

Evidence for the Generation of Singlet Molecular Oxygen during Dopa and Dopamine Peroxidation

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Participation of singlet molecular oxygen ($^1\text{O}_2$) in peroxidation of dopa and dopamine was studied by measurements of chemiluminescence spectra, the influence of solvents with various lifetime of O_2 ($^1\Delta_g$) and O_2 ($^1\Delta_g$)-quenchers on quantum yield of chemiluminescence.

A decrease of absorption and fluorescence intensities of 1,3-diphenylisobenzofuran (DPBF) was also studied in the presence of dopa and dopamine oxidized with alkaline H_2O_2 as a criterion for the involvement of $^1\text{O}_2$.

It is well known that low-level chemiluminescence (CL) accompanies to the autoxidation process of some biological important polyphenols [1–3]. The same chemiluminescent phenomena can be expected to occur under physiological conditions.

We have recently observed the light emission and the generation of potential cytotoxicity radicals such as $\text{O}_2^{\cdot-}$, HO^{\cdot} and H_2O_2 during oxidation of biogenic catecholamines [4, 5]. The spectroscopic data indicated that another highly reactive species *e.g.* singlet molecular oxygen, abbreviated $^1\text{O}_2$, is one of the CL emitters.

It is noteworthy that the catecholamine regulatory systems are widespread in the Animal Kingdom and particularly in Insects such as honeybees, where strong hyperglycemic responses have been evidenced as a characteristic consequence of the *in vivo* injection of aminergic derivatives [6]. In this case, it has been observed that rapid alterations of the injected solutions may occur and result in changes of the hyperglycemic response, involving the quenching of glucose increase and a delay in the trehalose peaking [7]. Obviously, if $^1\text{O}_2$ were to be generated in oxidation reactions of the natural aminergic hormones, and come across the regulatory process, then this species as well as their precursors ($\text{O}_2^{\cdot-}$, HO^{\cdot}) would influence the hyperglycemia level.

In this paper we describe further chemiluminescent-evidence for the generation of $^1\text{O}_2$ in dopa and dopamine peroxidation reactions.

Materials and Methods

Dopamine, was obtained from EGS-Chem. (Germany); D,L-dopa Aldrich; 1,3-diphenylisobenzofuran (DPBF) twice recrystallized from benzene was Fluka AG, Buchs; heavy water (D_2O) 99.8% was from IBI Świerk (Poland).

Compounds employed as quenchers of $^1\text{O}_2$ were obtained from Merck. Other reagent were of analytical grade from POCH, Gliwice (Poland). The pD values of the heavy water solutions were measured with a glass electrode using phosphate salts dissolved in D_2O and applying a correction of +0.4 pH. Universal buffer according to Britton and 0.15 M phosphate buffers were used. The solutions were prepared before experiments in redistilled water.

Chemiluminescence intensity was measured by means of a M12FQC51 photomultiplier with S20 cathode operating jointly with a K-200 recorder (G.D.R.). The quantum yields of CL were determined by the method of Stauff and Schmidkunz [8].

In spite of low quantum yields of dopa and dopamine chemiluminescence, conventional equipment could not be used in order to perform a spectral analysis of the emitted light. Therefore, a set of cut-off filters GOST9411–66 consisting of coloured glass and an EMI9558QB photomultiplier sensitive in the

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range 180–800 nm cooled to 203 K were used for that purpose according to the method described by Vassiliev [9]. The single photon counting technique was applied. The width of each rectangle is equal to the half spectral width of the difference in transmission of each pair of filters providing one experimental point.

Fluorescence spectra were measured with a Hitachi MPF-3 spectrofluorimeter and fluorescence kinetics with a spectrofluorimeter consisting of monochromators SPM-2, M12FQC51 photomultiplier and K-200 recorder.

The absorption measurements were made using a Zeiss Specord UV-VIS spectrophotometer.

All experiments were done at least in triplicate. Data are reported as the mean \pm S.D.

