

Activity *in Vivo* and Redox States *in Vitro* of Nitro- and Chlorodiphenyl Ether Herbicide Analogs¹

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ABSTRACT

Excised cucumber (*Cucumis sativus* L. cv 447 Wisconsin SMR 18) cotyledons were sensitive to acifluorfen-methyl (methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate) and MC-15608 (methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate). Injury was detected by monitoring efflux of 3-*O*-methyl-D-[U-¹⁴C]glucose from herbicide-treated tissue after exposure to light. Efflux kinetics of 3-*O*-methyl-[¹⁴C]glucose from cotyledons treated with either acifluorfen-methyl (AFM) or MC-15608 were similar. Neither herbicide was active in darkness.

Cyclic voltammetry was used to study redox behavior of AFM and MC-15608 in protolytic and aprotic solvents. In protic media reduction of AFM was chemically irreversible. Reduction of MC-15608 in protolytic solvent was not observed. In aprotic solvent, the electrode reaction of AFM was quasireversible. The voltammogram of MC-15608 in aprotic solvent was indicative of a multielectron, completely irreversible electrode reaction.

Although the physiological data indicate that AFM and MC-15608 have similar effects *in vivo*, the redox behavior of the two compounds is quite different. These results suggest that mechanisms involving direct reduction and reoxidation of the diphenyl ether molecule are probably not the basis for the action of these herbicides.

It has been proposed that DPE² herbicides are activated in light by yellow plant pigments and are then involved in the initiation of free radical chain reactions with the polyunsaturated fatty acid moieties of membrane phospholipids (4-6). In the model proposed (5), it was suggested that the photoactivated form of the AFM molecule (e.g. reduced DPE radical anion) is regenerated (i.e. reoxidized) following initiation of the lipophilic

radical reaction. The perturbations that follow radical initiation result in a loss of the membrane's selective permeability characteristics, thereby leading to cellular death.

Leong and Briggs (3) demonstrated that acifluorfen (sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate) affects the phototropic response of etiolated oat (*Avena sativa* L.) coleoptile by acting specifically at a blue light-sensitive, *b*-type Cyt-flavin complex. Orr and Hogan (7) found that nitrofen (2,4-dichlorophenyl *p*-nitrophenyl ether) enhances the production of superoxide radicals *in vitro* with and without riboflavin. Nitrofen can effect the light-dependent reduction of NBT and DCPIP anaerobically by transferring electrons directly to NBT and DCPIP through an O₂-independent mechanism or aerobically via superoxide radicals. The aerobic reduction of NBT and DCPIP was inhibited by superoxide dismutase (EC 1.15.1.1). These experiments demonstrated that nitrofen can participate in oxidation-reduction-type reactions following photoactivation and that toxic, activated O₂ species are involved.

We examined the redox behavior of nitroDPE herbicides using cyclic voltammetry (unpublished results). These experiments were undertaken to study the voltammetric properties of several permutations of chemical design built around the nitroDPE moiety. Orr and Hess (6) suggested that the relative activity differences of these compounds *in vivo* (4) might be related to the effect of various substituents on the molecules' ability to readily form highly reactive free radicals. The voltammetric properties of nitrofen were studied to determine its involvement (i.e. direct or indirect) in the redox chemistry of superoxide production *in vitro*. The electrode reaction of nitrofen was chemically irreversible in protolytic solvents (pH 5-7) and quasireversible in aprotic solvents (unpublished results). These results suggest the mechanism of nitroDPE action *in vitro* and *in vivo* could involve the direct oxidation-reduction of the herbicide molecule; i.e. the photoreduced nitroDPE radical anion could be the active phytotoxic agent.

The synthesis of the chlorodiphenyl ether analog of AFM, MC-15608, made it possible to study further the importance of the nitro group for DPE herbicidal activity. Experiments reported here were performed to study the activity *in vivo* and redox behavior *in vitro* of the chloro- and nitroDPE analogs, MC-15608 and AFM.

MATERIALS AND METHODS

Plant Material. Cucumber (*Cucumis sativus* L. cv 447 Wisconsin SMR 18) seed was obtained from Joseph Harris Co., Inc. (Rochester, NY). Seeds were planted in moistened vermiculite. Seedlings were grown in the dark at 23°C for 6 d.

