Rates of Reaction of Indoleacetic Acids with Horseradish Peroxidase Compound I and Their Dependence on the Redox Potentials†

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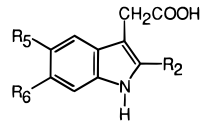
ABSTRACT: The rates of reaction of seven indole-3-acetic acid derivatives with horseradish peroxidase compound I at pH 5 were measured by stopped flow, and the reduction potentials and pK_a of their radical cations were determined by pulse radiolysis. Reasonable correlation of these properties with Hammett substituent parameters was found, but not with Brown−Okamoto (σ^+) parameters. The rates of reaction with compound I correlate well with the reduction potentials under the same conditions, with rates of reaction that increase by ca. 2.5 orders of magnitude with a 100 mV decrease in the reduction potential. This relationship is in agreement with that previously estimated for the reaction of compound I with phenols and anilines, suggesting that the rate of reaction depends solely on the reduction potential of the substrate radical, even for compounds of dissimilar structure.

Peroxidase from horseradish (EC 1.11.1.7; donor, hydrogen peroxide oxidoreductase) is a heme peroxidase that is able to catalyze the oxidation of a wide range of compounds, including phenols and amines (Dunford & Adeniran, 1986; Job & Dunford, 1976), phenylboronic acids (Sun et al., 1994), phenothiazines (Kelder et al., 1994), indoleacetic acids (Kobayashi et al., 1984; Ricard & Job, 1974), etc. In general, the catalytic cycle is described by the usual paradigm for heme peroxidases, in which the native (ferric) enzyme (HRP)[1] receives two oxidizing equivalents from hydrogen peroxide to form compound I (HRP-I), which in turn oxidizes the substrates by a sequence of two electron-transfer steps. However, the results of extensive studies suggest that the mechanism of the oxidation of indole-3-acetic acid may be more complex (Metodiewa et al., 1992; Smith et al., 1982). The involvement of free radicals has been demonstrated by electron spin resonance studies (Kobayashi et al., 1984; Mottley & Mason, 1986), and the chemistry of the indole-3-acetic acid radical cation has been elucidated in detail with the help of radiation chemistry techniques (Candeias et al., 1994). We have recently found that the rate of reaction of HRP-I with a series of indoleacetic acids in neutral solution is influenced by the substituents (Candeias et al., 1995). Previous studies on the structural effects on the rate of reaction of the enzyme intermediates with phenols and anilines have led to the suggestion that the rate of reaction depends only on the ease of oxidation of the substrates (Dunford & Adeniran, 1986; Job & Dunford, 1976). However, reliable determinations of the reduction potentials of the radicals of those compounds were lacking, and the mechanistic conclusions were based on an analysis of the substituent effects that has recently been criticized (Colclough & Smith, 1994).



1 R_2= R_5= R_6= H
2 R_2= H; R_5= Br; R_6= H
3 R_2= H; R_5= OH; R_6= H
4 R_2= H; R_5= CH_3O; R_6= H
5 R_2= CH_3; R_5= R_6= H
6 R_2= CH_3; R_5= CH_3O; R_6= H
7 R_2= CH_3; R_5= R_6= CH_3O

FIGURE 1: Indole-3-acetic acid derivatives used in this study.

With the advent of pulse radiolysis, reduction potentials involving species with lifetimes of less than milliseconds can be investigated (Jovanovic & Steenken, 1992; Shen et al., 1987; Wardman, 1989). In this technique, a short pulse of accelerated electrons generates known amounts of free radicals in solution, in times usually below 1 μs. Using spectrophotometric, conductometric, or other detection techniques, the formation and decay of radicals can be monitored (Tabata, 1990).

In the present study, we have used pulse radiolysis to study the reversible electron transfer between the radicals formed on one-electron oxidation of seven indole-3-acetic acid derivatives (Figure 1) and two redox standards, hexachloroiridate(III) and promethazine. On the basis of the experimentally determined equilibrium constants, we were able to determine the reduction potentials of the indoleacetic acid radicals. In addition, the rates of reaction of HRP-I with the same compounds were determined by stopped flow and correlated with the redox potentials.

MATERIALS AND METHODS

Materials. The indole-3-acetic acid derivatives, with the exception of 2-methyl-5,6-dimethoxyindole-3-acetic acid (**7**),

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[1] Abbreviations: HRP, horseradish peroxidase; HRP-I and HRP-II, horseradish peroxidase compounds I and II, respectively; PZ^+ and $PZ^{\bullet 2+}$, promethazine cation and radical dication, respectively.

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were purchased from Aldrich and used as received. Horseradish peroxidase type VI-A from Sigma was used, which is an essentially salt-free powder. All other reagents were of analytical grade. Solutions were freshly prepared with water purified by a Millipore Milli-Q system. For the stopped-flow experiments, stock solutions (0.1 mol dm^{-3}) of the indoleacetic acids in 10% ethanol were prepared, whereas for the radiolysis experiments, the indoles were dissolved in phosphate buffer at a concentration of 0.2 mmol dm^{-3} by being stirred with gentle heating (ca. 40 °C) under nitrogen and in the dark. Before irradiation, the solutions were saturated with zero grade nitrous oxide (oxygen content < 10 ppm) purchased from the British Oxygen Co.

