

Interaction of Chloroplasts with Inhibitors

EFFECTS OF TWO DIPHENYLETHER HERBICIDES, FOMESAFEN AND NITROFLUORFEN, ON ELECTRON TRANSPORT, AND SOME COMPARISONS WITH DIBROMOTHYMOQUINONE, DIURON, AND PARAQUAT

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ABSTRACT

Several effects on pea (*Pisum sativum* L. var Onwards) chloroplasts of a new diphenylether herbicide, fomesafen (5-[2-chloro-4-(trifluoromethyl)phenoxy]-*N*-methanesulfonyl-2-nitrobenzamide) have been compared with those of a herbicide of related structure, nitrofluorfen (2-chloro-1-[4-nitrophenoxy]-4-[trifluoromethyl]benzene). Although both compounds produce the same light-dependent symptoms of desiccation and chlorosis indicative of a common primary mechanism of action, this study is concerned with a more broadly based investigation of different effects on the electron transport system. Comparisons have also been made with other compounds interacting with the chloroplast. Unlike nitrofluorfen, fomesafen has little effect as an inhibitor of electron flow or energy transfer. Both compounds have the ability to stimulate superoxide production through a functional electron transport system, and this involves specifically the *p*-nitro substituent. The stimulation, which is not likely to be an essential part of the primary herbicidal effect, is diminished under conditions that remove the coupling factor. Evidence suggests that both diphenylethers may be able to bind to the coupling factor, and kinetic studies reveal this for dibromothymoquinone as well. Such a binding site might be an important feature in allowing the primary effect of the diphenylether herbicides to be expressed.

Diphenylether herbicides require light for their action (7, 14) and this has focused attention on the chloroplast for the elucidation of specific sites of attack. The ability to inhibit electron transport has been established for a wide range of diphenylethers (15, 30), and Moreland *et al.* (15) have proposed that the main site of action is associated with PSII. Bugg *et al.* (3) studying nitrofluorfen¹ and HOE 29152 have attempted to characterize the site in more detail and found that the point of inhibition lies between the two photosystems in the plastoquinone-Cyt *f* region. This site is similar to that inhibited by dibromothymoquinone (DBMIB)

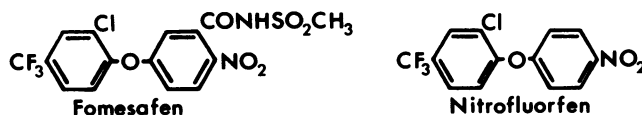
¹ Abbreviations: nitrofluorfen, 2-chloro-1-(4-nitrophenoxy)-4-(trifluoromethyl)benzene; HOE 29152, methyl-2-(4-[4-trifluoromethoxy]phenoxy)propanoate; PQ, plastoquinone; DBMIB or dibromothymoquinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, 2,4-dinitrophenylether of iodonitrothymol; acifluorfen, 5-(2-chloro-4-[trifluoromethyl]phenoxy)-2-nitrobenzoate; acifluorfen-methyl, methyl 5-(2-chloro-4-[trifluoro-methyl]phenoxy)-2-nitrobenzoate; fomesafen, 5-(2-chloro-4-trifluoromethylphenoxy)-*N*-methanesulfonyl-2-nitrobenzamide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; *I*₅₀, 50% inhibiting dose; MV, methyl viologen (paraquat); nitrofen, 2,4-dichlorophenyl-*p*-nitrophenyl ether; CF1, coupling factor 1; FeCN, potassium ferricyanide; oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene.

and DNP-INT (29), and more specifically the inhibition involves an interaction with the Cyt *b₆-f* complex close to the Rieske iron-sulfur center (13). Draber *et al.* (4) examined a number of diphenylethers for their ability to produce an inhibition pattern like DBMIB but some of these act at the same site as diuron (DCMU).

Lambert *et al.* (10) studying the mode of action of nitrofen find that the inhibition of electron transport in spinach chloroplasts is much greater than in chloroplasts from the microalga *Bumilleriopsis filiformis*. There is nevertheless severe inhibition of both cyclic and noncyclic photophosphorylation in chloroplasts from both species at 10 μM, a concentration that does not affect electron transport in *Bumilleriopsis*, and nitrofen acts as an energy transfer inhibitor in strict competition with ADP in the photophosphorylation reaction. Sandmann *et al.* (22) have compared a range of diphenylether structures with their differing abilities to inhibit electron transport, ATP production, and induce chlorosis in the green microalga *Scenedesmus acutus*.

One of the consequences reported for the action of diphenylether herbicides is an increase in permeability of cell membranes during illumination (16, 18, 31). The resulting tissue damage is essentially similar to that caused by paraquat rather than by inhibitors of electron transport, yet the bleaching effect is not reduced by Hill reaction inhibitors (14). Orr and Hess (16) reported the need for another diphenylether (acifluorfen-methyl) to be activated by carotenoid to initiate a free radical chain reaction (reviewed in 17), but it can do so in the presence of DCMU and DBMIB, which implies that the primary mechanism of action is not dependent on electron transport. Acifluorfen interferes with the blue-light-induced phototropic response and may act directly at the photoreceptor site (11).

The present study uses a new diphenylether herbicide, fomesafen, to compare several effects on electron transport with the structurally related herbicide nitrofluorfen, with a view to learning more about the interactions with chloroplasts (whether herbicidal or not) which are common to this chemical group. Although the two diphenylethers examined have different physical properties, they produce the same symptoms of chlorosis and desiccation in plants indicative of a common primary mechanism of action. The more wide-ranging information about effects on electron transport has been obtained through a comparison of the *in vitro* actions produced by DCMU, DBMIB, and paraquat.



