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# Pyrone derivatives: effective inhibitors of photosynthetic electron flow system

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The inhibitory effects of the pyrone derivatives, 6-($\omega$)-decenyl-2,3-dimethyl-$\gamma$-pyrone (DDP) and 6-farnesyl-2,3-dimethyl-$\gamma$-pyrone (FDP), on the photosynthetic electron flow system was investigated using the blue-green alga *Anabaena variabilis* and the green alga *Chlorella pyrenoidosa*.

Both reagents inhibited photosynthesis in intact cells; 50% inhibition occurred at $2.7 \times 10^{-5}$ M with DDP and at $4.3 \times 10^{-6}$ M with FDP in *Anabaena* photosynthesis. The reagents suppressed the photosystem II reaction [water to 2,6-dichlorophenol indophenol (DCIP)] of *Anabaena* membrane fragments, but were far less inhibitory on the photosystem I reaction ($DCIPH_2$ to methyl viologen). The kinetics of the fluorescence induction indicated that the reagents do not block Q-reduction, but do suppress the oxidation of reduced Q indirectly. Oxygen evolution under repetitive flashes at a low repetition rate (5 Hz) was insensitive to the reagents even at concentrations which induced more than 50% inhibition. These results are evidence that DDP and FDP inhibit the plastoquinone reaction by slowing down its turnover rate.

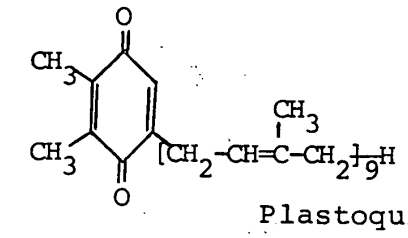
The advantages of pyrone derivatives are that they are inactive in the oxidation-reduction reaction and do not quench the fluorescence of chlorophyll in vivo.

**Key words:** Pyrone derivative — Photosynthesis inhibitor — Plastoquinone.

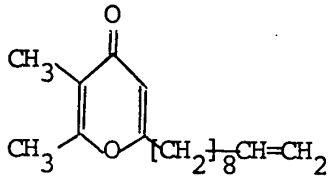
Reagents that inhibit the plastoquinone site in the photosynthetic electron flow system have been developed by finding active compounds chemically analogous to plastoquinones. The efforts of Trebst and his colleagues (*2*, *15*) revealed a potent inhibitor of this type, DBMIB, which commonly has been used to block the plastoquinone site in the photosynthetic electron flow system.

Besides DBMIB, many other types of quinone derivatives inhibit the plastoquinone site (*3*, *13*). However, these compounds themselves are reactive in the oxidation-reduction reaction. This reactivity induces an additional electron transfer reaction when they are present in a high concentration, so that their effects become more complex (*13*). This is due to their having the quinone structure. If we replace the quinone structure with a form that is inactive in oxidation-

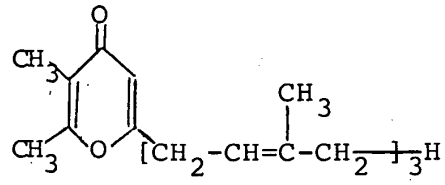
Abbreviations: DBMIB, dibromothymoquinone; DCIP, 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DDP, 6-($\omega$)-decenyl-2,3-dimethyl-$\gamma$-pyrone; FDP, 6-farnesyl-2,3-dimethyl-$\gamma$-pyrone; MV, methyl viologen.



