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Substituent Effect on the Oxidation of Phenols and Aromatic Amines by Horseradish Peroxidase Compound I

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A stopped-flow kinetic study shows that the reduction rate of horseradish peroxidase compound I by phenols and aromatic amines is greatly dependent upon the substituent effect on the benzene ring. Moreover it has been possible to relate the reduction rate constants with the ionization constants of monosubstituted substrates by a linear free-energy relationship (Hammett equation). The correlation of log (rate constants) with σ values (Hammett equation) and the absence of correlation with σ^+ values (Okamoto-Brown equation) can be explained by a mechanism of aromatic substrate oxidations, in which the substrate gives an electron to the enzyme compound I and simultaneously loses a proton. The analogy which has been made with oxidation potentials of phenols or anilines strengthens the view that the reaction is only dependent on the relative ease of oxidation of the substrate. The rate constant obtained for *p*-aminophenol indicates that a value of $2.3 \times 10^8\ M^{-1}\ s^{-1}$ probably approaches the diffusion-controlled limit for a bimolecular reaction involving compound I and an aromatic substrate.

One-electron oxidation of organic substrates (AH_2) catalyzed by peroxidase is ususally depicted by the following well-known mechanism [1–3]

$$E + H_2O_2 \xrightarrow{k_1} E\text{-}I \qquad (1)$$

$$E\text{-}I + AH_2 \xrightarrow{k_2} E\text{-}II + AH\cdot \qquad (2)$$

$$E\text{-}II + AH_2 \xrightarrow{k_3} E + AH\cdot \qquad (3)$$

in which E, E-I and E-II represent native horseradish peroxidase and its two spectroscopically and kinetically distinct intermediates compound I and compound II. Phenols [4, 4a] and aromatic amines [5] are known to react *via* such a mechanism, and the pH profiles of compound I reduction rate constants show almost the same behavior for these two kinds of substrates. pH-independent values for k_2 are obtained for pH values between 6 and 9. Shiga and Imaizumi [6] using an electron spin resonance flow technique have detected phenoxy radical formation from various substituted phenols during the H_2O_2 oxidation catalyzed by horseradish peroxidase. Furthermore, since phenoxy radical formation was not observed in the peroxidation of *p*-nitrophenol, these authors have advanced the hypothesis that the phenoxy radical formation occurs independently of the molecular sizes of phenols, but is dependent on their redox potentials.

Substituent effects in aromatic molecules upon rates of reaction and equilibrium constants are generally interpreted by using the Eqn (4), known as the Hammett equation [7]:

$$\log (k/k_0) = \sigma\varrho \qquad (4)$$

where k and k_0 are rate or equilibrium constants for reactions of the substituted and the unsubstituted compounds, respectively; σ is the substituent constant, which depends solely on the nature and position of the substituent, and ϱ is the reaction constant, which depends on the reaction, the conditions under which it takes place and the nature of the side-chain at which it occurs. The validity of Eqn (4) is restricted to substituents in the *meta* and the *para* positions of the benzene ring. Therefore, we planned to study the effects of *para* and *meta* substituents in phenol and aniline, on the reduction rate of compound I.

EXPERIMENTAL PROCEDURE

Materials

Horseradish peroxidase (lot 7394427) was purchased from Boehringer-Mannheim Corp. as an ammonium sulfate precipitate and was prepared for

This paper is no. 22 in a series.

Abbreviations. In equations, E represents horseradish peroxidase; E-I, horseradish peroxidase compound I; E-II, horseradish peroxidase compound II.

Enzyme. Peroxidase, donor: hydrogen-peroxide oxidoreductase (EC 1.11.1.7).

use by dialysis against water distilled five times. The purity of the enzyme prepared in this manner, as determined by the ratio of absorbances at 403 nm and 280 nm was 3.22. The concentration of peroxidase was determined spectrophotometrically at 403 nm using a molar absorption coefficient of 1.02×10^5 M^{-1} cm^{-1} [8]. The concentration of hydrogen peroxide was determined as described elsewhere [9]. Phenols and aromatic amines obtained from various sources (Sigma, BDH, Mallinckrodt, Fisher and Eastman Kodak), when reagent grade, were used without purification. Aniline and *m*-phenetidine were precipitated in ethyl ether with H_2SO_4, filtered and then recrystallized two or three times from a methanol/water solution. The water used for all solutions was distilled five times including two distillations from alkaline potassium permanganate.

Apparatus

The kinetic experiments were performed on a Gibson-Durrum stopped-flow spectrophotometer model D-110, equipped with a 2-cm cuvette, and mixing equal volumes of two solutions, the temperature was maintained at 27 $\pm$ 0.1 °C by circulating thermostated water. Usually, eight individual determinations of the rate constant were performed. The reaction was followed at the isosbestic wavelength between compound II and the native enzyme, that is 410.5 nm at pH 7.0. The absorbance measurements were performed on a Cary 14 spectrophotometer.

