Evidence for Hydroxyl Radicals as an Active Species Generated from Udenfriend's Reagent

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The inhibitory effect of six aliphatic alcohols, which are typical OH radical scavengers, on the yield of phenol in the oxidations of benzene with Fenton's ($Fe^{II} + H_2O_2$) and Udenfriend's (Fe^{II} + EDTA + O_2 + L-ascorbic acid) reagents has been examined. In both systems, these alcohols trap OH radicals effectively, as expected from the rate constants for the reactions of OH radical with benzene (k_B) and the alcohols (k_A), indicating that the active species generated from Udenfriend's reagent is OH radical. The Udenfriend oxidation of thymine in the presence of these alcohols gives further evidence for the generation of OH radical.

In 1954, Udenfriend and co-workers observed that a mixture of Fe^{II}, EDTA, ascorbic acid and dioxygen at neutral pH can hydroxylate aromatic compounds such as salicylic acid, thyramine and quinoline under mild conditions to yield products which are identical with those formed *in vivo*.[1,2] Despite a number of investigations,[3–6] the mechanism of hydroxylation by Udenfriend's and related reagents remains unresolved.[7] In one rationale, dioxygen is initially reduced with iron(II) ion to give H_2O_2, which then reacts with another iron(II) ion to generate hydroxyl radical (•OH), according to eqns. (1) and (2). Norman and Radda claimed that the active species

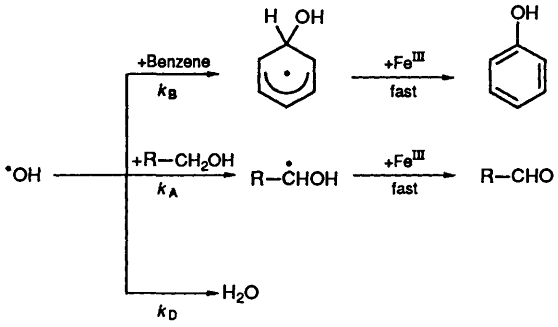
$$O_2 + 2Fe^{II} + 2H^+ = H_2O_2 + 2Fe^{III} \qquad (1)$$

$$H_2O_2 + Fe^{II} + H^+ = {}^{\bullet}OH + Fe^{III} + H_2O \qquad (2)$$

generated from Udenfriend's reagent is not hydroxyl radical, on the basis of the different isomer distributions obtained in the hydroxylation of anisole and chlorobenzene with Udenfriend's and Fenton's reagents.[8] Eberhardt pointed out that the distribution of three isomers in the oxidation of toluene with irradiated water varies markedly on changing the atmosphere from N_2 to O_2,[9] though the active species is undoubtedly hydroxyl radical. Since so-called 'activation' of dioxygen generates a variety of reactive species such as hydroxyl radical, superoxide anion ($O_2^{\bullet -}$), hydrogenperoxy radical ($HO_2^{\bullet}$), singlet oxygen and various oxo-metal complexes, it is difficult to determine unambiguously the active species generated from the hydroxylating agent in question. We have proposed a sophisticated kinetic method (which we named the 'OH-radical scavenging method') to identify the active species in question as a hydroxyl radical.[10] In the present study, the OH-radical scavenging method was applied to Fenton's and Udenfriend's systems.

Results and Discussion

Fenton Oxidation of Benzene.—Prior to the experiments on the Udenfriend oxidation, we confirmed, by means of 'the OH-radical scavenging method', that Fenton's reagent actually generates hydroxyl radicals according to reaction (2). In an oxidation which involves OH radical as an active species, a OH-scavenger such as methyl or ethyl alcohol should compete with benzene toward OH-radicals,[10,11] as illustrated in Scheme 1, and decrease the yield of phenol. The third reaction (k_D) in Scheme 1 is required to explain the yield of phenol being much less than 100% even in the absence of alcohols.† In Scheme 1, k_B and k_A are the rate constants for the reactions of hydroxyl radical with benzene and alcohol, respectively.



Scheme 1

Provided that the oxidation of benzene with hydroxyl radicals proceeds according to Scheme 1, the yield of phenol should be determined by five parameters, *i.e.* three rate constants (k_B, k_A, and k_D) and two concentrations ([Benzene] and [Alcohol]). Assuming that these concentrations are constant during the whole sequence of the Fenton oxidation, one can derive relationship (3) between the yield of phenol and

$$\frac{[PhOH]_0}{[PhOH]} = 1 + \frac{k_D}{k_B[Benzene]} + \frac{k_A[Alcohol]}{k_B[Benzene]} \qquad (3)$$

the concentration of the alcohol added where $[PhOH]_0$ and [PhOH] stand for the theoretical and observed concentrations of phenol, respectively. In the Fenton oxidation of benzene, $[PhOH]_0$ is equal to the concentration of hydrogen peroxide added. Under the present reaction conditions at 10 mmol dm^{-3} of iron(II) ions, the experimental value of $[PhOH]/[PhOH]_0$ was 0.69 ± 0.02 in the absence of alcohols.

