Inhibition of Chloroplast Electron Transport Reactions by Trifluralin and Diallate[1]

Steven J. Robinson, Charles F. Yocum, and Hiroshi Ikuma
Department of Cell and Molecular Biology, Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109

Fumihiko Hayashi
Criteria and Evaluation Division, Office of Pesticide Programs, United States Environmental Protection Agency, Washington, D.C. 20460

ABSTRACT

The herbicides trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine) and diallate (S-[2,3-dichloroallyl] diisopropylthiocarbamate) inhibit electron transport, ATP synthesis, and cytochrome f reduction by isolated spinach (*Spinacia oleracea* L.) chloroplasts. Both compounds inhibit noncyclic electron transport from H_2O to ferricyanide more than 90% in coupled chloroplasts at concentrations less than 50 μM. Neither herbicide inhibits electron transport in assays utilizing only photosystem I activity, and the photosystem II reaction elicited by addition of oxidized p-phenylenediamine or 2,5-dimethylquinone is only partially inhibited by herbicide concentrations which block electron flow from H_2O to ferricyanide. Inhibition of ATP synthesis parallels inhibition of electron flow in all noncyclic assay systems, and cyclic ATP synthesis catalyzed by either diaminodurene or phenazine methosulfate is susceptible to inhibition by both herbicides. These results indicate that trifluralin and diallate both inhibit electron flow in isolated chloroplasts at a point in the electron transport chain between the two photosystems.

Inhibitors of photosynthetic electron transport and associated phosphorylation are widely used as tools for the investigation of photosynthetic systems. Numerous inhibitors exist which depress noncyclic electron flow by blocking near PSII while having no effect on PSI reactions; the herbicide DCMU is an example (7, 19). Alternatively, PSI may be blocked with some specificity by cyanide treatment (13) or by exposure to mercuric chloride (9). There is, however, a dearth of inhibitors which act at an intermediate point between the two photosystems. The only such compound currently in use is the plastoquinone antagonist DBMIB[2] (4, 17). DMBIB has, until now, been considered to be a unique inhibitor in that it allows each photosystem to

function separately, but does not permit electron flow between the photosystems.

In surveying the effects on photosynthetic electron transport and energy conservation of 17 commercially available herbicides, we have encountered two compounds which appear to block electron flow between the photosystems, that is, in a manner distinctly different from DCMU (15). These two compounds, trifluralin and diallate, are soil-applied herbicides; trifluralin is used for control of many grasses and weeds (12), while diallate is used primarily for control of wild oats (3). Work concerning the biochemical modes of action of these herbicides is incomplete, and has seldom focused on inhibitory effects related to photosynthetic electron transport and phosphorylation. One notable exception is the work of Moreland *et al.* (12) who demonstrated that trifluralin inhibits the Hill reaction and noncyclic phosphorylation, although it is only marginally effective as an inhibitor of cyclic phosphorylation. This paper presents our observations on the efficacy of trifluralin and diallate as inhibitors of electron flow and phosphorylation in spinach chloroplasts.

MATERIALS AND METHODS

Chloroplasts. Nonswollen thylakoid membranes from leaves of spinach (*Spinacia oleracea* L.) were isolated and stored as previously described (6). The spectrophotometric method of Arnon (1) was used for Chl determinations.

Assay of Electron Transport and Phosphorylation. Electron transport rates were determined polarographically, by measuring O_2 evolution or consumption with a Clark electrode which monitored a continuously stirred and thermostatted (25 C) glass cuvette (1.6-ml capacity). A microscope lamp, or an Oriel light source (model 6325) provided white light at an intensity of 10^6 ergs·cm^{-2}·sec^{-1} at the reaction vessel surface. The reaction medium for O_2 evolution assays contained Tricine (12 mM, pH 8), NaCl (60 mM), ferricyanide (2.5 mM), and 15 to 25 μg of Chl. PSII activity was assayed by addition of 0.25 mM PD or DMQ to the above reaction mixture. O_2 consumption measurements were employed to assay noncyclic electron flow in a medium containing Tricine and NaCl, as above, with 0.12 mM methylviologen and 1.25 mM KCN; addition of 6 mM ascorbate, 0.25 mM DAD (or 0.06 mM DPIP), 3 μM DCMU, and omission of the KCN provided the medium for PSI assays. In all cases, uncoupling was induced by the addition of 9.4 mM methylamine.

