The Formation of Singlet Oxygen during Oxidation of Catechol Amines as Detected by Infrared Chemiluminescence and Spectrophotometric Method

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$^1\Delta_g \rightarrow {}^3\Sigma_g^-$ near infrared chemiluminescence from the peroxidation reaction of dopa, dopamine, noradrenaline and adrenaline was measured.

The spectrophotometric method based on the bleaching of *p*-nitrosodimethylaniline was applied to check the generation of singlet oxygen during oxidation of the above-mentioned catecholamines.

The generation of highly reactive oxygen radicals and electronically excited species have been observed during oxidation reactions of the biogenic catecholamines [1–3]. In our previous studies [2, 3] we have reported that the spectral distributions of the light emission from these reactions in visible region were similar to the spectrum of the dimol emission of singlet oxygen ($^1O_2$). Moreover, the measurements of the influence of solvents with various $O_2(^1\Delta_g)$-lifetimes and $^1O_2$-quenchers on quantum yield of chemiluminescence indicated the presence of this very reactive oxidant.

Since the observation of $^1O_2$ production by measurement of $O_2(^1\Delta_g \rightarrow {}^3\Sigma_g^-)$ emission at 1268 nm has been proved to be a very reliable and sensitive test [4–7], we have undertaken a study of this infrared chemiluminescence from dopa, dopamine, noradrenaline and adrenaline oxidation reactions. Additionally, we have also applied a sensitive spectrophotometric method for the detection of $^1O_2$ generated in these reactions.

Materials and Methods

D,L-Dopa and 1,4-diazabicyclo-[2,2,2]octane (DABCO) were obtained from Aldrich Chemical Co., dopamine was EGA Chem. (W. Germany), D,L-adrenaline was from B.D.H. Chem. Ltd. (England), noradrenaline from Fluka AG, Buchs. Compounds employed as quenchers of singlet oxygen, imidazole and *p*-nitrosodimethylaniline, were from Merck. Heavy water (D_2O) 99.8% was from I.B.J. Świerk (Poland). Other reagents were analytical grade from POCH Gliwice (Poland). The pD values of the heavy water solutions were measured with a glass electrode applying a correction of +0.4 pH. The solvent was redistilled water. All experiments were done in air-saturated 100 mM carbonate buffer pH and pD 10.8 at 295 K.

The spectrophotometric determination of singlet oxygen ($^1O_2$) was performed according to the method described by Kralijć and Mohsni [8]. This method is based on bleaching of *p*-nitrosodimethylaniline (RNO) caused by the intermediate product of the reaction of $^1O_2$ with imidazole (A), *i.e.* a transannular peroxide (AO_2):

$$^1O_2 + A \rightarrow AO_2$$

$$AO_2 + RNO \rightarrow - RNO + \text{products}.$$

The bleaching of RNO was followed by monitoring the decrease in optical density (O.D.) at 440 nm.

Chemiluminescence between 1000 and 1300 nm was measured by means of a specially selected, liquid nitrogen-cooled, M10FD29 photomultiplier VEB Werk für Fernsehelektronik (G.D.R.) responsive to 1300 nm and operating jointly with a K-200 Zeiss recorder (G.D.R.). The short-wavelength cut-off filter with the spectral transmittance in the range from 1000 nm to 2000 nm was placed in front of the photomultiplier to eliminate shorter wavelength light. Light from a cuvette to the photomultiplier passed

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through a filter holder which allowed to choose one of two interference filters with T_{max} at 1150 and 1257 nm (30 nm half-band widths).

Chemiluminescence measurements were repeated at least 5 times while those spectrophotometric were done in triplicate. Data are reported as the mean ± standard deviation (S.D.).