Methods

For the stopped-flow experiments one drive syringe contained the aromatic substrate and buffer (phosphate buffer, ionic strength 0.02) and the second drive syringe contained compound I. Both syringes contained potassium nitrate (0.1 M). The solution of compound I was prepared just prior to the kinetic experiment by adding to a peroxidase solution one molar equivalent of hydrogen peroxide. The two storage syringes were masked with opaque tape to avoid the photochemical conversion of compound I [10] and the photochemical oxidation of substrates (especially the amines).

When possible substrate concentrations in at least 10-fold excess of the compound I concentration (0.6 μM) were used to maintain pseudo-first-order conditions. The first-order kinetics were described by Eqn (5)

$$-\frac{d\,[E\text{-}I]}{dt} = k_{obs}\,[E\text{-}I] \qquad (5)$$

where k_{obs}, the pseudo-first-order rate constant including the constant concentration of the substrate, was determined as described by Roman *et al.* [11].

When the reactions were too fast to be studied under pseudo-first-order conditions, integrated rate expressions (6–8) for Reaction (2) were used.

$$\left| \ln \frac{[E\text{-}I]_0}{[AH_2]_0} \cdot \frac{[AH_2]_0 - [E\text{-}I]_t}{[E\text{-}I]_0 - [E\text{-}I]_t} \right| = kt([AH_2]_0 - [E\text{-}I]_0) = k_{obs}t \qquad (6)$$

when $[E\text{-}I]_0 < [AH_2]_0$.

$$\left| \ln \frac{[AH_2]_0}{[E\text{-}I]_0} \cdot \frac{[E\text{-}I]_0 - [E\text{-}I]_t}{[AH_2]_0 - [E\text{-}I]_t} \right| = kt([E\text{-}I]_0 - [AH_2]_0) = k_{obs}t \qquad (7)$$

when $[E\text{-}I]_0 > [AH_2]_0$ and

$$\frac{1}{[E\text{-}I]_t} = kt + \frac{1}{[E\text{-}I]_0} \qquad (8)$$

when $[E\text{-}I]_0 = [AH_2]_0$, where $[E\text{-}I]_0$ and $[E\text{-}I]_t$ are the concentrations of compound I at time zero and t. $[AH_2]_0$ is the concentration of substrate at time zero.

RESULTS

Experimental determinations of the rate constant using Eqns (6) and (7) for second-order kinetics are shown in Fig. 1 A and B. Fig. 2 shows an experimental result under pseudo-first-order conditions. The Hammett plots (at pH 7.0) for the phenols and the aromatic amines are shown in Fig. 3 and 4. The values of k_2 which define the plots of Fig. 3 and 4 are assembled in Table 1.

In order to ascertain that this result is primarily due to substituent effects in the substrate molecules, the rate measurements were also performed at two other pH values. As can be seen in Table 2, the values of k_2 obtained at pH 6.38 and pH 7.80 are nearly identical to those obtained at pH 7.0 listed in Table 1.

As expected, increasing σ (which results in a decrease in electron density) decreases the value of the reduction rate constant of compound I (oxidation rate constant of phenol or amine). All of the phenols and amines studied (except *p*-aminophenol) can only exchange one electron with compound I. This is also true of resorcinol, since the formation of a quinone is impossible.

p-Aminophenol

This substrate can exchange two electrons with compound I. This is shown in Fig. 5, since the absorption spectra of the equilibrium mixtures obtained at pH 7.0 after mixing compound I (7 μM) and *p*-aminophenol (3.5 μM or 1.75 μM) are assignable to mixtures composed mainly of native enzyme and compound I.

The kinetics of the reaction, followed at 427 nm (isosbestic wavelength between peroxidase and compound I), (Fig. 6) shows that the reaction between