Efflux Experiments. Efflux experiments were performed as described previously with minor modifications (4, 5). Cotyledons were excised, floated adaxial surface down on 40 ml of 5 mM KPO₄ (pH 7.0) with 200,000 cpm 3-*O*-methyl-D-[U-¹⁴C]glucose/

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² Abbreviations: DPE, diphenyl ether; NBT, *p*-nitro blue tetrazolium chloride; DCPIP, 2,6-dichlorophenol-indophenol; AFM, acifluorfen-methyl; PARC, Princeton Applied Research Corp.; *i*, current; E, potential; SCE, saturated calomel electrode; *E*(*v*) versus SCE, potential in volts relative to SCE reference electrode; TBAPF₆, tetra *n*-butylammonium hexafluorophosphate; DMF, dimethylformamide; *E*_{pc}, cathodic peak potential; *E*_{pa}, anodic peak potential; *i*_{pc}, cathodic peak current; *i*_{pa}, anodic peak current; *E*'₀, formal reduction potential; μ A, microamperes; ΔE_p , *E*_{pa} - *E*_{pc}; *n*, electron stoichiometry; *i*_p, peak current; *E*_{1/2}, measured half-wave potential; EC, heterogeneous electron transfer followed by homogeneous chemical reaction; P700*, photoactivated reaction center of PSI; P680*, photoactivated reaction center of PSII.

ml (Amersham Corp., Arlington Heights, IL), and allowed to absorb the radiolabeled sugar in low light ($75 \mu\text{E m}^{-2} \text{s}^{-1}$; PAR) at 23°C for 24 h. Cotyledons were then rinsed twice with distilled H_2O . Three cotyledons were floated adaxial surface down with aeration on 10 ml of efflux medium (5 mM KPO_4 , pH 7.0). At time zero, radiolabeled cotyledons were exposed to AFM or MC-15608 ($1 \mu\text{M}$ in 0.1% [v/v] ethanol) in high light ($600 \mu\text{E m}^{-2} \text{s}^{-1}$; PAR) or in darkness. Illumination was from above. The

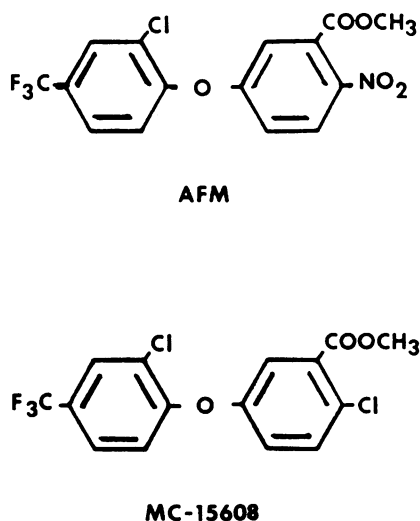


FIG. 1. Chemical structures of AFM and MC-15608. AFM, acifluorfen-methyl; MC-15608, coded chemical name originally designated by the manufacturer (Rhône-Poulenc Chemical Company).

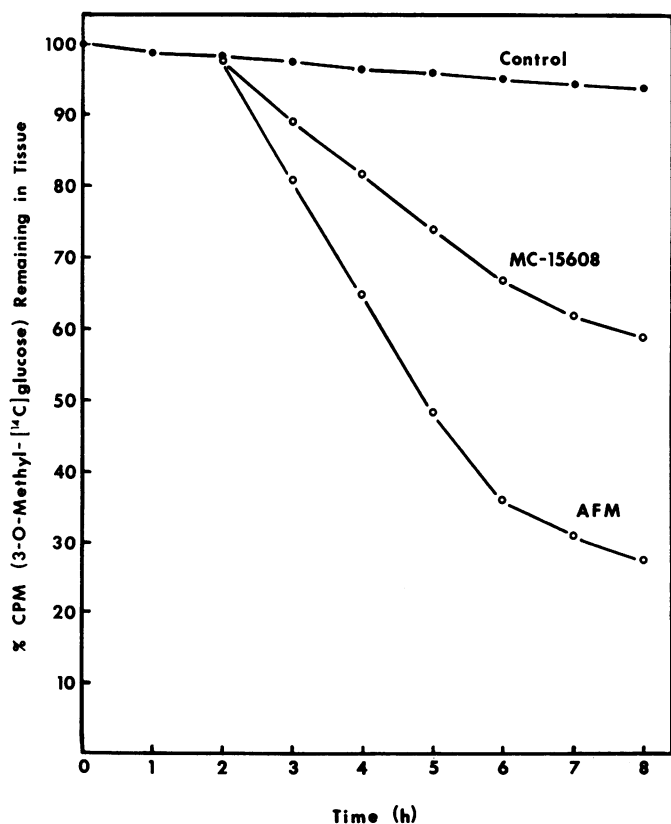


FIG. 2. Efflux of 3-O-methyl-[^{14}C]glucose from cucumber cotyledons treated at time zero with $1 \mu\text{M}$ AFM or MC-15608 in 0.1% (v/v) ethanol and exposed to high light ($600 \mu\text{E m}^{-2} \text{s}^{-1}$; PAR). Control, 0.1% (v/v) ethanol.