The ethyl ester of 2-methyl-5,6-dimethoxyindole-3-acetic acid was obtained by a modified literature method (Wynnewood & Childress, 1966). In our hands, the best yield (16%) was obtained when diethyl ether was used as a solvent for the reaction and diluted hydrochloric acid was added to the vigorously stirred reaction mixture in the isolation procedure.

*2-Methyl-5,6-dimethoxyindole-3-acetic Acid (**7**).* To a solution of the ethyl ester (0.48 g, 1.73 mmol) in ethanol (12 mL) was added sodium hydroxide solution (6 N, 2 mL) and the mixture stirred for 1 h. Water (6 mL) was then added and ethanol removed under vacuum. The aqueous solution was filtered and acidified with 6 N hydrochloric acid. The resulting solid was filtered off, washed with water, and dried under vacuum to give **7** as purple crystals (0.28 g, 65%): mp 95−97 °C; ν_{max} 3430, 1715, 1595 cm^{-1}; ($(CD_3)SO$) δ 2.10 (3H, s, 2-Me), 3.59 (2H, s, CH_2), 3.72 (6H, s, 2 × MeO), 6.78 (1H, s, ArH), 6.85 (1H, s, ArH), 8.19 (1H, br s, exchanged with D_2O, NH); HRMS (relative intensity) *m/z* 249 (M^+, 21), 219 (100), 204 (79). Anal. Found: C, 59.57; H, 6.46; N, 5.32. Calcd ($C_{13}H_{15}NO_4 \cdot {}^3/_4H_2O$): C, 59.43; H, 6.29; N, 5.33).

Methods. Stopped-flow experiments were performed with a single wavelength Hi-Tech SF 51 system with a mixing time of <2 ms. The optical path was either 2 or 10 mm, and the cell, load, and drive syringes were kept at 25 ± 0.2 °C by circulating water. Some experiments in the time scale of seconds used a Hewlett-Packard HP8452A diode array spectrophotometer fitted with a Hi-Tech SFA-11 fast kinetics accessory. The latter system has a time resolution of 0.1 s, determined by the spectrophotometer, but allows the rapid scan of absorption spectra.

Pulse radiolysis experiments were performed with a 4 MeV Van de Graaff accelerator as described previously (Candeias et al., 1993). Pulses of 10 ns were used which delivered doses of ca. 1 Gy, as determined by thiocyanate dosimetry (Bielski, 1993). Under these conditions, <1 μmol dm^{-3} radicals are generated, which minimizes the effects of reactions between radicals. The pulse radiolysis experiments were performed at room temperature (22 ± 2 °C). The radiolysis of N_2O-saturated solutions of potassium bromide (0.05 mol dm^{-3}) in phosphate buffer (2.5 mmol dm^{-3}) was used to generate the dibromine radical anion ($Br_2^{\bullet -}$) in <0.5 μs (Mamou et al., 1977; Zehavi & Rabani, 1972). This one-electron oxidant reacts with indole-3-acetic acid and derivatives with rate constants ∼5 × 10^8 dm^3 mol^{-1} s^{-1} (Candeias et al., 1994; Jovanovic & Steenken, 1992).

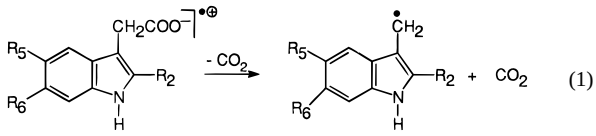
Table 1: pK_a Values of the Radical Cations of Indole-3-acetic Acid Derivatives at Room Temperature and an Ionic Strength of 0.05 mol dm^{-3}

compound	pK_a
1, indole-3-acetic acid	5.09 ± 0.02[a]
2, 5-bromoindole-3-acetic acid	<4
3, 5-hydroxyindole-3-acetic acid	<4
4, 5-methoxyindole-3-acetic acid	5.48 ± 0.04[a]
5, 2-methylindole-3-acetic acid	6.15 ± 0.02[a]
6, 2-methyl-5-methoxyindole-3-acetic acid	6.16 ± 0.03[a]
7, 2-methyl-5,6-dimethoxyindole-3-acetic acid	7.69 ± 0.01

[a] From Candeias et al. (1995); see also Jovanovic and Steenken (1992).

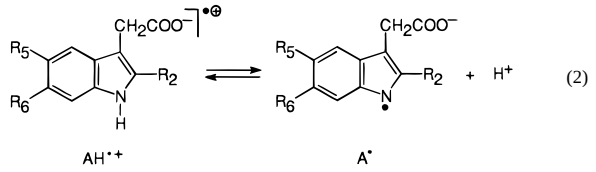
RESULTS

pK_a Values of the Radical Cations. The one-electron oxidation of indole-3-acetic acid and derivatives by the dibromine radical anion ($Br_2^{\bullet -}$) is known to generate the corresponding radical cations.[2] In acidic solution, the latter undergo spontaneous cleavage of the carbon−carbon bond of the acetic acid side chain to yield carbon dioxide and skatolyl radicals:



(1)

The rate constants of decarboxylation of the radical cations of various indole-3-acetic acid derivatives have recently been determined (Candeias et al., 1994; Jovanovic & Steenken, 1992). In neutral solution, the radical cations deprotonate rapidly and reversibly from the heterocyclic nitrogen to give indolyl radicals, which are stable with respect to decarboxylation:



(2)

In pulse radiolysis experiments, the deprotonation is visible as a shift in the maximum of the transient absorption from ∼560 nm at low pH to ∼520 nm in neutral or slightly alkaline solution (Posener et al., 1976). By monitoring the absorption at 560 nm at different pH values and at time scales such that the oxidation of the indole is complete but the decarboxylation has not yet taken place (typically 10−20 μs after the electron pulse), we could determine the pK_a's of the indolyl radical cations. The results obtained with compounds **1** and **4**−**6** were reported previously (Candeias et al., 1995), and here we extended our studies to a new indole-3-acetic acid derivative, 2-methyl-5,6-dimethoxyindole-3-acetic acid (**7**), to 5-bromoindole-3-acetic acid (**2**), and to 5-hydroxyindole-3-acetic acid (**3**). Whereas with compound **7**, the pK_a of the radical cation could be determined (Table 1 and Figure 2), the absorption of the radical of **2** at 520 nm was found to decay less than 20% on going from pH 7 to 4 and no significant increase of the

[2] Note that, due to the ionized side chain, the radical cations are in fact zwitterions. We use the term radical cation loosely.

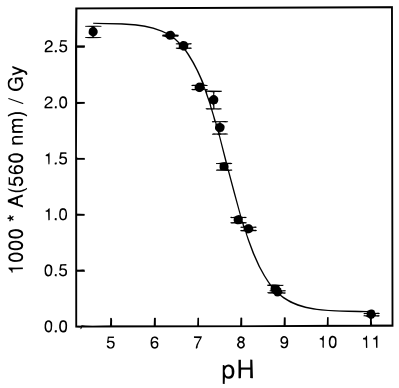
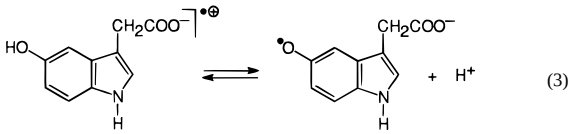


FIGURE 2: p*K* curve for the 2-methyl-5,6-dimethoxyindole-3-acetic acid (**7**) radical cation. The radical was generated by pulse radiolysis of N_2O solutions of the parent compound (0.2 mmol dm^{-3}) in KBr (0.05 mol dm^{-3}) at different pH values and the transient absorption at 560 nm, normalized for the dose, 10−20 μs after the pulse was measured. The error bars show the standard deviation of three measurements, and the line is an ideal p*K* curve fitted by the method of weighed nonlinear least squares.

absorption at 560 nm was observed. We therefore conclude that the radical of compound **2** exists in the deprotonated form over this pH range; i.e., the pK_a of the corresponding radical cation is <4. The hydroxylated compound 5-hydroxyindole-3-acetic acid (**3**) was also found to react with $Br_2^{\bullet-}$, but the absorption spectrum of the resulting radical was substantially different from that obtained with the other compounds. It had a maximum at 420 nm (ϵ = 4500 dm^3 mol^{-1} cm^{-1}). This result strongly suggests the formation of a phenoxyl radical by deprotonation of the radical from the 5-hydroxyl group:



(3)

The spectrum was found to be pH-independent in the range 4−7, showing that the radical does not protonate even at pH 4. Such low basicity is also characteristic of phenoxyl radicals (Neta & Fessenden, 1974).

Reduction Potentials of the Indoleacetic Acid Radicals. Even in neutral solution, the radicals of the indole-3-acetic acid derivatives are not sufficiently stable to allow the determination of their reduction potentials by conventional electrochemical methods. However, with the technique of pulse radiolysis, the establishment of electron-transfer equilibria between compounds of known redox potential (redox standards) and free radicals can be monitored, and therefore, reduction potentials involving short-lived radicals can be determined. We found that the hexachloroiridate(III) anion ($IrCl_6^{3-}$) is a convenient redox standard for the determination of the reduction potentials of the radicals of indole-3-acetic acid derivatives. This complex has no absorption above 350 nm, whereas its oxidized form, hexachloroiridate(IV) ($IrCl_6^{2-}$), exhibits an absorption maximum at 490 nm (ϵ = 4075 dm^3 mol^{-1} cm^{-1}). Its reduction potential is well-known from electrochemical measurements (Margerum et al., 1975) and its reactivity toward the dibromine anion ($k = 1.5 \times 10^7$ dm^3 mol^{-1} s^{-1}) is more than 10-fold lower than that of the indole-3-acetic acid derivatives (DeFelippis et al., 1989).

At pH 7.4, the radicals of the indoleacetic acids decayed by second order kinetics, i.e., by radical−radical reactions.

