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Sites of Inhibition by Disulfiram in Thylakoid Membranes[1]

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ABSTRACT

Disulfiram (tetraethylthiuram disulfide), a metal chelator, inhibits photosynthetic electron transport in broken chloroplasts. A major site of inhibition is detected on the electron-acceptor side of photosystem II between Q_A, the first plastoquinone electron-acceptor, and the second plastoquinone electron-acceptor, Q_B. This site of inhibition is shown by a severalfold increase in the half-time of Q_A^- oxidation, as monitored by the decay of the variable chlorophyll *a* flourescence after an actinic flash. Another site of inhibition is detected in the functioning of the reaction center of photosystem II; disulfiram is observed to quench the room temperature variable chlorophyll *a* fluorescence, as well as the intensity of the 695 nm peak, relative to the 685 nm peak, in the chlorophyll *a* fluorescence spectrum at 77 K. Electron transport from H_2O to the photosystem II electron-acceptor silicomolybdate is also inhibited. Disulfiram does not inhibit electron flow before the site(s) of donation by exogenous electron donors to photosystem II, and no inhibition is detected in the partial reactions associated with photosystem I.

Disulfiram (tetraethylthiuram disulfide) is an inhibitor of the cyanide-resistant respiratory pathway in plant mitochondria (2, 16) and of photosynthesis (19). Its chemistry is well-characterized (19): it is an effective metal chelator, is redox active with a midpoint potential at pH 6.3 of +0.33 V, and can act as a sulfhydryl reducing agent. These aspects of its chemistry may suggest possible mechanisms for inhibition of electron transport. Otherwise, nothing is known about the mode of interaction of disulfiram with the photosynthetic system. Thus, we have examined the disulfiram site(s) of action on the electron transport pathway of photosynthesis. Evidence is presented here that disulfiram inhibits the reoxidation of the first quinone acceptor Q_A^-,[3] and other reactions of PSII. No effect was observed on PSI reactions. Since disulfiram is a metal chelator, it is likely that the site of binding is the Fe^{2+} or the Q_A-Fe-Q_B complex of PSII.

MATERIALS AND METHODS

Materials. Disulfiram was obtained from Sigma Chemical Co. and was used without further purification. The compound is sparingly soluble in water, and even micromolar amounts tend to precipitate when added from an ethanol-based stock solution to thylakoids. To avoid artifacts associated with precipitation, the suspension buffer was homogenized with an excess of disulfiram, filtered through Whatman No. 1 paper, and then used for thylakoid suspension. The disulfiram concentration was measured spectrophotometrically after complexing with Cu, as described (1). The maximum concentration of disulfiram that could be obtained was about 130 μM at room temperature.

Thylakoids were obtained by grinding fresh leaves of market spinach for 10 s in a Sorvall omnimixer in a medium containing 20 mM Hepes (pH 7.5), 15 mM NaCl, 5 mM $MgCl_2$, 0.5% (w/v) BSA, and 1 mM EDTA. The BSA and EDTA were eliminated from the subsequent washing and resuspension of the thylkaoids. The homogenate was filtered through four layers of Miracloth and pelleted at 3500 *g* for 7 min. The thylakoids were washed once in the above medium (without BSA or EDTA) and resuspended in a minimum volume of the same medium. The Chl concentration was determined by the spectrophotometric method of MacKinney (20). Thylakoids were used fresh or were frozen in liquid N_2 until use.

Electron Transport. Rates of O_2 evolution or consumption were determined polarographically at 25°C using a Hansatech Pt/Ag-AgCl electrode, described by Delieu and Walker (9). Illumination, provided by a slide projector, was filtered through a Corning CS3-68 yellow filter and 2 inches of a 1% $CuSO_4$ solution. The light intensity reaching the sample chamber was 2.25×10^3 W m^{-2}, as measured by a Lambda Instruments LI-185 radiometer. Calibration of the signal was done with air-saturated water as described (9). Electron transport was measured from H_2O to silicomolybdate as described previously (12, 30) or from reduced diaminodurene to methyl viologen as described (13). The assay buffer contained 20 mM Hepes (pH 7.5), 15 mM NaCl and 5 mM $MgCl_2$. Other additions are listed in the legend to Table I.