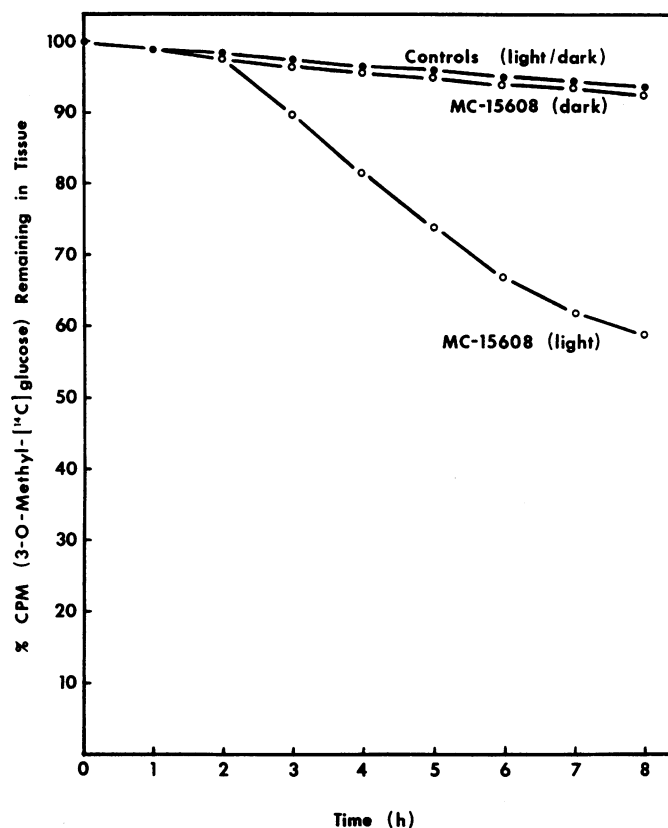


FIG. 3. Efflux of 3-O-methyl-[^{14}C]glucose from cucumber cotyledons exposed at time zero to $1 \mu\text{M}$ MC-15608 in 0.1% (v/v) ethanol in high light ($600 \mu\text{E m}^{-2} \text{s}^{-1}$; PAR) or in darkness. Controls, 0.1% (v/v) ethanol.

efflux solution was drained every hour and replaced with fresh medium plus herbicide. The amount of radioactivity present in the medium was determined using a Beckman LS 7500 microprocessor-controlled liquid scintillation counter. At the end of the experiment, the amount of radioactivity in the cotyledons was determined by measuring efflux after freezing and thawing the tissue two times. Data are expressed as per cent cpm remaining in the tissue over time. Experiments were repeated twice in duplicate. The SE of means (of raw data) were less than 10% of the mean of each experiment ($n = 4$).

Cyclic Voltammetry. Electrochemical measurements were made using a Princeton Applied Research (PARC) Model 173 potentiostat/galvanostat coupled to a PARC Model 175 universal programmer (2). Voltammograms (*i.e.* current-voltage [i - E] diagrams) were recorded on a Houston Omnigraph 2000 X-Y recorder. A glass electrochemical cell was used with a stationary, glassy carbon working electrode (Atomergic Chemetals Corp., Plainview, NY; area = 7 mm^2), a platinum wire auxiliary electrode, and a reference electrode (SCE). All potentials are reported as $E(v)$ versus SCE. Solutions in the electrochemical cell were deaerated with purified nitrogen. Measurements were made in unstirred solutions at room temperature. All solutions were freshly prepared prior to each experiment. Endpoint potentials were typically 0.5 and -2.0 v . Scans were initiated at 0.5 v in the forward direction (*i.e.* toward more negative potentials). Scan rates were 50, 100, 200, and 500 mv/s.

Electrochemical measurements made in aqueous protolytic solvent included 50% (v/v) ethanol buffered with 0.25 M sodium acetate (pH 5.0) or 0.1 M KPO_4 (pH 7.0). Reagents were used without further purification. Measurements made in an aprotic environment used TBAPF₆ as supporting electrolyte at 0.1 M in DMF. TBAPF₆ was prepared by metathesis of NH_4PF_6 and $n\text{-Bu}_4\text{NI}$ in aqueous solution followed by two recrystallizations