Plastoquinone



6-(ω)-Decenyl-2,3-dimethyl-γ-pyrone (DDP)



6-Farnesyl-2,3-dimethyl-γ-pyrone (FDP)

Fig. 1. *Chemical structures of plastoquinone, DDP and FDP.*

reduction, but is analogous to benzoquinone, the reagent becomes a more adequate inhibitor at the plastoquinone site. The pyrone structure may be one candidate that satisfies this requirement, like the dinitrophenol-ether of halogenated nitrothymol which inhibits the oxidation of reduced Q (*16*). A preliminary experiment done by Takahashi and his co-workers (*18*) with pyrone derivatives indicated that these compounds block the respiratory electron flow, probably at the ubiquinone site. To determine what action the pyrone derivatives have on the photosynthetic electron flow system, we examined the effects of two of them DDP and FDP (Fig. 1), on the photosynthetic electron flow systems in the green alga *Chlorella pyrenoidosa* and in the blue-green alga *Anabaena variabilis*. The results reported here indicate that both pyrone derivatives inhibit electron flow at the plastoquinone site and that they do not induce any additional reaction even at concentrations as high as $10^{-3}$ M.

## Materials and methods

The blue-green alga *Anabaena variabilis* (M-2) and the green alga *Chlorella pyrenoidosa* (C-106) were obtained from the Algal Collection at the Institute of Applied Microbiology, University of Tokyo and were grown autotrophically under continuous illumination (1,600 lux) at 26°C. Air containing 0.5% $CO_2$ was supplied continuously. Growth media MDM and M-1-N (cf. *17*) were used for the cultures of *Anabaena variabilis* and *Chlorella pyrenoidosa*, respectively. Cells at the late log growth phase or in the early linear growth phase were used for all experiments.

Membrane fragments of *Anabaena variabilis* were prepared by essentially the same method as that described by Fujita and Suzuki (*6*).

The photosynthetic activity of intact cells under continuous and repetitive flash illumination was measured as $O_2$-evolution with a Clark-type $O_2$-electrode as described previously (*10*); reaction temperature, 26°C. Cells were suspended in the culture medium described above. Continuous illumination was supplied by a halogen lamp (Ushio JC-12-130L) at an intensity of $6\times10^5$ erg/cm$^2$·sec. The flash source was a Sugawara Stroboscope MS-230 with an intensity of $3.8\times10^3$ erg/cm$^2$·flash.

The DCIP-Hill activity of the membrane fragments of *Anabaena variabilis* was measured as described previously (*9*). The reaction mixture (2.0 ml) contained 0.1 mM DCIP, 1 mM $MgCl_2$, 50 mM phosphate buffer (pH 7.0), 0.6 M sucrose and membrane fragments equivalent to 8.2 $\mu$M chlorophyll *a*. The reaction was followed by an absorption decrease at 600 nm under white light ($8.6\times10^6$ erg/cm$^2$·sec) at room temperature (21°C). Photosystem I-induced $O_2$-uptake was measured with the same $O_2$-electrode system used for the photosynthesis measurements. The reaction mixture (3.0 ml) contained 5 mM $MgCl_2$, 50 mM Tricine buffer (pH 8.0), 0.6 M sucrose and membrane fragments equivalent to 1.9 $\mu$M chlorophyll *a*.

The kinetics of fluorescence induction were measured with a laboratory-made apparatus. Excitation light (585 nm, $2\times10^3$ erg/cm$^2$·sec) was obtained from a Bausch & Lomb grating monochromator. Fluorescence was detected with a Hamamatsu TV R446 photomultiplier; a Toshiba VR 69 sharp cut filter was used as protection against the excitation light. Signals were amplified by a San-ei 6-L-5 DC amplifier and memorized by a Riken Denshi TCB 2000 transient converter.

DDP and FDP were synthesized by the method of Date et al. (*4*). DCMU was kindly supplied by Hodogaya Chemical Co. DCIP and MV were obtained from Tokyo Kasei Co.

