Interference by Herbicidal Inhibitors of Electron Transport with Phosphorylation and Permeability Properties of Chloroplast Membranes

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Many herbicides inhibit chloroplast electron transport by interfering with a proteinaceous component of the Q_B complex located in the appressed granal membrane. Certain of these herbicides, designated inhibitory uncouplers, also interfere with photophosphorylation and affect other chloroplast-mediated responses, some of which involve components located in the non-appressed granal membrane. The inhibitory uncouplers can be divided into dinoseb (phenolic) types which contain dissociable protons and dicryl (acylanilide) types which are nonionic. The dinoseb types can function as protonophores and shuttle protons across the thylakoid membrane at low concentrations and can alter the integrity of semipermeable membranes at higher concentrations. However, the dicryl types only alter the integrity of the membranes. The inhibitory uncouplers, but not the DCMU-types of electron transport inhibitors: stimulated electron transport from $DPIPH_2$ to methyl viologen; inhibited valinomycin-induced swelling of intact chloroplasts; increased the permeability of the chloroplast envelope to K^+ in the absence of an ionophore; prevented energization of the thylakoid membrane by PS I; and increased the permeability of phosphatidyl choline liposomes to protons. Chlorination response patterns obtained with isomers of N-phenyl-2-methylpentanamides in the above reactions, in general, were similar for interference with the Q_B complex, *i.e.*, in all assays, dichlorination in the 3,4 or 3,5 positions was associated with maximum inhibitory potency, whereas substitution in an *ortho* position decreased inhibitory activity. With a series of 1-alkyl-3-(α,α,α-trifluoro-*m*-tolyl)ureas, maximum inhibition of electron transport was obtained with the butyl derivative, whereas maximum responses for uncoupling and membrane disturbances were obtained with the hexyl or octyl derivatives. Some of the interferences produced by inhibitory uncouplers may result from interactions with the lipoidal components of chloroplast membranes.

Introduction

Approximately one-half of all commercial herbicides and their derivatives interfere with electron transport and the associated production of ATP in isolated chloroplasts. The herbicides inhibit by interacting with a component of the Q_B-protein complex [1–3]. According to current concepts of the compositional arrangement of thylakoids, the Q_B-protein complex is concentrated in the appressed granal membranes [4]. The inhibitory herbicides have been divided into two major groups: electron transport inhibitors and inhibitory uncouplers [5]. In this grouping, the DCMU-type inhibitors are classified as electron transport inhibitors (biscarbamates, chlorinated phenylureas, pyridazinones, *s*-triazines, triazinones, uracils, and urea-carbamates). They interfere primarily with the Q_B-protein complex. The inhibitory uncouplers have in turn been subdivided into two groups: dinoseb types and dicryl types [6]. Classified as being dinoseb types are the phenolics (benzonitriles and dinitrophenols) and benzimidazoles, which possess dissociable protons and are weak acids with pK_a's below 7. Included as dicryl types are the various acylanilides (propanil, chlorpropham) which are nonionic. The inhibitory uncouplers, in addition to interfering with electron transport at Q_B, also inhibit photophosphorylation.

Whereas interference of electron transport has been shown to involve interaction with a proteinaceous component of the thylakoid, interference

Abbreviations: Bromofenoxim, 3,5-dibromo-4-hydroxybenzaldehyde-2,4-dinitrophenyloxime; Chl, chlorophyll; chlorpropham, isopropyl-N-(3-chlorophenyl)carbamate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; desmethyl fluometuron, 1-methyl-3-(α,α,α-trifluoro-*m*-tolyl)-urea; dinoseb, 2,4-dinitro-6-*sec*-butylphenol; DPIP, 2,6-dichlorophenolindophenol; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; karsil, N-(3,4-dichlorophenyl)-2-methylpentanamide; PMS, phenazine methosulfate; propanil, N-(3,4-dichlorophenyl)propionamide; PS, photosystem; Q_B, secondary quinone electron acceptor of photosystem II.

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with photophosphorylation has been postulated to involve interaction with lipoidal constituents of the membrane [7]. The inhibitory uncouplers, but not the DCMU-types of inhibitors, also uncouple oxidative phosphorylation [1, 7, 8].