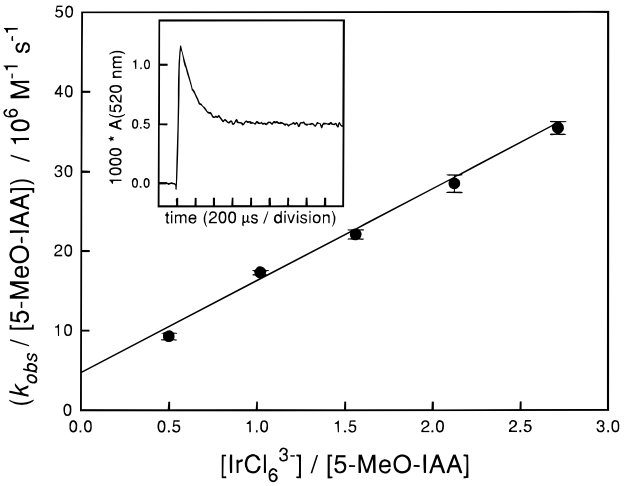


FIGURE 3: Determination of the electron-transfer equilibrium constant between the radical of 5-methoxyindole-3-acetic acid (**4**) and hexachloroiridate(III). The ratio of the observed rate of decay at 520 nm over the concentration of the indoleacetic acid is plotted against the ratio of the concentrations of the iridium complex and the indoleacetic acid. The error bars show the average of three measurements. Insert: transient absorption at 520 nm in a solution containing 183 μmol dm^{-3} 5-methoxyindole-3-acetic acid and 497 μmol dm^{-3} $IrCl_6^{3-}$, after a pulse of ca. 1 Gy.

In the presence of $IrCl_6^{3-}$ (Figure 3), their decay turned to first order kinetics (except with compounds **3** and **7**, see below). Simultaneously, an increase of the absorption at 490 nm could be observed, showing the formation of hexachloroiridate(IV) and suggesting that indolyl radicals were oxidizing the iridium(III) complex:

$$A^{\bullet} + IrCl_6^{3-} + H^+ \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AH + IrCl_6 \qquad (4)$$

(here, AH and $A^{\bullet}$ represent the indole-3-acetic acid derivatives and their deprotonated radicals, respectively). The observed rate of reaction 4 (k_{obs}) was dependent not only on the concentration of $IrCl_6^{3-}$ but also on the concentration of the indoleacetic acid, which is a product of the reaction, clearly indicating the reversibility of reaction 4. Integration of the kinetic differential equations, with the approximations of negligible radical decay during the establishment of the equilibrium ($[A^{\bullet}] + [IrCl_6^{2-}]$ = constant) and low radical concentration ($[A^{\bullet}] \ll [AH]$ and $[IrCl_6^{2-}] \ll [IrCl_6^{3-}]$), leads to a linear relation between k_{obs}/[AH] and the concentration ratio $[IrCl_6^{3-}]$/[AH]:

$$k_{obs}/[AH] = k_f([IrCl_6^{3-}]/[AH]) + k_r \qquad (5)$$

where k_f and k_r are the rates of the forward and reverse reactions in equilibrium 4, respectively. Further details of the method can be found elsewhere (Wardman, 1989). Several series of experiments were performed where the rates of decay of the indolyl radicals were determined (from the transient absorption at 520 nm) in the presence of variable concentrations of $IrCl_6^{3-}$ and the parent indoleacetic acid. The increase of absorption at 490 nm, associated with the formation of $IrCl_6^{2-}$, was largely concealed by the considerable absorption of the indolyl radicals at this wavelength that resulted in small changes of absorption during the establishment of the equilibrium. For this reason, only measurements at 520 nm were used. Fitting of eq 5 to the experimental data yielded the rate and equilibrium constants ($K = k_f/k_r$)

Table 2: Electron-Transfer Equilibrium Constants at pH 7.4, Room Temperature, and an Ionic Strength of 0.05 mol dm^{-3}

$$A^{\bullet} + S + H^{+} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AH + S^{\bullet+}$$

AH	S	wavelength (nm)	k_f (dm^3 mol^{-1} s^{-1})[a]	k_r (dm^3 mol^{-1} s^{-1}	K	$E_{7.4}$ (V)
1, indole-3-acetic acid	PZ^+	560	$(2.2 \pm 0.3) \times 10^8$	$(1.7 \pm 0.4) \times 10^7$	13.1 ± 3.4	0.922
	$IrCl_6^{3-}$	520	$(7.4 \pm 0.2) \times 10^6$	$(3.6 \pm 0.3) \times 10^6$	2.1 ± 0.1	0.913
2, 5-bromoindole-3-acetic acid	$IrCl_6^{3-}$	520	$(8.9 \pm 0.4) \times 10^6$	$(2.8 \pm 0.2) \times 10^6$	3.2 ± 0.2	0.924
3, 5-hydroxyindole-3-acetic acid	PZ^+	420	$(2.1 \pm 0.2) \times 10^7$	$(2.5 \pm 0.1) \times 10^8$	0.082 ± 0.010	0.792
4, 5-methoxyindole-3-acetic acid	PZ^+	515	$(3.2 \pm 0.2) \times 10^8$	$(3.3 \pm 0.2) \times 10^7$	9.6 ± 0.9	0.914
	$IrCl_6^{3-}$	520	$(1.16 \pm 0.04) \times 10^7$	$(4.7 \pm 0.4) \times 10^6$	2.5 ± 0.2	0.917
5, 2-methylindole-3-acetic acid	$IrCl_6^{3-}$	520	$(8.3 \pm 0.4) \times 10^6$	$(1.1 \pm 0.1) \times 10^7$	0.78 ± 0.10	0.888
6, 2-methyl-5-methoxyindole-3-acetic acid	$IrCl_6^{3-}$	520	$(4.7 \pm 0.3) \times 10^6$	$(1.31 \pm 0.06) \times 10^7$	0.36 ± 0.03	0.867
7, 2-methyl-5,6-dimethoxyindole-3-acetic acid	PZ^+	500	$(4.2 \pm 1.0) \times 10^8$	$(9.2 \pm 0.5) \times 10^8$	0.46 ± 0.12	0.836
PZ^+, promethazine	$IrCl_6^{3-}$	525	$(1.7 \pm 0.1) \times 10^6$	$(7.3 \pm 0.2) \times 10^6$	0.23 ± 0.02	0.856