Electron Flow out of Q_A^-: Decay of the Variable Chl *a* Fluorescence. The yield of the 685 nm Chl *a* fluorescence was measured during a weak flash (about 1% of the PSII centers sampled), given at a programmed time interval after a saturating actinic flash. Several such measurements, made on fresh aliquots at varying time intervals and run under computer control, produced the decay curve of the variable Chl *a* fluorescence. Details of the instrument (11) and experimental protocol (26) have been described.

Probing the Activity of the PSII Reaction Center: Fluorescence Transient and Spectra. Fluorescence transients were measured with the fluorometer described earlier (22) with modification. The output signal was digitized with 8-bit precision by a Biomation Model 805 waveform recorder and stored on an LSI 11 minicomputer (Digital Equipment Corporation). A program developed by us permitted display and printout of the transient. Illumination, provided by a General Electric DDY 750 W, 120 V lamp, was filtered through 5 cm of water and Corning CS5-56 and CS4-76 blue filters. The fluorescence was filtered with a Corning CS2-61 red filter before entering the monochromator

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[3] Abbreviations: Q_A, first plastoquinone electron-acceptor of PSII; Q_B, second plastoquinone electron-acceptor of PSII; F_{685}, F_{695}, F_{735}, Chl *a* fluorescence bands with peaks at 685, 695, and 735 nm at 77 K; F_o, F_I, F_{max}, original, intermediate, and maximum Chl *a* fluorescence levels after the onset of illumination; Pheo, pheophytin.

(slit widths: 4 mm; band pass: 13.2 nm). A S-20 EM I (9558 B) photomultiplier was used as a photo-detector.

Emission spectra were measured on the same apparatus described above. The slit widths of the monochromator (Bausch & Lomb 33-86-45, 700 nm blaze) were 1 mm (bandpass: 3.3 nm) for maximum resolution of the F_{685} and F_{695} peaks. A motor moved the grating of the monochromator at a predetermined rate, and a real-time clock was used to control the sampling rate of the waveform recorder. Emission spectra, presented here, were not corrected for the 700 nm blaze monochromator and the S-20 photomultiplier used. The thylakoid suspension was introduced onto two layers of cheesecloth, held down by a Teflon ring, in the bottom of the Dewar flask sample chamber and liquid N_2 was poured on top. An internal standard of 5 μM fluorescein permitted normalization of the spectra in order to correct for variations in sample thickness. The exciting lamp was filtered with Corning CS7-59 and CS4-76 blue filters, and the emission was filtered with a Corning CS3-69 yellow filter.

RESULTS AND DISCUSSION

In order to study the site of action of an inhibitor on the electron transport chain of photosynthesis, the following framework is useful (see self-explanatory scheme in Fig. 1). The PSI reactions were measured as electron transport from reduced diaminodurene to methyl viologen, monitored as oxygen uptake mediated by the reduced methyl viologen. Intersystem electron flow from the reduced Q_A (Q_A^-), the first quinone electron acceptor of PSII, to Q_B, the second quinone electron acceptor of PSII, was monitored as the decay of variable Chl *a* fluorescence: a strong and brief light flash was given to convert all Q_A to Q_A^-, and this was followed by a weak measuring beam given at different times after the actinic flash to monitor the level of Q_A^-. The Chl *a* fluorescence yield is an indicator of $[Q_A^-]$ (4, 10, 14). PSII electron transport, from H_2O through Q_A, with silicomolybdate as electron acceptor and in the presence of diuron, was measured as oxygen evolution. The activity of the PSII reaction center was probed by measurement of the fast Chl *a* fluorescence transient and of the emission spectra at 685 nm and 696 nm at 77 K (from PSII pigment-protein components *in vivo*) (see, *e.g.*, Ref. 14). Finally, the reactions on the electron donor side of PSII were probed by comparing the fluorescence transients with and without artificial electron donors to PSII.