The inhibitory uncouplers conceivably interact with the coupling factor (F_0-F_1) complex and possibly reactions mediated by the PS I complex which are considered to be concentrated in the nonappressed membranes of thylakoids [4]. Appressed and nonappressed membranes exhibit, in addition to different protein composition, differences in lipid composition and in fluidity [4]. The appressed membranes are somewhat less fluid than the nonappressed membranes, primarily because of a larger protein/lipid ratio.

Previous structure-activity studies conducted with herbicides have focused, for the most part, on interactions with the Q_B (appressed membrane) proteinaceous site [1]. Hence, the objectives of this study were to examine structural requirements for interactions (a) with complexes located in the nonappressed thylakoid membrane and (b) with chloroplast envelopes.

Materials and Methods

Intact chloroplasts and thylakoids were isolated from freshly harvested growth chamber-grown spinach (*Spinacia oleracea* L.) as described previously [7].

Responses induced by herbicides were measured on several assays. Inhibition of ferricyanide reduction with water as oxidant (noncyclic electron transport) and the coupled phosphorylation were performed as described previously [7] and reflect effects imposed on PS II at Q_B.

Three assays were used that involved PS I. Interference with phosphorylation and membrane energization responses were monitored by measuring PMS-mediated cyclic phosphorylation [9], electron transport from $DPIPH_2$ to methyl viologen [10], and relief of light-induced quenching of atebrin fluorescence [7]. The specific activity of the $DPIPH_2$ reaction, measured as oxygen uptake, averaged 120 µmoles of oxygen consumed/mg Chl/h in the absence of test compounds. Uncouplers stimulate the reaction, hence, results are presented as the concentrations required to double the rate of oxygen consumption (S_{100} or pS_{100}).

Alterations induced to the cation permeability of chloroplast membranes were monitored with osmotic swelling techniques [7]. Chloroplast envelopes and thylakoid membranes are relatively impermeable to cations such as H^+ and K^+, but freely permeable to the lipophilic anion SCN^- [11, 12]. Hence, the organelles are osmotically stable when suspended in isotonic KSCN unless the permeability of K^+ is altered. An increase in internal K^+ will be accompanied by the diffusion of SCN^-, the counter ion, across the membrane to maintain electroneutrality. The resultant increase in internal solute concentration will result in an influx of water and swelling will occur (passive swelling). Swelling was monitored as a change in absorbance at 550 nm. Results are expressed as the concentration of test chemicals required to induce a swelling rate (ΔA_{550}) of 0.02 A/min. The mobile K^+-transporting ionophore, valinomycin, also induces swelling [13]. Valinomycin-induced swelling is inhibited by uncouplers. Chloroplast envelopes and thylakoids are affected to the same extent [7]. In this report, only data obtained with intact chloroplasts are presented because the magnitude of swelling changes were 3 to 4 times larger than that obtained with thylakoids and thus could be measured more readily.

Herbicide-induced increases in proton permeability also were measured with soybean lecithin liposomes prepared and assayed as described previously [7]. In this system, the lipophilic electron carrier, ferrocene mediates the reduction of internally entrapped ferricyanide by external ascorbate. Diffusion of protons across the membrane is required to maintain electroneutrality [14]. The no-herbicide control rate of ferricyanide reduction averaged 40 nmoles/min. Results obtained with the test compounds are presented as the concentrations required to double the rate of ferricyanide reduction (S_{100} or pS_{100}). Uncouplers have been shown to accelerate the reduction of ferricyanide, presumably by increasing proton permeability.

Stock solutions of the desired concentrations of test chemicals were prepared in acetone. Solvent concentration was held constant at 1% (v/v) in all assays including controls. Data presented represent averages of results obtained in a minimum of three separate isolations and replications of chloroplasts, thylakoids, or liposomes. Data are expressed as I_{50} and pI_{50} values except as noted above.

Results

Shown in Fig. 1 are interference profiles that reflect molar concentrations required for some inhibitory uncoupler herbicides and the uncoupler FCCP to affect the different assays used in this study. Results obtained in the three assays that involved PS I-mediated reactions (cyclic phosphorylation, oxidation of $DPIPH_2$, and restoration of the quenched fluorescence of atebrin) have been combined into a concentration range. Results obtained in the two assays that involved changes in membrane permeability (passive swelling and valinomycin-induced swelling) also are presented as a concentration range.