Results

In our previous papers [2, 3] we have shown that the oxidation of alkaline air-saturated solutions of catecholamines (CAT) catalyzed by transition metal ions (Me^n) is accompanied by chemiluminescence. The total quantum yields of chemiluminescence of the CAT + Me^n + HO^- system detected using a M12FQC51 photomultiplier with S-20 cathode, sensitive in the range 180–850 nm were found to be of the order of 10^{-12} photon/catecholamine molecule, and the addition of 1 mM H_2O_2 enhanced their values by a factor of 10^3. The values of the quantum yields were also strongly increased in solvents in which $O_2(^1\Delta_g)$ has a longer lifetime than in water, and they were reduced by $^1O_2$-quenchers. The chemiluminescence spectral distributions from the autoxidation and peroxidation reactions (estimated with a set of glass cut-off filters and an EMI9558QB photomultiplier sensitive in the range 180–800 nm) revealed maxima at 580, 640 and 700 nm corresponding to the $2(^1\Delta_g) \rightarrow 2(^3\Sigma_g^-)$ transition from $^1O_2$-dimoles. The spectra contained also the band at 480–500 nm, which was attributed to the fluorescence of the catecholamines oxidation products. Using a M12FQC51 photomultiplier we also observed a two-phase decay of kinetic curves of chemiluminescence accompanying the peroxidation of catecholamines with a short half-life time which corresponded to the first phase.

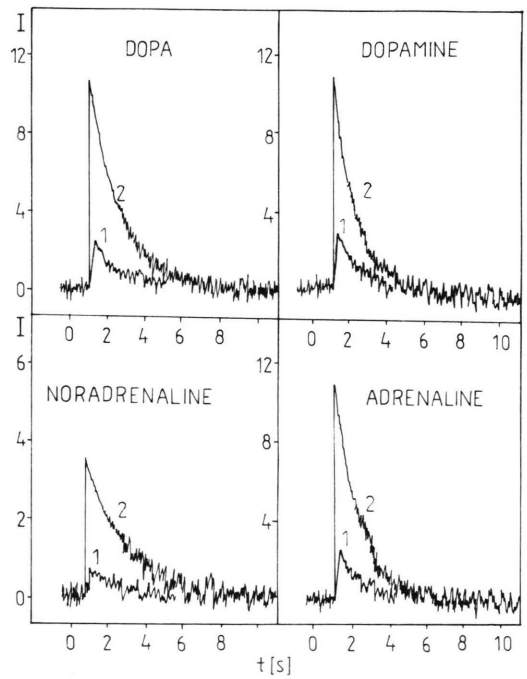

Fig. 1. Kinetics of infrared chemiluminescence at 1257 nm from the CAT + Co^{2+} + HO^- + H_2O_2 system in H_2O (curves 1) and D_2O containing 17% H_2O (curves 2). Reaction mixtures contained: 1.7 mM CAT, 0.8 mM $CoCl_2$, 160 mM H_2O_2. The size of the noise approximates the S.D.

In this paper we present the kinetic curves of chemiluminescence emitted during peroxidation of catecholamines but detected using of a M12FD29 photomultiplier (Fig. 1). Light emission stared immediately after the addition of H_2O_2. The decay of the infrared emission in all system fits well to a single exponential behaviour. No light emission at 1257 nm was detected when catecholamine or metal ions or H_2O_2 were absent from the system. This results from a low sensitivity of the apparatus used. As seen from Fig. 1 the emission was enhanced by a factor of about 5 for all catecholamines in D_2O compared to that found in H_2O. The observed increases in I_{max} are in good agreement with those expected for the lifetimes ratios in these solvents (D_2O solutions contained 17% H_2O and lifetime (τ) of $^1\Delta_g$ in this mixture was found to be 14.9 μs. Using a value of 3.1 μs for τ in H_2O [9] a value of $\tau_{D_2O-H_2O}/\tau_{H_2O}$ was calculated to be 4.8).

Spectral distributions of the infrared chemiluminescence from the reaction of catecholamines peroxi-

Table I. Spectral distribution of infrared chemiluminescence. Assay conditions for catecholamines as in Fig. 1. Concentrations of remain substrates were: 16 mM H_2O_2, 16 mM NaOCl. The chemiluminescence is expressed as relative to H_2O_2 + NaOCl system. Emission intensities were corrected for the filters transmission and the detector efficiency.