## Results and discussion

*Inhibition of photosynthesis*

Both DDP and FDP inhibited the photosynthesis of *Anabaena variabilis* (Fig. 2 and Table 1), but only DDP acted on the photosynthesis of *Chlorella pyrenoidosa*; inhibition required a high concentration of the reagents. The concentrations needed to induce 50% inhibition were two (FDP) or three orders (DDP) above the DCMU concentration (Fig. 2), but were almost the same as that of DBMIB ($10^{-5}$ M) in *Anabaena* photosynthesis. FDP (Fig. 2, crosses and Table 1) and DDP (Table 1) inhibited the DCIP-Hill reaction of *Anabaena* membrane fragments. The effects of FDP and DDP on the Hill reaction were two and three times stronger, respectively, than on photosynthesis. In the membrane system, pyrone derivatives probably reach their targets more easily than in intact cells; thus, the observed photosynthesis inhibition may be attributed primarily to their action in blocking electron flow in the manner of quinone derivatives. The quinone derivatives cause an additional oxidation-reduction reaction at a high concentration (*2*). However, the pyrone derivatives do not; their inhibition of the DCIP-Hill reaction followed a simple concentration dependency.

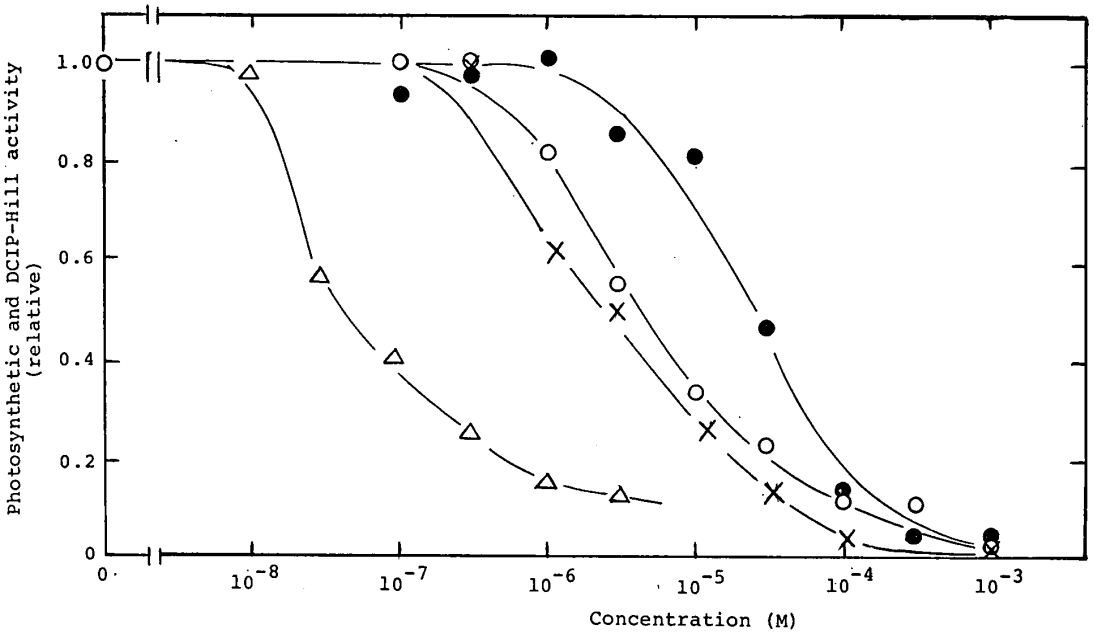



Fig. 2. *Inhibitory effects of DDP, FDP and DCMU on photosynthesis in intact cells and on the DCIP-Hill reaction in membrane fragments of* Anabaena variabilis. Relative activities are plotted against concentrations of inhibitors; closed circles: DDP-, open circles: FDP- and open triangles: DCMU-effect on photosynthesis. Cell concentrations were equivalent to 1.92 μM chlorophyll *a* for DDP and FDP and 1.20 μM for DCMU. Control activities were 253 and 308 μmoles $O_2$ evolved/mg chlorophyll *a*·hr in the DDP and FDP experiments, and in the DCMU experiments, respectively. The FDP effect on the DCIP-Hill reaction is shown with crosses. The concentration of membrane fragments was equivalent to 8.2 μM chlorophyll *a*. The activity of the control was 109 μmoles DCIP reduced/mg chlorophyll *a*·hr.