MATERIALS AND METHODS

Plant Material. Chloroplasts were isolated from pea leaves (*Pisum sativum* L. var Onwards). Plants were grown for 17 to 20

d in a growth room at 14°C under 40 $\mu\text{E}/\text{m}^2 \cdot \text{s}$ light energy from nine 30-w strip lights (six white lights and three 'Grolux' lights).

Isolation of Chloroplasts. Isolation of intact chloroplasts from pea seedlings was based on methods developed in Walker's laboratory (12, 21, 24). About 40 young pea shoots were homogenized for 5 to 10 s by an Ultraturrex blender in 250 ml of isolation medium (21); for experiments on energy transfer, the Pi buffer was replaced by Tricine at pH 7.1. After filtration through muslin and 50- μm mesh cloth (19), the filtrate was centrifuged at 2,000g for 20 s. The pellet was resuspended with a soft quill brush in a wash medium (containing 330 mM glucose, 5 mM MgCl_2 , and 2 ml of double-strength resuspension medium, in 50 ml) and centrifuged at 2,000g for 20 s. The chloroplast pellet was finally taken up in resuspension medium containing the following final concentrations: 330 mM sorbitol, 50 mM Hepes, 2 mM MgCl_2 , 2 mM EDTA, 5 mM K_2HPO_4 , and 0.1% BSA adjusted to pH 7.8. (EDTA was omitted from energy transfer experiments, and Pi was omitted in experiments involving a Pi dose response.) Total Chl was estimated by the method of Arnon (1).

PSII Activity and Energy Transfer. O_2 evolution in the presence of FeCN as a Hill reaction acceptor was measured in a water-jacketed electrode (Hansatech Ltd., King's Lynn, Norfolk) maintained at 20°C. Intact chloroplasts equivalent to a final concentration of 25 μg Chl/ml were ruptured osmotically in water in the electrode cell and then double-strength resuspension medium added, together with 1 mM FeCN, 5 mM K_2HPO_4 , 500 μM ADP, and inhibitor doses as indicated in figures, giving a final volume of 1 ml. The pH was 7.8 which is in the middle of the plateau for the pH-dependence of ATP formation (23). The electrode cell was illuminated from a 150-w tungsten-iodide projector lamp with the light beam passing through a Calflex C interference heat filter and giving 3,200 $\mu\text{E}/\text{m}^2 \cdot \text{s}$ light energy at the cell surface.

For measuring the kinetics of energy transfer, a coupled nonphosphorylating basal rate (V_o) was initially recorded for 2 to 3 min, and then either ADP (50 or 60–400 μM) or Pi (100–1500 μM) added to promote State 3 electron flow (V). The stimulation of electron flow ($V-V_o$) was used for kinetic analysis. Phosphate (5 mM) was present in the reaction mixture before illumination when varying concentrations of ADP were used to initiate State 3 electron transport. Conversely, 500 μM ADP was initially present when varying concentrations of Pi were used to initiate State 3 electron flow during illumination. Inhibitors were always added to the reaction mixture before the start of illumination.

Each complete set of kinetic data for energy transfer (consisting of a series of controls and a series with one dose of inhibitor) takes between 3 and 4 h to generate with each substrate dose replicated two or three times. To off-set any fall-off in the activity of PSII during the analysis period, each chloroplast sampling for a control was followed by a chloroplast sampling that was inhibitor-treated. Concurrent analysis was found to produce more reliable data than consecutive analysis. Thus, each inhibitor dose had its own control, and only one inhibitor dose was examined in any one experiment, but a kinetic analysis for each dose was repeated two or three times. Determination of Michaelis-Menten kinetics for electron transport stimulation ($V-V_o$) versus ADP or Pi (where V is the State 3 rate and V_o is the basal rate) was obtained by an iterative fit using a microcomputer, which also generated the reciprocal plots from the calculated K_m and V_{max} values.

PSI Activity. PSI activity was measured as O_2 uptake in the presence of an artificial electron donor and DCMU. The reaction mixture (2 ml) contained resuspension medium, 100 μM TMPD, 1 mM Na isoascorbate, 2.5 μM DCMU, 500 μM NaN_3 (final concentrations), and ruptured chloroplasts equivalent to 50 μg Chl.

Pseudocyclic Electron Transport. Intact chloroplasts were ruptured osmotically in the O_2 electrode cell as described above, and the net uptake of O_2 during illumination was recorded. The

reaction mixture (2 ml) contained resuspension medium, 500 μM ADP, and 500 μM NaN_3 (final concentrations), and 50 μg Chl. Inhibitors were added before the start of illumination.

Measurement of Superoxide. The superoxide free radical ion oxidizes hydroxylamine to NO_2^- (6) and the accumulated NO_2^- was determined by a method based on that of Elstner and Heupel (5). The reaction mixture (4 ml) for the production of superoxide contained the following concentrations: 25 mM Tricine (pH 7.8), 2 mM NH_4Cl , 2 mM MgCl_2 , 12.5 μM NH_2OH , 1 mM NaN_3 , chloroplasts equivalent to 100 μg Chl, and in the test sample, 25 μM diphenylether. The mixture was illuminated with saturating light at 20°C, and 200- μl samples were removed at intervals for estimation of NO_2^- .

Chemicals. Fomesafen, nitrofluorfen, DBMIB, and DCMU were synthesized at Jealott's Hill. Stock solutions of fomesafen were prepared as the Na salt in water. Stock solutions of nitrofluorfen, DBMIB, and DCMU were prepared in ethanol, but the final concentration of ethanol in any reaction mixture never exceeded 0.3%. Trisodium thiophosphate was obtained from Thiokol Corporation, Danvers, MA.

