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Short Communication

Disulfiram Metabolism in Isolated Mesophyll Cells and Inhibition of Photosynthesis and Cyanide-Resistant Respiration[1]

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ABSTRACT

Tetraethylthiuram disulfide (disulfiram) stimulated medium acidification when added at a concentration of 0.4 millimolar to illuminated or nonilluminated suspensions of *Asparagus sprengeri* Regel mesophyll cells. Similar concentrations inhibited photosynthesis and cyanide-resistant respiration. The reduction product of disulfiram, diethyldithiocarbamic acid, accumulated in concentrations sufficient to account for the observed acidification.

Disulfiram (tetraethylthiuram disulfide) is a potent inhibitor of cyanide-resistant respiration in isolated mitochondria (7) or intact mesophyll cells of *Asparagus sprengeri* Regel (17). In addition, disulfiram and other thiuram disulfides inhibit photosynthesis (13, 14). The mechanisms by which disulfiram inhibits these processes are not understood. Inhibition in both cases may result from a reaction between the disulfide component of disulfiram and enzyme thiol groups essential for the cyanide-resistant pathway or photosynthesis (7, 14, 16). Another possible mechanism of inhibition involves chelation by disulfiram of transition metals located in enzyme systems (14). Inhibition may also be mediated by disulfiram's ability to function as a free radical scavenger and react with fatty acid peroxy radicals (18). In the present paper, the ability of isolated *Asparagus* mesophyll cells to rapidly reduce disulfiram to DIECA[2] is demonstrated. The possible involvement of DIECA production in inhibition of either process is investigated.

MATERIALS AND METHODS

Asparagus sprengeri Regel was grown and mesophyll cells isolated using previously described methods (3, 4). Chl content of cell suspensions was determined by the method of Arnon (2) and cell numbers measured with a hemocytometer. A mean value of 36 ± 20 (SD) μg Chl/10^6 cells was obtained.

Rates of acidification in aerated and stirred cell suspensions maintained at 30°C in 10 ml unbuffered salt solutions, containing 5 mM KCl, 5 mM NaCl, and 0.2 mM $CaCl_2$, were determined with a recording pH meter (3). Rates were calculated as nmol $H^+/10^6$ cells·min or nmol H^+/mg Chl·min.

DIECA in the suspending medium was determined by measuring the absorbance of a copper (II) DIECA complex at 433 nm (1).

Rates of O_2 production in the light or O_2 consumption in the dark were determined with a calibrated O_2 electrode (YSI 4004) using cells suspended in 7.5 ml of a 30°C buffered medium contained witin a closed system (3). A concentration of $NaHCO_3$ saturating for photosynthesis (4) was added to the cell suspension immediately after illumination with a 300-w reflector lamp (Sylvania) began. Irradiance at the surface of the vessel was 1200×10^{-5} w cm^{-2}. Rates were corrected for consumption by the O_2 electrode and expressed as μmol O_2/mg Chl·h or μmol $O_2/10^6$ cells·h.

Stock solutions of DIECA and disulfiram were made using 80% (v/v) ethanol and volumes of KCN, ethanol, DIECA, or disulfiram were dispensed with Hamilton syringes to give the concentrations indicated.

RESULTS

Measurements of net rates of H^+ efflux were initiated by the addition of 9 to 12×10^6 cells suspended in 3 ml unbuffered salt solution to 7 ml of similar medium. The resulting 10 ml cell suspension was stirred and aerated. The initial value for the net rate of H^+ efflux varied between 0.3 and 1.8 nmol $H^+/10^6$ cells·min and the mean value expressed on a Chl basis was 31.6 nmol H^+/mg Chl·min. Disulfiram stimulated acidification with no apparent lag (Fig. 1). In the light, 0.4 mM disulfiram stimulated acidification to a mean value of 18 nmol $H^+/10^6$ cells·min and the overall decline in pH was equivalent to 396 ± 83 (SD) nmol H^+. The molar ratio of H^+ appearing in the medium to disulfiram added was 0.1. In the dark, 0.4 mM disulfiram stimulated the acidification rate to 8.6 nmol $H^+/10^6$ cells·min and the pH decrease was equivalent to 210 ± 64 (SD) nmol H^+ (Fig. 1). The rate or extent of acidification was not significantly reduced until the disulfiram concentration was decreased to 10 μM. When the normal rate of H^+ efflux was eliminated with 1 μg/ml oligomycin acidification in response to disulfiram was still observed. Control experiments showed that the disulfiram solvent ethanol did not stimulate acidification and in the absence of cells neither did disulfiram. The Na^+ salt of DIECA did not stimulate acidification, and concentrations greater than 40 μM resulted in alkanization of the cell suspension medium.