The effective concentration was lower with FDP than with DDP in *Anabaena* photosynthesis, as in inhibition of the DCIP-Hill reaction by membrane fragments (Table 1). However, *Chlorella* photosynthesis was inhibited only by DDP. The two pyrone derivatives differ only in the $C_3$ side chain; DDP has ω-decenyl whereas FDP has a farnesyl chain (Fig. 1). Thus, the difference in the activities of the two derivatives may be attributed to the structure of the side chain. Inhibitory activity and lipophilicity are correlated in quinone derivatives (*13*). Lipophilicity primarily is due to the structure of the side chains or substituted groups and may determine their solubility in thylakoid membranes in which the target(s) is present. If the same relationship holds for pyrone derivatives, the difference in inhibitory activities

Table 1 *Inhibitory effects of FDP and DDP on photosynthesis in intact cells and on the DCIP-Hill reaction in membrane fragments of* Anabaena variabilis

| | Concentrations for 50% inhibition (μM) | |
|---|---|---|
| | FDP | DDP |
| Photosynthesis | 4.6 | 27 |
| DCIP-Hill activity | 2.3 | 8.6 |

must be due mainly to their solubilities in thylakoid membranes. Cytoplasmic membranes and, in the case of *Chlorella*, chloroplast envelopes may constitute another barrier against the penetration of exogenous reagents and, thus, produce the difference in the effects of the added reagents.

*Determination of the inhibition site*

To determine the site inhibited by DDP and FDP, we compared their effects on the DCIP-Hill reaction and photosystem I-dependent $O_2$-uptake (Table 2). As described above, both derivatives are potent inhibitors of the Hill reaction (see also Table 1); complete inhibition occurs at $3\times10^{-4}$ M with both. However, the photosystem I reaction measured as $O_2$-uptake was far less sensitive; even when the Hill reaction was completely suppressed, inhibition was less than 30%. Membrane fragments of this type reduce most of the DCIP under the action of photosystem II alone (*5*), and the reduced DCIP feeds electrons to photosystem I via plastocyanin (*9*). Thus, our results indicate that the site(s) inhibited by these derivatives is located in the electron flow system before plastocyanin. The slight inhibition of the photosystem I reaction suggests that reduced DCIP feeds electrons to reaction center I, not only *via* plastocyanin but also partly *via* the component acting before plastocyanin, which is sensitive to pyrone derivatives.

A more detailed determination of the inhibition-site was made with the kinetics of the fluorescence induction observed at room temperature. Chlorophyll *a* fluorescence from the dark-adapted *Anabaena* cells showed induction kinetics under the 585 nm excitation (phycocyanin-excitation, Fig. 3). DCMU strongly enhanced the size of the increase in variable fluorescence ($F_v$, Fig. 3B-c). FDP also caused an increase in the $F_v$ size (Fig. 3b). However, the enhancement of $F_v$ was less for the latter although photosynthesis inhibition was greater. The kinetics of the $F_v$ increase were biphasic for both DCMU and FDP, and both enhanced the rate in the rapid phase (Fig. 3B-b and c). Similar results were obtained with an addition of DDP. Quinone derivatives are known to quench the fluorescence of chlorophyll *a* in vivo (*1*). However, $F_0$ was not affected by additions of FDP and DDP (Fig.

Table 2 *DDP- and FDP-inhibition of photosystem I and II reactions in membrane fragments of* Anabaena variabilis

| | DCIP-Hill activity | $O_2$-uptake activity |
|---|---|---|
| | Inhibition | (%) |
| DDP (30 μM) | 65 | 16 |
| DDP (0.3 mM) | 100 | 31 |
| FDP (28 μM) | 81 | 13 |
| FDP (0.3 mM) | 100 | 26 |

The photosystem I reaction was measured as $O_2$-uptake in the presence of MV (0.1 mM). The intensities of continuous light were $6\times10^5$ erg/cm$^2$·sec. DCIP (0.1 mM) and ascorbate (5 mM) as the electron donor were added to the basal reaction mixture (2.0 ml) containing 0.6 M sucrose, 20 mM NaCl, 1 mM $MgCl_2$ and 25 mM Tricine-NaOH buffer (pH 8.0). Activities were measured at 26°C. The $O_2$-uptake activity of the control was 530 μmoles $O_2$/mg chlorophyll *a*·hr. Concentrations of membrane fragments were equivalent to 1.9 μM chlorophyll *a*. The photosystem II reaction was measured as shown in Fig. 2 and the text.