Results

The main criterion for the involvement of $^1\text{O}_2$ in chemical and biological systems apart from spectral analysis is a demonstration of an enhancement of chemiluminescence quantum yield (θ_{CL}) as well as maximum intensity (I_{max}) in solvent in which $\text{O}_2(^1\Delta_{\text{g}})$ has longer lifetime (τ) than in water [10, 11].

The second very important criterion for the presence of this very reactive species is the inhibition of the above mentioned parameters of CL by the specific quenchers of $^1\text{O}_2$ [12, 13]. We have used both criteria as a diagnostic test of the intermediacy of $^1\text{O}_2$.

Quantum yields

The autoxidation of alkaline solutions of dopa and dopamine in the presence of molecular oxygen is accompanied by CL. The quantum yields of these reac-

tions were estimated to be about 10^{-12} photons/catecholamine molecule. The addition of H_2O_2 to the above solutions of dopa and dopamine leads to a large increase in the light intensity as well as the quantum yields. Both values have been measured in several solvents, and these values are collected in Table I. The data obtained indicate the increase of luminescence in methanol and ethanol in comparison with water by the factors which correlate with lifetimes of $^1\text{O}_2$ in these solvents [10]. In the case of D_2O the observed increase in emission intensity and quantum yield is lower than that expected from the $\tau_{\text{D}_2\text{O}}/\tau_{\text{H}_2\text{O}}$ ratio. These values are clearly destored due to the following factors: D_2O solutions contained more than 0.5% H_2O . The lifetime of $\text{O}_2(^1\Delta_{\text{g}})$ ranges from 3.1 μs in H_2O to approximately 67.0 μs in D_2O [14], so radiationless decay of $\text{O}_2(^1\Delta_{\text{g}})$ is very fast in water. Moreover, the rate constant of the H_2O_2 decomposition is less in D_2O than in H_2O as well as the amounts of formed $^1\text{O}_2$ escaping into gas bubbles may be also different in both solvents [10]. This can explain why the observed increase in the CL intensity is not such high as it results from the prolonged lifetime of $^1\text{O}_2$ in deuterated solvent.

If the measured emission comes from radiative deactivation of $^1\text{O}_2$ -dimols the influence of $\text{O}_2(^1\Delta_{\text{g}})$ lifetime (τ) in different solvents on the quantum yield (θ_{CL}) could be described by the following expression [15];

$$\sqrt{\theta_{\text{CL}}} = \alpha \cdot \tau,$$

where $\alpha = \text{const.}$ for a given substrate system.

The plot of $\sqrt{\theta_{\text{CL}}}$ vs. lifetime of $\text{O}_2(^1\Delta_{\text{g}})$ in the linear scale is represented by a straight line for both investigated systems (Fig. 1). The results of these kinetic experiments confirm the generation of $^1\text{O}_2$ as the major light-emitting species.

Table I. The influence of solvents on the maximum intensity (I_{max}) and the quantum yield (θ) of chemiluminescence. Conditions: 1 mM dopa, 1 mM dopamine, 0.5 mM CoCl_2 , 10 mM H_2O_2 , pH 11.2, temperature 310 K. Lifetimes of $\text{O}_2(^1\Delta_{\text{g}})$ were taken from [10, 14]. AH denotes dopa or dopamine molecule.

Solvent	$\tau [\mu\text{s}]$	I_{max}		$\theta \times 10^{10}$		hv/AH	
		Dopa	Dopamine	Dopa	Dopamine	Dopa	Dopamine
H_2O	3.1 ± 0.3	96 ± 7	236 ± 17	7.3 ± 0.7	78.0 ± 8		
CH_3OH	7.0 ± 0.7	556 ± 21	3066 ± 140	288.0 ± 15	229.0 ± 12		
$\text{C}_2\text{H}_5\text{OH}$	10.8 ± 1.0	2710 ± 85	25055 ± 1100	1210.0 ± 60	322.0 ± 16		
D_2O	67.0 ± 7.0	296 ± 14	610 ± 53	9.9 ± 1.0	108.0 ± 10		

