The pK_a and pH Dependence of the Formation of Nitroxide Radicals from Some Drug Substances with an Aliphatic Secondary Amino Group by Oxidation with Hydrogen Peroxide. An Electron Spin Resonance (ESR) Study

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The pK_a and pH dependence of the formation of nitroxide radicals from the following drugs that contain an aliphatic secondary amino group, by oxidation with hydrogen peroxide, have been studied by ESR spectroscopy: Ephedrine, $(1R,2S)$-1-phenyl-2-methyl-aminopropanol (1), timolol, (S)-1-(tert-butyl-amino)-3-[(4-morpholino-1,2,3-thiadiazol-3-yl)oxy]-2-propanol (2), metoprolol, 1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]-2-propanol (3) and te-rodiline, N-tert-butyl-3,3-diphenyl-1-methylpropylamine (4). Radicals were for-med from the non-ionized base only. Therefore, the pK_a value of the amine and the pH of the reaction mixture is of crucial importance for the yield of nitroxide radical. At 37 °C the pK_a values of 1–3 are about 9.2, and of 4 about 9.6, which means that 1.5 % of 1–3, and 0.6 % of 4, are present in the reactive base form at the physiological pH of 7.4. Horse-radish peroxidase was found both to enhance radical production and to decrease the life-time of the radicals formed in the reaction with hydrogen peroxide.

Free radicals are formed in the metabolism of a large number of drug substances and xenobiotics.[1-3] Among the many substances investigated, aromatic amines were found to give rise to nitroxide radicals when incubated with liver microsomes.[2-4] In connection with such studies a number of aliphatic amines were also tested. No radicals could be observed with amines such as 1,2-di-amino-ethane, 2-amino-2-methylpropane and pi-peridine. Any aliphatic nitroxides formed were considered to be reactive and too short-lived to be detected by the ESR technique used.[4-7]

However, many nitroxide radicals derived from aliphatic secondary amines are persistent enough to be detected by ESR spectroscopy when prepared by oxidation of the parent compound with 3-chloroperbenzoic acid or hydrogen peroxide.[8-11] In view of this fact and possible adverse effects of metabolically formed nitroxide radicals, it is of interest to study the reaction conditions for the formation of such radicals, especially from aliphatic secondary amines since a number of therapeutic drugs have aliphatic side chains carrying secondary amino groups.

It has now been found by ESR spectroscopy that nitroxide radicals are indeed formed from drugs that have aliphatic side chains containing a secondary amino group. The study has been limited to the nitroxide radicals formed from ephe-drine, $(1R,2S)$-1-phenyl-2-methylaminopropanol (1), timolol, (S)-1-(tert-butylamino)-3-[(4-morpholino-1,2,3-thiadiazol-3-yl)oxy]-2-propan-ol (2), metoprolol, 1-[4-(2-methoxyethyl)phen-oxy]-3-[(1-methylethyl)-amino]-2-propanol (3) and terodiline, N-tert-butyl-3,3-diphenyl-1-methyl-propylamine (4) on oxidation of the parent compounds with hydrogen peroxide, a natu-ral product of cell metabolism.[12] It was found that the yield of radical is dependent on the pK_a value of the parent substance, and consequently on the
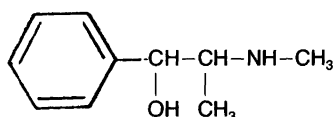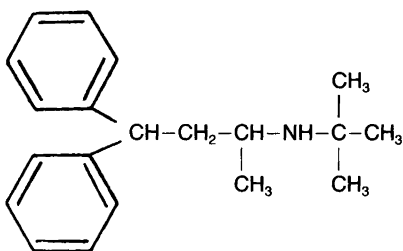
Ephedrine (1)
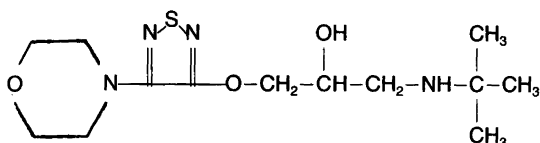
Timolol (2)
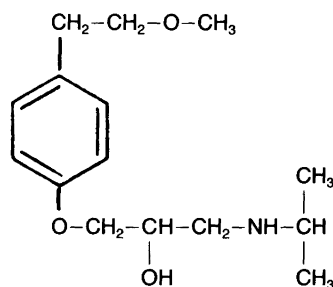
Metoprolol (3)

Terodiline (4)

pH of the reaction mixture since radicals are formed from the free bases only.