Photophosphorylation was assayed by measuring ^{32}P-orthophosphate incorporation into ATP after extraction of unincorporated ^{32}P by the method of Avron (2). Reactions were

[2] Abbreviations used are: DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; PD: p-phenylenediamine; PD$_{ox}$: p-phenylenediimine; DMQ: 2,5-dimethyl-p-benzoquinone; DAD: 2,3,5,6-tetramethyl-p-phenylenediamine; DAD$_{ox}$: 2,3,5,6-tetramethyl-p-phenylenediimine; DPIP: 2,6-dichlorophenolindophenol; PMS: phenazine methosulfate; P/e$_2$: ratio of ATP molecules synthesized to electron pairs transferred; EDAC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; I$_{50}$: herbicide concentration required for 50% inhibition of activity.

terminated and counting performed as previously described (20). For measurement of noncyclic, site II and site I phosphorylation, the reaction media, as described above for polarographic measurements, were supplemented with 3 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM ADP, and ^{32}P to 10^6 cpm, and the Tricine concentration was increased to 40 mM. Cyclic photophosphorylation experiments were performed in test tubes containing 1.6 ml of medium with $MgCl_2$, NaH_2PO_4, ADP, ^{32}P, and Tricine, as above, with 6.25 μM DCMU and 62.5 μM PMS or 0.625 mM DAD added. Illumination was provided for 1 min by photoflood lamps at an intensity of 10^6 ergs·cm^{-2}·sec^{-1}.

Exchange and Mg^{2+}-ATPase. Activation of the light- and sulfhydryl-induced ATP-Pi exchange and Mg^{2+}-ATPase activities was done according to the method of McCarty and Racker (11), except that a higher concentration (50 mM) of DTT was used. Following activation, ATP-Pi exchange activity was determined by withdrawing an aliquot of the activation mixture and placing it into an incubation medium of 50 mM Tricine (pH 8), 10 mM ATP, 10 mM $MgCl_2$, 0.5 mM NaH_2PO_4, and 10^6 cpm ^{32}P; after a 30-min incubation at 37 C, ^{32}P-ATP was assayed as previously described. For assay of Mg^{2+}-ATPase, an aliquot of the activation mixture was incubated in 50 mM Tricine, 10 mM ATP, and 10 mM $MgCl_2$ for 30 min. The inorganic phosphate released was determined by the method of Taussky and Schorr (16).

Cytochrome f Oxidation. Changes in the redox state of Cyt f were observed using an Aminco DW-2 dual wavelength spectrophotometer equipped with a side illumination apparatus. Interference filters were used to provide illumination (640 nm or 720 nm at intensities of 0.9 or 1.1 × 10^4 ergs cm^{-2} sec^{-1}). The phototube was shielded from the actinic beam by a blue filter (Corning No. CS4-96). Absorption changes were followed at 554 nm, using 540 nm as the reference wavelength.

Chemicals. Trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine) was obtained from Eli Lilly; diallate (S-[2,3-dichloroallyl]diisopropylthiocarbamate) from Monsanto. Both herbicides were purified by recrystallization from methanol after adsorption of impurities on charcoal. Stock solutions of trifluralin and diallate were prepared in dimethylsulfoxide; microliter quantities of these solutions were added to assay mixtures so that the final dimethylsulfoxide concentration was in the range of 0.5 to 3%, a solvent concentration which by itself produced no observable effects on the photosynthetic reactions studied. Tricine, ADP, PD, and PMS were obtained from Sigma, DMQ from Eastman, and DAD from Research Inorganics.