RESULTS

Dose Responses for the Inhibition of Electron Transport. Dose responses have been recorded as FeCN-dependent O_2 evolution which measures coupled nonphosphorylating basal electron transport (V_o) and phosphorylating State 3 electron transport (V). As seen in Figure 1A, the new herbicide, fomesafen, registers only a limited effect on V_o and V at doses up to 300 μM when it inhibits 14% and 19%, respectively; the inhibition is almost entirely reversed when electron flow is uncoupled. The structure/activity data reported by Sandmann *et al.* (22) showed less inhibition of electron flow when there is a charged group in the 3' position. As expected, therefore, an inhibition of electron transport is not a primary herbicidal action of fomesafen. Nitrofluorfen, by comparison, is clearly a good inhibitor (shown in Fig. 1B over a limited range up to 10 μM) giving a rather greater inhibition of V

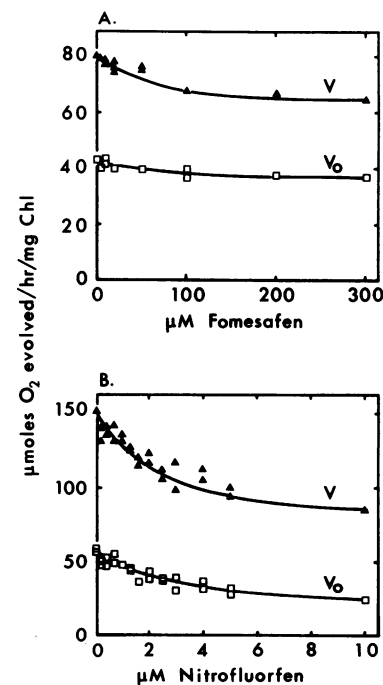


FIG. 1. Dose responses for the inhibition of coupled nonphosphorylating basal rate (V_o) and phosphorylating State 3 (V) electron transport by (A) fomesafen, and (B) nitrofluorfen, recorded as rates of O_2 evolution in the presence of FeCN.

over that of V_o , particularly at the lower doses; from wider range dose responses (not shown) the I_{50} for V is $8.5 \mu\text{M}$ and for V_o is $11.8 \mu\text{M}$. These values are in general agreement with those reported by Bugg *et al.* (3) for electron transport from H_2O to MV, but these authors found no difference between the coupled and uncoupled systems, whereas in the experiments reported here, the inhibition (H_2O to FeCN) is partially relieved by an uncoupler (NH_4Cl) giving an I_{50} of $21.5 \mu\text{M}$ (data not shown). The inhibition by DCMU (in a low dose response up to $0.25 \mu\text{M}$) differs from nitrofluorfen in showing a distinct lag before there is any inhibition of basal rate electron transport (V_o), but State 3 (V) is inhibited to varying degrees by all the doses across the range (Fig. 2). Inhibition by DCMU or DBMIB is not relieved by uncouplers, in fact DCMU is more inhibitory to uncoupled electron transport (at $28 \mu\text{M}$ Chl, the I_{50} for coupled electron flow is $0.13 \mu\text{M}$, and for

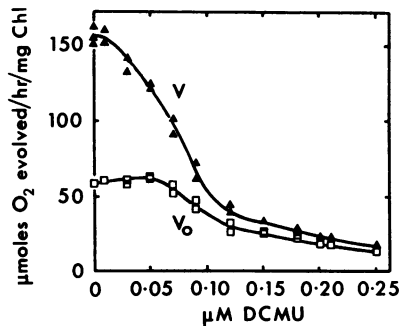


FIG. 2. Low concentration dose responses for the inhibition by DCMU of basal rate (V_o) and State 3 rate (V) electron transport.

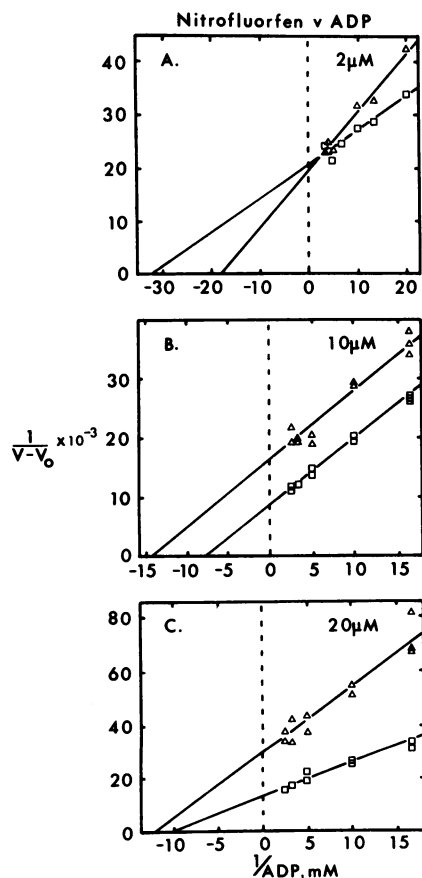


FIG. 3. Kinetic analyses for inhibition of ADP-stimulated energy transfer ($V-V_o$) by nitrofluorfen. A, Competitive inhibition by $2 \mu\text{M}$; B, uncompetitive or mixed inhibition by $10 \mu\text{M}$; C, noncompetitive inhibition by $20 \mu\text{M}$. (Δ), Nitrofluorfen; (\square), control. $V-V_o = \mu\text{mol O}_2/\text{h}\cdot\text{mg Chl}$.

the uncoupled system it is $0.038 \mu\text{M}$).