Materials and methods

Materials. Ephedrine, ephedrine hydrochloride, timolol maleate, adenosine diphosphate, horse-radish peroxidase and horse-radish peroxidase immobilized on beaded agarose were obtained from Sigma Chemical Company. Metoprolol tartrate was a gift from AB Hässle, Mölndal, and terodiline hydrochloride a gift from AB Kabi, Stockholm. Potassium peroxylamine disulfonate was obtained from Aldrich. Buffer substances and 30 % hydrogen peroxide were of analytical grade.

Electron spin resonance (ESR) measurements. The ESR spectra were recorded using a Varian E-9 spectrometer. The radical concentration was measured at 20 °C as a function of the reaction time when the samples in aqueous solution were contained in a flat cell in the ESR cavity. Unless otherwise stated, the radical concentration curves were recorded with a microwave power of 20 mW and a 100 kHz modulation amplitude of 1 G. Peak-to-peak heights of the spectral components were used as a measure of the radical concentration, which is given in arbitrary units in Figs. 3–7. As the scale factor is the same only for the curves of each individual figure, quantitative comparison between curves of different figures is not possible. Hyperfine splitting (hfs) constants were evaluated by use of peroxylamine disulfo-

nate (Fremy's radical, $a_N = 13.0$ gauss). Radical concentrations were estimated by comparison with freshly prepared solutions of peroxylamine disulfonate in 0.1 N NaOH.

Measurement of pK_a values. The pK_a values of the substances of this series were evaluated by potentiometric titration (0.1 and 1.0 mmol) in 50 ml of solvent [H_2O or ethanol/H_2O 1:1 (v/v)] with 0.01 and 0.1 M HCl and NaOH at 20 and 37 °C.

Results and discussion

ESR spectra of the radicals. The ^{14}N and hydrogen atom splittings in the spectra of the radicals derived from 1–4 by oxidation with hydrogen peroxide in the reaction

$$R^1-N(H)-R^2 \rightarrow R^1-N(O\cdot)-R^2$$
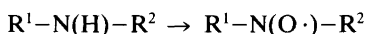
are collected un Table 1. The radicals derived from 1 give rise to 24 theoretical hyperfine lines (3×4×2) by interaction of the unpaired electron with the three equivalent hydrogen atoms of the N-methyl group and the single α-hydrogen atom, in addition to the ^{14}N nucleus. The hyperfine lines, of which at least 22 are resolved (Fig. 1), are arranged in 6 groups (E1–E6). The radicals derived from 4 give rise to a simple 3×2 line spectrum (T1, T2 and T3) by interaction with the ^{14}N nucleus and the single α-hydrogen atom.

Compound 2 gave rise to a 3×2×2 line spectrum, indicating that the hydrogen atoms of the

Table 1. Coupling constants (G) of the nitroxide radicals formed from 1–4 by oxidation with hydrogen peroxide.

Parent compound	a_N	$a_H{}^a$	$a_H{}^a$	$a_H{}^a$
Ephedrine (1)	15.9	14.8 q	4.1 d	
Timolol (2)	16.1	14.0 d	8.3 d	
Metoprolol (3)	16.1	13.1 d	7.8 d	5.2 d
Terodiline (4)	16.6	1.8 d		

^{a}q = quartet 1:3:3:1; d = doublet 1:1.

α-methylene group are non-equivalent. The radicals derived from 3 also exhibit non-equivalent hydrogen atoms of the α-methylene group, giving rise to a 3×2×2×2 line spectrum. However, the spectrum was incompletely resolved due to overlapping hfs lines. Non-equivalence of the hydrogen atoms of the α-situated (N-situated) methylene groups has been observed for a number of nitroxide radicals, and is in general due to steric hindrance.[13]