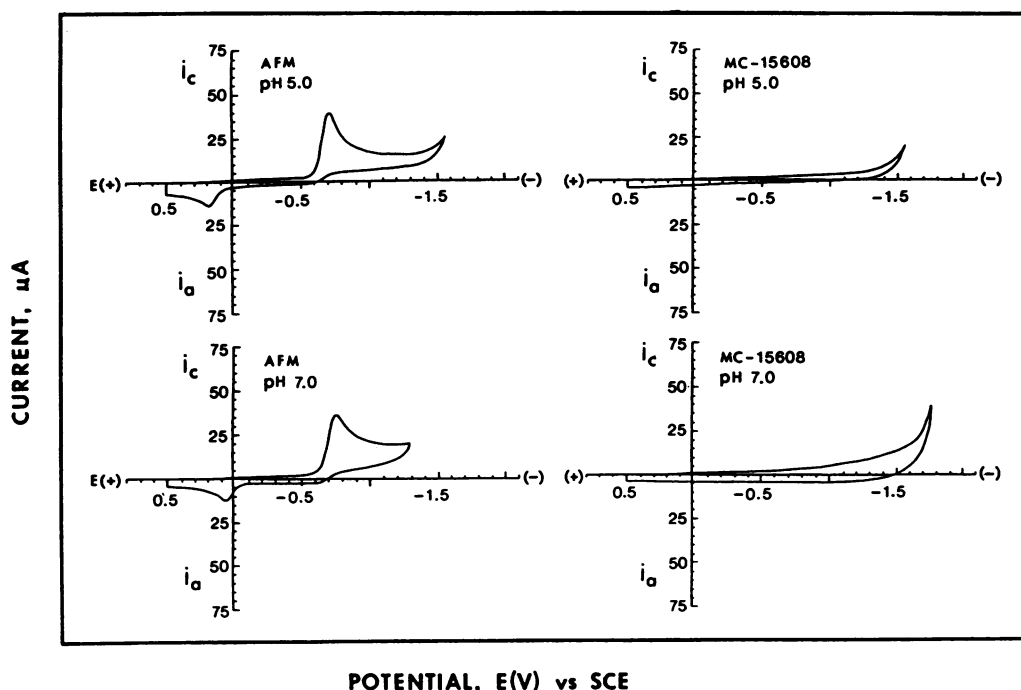


FIG. 4. Cyclic voltammograms of 1 mM AFM and MC-15608 in 0.25 M sodium acetate (pH 5.0)/50% (v/v) ethanol and 0.1 M KPO_4 (pH 7.0)/50% (v/v) ethanol. Scans were initiated at 0.5 V in the negative direction at 100 mV/s.

from ethanol and finally vacuum dried. DMF ('Distilled in Glass' grade, Burdick and Jackson, Muskegan, MI) was used without further purification. For aqueous solutions, electrochemical breakdown of the solvent, supporting electrolytes, or both occurred starting at about -1.5 V. This phenomenon was not observed for the aprotic solvent system within the potential range used for most of the experiments reported here.

Herbicides. AFM and MC-15608 were contributed by Rhône-Poulenc Chemical Company, Monmouth Junction, NJ. The coded chemical name for MC-15608 now designated by the manufacturer is LS 82.0340.

RESULTS AND DISCUSSION

The chemical structures of AFM and MC-15608 are shown in Figure 1. MC-15608 is the chloro analog of the nitroDPE AFM. The two compounds differ only with respect to the substituent at the 4'-position of the DPE moiety.

Activities *in vivo* of AFM and MC-15608 were similar. The placement of cucumber cotyledons greened for 24 h in low light ($75 \mu\text{E m}^{-2} \text{s}^{-1}$; PAR) into high light ($600 \mu\text{E m}^{-2} \text{s}^{-1}$; PAR) with $1 \mu\text{M}$ AFM or MC-15608 in 0.1% (v/v) ethanol resulted in nearly identical visual injury symptoms (not shown). Injury symptoms of AFM activity have been described elsewhere (6). Efflux kinetics of 3-*O*-methyl- ^{14}C glucose from cotyledons treated with either AFM or MC-15608 are also similar (Fig. 2). There was a significant increase in the efflux of 3-*O*-methyl- ^{14}C glucose from herbicide-treated cotyledons after 2 h in high light. The activity of MC-15608 was somewhat less than AFM.

The herbicidal activity of AFM is light-dependent (4). MC-15608 also required light for activity (Fig. 3). In darkness, the efflux of 3-*O*-methyl- ^{14}C glucose from cotyledons treated with $1 \mu\text{M}$ MC-15608 was no different from the controls (light or dark).

The cyclic voltammograms of AFM and MC-15608 in 0.25 M sodium acetate (pH 5.0)/50% (v/v) ethanol and 0.1 M KPO_4 (pH 7.0)/50% (v/v) ethanol are presented in Figure 4. The voltammograms were obtained by measuring the current i at the working electrode as a function of the potential $E(v)$ versus SCE. A

scan of the potential was initiated at 0.5 V in the forward direction at 100 mV/s. One cathodic peak was observed for AFM during the initial forward scan and a smaller, single anodic peak was seen during the reverse scan. Cathodic peak potentials E_{pc} for AFM in protolytic solvent at pH 5.0 and 7.0 are -0.720 and -0.770 V, respectively. These voltammograms are indicative of a chemically irreversible electrode reaction (1). The initial reduction of AFM under these conditions is followed by a rapid irreversible chemical step (1). The reaction mechanism is complex and most likely is similar to that of the simplest nitroDPE herbicide nitrofen in protolytic solvent (unpublished). The redox mechanism of nitrofen is characteristic of an EC reaction (1): heterogeneous electron transfer followed by a homogeneous chemical reaction. The initial reduction of nitrofen in protic media appears to be a multielectron process centered at the nitro group. Chemical instability of the initial reduction product of AFM and the irreversible nature of these reactions disallow calculation of any thermodynamic parameters (e.g. formal reduction potential $E^{\circ'}$). In contrast to AFM, MC-15608 was not electroactive within the potential range accessible in protolytic solvent. Electrochemical breakdown of the solvent/supporting electrolyte system occurred before reduction of MC-15608 at both pH 5.0 and 7.0. This was determined by scanning the potential of the solvent blank in the absence of herbicide (not shown). This observation also suggests the site of redox activity in AFM is the nitro group.