Kinetic Analyses of Energy Transfer Inhibition. Partial or complete reversal of electron transport inhibition by the diphenyl ethers when the system is uncoupled is typical of the effect found with energy transfer inhibitors (8). Kinetic analyses have been carried out for the inhibition of the ADP-dependent and Pi-dependent stimulation of electron flow: that is the State 3 rate (V) minus the basal rate (V_o). The stimulation of electron transport ($V-V_o$) is a more realistic measure of energy transfer than V alone, and provides a better distinction between the effects on energy transfer caused by direct interference with CF1 and the indirect effects of inhibiting electron transport (and hence, the proton gradient on which the activation of CF1 depends). K_m and V_{max} values in untreated (control) chloroplasts varied from one experiment to another but, typically, they were in the following ranges: $K_m(\text{ADP}) = 94$ to $126 \mu\text{M}$; $V_{max}(\text{ADP}) = 60$ to $113 \mu\text{mol O}_2$ evolved/h·mg Chl; $K_m(\text{Pi}) = 220$ to $275 \mu\text{M}$; $V_{max}(\text{Pi}) = 89$ to $98 \mu\text{mol O}_2$ evolved/h·mg Chl. The $K_m(\text{ADP})$ values are close to those found in direct measurements of photophosphorylation by Selman and Selman-Reimer (25), but the $K_m(\text{Pi})$ values are much lower.

A range of doses of fomesafen up to $50 \mu\text{M}$ fails to show any inhibition of energy transfer, thus precluding this mechanism as a possible cause of herbicidal action. Nitrofluorfen, on the other hand, inhibits energy transfer at doses between 1 and $20 \mu\text{M}$, but the kinetic analyses with respect to ADP show changes in the inhibition characteristics with dose in double reciprocal plots (Fig. 3). Thus, $2 \mu\text{M}$ nitrofluorfen shows competitive inhibition (Fig. 3A), which agrees with the reported inhibition of nitrofen at this dose (10). At $10 \mu\text{M}$, nitrofluorfen shows uncompetitive inhibition (the lines are parallel here) (Fig. 3B), and $20 \mu\text{M}$ shows noncompetitive inhibition (Fig. 3C). These results suggest that there is a direct interaction of nitrofluorfen with CF1, as well as an indirect effect on energy transfer through inhibition of electron transport. Table I shows that, at these doses, basal rate electron transport (V_o) is inhibited more than the degree of ADP stimulation ($V-V_o$). With respect to Pi, nitrofluorfen shows the characteristics of noncompetitive inhibition at all these doses (data not shown).

A range of doses of DBMIB also shows a changing pattern of inhibition characteristics with respect to ADP in double reciprocal

Table I. Influence of Nitrofluorfen on Basal and ADP-Stimulated Electron Transport

Control basal rates (V_o) averaged $41 \mu\text{mol O}_2/\text{h}\cdot\text{mg Chl}$. Percentages were calculated from the experiments shown in Figure 3.

Dose	Basal Rate (V_o)	Stimulation ($V-V_o$)
μM	avg. % of controls	
0	100	100
2	82	86
10	54	67
20	40	47

Table II. Influence of DBMIB on Basal and ADP-Stimulated Electron Transport

Control basal rates (V_o) averaged $33 \mu\text{mol O}_2/\text{h}\cdot\text{mg Chl}$. Percentages were calculated from the experiments shown in Figure 4.

Dose	Basal Rate (V_o)	Stimulation ($V-V_o$)
μM	avg. % of controls	
0	100	100
0.1	89	86
0.2	86	66
0.4	76	43

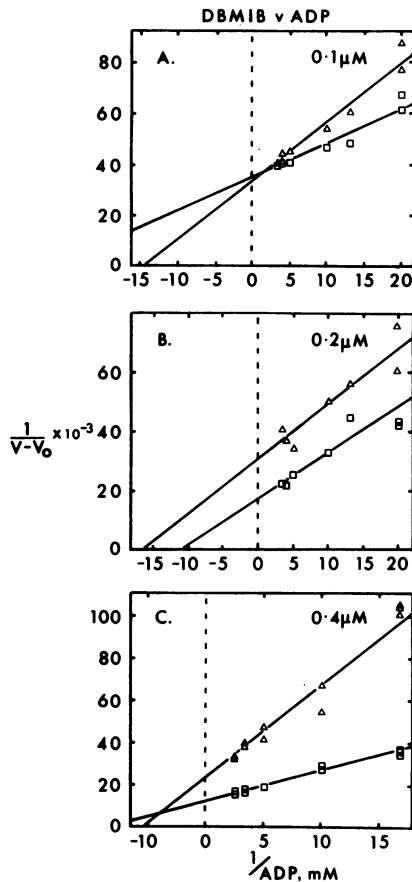


FIG. 4. Kinetic analyses for inhibition of ADP-stimulated energy transfer ($V-V_o$) by DBMIB. A, Competitive inhibition by 0.1 μM ; B, mixed inhibition by 0.2 μM ; C, noncompetitive inhibition by 0.4 μM . (Δ), DBMIB; (\square), control. $V-V_o = \mu mol O_2/h \cdot mg Chl$.

plots (Fig. 4). This is similar to that found for nitrofluorfen when the extent of the inhibitions of stimulated electron flow ($V-V_o$) by the two compounds are matched rather than inhibitions of basal electron transport; this requires DBMIB concentrations to be up to 50-fold less than nitrofluorfen (Table II, and compare with Table I). DBMIB (0.1 μM) gives competitive inhibition (Fig. 4A), 0.2 μM gives mixed inhibition (the lines are not parallel here) (Fig. 4B), and 0.4 μM gives noncompetitive inhibition (Fig. 4C). At the lowest dose (0.1 μM), DBMIB must be reacting directly with CF1 (competitive inhibition was also found in electron transport from H_2O to MV but at a dose of 1 μM which did not inhibit basal electron flow in this particular sequence). At higher doses of DBMIB, the effects are more related to inhibition of electron transport, presumably through a direct interference with the generation of a proton gradient in competition with plastoquinone. This perhaps helps to clarify the apparent uncompetitive inhibition by 10 μM nitrofluorfen since the inhibition is in reality caused by multiple effects which change both the K_m and V_{max} , and the lines are only coincidentally parallel (Fig. 3B). DBMIB differs from nitrofluorfen in causing a much greater inhibition of the ADP-stimulated electron flow ($V-V_o$) over that of basal electron transport (V_o) (Table II). However, with respect to Pi, DBMIB is similar in showing the characteristics of noncompetitive inhibition at these doses (data not shown).