The hfs lines of the ESR spectra of the radicals derived from 1 and 4 are situated such that it was possible to obtain a spectrum of a mixture of the radicals from these two substances without the occurrence of interfering overlap with T1 of the 4 radicals situated in the space between E2 and E3 of the 1 radicals, etc. Combined spectra of the 1 and 4 radicals were used for the simultaneous recording of the pH-dependent formation of these two radicals (Fig. 2).
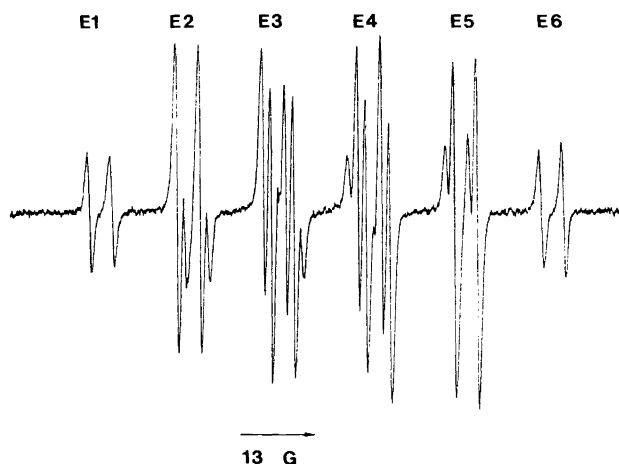

E1 E2 E3 E4 E5 E6

13 G

Fig. 1. ESR spectrum of the nitroxide radicals formed from 1 (0.04 M) by oxidation with hydrogen peroxide (1.76 M) at pH 8.5. Coupling constants: see Table 1. The hyperfine lines are distributed in six groups: E1–E6 (see text). Microwave power 1 mW, 100 kHz modulation amplitude 0.5 G.

Fig. 2. The ESR overlap spectrum of the nitroxide radicals formed from a mixture of **1** (0.04 M) and **4** (0.04 M) by oxidation with hydrogen peroxide (1.75 M) at pH 8.3. The spectrum was recorded after a reaction time of 2 h. The spectral component T1 of **4** is situated in the space between E2 and E3 of **1**, etc.
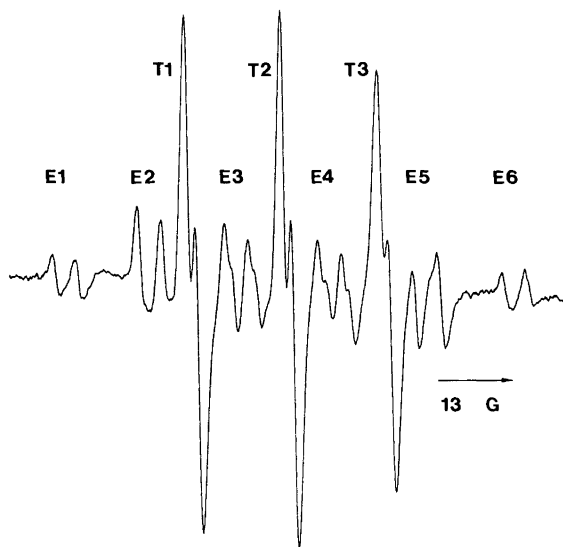
The pK_a *and pH dependence of the radical yield.* In conformity with findings made with several other nitroxides,[8–11] it was observed that the yield of the radicals of this series was increased in alkaline solutions. Therefore, a closer study of the pK_a and pH dependence of the radical formation was made. The pK_a values of **1–4** were estimated by titration (Table 2). The pK_a value of **4** could not be established by titration of a water solution of the hydrochloride. The low solubility of the base in water gave rise to precipitation which occurred at a pH of about 9.21 (0.002 M, 20 °C). As **4** is soluble in ethanol/H$_2$O[1:1 (*v/v*)] in the range of pH of the measurements, an indirect estimate of the pK_a value of this substance was made by comparison with **1** dissolved in water and in 1:1 ethanol/H$_2$O (Table 2).[14–16] This extrapolation from **1** gave the following values for **4** in water:

9.72+0.51 = 10.23 at 20 °C, and 9.38+0.25 = 9.63 at 37 °C.

As the ESR spectra of **1** and **4** radicals were convenient for a comparison (cf. Fig. 2), this study was focused on these radicals. Figs. 3 and 4 show the relative concentrations of the radicals formed from **1** and **4** by oxidation with hydrogen peroxide plotted against the reaction time at different pH values. The **1** radicals reached a maximum value after about only half an hour. After this time there was a decrease in the radical concentration. Both radical yield and rate of radical disappearance increased with increasing pH (Fig. 3). The radicals derived from **4** exhibited a much slower rate of formation. At pH 8.0 the maximum yield was still not reached after 8 h (Fig. 4).