The interference profile obtained with the classical uncoupler FCCP is shown for comparative purposes. With FCCP, the liposome assay was the most sensitive followed by interference of noncyclic phosphorylation, and the assays that involved PS I.

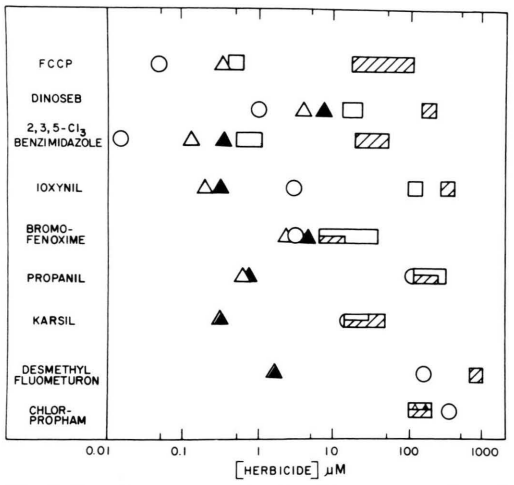

Fig. 1. Interference profiles for FCCP and selected herbicides that represent effective molar concentrations obtained for responses for assays that involved lecithin liposomes and spinach chloroplasts. Legend: ○, concentration required to double the control rate of reduction (S_{100}) of ferricyanide entrapped in lecithin liposomes; △, I_{50} for inhibition of noncyclic photophosphorylation; ▲, I_{50} for inhibition of noncyclic electron transport; □, concentration range for interferences with PS I-mediated assays (I_{50} for inhibition of cyclic phosphorylation, I_{50} for inhibition of light-induced quenching of atebrin fluorescence, S_{100} for stimulation of PS I electron transport); ▨, concentration range for interferences with the semipermeability of chloroplast membranes (I_{50} for inhibition of valinomycin-induced swelling, concentration required to induce a swelling rate of 0.02 A/min). Assay conditions were as described in Materials and Methods.

These responses can be attributed to the protonophoric action of FCCP. FCCP does not inhibit PS II electron transport at concentrations up to 500 μM. Effects on assays that reflect alterations to the permeability properties of the chloroplast envelope occur at concentrations two orders of magnitude higher than the other reactions. Hence, FCCP appears to express two effects. At low molar concentrations, a protonophoric action is manifested, whereas at higher concentrations, alterations are induced to the permeability properties of the membranes, *i.e.*, the membranes have become permeable to protons and cations.

The interference profiles of dinoseb and the benzimidazole (Fig. 1) parallel FCCP's profile with the exception that the herbicides also inhibited noncyclic electron transport. At low molar concentrations, the interferences produced to liposomes, phosphorylation, and PS I reactions by these two compounds can be ascribed to protonophoric activity. However, at higher concentrations a general disruption of the chloroplast membrane was induced.

With ioxynil, coupled noncyclic phosphorylation and electron transport were most sensitive with the liposome assay being intermediate in sensitivity. These interferences can be attributed to the protonophoric action of ioxynil. Interference with the PS I assays and permeability disturbances occurred at concentrations almost two orders of magnitude higher than that required to interfere with the liposome assay.

For bromofenoxim, all responses occurred within an approximate single order of magnitude. The concentration range over which protonophoric action and permeability disturbances were expressed was very narrow.

For three of the four acylanilides [propanil, karsil (a methylpentanamide), and desmethyl fluometuron] noncyclic phosphorylation was only slightly more sensitive than electron transport. Interactions with the liposome system, PS I assays, and the permeability responses occurred at concentrations two orders of magnitude higher. Desmethyl fluometuron (a phenylurea), in contrast to the other acylanilides, had no affect on photosystem I assays up to 1.0 mM. Chlorpropham was a very poor inhibitor of the noncyclic reactions. The concentrations at which electron transport and phosphorylation were inhibited overlapped with its actions on PS I and the permeability disturbances.