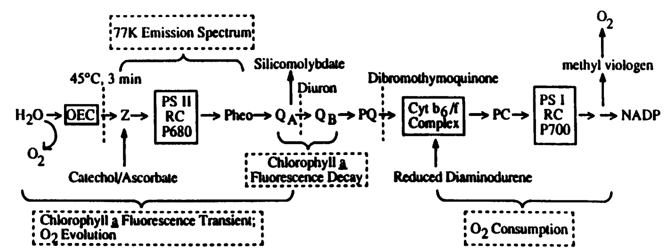

FIG. 1. Scheme for the linear electron transfer pathway of photosynthesis. The vertical arrows point to sites of electron donation or acceptance by exogenous redox agents. The dashed boxes indicate measurements used to probe those sections of the pathway within the brackets. Treatments which block electron flow are shown with a dashed line to indicate the step affected. Abbreviations: RC stands for the reaction center Chl *a* molecules P_{680} of PSII or P_{700} of PSI, which initiate electron flow after absorption of light energy; OEC stands for oxygen evolving complex; Z represents the physiological donor to P_{680}; Pheo is pheophytin; Q_A, Q_B, and PQ are plastoquinone molecules; and PC is plastocyanin. NADP, the terminal physiological acceptor, is absent from isolated thylakoids.

PSI Reaction. As shown in Table I, electron transport from reduced diaminodurene to methyl viologen was only slightly affected by suspension in a buffer saturated with disulfiram (approximately 130 μM). This is in marked contrast to electron transport from H_2O to silicomolybdate, which was inhibited 77% by the same buffer. Other PSII reactions, discussed below, are similarly inhibited at this concentration of disulfiram.

Q_A^- to Q_B Electron Flow. To see if disulfiram has an inhibitory effect on the electron acceptor side of PSII, the Chl *a* fluorescence decay after an actinic flash was measured (Fig. 2). This measurement is an indicator of the kinetics of the reoxidation of Q_A^-. It is clear that disulfiram does indeed inhibit the oxidation of Q_A^-, by eliminating the fast component of the decay. This is similar to the effect of HCO_3^- depletion (11, 17, 27).

Figure 3 shows the fluorescence, as a function of flash number, at various times after the flash. In the absence of disulfiram, a binary oscillation is observed (*i.e.*, 220 μs after the flash), which is normal (8, 14, 29). An oscillation of period four, due to the turnover of the O_2-evolving system, is superimposed on the binary oscillation to give complex kinetics. What is of interest is how rapidly the oscillations are dampened in the presence of disulfiram. This is explained by inhibition of turnover of the PSII reaction center. Figure 3 also confirms that the oxidation of Q_A^- is inhibited after all flashes.

Although an inhibitory effect on Chl *a* fluorescence decay (measuring Q_A^- oxidation) is clearly shown in Figure 2, this effect does not explain the quenching of normalized variable fluorescence $(F - F_o)/F_o$, from a value of 3.5 to 2.7 at times close to zero after the actinic flash, representing maximum variable fluorescence. This quenching indicates that disulfiram inhibited the accumulation of Q_A^-. Since disulfiram has a redox potential of +0.33 V (19), there seemed the possibility that it might have been siphoning electrons from Q_A^- to keep a significant proportion of Q_A oxidized. However, as shown in Figure 2, Chl *a* fluorescence, and thus Q_A^-, remains high at times 500 μs and

Table I. *Effects of Disulfiram on Electron Transport in PSII or PSI*

The reaction mixture, without disulfiram or saturated with disulfiram, (130 μM), contained 20 mM Hepes (pH 7.5), 15 mM NaCl, 5 mM $MgCl_2$, and 13 μg of Chl/mL of thylakoid suspension. The saturating light intensity was 2.25×10^3 W m^{-2}.

	Electron Transport Reaction[a]	Electron Transfer		
		− Disulfiram	+ Disulfiram	Inhibition
			μeq/mg Chl·h	%
(1)	$H_2O \rightarrow$ silicomolybdate	140	32	77
(2)	Reduced diaminodurene → methyl viologen	1960	1860	5

[a] Additions to the reaction mixture were: (1) 10 μM diuron (added in the light), 0.1 mM silicomolybdate (added in the light, after diuron) and 10 mM $CH_3NH_2 \cdot HCl$; and (2) 1 mM diaminodurene, 3 mM Na ascorbate, 2 μM dibromothymoquinone, 10 mM $CH_3NH_2 \cdot HCl$, 0.1 mM methyl viologen, 225 units/mL superoxide dismutase and 1 mM NaN_3.