System	Relative light emission Filter	
	1143 nm	1257 nm
H_2O_2 + NaOCl	0.05 ± 0.03	1.00 ± 0.1
DOPA	0.11 ± 0.09	0.66 ± 0.1
Dopamine	0.15 ± 0.08	0.69 ± 0.1
Noradrenaline	0.07 ± 0.04	0.31 ± 0.09
Adrenaline	0.17 ± 0.09	0.70 ± 0.09

Table II. The influence of singlet oxygen quenchers on infrared chemiluminescence from catecholamines peroxidation. Conditions: 1.7 mM CAT, 0.8 mM $CoCl_2$, 160 mM H_2O_2, solvent: D_2O containing 17% H_2O. Quencher was added to the reaction mixture before the addition of H_2O_2. Abbreviations used: I_o, maximum intensity of chemiluminescence without quencher; I, maximum intensity with quencher; DABCO, 1,4-diazabicyclo-[2,2,2]octane.

Quencher [mM]	$\frac{I_o - I}{I_o} \times 100\%$ Dopamine	Adrenaline
3.4 Sodium azide	68 ± 8	79 ± 6
1.7 Methionine	71 ± 7	69 ± 5
3.4 Histidine	70 ± 3	74 ± 6
1.7 Tyrosine	76 ± 5	82 ± 5
1.7 Tryptophane	69 ± 6	71 ± 4
10 DABCO	0	0

dation and from NaOCl + H_2O_2 system are presented in Table I. As seen from this table the light emission from all catecholamines has a maximum centered at about 1270 nm and is similar to that resulting from the NaOCl + H_2O_2 reaction. The last emission does not differ significantly from that reported by other authors [5, 7].

The band at 1270 nm corresponding to the $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ transition with vibrational quantum number (0.0) is a direct evidence for the generation of $^1O_2$ in the $^1\Delta_g$ state.

The chemiluminescence intensity was evidently decreased by the addition of $^1O_2$-quenchers *i.e.* sodium azide, methionine, histidine, tyrosine and tryptophane. Table II lists representative data for dopamine and adrenaline. As seen from the table DABCO, which is known to enhance the emission from $^1O_2$ [10], seems to be ineffective in quenching of the light emission. The above fact is consistent with the recent tendency in questioning of the use of DABCO as a test for the presence of $^1O_2$ [11].

In order to demonstrate additionally the participation of the $^1O_2$ mechanism in the oxidation process of catecholamines we have also applied a spectrophotometric method [8]. Since this method appears to be very sensitive it can be used even for autoxidation reactions. Fig. 2 shows the bleaching of *p*-nitrosodimethylaniline (RNO) in the RNO + imidazole (A) + catecholamine (CAT) + Co^{2+} + HO^- system (curves 1) and in the RNO + A + CAT + Co^{2+} + HO^- + H_2O_2 system (curves 2). In the absence of imidazol the loss of RNO was not measurable. Thus neither H_2O_2 nor $^1O_2$ formed during the decomposition of

