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Report

Are Reduction Potentials of Antifungal Agents Relevant to Activity?

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Cyclic voltammetry data were obtained for several categories of fungicidal agents including quinones (akrobomycin, podosporin A), iminium ions and precursors (pyridazines, 15-azahomosterol, griseofulvin-4′-oxime), and metal derivatives of chelators (pyridine-2-aldehyde thiosemicarbazones). The reductions usually occurred in the range of −0.7 to +0.3 V. Reduction potentials provide information on the feasibility of electron transfer *in vivo*. Catalytic production of oxidative stress from redox cycling is a possible mode of action. Alternatively, there may be interference with normal electron transport chains.

KEY WORDS: fungicides; mechanism; electron transfer; oxidative stress; electrochemistry; iminium; quinones; metal complexes.

INTRODUCTION

Oxy radical generation and electron transfer (ET) have been increasingly implicated as mechanistic pathways for a variety of xenobiotics and in human diseases. Hypothetically, the active form of the drug is thought to function catalytically by passing electrons from a donor, e.g., DNA, protein or a normal ET chain, to an acceptor, such as oxygen or a cellular constituent. In our laboratory, application of this general theme has been applied to carcinogens (1), anticancer agents (2), antibacterial agents (3), amebicides (4), antimalarials (5), anthelmintics (6), β-lactam antibiotics (7), and antimycobacterials (8). There appear to be several main classes of ET agents (9): quinoidal, metal complexes, aromatic nitro, and iminium ions (1–8). A similar unifying approach has been advanced by others in the anticancer (9,10) and antimalarial (11) areas.

We have now expanded this theme to include fungicides. The possibility of ET involvement *in vivo* was examined by means of electrochemical data gathered through cyclic voltammetry. Theoretically, if the reduction potential of the antifungal agent is relatively positive, it is energetically possible for the agent to be reduced, followed by formation of toxic oxy radicals via superoxide *in vivo*. Alternatively, the drug may interfere with normal electron transport chains (12). The discussed drugs, all of which incorporate ET functionalities, include quinones (akrobomycin, 1; podosporin A, 2;) iminium, 3, and precursors (benzothiazole *p*-hydroxybenzaldehyde imine, 4; 15-azahomosterol, 5; griseofulvin-4′-oxime, 6; pyridazomycin, 7; the pyridazine, 8; gentian violet, 9) and metal complexes of pyridine 2-aldehyde thiosemicarbazone, 10a; vanillin thiosemicarbazone, 10b; and 2-acetamido-4-acetyl-5-dimethyl-Δ^2-1,3,4-thiadiazoline, 11.

MATERIALS AND METHODS

Fungicidal agents and other chemicals were obtained from the Aldrich Chemical Company (Milwaukee, WI), except for azahomosterol, pyridazine (Lilly Research Laboratories, Indianapolis, IN), pyridazomycin (Dr. A. Zeeck, Göttingen, FRG), griseofulvin oxime (Dr. B. L. Currie, University of Illinois, Chicago, IL), akrobomycin (Kirin Brewery Co. LTD., Maebashi, Japan), and podosporin A (Dr. J. B. Gloer, Department of Chemistry, University of Iowa, Iowa City).

Compound 11 was prepared according to the method of Kubota *et al.* (13) (m.p. 194–196°C, lit. m.p. 195–196°C); calc. for: $C_8H_{13}N_3O_3$: C, 48.3; H, 6.5; N, 21.1; found C, 48.5; H, 6.1, N, 21.3. Pyridine-2-aldehyde thiosemicarbazone was prepared according to a literature procedure (m.p. 205–206°C, lit. m.p. 203–204°C) (14). Benzothiazole *p*-hydroxybenzaldehyde imine was prepared by the method of Dash *et al.* (15) (m.p. 183–185°C, lit. m.p. 185°C); calc. for: $C_{14}H_{10}N_2OS$: C, 66.1; H, 3.9; N, 11.0; found C, 65.8; H, 3.7; N, 11.3. Metal complexes were obtained according to literature methods (14,16,17).

The electrolyte used in the nonbuffered electrochemical studies was tetraethylammonium perchlorate (0.1 *M*) (G. F. Smith, Columbus, OH). Dimethylformamide (DMF) was purchased in the highest available purity and then distilled from triphenylsilyl chloride to insure complete dryness. Absolute ethanol (U.S. Industrial, Tuscola, IL) was used to prepare the aqueous solutions. All compounds were investigated at a concentration of 0.5 m*M*.