The kinetic analyses of each inhibitor dose have been repeated in separate experiments with the same result each time. However, it was still considered necessary to check on the reliability of these kinetic characteristics because the site at which the effects are measured (O_2 evolution at PSII) is several steps removed from the site at which ADP induces the stimulation of electron flow (the

coupling factor), and only one inhibitor dose was used in each experiment. DCMU was used as an inhibitor directly affecting only electron transport but acting close to the site of O_2 evolution. Trisodium thiophosphate, a competitive inhibitor of Pi (2), was used as an inhibitor reacting only with CF1 and, therefore, at a site which is at the far end of the process influencing the rate of O_2 evolution. DCMU gives noncompetitive inhibition with respect to both ADP and Pi over a range of doses up to 0.2 μM (data not shown). This is to be expected because a direct inhibition of electron transport indirectly lowers the proton gradient required to allow ADP (and Pi) to bind to CF1, and so acts like an allosteric inhibitor. The inhibition with respect to ADP by a low dose (0.05 μM), which does not affect basal electron transport (Fig. 2) but inhibits ADP-stimulated electron flow ($V-V_o$) by an average of 42%, also gives noncompetitive inhibition (Fig. 5). Five mM trisodium thiophosphate does not inhibit electron transport but inhibits ADP-stimulated electron flow ($V-V_o$) by an average of 38%. It shows the characteristics of uncompetitive inhibition with respect to ADP (Fig. 6), which agrees with the inhibition kinetics found by Selman and Selman-Reimer (25) in direct measurements of PSI-driven photophosphorylation from which they established a compulsory order of binding to CF1 with ADP binding first. These results provide reasonable grounds for believing that factors affecting energy transfer in different ways can be reliably measured by means of analysis of O_2 evolution kinetics.

Interaction with PSI. Fomesafen and nitrofluorfen at doses up to 50 μM show no inhibition of O_2 uptake in a PSI reaction with 1 μM MV as electron acceptor and reduced TMPD as donor. However, in the absence of MV, both diphenylethers induce a stimulation in the rate of O_2 uptake, and the addition of superoxide dismutase and catalase diminishes this by about a half (Table III), indicating that both superoxide and H_2O_2 are being generated. It is, therefore, possible that part of the herbicidal action of fomesafen and related diphenylethers lies in the generation of superoxide, which would be in keeping with the observed symptomatology. This aspect has been examined in more detail.

Many experiments with fomesafen have suggested that the age

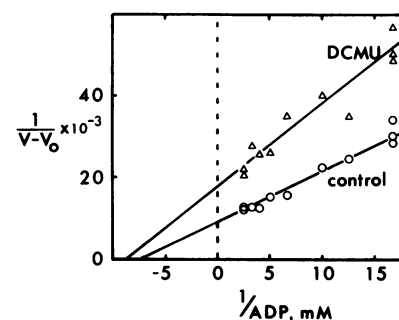


FIG. 5. Kinetics of noncompetitive inhibition by 0.05 μM DCMU with respect to ADP-stimulated energy transfer ($V-V_o = \mu mol O_2/h \cdot mg Chl$).

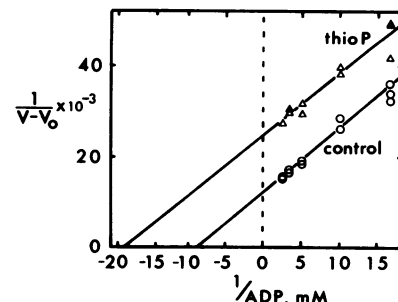


FIG. 6. Kinetics of uncompetitive inhibition by 5 mM trisodium thiophosphate with respect to ADP-stimulated energy transfer ($V-V_o = \mu mol O_2/h \cdot mg Chl$).

Table III. Action of Superoxide Dismutase and Catalase on the Stimulation of O₂ Uptake Induced by Fomesafen in a PSI Reaction

Reaction medium contained chloroplasts equivalent to 100 μg Chl. NaN₃ was omitted. Additions during illumination were made at 2.5-min intervals.

Additions during Illumination	O ₂ Uptake μmol/mg Chl·h
Experiment 1	
Base rate	11
+ Fomesafen (33 μM)	32
+ SOD (250 units)	14
+ Catalase (250 units)	7
Experiment 2	
Base rate	14
+ Fomesafen (33 μM)	31
+ Catalase (250 units)	15
+ SOD (250 units)	8

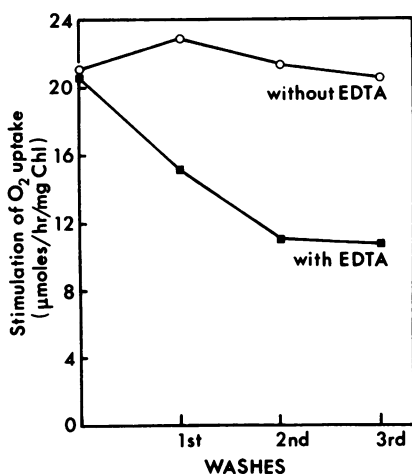


FIG. 7. Effects of successive washings of the chloroplast lamellae with EDTA on the ability of fomesafen to stimulate O₂ uptake in a PSI reaction. A suspension of intact chloroplasts (220 μg Chl/ml) was disrupted by homogenization in a cooled Potter-Elvehjem grinder. The suspension was divided into two portions ('a' and 'b'), and after centrifugation, 'a' was resuspended in 50 mM Hepes buffer (pH 7.5) (control), and 'b' in 1 mM EDTA in 50 mM Hepes (pH 7.5). The stimulation of O₂ uptake induced by 50 μM fomesafen was recorded in a PSI reaction in an aliquot from each preparation, and Chl concentration also determined. The two suspensions of chloroplasts were sedimented and again resuspended in (a) Hepes buffer, and (b) EDTA-Hepes buffer and homogenized (1st wash) giving 150 μg Chl/ml in each. After further sampling to test PSI activity and Chl concentration, the washing procedure and sampling were repeated twice more giving a total of three washes. All points are the average of duplicates.