RESULTS

Electron Transfer Inhibition. Titration experiments were performed to determine the effects of trifluralin and diallate on photosynthetic electron transport using the Hill reaction (water → ferricyanide) as well as PSII (H_2O → PD_{ox}) and PSI (ascorbate/DAD → methylviologen) partial reactions. Trifluralin effectively inhibits electron flow from water to ferricyanide, showing half-maximal inhibition (I_{50}) at 10 to 20 μM; methylamine uncoupling produced a titration curve with very similar characteristics (Fig. 1). In both the coupled and the uncoupled systems, complete inhibition is never obtained; approximately 10 to 15% of the control activity remains even at saturating concentrations of trifluralin. If noncyclic electron flow is assayed using methylviologen as the acceptor, the same results are obtained; half-maximal inhibition at 10 to 15 μM, a residual activity of 10% of the control. Trifluralin does not appear to affect PSI electron flow as assayed by ascorbate/DAD or ascorbate/DPIP to methylviologen reactions. No PSI inhibition was noted even when chloroplasts were incubated in high concentrations of trifluralin (>200 μM) for periods up to 1 hr.
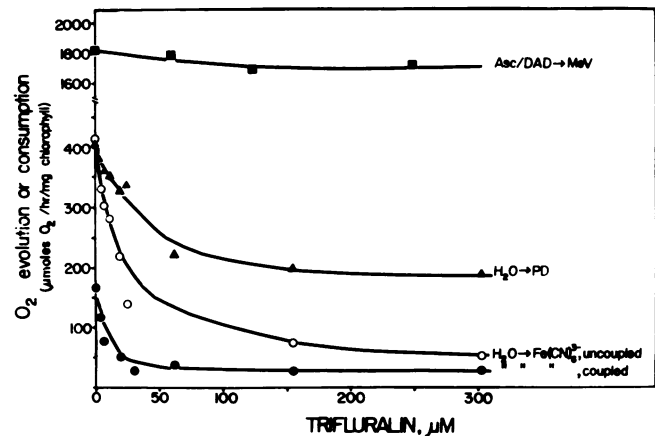

Fig. 1. Effect of trifluralin concentration on photosynthetic electron transport. O_2 evolution was used to measured H_2O → ferricyanide and H_2O → PD_{ox} reactions; O_2 consumption was assayed for the ascorbate/DAD → methylviologen reaction.
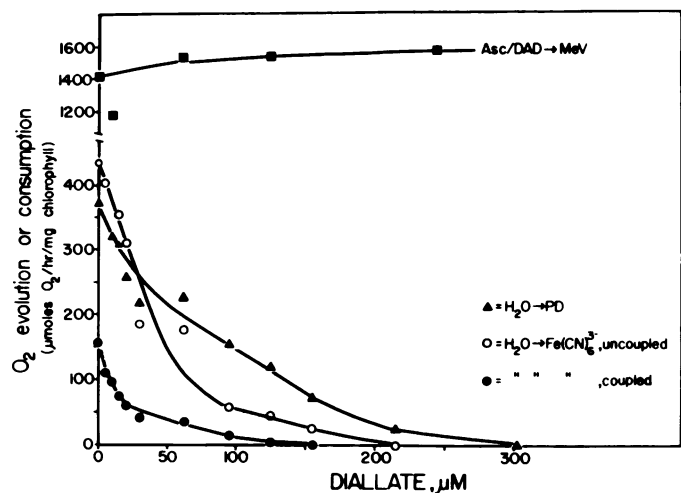

Fig. 2. Effect of diallate concentration on photosynthetic electron transport.

Finally, PSII electron flow is only weakly inhibited in the presence of the class III acceptor PD_{ox}; maximal inhibition is only 50% even at the highest trifluralin concentrations. These results indicate that trifluralin blocks neither PSII nor PSI directly, but rather that inhibition is exerted somewhere between the two photosystems. Diallate shows certain inhibitory effects similar to those evinced by trifluralin (Fig. 2); namely, noncyclic electron transport (water → ferricyanide) is inhibited half-maximally in the micromolar range (I_{50} = 20-30 μM), and PSI is not noticeably inhibited. However, there are dissimilarities in the inhibitory effects elicited by the two compounds. Diallate is a more effective inhibitor of PSII than is trifluralin, causing complete cessation of PSII (H_2O → PD_{ox}) electron flow at 300 μM, with half-maximal inhibition at 75 μM. Furthermore, diallate inhibition of the Hill reaction appears to be partially reversed by methylamine uncoupling, suggesting that under these assay conditions diallate may act to a limited extent as an energy transfer inhibitor. No such function is ascribable to trifluralin on the basis of our observations.