DIECA in the 10-ml suspension medium was determined after acidification, in response to 0.4 mM disulfiram, was complete and cells were removed by centrifugation. With illumination, a mean value of 1,010 nmol DIECA were produced; without illumination, 960 nmol of DIECA were obtained. These values indicate an approximate 0.1 mM DIECA concentration in the 10-ml suspension medium. The molar ratio of DIECA production to disulfiram employed was 0.25. The ratio of H^+ to DIECA

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[2] Abbreviations: DIECA, diethyldithiocarbamic acid; DMO, 5,5-dimethyloxazolidine-2,4-dione; SHAM, salicylhydroxamic acid; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone.

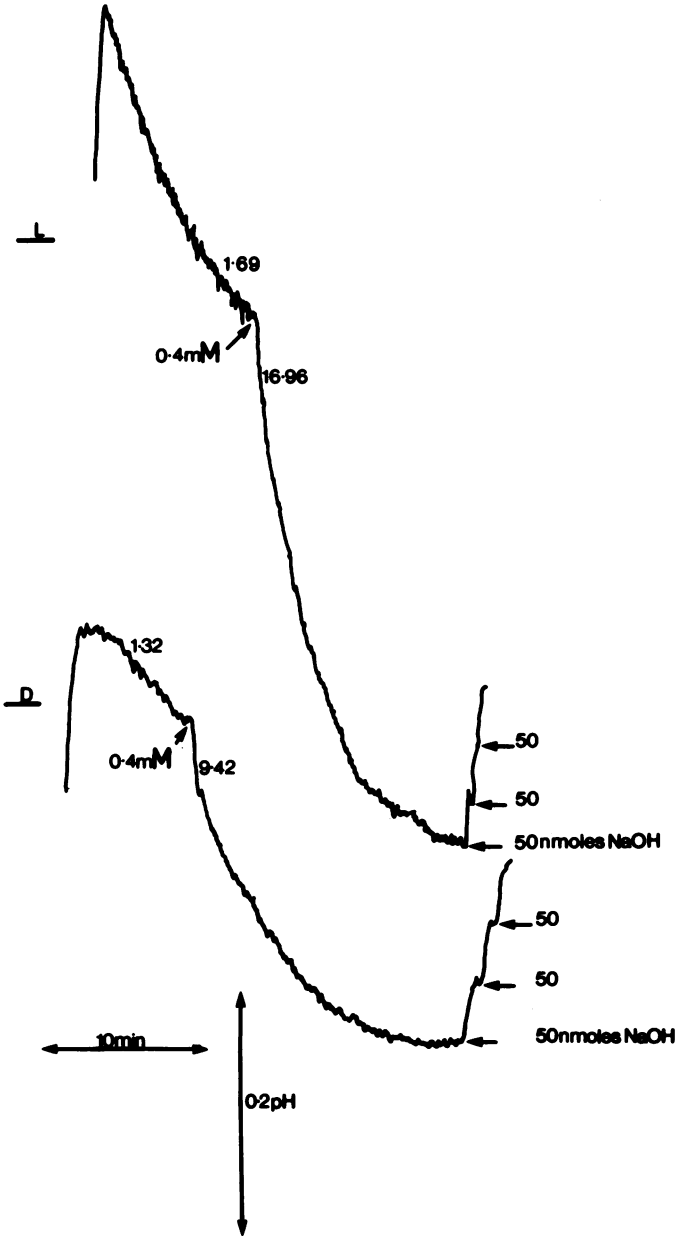

FIG. 1. Stimulation of medium acidification by disulfiram. Recordings of pH change with cells suspended in unbuffered 5 mM KCl, 5 mM NaCl, and 0.2 mM $CaCl_2$. Recordings obtained in the dark (D) or with illumination (L). Rates of acidification are expressed as nmol $H^+/10^6$ cells·min.