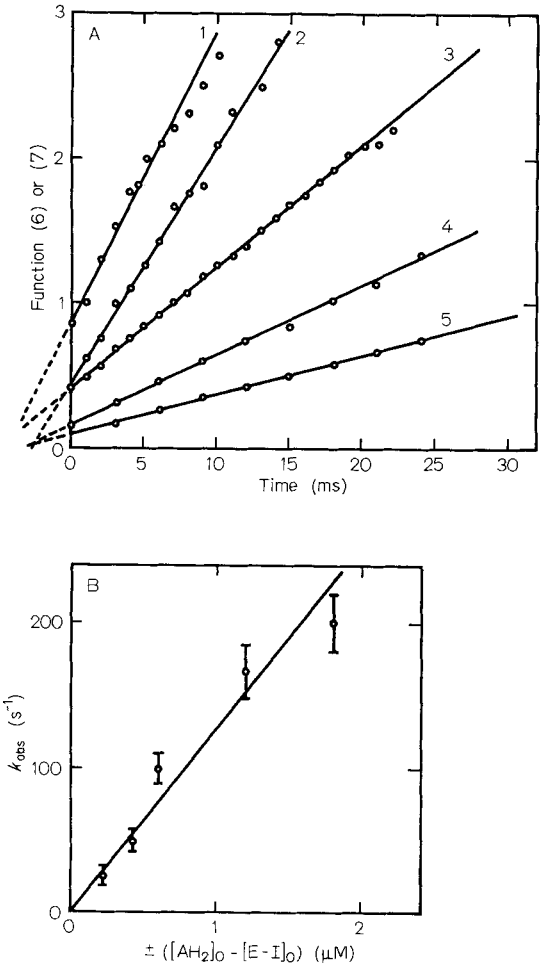

Fig. 1. *(A) Application of Eqns (6) and (7) to analyse the voltage changes observed after the rapid mixing of a solution of compound I (1.2 μM)) and a solution of* p-*methoxyphenol at various concentrations. (B) Determination of the second-order rate constant corresponding to the reaction between compound I and* p-*methoxyphenol.* The experimental conditions are described in Methods. (A) *p*-Methoxyphenol concentration: (1) 4.8 μM, (2) 3.62 μM, (3) 2.42 μM, (4) 0.30 μM, (5) 0.754 μM. The plots are linear over 3 or 4 half-lives. (----) Part of the reactions included in the dead-time of the stopped-flow apparatus (3 ms)

$$\text{Function (6)} = \ln\left(\frac{[\text{E-I}]_0}{[\text{AH}_2]_0}\,\frac{[\text{AH}_2]_0 - [\text{E-I}]_t}{[\text{E-I}]_0 - [\text{E-I}]_t}\right)$$

$$\text{Function (7)} = \ln\left(\frac{[\text{AH}_2]_0}{[\text{E-I}]_0}\,\frac{[\text{E-I}]_0 - [\text{E-I}]_t}{[\text{AH}_2]_0 - [\text{E-I}]_t}\right)$$

(B) k_{obs} is either $k([AH_2]_0 - [E\text{-}I]_0)$ or $k([E\text{-}I]_0 - [AH_2]_0$

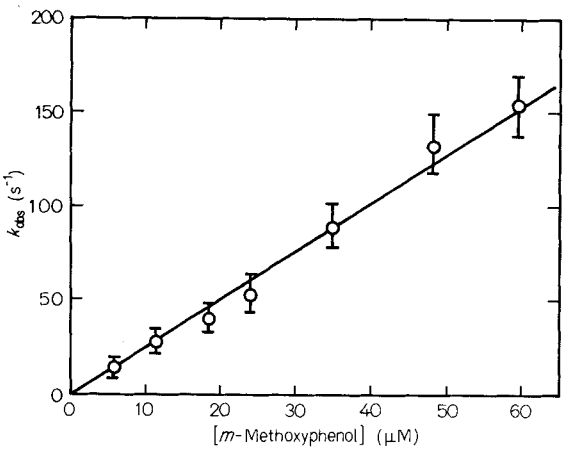

Fig. 2. *Example of linear-order plot obtained under pseudo-first-order conditions: the reaction between compound I and* p-*methoxyphenol at pH 7.0 and 27 °C*

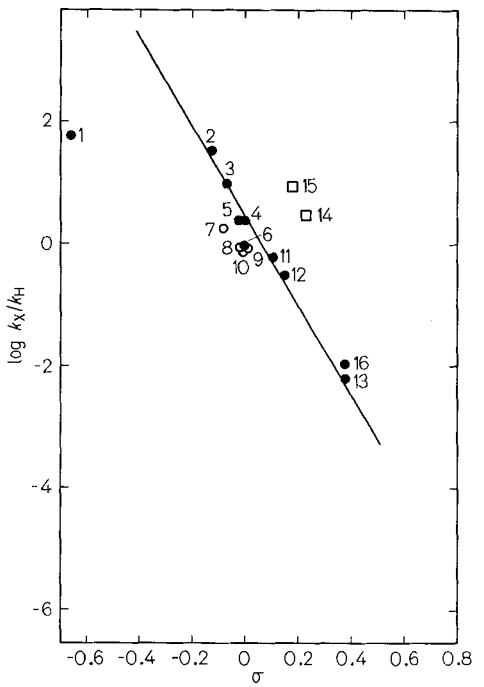

Fig. 3. *Hammett plot for the rate constants of compound I reduction by various substituted phenols at pH 7.0 and 27 °C.* The rate constants and the σ values are those listed in Table 1. Phenols: (1) *p*-NH_2; (2) *p*-OCH_3; (3) *p*-CH_3; (4) *m*-OH; (5) *m*-CH_3; (6) H; (7) 3,4-$(CH_3)_2$; (8) 3,5-$(CH_3)_2$; (9) 3-OH,5-CH_3; (10) 3-C_2H_5,5-CH_3; (11) *m*-OCH_3; (12) *m*-OC_2H_5; (13) *p*-SO_3^-; (14) *p*-Cl; (15) 3-CH_3, 4-Cl; (16) *m*-CHO

compound I and *p*-aminophenol proceeds in two different steps.