To determine more precisely the sites of trifluralin and diallate inhibition of electron transport, the light-induced oxidation and reduction changes of Cyt f were observed by dual wavelength spectrophotometry. Neither herbicide was found to alter the characteristics of Cyt f oxidation when excited by PSI light (720 nm). In a system with methylviologen as the electron acceptor
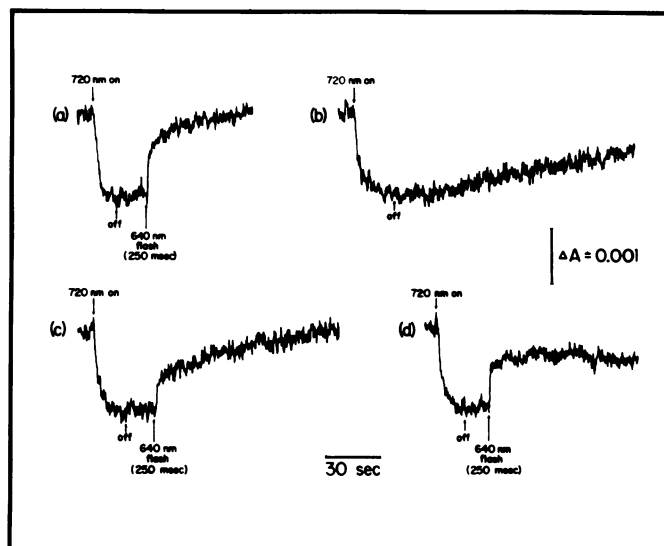
FIG. 3. Effects of trifluralin and diallate on the light-induced absorption changes of Cyt *f*. Absorption changes were observed as described under "Materials and Methods" using a Chl concentration of 65 μg/ml. The 1-ml reaction mixture contained 50 mM Tricine (pH 8), 50 mM NaCl, 3 mM $MgCl_2$, 1 mM ADP, 5 mM NaH_2PO_4, and 125 μM methylviologen. The measuring beam pathlength was 1 cm; the actinic pathlength was 0.5 cm (a) Control (no herbicide addition) showing Cyt *f* oxidation by PSI (720 nm illumination) and subsequent reduction by PSII (640 nm); (b) control (no herbicide addition) showing Cyt *f* oxidation by PSI and subsequent slow reduction in the dark; (c) effect of 200 μM trifluralin added prior to illumination; (d) effect of 100 μM diallate added prior to illumination. Illumination provided and terminated as indicated by arrows.

(Fig. 3), Cyt *f* oxidation by PSI (720 nm illumination) is unhindered by trifluralin and diallate, but the rereduction of photooxidized Cyt *f* by PSII (640 nm illumination) is slowed by the presence of either herbicide. Both inhibitors seem to influence photosynthetic electron flow on the PSII side of Cyt *f*, and neither inhibitor appears to block near PSI.

Inhibition of Photophosphorylation. The effects of trifluralin and diallate on noncyclic photophosphorylation were investigated by assaying ATP formation associated with electron transport through the entire chain ($H_2O \rightarrow$ ferricyanide), PSII (site II phosphorylation, $H_2O \rightarrow$ DMQ), and PSI (site I, ascorbate/DAD $\rightarrow$ methylviologen). The results presented above for electron transport inhibition by diallate and trifluralin are supported by the data for inhibition of phosphorylation. Figure 4 depicts the results obtained with trifluralin. Noncyclic photophosphorylation is inhibited half-maximally at approximately 50 μM, while neither site II nor site I phosphorylation is significantly affected. In all cases the phosphorylation efficiency is constant regardless of the presence of varied concentrations of trifluralin; the P/e$_2$ values for these processes fall within the ranges of: 0.79 to 1.20 for noncyclic, 0.44 to 0.50 for site II, and 0.14 to 0.20 for site I. In the case of diallate, both complete noncyclic and site II phosphorylation are inhibited, while site I is unaffected (Fig. 5). Half-maximal inhibition values for noncyclic (I_{50} = 25 μM) and site II (I_{50} = 100 μM) are close to what is expected on the basis of electron transfer inhibition results. Again, the phosphorylation efficiencies remain constant, and are similar to the values obtained with trifluralin.