The cyclic voltametric measurements were performed

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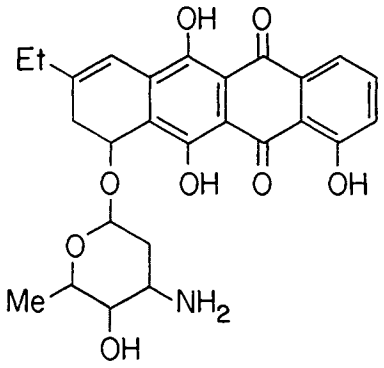
at ambient temperature with IBM Corporation model EC 225 voltametric analyzer associated with a Houston Instrument Model 200 *X-Y* recorder. The operation of the instrument and the electrodes was checked against a benzil standard before each use. The scan rates generally ranged from 20 to 200 mV/sec. Solutions were purged of oxygen for 15 min with prepurified nitrogen (30 min for metal complexes) that was passed through an oxygen scrubbing system. The working electrodes consisted of either a platinum flag (Sargent-Welsh, Skokie, IL) or a hanging mercury drop electrode (HMDE). A platinum wire was used as the counter and saturated calomel (SCE) was the reference electrode. Observed potentials (our work and literature values) were converted to the normal hydrogen electrode (NHE) reference by adding 0.24 V to the SCE values. The reported data are an average of two or more measurements involving freshly made solutions. The following equations were used for the half-wave potentials, the difference in potentials, the current function, and the current ratio: $E^{\circ\prime} = (E_{pc} + E_{pa})/2$, $E_{pp/2} = |E_{pc} - E_{pc/2}|$, $CF = i_p/(v^{1/2} \times C)$, and $i_{pa}/i_{pc} = (i_{pa})_o/i_{pc} + 0.485\,(i_{sp})_o/i_{pc} + 0.086$.

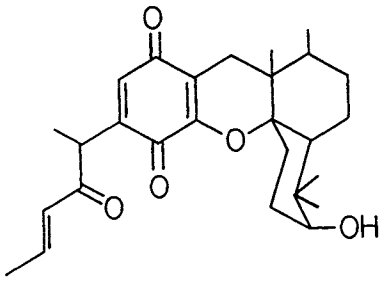
RESULTS AND DISCUSSION

Quinones

Members of this category demonstrate activity of diverse types, including fungicidal agents (18). Previous work with antitumor anthraquinones and related substances has resulted in advances in our understanding of how these agents operate (19). One electron reduction of the species bound to DNA apparently occurs to yield the semiquinone state, which, in turn, can reduce oxygen to superoxide resulting in quinone regeneration. Inhibition of the rate of DNA cleavage has been observed for certain members with the addition of superoxide dismutase (SOD) or radical scavengers. Activated oxygen evidently arises via redox cycling (6). The whole process has been termed site-specific free radical generation (20). Metal complexation may also be significant (3). For some antibacterial (6) and fungicidal quinones (18), there appears to be a correlation between reduction potential and activity; the more positive, the more active. Anthraquinones with phenolic hydroxyl groups have been observed to uncouple respiration (21) and may do so by ET. The electrochemistry of two recent, powerful fungicidal



Scheme 1



Scheme 2

quinones, akrobomycin (1) and podosporin A (2) (23), was examined (Schemes 1 and 2).

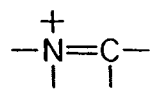
1. Akrobomycin (1). In our electrochemical studies a favorable reduction potential ($E^{\circ\prime}$) of −0.68 V was obtained. The reduction was quasireversible and diffusion controlled. The CF ratio of compound 1 to benzil, a compound known to undergo one electron reduction, was 1.1, denoting one electron reduction to the semiquinone. The reduced solution turned deep purple, indicating the presence of semiquinone. This intermediate showed good stability with an *i*(pa)/*i*(pc) value of 0.83 at a scan rate of 100 mV/sec and 0.91 at 200 mV/sec. The reduction was not reversible in an aqueous/ethanol medium, presumably due to reaction of the reduced species with solvent. Binding with DNA has been shown to be facilitated by the basic sugar moiety for related members (24).

2. Podosporin A (2). Compound 2 exhibited an even more favorable quasireversible diffusion-controlled reduction, $E^{\circ\prime}$ of −0.13 to −0.29 V, with 61% reversibility (current ratio × 100%) accompanied by a broad irreversible wave at −0.9 V. A one-electron reduction to the semiquinone is also indicated.