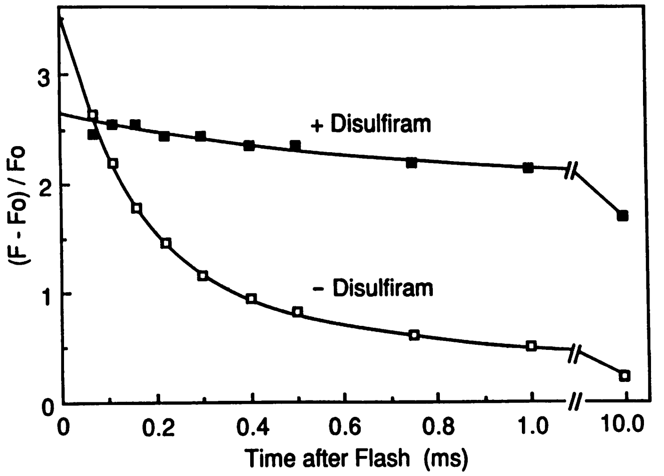

FIG. 2. Decay of the variable Chl *a* fluorescence of spinach thylakoids after an actinic flash in the presence and absence of disulfiram. The thylakoids were suspended to a Chl concentration of 5 μg/ml in a solution of 50 mM Na phosphate (pH 7.2), 100 μM methyl viologen, 0.1 μM gramicidin, and containing no disulfiram (lower curve) or saturated with disulfiram (130 μM). F_o is the Chl *a* fluorescence yield from the measuring flash with all Q_A oxidized (dark-adapted thylakoids), and F is the yield at the indicated time after the actinic flash.

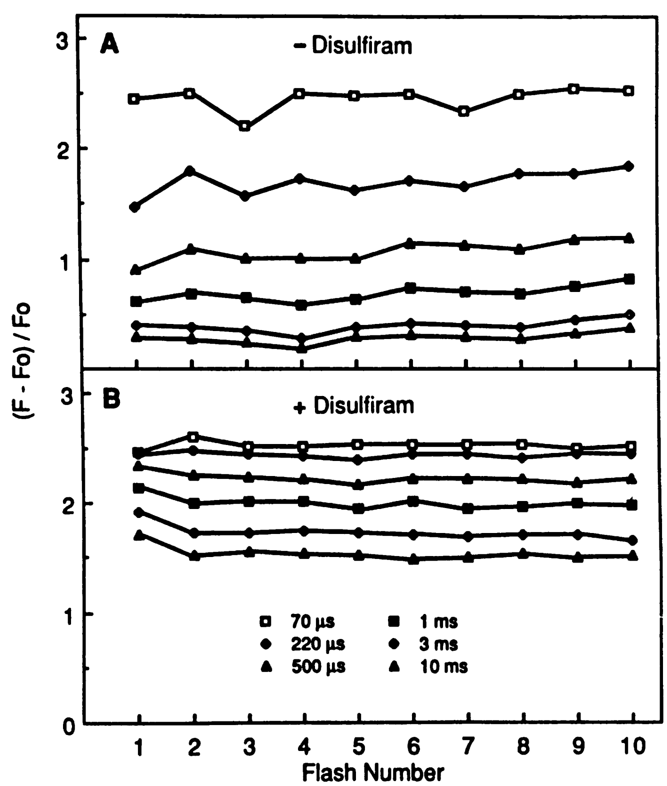

FIG. 3. Variable Chl *a* fluorescence as a function of flash number, in the absence (A) and in the presence (B) of 130 μM disulfiram. All other details are as in Figure 2. The actinic flash frequency was 1 Hz. The times indicated are when the measuring flash was fired.

beyond after the flash. Furthermore, no O_2 evolution could be detected when disulfiram was present without additional electron acceptors, even though some basal activity appeared when the electron acceptor ferricyanide was added on top of the disulfiram (data not shown). Therefore, disulfiram is not acting as an electron acceptor from Q_A^-. Table I shows the inhibitory effect of disulfiram on the photosynthetic reduction of silicomolybdate.