As shown in Fig. 1, the plots of $[PhOH]_0/[PhOH]$ against the initial concentration of the six alcohols (methyl, ethyl, propyl, isopropyl, isobutyl, and *tert*-butyl alcohols) gave straight lines with a constant intercept of 1.45 ± 0.03, in accordance with eqn. (3). The slopes in Fig. 1 are a measure of the inhibiting effect of the alcohols and correspond to k_A/k_B[Benzene] in eqn. (3). Accordingly, one can obtain the relative rate constant k_A/k_B from the numerical values of the

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† Possible deactivation reactions of hydroxyl radicals are those with Fe^{II} ions and with phenol. k_D stands for the sum of the pseudo-first order rate constants for these reactions.[12]

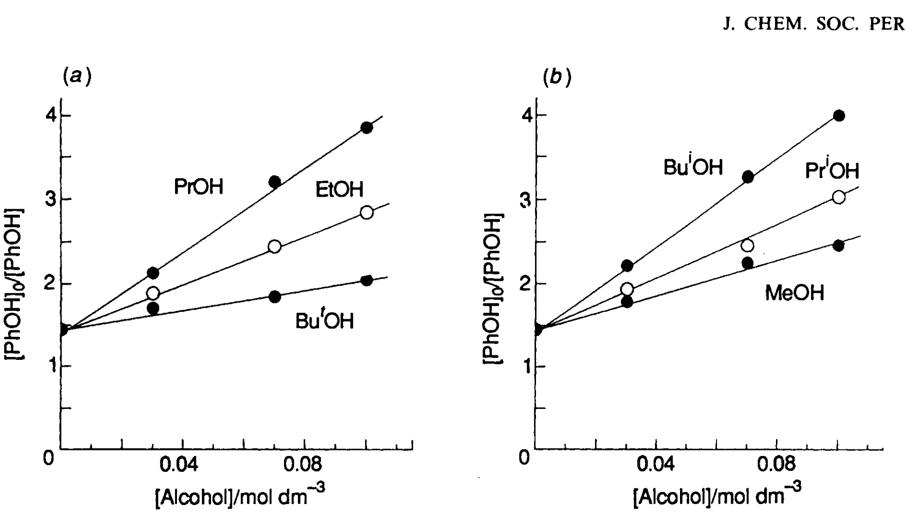


Fig. 1 Linear relationships between $[PhOH]_0/[PhOH]$ and [Alcohol] in the Fenton oxidation of benzene under the standard reaction conditions (see Experimental section)

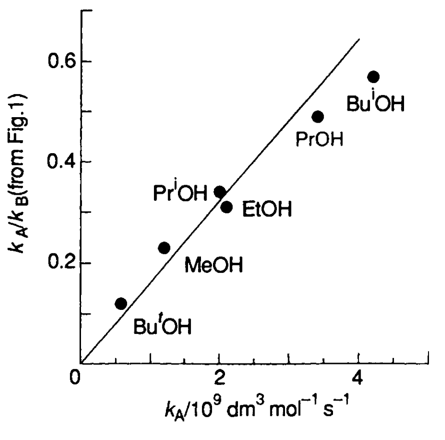


Fig. 2 Plot of k_A/k_B obtained from Fig. 1 against the authentic k_A reported by Walling.[11] The solid line was calculated assuming $k_B = 6.2 \times 10^9\ dm^3\ mol^{-1}\ s^{-1}$.

slopes for each alcohol and the concentration of benzene (0.023 mol dm^{-3}) in saturated aqueous solutions. The k_A/k_B values thus calculated for methyl, ethyl, propyl, isopropyl, isobutyl, and *tert*-butyl alcohols were 0.23, 0.31, 0.49, 0.34, 0.57 and 0.12, respectively.