Experiments were conducted to determine the effects of trifluralin and diallate on cyclic photophosphorylation (Table I). It was found that while diallate clearly inhibits PMS cyclic phosphorylation (I_{50} = 50 μM), trifluralin appears to have no effect. This inhibitory effect of diallate was also observed in the
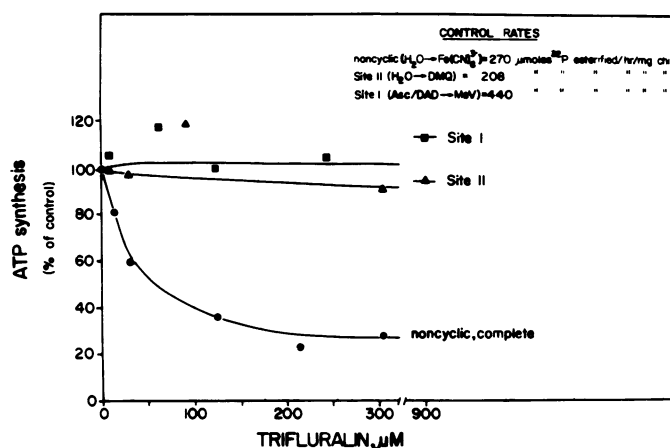

FIG. 4. Effect of trifluralin concentration on photophosphorylation associated with noncyclic electron flow and with the partial reactions of site II and site I. Control rates of ATP synthesis in the absence of trifluralin are given in the inset.
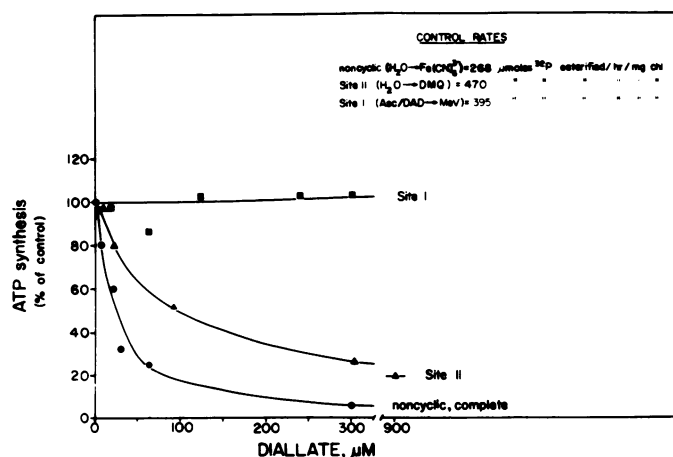

FIG. 5. Effect of diallate concentration on photophosphorylation associated with noncyclic electron flow and with the partial reactions of site II and site I. Control rates of ATP synthesis in- the absence of diallate are given in the inset.

Table I.

Effects of Trifluralin and Diallate on Cyclic Photophosphorylation.

Control rates (μmoles ATP·hr^{-1}·mg chl^{-1}) are 901 for the PMS experiments and 543 for the DAD experiments. See "Materials and Methods" for details.

INHIBITOR	Concentration	INHIBITION	
		PMS	DAD
	(μM)	(%)	
Trifluralin	0	0	0
	10	4	6
	25	16	24
	50	18	28
	100	16	17
	200	20	6
Diallate	0	0	0
	10	8	8
	25	25	32
	50	54	48
	100	52	20
	200	66	32

absence of DCMU in white light, as well as in red light, and in red light in the presence of 0.33 mM ascorbate (data not shown). Diallate does not directly interfere with PMS, since this redox cofactor has the same spectral properties in the presence or absence of diallate (as determined in a Cary 17

ratio recording spectrophotometer). To determine whether the diallate effect on cyclic phosphorylation is specific to the PMS and pyocyanine systems, phosphorylation using DAD as a cyclic electron mediator was also examined (Table I). These results are more difficult to interpret. The DAD-catalyzed reaction is inhibited in a manner nearly identical to the inhibition of PMS cyclic at diallate concentrations of 50 μM or less; however, at 100 μM and above, there appears to be a partial release from inhibition. Inhibition of the DAD cyclic reaction by trifluralin appears to exhibit a similar behavior, with greatest inhibition at intermediate concentrations of trifluralin. The inhibition of cyclic phosphorylation by trifluralin never attains the severity imposed upon the same system by diallate.