[a] Not considering H^+, when involved.

listed in Table 2. An example of the data is shown in Figure 3. An alternative method of determining the equilibrium constant of reaction 5 would be to measure the absorbance at the time of the establishment of equilibrium. Ideally, both approaches should yield the same equilibrium constants. However, the method of absorbance at equilibrium is affected by the bimolecular decay of the free radicals that competes with the establishment of electron-transfer equilibrium, even at the low radiation doses used in this study (~1 Gy per pulse). In the kinetic method, the rate of approach to the equilibrium was determined from the initial part of the exponential curves and the results are therefore much less sensitive to the bimolecular decay of the radicals. Therefore, only the results obtained by the latter method were used.

The equilibrium constants obtained, in conjunction with the Nernst equation, allow the calculation of the reduction potentials of the indoleacetic acid radicals:

$$\Delta E = E(A^{\bullet},H^{+}/AH) - E(IrCl_6^{2-}/IrCl_6^{3-}) = (RT/F) \ln K \quad (6)$$

(R is the gas constant, T the absolute temperature, and F the Faraday constant). However, some preliminary considerations about the reduction potential of the standard couple are required. Reliable electrochemical measurements have yielded the value $E°(IrCl_6^{2-}/IrCl_6^{3-}) = 0.876$ V at 0 ionic strength. Due to the charges on both oxidized and reduced forms, this potential is strongly dependent on the ionic strength (μ). For moderate ionic strengths ($\mu \leq 0.1$ mol dm^{-3}), we can apply a correction by inserting in the Nernst equation the Debye−Hückel expression for the activity coefficients (γ):

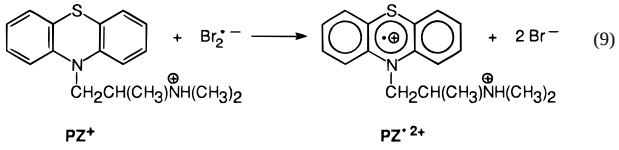
$$\log \gamma = -0.509 z^2 \mu^{1/2}/(1 + \mu^{1/2}) \quad (7)$$

where z represents the charge of the ion. For a generic redox couple at 25 °C, the following expression is obtained:

$$E_\mu = E_{\mu=0} - 0.030(z_{ox}^{\ 2} - z_{red}^{\ 2})\mu^{1/2}/(1 + \mu^{1/2}) \quad (8)$$

where z_{ox} and z_{red} are the charges of the oxidized and reduced forms, respectively, and E_μ and $E_{\mu=0}$ represent the redox potentials at ionic strength μ and 0, respectively. It can be verified that the reduction potential of hexachloroiridate(IV) at $\mu = 0.1$ mol dm^{-3} calculated by eq 8 is in good agreement with the experimentally determined value (0.892 V) (Margerum et al., 1975). On this basis, we estimate for the reduction potential of hexachloroiridate(IV) $E_{\mu=0.05}(IrCl_6^{2-}/IrCl_6^{3-}) = 0.894$ V at an ionic strength of 0.05 mol dm^{-3}. This value was used to calculate the reduction potentials of the indoleacetic acid radicals listed in Table 2.

The radicals of compounds **3** and **7** did not exhibit measurable reactivity toward $IrCl_6^{3-}$, and therefore, a second redox standard, promethazine (PZ^+), was used. This compound reacts with $Br_2^{\bullet-}$ to yield the radical dication $PZ^{\bullet 2+}$ (eq 9) (Bahnemann & Asmus, 1983):



(9)

The latter was found to react reversibly with 5-hydroxyindole-3-acetic acid (**3**), as concluded from measurements of the decay of $PZ^{\bullet 2+}$ at 515 nm or the buildup of the radical of **3** at 420 nm. These measurements were used to determine the electron-transfer equilibrium constant by the method outlined above, and the results are presented in Table 2. The promethazine radical dication was also found to react reversibly with compound **7**, but unfortunately, its spectrum almost overlaps that of the indolyl radical. However, the difference of extinction coefficients at 500 nm was found to be sufficient to allow the establishment of equilibrium to be monitored. Reversible electron-transfer equilibrium between $PZ^{\bullet 2+}$ and compounds **1** and **4** could also be demonstrated, but in these cases, the reaction was found to proceed predominantly in the opposite direction; i.e., at equal concentrations of PZ^+ and indoleacetic acid, the radicals of the latter were found to oxidize the former. In the experiments with PZ^+, the observed rates of approach to equilibrium were higher than with $IrCl_6^{3-}$ and the equilibrium constants could also be measured from the absorption at equilibrium. The obtained values led to reduction potentials that differ by ≤10 mV from those obtained from the kinetic method.