of the chloroplasts after isolation and the condition of the lamellae (depending on how intact the plastids are before breaking) is important in obtaining the maximum stimulation of O₂ uptake in a PSI reaction; this is generally not the case with paraquat. A more definitive experiment has been carried out to investigate the dependence of fomesafen action on the state of the chloroplasts: isolated intact chloroplasts have been broken osmotically and then treated by washing successively with either 50 mM Hepes buffer at pH 7.5, or buffer containing 1 mM EDTA to provide a progressive removal of components bound to the surface of the lamellae (Fig. 7). The ability of 50 μM fomesafen to induce a stimulation of O₂ uptake in a PSI reaction is diminished by 50% following three

washings with EDTA-buffer, but is unaffected by buffer alone; the rate of O₂ uptake in the absence of fomesafen is not affected by the EDTA treatment. By contrast, the stimulation of O₂ uptake by 0.5 μM paraquat is diminished by less than 5% following three washings with EDTA-buffer solution; the stimulation was 70.3 μmol O₂/h·mg Chl when no EDTA is present, and 67.1 μmol O₂/h·mg Chl when EDTA is included in the washing medium. This experiment shows that a component attached to the lamellar membrane by ionic interaction is involved in the ability of fomesafen to stimulate O₂ uptake, but this is not true of paraquat.

Effects on Pseudocyclic Electron Flow. The rate of O₂ uptake in pseudocyclic electron transport (with H₂O as electron donor and O₂ as acceptor) is also stimulated by both fomesafen and nitrofluorfen, but the dose responses for coupled and uncoupled electron transport show marked differences between the two diphenylethers. In coupled electron transport, fomesafen increases O₂ uptake between 5 and 100 μM, but thereafter no more stimulation occurs at doses up to 300 μM (Fig. 8A). For comparison, the rate of coupled electron flow in the presence of paraquat is shown in Figure 8A. The rate of O₂ uptake reaches a plateau at 10 μM paraquat and is almost 1.5 times the rate in the corresponding plateau with fomesafen. If pseudocyclic electron transport is uncoupled with NH₄Cl, the rate of O₂ uptake with fomesafen is increased over that in the coupled system. No limit is reached at concentrations up to 300 μM, suggesting that the proton gradient, rather than direct inhibition, is limiting the rate of coupled electron transport at doses greater than 100 μM. (The uncoupled rate with 300 μM fomesafen is not shown in Figure 8A, but is 97 μmol O₂ consumed/h·mg Chl.) Again for comparison, the rate of uncoupled electron flow in the presence of paraquat rises from 62 to 250 μmol O₂ consumed/h·mg Chl with concentrations from 0.25 to 25 μM, and thereafter the rate remains constant up to 300 μM paraquat.

In contrast, nitrofluorfen progressively inhibits coupled pseudocyclic electron transport at concentrations from 5 μM (6%) to 50 μM (48%) but gives a plateau at doses between 75 and 300 μM, which represents 36% inhibition of the rate of pseudocyclic electron transport with no inhibitor present (Fig. 8B). The greatest degree of inhibition over the concentration range 5 to 300 μM nitrofluorfen is 48% observed with 50 μM. This differs from an I₅₀ of 8.5 μM nitrofluorfen in coupled electron transport with FeCN as electron acceptor (Fig. 1B). The rate of O₂ uptake in uncoupled

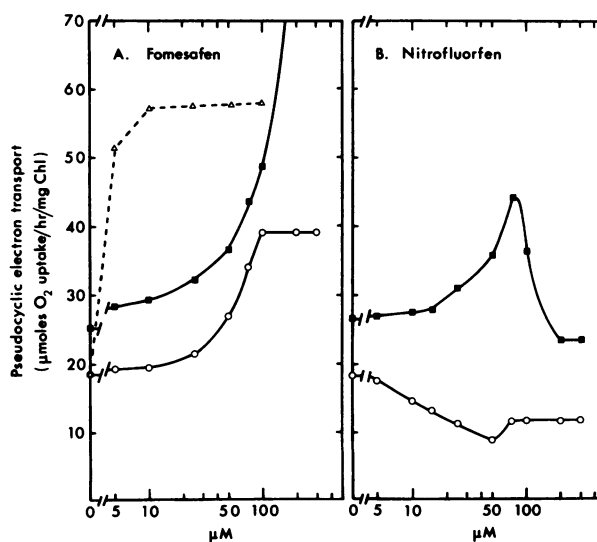


FIG. 8. Effects of (A) fomesafen and (B) nitrofluorfen on O₂ uptake in coupled (○) and uncoupled (■) pseudocyclic electron transport, and comparison with paraquat on the coupled system (Δ--Δ). Ammonium chloride was added to a final concentration of 4 mM after 2.5 min illumination to obtain the uncoupled rates.

pseudocyclic electron flow is greater than in the coupled system and is progressively stimulated by nitrofluorfen at concentrations between 5 and 75 μM . The degree of stimulation is very similar to that induced by fomesafen over the same range, so it is interesting to note that there can be no inhibition of uncoupled pseudocyclic electron flow by nitrofluorfen over this dose range. (Again, this contrasts with an inhibition of uncoupled electron flow when FeCN is the acceptor, giving an I_{50} of 22 μM .) Doses of nitrofluorfen greater than 75 μM then show a fall off in the rate of O_2 uptake stimulation and, in this respect, the action differs from that of fomesafen. A level is reached at doses between 200 and 300 μM where the rates are close to that in the control; this plateau is likely to be due to nitrofluorfen being at the limit of its solubility under the conditions used.