(a) The first step is fast (half-life < 2 ms) causing a decrease in the transmittance corresponding to compound II formation.

(b) The second step is slower (half-life ≈ 10 ms) and an increase in the transmittance suggests in this case the transition from compound I to the ferric enzyme.

The transient appearance of compound II during the reaction between compound I and *p*-aminophenol makes it possible to relate the rate constant determined in this particular case to those rate constants determined for all other phenols and amines which only can exchange one electron.

It was also observed that at pH 10.5 the reaction between compound I and *p*-aminophenol gives compound II. In the case of *p*-nitrophenol no reaction was

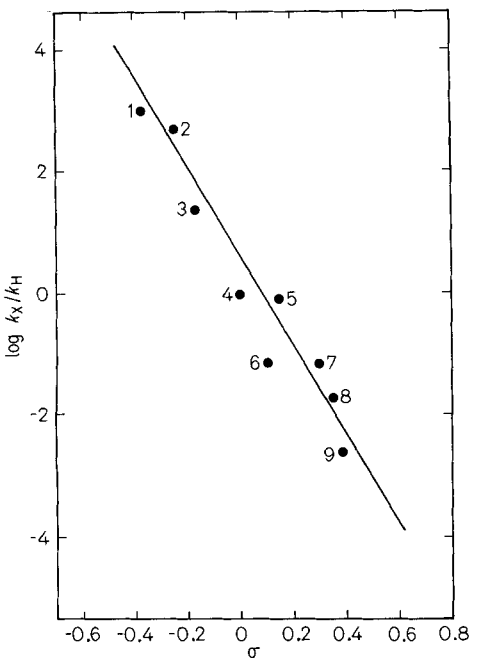

Fig. 4. *Hammett plot for the rate constants of compound I reduction by various substituted anilines at pH 7.0 and 27 °C.* The rate constants and the σ values are those listed in Table 1. Anilines: (1) *p*-OH; (2) *p*-OC_2H_5; (3) *p*-CH_3; (4) H; (5) *m*-OC_2H_5; (5) *m*-COO^-; (7) *m*-$COCH_3$; (8) *p*-COO^-; (9) *p*-SO_3^-

detected, in agreement with electron spin resonance experiments of Shiga and Imaizumi [6].

DISCUSSION

Meta *or* Para *Monosubstituted Derivatives*

As can be seen in Fig. 3 and 4, Hammett's rules apply for compound I reduction by monosubstituted phenols and anilines with different substituents in the *meta* or *para* positions.

At pH 7.0, using the treatment given by Jaffé [12] and van Bekklum *et al.* [16], the following relations are obtained

$$\text{phenols} \quad \log k_X/k_H = -6.92\,\sigma \tag{9}$$

$$\text{anilines} \quad \log k_X/k_H = -7.00\,\sigma \tag{10}$$

where k_H and k_X are the second-order rate constants for the reaction between compound I and an unsubstituted phenol or an aromatic amine, and with the substituent X in the *meta* or *para* positions. The correlation coefficients are 0.97 and 0.94 respectively for phenols and anilines plots. As can be seen on Fig. 3 the value of the slope obtained for the phenols ($\varrho = -6.92$) allows us to explain why there is no

Table 1. *Values of* k_2 *at pH 7.0 which define the Hammett plots of Fig. 3 and 4*