To determine the effects of trifluralin and diallate on coupling factor functions in chloroplasts, ATP-Pi exchange and ATPase activities were determined in the presence of varying concentrations of the two herbicides. Figure 6 shows the titration for ATP-Pi exchange. Both trifluralin and diallate inhibit this activity half-maximally in the range of 20 to 30 μM. At high concentrations (200 μM), each herbicide is able to inhibit exchange activity to the level of the unactivated dark control. Finally, both compounds are able to exert their influence whether present in the activation stage (necessary to trigger the exchange activity) or during incubation (in the presence of
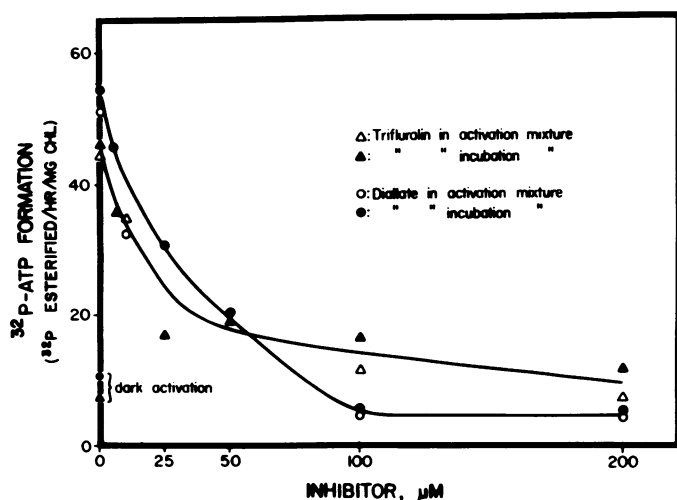

Fig. 6. Effect of trifluralin and diallate on the light- and sulfhydryl-induced ATP-Pi exchange activity. Herbicides were present either during the activation in the light, or during the subsequent incubation at 37 C, as indicated in the figure.

Table II.

Effects of dithiothreitol on diallate and trifluralin inhibition of photosynthetic electron transport.

Chloroplasts (10-20 ug Chl/ml) were incubated at 25 C with dithiotreitol, trifluralin, and diallate, as indicated. The rate of oxygen evolution (H_2O → ferricyanide) was measured as described in "Materials and Methods".

TREATMENT	RATE OF OXYGEN EVOLUTION	
	basal	uncoupled by methylamine
	(μmol O_2/hr·mg Chl)	
untreated	79	288
+0.31 mM DTT	78	329
+62.5 μM trifluralin	17	62
" " " +0.31 mM DTT, simultaneously	11	64
+62.5 μM diallate	29	105
" " " +0.31 mM DTT, simultaneously	22	97
+125 μM diallate	12	40
" " " +0.31 mM DTT, simultaneously	15	30
5 min chlplst dark preincubation with 0.31 mM DTT; 1 min dark with 62.5 um trifluralin	15	60
5 min chlplst dark preincubation with 0.31 mM DTT; 1 min dark with 125 μM diallate	15	30

ATP). Similar results were obtained for Mg^{2+}-ATPase activity, except that the values for half-maximal inhibition were somewhat higher (65 μM for diallate, 90 μM for trifluralin). Thus, both diallate and trifluralin are able to inhibit coupling factor functions not directly related to coupled photophosphorylation.

Effects of Thiol Reagents on Trifluralin and Diallate Inhibition. Using the thiol reagent DTT, experiments were conducted to determine whether trifluralin or diallate inhibition can be abrogated by thiol reagents, as is the case for DBMIB (14). The data, presented in Table II, show that a 5-fold excess of DTT, which is clearly effective in reversing DBMIB inhibition (14), has no effect on trifluralin or diallate inhibition of electron flow. This is true either when DTT and the specified herbicide are simultaneously added to the reaction vessel before chloroplast addition, or when the chloroplasts are preincubated with DTT for a 5-min dark period. This inability of DTT to reverse trifluralin and diallate inhibition contrasts sharply with the reversal of DBMIB inhibition by thiol compounds.