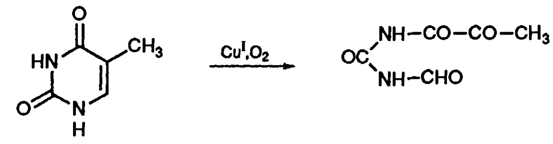
Since Walling and co-workers reported the relative rate constants k_A/k_F for these alcohols,[11] where k_F is the rate constant for the reaction of hydroxyl radical with iron(II) ion [eqn. (4)] one can calculate the values of k_A assuming that k_F

$$^{\bullet}OH + Fe^{II} + H^+ = H_2O + Fe^{III} \qquad (4)$$

is equal to 3×10^8 dm^3 mol^{-1} s^{-1}. The values of k_A thus calculated are 1.2, 2.1, 3.4, 2.0, 4.2 and 0.57 × 10^9 dm^3 mol^{-1} s^{-1} for methyl, ethyl, propyl, isopropyl, isobutyl and *tert*-butyl alcohols, respectively. The plots of k_A/k_B values obtained from the slopes in Fig. 1 against the k_A values reported by Walling[11] showed a linear relationship (Fig. 2) with a slope of $(1.66 \pm 0.29) \times 10^{-10}$ s mol dm^{-3}, which corresponds to $1/k_B$. The k_B value thus obtained was $(6.2 \pm 1.1) \times 10^9$ dm^3 mol^{-1} s^{-1}, which is in good agreement with the reported values, 7.7×10^9 (by Neta and Dorfman),[13] 4.3×10^9 (by Dorfman and co-workers)[14] and 6.8×10^9 dm^3 mol^{-1} s^{-1} (by the present authors).[10]

In summary, 'the OH-radical scavenging method' described above can identify hydroxyl radical as an active species by the following three criteria: (i) linearity in the plot of $[PhOH]_0/[PhOH]$ against [Alcohol] (Plot I); (ii) linearity in the plot of the slope of Plot I against the rate constant k_A determined by Walling (Plot II); (iii) agreement between the authentic k_B value $[(6.0 \pm 1.7) \times 10^9\ dm^3\ mol^{-1}\ s^{-1}]$ and that calculated from the slope of Plot II. In the next section, the OH-radical scavenging method will be applied to Udenfriend's system.

Udenfriend Oxidation of Thymine.—Since Udenfriend's reagent utilizes molecular oxygen instead of hydrogen peroxide, one cannot determine the theoretical yield of phenol ($[PhOH]_0$) on the basis of hydrogen peroxide. Alternatively, the theoretical yield on the basis of benzene can be used, though the accurate determination of the actual concentration of benzene suspended in an aqueous solution is difficult, mainly because of its volatility. In previous papers,[15,16] we have demonstrated that the oxidation of thymine **1** with molecular oxygen promoted by copper(I) ions gave *N*-formylpyruvylurea (FPU) **2** in 70% yield based on the amount of thymine consumed. Since thymine and FPU are accurately and conveniently determined by HPLC, we used thymine as a preferable substrate for the Udenfriend oxidation.



1 **2**

In preference to the OH-radical scavenging experiment, we examined the role of the L-ascorbic acid in Udenfriend's reagent. Though Udenfriend and co-workers showed that L-ascorbic acid is indispensable for the hydroxylation of salicylic acid, thyramine, antipyrine and so on,[1] the initial concentration of iron(II) sulfate (0.015 mmol dm^{-3}) was much smaller than those of L-ascorbic acid (0.142 mmol dm^{-3}) and the substrate (0.06 mmol dm^{-3}) in their experiment. In the aerial oxidation of thymine at pH 7.0 in the presence of 5 mmol dm^{-3} (1.25 mmol) of iron(II) sulfate and 11 mmol dm^{-3} of EDTA, an appreciable amount of FPU (0.382 mmol dm^{-3}) was formed even in the absence of L-ascorbic acid (Table 1). The yield of FPU, calculated from the stoichiometry in eqn. (5) was 31% based on

$$\text{Thymine} + 2O_2 + 4Fe^{II} + 4H^+ = \text{FPU} + 4Fe^{III} + 2H_2O \qquad (5)$$

the Fe^{II} added. Likewise, phenol and hydroquinone were obtained in yields of 29 and 30% (based on Fe^{II} added), respectively, in the oxidation of benzene with the Fe^{II}/EDTA/air