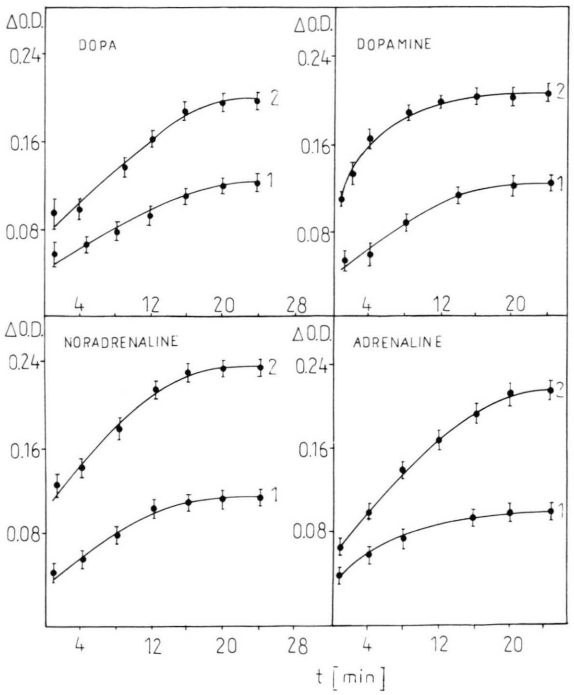

Fig. 2. Time course of the bleaching of RNO in the RNO + A + CAT + Co^{2+} + HO^- system (curves 1) and in the RNO + A + CAT + Co^{2+} + HO^- + H_2O_2 system (curves 2). Reaction conditions: 0.45 mM RNO, 1 mM CAT, 60 mM A, 0.5 mM $CoCl_2$, 10 mM H_2O_2. ΔO.D. represents the difference in optical density of the above systems and CAT + Co^{2+} system. Bleaching of RNO was followed at 440 nm, an absorption maximum of RNO (0.1 cm cell). The size of the bars approximates the S.D.

H_2O_2 could cause the bleaching of RNO. This indicates that this method can be used to the detection of $^1O_2$ in CAT peroxidation reactions.

It is evident that during peroxidation of catecholamines the bleaching of RNO was increased. This result suggesting the formation of $^1O_2$ at much higher concentration is consistent with the observed increase of the light intensity and quantum yield of chemiluminescence in the presence of H_2O_2 [2].

The bleaching of RNO is strongly dependent on both the initial concentration of catecholamine and H_2O_2 as well as on the time of reaction. Fig. 3 shows, as an example, the changes of ΔO.D. in the presence of adrenaline. The bleaching of RNO was almost maximal with 8 mM adrenaline (part A). In contrast to adrenaline, 100 mM H_2O_2 did not cause the saturation (part B). Furthermore, the value of ΔO.D. (0.32) for $[H_2O_2]$ = 100 mM and 1 mM adrenaline at t = 660 s was 4 − times less than for $[H_2O_2]$ = 10 mM

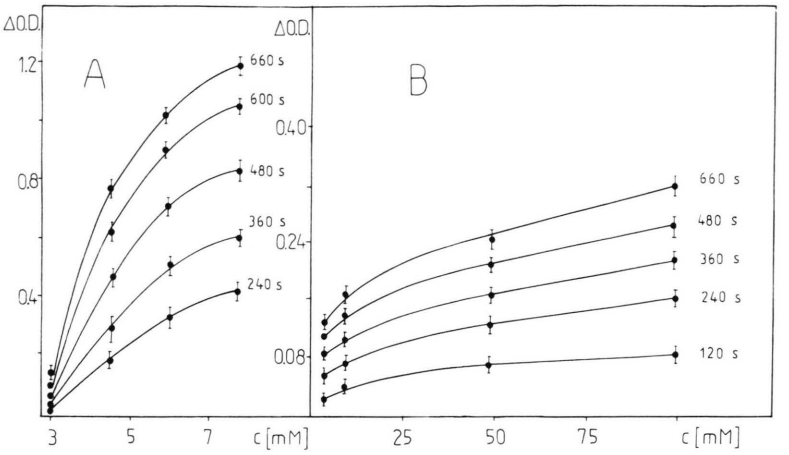

Fig. 3. Effect of the adrenaline (part A) and H_2O_2 concentrations (part B) on the bleaching of RNO in the RNO + A + adrenaline + Co^{2+} + H_2O_2 system. Experimental conditions were as described in Fig. 2.

and 8 mM adrenaline (ΔO.D. = 1.2). These results suggest that the decrease of [RNO] is more sensitive to adrenaline concentration that to $[H_2O_2]$.