Substance	k_2	Concentration	σ	Reference source for σ
	$M^{-1}\,s^{-1}$	M		
Phenols				
p-NH_2	(2.26 ± 0.40) 10^8	(1.2–6) 10^{-7}	−0.66	[7,12,15]
p-OCH_3	(1.30 ± 0.20) 10^8	(0.3–2.5) 10^{-6}	−0.13	[14]
p-CH_3 [a]	(4.20 ± 0.20) 10^7		−0.076	[13]
m-CH_3	(7.88 ± 0.90) 10^6	(0.5–3.5) 10^{-5}	−0.007	[13]
m-OH	(8.00 ± 0.90) 10^6	(0.7–5.5) 10^{-5}	−0.002	[12]
H	(2.76 ± 0.30) 10^6	(0.6–8) 10^{-5}	0	
m-OCH_3	(2.44 ± 0.28) 10^6	(0.6–6) 10^{-5}	0.115	[7,12,15]
m-OC_2H_5	(1.25 ± 0.15) 10^6	(0.6–6) 10^{-5}	0.15	[7,12,15]
p-SO_3^-	(1.97 ± 0.25) 10^4	(0.2–2) 10^{-3}	0.38	[12]
m-CHO	(4.15 ± 0.35) 10^4	(0.2–1.5) 10^{-3}	0.381	[7]
3,4-$(CH_3)_2$	(6.85 ± 0.75) 10^6	(0.6–3.5) 10^{-5}	−0.083	[13]
3,5-$(CH_3)_2$	(2.90 ± 0.20) 10^6	(0.6–2.5) 10^{-5}	−0.014	[13]
3-OH,5-CH_3	(3.58 ± 0.33) 10^6	(1–6) 10^{-5}	−0.009	[12,13]
p-Cl	(1.13 ± 0.16) 10^7	(0.6–3.5) 10^{-5}	0.227	[7,12,15]
4-Cl,3-CH_3	(3.52 ± 0.90) 10^7	(0.6–2.5) 10^{-5}	0.22	[7,13]
Anilines				
p-OH	(2.26 ± 0.40) 10^8	(1.2–6) 10^{-7}	−0.357	[12]
p-OC_2H_5	(1.24 ± 0.20) 10^8	(0.2–2.4) 10^{-6}	−0.25	[12,14]
p-CH_3	(5.25 ± 0.30) 10^6	(0.6–3) 10^{-5}	−0.17	[7,12]
H	(2.43 ± 0.12) 10^5	(0.2–1) 10^{-3}	0	
m-COO^-	(1.93 ± 0.30) 10^4	(0.5–1.6) 10^{-3}	0.103	[12]
m-$COCH_3$	(1.74 ± 0.20) 10^4	(0.3–1.5) 10^{-3}	0.306	[7,12]
p-COO^- [b]	(4.70 ± 0.10) 10^3		0.35	[12]
p-SO_3^-	(3.90 ± 0.60) 10^2	(0.4–1.5) 10^{-3}	0.38	[12]
m-OC_2H_5	(2.36 ± 0.36) 10^5	(1–6.5) 10^{-4}	0.15	[12,14]

[a] Values reported in [4a].
[b] Values reported in [5].

reaction between compound I and *p*-nitrophenol ($\sigma = +1.27$).

The diffusion-controlled limit rule for bimolecular reactions [17] can be used to show that the neutral (unionized) forms of the more rapidly reacting phenols are the reactive species.

Adherence to the Hammett rule for *meta* or *para* monosubstituted phenols indicates that all phenols are oxidized by compound I by the same mechanism and it therefore follows that in all cases the neutral forms of the phenols are reacting. The same situation applies for the *meta* or *para* monosubstituted anilines.

Briggs and Robinson [14] have reported relations for the ionization of phenols. When they are expressed in terms of basicity of the phenoxide ions they become

$$\log K_X/K_H = -2.229\,\sigma \qquad (11)$$

Table 2. *Reduction rate constant of horseradish peroxidase compound I (k_2) by various phenols and aromatic amines at pH 6.38 and 7.80*

Substance	k_2	
	pH 6.38	pH 7.80
	$M^{-1}\,s^{-1}$	
Phenols		
p-CH_3[a]	(4.2 ± 0.2) 10^7	(4.3 ± 0.20) 10^7
m-CH_3	(1.12 ± 0.08) 10^7	(9.33 ± 0.80) 10^6
m-OH	(1.03 ± 0.10) 10^7	(1.09 ± 0.15) 10^7
H	(2.75 ± 0.18) 10^6	(2.57 ± 0.28) 10^6
m-OCH_3	(2.62 ± 0.32) 10^6	(2.80 ± 0.20) 10^6
m-OC_2H_5	(1.49 ± 0.07) 10^6	(1.51 ± 0.10) 10^6
p-SO_3^-	(2.36 ± 0.35) 10^4	(1.92 ± 0.13) 10^4
m-CHO	(3.98 ± 0.28) 10^4	(3.75 ± 0.20) 10^4
3,5-$(CH_3)_2$	(2.96 ± 0.26) 10^6	(3.02 ± 0.22) 10^6
3-OH,5-CH_3	(3.31 ± 0.27) 10^6	(3.75 ± 0.22) 10^6
p-Cl	(1.20 ± 0.18) 10^7	(1.08 ± 0.3) 10^7
4-Cl,3-CH_3	(3.85 ± 0.87) 10^7	(3.46 ± 0.58) 10^7
Anilines		
H	(3.08 ± 0.20) 10^5	(1.91 ± 0.08) 10^5
m-$COCH_3$	(1.95 ± 0.20) 10^4	(1.37 ± 0.25) 10^4
p-COO^-[b]	(6.2 ± 0.20) 10^3	(4.3 ± 0.1) 10^3
m-COO^-	(1.91 ± 0.09) 10^4	(1.80 ± 0.10) 10^3
p-SO_3^-	(4.85 ± 0.15) 10^2	(3.40 ± 0.40) 10^2
m-OC_2H_5	(3.22 ± 0.40) 10^5	(4.05 ± 0.30) 10^5
p-CH_3	(6.43 ± 0.80) 10^6	(4.14 ± 0.40) 10^6

[a] Values reported in [4a].
[b] Values reported in [5].