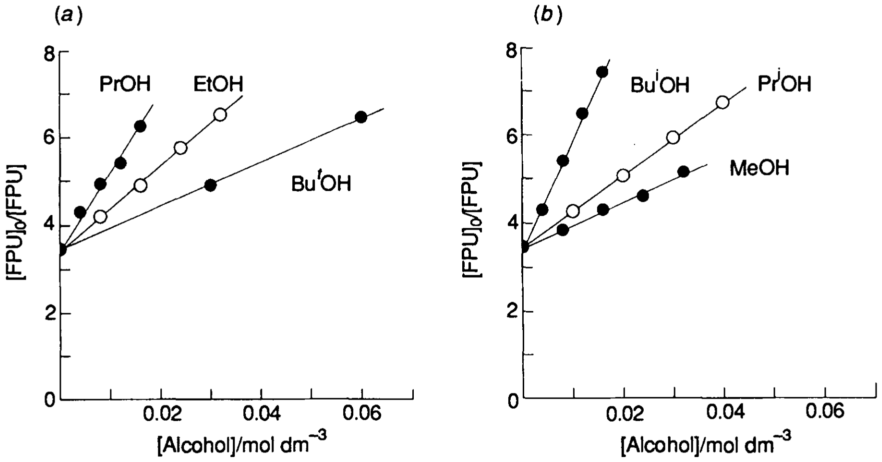
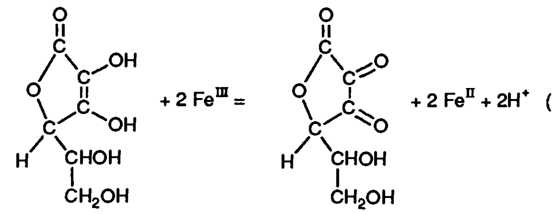


Fig. 3 Linear relationships between $[FPU]_0/[FPU]$ and [Alcohol] in the Udenfriend oxidation of thymine under the standard reaction conditions (see Experimental section)

Table 1 Udenfriend oxidation of thymine and benzene[a]

Reactant	$[Fe^{II}]$/ mmol dm^{-3}	[Ascorbic acid]/ mmol dm^{-3}	Product	Yield[b]/ mmol dm^{-3}
Thymine	5	0	FPU	0.382 (31)
Thymine	5	2.5	FPU	0.780 (31)
Thymine	1	0	FPU	0.141 (56)
Thymine	1	0.75	FPU	0.376 (60)
Thymine	1	3.75	FPU	0.937 (50)
Benzene	5	0	Phenol Hydroquinone	0.366 (29) 0.378 (30)
Benzene[c]	5	0	Phenol Hydroquinone	0.243 (19) 0.491 (39)

[a] Solvent: 25 cm^3 of 0.1 mol dm^{-3} phosphate buffer (pH 7.0); $[FeSO_4]$, 5 mmol dm^{-3}; [EDTA], 11 mmol dm^{-3}; [reactant], 5 mmol dm^{-3} of thymine or 0.75 cm^3 (8.4 mmol) of benzene; Atmosphere: air; T, 25 °C; reaction time: 90 min (thymine), 60 min (benzene). [b] The values in parentheses are the yields of the products based on 'the total concentration of Fe^{II}' calculated from eqn. (7). [c] Under O_2 atmosphere.



(6)

system. When 2.5 mmol dm^{-3} of ascorbic acid was added to the Fe^{II}/EDTA/air/thymine system, the yield of FPU was doubled (0.780 mmol dm^{-3}). The increase of the yield can be readily explained by the regeneration of iron(II) ions according to reaction (6), and the yield of FPU based on 'the total concentration of Fe^{II}', which can be calculated from eqn. (7) was

$$[Fe^{II}]_{total} = [Fe^{II}]_{added} + 2[\text{Ascorbic acid}] \quad (7)$$

essentially the same in the absence and presence of ascorbic acid. At 1.0 mmol dm^{-3} of Fe^{II} added, the yield of FPU based on 'the total concentration of Fe^{II}' was constant (55 ± 5%) regardless of the amount of ascorbic acid added, as seen in Table 1. The result in Table 1 indicates that the role of ascorbic acid in the Udenfriend oxidation is merely as a reductant of Fe^{III} to regenerate Fe^{II} ions according to reaction (6). In the following experiment, therefore, L-ascorbic acid was not added to the reaction system.