A direct comparison of the radical yield from the two substances was made by simultaneously

Table 2. pK$_a$ values of 1–4.

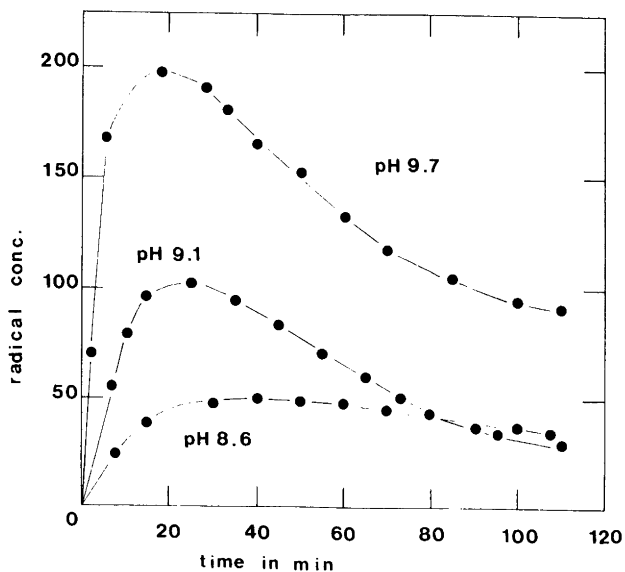|	In H$_2$O at			In ethanol/H$_2$O (1:1) at	
	20 °C	37 °C		20 °C	37 °C
Ephedrine (**1**)	9.67	9.23		9.16	8.98
Timolol (**2**)	9.70	–		–	–
Metoprolol (**3**)	9.60	–		–	–
Terodiline (**4**)	pptd.	pptd.		9.72	9.38

Fig. 3. The concentration of the radicals formed from **1** (0.04 M) by oxidation with hydrogen peroxide (1.76 M) plotted against the reaction time at pH 9.7, 9.1 and 8.6.

recording the ESR spectra (cf. Fig. 2) derived from equimolar (0.04 M) solutions of **1** and **4** at different pH's in the range between 7.4 and 8.3 after the addition of an excess of hydrogen per-oxide (1.76 M). In these experiments it was necessary to add ethanol 1:20 (v/v) to dissolve **4**. The sum of the peak-to-peak heights of the spectral components E2 and E3 of the **1** spectrum,
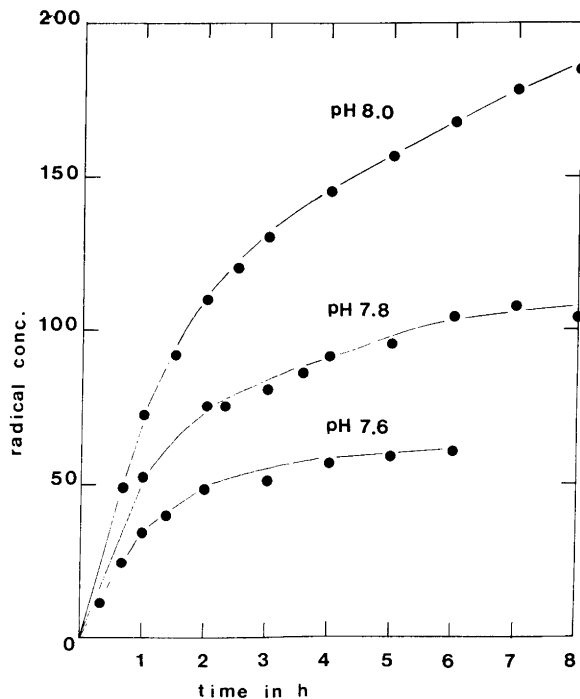