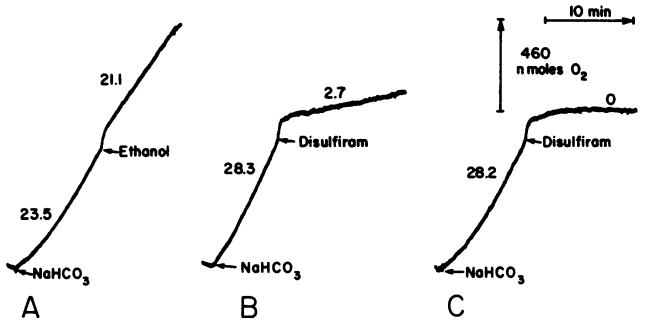

FIG. 2. Inhibition of photosynthesis by disulfiram. Recordings of O_2 evolution were obtained with cells suspended in 0.05 Hepes buffer (pH 7.2) (A and C) or 0.05 M phosphate buffer (pH 6.2) (B). The final concentration of disulfiram was 50 μM and the final concentration of ethanol 0.8% (v/v). Rates of photosynthesis are expressed as μmol $O_2/10^6$ cells·h.

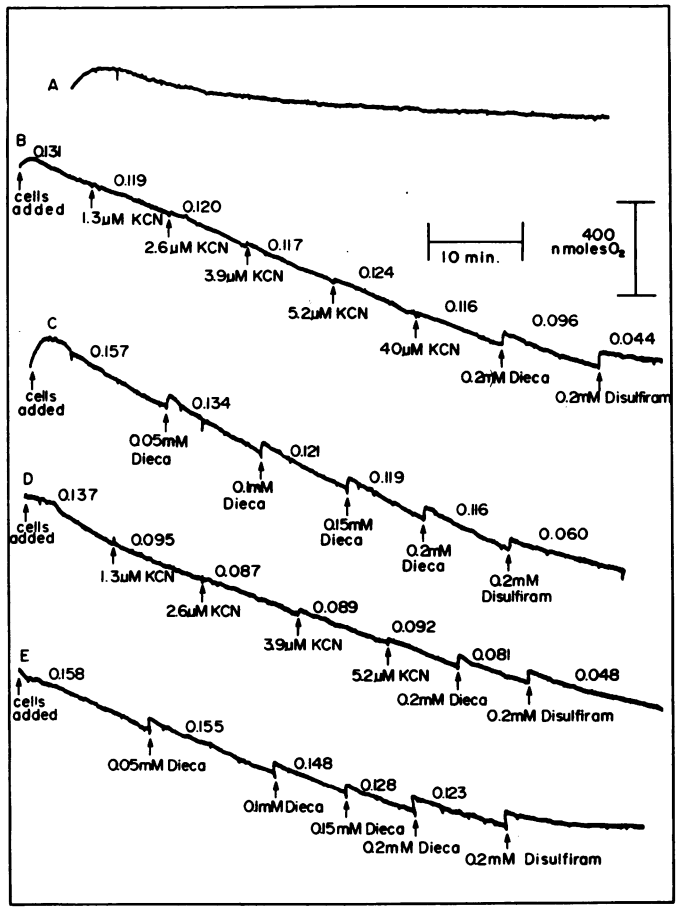

FIG. 3. Inhibition of cyanide-resistant respiration by difulfiram. All recordings were obtained using 0.05 M Hepes buffer (pH 7.2). Trace A indicates changes in O_2 concentration in the absence of cells. Additions of KCN, DIECA, and disulfiram are indicated. Rates of O_2 consumption after each addition are expressed as μmol $O_2/10^6$ cells·h.

generated in response to disulfiram was 0.40 in the light and 0.21 in the dark. DIECA was not detected when disulfiram was added to aerated and stirred suspension medium lacking cells.

Photosynthetic O_2 production was measured using saturating light and bicarbonate concentrations with 3 to 4 × 10^6 cells suspended in7.5 ml of 50 mM Hepes buffer adjusted to pH 7.5 with KOH. The mean rate obtained was 32 ± 6.4 (SD) μmol O_2/mg Chl·h or 1.10 ± 0.22 (SD) μmol $O_2/10^6$ cells·h. Addition of 50 μM disulfiram resulted in complete inhibition of O_2 evolution within 2 min (Fig. 2), and 10 μM disulfiram inhibited evolution by approximately 50%. Similar results were obtained with cells suspended in 50 mM K-phosphate (pH 6.2) (Fig. 2). The concentration of ethanol used in these studies had little or no effect on photosynthesis indicating that inhibition was due to disulfiram, not its solvent (Fig. 2). The Na^+ salt of DIECA at a 0.4 mM concentration had no significant effect on photosynthetic oxygen production.