The reduction of 2 mM AFM was shifted to more negative potentials in the absence of protons (Fig. 5). The mechanism of reduction under these conditions is quite different from that in water. The electrode reaction depicted by the voltammogram of AFM in DMF is characteristic of a quasireversible electron transfer process (1). Cathodic E_{pc} and anodic E_{pa} peak potentials are -1.05 and -0.940 V, respectively. The peak potential separation $\Delta E_p = E_{pa} - E_{pc}$ is 0.110 V and the measured half-wave potential $E_{1/2} = (E_{pa} + E_{pc})/2$ is -0.995 V. The reduction product was chemically stable on the time scale of the experiment ($i_{pc}/i_{pa} \approx 1.0$). Linear sweep voltammetry employing a rotating disk electrode (1) indicated the electron stoichiometry n for the re-

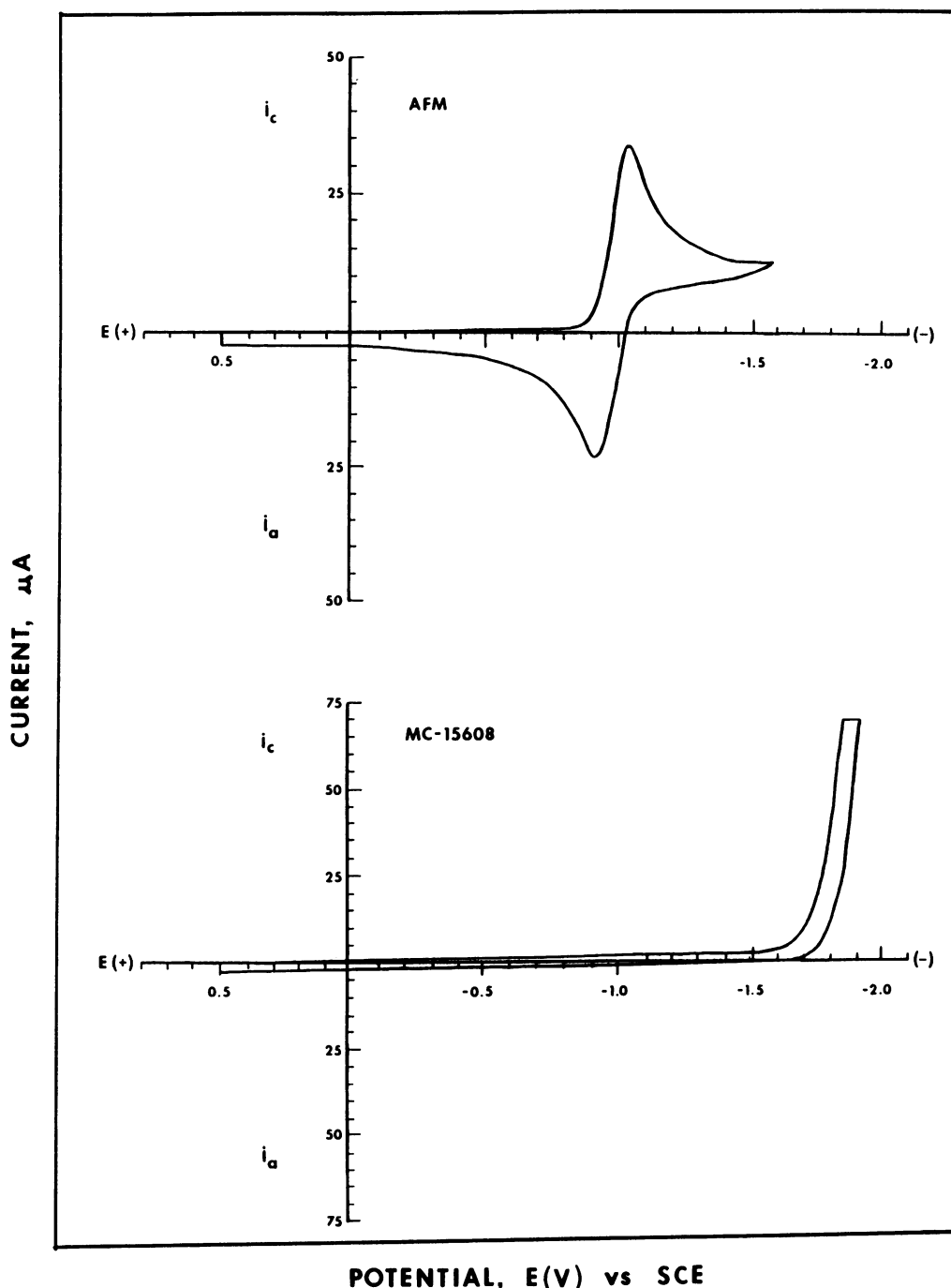


FIG. 5. Cyclic voltammograms of 2 mM AFM and MC-15608 in DMF. Supporting electrolyte was 0.1 M TBAPF₆ in DMF. Scans were initiated at 0.5 v in the negative direction at 100 mv/s.

duction of nitrofen in aprotic solvent was one (unpublished results). Based on comparisons with nitrofen and other nitroaromatics (8), the reduction of AFM under aprotic conditions is most likely a one-electron reduction to the radical anion (1). Thus, $E_{1/2}$ is a good approximation of E° for the quasireversible one-electron reduction process of AFM.