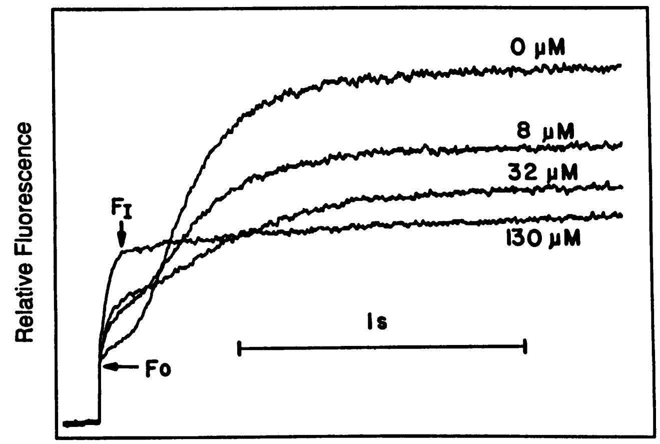

FIG. 4. Effect of increasing concentrations of disulfiram on the Chl *a* fluorescence transient of spinach thylakoids. The thylakoids were suspended to a Chl concentration of 25 μg/ml in solutions of 50 mM Na phosphate (pH 7.2) containing the indicated concentration of disulfiram. The thylakoids were dark adapted 5 min before measuring the transient. F_o is the initial fluorescence level immediately upon illumination.

If silicomolybdate is presumed to accept electrons directly from Q_A^- (12, 28) (however, see Ref. 15), then this result would indicate another inhibitory site prior to Q_A and this would account for the quenching of F_{max}.

Chl *a* Fluorescence Transient. Figure 4 shows the effect of increasing concentrations of disulfiram on the Chl *a* fluorescence transient. The maximum level of fluorescence, F_{max}, is quenched considerably by the disulfiram, but there is no effect on the initial fluorescence level, F_o. The effect is almost saturated at 130 μM, the highest concentration that can be obtained in solution. The absence of any effect on F_o suggests that the quenching is not due to nonphotochemical quenching, but is due to a diminished $[Q_A^-]$, as discussed above in connection with the Chl *a* fluorescence decay. It was similarly observed that disulfiram has no quenching effect on the fluorescence of a Chl solution (data not shown).

Several explanations of the quenching of the variable Chl *a* fluorescence are possible: (a) a block on the donor side of PSII: if no electrons flow from the H_2O side, Q_A cannot be reduced to Q_A^- and fluorescence will remain low; (b) enhanced electron transfer: rapid removal of electrons from Q_A^- could keep Q_A in the oxidized state and fluorescence would be low (this possibility has already been discussed and rejected); and (c) an accumulation of the oxidized form of the Chl *a* of the reaction center II (P_{680}^+), or the reduced form of pheophytin ($Pheo^-$): both are known to quench Chl *a* fluorescence (5, 6, 18). Alternatives (a) and (c) were tested further.

Although F_{max} is quenched, the intermediate fluorescence level F_I is increased (Fig. 4). The reason for this is not known, but is common in treatments that are known to inhibit the electron-donor side of PSII (14). However, the F_I level in the thylakoids maximally inhibited by disulfiram is higher than usual for treatments that block on the electron-donor side (c.f. Fig. 5A), which is consistent with one site of inhibition being between Q_A^- and Q_B. Other compounds which are known to block the oxidation of Q_A^-, such as the herbicide diuron, induce an extreme acceleration of the fluorescence rise to F_{max}. Even when the electron-donor side is inhibited, diuron still induces a high-fluorescent state (Fig. 5A). At 130 μM, disulfiram similarly induces an accelerated fluorescence rise (Fig. 4), but has the additional effect of a large quenching of F_{max}, which persists even in the presence of diuron (Fig. 5B). This suggests that the fluorescence quenching is not due to an inhibition on the electron-donor side (alternative