Fig. 4. The concentration of radicals formed from **4** (0.016 M) by oxidation with hydrogen peroxide (1.76 M) plotted against the reaction time at pH 8.0, 7.8 and 7.6.
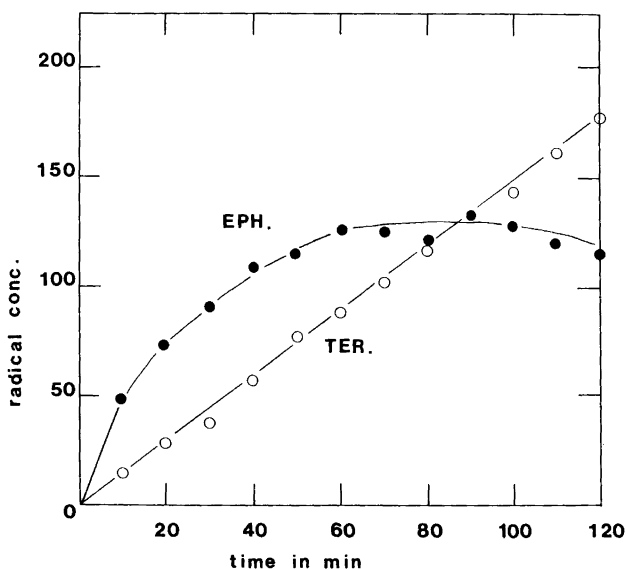
Fig. 5. The concentration of the radicals formed from an equimolar mixture of **1** and **4** (0.04 M) by oxidation with hydrogen peroxide (1.76 M) plotted against the reaction time at pH 7.8.

and the sum of the heights of the components T1 and T2 of the **4** spectrum were used as relative measures of the radical concentrations. From a stick diagram of the spectra, it can be shown that these components involve 11/24 of the total spectral intensity of the **1** spectrum and 2/3 of the intensity of the **4** spectrum. Therefore, the measured values for the **1** radicals were corrected by multiplying with the factor 1.45 (48/33) in order to obtain a comparison of the concentrations of

the **1** and **4** radicals. Fig. 5 shows the relative concentrations of the two radical species obtained by oxidation of a mixture of **1** and **4** with hydrogen peroxide at pH 7.8 plotted against the reaction time. The **1** radicals reached their maximum concentration rather rapidly, whereas the concentration of the **4** radicals showed a slower increase and exceeded the concentration of the **1** radicals after about 90 min.

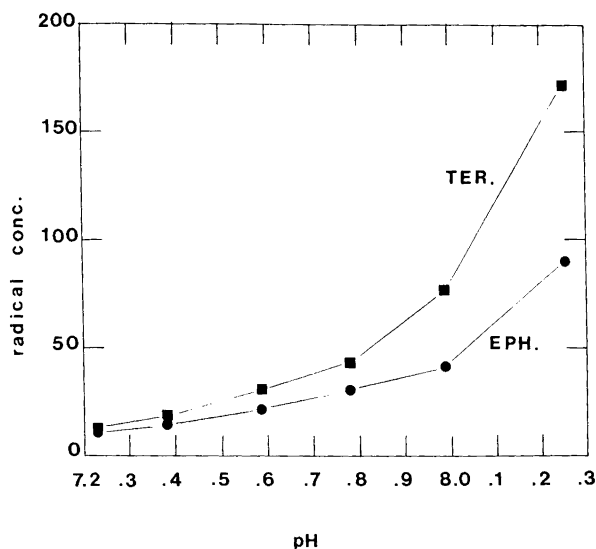Fig. 6 shows the relative concentrations of **1**

Fig. 6. The concentration of the nitroxide radicals formed from equimolar amounts of **1** and **4** (0.04 M) by oxidation with hydrogen peroxide (1.76 M) plotted against the pH of the reaction mixture. The concentrations correspond to the values recorded after a reaction time of 2 h.

and **4** radicals plotted against the pH of the reaction mixture in the range between 7.4 and 8.3. The radical concentrations were taken from time curves similar to those of Fig. 5, and represent the values observed after a reaction time of 120 min. The radical yield for both **1** and **4** increased with increasing pH. The yield of **4** radicals was larger than that obtained with **1**, and the difference between the yields of the two species increased at the higher pH. However, the curves converge at the lower pH. Curves similar to those presented in Fig. 6 were also obtained when the maximum values from the concentration vs. time curves were used in place of the concentrations recorded at 120 min.

The results indicate that the radicals of this series formed in the reaction with hydrogen peroxide are produced from the base only, and not from the protonated forms of the secondary amines. At 20°C the pK_a values indicate that about 0.6 % of the amounts of **1–3** and 0.14 % of **4** are present as the reactive base form at pH 7.4. The pK_a values decrease with increasing temperature.[14–16] This implies that the fraction of reactive base is about 1.5 % for the former substances, and about 0.6 % for the latter at 37°C.

