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The Catalytic Role of a Flavin Analogue for Aromatic Hydroxylation with the Udenfriend System

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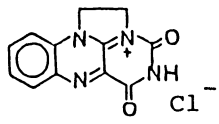
A new combination of the Udenfriend reagent and a flavin analogue was found to hydroxylate benzene to phenol. Effects of temperature and concentrations of reaction components, i.e., iron(II), flavin, and ascorbate, on the phenol yield were examined. In particular, decreasing ascorbate concentration increased the yield of phenol. Thus, 68% of the yield was obtained under better conditions. The mechanism of action of ascorbic acid and the flavin was discussed.

The Udenfriend system (Fe^{2+}/EDTA/ascorbate/O_2),[1] a model peroxidase, is a general method for the addition of a hydroxyl group into an aromatic substrate. Grinstead has examined the hydroxylation of benzoic and salicylic acids and has suggested a chain mechanism in which a hydroxyl radical is the reactive species that takes part in hydroxylation.[2] The reaction is relatively mild compared to other methods,[3] and the products obtained with the Udenfriend system in vitro are identical with that from the same compound in vivo. From a biomimetic or mechanistic viewpoint, the Udenfriend and related systems are still worthy of further investigations.[4]

Nevertheless, these systems usually afford only 3 to 25% yields depending on the substrate, the metal ion, and the complexing agent. Particularly, the hydroxylation of benzene gave rise to only less than 5% yield of phenol even under better conditions. This suggests that the Udenfriend system itself has yet a critical disadvantage of electron accepting/transferring relay in the catalytic cycle.

We report here that the 1,10-ethanoisoalloxazinium cation (a flavin analogue **1**)[5] markedly enhances the catalytic efficiency of the Udenfriend system. In this work, benzene was selected as a diagnostic substrate and for ease of the quantitative analysis of the product phenol.



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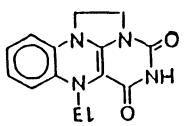

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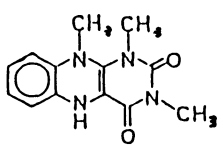

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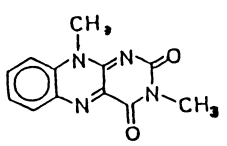

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Experimental

Materials. EDTA, ascorbic acid, 1,10-phenanthroline, iron(II) sulfate were purchased from commercial suppliers and used without further purification. Flavins (**1**—**4**) were prepared by literature procedures.[6]

Hydroxylation. The standard hydroxylation was performed in a benzene (1 ml)–water (50 ml) two-phase system, containing 2 mM (1 M=1 mmol dm^{-3}) of Fe^{2+}, 2 mM of **1**, and 20 mM of ascorbic acid, at 50 °C under constant bubbling of oxygen through two needles with mechanical shaking. The pH of the reaction medium was initially adjusted to 3.0 with H_2SO_4. As the rate of the reaction is slow compared to dissolution of oxygen, the solution was assumed to be saturated with oxygen at all times. Benzene in excess of saturation was employed to keep the benzene concentration in the aqueous layer constant. The amount of phenol produced was determined with HPLC by using anisole as an internal standard as described previously.[7] All the percent yields are based on the added amount of ascorbate. Under the standard conditions (90 min), the yield was 31.0%. The results thus obtained are summarized in Figs. 1—4 and Tables 1—3. Unlike the original Fenton

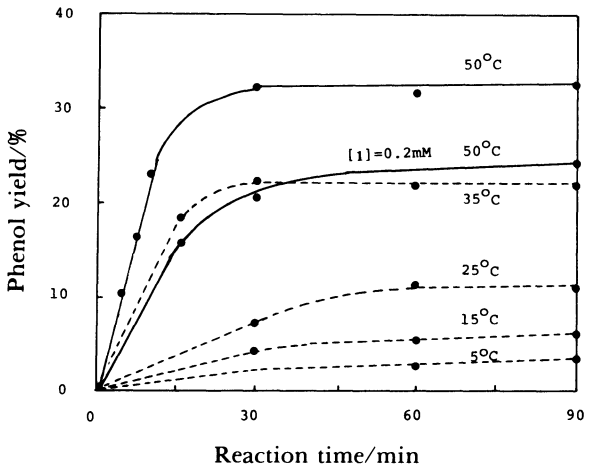


Fig. 1. Time courses of hydroxylation with Fe^{2+}/**1**/AsH_2/O_2.
Conditions: Fe^{2+}(2 mM)/**1**(2 mM)/AsH_2(20 mM) under O_2 at pH 3.

system, no biphenyl was detected by HPLC analysis in any cases. However, 10% of hydroquinone was produced.