The dose response patterns for the effects of nitrofluorfen on coupled and uncoupled electron transport have been reproduced in three separate experiments with each point assessed in triplicate to ensure that the inflections in the curves are genuine. Therefore, the action on overall electron transport generates patterns that are a reflection of nitrofluorfen's ability both to stimulate O_2 consumption and partially inhibit electron transport at the same time.

Production of Superoxide. The production of superoxide has been measured directly at intervals during a 15-min period of illumination of osmotically ruptured chloroplasts. Figure 9 shows that, under the experimental conditions of pseudocyclic electron flow, superoxide is produced by untreated chloroplasts. In the presence of 25 μM of fomesafen or nitrofluorfen, the production is seen to be stimulated, with a rather greater effect from the former. When 5 μM DCMU is also present with fomesafen, no superoxide is produced at all. This shows that the superoxide generation seen here (Fig. 9) depends on a functioning electron transport system. An experimental analog of fomesafen, in which the *p*-nitro substituent is replaced by a *p*-chloro, is as herbicidally active as fomesafen itself and produces identical symptoms. However, it appears to stimulate superoxide production only slightly within the duration of this experiment (Fig. 9), which implies that it is the *p*-nitro group that is responsible for the effect under these conditions.

DISCUSSION

The primary mechanism of action of the diphenylether herbicides may be related to the initiation of a radical chain reaction

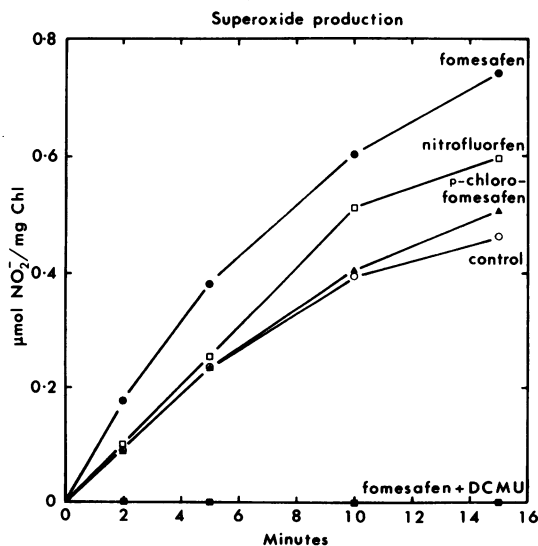


FIG. 9. Effect of 25 μM fomesafen, nitrofluorfen, and the *p*-chloro analog of fomesafen, and also 25 μM fomesafen + 5 μM DCMU, on the production of superoxide during uncoupled pseudocyclic electron transport.

through activation of the herbicides by carotenoids (17). There is no reason to suppose that the new herbicide, fomesafen, behaves differently in its primary herbicidal effect from other diphenylethers of related structure such as acifluorfen-methyl or nitrofluorfen, since these compounds produce similar effects on whole plants (including peas). The main symptoms are chlorosis and desiccation, which are typically caused by toxic O_2 species, but the primary action leading to these symptoms by the two particular molecules examined here must be independent of the differences in properties conferred by the presence of the charged 3'-substituent. Although the light-dependence of the herbicidal action has focused attention on the chloroplast, the studies reported in this paper have been concerned with a more broadly based investigation of different effects on the electron transport system. This has involved a comparison with other inhibitors of different structural types to look for any similarities and differences in biochemical effects that might add to our understanding of the interaction of chloroplasts with inhibitors. The work has raised questions about a possible binding site that may in itself be of importance in the expression of the primary herbicidal effect.

Fomesafen is a very weak inhibitor of the FeCN-dependent Hill reaction (Fig. 1A) and appears not to inhibit pseudocyclic electron flow at all either in the coupled or uncoupled state (Fig. 8A). In contrast, nitrofluorfen is quite a good inhibitor of coupled and uncoupled electron transport in a FeCN-dependent Hill reaction (Fig. 1B), although it differs at low doses from the action of DCMU on basal (V_o) and State 3 (V) electron transport (Fig. 2). (This is a reflection of their different inhibition sites [DCMU acting before PQ function and nitrofluorfen acting after it (3)], and the different influences this has on the proton gradient; the action of DCMU that explains the lag in the inhibition of V_o in Figure 2 has been studied by Siggel *et al.* [26]). In pseudocyclic electron transport, a rather weak inhibition can be seen in the coupled system, but there is no inhibition at all with doses of nitrofluorfen up to 75 μM in uncoupled electron transport (Fig. 8B); an adequate explanation for this will require further study. The difference in response to effects on electron transport by the two diphenylethers may be accounted for by differences in physical properties and hence membrane partitioning, but nevertheless it does rule out a block in electron transport as a primary mode of action (as suggested in earlier research on other diphenylether herbicides [15]) in agreement with recent findings (17).

In more general studies on the diphenylether herbicides, the possibility that separate inhibition sites might be distinguished at different doses of nitrofluorfen using only energy transfer kinetic data generated in the O_2 electrode has been examined. Lambert *et al.* (10), using low doses of nitrofen on chloroplasts from *Bumilleriopsis filiformis*, were able to distinguish between doses causing direct inhibition of coupled nonphosphorylating electron transport (>10 μM) and doses causing direct inhibition of photophosphorylation (<10 μM).