Hydrogen peroxide added to the RNO + A + Co^{2+} + HO^- system also causes the bleaching of RNO (Fig. 4A). The bleaching depends on the initial concentration of H_2O_2 (Fig. 4B) and pH (the data are not shown). The decomposition of H_2O_2 under these conditions was found to be accompanied by chemiluminescence with a quantum yield of about 10^{-12} hν/H_2O_2 molecule (estimated in the 400–850 nm region), and $^1O_2$ was proposed to be an emitter [12]. The dependence of the RNO bleaching on the concentration of H_2O_2 was the same as the dependence of quantum yield of chemiluminescence *versus* $[H_2O_2]$ found in [12]. Unfortunately, no emission in the 1000–1300 nm region was observed from H_2O_2 + Co^{2+} + HO^- reaction, since the light intensity emitted was below the sensitivity of our apparatus.

The data presented in Fig. 2 and Fig. 4A show that maximum values of the RNO bleaching (measured 22 min after the beginning of the reaction) in the RNO + A + CAT + Co^{2+} + HO^- + H_2O_2 system were on average 2 times greater than that in the RNO + A + Co^{2+} + HO^- + H_2O_2 system under the same conditions.

The spectrophotometric observations strongly support the important role of catecholamines in the generation of $^1O_2$ in both autoxidation and peroxidation reactions.

Discussion

The results of this paper reveal two basic aspects, namely, a) a generation of $^1O_2$ in the oxidation reactions of CAT by the molecular oxygen ($^3O_2$), and b) the formation of this reactive species during catecholamines oxidation by H_2O_2.

Singlet oxygen generated in both cases is believed to be a product of the interaction between the reduced oxygen intermediates such like: superoxide anion radical ($O_2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$) and H_2O_2.

The reaction predicted by the theory, according to the Misra and Fridovich mechanism for adrenaline

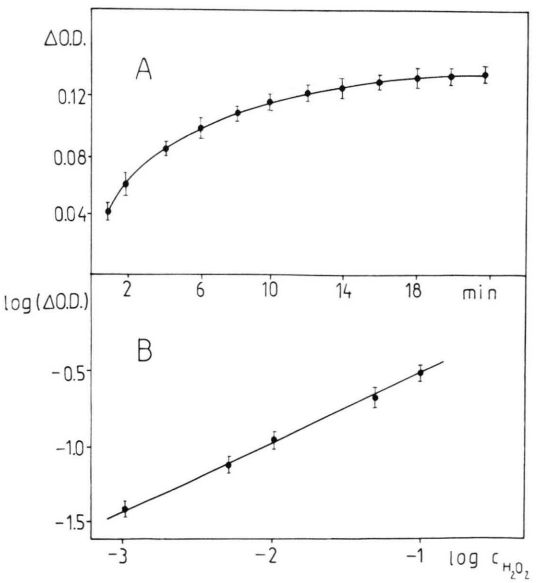

Fig. 4. Dependence of the RNO bleaching on the time (part A) and on the concentration of H_2O_2 (part B) in the RNO + A + Co^{2+} + HO^- + H_2O_2 system. ΔO.D. in part B were measured for t = 11 min. Assay conditions as in Fig. 2.

autoxidation [13], in which the oxygen intermediates can be generated, may be listed as follows:

$$CAT + HO^- \longrightarrow CAT^- + H_2O \quad (1)$$
$$Co(II) + O_2 \longrightarrow Co(III) + O_2^{\cdot} \quad (2)$$
$$CAT^- + Co(III) \longrightarrow SQ(CAT)^{\cdot} + Co(II) \quad (3)$$
$$SQ(CAT)^{\cdot} + O_2 \longrightarrow Q(CAT) + O_2^{\cdot} + H^+ \quad (4)$$
$$CAT^- + O_2^{\cdot} + 2H^+ \longrightarrow SQ(CAT)^{\cdot} + H_2O_2 \quad (5)$$
$$CAT + O_2 \longrightarrow SQ(CAT)^{\cdot} + O_2^{\cdot} + H^+ \quad (6)$$

where CAT is the catecholamine molecule, $SQ(CAT)^{\cdot}$ denotes semiquinone free radical of CAT and Q(CAT) is the quinone of CAT.