Kinetics. The rate of reaction of **1** with ascorbic acid under N_2 at 25 °C was followed by the disappearance of the absorption peak due to **1** (λ_{max} 365 nm; ε 13500 in H_2O). Typical UV traces are shown in Fig. 5.

Results and Discussion

Figure 1 depicts the time plots for the benzene hydroxylation with the Fe^{2+}/**1**/ascorbic acid/O_2 system at various temperatures. The reaction is strongly temperature-dependent, and the phenol yield increases steadily with reaction time and reaches a limiting value at each reaction temperature. Table 1 compares the effect of changing structures of flavins on the yield under the standard conditions. The phenol yield for unblocked flavin **4** is only 1.1% and as poor as that for the control reaction in the absence of any flavins. On the other hand, flavin **1**, blocked with an ethano bridge at N-1 and N-10, afforded more than 30% yield. Further blocking at N-5 position (**2**) is far less effective than is the blocking at N-1 and N-10. Hemmerich and Massey[8] have studied the flavin-dependent molecular oxygen activation and have concluded that: (1) the more planar is the structure of a flavin molecule, the greater the reactivity toward O_2, (2) the substitution of 5-position with an alkyl group lowers the catalytic activity. On the basis of these criteria, the catalytic activity is expected to be in the order, **1**>**2**>**3**, just as was observed here.

A mechanism shown in Scheme 1 can be tentatively proposed for this reaction on the basis of this and other research. There are two catalytic cycles in the sequence; one is for the generation of hydrogen peroxide (Reactions 1, 2), the other for the production of phenol (Reactions 3—6). Although this mechanism is basically analogous to that previously reported for the nonclassical Fenton reaction (Fe^{3+}/MPH/O_2/NADH system),[9] the role of ascorbic acid (AsH_2) is somewhat different from that of NADH which has been proposed to reduce solely 5-methylphenazinium cation (MP^+). The details will be discussed later. In this mechanism, we presume the catalytic function of a flavin as follows; its fully reduced form (Fl_{red}) would be involved in formation of H_2O_2 from molecular oxygen via Reaction 2, while the oxidized form (Fl_{ox}) would be involved in phenol formation via Reaction 6 in which Fl_{ox} is converted to Fl_{sem} by abstracting one electron from the 1-hydroxycyclohexadienyl radical (**5**). The carbon cation thus formed is converted into phenol immediately after its formation (Reaction 6).[10] The Fl_{ox} could be reconverted to Fl_{red} either via disproportionation of Fl_{sem} (Reaction 7) or reduction with ascorbic acid (Reaction 1).

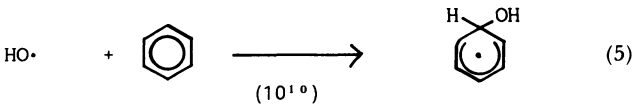
Thus, the lack of catalytic activity of unblocked flavin **4** is explained by its lower redox potential than that of ascorbic acid; hence, Reaction 1 is substantially endothermic. An altenative explanation may be possible. Reaction 6 would be also too endothermic to propagate the radical chain reaction, for the redox potential of **4** (Fl_{ox}) was estimated to be much lower than that of the hexadienyl radical.[7]

$$Fl_{ox} + AsH_2 \underset{(0.5)}{\overset{-H^+}{\rightleftharpoons}} Fl_{red}\cdot + dAs \quad (1)$$

$$Fl_{red} + O_2 \xrightarrow[(10^3)^{6)}]{+H^+} Fl_{ox} + H_2O_2 \quad (2)$$

$$2\ Fe(III) + AsH_2 \xrightarrow[(10^2)^{2)}]{-2\ H^+} 2\ Fe(II) + dAs \quad (3)$$

$$Fe(II) + H_2O_2 \xrightarrow[(50)^{13)}]{+H^+} Fe(III) + HO\cdot \quad (4)$$



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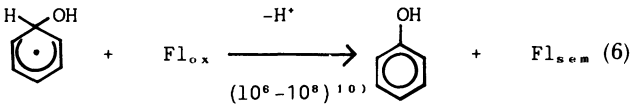


(6)

$$2\ Fl_{sem} \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} Fl_{ox} + Fl_{red} \quad (7)$$

Scheme 1. Figures in parentheses are the 2nd-order rate constants, $M^{-1}\,s^{-1}$, where a flavin is **1**.