When thymine was oxidized with Udenfriend's reagent in the presence of various amounts of alcohols, the yield of FPU ([FPU]) decreased with increasing concentration of alcohols in a similar manner as in the Fenton oxidation. Since the initial concentration of iron(II) ions was fixed at 5.0 mmol dm^{-3} in 'the OH-radical scavenging experiment', the theoretical yield of FPU, ($[FPU]_0$), is 1.25 mmol dm^{-3} according to eqn. (4). The plots of $[FPU]_0/[FPU]$ against the concentrations of six alcohols added gave straight lines as demonstrated in Fig. 3. The result in Fig. 3 (Plot I for the oxidation of thymine) indicates that eqn. (8) holds for Udenfriend's system as well as

$$\frac{[FPU]_0}{[FPU]} = 1 + \frac{k_D'}{k_T[\text{Thymine}]} + \frac{k_A[\text{Alcohol}]}{k_T[\text{Thymine}]} \quad (8)$$

for Fenton's system where k_T and k_D' are the rate constants for the reaction of hydroxyl radical with thymine and for the deactivation reaction of hydroxyl radical,[12] respectively. The slopes of Plot I (Fig. 3) correspond to $k_A/k_T[\text{Thymine}]$, provided that the active species generated from Udenfriend's reagent is hydroxyl radical. The plots of the k_A/k_T values obtained from the slopes in Fig. 3 and the concentration of thymine (5 mmol dm^{-3}) against the authentic rate constants k_A[11] gave a linear relationship as shown in Fig. 4. The slope of Plot II in Fig. 4 was $(0.26 \pm 0.04) \times 10^{-10}$ s mol dm^{-3}, which corresponds to $1/k_T$. The k_T value thus obtained was $(3.9 \pm 0.6) \times 10^9$ dm^3 mol^{-1} s^{-1}, which is in excellent agreement with the reported values of k_T, $(3.4 \pm 1.4) \times 10^9$ by Anbar and Neta[17] and $(5.0 \pm 0.3) \times 10^9$ mol^{-1} dm^3 s^{-1} by Shinohara and co-workers.[18]

The result of the OH-radical scavenging experiment for the Udenfriend oxidation of thymine can be summarized as follows: (i) good linearity of Plot I; (ii) good linearity of Plot II; and (iii) excellent agreement between the authentic and observed k_T values. Accordingly, the active species generated from

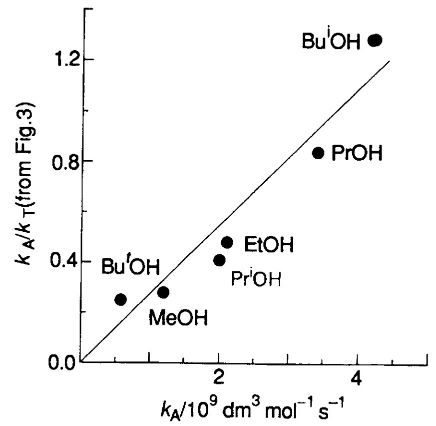


Fig. 4 Plot of k_A/k_T obtained from Fig. 3 against the authentic k_A reported by Walling.[11] The solid line was calculated assuming k_T = 3.6 × 10^9 dm^3 mol^{-1} s^{-1}.

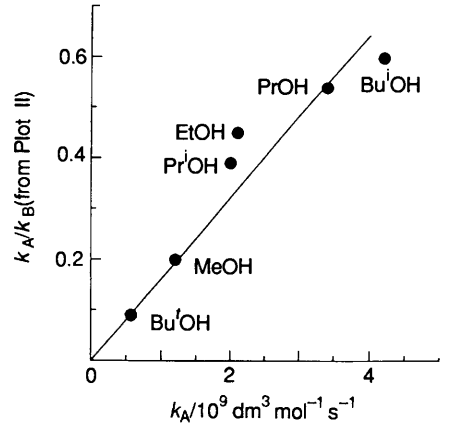


Fig. 5 Plot of k_A/k_B obtained from Plot II for the Udenfriend oxidation of benzene against the authentic k_A reported by Walling.[11] The solid line was calculated assuming k_B = 6.2 × 10^9 dm^3 mol^{-1} s^{-1}.

Udenfriend's reagent should be hydroxyl radical in the same sense as Fenton's reagent generates hydroxyl radicals.

Udenfriend Oxidation of Benzene.—To confirm the above conclusion, a further OH-radical scavenging experiment was carried out for the hydroxylation of benzene with Udenfriend's reagent under the reaction conditions shown in Table 1. The theoretical yield of phenol $[PhOH]_0$ is 1.25 mmol dm^{-3} for the initial concentration of 5 mmol dm^{-3} of iron(II) sulfate. The plots of $[PhOH]_0/[PhOH]$ against the concentration of six alcohols gave six straight lines, the slopes of which were proportional to the rate constant k_A,[11] as shown in Fig. 5. The slope obtained from Fig. 5 was (1.7 ± 0.3) × 10^{-10} s mol dm^{-3}, which corresponds to $1/k_B$. Thus one can obtain k_B = (6.2 ± 1.2) × 10^9 dm^3 mol^{-1} s^{-1}, which is in excellent agreement with the reported values,[10,13,14] (6.0 ± 1.7) × 10^9 dm^3 mol^{-1} s^{-1}. Fig. 5 (for the Udenfriend oxidation) and Fig. 2 (for the Fenton oxidation of benzene) can be completely superposed upon each other.