Second-order rate constants, k_2. Assuming that the oxidation of the parent substances of this series with hydrogen peroxide is a second-order reaction, eqn. 1 is valid, where V is the initial

$$V = k_2 \cdot [H_2O_2] \cdot [S \cdot B] \qquad (1)$$

velocity (M s^{-1}), [H$_2$O$_2$] the concentration of hydrogen peroxide, and [$S \cdot B$] the concentration of the parent compound in the base form, where S is the total concentration of the parent compound and B the fraction of base at the pH of the reaction mixture. B was obtained from eqn. 2.

$$pH = pK_a + \log \frac{B}{1 - B} \qquad (2)$$

Table 3. Second order rate constants, k_2, for the formation of nitroxide radicals from ephedrine (**1**) and terodiline (**4**) by oxidation with hydrogen peroxide at different pH. [Ephedrine]: 0.04 M, [Terodiline]: 0.016 M, [H$_2$O$_2$]: 1.76 M. Temperature: 20°C.

Ephedrine

pH	$V/10^{-10}$ M s^{-1} [a]	Product of concentrations $S \cdot B$/mM	$k_2/10^{-8}$ M^{-1} s^{-1}
9.1	18.7 ±0.30	0.04×0.24	11.6±0.19
8.6	8.06±0.17	0.04×0.09	12.8±0.28
8.0	2.19±0.08	0.04×0.025	12.4±0.43
		mean value	12.3±0.61

Terodiline

pH	$V/10^{-11}$0 M s^{-1} [a]	Product of concentrations $S \cdot B$/mM	$k_2/10^{-8}$ M^{-1} s^{-1}
8.0	5.73±0.26	0.016×0.0059	34.4±1.58
7.8	4.28±0.13	0.016×0.0037	41.1±1.25
7.6	2.72±0.13	0.016×0.0023	41.1±1.98
		mean value	38.9±3.88

[a]The velocities V are the mean values of three separate determinations.

Table 3 gives the second-order rate constants, k_2, obtained for **1** and **4** together with the initial velocities (V) and the fraction of the base form (B) at different pH values. Rather good agreement was obtained for the k_2 values evaluated for the different pH's. As seen from Table 3, the initial velocity of the reaction of **1** at pH 9.1 is about 8.5 times greater than at pH 8.0, whereas the k_2 values are 11.6 and 12.4×10^{-8} M^{-1} s^{-1}, a difference of only about 7 %. Similar results were obtained for the formation of the **4** radicals. These findings support the statement that the reactive form of the parent compounds is the non-ionized base.

As the fraction of non-ionized base, B, is dependent on the pK_a value (eqn. 2), and k_2 is inversely proportional to B (eqn. 1), an inaccurate pK_a value will introduce systematic errors into the absolute values of the second-order rate constants obtained at different pH values. Such an error may be present in the k_2 values obtained for **4**, since the pK_a of this substance could only be indirectly estimated owing to the low solubility of **4** in water (see above). However, it seems difficult to estimate the importance of such a possible error in the pK_a value in relation to other experimental errors inherent in the measurement of low concentrations of radicals from ESR spectra that exhibit incompletely resolved hyperfine structures.

From Table 3 it is seen that the k_2 values for **4** are about 3 times larger than the k_2 values obtained for **1**. A reasonable explanation for this difference is not obvious. The difference might be associated with higher stability of the **4** radicals, as manifested by the higher yield of **4** radicals shown in Fig. 6. It should also be pointed out that a pK_a value for **4** lower than that indirectly estimated by comparison with **1**, and used for evaluation of the k_2 values of Table 3, would have reduced these values.

Effect of · OH radicals. It has been suggested that · OH radicals are involved in the formation of nitroxide radicals in the reaction between secondary amines and hydrogen peroxide.[9] Some experiments were performed both with photochemically generated · OH radicals, and with · OH radicals generated in a Fenton reaction in which Fe(II) is complexed with adenosine diphosphate.[17] No effect on the yield of nitroxide radicals could be detected in the reaction of the substances of this series.