Table 1. Hydroxylation of Benzene by Fe^{2+}–AsH_2–O_2 in the Presence of Various Flavin Analogs[a]

Flavin	Phenol yield/%[b]
None (Udenfriend's conditions)	1.1
1	31.9
2	7.1
3	16.2
4	1.8

a) Conditions: Fe^{2+}(2.0 mM)/flavin(2.0 mM)/AsH_2(20 mM); reaction pH 3, 50 °C under O_2 for 90 min. b) Phenol yield based on added AsH_2.

In order to obtain better yields of phenol and to establish the reaction mechanism further, studies of variation of pH and concentrations of reaction components such as iron(II), flavin **1**, and ascorbic acid, and of effect of coordinating additives were performed. As **1** was found to be the most effective of all flavins at pH 3 (Table 2), only **1** was used for

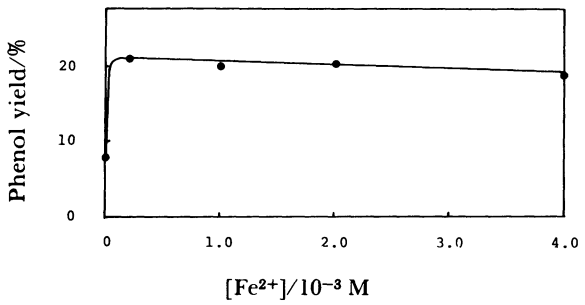


Fig. 2. Effect of Fe^{2+} concentration on phenol yield. Conditions: **1**(2 mM)/AsH_2(20 mM) under O_2 at pH 3 for 90 min.

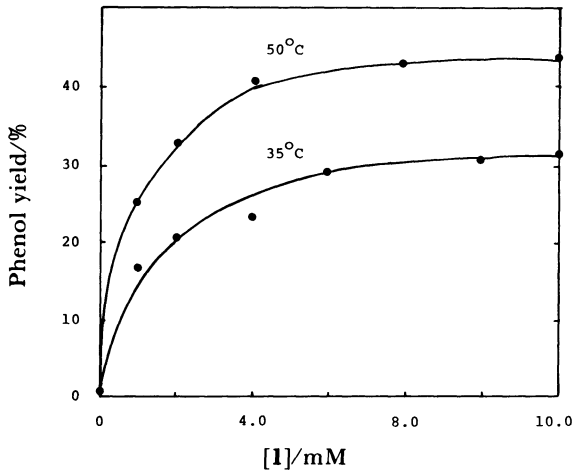


Fig. 3. Effect of concentration of **1** on phenol yield. Conditions: Fe^{2+}(2 mM)/AsH_2(20 mM) under O_2 for 90 min.

Table 2. Dependence of Phenol Yield on Reaction pH

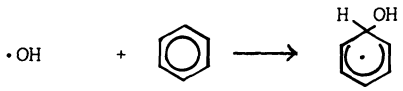
pH\Flavin	Yield/%		
	1	**2**	**3**
1	23.3	6.0	24.0
3	31.9	7.1	16.2
5	11.8	2.6	9.2

Conditions: Fe^{2+}(2.0 mM)/flavin(2.0 mM)/AsH_2 (20.0 mM) under O_2 at 50 °C for 90 min.

further investigations. The results are collected in Figs. 2 and 3. Figure 2 shows that the yield appears to be virtually independent of Fe^{2+} concentrations beyond 0.1 mM, indicating that neither the reduction (Eq. 3) nor the oxidation of metal ion (Eq. 4)[11] is rate-determining under the standard conditions. Moreover, the same Figure shows that even adventitious iron ion (no added Fe^{2+}), although less efficient, is capable of continuing the catalytic chain cycle. In these respects the present reaction follows a reaction pattern characteristic of the Fe^{3+}/MPH/O_2/NADH system.[9]