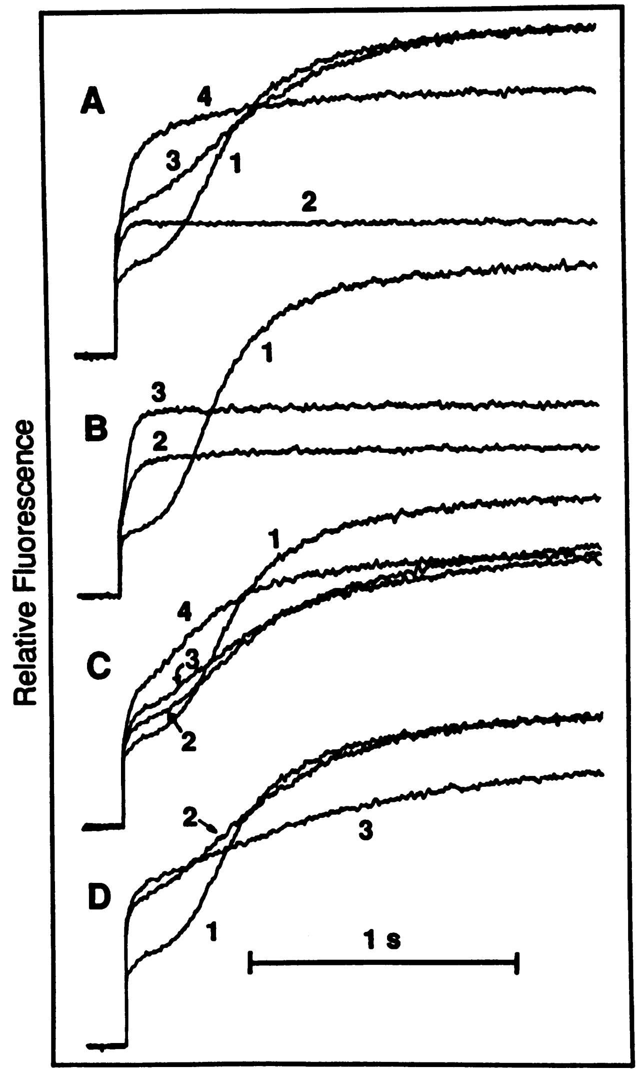

FIG. 5. The Chl *a* fluorescence transients of regular, heat-treated, and diuron-treated thylakoids in the presence and absence of disulfiram. Spinach thylakoids, either untreated or heated in a water bath at 45°C for 3 min to impair the O_2 evolving complex, were suspended to a Chl concentration of 25 μg/ml in 50 mM Na phosphate (pH 7.2), with or without 8 μM or 130 μM disulfiram. Where present, 10 μM diuron or 0.5 mM catechol and 3 mM ascorbate were added from a 100× stock solution. (A) Trace 1, control thylakoids; trace 2, heat-treated thylakoids; trace 3, same as trace 2, + catechol/ascorbate; trace 4, same as trace 2, + diuron. (B) Trace 1, control thylakoids; trace 2, regular thylakoids + 130 μM disulfiram; trace 3, same as trace 2, + diuron; (C) Trace 1, control thylakoids; trace 2, regular thylakoids + 8 μM disulfiram; trace 3, same as trace 2, + catechol/ascorbate; trace 4, same as trace 3, after a second 5 min dark adaptation. (D) Trace 1, control thylakoids; trace 2, heat-treated thylakoids + catechol/ascorbate; trace 3, same as trace 2, + 8 μM disulfiram.

a, above), but is more likely due to an accumulation of $Pheo^-$ (alternative c).

Alternative (a) for the quenching of F_{max} was tested as follows. Figure 5A shows the effect on the Chl *a* fluorescence transient of a mild heating of the thylakoids at 45°C for 3 min, a treatment that is known to selectively inhibit the O_2 evolving complex (see *e.g.*, Ref. 7). The addition of catechol/ascorbate, an artificial electron-donor system to PSII, restores the variable fluorescence to these thylakoids, as is well known (4, 14). The original F_{max} level is obtained, although a high F_I level remains. The reason for including these results here is to show that the catechol/ascorbate electron-donor pair was indeed functioning in our system. The catechol/ascorbate did not relieve the quenching effect of a subsaturating disulfiram concentration (Fig. 5C), which suggests that the site of inhibition is after the site of electron donation by catechol/ascorbate (*i.e.*, after the primary electron-donor to P_{680}, Z). Similarly, Figure 5D shows that disulfiram still quenches the F_{max}, even after the variable fluorescence has been restored to heat-treated thylakoids by catechol/ascorbate. Thus, disulfiram does not appear to inhibit photosynthesis by inhibiting the O_2 evolving complex.