The reduction of 2 mM MC-15608 in DMF occurred at a more negative potential than was observed for AFM. The voltammogram of MC-15608 (Fig. 5) is indicative of a multielectron, completely irreversible electrode reaction (1). Several ill-resolved cathodic processes ($E_{pc} < -1.89$ v) were observed during the initial forward scan (not shown). The current increased sharply at more negative potentials, producing a large current relative to that seen for AFM. There was no anodic peak. This behavior is

indicative of highly chemically irreversible processes involving large numbers of equivalents of electrons (1). Our linear sweep voltammetry experiments at a rotating disk electrode with MC-15608 (unpublished results) also confirmed that the electrode reaction was multielectron. From this experiment, there appeared to be two barely resolvable one-electron waves followed by significant decomposition of the compound at more negative potentials ($E[v]$ versus SCE < -2.2 v). It was impossible to resolve the multielectron processes further. The reaction mechanism and the reduction and apparent subsequent decomposition of MC-15608 are unknown. In any event, our results demonstrate that in terms of i - E relationships, electron stoichiometries, and product stabilities the redox processes of AFM and MC-15608 in aprotic solvent are quite different.

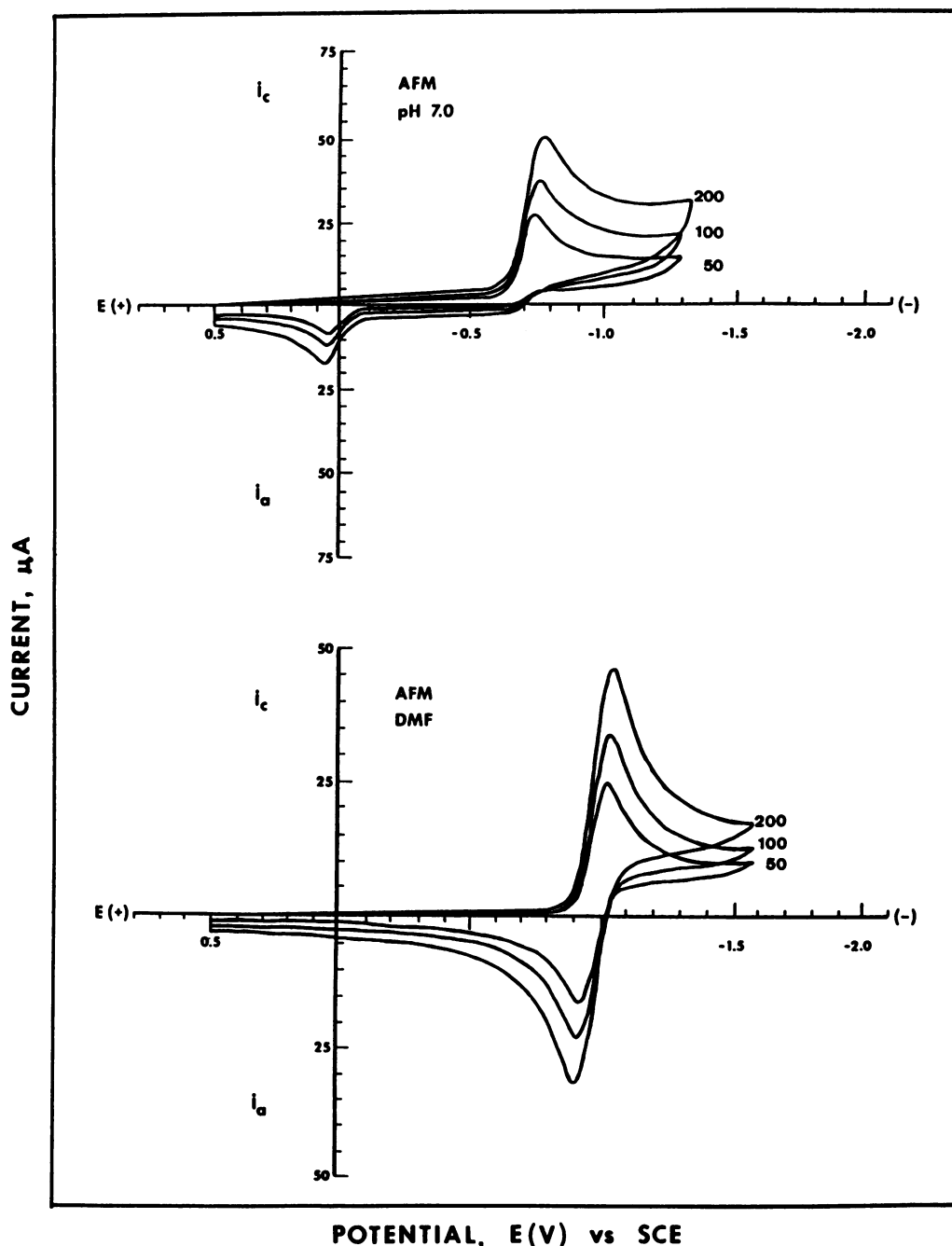


FIG. 6. Cyclic voltammograms of 1 mM AFM in 0.1 M KPO_4 (pH 7.0)/50% (v/v) ethanol and 2 mM AFM in DMF at three different scan rates. Supporting electrolyte in DMF was TBAPF_6 at 0.1 M. Scans were initiated at 0.5 V in the negative direction at 50, 100, and 200 mV/s.

The voltammograms of AFM in protolytic (pH 7.0) and aprotic (DMF) solvents at three different scan rates ν (50, 100, and 200 mV/s) are shown in Figure 6. The magnitude of the peak height i_p is a function of the scan rate ν in both systems. Plots of i_{pc} versus $\nu^{1/2}$ for AFM at pH 7.0 and