As can be seen from Table 3, the Fe^{2+}/**1**/H_2O_2 hydroxylating system, in which hydrogen peroxide is itself a reactant, is inhibited by addition of metal-complexing agents such as EDTA and 1,10-phenanthroline. Particularly, effect of EDTA is quite interesting. As was reported in the previous paper,[7] the mechanism for that hydroxylation is as indicated by the reaction sequence in Scheme 2. EDTA prevents the reaction of Fl_{red} (Fl_{sem}) with Fe^{3+} (Eq. 11) by markedly lowering the redox potential of Fe^{3+} below that of Fl_{red} (Fl_{sem}).

$$Fe(II) + H_2O_2 \underset{-H_2O}{\overset{+H^+}{\longrightarrow}} Fe(III) + \cdot OH \quad (8)$$



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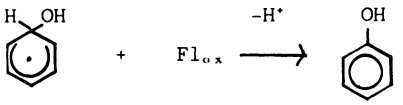


(10)

$$Fl_{red}(Fl_{sem}) + Fe(III) \longrightarrow Fl_{sem}(Fl_{ox}) + Fe(II) \quad (11)$$

$$2\,Fl_{sem} \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} Fl_{red} + Fl_{ox} \quad (12)$$

On the other hand, EDTA in the present system had a little or no inhibitory effect on the phenol production, indicating that ascorbate must be used for

Table 3. Effect of Metal Ion-Chelating Agents (pH 3; 50 °C)

Hydroxylating system	Fe^{2+}	**1**	% Phenol yield			
	mM	mM	None	EDTA[e]	DETAPAC[f]	Phen[g]
Present (a)	0.05	2.0	31.0	34.5	—	4.0
(b)	1.0	2.0	31.9	30.8	38.7	27.1
Udenfriend[c]	1.0	0.0	0.8	4.4	1.3	0.7
Fe^{2+}/**1**/H_2O_2[d]	1.0	2.0	41.0	7.3	6.5	4.8

a) Fe^{2+}/**1**/AsH_2(20 mM)/chelating agent(1 mM) under O_2 for 1.5 h. b) Fe^{2+}/**1**/AsH_2(20 mM)/chelating agent(2 mM) under O_2 for 1.5 h. c) No **1** was added. Fe^{2+}/AsH_2(20 mM)/chelating agent(2 mM) under O_2 for 1.5 h. d) Fe^{2+}/**1**/H_2O_2(1 mM)/chelating agent(2 mM) at 25 °C under N_2 for 2.5 h. e) Ethylenediaminetetraacetic acid. f) Diethylenetriaminepentaacetic acid. g) 1,10-Phenanthroline.

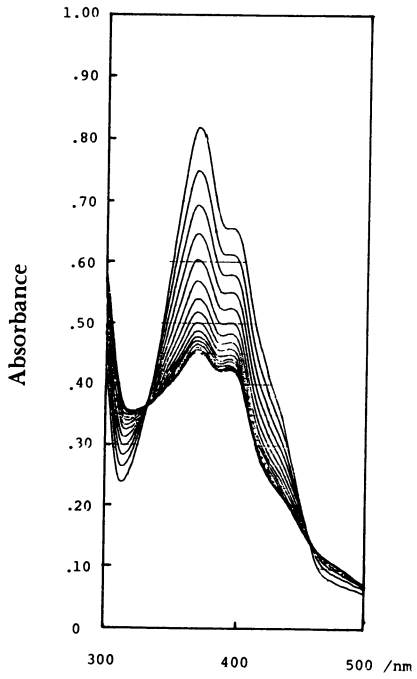


Fig. 4. UV traces for reaction of **1** with AsH_2 under N_2 at 25 °C at pH 3. The time interval is kept constant at 3 min. [**1**]=0.067 mM; $[AsH_2]_0$=2.68 mM.

reducing not only Fe^{3+} but also even the Fe(III)-EDTA chelate (Eq. 3). The Fe(II)-EDTA chelate have been well-known to react with H_2O_2 much more rapidly than the haxaaquairon(II) ion to produce reactive oxidants (Eq. 4).[11] As opposed to EDTA, 1,10-phenanthroline almost completely inhibited the production of phenol. This is probably because complexation of Fe^{3+} with 1,10-phenanthroline significantly stabilizes the Fe(II) rather than Fe(III) state and hence surpresses the generation of OH radical (Eq. 13).