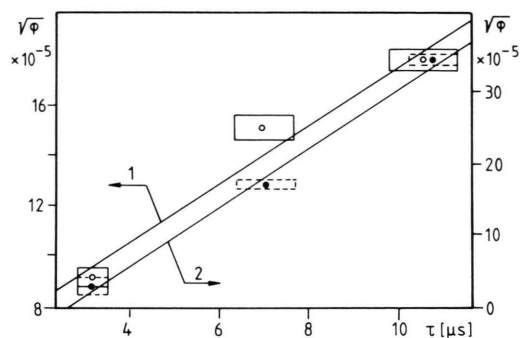


Fig. 1. Plots of $\sqrt{\phi}$ vs. lifetime of solvent for dopamine (1) and dopa (2). Under the same conditions as in Table I. Horizontal segments approximate the experimental errors of lifetimes determination taken from [10, 14]. Vertical segments represent standard deviation ($n=5$).

Spectral distribution of chemiluminescence

The spectral distribution of CL from dopa and dopamine was measured in different solvents to check the enhancement of $^1\text{O}_2$ -dimols emission due to the solvent kind (Fig. 2). The spectra are spread through the full visible region and have four emission maxima at around 480–500, 580, 640 and 700 nm. The fluorescence bands of the reaction mixture match only the broad blue-green band of CL spectra

(depicted as a dotted line). General properties of CL from dopa and dopamine peroxidation in water and D_2O were described in previous papers [4, 5], and the blue-green bands were attributed to the fluorescence of the dopa and dopamine oxidation products. Also an emission from excited singlet and/or triplet carbonyl-containing products formed during the decomposition of a dioxetane intermediate may also be involved in the 440–560 nm region [16]. As is well known, excited triplet states are strongly quenched by molecular oxygen, this, indeed, was present at large concentration in the investigated reactions. Hence, one can suppose that the contribution of this emitter to the 480 nm band is rather small. The contribution to a total emission of the red light CL bands around 640 and 700 nm depends on the kind of the solvent used and it reaches the greatest value in ethanol. The red bands intensity increase distinctly with lifetime of $\text{O}_2(^1\Delta_g)$ in applied solvents. Emission bands at 580, 640 and 700 nm are found to be the luminescence from $^1\text{O}_2$ -dimols $2\ ^1\Delta_g \rightarrow 2\ ^3\Sigma_g^-$. Additionally, a weak CL can participate at 480 nm, which corresponds to the $^1\Delta_g, ^1\Sigma_g^+ \rightarrow 2\ ^3\Sigma_g^-$ transition.

The effect of $^1\text{O}_2$ -quenchers

In order to elucidate the contribution of $^1\text{O}_2$ to the observed CL from our systems, the influence of some