Previously, it has been noticed that the reduction potential of the promethazine radical dication can exhibit considerable variation, depending on the experimental conditions, and its calibration has been recommended (Jonsson et al., 1994). Therefore, we decided to measure the equilibrium constant for the reaction between $PZ^{\bullet 2+}$ and $IrCl_6^{3-}$. On the basis of

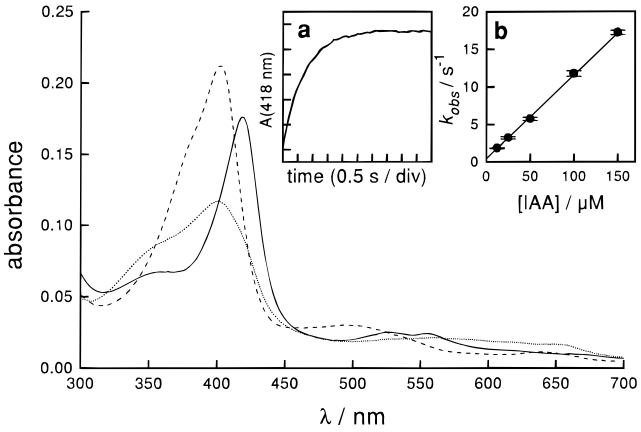


FIGURE 4: Absoprtion spectra of horseradish peroxidase in the ferric state (dashed line), after addition of 0.5 mmol dm^{-3} hydrogen peroxide (HRP-I, dotted line), and after subsequent addition of 12.5 μmol dm^{-3} indole-3-acetic acid (**1**) (solid line). Insert a: observed increase of absorption at 418 nm on reaction of HRP-I with 12.5 μmol dm^{-3} indole-3-acetic acid. Insert b: linear dependence of the observed rate of buildup at 418 nm as a function of the indole-3-acetic acid concentration; error bars represent the standard deviation of five measurements. The experiments were carried out in 0.05 mol dm^{-3} KBr and 2.5 mmol dm^{-3} phosphate at pH 5.0 and 20 °C.

the obtained constant (Table 2), we estimate for the reduction potential of the promethazine radical dication the value $E_{\mu=0.05}(PZ^{\bullet 2+}/PZ^{+}) = 0.856$ V at an ionic strength of 0.05 mol dm^{-3}. We note that this value is much lower than either of the discordant values obtained previously by pulse radiolysis (Jonsson et al., 1994; Jovanovic et al., 1990) but is close to the value recommended in a previous compilation (Wardman, 1989). Using this value, the reduction potentials of the indoleacetic radicals were calculated, as shown in Table 2. With compounds **1** and **4**, reversible electron transfer with both standards could be achieved and the potentials obtained with either of the standards agree within 10 mV, showing the internal consistency of our data. Ultimately, all the values determined in the present study are based on the reduction potential of $IrCl_6^{3-}$.

Reaction with Horseradish Peroxidase Compound I. Horseradish peroxidase (HRP) catalyzes the oxidation of indole-3-acetic acid in the presence of hydrogen peroxide by a complex mechanism. Hydrogen peroxide reacts with the native (ferric) form of the enzyme to yield compound I (HRP-I, eq 10), which contains iron(IV) coordinated to an oxygen atom and a porphyrin radical cation. This fast reaction ($k = 2.3 \times 10^7$ dm^3 mol^{-1} s^{-1}) can be monitored by the change of the absorption spectrum of the enzyme, as shown in Figure 4 (Dolman et al., 1975). Compound I reacts rapidly with indole-3-acetic acid to yield compound II with an absorption maximum at 418 nm and in which the porphyrin radical cation has been neutralized. The radical cation of indole-3-acetic acid is believed to be involved as a reaction intermediate:

$$HRP + H_2O_2 \rightarrow HRP\text{-}I + H_2O \quad (10)$$

$$HRP\text{-}I + AH \rightarrow HRP\text{-}II + AH^{\bullet +} \quad (11)$$

In this study, we used the technique of stopped flow to determine the rate of reaction of HRP-I with the indole-3-acetic derivatives listed in Figure 1. The experiments were performed at pH 5.0, where the enzyme is considerably more reactive than in neutral solution. Solutions of variable concentrations of those compounds containing hydrogen peroxide were rapidly mixed with solutions of the enzyme in the ferric state. The concentration of hydrogen peroxide (0.5 mmol dm^{-3} after mixing) was such that the formation of HRP-I was complete within the mixing time (≤2 ms), whereas the concentration of enzyme (∼1 μmol dm^{-3} after mixing) was adjusted to give an easily measurable absorbance ($A \sim 0.2$ cm^{-1}) and the indoleacetic acid was in 10-fold excess, at least. To allow a direct correlation with the reduction potentials measured by pulse radiolysis, a similar reaction medium was used, i.e., 0.05 mol dm^{-3} KBr and 2.5 mmol dm^{-3} phosphate. Under these conditions, the formation of compound II on reaction of HRP-I with the indoleacetic acid could be monitored as an increase of absorption at 418 nm. Following this reaction, a slow decay of absorbance was observed, which can be attributed to the reaction of compound II with the indoleacetic acid and/or with hydrogen peroxide. However, this second process was too slow to interfere with the formation of compound II, the rate of which could be determined by fitting of an exponential curve. The observed rates (≤500 s^{-1}) were found to be linearly dependent on the concentration of the indoleacetic acid (Figure 4), and no evidence for saturation kinetics was found, indicating that either there is no enzyme−substrate binding or the dissociation of such a complex is very fast. The rate constants for the reaction of HRP-I with compounds **1**−**7**, determined from the slopes of the linear dependencies of the observed rate on the indoleacetic acid concentration, are listed in Table 3.