$$\underset{E°(V)\ 1.03}{\text{Fe(II)-phen}} + H_2O_2 \not\longrightarrow \underset{0.72}{\text{Fe(III)-phen}} + HO\cdot \qquad (13)$$

Figure 3 illustrates the plots of the phenol yield as a function of varying concentrations of **1** over its concentration range 0—10 mM under the standard conditions, unless otherwise stated. With increase of the flavin concentrations, the yield increases steadily and approaches a limiting value of 32% at flavin concentrations greater than 4 mM. Similarly, comparison of time plots at 50 °C, shown as solid lines (a and b) in Fig. 1, reveals that the hydroxylation rate is proportional to the concentration of the catalyst flavin. These findings suggest again that either step associated with the formation of H_2O_2, i.e., Reaction 1 or 2, seems rate-determining. According to Bruice's calculation of the Gibbs free energy,[6] Reaction 2 is significantly exothermic as opposed to Reaction 1. Thus, the rate-determining step should be Reaction 1 rather than 2. Actually, this is evidenced further by

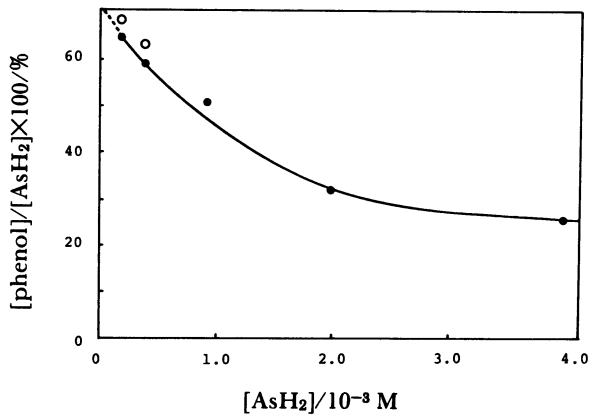


Fig. 5. Plots of phenyl yield vs. initial concentration of AsH_2. The yield is based on the initially added amount of AsH_2.
Conditions: (●) Fe^{2+}(2 mM)/**1**(2 mM); (○) Fe^{2+}(0.2 mM)/**1**(2 mM) under O_2 at pH 3 for 90 min.

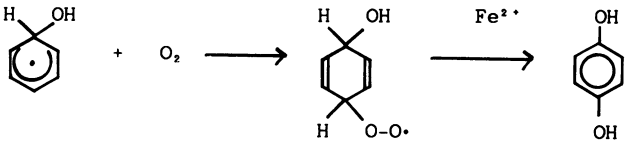
Fig. 4, which illustrates the kinetic feature of reduction of Fl_{ox} with ascorbic acid in a 40-fold excess under nitrogen. The absorbance at λ_{max} 365 nm due to **1** decreases and finally the equilibrium is attained; as would be expected, the decreased peak returns to the original peak as soon as air has been introduced into the solution. The apparent 2nd-order rate constant for the reduction of Fl_{ox} thus obtained is 0.5 $M^{-1}\,s^{-1}$. This figure shows that the reduction in question is the slowest of all the reactions in Scheme 1. It is quite probable, therefore, that Reaction 1 is the rate-limiting step of the hydroxylation.

Meanwhile, the effect of altering ascorbic acid concentrations is particularly suggestive (Fig. 5). The yield of phenol increased steadily as the concentration of ascorbic acid is decreased; namely, the eight-fold decrease of ascorbic acid increases the yield up to 67.7%. This means that the presence of too much ascorbic acid in solution is a major important factor diminishing the phenol yield, where side reactions such as Reactions 14 and 15 might participate.

$$AsH_2 + \cdot OH \longrightarrow AsH\cdot + H_2O \qquad (14)$$

$$AsH_2 + H_2O_2 \longrightarrow dAs + 2\,H_2O \qquad (15)$$

Another inevitable side reaction would be ascribed to the reaction of the 1-hydroxycyclohexadienyl radical itself. As have often been reported by many workers,[12] the cyclohexadienyl radical will be trapped by molecular oxygen to give eventually hydroquinone. In fact, 10.0% of hydroquinone was produced under the standard conditions.



In conclusion, we have found that certain flavins markedly improve the Udenfriend hydroxylation system; a flavin plays versatile roles as catalyst for accepting and transferring electron(s). Ascorbic acid is merely the reductant toward Fl_{ox} and Fe^{3+}.

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