Acylanilides monochlorinated in the 3 or 4 position are, in general, more inhibitory to electron transport than the unsubstituted parent compound by an order of magnitude [15]. Dichlorination in the 3,4 or 3,5 positions is associated with increased inhibitory potency of another order of magnitude. However, chlorination in an *ortho* position, in general, has been associated with a decrease or negation of inhibitory activity. Shown in Fig. 2, plotted as pI_{50} or pS_{100}, are responses obtained with N-phenyl-2-methylpentamide and its chlorinated isomers on some of the chloroplast and liposome assays used in developing the inhibition profiles.

In the uncoupled reductive reaction, the isomers can be placed in the approximate following order of decreasing effectiveness, with the most inhibitory isomer listed first (Fig. 2):

$$3,4 > 3,5 = 4 > 3 > H > 2,3 > 2,4 > 2 > 2,5 = 2,6 .$$

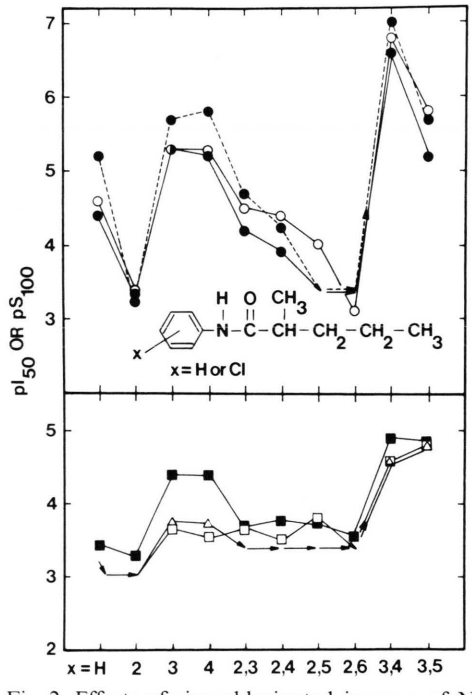

Fig. 2. Effects of ring chlorinated isomers of N-phenyl-2-methylpentanamide on assays that involved spinach chloroplasts and lecithin liposomes. Legend: pI_{50} values for uncoupled noncyclic ferricyanide reduction (●– – –●), coupled noncyclic ferricyanide reduction (●——●) and phosphorylation (○——○), light-induced quenching of atebrin fluorescence (□——□), and valinomycin-induced swelling (△——△); pS_{100} for reduction of ferricyanide entrapped in lecithin liposomes (■——■). Arrows indicate saturation of the isomer in the assay medium. Assay conditions were as described in Materials and Methods.

The same general trend prevailed for interference by the isomers with the noncyclic reductive reaction and the coupled phosphorylation reaction. All three reactions directly or indirectly involved the Q_B complex, hence, reflect the affinity of the Q_B complex for the isomers.

The phosphorylation reaction was more sensitive (lower pI_{50} values) than the coupled reductive reaction for all of the isomers. The difference in pI_{50}'s was slight for most isomers, but approached an order of magnitude for the 2,3; 2,4; and 2,5 – dichlorinated isomers. The differential response of the phosphorylation reaction may reflect the affinity of the isomers for the coupling factor complex.

In the assays involving liposomes, valinomycin, and atebrin, the pI_{50} and pS_{100} values were considerably lower, and the spread between values for the isomers was not as great as reflected in the assays that involved the Q_B complex. Additionally, the isomers saturated the reaction mixtures at approximately 400 μM ($pI_{50} = 3.4$). In the liposome assay, which reflects interaction with purely lipid bilayers, the 3,4; 3,5; 3; and 4 isomers were most active. There was little differentiation between the 2,3; 2,4; 2,5; 2,6; and unsubstituted isomers. The 2-chloro isomer was least active.

In the atebrin assay, which measured the ability of the isomers to dissipate energization of the thylakoid membrane by PS I, and the inhibition of valinomycin-induced swelling assay, the 3,5 and 3,4 isomers were considerably more active than the other isomers.