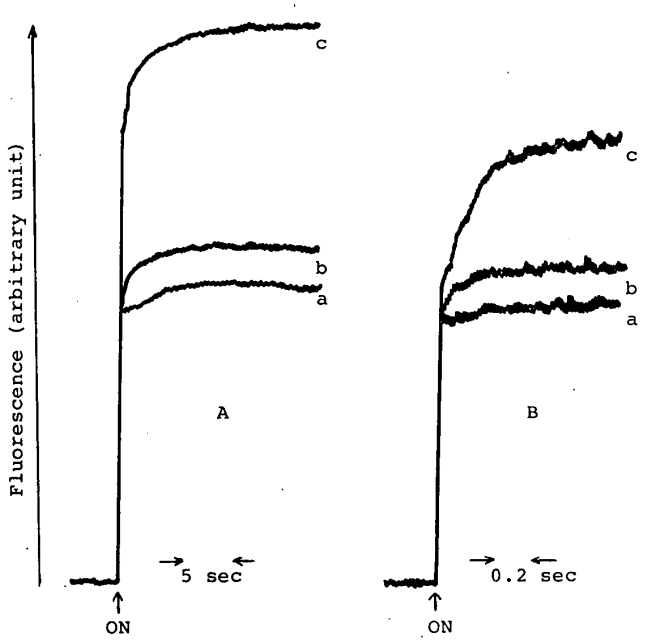



Fig. 3. *Induction kinetics of chlorophyll* a *fluorescence from dark-adapted* Anabaena variabilis. After dark-adaptation for 20 sec, cells were excited by 585 nm light ($2\%10^3$ erg/cm$^2$·sec). Fluorescence at longer than 670 nm was monitored at room temperature. In A, the kinetics for a long span are presented; a: control, b: with FDP (50 $\mu$M) and c: with DCMU (0.1 $\mu$M). Inhibition of photosynthesis by FDP and DCMU were 60.4 and 40.0%, respectively. In B, the kinetics for a short span are presented; samples were the same as in A.

3B-a vs 3B-b). Nor were $F_0$ and $F_v$, in the presence of DCMU at concentrations for complete inhibition, reduced by further additions of FDP or DDP. These features indicate that pyrone derivatives do not have quenching action, at least at the concentrations used. Thus, our observations on fluorescence induction may simply reflect the oxidation-reduction of Q. The enhancement of both the rate of increase and the size of $F_v$ by DDP and FDP indicates that these compounds do not inhibit the reduction of Q, but do suppress its oxidation. Judging from the fluorescence enhancement, we can say that the accumulation of reduced Q under FDP- or DDP-inhibition always was less than that under DCMU-inhibition. The pyrone derivatives probably do not directly suppress the oxidation of reduced Q. The reaction(s) associated with plastoquinone is the most likely site that is sensitive to pyrone derivatives.

As shown in Table 3, another characteristic mode of inhibition was found in the photosyntheses under continuous and repetitive short flash illuminations (repetitive rate, 5 Hz). DCMU-inhibition was approximately equal in both photosyntheses. However, the effect of DDP was selective, being far less inhibitory on the reaction under repetitive flashes. The inhibition became negligible when the repetitive rate was lower than 20 Hz. Under the experimental conditions we used for repetitive flashes, the yield of $O_2$-evolution corresponds to the maximum number of open reaction centers II (cf. *10*). Thus, pyrone derivatives inhibit electron flow without reducing the number of open reaction centers II. Since DCMU, which directly blocks the oxidation of reduced Q, closes reaction center II, our results are evidence that pyrone derivatives do not directly block the oxidation of reduced Q,