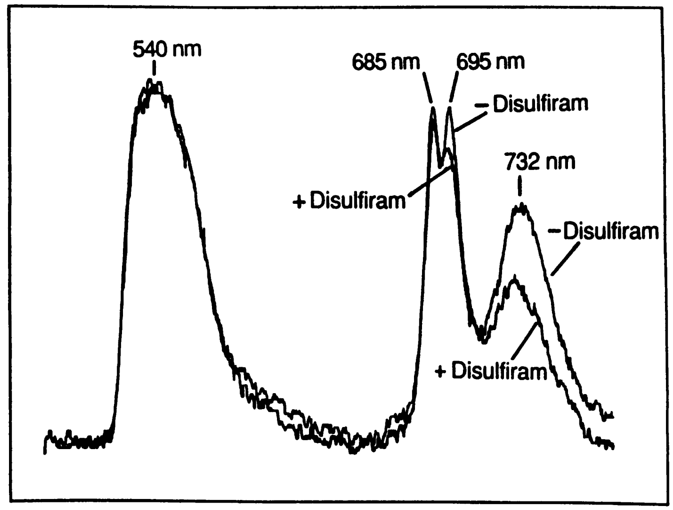

FIG. 6. Chl *a* fluorescence spectrum (uncorrected) at 77 K in the absence and in the presence of 130 μM disulfiram, showing the specific quenching of the F_{695} and F_{735} peaks, relative to F_{685}. The exciting light was filtered through 5 cm water and Corning Cs7-59 and Cs4-76 blue filters. The emission was filtered through a Corning Cs3-69 yellow filter. Fluorescein (5 μM) was present in both traces as an internal standard. The spectra are normalized with respect to the fluorescein peak at 540 nm.

Fluorescence Spectra at 77 K. The effect of disulfiram on the fluorescence spectrum at 77 K is shown in Figure 6. The thylakoids contained 5 μM fluorescein as an internal standard, to which the spectra are normalized. Disulfiram causes a specific quenching of the F_{695} and F_{735} peaks, but no quenching of the F_{685} peak. (For a discussion of the fluorescence peaks, see Refs. 4, 14, and 23). A specific quenching of the F_{695} peak by $Pheo^-$ was predicted (3) and later demonstrated to occur (25) under conditions in which PSII centers had accumulated in the state $Z \cdot P_{680} \cdot Pheo^-$. This effect, however, must be indirect, since F_{695} is believed to originate in the Chl *a*-protein complex CP-47 (considered to be an antenna system, although it is closely associated with the PSII reaction center proteins, D_1 and D_2 (24, 28)). On the basis of data accumulated in this paper, it is considered likely that disulfiram inhibits the $Pheo^-$ to Q_A electron transfer, in addition to slowing down the oxidation of Q_A^-. The quenching of the F_{735} peak, which originates in the pigment protein complex of PSI, may indicate a decreased energy transfer from PSII to PSI, since excitation of the sample was mostly in PSII, and the PSI reaction (under saturating light) was unaffected by disulfiram (Table I).

In summary, three apparent effects of disulfiram on photosynthesis have been identified: (a) electron transfer is blocked between Q_A^- and Q_B (b) the $Pheo^-$ to Q_A electron transfer is inhibited, and (c) energy transfer from PSII to PSI appears to be decreased. There was no evidence that disulfiram inhibited PSI electron transport or reactions on the electron donor side of PSII. Disulfiram is a potent metal chelator and could be complexing with the Fe^{2+} of the PSII reaction center. The Fe^{2+} is structurally important and lies between Q_A and Q_B (21). The complexing of disulfiram with the Fe^{2+} could cause sufficient structural changes to account for each of the effects observed.

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