Table 3: Rate Constants for the Reaction of Horseradish Peroxidase Compound I with Indole-3-acetic Acid Derivatives at pH 5.0, 20 °C, and an Ionic Strength of 0.05 mol dm^{-3}

	compound	$k_{HRP\text{-}I}$ ($dm^3 mol^{-1} s^{-1}$)
1,	indole-3-acetic acid	$(1.12 \pm 0.02) \times 10^5$
2,	5-bromoindole-3-acetic acid	$(2.58 \pm 0.10) \times 10^4$
3,	5-hydroxyindole-3-acetic acid	$(2.08 \pm 0.17) \times 10^7$
4,	5-methoxyindole-3-acetic acid	$(6.43 \pm 0.14) \times 10^5$
5,	2-methylindole-3-acetic acid	$(9.72 \pm 0.49) \times 10^6$
6,	2-methyl-5-methoxyindole-3-acetic acid	$(1.95 \pm 0.20) \times 10^7$
7,	2-methyl-5,6-dimethoxyindole-3-acetic acid	$(3.45 \pm 0.48) \times 10^7$

DISCUSSION

As pointed out in previous studies, the acid−base properties of the radical cations of indole-3-acetic acid derivatives are dependent on the substituents (Candeias et al., 1995). The pK_a values of the radical cations of compounds **1** and **4**−**6** were examined in terms of Hammett substituent parameters (σ values), but only a poor correlation was found. The new indoleacetic acid **7**, carrying three electron-donating substituents, allows a reexamination of the substituent effect over a wider range. As shown in Figure 5a, while substantial deviations can still be observed, there is some correlation with Hammett σ values (correlation coefficient of −0.94); a worse correlation with Brown−Okamoto σ^+ values was found (correlation coefficient of −0.87, not shown).

Using the technique of pulse radiolysis, we were also able to determine the reduction potentials of the indoleacetic acid radicals at pH 7.4 (Table 2). The values obtained are in the range 0.79−0.92 V, showing some effect of the substituent. A previous attempt to determine the redox potentials of

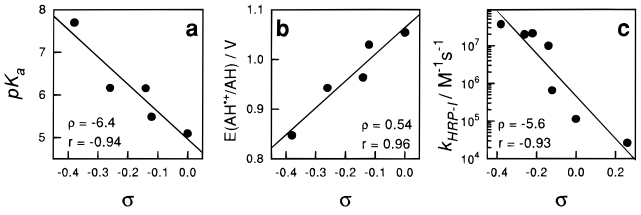


FIGURE 5: Correlation with Hammett substituent parameters of the pK_a values (a) and reduction potentials (b) of the radical cations of the indoleacetic acids and of their rate of reaction with horseradish peroxidase compound I (c). The correlation coefficients (ρ) and slopes of the best fit straight lines (r) are indicated in the figure.

Table 4: Hammett Substituent Parameters, Calculated Reduction Potentials of the Indoleacetic Acid Radical Cations, and Reduction Potentials at pH 5.0, Room Temperature, and an Ionic Strength of 0.05 mol dm^{-3}

compound	$\sum\sigma^a$	E(AH$^{\bullet+}$/AH) (V)	E_5 (V)
1, indole-3-acetic acid	0	1.054	1.039
2, 5-bromoindole-3-acetic acid	0.26	−	1.066
3, 5-hydroxyindole-3-acetic acid	−0.22	−	0.934
4, 5-methoxyindole-3-acetic acid	−0.12	1.029	1.022
5, 2-methylindole-3-acetic acid	−0.14	0.963	0.962
6, 2-methyl-5-methoxy indole-3-acetic acid	−0.26	0.942	0.940
7, 2-methyl-5,6-dimethoxy indole-3-acetic acid	−0.38	0.847	0.847

[a] σ values from Ehrenson et al. (1973).

compounds **1** and **6** yielded slightly higher values, which may be due to the use of a different value for the potential of the redox standard promethazine and to the different ionic strength conditions (Jovanovic & Steenken, 1992). However, both sets of data appear internally consistent.

The redox half-reaction describing the reduction of the indoleacetic acid radicals involves 1 equiv of H^+. Consequently, on the basis of the Nernst equation (eq 6), the reduction potentials can be predicted to vary by 59 mV per pH unit, above the pK_a of the respective radical cation. In fact, the combination of the reduction potentials at pH 7.4 and the pK_a values of the radical cations of compounds **1** and **4**−**7** allows the calculation of the redox potentials at any pH value between 4 and 7. Therefore, even the reduction potentials of the indoleacetic radical cations, which cannot be directly determined due to the fast decarboxylation (eq 1), can be calculated. Not surprisingly, the reduction potentials of the radical cations are increased by electron-donating substituents; they exhibit an acceptable correlation with Hammett σ values (correlation coefficient of 0.96, Figure 5b) but a poorer correlation with σ^+ (correlation coefficient of 0.86, not shown). This is in disagreement with a previous analysis of the substituent effects on the redox potentials of indoles (Jovanovic & Steenken, 1992). However, in the same study, the indoleacetic acids (compounds **1** and **6**) were found to deviate from the general correlation.