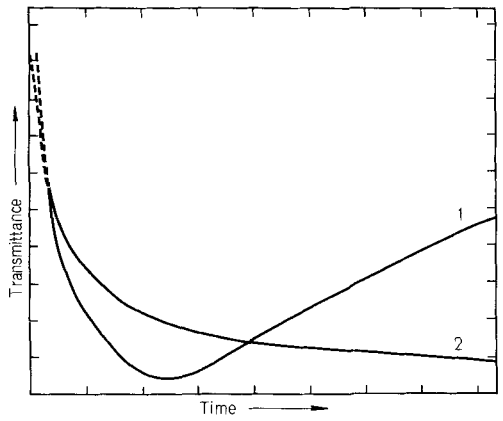

Fig. 6. *Stopped-flow oscilloscope traces of the reaction of compound I (0.6 μM) with* p-*aminophenol (0.55 μM) at pH 7.0.* Trace 1: wavelength 427 nm; ordinate: 100 mV/division; abscissa: 5 ms/division; RC time constant: 0.5 ms. Trace 2: Wavelength 410.5 nm; ordinate: 200 mV/division; abscissa: 5 ms/division; RC time constant: 0.5 ms

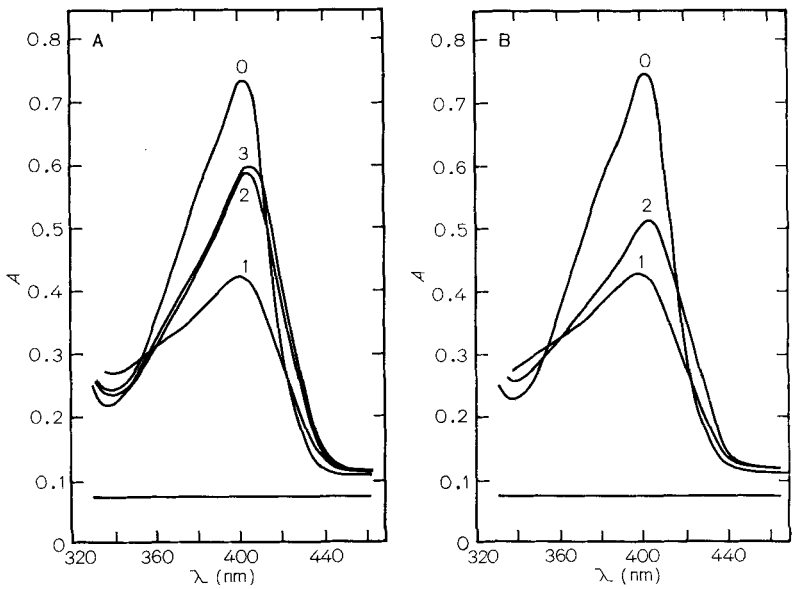

Fig. 5. *Spectra obtained after the reaction of compound I and* p-*aminophenol.* (A, B) (0) Spectrum of peroxidase (6.37 μM) at pH 7.0; (1) spectrum of compound I obtained after mixing peroxidase with H_2O_2 (7 μM). The H_2O base-lines are included. (A) (2) Spectrum obtained after mixing compound I with *p*-aminophenol (3.5 μM); (3) same as (2) but 5 min later. (B) (2) Spectrum obtained after mixing compound I with *p*-aminophenol (1.75 μM)