Respiration rates were measured with nonilluminated cells suspended in either 50 mM Hepes buffer (pH 7.5) or 50 mM K-phosphate (pH 6.2). Rates were linear with time and varied between 0.12 and 0.22 μmol $O_2/10^6$ cells·h; the mean rate when expressed on Chl basis was 1.4 μmol O_2/mg Chl·h. Sequential increments in the DIECA sodium salt concentrations up to 0.2

mM resulted in 24% and 28% mean inhibition of the initial rates in the Hepes and phosphate buffers, respectively. Subsequent addition of 0.2 mM disulfiram resulted in 58% and 65% mean inhibition of the remaining respiration, indicating that disulfiram is a more potent inhibition than DIECA (Fig. 3). Addition of KCN concentrations up to 100 μM demonstrated that respiration was resistant to cyanide and in some cases respiration was stimulated. The cyanide-resistant respiration was inhibited by a mean value of 14% by 0.2 mM DIECA. Subsequent addition of 0.2 mM disulfiram, however, reduced rates by approximately 50% (Fig. 3).

DISCUSSION

Rapid inhibition of photosynthesis and cyanide-resistant respiration by disulfiram indicate that it passes readily into the cell (Figs. 2 and 3). A previous report concluded that, while disulfiram was a potent inhibitor of the alternative pathway in isolated mitochondria, it was without effect on intact tissues or cells (7). However, rapid entry is expected with a nonpolar compound having four ethyl groups when stirring reduces diffusion distances to a minimum. The reduction of disulfiram to DIECA will result in the generation of a free acid group (pK 3.9) which will be largely dissociated at cytoplasmic pH values. Accumulation of DIECA in the unbuffered suspension medium indicates that acidification of this medium (Fig. 1) results from equilibration of undissociated uncharged DIECA across the plasma membrane and with the internal and external pools of the corresponding anions. The molar excess of DIECA over H^+ generated in the suspension medium indicates that measurements of net H^+ efflux may be reduced by H^+ reflux.

Reduction of disulfiram to DIECA could involve a reaction with protein sulfhydryl groups resulting in the production of a protein-mixed disulfide complex and one molecule of DIECA (7, 16). Alternatively, reduction may be catalyzed by an enzyme system such as NADP-linked glutathione reductase or thioredoxin reductase which reduce disulfide compounds (9). In this case, one disulfiram molecule would generate two DIECA molecules. Photosynthetically driven reduction of disulfiram may explain the faster acidification rate in the light (Fig. 1), although the process was not inhibited by DCMU.

The response to disulfiram is strikingly similar to the response of *Asparagus* cells to the addition of lipophilic carboxyester compounds (5). In both cases, rapid metabolism generates an acidic compound, acidification of the medium occurs, photosynthesis is inhibited, and addition of the metabolic products does not inhibit photosynthesis. Inhibition of photosynthesis may arise through an intracellular acidification process which reduces the intracellular pH. Whereas a stromal pH of 7.0 inhibits CO_2 fixation, optimum activity is found at pH 8.0 (19). Acidification could result from the reduction of disulfiram within the stroma or from entry and dissociation of weak acids generated in the cytosol as a result of protonation at a reduced cytosolic pH (6). This interpretation is supported by preliminary experiments in which addition of 10 mM NH_4Cl resulted in a rapid 37% recovery of disulfiram-inhibited O_2 evolution. Similar experiments with concentrations of CCCP sufficient to uncouple photophosphorylation (8) did not result in a renewal of O_2 evolution. These data suggest that the effect of NH_4Cl is not due to an uncoupling of photophosphorylation but are consistent with a reversal of intracellular acidification when membrane-permanent NH_3 is protonated. In addition, the similarity in the responses of *Asparagus* cells to disulfiram and lipophilic carboxyesters which lack a disulfide group (5) suggests that inhibition of photosynthesis may not involve an interaction between functional thiol and disulfide groups. This interpretation contrasts with a previous paper which demonstrated disulfiram reduction to DIECA, but which did not consider intracellular acidification as a possible mechanism by which photosynthesis is inhibited (14).