The effects of increasing alkyl chain length, with the ring substituent being held constant, were measured with a series of 1-alkyl-3-(α,α,α-trifluoro-*m*-tolyl)ureas (Fig. 3). Plotted on the ordinate (Fig. 3) is the response measured as pI_{50} or pS_{100}, as a function of alkyl chain length. The methyl derivative is desmethyl fluometuron. For inhibition of uncoupled electron transport with ferricyanide as the electron acceptor, the butyl derivative was most inhibitory with the methyl derivative being the second most inhibitory isomer. Diminished inhibitory activity was associated with alkyl lengths greater than 4 carbons. The relative order of activity of the effect of alkyl chain length with inhibition of the Hill reaction agrees with results reported previously [15]. Conceivably, the results reflect the steric requirements for accessibility and binding to the Q_B complex.

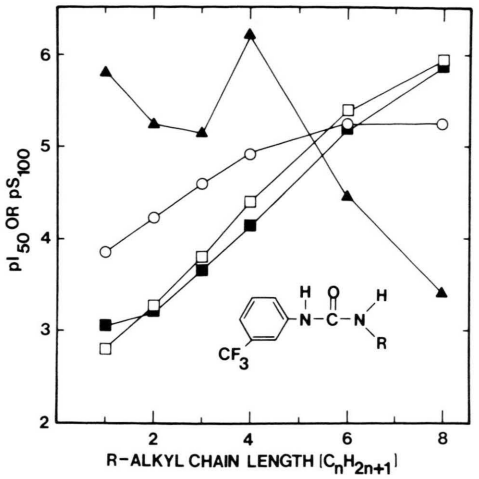

Fig. 3. Effects of 1-alkyl-3-(α,α,α-trifluoro-*m*-tolyl)ureas on reactions that involved spinach chloroplasts and lecithin liposomes. Legend: pI_{50} values for uncoupled noncyclic ferricyanide reduction (▲——▲), light-induced quenching of atebrin fluorescence (□——□), and valinomycin-induced swelling (■——■); pS_{100} for reduction of ferricyanide entrapped in lecithin liposomes (○——○). Assay conditions were as described in Materials and Methods.

For the assays that involved dissipation of the energized state of the thylakoid membrane imposed by PS I (atebrin assay) and alteration to the permeability properties of the envelope and thylakoids (valinomycin assay), activity increased linearly with increasing alkyl chain length. The same trend was shown with the purely lipid bilayers except that activity leveled off, but did not decrease, with alkyl groups longer than 4 or possibly the extrapolated 5 carbons.

Discussion

Results obtained in the studies reported herein supported the previous subdivision of inhibitory uncouplers into two groups: dinoseb types and dicryl (acylanilide) types [6]. The inhibitory uncouplers, but not electron transport inhibitors (DCMU-types) inhibited reactions associated with components located in the nonappressed thylakoid membranes. The dinoseb types, like FCCP, appeared to increase the permeability of membranes to protons in two ways, *i.e.*, at low concentrations they acted as protonophores and shuttled protons across the membrane, whereas at high concentrations they altered the permeability and integrity of membranes. The dicryl types only altered the semipermeability of the membranes.

The chlorination response pattern (Fig. 2) and responses to alkyl side chain length (Fig. 3) were essentially similar for assays that measured membrane perturbations (inhibition of valinomycin-induced swelling), dissipation of membrane energization by PS I (atebrin assay), and a purely lipid system (lecithin liposomes). None of the systems directly involved PS II. However, the chlorination response pattern was similar to that shown for interference with PS II-associated electron transport (the Q_B complex). However, derivatives with long alkyl side chains were not inhibitory to the Q_B-mediated reaction.

Conceivably, the inhibitory uncouplers may perturb all cellular membranes (plasmalemma, tonoplast, nuclear, and endoplasmic reticulum in addition to the chloroplast and mitochondrial membranes). However, marker systems that can be monitored readily which reflect the perturbations remain to be identified.

The physiochemical interaction of the inhibitory uncouplers with lipids can be expected to alter the many transport, biosynthetic, and regulatory activities associated with cellular membranes. The relation between membrane perturbations and the expression of phytotoxicity remains to be identified. The interaction with membranes may explain the phytotoxicity of some of the herbicides in the absence of light, in nonchlorophyllous tissue, or when PS II has been inhibited. At this time, it is not possible to determine the impact of alterations to the integrity and permeability of organelle membranes on the physiological status of plants, but small changes, coupled with a reduction in the availability of chloroplast and mitochondrially generated ATP, could have a significant effect over a time span of many hours or several days.

Acknowledgements

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