i_{pc} and i_{pa} versus $\nu^{1/2}$ for AFM in DMF are shown in Figure 7, A and B, respectively. The peak current i_p is a linear function of the square root of the scan rate $\nu^{1/2}$ for both the irreversible and quasireversible electrode reaction. This relationship indicates that reduction is diffusion controlled and that there is no specific interaction of AFM with the electrode surface, e.g. chemisorption (1). Thus, homogeneous redox reagents having formal reduction potentials E° less negative (i.e. weaker reducing agents) than E_{pc} or $E_{1/2}$ would be incapable of transferring electrons to AFM under similar solution conditions.

Results of the experiments reported here pose some interesting questions. (a) Do AFM and MC-15608 behave identically *in vivo*? (b) Is the mechanism of AFM-induced radical initiation proposed by Orr and Hess (5) accurate? (c) Is knowledge of the redox behavior of DPEs meaningful for the interpretation of mechanism of herbicide action? (d) Could DPEs have an indirect effect on oxidation-reduction reactions *in vivo* (e.g. indirectly generate toxic activated O_2 species upon photoactivation without the DPE molecule itself undergoing oxidation-reduction) and yet still be involved in the initiation of lipophilic free radicals? (e) Could the binding of DPEs, both chloro- and nitroDPEs, to some cellular component effectively shift the reduction of the molecule to less negative potentials (effectively an inner sphere process), thus permitting a biological system to do this kind of redox chemistry?

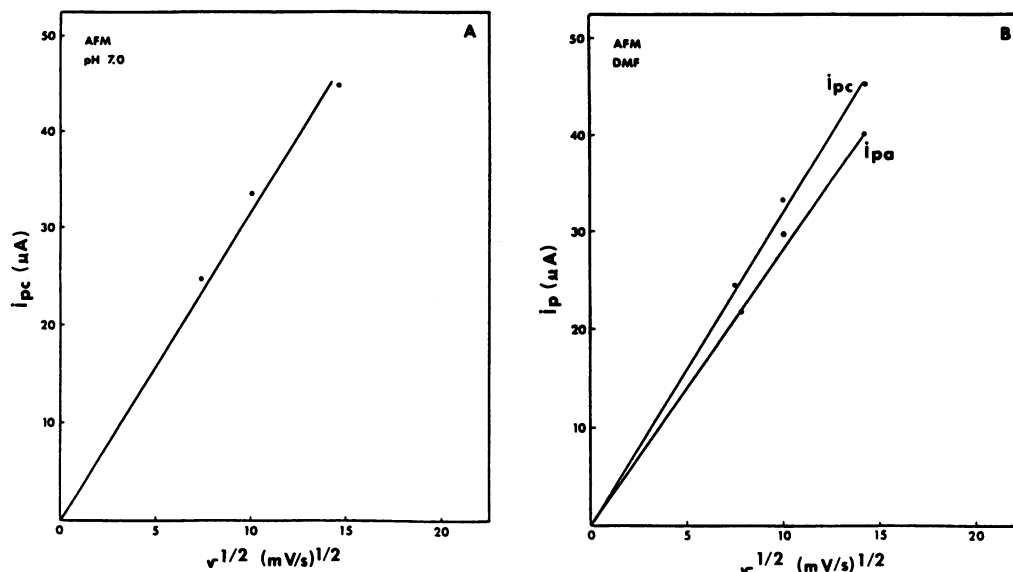


FIG. 7. A, Plot of cathodic peak current i_{pc} as a function of the square root of the scan rate $v^{1/2}$ for 1 mM AFM in 0.1 M KPO_4 (pH 7.0)/50% (v/v) ethanol; B, plot of cathodic i_{pc} and anodic i_{pa} peak currents as a function of the square root of the scan rate $v^{1/2}$ for 2 mM AFM in DMF. Voltammetric data were obtained from the voltammograms of Figure 6.