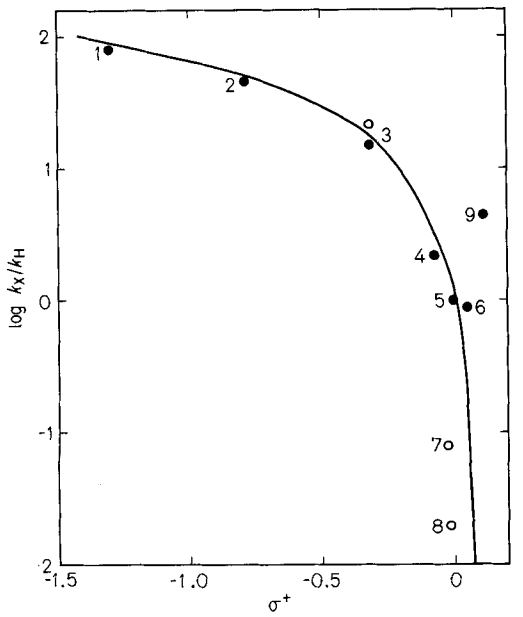

Fig. 7. *Okamoto-Brown plot for the rate constants of compound I reduction by various substituted phenols (●) and anilines (○) at pH 7.0 and 27 °C.* The rate constants are those listed in Table 1 and the σ^+ values are those given by Brown and Okamoto [18]. The line connecting the data points has no theoretical significance. Substituent: (1) *p*-NH_2; (2) *p*-OCH_3; (3) *p*-CH_3; (4) *m*-CH_3; (5) H; (6) *m*-OCH_3; (7) *m*-COO^-; (8) *p*-COO^-; (9) *p*-Cl

and for the basicity of the anilines

$$\log K_X/K_H = -2.889\,\sigma. \qquad (12)$$

Thus, *meta* or *para* substituents produce nearly the same effect upon the addition of a proton to the O^- or the NH_2 group. One can also see from Eqns (9) and (10) that *meta* or *para* substituents produce nearly the same effect upon the oxidation rate by compound I of phenols and anilines. This suggests that the rate-determining step in the oxidation of a phenol or an aromatic amine by compound I is the removal of one electron. This agrees with the results of Shiga and Imaizumi [6] showing the formation of phenoxy radicals during the peroxidation of various substituted phenols. Removing one electron from phenols or anilines by compound I would generate a positive charge in the substrate molecule. Furthermore, reactions involving generation of positive charge capable of resonance interaction with a substituent have been shown to follow the Okamoto-Brown equation (13) [18,19]

$$\log k_X/k_H = \varrho\,\sigma^+. \qquad (13)$$

Nevertheless, we observed that plotting the rate constants given in Table 1 in the form of Eqn (13) does not lead to a linear correlation (Fig. 7). An explanation of our good correlation with the σ Hammett values could be found in a mechanism in which the neutral substrate molecule gives an electron to compound I thus forming a free-radical and simultaneously loses a proton, avoiding the formation of a positive charge. Saunders and Mann [20] in 1940 suggested such a mechanism, based on a different line of reasoning. One explanation for a non-linear plot of the type presented in Fig. 7 is that the rate constants approach the diffusion-controlled limit [21]. However, deviation from a straight line in Fig. 7 occurs for rate constant values as small as $10^5\ M^{-1}\ s^{-1}$, which are far too small to be attributed to diffusion-controlled rate constants [22].

Most of the monophenol (and resorcinol) oxidations are irreversible because of the existence of further fast reactions involving the phenoxy radicals, and therefore thermodynamic redox potentials for such systems cannot be determined experimentally. Nevertheless, in 1930 Fieser [23] introduced the concept of 'critical oxidation potential' or 'apparent oxidation potential' E_c of phenols and showed that in aqueous solutions this critical potential E_c is related to the normal oxidation potential by:

$$E_0 = E_c + 0.136\ V\ \text{(monophenols)}. \qquad (14)$$

Moreover, Cook *et al.* [24] have observed that a rough but significant correlation exists between Hammett's σ-values and Fieser's critical potentials. Plotting the data of Fieser [23] in the form E_c *vs* σ gives:

$$E_c = 0.45\,\sigma + 1.089. \qquad (15)$$

The existence of Eqns (15) and (9) supports the conclusions of Shiga and Imaizumi [6], because if the phenoxy radical formation occurs dependently on the redox potentials of phenols, it should be possible to relate both their oxidation potentials and their rate constants for oxidation by compound I with Hammett rules.

Furthermore, Fieser [23] has attempted to relate critical potentials of phenols and aromatic amines by the relation

$$E_c^{\text{phenol-X}} = E_c^{\text{amines-X}} - 0.044 \qquad (16)$$

where phenol-X and amine-X are respectively the phenol and the corresponding aromatic amine carrying the same substituent X. Eqn (16) implies two important deductions.

(a) If the rate-determining step for the oxidation of phenols and aromatic amines is the removal of one electron, the ϱ values obtained from the two Hammett plots of Fig. 3 and 4 should be identical.

(b) All values of the rate constant k_2 obtained with aromatic amines should be lower than those obtained for the corresponding phenols, since aromatic amines become oxidized only at a higher potential than that required for the corresponding phenols. One can see from Eqns (9) and (10) and from Tables 1 and 2 that these two effects are observed.

It should be noted that the gas-phase ionization potential of aniline is lower than that of phenol [25] in contrast to the ease of oxidation observed in aqueous solution. Furthermore, the oxidation potentials of anilines in non-aqueous media correlate with the σ^+ function [26]. These suggest that in the gas-phase or in an aprotic solvent, the step in which ionization occurs does not include loss of a proton. (The proton could be lost in a subsequent step.)

