSEL, most probably as a hydrogen donor in the reaction. Thus, the conclusions reached by WESSEL would not apply to the photo-oxidation of ascorbate via DPIP with chloroplasts in aqueous media.

In the absence of excess ascorbic acid, $DPIPH_2$ was only slightly photo-oxidized by chloroplasts, while the addition of excess ascorbate caused an immediate photo-oxidation of the added ascorbate. As shown previously[5], removal of the DPIP from the DPIP-ascorbate system caused a complete loss of activity showing that the ascorbate was being oxidized through the intervention of DPIP. The rates of photo-oxidation under these two conditions (with exactly reduced DPIP and with excess ascorbate) were compared using both leaf homogenate and cell-free extracts of *R. rubrum* and *Rsp. spheroides*. The results given in Table IV show the rate of photo-

TABLE IV

COMPARISON OF PHOTO-OXIDATION RATES OF DPIP-ASCORBATE COUPLE AND EXACTLY REDUCED DPIP

For the experiments with excess ascorbate, the composition was the same as for Table I with no FMN present. Leaf homogenate or bacterial extract was added as noted. For the experiments with reduced DPIP, ascorbic acid was omitted and the DPIP added was exactly reduced with ascorbic acid just prior to the experiment. The reduced DPIP oxidized was calculated from the optical density difference at 590 mμ between light and dark tubes. With excess ascorbate present, the ascorbate oxidized was calculated by titration with standard DPIP solution. Method of exposure is given in METHODS.

Photosynthetic material	*Chlorophyll content* mg	*Ascorbate oxidized with excess ascorbate present* μmoles (*A*)	*DPIP oxidized with exactly reduced DPIP* μmoles (*B*)	*Ratio A/B*	*Exposure time*
Sugar beet leaf homogenate	0.046	2.21	0.04	55	20 min
Rsp. spheroides extract	0.090	2.10	0.04	52	6 min
R. rubrum extract	0.065	2.64	0.15	17	20 min

References p. 123.

oxidation with excess ascorbic acid to be greater by a factor of at least 17 in all cases, which emphasizes the necessity of keeping DPIP completely reduced to allow the photo-oxidation to proceed at the maximum rate.

DISCUSSION

The data obtained on the photo-oxidation of $DPIPH_2$ with and without excess ascorbate suggest that while the mechanism proposed by WESSEL[10] may well be operative in ethanolic solutions of $DPIPH_2$, it does not apply to $DPIPH_2$ in aqueous media. This conclusion is reached upon consideration of the following points.

(1) Whereas isolated chlorophyll *a* catalyzed the photo-oxidation of $DPIPH_2$ in ethanolic solutions, this ability decreased rapidly as the water content of the system increased. It would be difficult to ascribe the photo-oxidation of DPIP-ascorbate couple by chloroplasts in aqueous media to a mechanism that operates in alcohol solutions.

(2) The photo-oxidation of the DPIP-ascorbate couple by leaf homogenates and bacterial extracts was at least 17 times as great as the rate of $DPIPH_2$ photo-oxidation. This difference in rates is difficult to explain in terms of a mass action effect due to the decreased concentration of $DPIPH_2$ as the oxidation proceeds in the absence of excess ascorbate, since only a fraction of the $DPIPH_2$ available was oxidized during the illumination period. Rather, it is probably due to the fact that when oxidized DPIP was allowed to accumulate in the system, it was reduced by the photochemical [H] system in the regular Hill reaction, resulting in a sort of "short circuit" between the photochemical [H] and [OH] systems.

(3) The photo-oxidation of the DPIP-ascorbate couple was stimulated by the addition of FMN or IC to the system containing leaf homogenate, but not when chlorophyll *a* was substituted for the chloroplasts. This is best explained in terms of more efficient interaction of oxygen with the photochemical [H] system when FMN and IC were present.

(4) If WESSEL's theory were correct and applied to chlorophyll in the organized chloroplast, it is difficult to see how one would expect to obtain a Hill reaction at all with DPIP, since the reduction of the DPIP by the photochemical [H] system would be countered by the oxidation of the dye by the mechanism he proposed.

The photo-oxidation of both $DPIPH_2$ and reduced cytochrome *c* by FMN presents a reaction that should be considered in photochemical studies involving flavin nucleotides. The photo-oxidation takes place at an appreciable rate at flavin concentrations above $10^{-5}\,M$, and thus many of the photochemical oxidations involving high light intensities and long illumination periods, could well be due to the presence of FMN or other flavin nucleotides. The mechanism of the photo-oxidation is not known, but probably involves the interaction of the activated flavin with $DPIPH_2$ according to the following scheme:

$$FMN + h\nu \longrightarrow FMN^* \quad (10)$$

$$FMN^* + DPIPH_2 \longrightarrow FMNH_2 + DPIP \quad (11)$$

$$FMNH_2 + O_2 \longrightarrow FMN + H_2O_2 \quad (12)$$

The peroxide generated could then oxidize the $DPIPH_2$ peroxidatively in the presence of catalase or peroxidase, but would not oxidize the cytochrome *c*. This oxidation of

References p. 123.

cytochrome *c* by FMN is distinct from the photo-oxidation catalyzed by sonic extracts of *R. rubrum*[11], since the system responsible for the latter reaction is heat-labile, and is presumably connected to the bacterial photosynthetic mechanism.

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SUMMARY

1. The photo-oxidation of the 2,6-dichlorophenolindophenol-ascorbate couple by sugar beet leaf homogenate is stimulated by the addition of FMN, indigo carmine or 1,2-naphthoquinone-4-sulfonic acid. This stimulation is probably due to interaction of these compounds with the photochemical [H] system.

2. The 2,6-dichlorophenolindophenol-ascorbate couple was photo-oxidized at least 17 times as fast as exactly reduced 2,6-dichlorophenolindophenol by sugar beet leaf homogenate or sonic extracts of *R. rubrum* and *Rsp. spheroides*.

3. Reduced 2,6-dichlorophenolindophenol was photo-oxidized by chlorophyll *a* in ethanolic solutions, but the rate fell off rapidly as the water content increased in the system.

4. Reduced 2,6-dichlorophenolindophenol and reduced cytochrome *c* were photo-oxidized rapidly by FMN in aqueous media. The photo-oxidation of reduced 2,6-dichlorophenolindophenol was further stimulated by added catalase or peroxidase. These reagents did not affect cytochrome *c* photo-oxidation.

5. These data are discussed in relation to two proposed mechanisms for oxidation of the 2,6-dichlorophenolindophenol-ascorbate couple by chloroplasts.

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