Moreover, CAT undergo ring closure forming 5,6-dihydroxyindoles compounds as well as the corresponding semiquinone radical (SQ) and quinones (Q) of these compounds [2]. Thus, the similar reactions generating $O_2^{\cdot}$ and H_2O_2 molecules can be repeated for the above-mentioned intermediates.

$$Q(CAT) + O_2^{\cdot} + H^+ \longrightarrow SQ^{\cdot} + H_2O_2 \quad (7)$$
$$SQ^{\cdot} + O_2 \longrightarrow Q + O_2^{\cdot} + H^+. \quad (8)$$

The first step is the dissociation of the hydroxyl group of phenolic ring of catecholamine into its anionic form, and electron transfer from formed anion to the ground state of oxygen molecule ($^3O_2$) yielding $O_2^{\cdot}$. These reactions constitute a reaction chain in which $O_2^{\cdot}$ and $SQ^{\cdot}$ are the propagating species. The superoxide anion radical can also be generated during the autoxidation of catecholamines (reaction 6). It was established that the reactions generating $O_2^{\cdot}$ were the rate-limiting step because addition of superoxide dismutase inhibited the rate of catecholamines oxidation and the light emission [2].

It is also worth-while to notice that the chemiluminescence intensity was decreased by the introduction of a nitrogen stream and it was highly enhanced by the introduction of an $^3O_2$ stream.

Hydrogen peroxide produced during autoxidation of catecholamines in the presence of metal ions generates another very reactive oxygen species such as the $HO^{\cdot}$ radical [14, 15].

$$H_2O_2 + Co(II) \longrightarrow Co(III) + HO^- + HO^{\cdot}$$
$$Co(III) + H_2O_2 \longrightarrow Co(II)OOH^- + H^+.$$

Our previous experiments showed that H_2O_2 also plays a very important role in the autoxidation of catecholamines since catalase and $HO^{\cdot}$-inhibitors clearly diminished catecholamine oxidation [2].

The second aspect of this work refers to the peroxidation of catecholamines. As it was mentioned in the section describing the results the addition of H_2O_2 to the $CAT + Co^{2+} + HO^- + {}^3O_2$ system increased both the rate of catecholamines oxidation as well as the light emission, especially in the red region of the chemiluminescence spectrum. The correlation between the increase of the light emission and H_2O_2 concentration was observed. Next, chemiluminescence spectra measured during the autoxidation and peroxidation of catecholamines contained the same bands corresponding to the emission from $^1O_2$ dimoles.

The experimental results and the literature data allow to suggest the mechanism of the $^1O_2$ generation which is common for both the catecholamine autoxidation and peroxidation reaction:

a) The base catalyzed disproportionation of H_2O_2 [14]

$$H_2O_2 + HO^- \longrightarrow HOO^- + H_2O$$
$$H_2O_2 + HOO^- \longrightarrow H_2O + HO^- + {}^1O_2;$$

b) the interaction of $O_2^{\cdot}$ and H_2O_2 in the presence of transition metal ion through the modificated Haber-Weiss reaction [16, 17]

$$O_2^{\cdot} + H_2O_2 \longrightarrow HO^- + HO^{\cdot} + {}^1O_2;$$

c) the interaction of $O_2^{\cdot}$ and $HO^{\cdot}$ [18]

$$O_2^{\cdot} + HO^{\cdot} \longrightarrow HO^- + {}^1O_2;$$

d) the interaction of $O_2^{\cdot}$ and $HO_2^{\cdot}$ [19]

$$O_2^{\cdot} + HO_2^{\cdot} \longrightarrow HOO^- + {}^1O_2;$$

e) the spontaneous and catalyzed disproportionation of $O_2^{\cdot}$ [20, 21]

$$O_2^{\cdot} + O_2^{\cdot} \xrightarrow{+\,2H^+} H_2O_2 + {}^1O_2$$
$$^1O_2 + {}^1O_2 \longrightarrow (^1O_2)_2.$$

In summary, we have presented the results of the first measurement of (0.0) ($^1\Delta_g \rightarrow {}^3\Sigma_g^-$) chemiluminescence during reactions of catecholamine peroxidation as an evidence for the generation of $^1O_2$.

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