On the basis of eq 6 and the dissociation constants of the indoleacetic acid radicals, the reduction potentials of the radicals of the indoleacetic acids at pH 5 can be calculated (Table 4) and correlated with the rates of reaction of these compounds with HRP-I. Those rates (Table 3) are nearly 2 orders of magnitude higher than those measured previously at pH 7.4 (Candeias et al., 1995), in agreement with the pronounced pH effect on this reaction reported earlier (Kobayashi et al., 1984). As the dissociation constants and

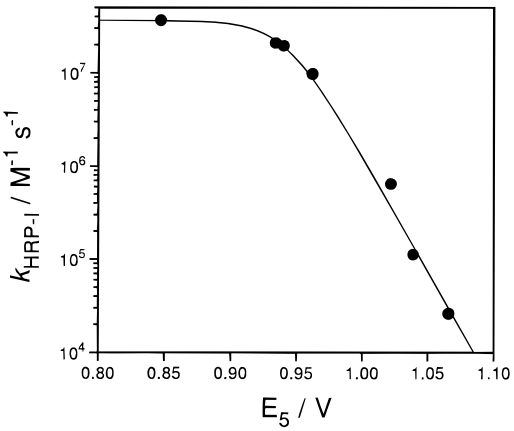


FIGURE 6: Relation of the rate of reaction of horseradish peroxidase compound I with indoleacetic acids and the reduction potentials of the respective radicals at pH 5. The straight line is the best fit to a linear relation with correction for diffusion limit.

reduction potentials of the radicals, the rates of reaction with HRP-I correlate approximately with the Hammett substituent parameters (Figure 5c) but poorly with Brown−Okamoto parameters (correlation coefficient of −0.81, not shown). This is in agreement with the conclusion of a previous study on the reaction of HRP-I with phenols and anilines; the correlation with σ, but not σ^+, was taken as evidence for electron transfer between the substrate and enzyme with simultaneous loss of a proton (Job & Dunford, 1976). On this basis, a correlation between rate of reaction and the reduction potential measurements of the radicals of the substrates in question was predicted. Unfortunately, reliable measurements of those potentials were not available at the time. In the present study, we have been able to determine the rates of reaction of HRP-I with indole-3-acetic acid and the reduction potentials of the corresponding radicals under the same conditions. The correlation between these two quantities is shown in Figure 6.

For compounds **1**−**6**, there is a good linear correlation (correlation coefficient of −0.99) between the logarithm of the rate of reaction with HRP-I and the reduction potentials. This is clearly better than the correlation with σ, providing a further argument against the applicability of the usual substituent parameters to indole-3-acetic acid derivatives. The slope of the linear relation (-25 ± 3 V^{-1}) is noteworthy; it implies that a 100 mV decrease of reduction potentials causes an increase of the rate of reaction with HRP-I of more than 2 orders of magnitude. Interestingly, this is within the range previously estimated for the relation between the rate of reaction of HRP-I with phenols and anilines and the reduction potentials of the respective radicals (Job & Dunford, 1976). In this context, it is interesting that 5-hydroxyindole-3-acetic acid (**3**), which upon oxidation yields phenoxyl radicals and can therefore be considered a phenolic compound, shows a rate of reaction with HRP-I consistent with those of the other indoleacetic acids. This gives another indication that HRP-I reacts with substrates, with the rate determined by the reduction potentials of their radicals and not by their structure.

However, the rate obtained with compound **7** is lower than expected on the basis of the linear correlation. A possible explanation is that the reaction of this compound with HRP-I is diffusion-limited. In view of this possibility, a curve was fitted to the experimental data describing a linear relation

between the logarithm of the rate of reaction with HRP-I and the redox potentials, but including a correction for the diffusion limit through the formula:

$$1/k_{\text{HRP-I}} = 1/k_{\text{ET}} + 1/k_{\text{diff}} \quad (12)$$

where $k_{\text{HRP-I}}$, k_{ET}, and k_{diff} represent the observed rate, the rate of electron transfer, and the diffusion rate, respectively. As shown in Figure 6, a good fit is obtained with $k_{\text{diff}} = (3.6 \pm 0.1) \times 10^7$ dm^3 mol^{-1} s^{-1}. With phenols and anilines, rates of reaction of HRP-I of up to $\sim 2 \times 10^8$ dm^3 mol^{-1} s^{-1} have been reported, which may indicate a lower diffusion limit in the case of the indoleacetic acids.

In conclusion, the rates of reaction of HRP-I with indole-3-acetic acid derivatives at pH 5.0 are related to the reduction potential of the respective radicals calculated for the same conditions on the basis of pulse radiolysis measurements at pH 7.4. For redox potentials ≥ 0.93 V, the rate of reaction increases by 2.5 orders of magnitude for a 100 mV decrease in reduction potential, a relation similar to that estimated for the reaction of HRP-I with phenols and anilines. These results support the hypothesis that the reaction of the enzyme intermediate with substrates is an electron-transfer reaction, which occurs simultaneously with a proton release, at pH values above the pK_a of the incipient radical cation.

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