From the experimental results described above, one can conclude that the effect of the six alcohols on the yield of oxidation products is essentially the same for the Fenton and Udenfriend oxidations of benzene, and that the active species generated from Udenfriend's reagent is hydroxyl radical. This conclusion is consistent with the recent results of an EPR study on the species generated by the reaction of O_2 with Fe^{II}–EDTA complex.[19] The OH-radical scavenging method was found to be useful for the identification of hydroxyl radical as the active species generated from Fenton's and Udenfriend's reagents as well as from the copper(I) chloride/O_2 system.[10]

Experimental

Materials.—All the chemicals were commercial reagent grade and were used without further purification. Hydrogen peroxide (35 wt%) was obtained from Katayama Kagaku Kogyou.

Analysis.—Phenol and hydroquinone were determined by reversed-phase HPLC using a JASCO Intelligent HPLC pump (880-PU) and a JASCO Intelligent UV–VIS detector (875-UV) coupled with a Shimadzu Chromatopac C-R6A. The separation column was a Cica-MERCK Hibar Lichrosorb RP-18 (5 μm) and the eluent was a mixture of acetonitrile (14 vol%) and a phosphate buffer (86 vol%, pH 3.5). In the oxidation of thymine, FPU and thymine were determined likewise except using a eluent of a phosphate buffer (100 mmol dm^{-3}, pH 3.5).[15,16] Hydrogen peroxide was determined spectroscopically (titanium sulfate method)[20] by use of a JASCO 610 spectrophotometer.

Fenton Oxidation of Benzene.—The standard procedure for the Fenton oxidation of benzene was as follows. Into a 100 cm^3 Erlenmeyer flask with a rubber stopper fitted with four glass tubes, was placed 25 cm^3 of H_2SO_4 (50 mmol dm^{-3}) containing $FeSO_4$ (10 mmol dm^{-3}), $CuSO_4$ (30 mmol dm^{-3}) and alcohol (10–100 mmol dm^{-3}). After purging the air in the flask with N_2 through two glass tubes fitted to the rubber stopper, 0.75 cm^3 (8.4 mmol) of benzene was added through the third tube using a microfeeder (Azuma Denki Kogyou, KF-2). With vigorous magnetic stirring at 1000–1100 rpm, H_2O_2 (100 mmol dm^{-3}) in H_2SO_4 (50 mmol dm^{-3}) was added dropwise using another microfeeder (Azuma Denki Kogyou, KF-2) at a rate of 0.2759 cm^3 min^{-1}. After addition of the pre-calculated amount (0.28–0.30 mmol) of H_2O_2 at 25 °C, the reaction mixture was allowed to stand for 30 min with stirring. After evaporating the remaining benzene with a stream of N_2, 5 cm^3 of the reaction mixture was diluted to 50 cm^3 with EDTA (25 mmol dm^{-3}). An aliquot of the diluted reaction mixture was subjected to HPLC analysis.

Udenfriend Oxidation of Thymine.—Into a 100 cm^3 Erlenmeyer flask with a glass stopper, was placed 47.5 cm^3 of a phosphate buffer solution (100 mmol dm^{-3}, pH 7.0) containing thymine (0.25 mmol), EDTA (0.55 mmol) and alcohols (0.2–6.0 mmol). After addition of 2.5 cm^3 of H_2SO_4 (50 mmol dm^{-3}) containing iron(II) ammonium sulfate (0.25 mmol), the flask was shaken for 1 h at 170 oscilations per minute under air atmosphere at 25 °C. The reaction mixture was subjected to HPLC analysis after dilution of the reaction mixture (1 cm^3) with 1.5 mmol dm^{-3} EDTA (4 cm^3). Some experiments were done in the presence of 0.75–10 mmol dm^{-3} L-ascorbic acid.

Udenfriend Oxidation of Benzene.—Instead of thymine, 0.75 cm^3 (8.4 mmol) of benzene was added to 47.5 cm^3 of the phosphate buffer as described above. The procedure was the same as for the oxidation of thymine.

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