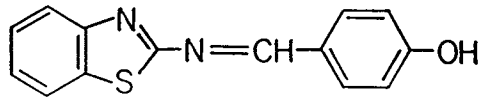
3. Others. There is evidence that other agents in this category operate by ET. Chloroneb, a commonly used fungicide which is a quinone precursor, is known to stimulate lipid peroxidation (25). The antifungal activity is moderated by the addition of radical scavengers (26). Electron transfer to oxygen is strongly implicated as a likely mode of action. Shapovalov has shown a correlation between the fungitoxicity of several quinoidal species and their ability to act as redox mediators in a peroxidase reaction producing hydrogen peroxide, which is a precursor of hydroxyl radicals (27). Another important quinone antifungal drug, tetrachlorobenzoquinone (chloranil), is reduced to the semiquinone radical in a fungal culture (28). It is relevant that chloranil reduces within the physiological range ($E_{1/2}$ of +0.29 V) (29).

Iminium, Imine, and Related Species

The iminium class (3) has not enjoyed recognition as a prevalent ET type *in vivo,* even though the functionality is present as such or in a precursor form in many physiologically active agents. There are several subdivisions for the ion and precursors including Schiff base imines, heterocyclic



Scheme 3



Scheme 4

iminium species, oximes, pyridazines, and basic triarylmethane dyes (Schemes 4–9) (30).

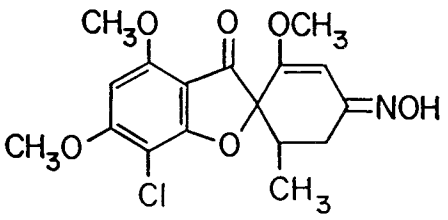
1. Schiff Bases. Benzothiazole *p*-hydroxybenzaldehyde imine (4) is a moderately potent antifungal agent (15). The parent free base showed no reduction before −1.0 V. However, the protonated ion may occur *in vivo*. Upon the addition of perchloric acid, a strong positive shift in the one electron reduction was observed, due to the involvement of the iminium species ($E^{\circ\prime}$ of −0.61 V). There was also a large increase in the positive direction with the weaker acetic acid (E_p of −0.79 V). The latter case was not reversible, presumably due to kinetic or other complications.

2. Heterocyclic Species. 15-Azahomosterol (5), a potent antifungal agent, is known to inhibit ergosterol biosynthesis (31). Dolle has suggested that the iminium form of 5 is formed at the active site of Δ^{14}-sterol reductase. The drug gave an irreversible diffusion-controlled wave which also was a function of pH (E_p = −0.78–0.023 pH V, pH 1.0 to 7.0). The $E_{pp/2}$ for the protonated form of 5 was 65 to 70 mV, which is close to the theoretical value (56.6 mV) for a one-electron process. Moreover, the E_p changed by 20 mV upon a decade increase in scan rate, indicating that ET was followed by a rapid unknown chemical reaction.

Since the potential at which compound 5 reduces is quite negative, it is unlikely that there is involvement of ET *in vivo*. Olefin bonds in sterols are known to undergo isomerization *in vivo* (32). If the Δ^8 *trans* double bond is isomerized to the Δ^7 *cis* configuration by Δ^7–Δ^8 isomerase *in vivo*, then one can expect approximately a 0.2-V positive shift in the reduction potential (33), making ET processes more likely.

3. Oximes. Griseofulvin, a well-known fungistatic agent, also exhibits teratogenic and carcinogenic activity (30). The site of action is apparently related to nucleic acids (34) and DNA replication (30). It is also known to produce lipid peroxidation (35). The oxime derivative 6 of griseofulvin is more fungistatic than its parent (36). The oxime form of the drug did not reduce before −1.0 V in neutral media. Under protic conditions the reduction potential shifted to a more positive value and exhibited a one-electron quasireversible wave at −0.74 and −0.89 V with one equivalent of perchloric or acetic acid, respectively.

4. Pyridazines. Pyridazomycin (7), a highly effective fungicidal agent, is the first naturally occurring antibiotic with a pyridazine core to be discovered (37). Compound 7



Scheme 6

underwent quasireversible reductions in both neutral and acidic media (pH 3.5), providing reduction potentials of −0.77 and −0.53 V, respectively.