Influence of horse-radish peroxidase. Soluble horse-radish peroxidase increased the rate of radical formation from **1** in the reaction with hydrogen peroxide. The radicals also disappeared very rapidly in the presence of the enzyme, so that the

Fig. 7. The concentration of the nitroxide radicals derived from **1** plotted against the reaction time. **1** (0.05 M) was oxidized with an equimolar amount of hydrogen peroxide at pH 9.6 in the presence of horse-radish peroxidase immobilized on beaded agarose. The reference curve with no enzyme is the mean of six separate recordings. The curves obtained with three different concentrations of immobilized peroxidase (3, 5 and 10×10^{-3} units μl^{-1} of reaction mixture) are shown; one unit will produce 1.0 μmol min^{-1} of purpurogallin from pyrogallol at pH 6.0 and 30 °C (Sigma Chemical Company).
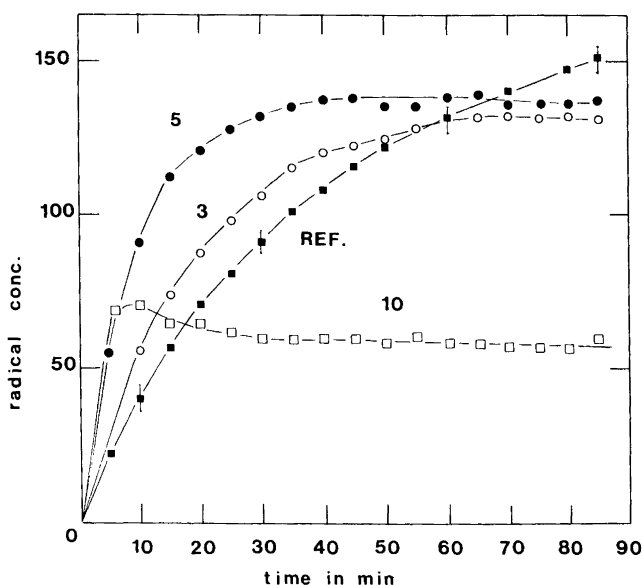
radical concentration could not be followed by the static measurement technique used in this study. However, some radical concentration-time curves could be obtained by using horse-radish peroxidase immobilized on beaded agarose. Evidently, some of the enzymatically generated radicals diffused from the immobilized peroxidase, thus escaping further reactions leading to the disappearance of the radical state.

Equal concentrations of 1 and hydrogen peroxide (0.05 M) were used in the experiments with insoluble horse-radish peroxidase which were performed at pH 9.6 and 20 °C. Fig. 7 shows the relative radical concentration plotted against the reaction time for three different concentrations of insoluble peroxidase, together with the reference with no enzyme. The initial slope of the curves increased with the peroxidase concentration, indicating an increased reaction velocity. The highest peroxidase concentration in these experiments ("10") brought about a four-fold increase in the reaction velocity relative to the reference. The maximum radical concentration was also reached in a shorter time in the presence of peroxidase than with the reference. However, the maximum concentrations obtained with peroxidase are smaller than those with the reference, which indicates that the increased rate of formation is accompanied by radical destruction.

Conclusions

The base form of the secondary amines of this series constitutes the reactive species that is oxidized with hydrogen peroxide to nitroxide radicals. Therefore, the pK_a values of the substances and the pH values of the reaction mixtures are of importance for radical formation. The pK_a values at 20 °C for 1−3 indicate that about 0.68 % of these substances, and about 0.14 % of 4 are present in the reactive base form at the physiological pH of 7.4. However, the pK_a values of amines decrease with increasing temperature (Table 2), which means that the fractions of the reactive base forms are larger at higher temperatures. Thus, the fraction of reactive base is about 1.5 % for 1−3, and about 0.6 % for 4 at 37 °C and pH 7.4. If we disregard other factors such as enzymatic enhancement, the fractions of reactive base seem to indicate that the production of nitroxide radicals by oxidation with physiologically produced hydrogen peroxide in the cell is small

for the substances in this series. It should be pointed out that possible detrimental effects of metabolically formed nitroxides are not only dependent on their bulk concentrations, but also on the site of formation in the living cell. Consequently, radical formation from the substances in this series *in vivo* should not be entirely disregarded. The high yield of nitroxide radicals observed with a number of aromatic amines (see above) seems, in part, to be a consequence of their low pK_a values (cf. *N*-methylaniline: pK_a = 4.25, 25 °C[14,15]). Thus, these substances are present mainly in the base form at pH 7.4.

The results obtained so far with immobilized horse-radish peroxidase indicate that the oxidation of 1 with hydrogen peroxide is enzymatically enhanced. However, more experiments are needed to elucidate these reactions.

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