We now attempt to discuss the numerical values of the slopes in Eqns (9) and (10). The relative ease of oxidation of phenols to phenoxy radicals has been estimated from relative oxidation potentials corresponding to the half-wave potentials determined by polarographic methods [27,28]. However, since it was shown [27,29,30] that polarographic half-wave potentials for reversible systems are comparable, at least in their sequence, with the thermodynamic redox potential, it is possible to use half-wave oxidation potentials ($E_{1/2}$) of irreversible phenol oxidations, as a measure of phenol oxidizability. It was found that for phenols carrying substituents X in *para* position [31]

$$E_{1/2}^{\text{phenol-X}} = 0.52\,\sigma + 0.53 \qquad (17)$$

and for anilines carrying a substituent X in *para* or *meta* positions [32].

$$E_{1/2}^{\text{amine-X}} = 0.50\,\sigma + 0.78 \qquad (18)$$

which is in good agreement with the results obtained by Fieser [23] [Eqns (15) and (16)]. Bezuglyi and Beilis [32] have in fact reported the following relation

$$E_{1/2}^{\text{amine-X}} = 0.40\,\sigma + 0.72$$

for *meta* monosubstituted anilines. Including the $E_{1/2}$ values of the *para* derivatives (with the exceptions of *p*-aminophenol and *p*-phenyleneamine) found by these authors, in the $E_{1/2}$ *vs* σ plot, leads to the rough but significant Eqn (18).

In the case of irreversible systems the correlation between rate constants and potentials of polarographic oxidations has been expressed as

$$E_{1/2}^{X} - E_{1/2}^{H} = -\frac{RT}{\alpha n F} \ln \frac{k_X}{k_H} \qquad (19)$$

where R is the gas constant, T is the absolute temperature, α is the transfer coefficient of the reaction, n is the number of electrons transferred in the process, F is the Faraday, k_X is the rate constant of the process related to the phenol (or amine) carrying the substituent X and k_H is the rate constant for the unsubstituted phenol (or amine).

Comparing (19) and (17) or (18) gives:

$$\ln k_X = -\left(\varrho \frac{\alpha n F}{RT}\right) \sigma + \ln k_H. \qquad (20)$$

Comparing now (20) with (9) or (10) gives

$$n\alpha^{\text{phenols}} = 0.78$$

$$n\alpha^{\text{amines}} = 0.82$$

using $\varrho = 0.52$ for the phenols and $\varrho = 0.50$ for the amines. These calculations are in good agreement with the values reported by Bezuglyi and Beilis [32]

$$0.71 < n\alpha^{\text{amines}} \leq 1.18$$

and by Nash *et al.* [31]

$$0.81 < n\alpha^{\text{phenols}} < 1.15$$

calculating the $n\alpha$ values directly by the logarithmic analysis of the shape of the wave.

In conclusion there are many arguments favoring the idea that the reaction between compound I and *para* or *meta* substituted phenols or aromatic amines is only dependent of their relative ease of oxidation. The plots presented in Fig. 3 and 4 should allow us to know whether the removal of the first electron of *p*-aminophenol corresponds to its reaction as a phenol or an aromatic amine. Although the value obtained for the rate constant seems to fit better with the plot obtained with aromatic amines than the plot obtained with phenols, the possibility cannot be excluded that the reaction of *p*-aminophenol was diffusion controlled, and thus the value of $\log (k_{p\text{-NH}_2}/k_H)$ was smaller than that predicted by the slope of the phenol plot.

As can be seen in Fig. 3, the values of the rate constants obtained for the chloro derivatives of phenols are greater than those expected from the positive σ values. In fact, in a compilation by Zuman [33] of 91 reactions which obey the Hammett rule, 18 of them present deviations in the case of halogen derivatives. It has been shown [33] that these deviations are often accompanied by changes in the value of the transfer coefficient, and the validity of Eqn (20) implies a constant value for the transfer coefficient. Nevertheless, 3-methyl-4-chlorophenol reacted more rapidly with compound I than the *p*-chlorophenol, which was expected from the σ values.

Although the σ values are usually reported to be additives for multisubstituted derivatives [12,13], a systematic deviation occurs with all disubstituted phenols in which lower rate constants than expected from the σ values are obtained. Thus, 3,4-dimethylphenol reduces compound I more slowly than does *p*-cresol, while 3,5-dimethylphenol, 3-ethyl-5-methylphenol and orcinol reduce compound I more slowly than do *m*-cresol and resorcinol. Perhaps this could arise because of different steric interactions between the disubstituted phenols and enzyme. Such an effect has been recently observed by Baker and Saunders [34].

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