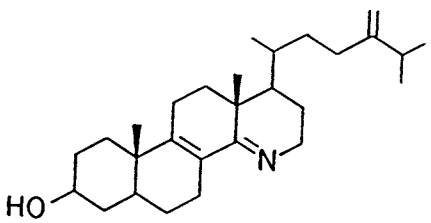
The pyridazine 8, a synthetic member of this class, also possesses antifungal activity (38). The free base exhibited quasireversible one electron reduction ($E^{\circ\prime}$ of −0.80 V). Kinetic or other complications were indicated by an i(pa)/i(pc) value of 0.54 at a scan rate of 100 mV/sec and 0.62 at a scan rate of 200 mV/sec. The reaction became less reversible at lower scan rates. The protonated form of the agent exhibited reversible, diffusion-controlled behavior with a cathodic shift of the wave as the pH decreased; at pH 3.5, $E^{\circ\prime}$ was −0.47 V. The process apparently involves one electron transfer from comparison of the current function of 8 to that of benzil (Table I). Since both compounds are relatively easy to reduce in ionic form, this class represents a new category of potential ET agent.

5. Basic Triarylmethane Dyes. An example which incorporates the iminium functionality is gentian violet 9, $E^{\circ\prime}$ of −0.55 V (3). Drug 9 is used as a topical antibacterial, anthelmintic, and antifungal agent (3,30). Synthetic dyes are known to produce toxic oxygen species through redox cycling (3). The one-electron reduction product of 9, detected *in vivo* by ESR spectroscopy (3), can react with oxygen to form a much more reactive peroxyl radical. Gentian violet is also known to damage DNA (3). This class, which bears structural similarity to quinones, is one of the best-supported examples of involvement of iminium species in redox cycling to produce toxic oxygen entities.

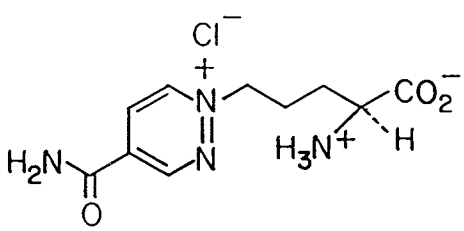
METAL COMPLEXES AND COMPLEXORS

A number of fungicidal agents appear to function via complexation with metal ions (Schemes 10 and 11) (30). We performed electrochemical studies on various antifungal ligands and their metal chelates in order to determine the feasibility of ET *in vivo*. The data are summarized in Table II.

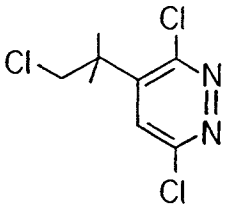
1. Thiosemicarbazones. Members of this class show activity as antimalarial, antiviral, and antifungal agents (5). Electroreduction was performed on the parent ligand, pro-



Scheme 5



Scheme 7

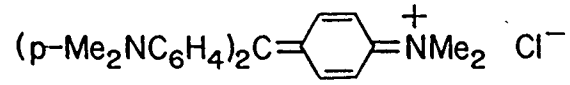


Scheme 8

tonated form (iminium type) and complexes with Cu and Fe, since these are two of the most important metals *in vivo*.

The free base of pyridine-2-aldehyde thiosemicarbazone (**10a**), as well as the copper and iron complexes, shows moderate fungicidal activity (14). The free base exhibited no reduction before −1.0 V. The addition of one equivalent of perchloric acid produced a large positive shift in the wave to give an irreversible reduction at −0.75 V. The most encouraging results were obtained with the metal complexes. The Cu(II) coordination compound gave irreversible reductions at +0.28 and −0.39 V, with a very broad anodic peak ($E_{pp/2}$ = 170 mV). The *bis* Fe(III) complex produced a small irreversible reduction at +0.04 V and a quasireversible process at +0.35 V. The 3-formyl analogue shows no antifungal activity (14). Structural alterations that abolish affinity for cations also tend to eliminate biological activity, presumably due to the difficulty in forming complexes *in vivo* (39).

Vanillin thiosemicarbazone (**10b**) has been studied for its fungicidal and antitubercular activity (16). The free base gave no reduction before −1.5 V. The addition of one equivalent of perchloric acid resulted in no reaction before −1.0



Scheme 9

V. The Cu(II) bidentate complex produced an irreversible wave at +0.18 V and a one-electron diffusion-controlled quasireversible reduction at −0.33 V. The Fe(III) complex was also investigated ($E^{\circ\prime}$ of −0.37 V). Cu(I) thiosemicarbazone complexes are known to be autoxidizable by oxygen (4).