Table 3 *DDP- and DCMU-inhibition of the photosynthesis of* Chlorella pyrenoidosa *under continuous and repetitive flash illumination*

| | Inhibition (%) | |
|---|---|---|
| | Continuous light | Repetitive flash |
| DDP (10 μM) | 52 | 6 |
| DBMIB (10 μM) | 53 | 34 |
| DCMU (8.2 μM) | 58 | 57 |

Intensities of the repetitive flashes (5 Hz) and of continuous light were $3.8 \times 10^3$ erg/cm$^2$·flash and $6 \times 10^5$ erg/cm$^2$·sec, respectively. Activities were measured at 26°C. Photosynthetic activities of the control runs were 0.655 mmole $O_2$/mole chlorophyll·flash under repetitive flashes and 418 μmoles $O_2$/mg chlorophyll·hr under continuous light. Cell concentrations were equivalent to 5.1 and 5.2 μM chlorophyll.

but do suppress the reaction(s) associated with the electron accepting system of the reduced Q.

DBMIB showed a mode of inhibition different from that of pyrone derivatives but similar to DCMU; inhibition under repetitive flashes was close to that under continuous illumination (Table 3). According to Haehnel (*7*), this reagent does not directly suppress electron flow within the plastoquinone pool, but does block the site for oxidation of the plastoquinone pool. He estimated that the target component is present at less than two per one reaction center. If so, the component may differ from the bulk of the plastoquinones which form the electron pool. Koike et al. also proposed a similar idea for the site attacked by DBMIB (*11*). DBMIB may inactivate the component mediating electrons from the plastoquinone pool to photosystem I, so that it reduces the number of active electron flow systems consisting of two reaction centers, as observed under repetitive flashes.

Inhibition by pyrone derivatives is not accompanied by a reduction in the number of open reaction center II, indicating that pyrone derivatives slow down the rates of electron flow in all systems uniformly, but not reduce the number of active systems. This mode of inhibition can be expected when multiple molecules of the target are present in one electron flow system, or when the inhibitor does not inactivate its target, but reduces the turnover rate at the target. Although pyrone derivatives are analogues of plastoquinones, they are inactive in the oxidation-reduction reaction. They may be dissolved in the thylakoid membranes, especially in areas where plastoquinones are forming the electron pools for photosystem II. If so, the derivatives settled in the field for the plastoquinone pool may become a barrier against electron flow within the plastoquinone pool, and may produce a geometric hindrance of the electron transport by plastoquinones across the thylakoid membranes (*8*) or they may inhibit the reduction of the plastoquinone pool by photosystem II or oxidation by photosystem I competitively.

The main targets attacked by quinone derivatives are the sites for the reduction and oxidation of the plastoquinone pool, but not the electron flow within the pool (cf. *12* and *14*). Thus, the inhibitory action of pyrones probably differs from that of quinones. However, quinones developed as photosynthesis inhibitors always have a halogen-substituent, whereas the pyrones used here have substituent groups

similar to those of plastoquinone (Fig. 1). This difference in substituent groups may account for the difference in inhibitory action. There is a general tendency that halogen-substituted quinones block at the site for oxidation of the plastoquinone pool as does DBMIB, whereas halogen-substituted nitroquinones act in a way analogous to DCMU (cf. *14*). The difference in inhibitory action between pyrones and quinones may be due to their substituent groups, not to their ring structures. The characteristic actions of DDP and FDP may be due to the fact that their substituent groups are similar to those of plastoquinone. Thus, pyrones with substituent groups similar to those of quinones that are functionally analogous to DBMIB or DCMU may act like quinones. If so, pyrone derivatives would provide a new inhibitory system to block electron flow around the plastoquinone pool. This system, once established, might be used to improve the study of the photosynthetic electron flow mechanism, because pyrones are inactive in the oxidation-reduction reaction and in quenching chlorophyll *a* fluorescence in vivo.

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