Most work on cyanide-resistant respiration has focused on tissue slices or mitochondria from fruits or storage organs (12). Two recent reports, however, demonstrate the presence of cyanide-resistant SHAM-sensitive respiration in leaf tissue (11) and cyanide-resistant disulfiram-sensitive respiration in isolated *Asparagus* mesophyll cells (17). Lipophilic carboxyesters had no influence on cyanide-resistant respiration suggesting that disulfiram inhibition does not involve intracellular acidification. The generation of 0.1 mM DIECA concentrations on the addition of 0.4 mM disulfiram indicates that inhibition may involve DIECA. However, disulfiram was a far more potent inhibitor of cyanide-resistant respiration than an equal concentration of DIECA (Fig. 3). The small inhibition observed on addition of DIECA may in fact be due to disulfiram if a reversible redox reaction produces an equilibrium ratio of disulfiram and DIECA (10). The evidence suggests that inhibition does not depend on production of DIECA, and that disulfiram is the inhibitory species. A similar conclusion was reached when disulfiram was used to inhibit cyanide-resistant respiration in isolated mitochondria (7). The residual respiration (Fig. 3) may represent lipoxygenase activity which is insensitive to cyanide and disulfiram (15).

LITERATURE CITED

1. AKERSTRÖM S, PEB LINDAHL 1962 A convenient method for determination of tetramethylthiuram disulfide. Acta Chem Scand 16: 1206–1211
2. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
3. BOWN AW 1982 An investigation into the roles of photosynthesis and respiration in H^+ efflux from aerated suspensions of *Asparagus* mesophyll cells. Plant Physiol 70: 803–810
4. COLMAN B, BT MAWSON, GS ESPIE 1979 The rapid isolation of photosynthetically active mesophyll cells from *Asparagus* cladophylls. Can J Bot 57: 1505–1510
5. DUHAIME DE, AW BOWN 1983 Stimulation of H^+ efflux and inhibition of photosynthesis by esters of carboxylic acids. Plant Physiol 73: 828–833
6. FLUGG UI, M FREISL, HW HELDT 1980 The mechanism of the control of carbon fixation by the pH in the chloroplast stroma: Studies with acid mediated proton transfer across the envelope. Planta 149: 48–51
7. GROVER SD, GG LATIES 1981 Disulfiram inhibition of the alternative respiratory pathway in plant mitochondria. Plant Physiol 68: 393–400
8. IZAWA S, NE GOOD 1972 Inhibition of photosynthetic electron transport and photophosphorylation. Methods Enzymol 24: 355–377
9. JOHNSTON CD 1953 The *in vitro* reaction between tetraethylthiuram disulfide (Antabuse) and glutathione. Arch Biochem Biophys 44: 249–251
10. KAARS SIJPESTEIJN A, J KASLANDER, GJM VAN DER KERK 1962 On the conversion of sodium dimethyldithiocarbamate into its α-aminobutyric acid derivative by microorganisms. Biochim Biophys Acta 62: 587–589
11. LAMBERS N, DA DAY, J AZCON-BIETO 1983 Cyanide-resistant respiration in roots and leaves. Measurements with intact tissues and isolated mitochondria. Physiol Plant 58: 148–154
12. LATIES GG 1982 The cyanide resistant alternative path in higher plant respiration. Annu Rev Plant Physiol 33: 519–555
13. LINDAHL PEB 1961 Inhibition of growth and photosynthesis in submerged plants with tetramethylthiuram disulfide and sodium dimethyldithiocarbamate. Nature 191: 51–53
14. LINDAHL PEB, S AKERSTROM 1965 On the mechanism of inhibition of photosynthesis by N-disubstituted dithiocarbamates and corresponding thiuram disulfides. Lantbrukshogsk Ann 31: 459–503
15. MILLER MG, RL OBENDORF 1981 Use of tetraethylthiuram disulfide to discriminate between alternative respiration and lipoxygenase. Plant Physiol 67: 962–964
16. NEIMS AH, DS COFFEY, L HELLERMAN 1966 Interaction between tetraethylthiuram disulfide and the sulfhydryl groups of D-amino acid oxidase and of hemoglobin. J Biol Chem 241: 5941–5948
17. SHADEED N, AW BOWN 1984 Cyanide resistant respiration in isolated *Asparagus* mesophyll cells. Can J Bot 62: 1122–1126
18. RUSTIN P, J DUPONT, C LANCE 1983 A role for fatty acid peroxy radicals in the cyanide-insensitive pathway of plant mitochondria. TIBS 8: 155–157
19. WERDAN K, HW HELDT, M MILOVANCEV 1975 The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and in the dark. Biochim Biophys Acta 392: 276–292