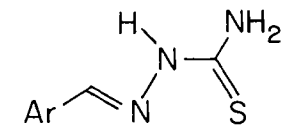
2. Thiadiazoles. Derivatives of this class are reported to possess antiviral, antibacterial, fungicidal, and analgesic activities (17). The antifungal thiadiazoline **11** and its metal chelates were chosen for electrochemical study (17). The parent ligand exhibited no reduction before −1.0 V. The synthesized Cu(II) and Fe(II) chelates produced favorable reductions of E_p = 0.35 and $E^{\circ\prime}$ of −0.57 V, respectively.

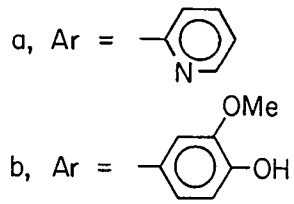
3. Others. Organometallic derivatives of mercury (30) and tin (12) have long been known to possess fungistatic activity. In electrochemical studies of active members, tribenzyltin chloride and benzylmercury chloride displayed reversible reduction at −0.61 and −0.23 V, respectively (40). In general, members of this class reduce within the physiological range. Dithiocarbamates, which are fungicidal, are known metal chelators *in vivo* (8). Changes in the oxidation states of copper and iron coordinated by dithiocarbamates have been observed by ESR spectroscopy in live fungal cultures (28). The reduction potential of the Cu(II) complex of the diethyl derivative has been reported ($E_{1/2}$ of −0.49 V) (8).

Table I. Electroreduction of Antifungal Agents[a]

Agent	Solvent and additives	Electrode	$E^{\circ\prime}$ or E_p(V)	Wave type	CF ratio[b]	i(pa)/i(pc)
1	DMF	Pt	−0.68	QR	1.1	0.83
1	EtOH/H_2O	Pt	−0.79[c]	IR	—	—
2	DMF	Pt	−0.29	QR	0.82	0.61
2	EtOH/H_2O	Pt	−0.13	QR	0.73	0.73
4	DMF	HMDE	−1.4[c]	IR	—	—
4	EtOH/H_2O, 1 eq $HClO_4$	HMDE	−0.61	QR	0.94	0.51
4	EtOH/H_2O, 1 eq HOAc	HMDE	−0.79[c]	IR	—	—
5	EtOH/H_2O, pH 1.3–7.0	HMDE	−0.78 −0.023pH[c]	IR	—	—
6	EtOH/H_2O	Pt	−1.12[c]	IR	—	—
6	EtOH/H_2O, 1 eq $HClO_4$	Pt	−0.74	QR	0.87	0.62
6	EtOH/H_2O, 1 eq HOAc	Pt	−0.89	QR	0.91	0.70
7	EtOH/H_2O	Pt	−0.77	QR	1.3	0.51
7	EtOH/H_2O, pH 3.5	HMDE	−0.53	QR	0.97	0.83
8	EtOH/H_2O	HMDE	−0.80	QR	1.1	0.54
8	EtOH/H_2O, pH 3.5	Pt	−0.47	R	0.90	0.97

[a] 100 MV/sec; TEAP (0.10 *M*); substrate (0.5 m*M*).
[b] CF_{benzil} = 16.27 (0.5 m*M*, Pt); 0.185 (0.5 m*M*, HMDE).
[c] E_p.



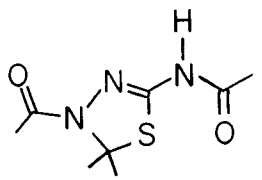


Scheme 10

Many metal complexes are known to inhibit respiration, e.g., those of copper, mercury, arsenic, and tin (12); ET may play a role. Complexes of metals which participate in Fenton reactions are known to generate toxic hydroxyl radicals *in vivo,* leading to lipid peroxidation (41). The chemical behavior of these compounds may be similar to that of copper and iron metalloenzymes which are involved in normal ET processes (42).