Nitrofluorfen inhibits energy transfer (electron transport stimulated by ADP and Pi), and the kinetics at different doses do indeed reveal multiple effects because, where the electron transport system is only rather weakly inhibited, nitrofluorfen has one binding site on CF1 that is in competition with the binding of ADP. As the basal rate of electron transport becomes more inhibited due to nitrofluorfen binding to a second site, the indirect effect on the build-up of a proton gradient (and subsequent binding of ADP and Pi) is revealed through noncompetitive inhibition kinetics (Fig. 3C); a similar indirect effect is seen with DCMU (Fig. 5). At an intermediate dose (10 μM), the kinetic pattern reveals changes in both K_m and V_{max} and gives parallel lines typical of classical uncompetitive inhibition (Fig. 3B) which should indicate a binding of nitrofluorfen to the ADP-CF1 complex. It is more likely though that the lines are coincidentally parallel and the result is the mixed effect of direct and indirect

inhibition of energy transfer. The finding of two separate binding sites for nitrofluorfen is in general agreement with that of Lambert *et al.* (10) for the binding of nitrofen. Clearly, fomesafen acts differently since it does not inhibit energy transfer at doses up to 50 μM ; such a no-effect level precludes this as a primary herbicidal mechanism for fomesafen.

DBMIB is believed to inhibit electron transport at the same point as nitrofluorfen (3), and indeed it shows a similar change in the kinetic characteristics of reciprocal plots at varying doses (Fig. 4), but at much lower concentrations than nitrofluorfen. There is a greater comparative effect on stimulated electron transport ($V - V_0$) than on basal electron flow (V) (Table II) in accordance with a more direct action on the proton gradient through competition with plastoquinol for oxidation by the Cyt *b-f* complex (reviewed in 28). Nitrofluorfen shows a greater effect on basal electron transport (Table I) and so reveals an essential difference in the inhibition by these two compounds. The competitive inhibition by a low dose (0.1 μM) of DBMIB with respect to ADP (Fig. 4A) is the first intimation that DBMIB can bind directly to CF1, although this conflicts with the report that higher doses do not inhibit cyclic photophosphorylation dependent upon cofactors that by-pass plastoquinone (28). A higher dose of DBMIB (0.4 μM) has its primary effect as a plastoquinone antagonist as reflected in noncompetitive inhibition kinetics (Fig. 4C), and an intermediate dose (0.2 μM) again shows shifts in both K_m and V_{max} (Fig. 4B) but which are surely indicative of the mixed effects resulting from the two inhibition sites. Confidence in the results from the use of only one inhibitor dose in each experiment has been strengthened when the expected inhibition kinetic characteristics were obtained against ADP by DCMU (Fig. 5) and trisodium thiophosphate (Fig. 6).

A feature common to both the diphenylethers examined here is the ability to induce a stimulation of O_2 uptake in pseudocyclic electron transport. This is associated with a production of superoxide (Fig. 9) which is known to lead to the peroxidation of lipids. Although such a finding would be in keeping with a rapid increase in cell membrane permeability observed with another diphenylether of closely related structure, oxyfluorfen (18, 31), the results of the experiment in Figure 9 clearly show that it is the *p*-nitro substituent that is essential for the production of superoxide. The *p*-chloro analog of fomesafen shows a similar level of herbicidal activity as fomesafen itself and the symptoms are identical (Ridley, unpublished), but it does not appear to stimulate the production of significant amounts of superoxide via noncyclic electron transport (Fig. 9). Furthermore, 5 μM DCMU completely abolishes the generation of superoxide by fomesafen (Fig. 9) confirming that, in this particular case, it is dependent on a functional electron transport system, but the herbicidal action of the diphenylethers has been shown to be independent of electron transport (14, 16).

These results show that there is unlikely to be an association between the symptoms of chlorosis and desiccation *in vivo*, and the mechanism for O_2 uptake *in vitro* shown here (Fig. 9). Whatever the mechanism for stimulating O_2 uptake, it must differ from that induced by paraquat since (unlike paraquat) it is greatly diminished when the lamellae are washed with EDTA (Fig. 7). This treatment causes a partial removal of a component which must be fairly tightly bound to the lamellae by ionic interaction, inasmuch as successive washings with 1 mM EDTA are needed to achieve it.

Perhaps the true value of these observations is not so much the stimulation of O_2 uptake *per se* as the fact that it has allowed a binding site to be located within the chloroplast which could be essential for inducing the desiccation effects in whole plants, and hence be related to the true mechanism of action of the diphenylether herbicides. The direct generation of superoxide (Fig. 9; Table III) may still play a role in the action of the *p*-nitrodiphenylethers. The identity of the component removed by

EDTA-treatment (Fig. 7) is as yet unknown, but clearly a primary candidate must be CF1 itself. Nitrofluorfen must bind to CF1 in order to give competitive inhibition with respect to ADP. Fomesafen may also bind to CF1 even though it does not interfere directly with the energy transfer process. The degree of stimulation of O_2 uptake by fomesafen is diminished to a level of about 50% when the lamellae are washed with EDTA (Fig. 7); it is known that only 50% of CF1 is removed by treatment with EDTA (reviewed in 9).

By binding to CF1, the diphenylethers may be able to interact with a component necessary to initiate a light-dependent free radical chain reaction. The coupling factor is closely associated with PSI in the thylakoid membrane in the vicinity of the Fd-NADP and Fd-thioredoxin reductases (27). Orr and Hess (17) have raised the possibility that the diphenylethers could be activated by flavin. The environment of the flavin moiety of the Fd-NADP reductases may be sufficiently disturbed by these herbicides to allow a direct interaction of the flavin with O_2 , which could also account for interference with the blue-light response (11). Alternatively, the diphenylethers may react directly with P_{700} and its associated carotenoids, since it has already been established that photodestruction of P_{700} can be induced by paraquat without the need of a functional noncyclic electron transport system (reviewed in 20), and the diphenylethers may behave in a similar manner.

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