DISCUSSION

Previous investigations on the role of trifluralin in isolated spinach chloroplasts have been reported only by Moreland et al. (12) who found that trifluralin inhibited ferricyanide reduction and associated phosphorylation, and that PMS cyclic phosphorylation was only slightly affected by this herbicide. Our work confirms these results and further directs attention to a specific site of trifluralin inhibition between the two photosystems. We find that concentrations of trifluralin which strongly inhibit ferricyanide reduction and which block the rereduction of photooxidized Cyt f do not effectively prevent the reduction of lipophilic electron acceptors and have no effect on PSI reactions. Amine uncoupling fails to alter inhibition by trifluralin. The coupling of electron transport to phosphorylation is unaffected by trifluralin, since the P/e_2 ratios of the various systems assayed remained constant over a wide range of inhibitor concentrations.

The results reported here with trifluralin are reminiscent of effects observed with DBMIB (8, 18), most notably the reversal by class III acceptors of the inhibition of ferricyanide reduction. There are, however, differences between DBMIB and trifluralin. One such difference is purely procedural: while DBMIB must be synthesized in the laboratory, trifluralin is a commercially available compound that can be quickly purified. A second difference relates to the observation that DBMIB, at concentrations greater than 10 μM, acts as a class III acceptor (5, 8, 10). The complication of using an inhibitor that may act as an electron acceptor near its site of inhibition is avoided with trifluralin since none of our experiments indicate that this herbicide can act as a class III acceptor. The final notable difference between trifluralin and DBMIB concerns reactivity with thiol reagents. The observation that DBMIB inhibition may be reversed by DTT led Reimer and Trebst (14) to suggest that DBMIB might react with —SH groups in the thylakoid membrane to exert its inhibitory effects. Trifluralin inhibition shows no such thiol reversibility. While this finding yields no information as to the mechanism of trifluralin inhibition, it does suggest that this mechanism is likely to be different from the mechanism operative for inhibition by DBMIB.

On the basis of the data presented here, and by analogy to the behavior of DBMIB, it seems clear that trifluralin inhibits electron flow at a site between the two photosystems, on the PSII side of Cyt f. Since trifluralin is more easily obtainable than DBMIB and since it does not exhibit the complicating behavior of acting as a class III acceptor, it should prove a useful inhibitor for blocking electron flow between PSII and PSI.

Diallate exhibits a more complex set of inhibitory characteristics than does trifluralin. Diallate is clearly an effective inhibitor of noncyclic electron flow; however, this inhibition is altered by both methylamine and PD_{ox}. The ability of methylamine to

reverse diallate inhibition is suggestive of weak energy transfer inhibition (7). The partial reversal of diallate inhibition by PD_{ox} indicates that one site of diallate inhibition may occur after the site of electron withdrawal from PSII by class III acceptors. However, the fact that PD_{ox} cannot sustain electron flow at high diallate concentrations indicates that a second site of diallate blockage may occur closer to PSII. Thus, at low concentrations diallate may block primarily at a site near that suggested above for trifluralin, while at higher concentrations, a secondary site of inhibition, nearer PSII, may limit activity. In any case, diallate inhibition is exerted on the PSII side of Cyt f.

Although diallate apparently does not affect PSI noncyclic electron flow or associated phosphorylation, it does inhibit cyclic photophosphorylation catalyzed by PMS or DAD. This result is puzzling: since these cyclic systems depend on components associated with PSI, one would expect that both cyclic and noncyclic electron flow requiring PSI would be similarly affected by diallate. It is possible that the site of diallate inhibition in the noncyclic electron transfer pathway is also operative in the inhibition of the cyclic systems, that is, that some portion of the electron transport chain prior to Cyt f is common to both the cyclic and the noncyclic systems and susceptible to diallate inhibition. This seems difficult to reconcile with the observation that trifluralin, which seems to inhibit the same area of the electron transport chain as diallate, does not depress cyclic electron flow. Nevertheless, alternative explanations for this unexpected behavior of diallate do not come to mind.

The multiple effects elicited by diallate in inhibiting photosynthetic electron flow and photophosphorylation make it a less easily understandable and, therefore, perhaps a less immediately useful inhibitor than trifluralin. Nevertheless, it is clear that both of these herbicides block electron flow between the two photosystems and are thus unusual and interesting inhibitors.

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