Other Agents

Aromatic nitro compounds comprise one of the main categories of ET agents. A positive correlation between reduction potential and activity has been shown for some members (43). Several representatives are in agricultural use (25). Pentachloronitrobenzene (fungicide) is known to inhibit respiration and promote lipid peroxidation. Radical scavengers were shown to decrease the extent of lipid peroxidation (25). A one-electron reduction at -0.69 V (E_p) to the radical anion has been reported (44). In an aerobic environment the nitro radical anion readily conveys an electron to oxygen (4).



Scheme 11

Several other categories of drugs are effective against fungi, all of which have been implicated in the formation of active oxygen species. These include pentachlorophenol (3,4,25) and the well-known dicarboximides (46).

Cyclic peroxides show an unusually high activity in antibacterial (3), antimalarial (5), and antifungal (3) areas. For this class, activated oxygen is already incorporated within the structure. Therefore, ET reactions need not be initially involved. A reasonable suggestion is that the peroxide acts as an initiator in a free radical chain reaction leading to oxidative stress (5,45).

OTHER CONSIDERATIONS

In our prior discussion evidence has been cited supporting the formation of radical intermediates and involvement of activated oxygen species. Application of the ET concept in a broad manner to the action of medicinals is not novel (4,9). Several authors have drawn a connection between drug toxicity and oxidative stress (1–6). Alternatively, the drug may interfere with normal ET chains, thus increasing electron flux *in vivo*. Lyr has suggested that lipid peroxidation within the inner mitochondrial membrane can inhibit respiration and ribosomal protein synthesis without site specific action (25). Also, the drug may act as a foreign mediator with redox active enzymes (27).

It should be emphasized that the activity of fungicides can be displayed by various pathways. Moreover, several routes may operate in concert for certain agents. Any attack

Table II. Electroreduction of Metal Chelators and Their Complexes[a]

Agent	Solvent and additives	Electrode	$E^{\circ\prime}$ or E_p(V)	Wave type	ΔE_p (mV)
10a	EtOH/H_2O	Pt	-1.31^b	IR	—
10a	EtOH/H_2O, 1 eq $HClO_4$	Pt	-0.75^b	IR	—
(**10a**)$_2$ · Cu(II)Cl_2	DMF	HMDE	$+0.28^b$	IR	—
			-0.39^b	IR	170[d]
(**10a**)$_2$ · Fe(III)Cl_3	DMF	HMDE	$+0.35^c$	QR	95
10b	DMF	Pt	$>-1.5^b$	IR	—
(**10b**)$_2$ · Cu(II)Cl_2	EtOH/H_2O	HMDE	$+0.18^b$	IR	—
			-0.33^c	QR	75
(**10b**)$_2$ · Fe(III)Cl_2	DMF	HMDE	-0.37^c	QR	71
11	DMF	HMDE	-1.3^b	IR	—
(**11**) · Cu(II)Cl_2	DMF	HMDE	-0.35^b	IR	>150[d]
(**11**) · Fe(II)Cl_2	DMF	HMDE	-0.57^c	R	76
$CuCl_2$	EtOH/H_2O	Pt	$+0.28^b$	—	—
$FeCl_3$	EtOH/H_2O	Pt	$+0.41^b$	—	—

[a] 100 mV/sec; TEAP (0.10 *M*); substrate (0.5 m*M*); see Refs. 14, 16, and 17 for characterization.
[b] E_p.
[c] $E^{\circ\prime}$.
[d] $E_{pp/2}$.

on the energy function of an organism results in an immediate disturbance of the entire metabolism. If energy production falls below that required for maintenance of essential processes, lysis can occur (12). In this scenario it would become more difficult for the organism to battle the invading xenobiotic.

One component of the ET theory requires catalytic action of the agents. Some of the electroreductions lacked a reoxidation wave. In many systems reversibility is affected by scan rate and solvent. Therefore, reversibility is a function of kinetics of the follow-up reaction(s) between the reduced species and/or solvent molecules (3). Reversibility can be more favorable *in vitro* because follow-up chemistry would be prevented when the reduced species is site bound. Another component of ET theory requires that the active agent have a relatively positive reduction potential. The E value of a bound agent may be more positive *in vivo* (6).

One should not expect a direct correlation between reduction potential and activity in all cases, because other factors, such as solubility, metabolism, site binding, and stereochemistry complicate the situation *in vivo*. However, a correlation between redox potential and fungitoxicity has been noted for the quinoidal category (47). Correlations between reduction potential and activity exist in other drug categories (4). Many of the discussed fungicides or their analogues show activity in several other medicinal areas (30), which may also entail ET (1–6).

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