The light-dependent reduction of the lipophilic nitroDPE AFM by plant pigments to the nitro anion radical in the potentially aprotic interior of cellular membranes is a plausible, if not correct, mechanism for the phytotoxic expression of AFM activity. That is, the photoactivated species initiating the proposed lipophilic free radical reactions (5) ultimately leading to cellular death could be the nitroDPE anion radical. But even without information of MC-15608 redox chemistry the mechanism is tentative. The potentials required for the reduction of DPEs at first appear to be quite negative. The redox chemistry necessary may not be within the ability of a biological system. We are unable to calculate $E^{\circ'}$ and without this information it is difficult to predict accurately the feasibility of this chemistry *in vivo*.

It is impossible to make strict thermodynamic comparisons for irreversible processes (1), e.g. for AFM in protolytic solvent and for MC-15608 in aprotic solvent. Within the limits that the electrode behaves as an outer sphere redox reagent, however, relative kinetic arguments can be developed. In aprotic solvent, the quasireversible one-electron reduction of AFM with $E_{1/2}$ of -0.995 v can be thought of as the lower potential limit of AFM reduction. The presence of protons provides a mechanism(s) by which rapid multielectron reduction of AFM can occur at more positive potentials, and thus more easily than the one-electron reduction to the radical anion in the absence of protons. The relevance of these observations to homogeneous reactions *in vivo* is that (a) outer sphere electron transfer to AFM is probably fast with reducing agents having $E^{\circ'} < -0.995$ v (measured under the same relative conditions as AFM) irrespective of the availability of protons, and (b) the availability of protons provides other mechanisms by which the reduction process can proceed at even higher potentials than does the one-electron reduction, just as E_{pc} in proteolytic solvent is more positive than E_{pc} in aprotic solvent.

The standard redox potentials for the photoactivated pigments P700* of PSI and P680* of PSII are -1.35 and -1.05 v, respectively (9). These values are 0.241 v more negative when corrected to the formal reduction potential $E^{\circ'}$ versus SCE. Obviously, P700 and P680 absorb more energy than is normally required

for photosynthetic electron transfer. Is it possible this excess energy could be used to photoreduce nitroDPEs?

Perhaps the reduction of AFM by a biological system is plausible. However, the option of direct outer sphere reduction *in vivo* does not overtly appear to be available for the chloroDPE MC-15608. In comparison with AFM, the reduction of MC-15608 in protolytic solvent is less than -1.5 v and the potential for the onset of measurable reduction current in aprotic solvent is approximately -1.7 v. Although further experimentation is required, the similarity of AFM and MC-15608 activity *in vivo* suggests that nitro- and chloroDPE herbicides have a common mechanism of action. Thus, the results of the experiments presented here suggest it is unlikely the mechanism of herbicide action involves the direct reduction and reoxidation of the DPE molecule.

LITERATURE CITED

- BARD AJ, LR FAULKNER 1980 Electrochemical Methods. Fundamentals and Applications. John Wiley and Sons, New York
- ELLIOTT CM, E HERSHENHART, RG FINKE, BL SMITH 1981 Coenzyme B_{12} model studies: an electrochemical comparison of cobaloxime and $Co(C_2(DO)(DOH)ph)$ complexes to coenzyme B_{12} . *J Am Chem Soc* 103: 5558-5560
- LEONG TY, WR BRIGGS 1982 Evidence from studies with acifluorfen for participation of a flavin-cytochrome complex in blue light photoreception for phototropism of oat coleoptiles. *Plant Physiol* 70: 875-881
- ORR GL, FD HESS 1981 Characterization of herbicidal injury by acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. *Pestic Biochem Physiol* 16: 171-178
- ORR GL, FD HESS 1982 Mechanism of action of the diphenyl ether herbicide acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. *Plant Physiol* 69: 502-507
- ORR GL, FD HESS 1982 Proposed site(s) of action of new diphenyl ether herbicides. In DE Moreland, JB St John, FD Hess, eds, *Biochemical Responses Induced by Herbicides*. 181 ACS Symposium Series, Washington DC, pp 131-152
- ORR GL, ME HOGAN 1983 Enhancement of superoxide production *in vitro* by the diphenyl ether herbicide nitrofen. *Pestic Biochem Physiol*. In press
- SMITH WH, AJ BARD 1975 Electrochemical reactions of organic compounds in liquid ammonia. II. Nitrobenzene and nitrosobenzene. *J Am Chem Soc* 97: 5203-5210
- WITT HT 1976 Primary actions of excited molecules in the functional membrane of photosynthesis. In JB Birks, ed, *Excited States of Biological Molecules*. John Wiley and Sons, New York, pp 245-261