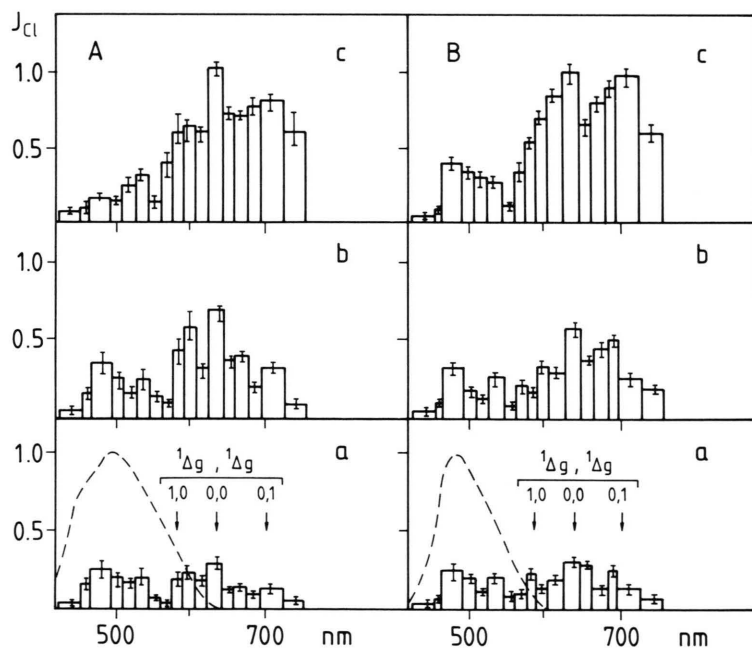


Fig. 2. Spectral distribution of chemiluminescence and fluorescence from the reaction of dopa (A) and dopamine (B) with H_2O_2 in H_2O (a), D_2O (b), $\text{C}_2\text{H}_5\text{OH}$ (c). Experimental conditions as in Table I. The spectra were measured after 20 min from the beginning of dopa and dopamine peroxidation. The height of the rectangle corresponds to the mean value of 10 measurement of the relative intensity. The size of the bars approximates the S.D.

$^1\text{O}_2$ -quenchers has been investigated quantitatively. The quenchers added to the reaction mixtures reduced the CL intensity and the quantum yield according to the Stern-Volmer equation. The rate constants for the quenching of $^1\text{O}_2$ (k_q) are summarized in Table II. The values for k_q determined in this work do not differ significantly from those reported by the other authors [13]. The obvious conclusion is that the quenchers reactivity is similar in each of the investigated systems (with dopa and dopamine), but that the precision for the determination of k_q by our method is not excellent. Other quenchers of $^1\text{O}_2$ as DPBF, hydroquinone, tryptofan, also quench clearly CL of dopa and dopamine, but their effect has been studied only qualitatively.

DPBF is widely used as the $^1\text{O}_2$ scavenger. Its usage is based on the decrease of the absorption as well as fluorescence due to the oxidation by $^1\text{O}_2$ [17]. The rate of disappearance of DPBF was followed by monitoring the decrease in absorption at 415 nm and fluorescence intensity at 458 nm in the presence of different reactants of CL mixture as a function of time (Fig. 3). The rate of DPBF bleaching depends on the presence of particular reactants and it is the highest for the systems exhibiting the greatest values of the maximum intensity and the quantum yield of CL *e.g.* for the dopa or dopamine + Co^{2+} + H_2O_2 + pH system.

The system of Co^{2+} + H_2O_2 + pH also causes the bleaching of the DPBF absorption as well as the fluorescence quenching of DPBF, since it emits CL

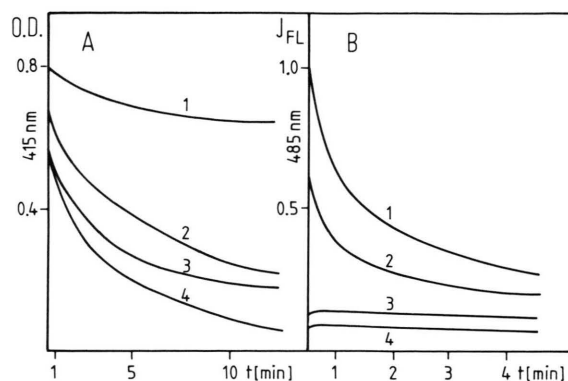


Fig. 3. Changes of absorption (A) and fluorescence (B) intensity during 1,3-diphenylisobenzofuran (DPBF) oxidation in the presence of different substrates combination. Curves: 1-DPBF alone; 2-DPBF + Co^{2+} + H_2O_2 ; 3-DPBF + Co^{2+} + H_2O_2 + dopamine; 4-DPBF + Co^{2+} + H_2O_2 + dopa. The initial concentrations of DPBF were: 0.1 mM during absorption measurements (A) and 5 μM during kinetics of fluorescence (B). Concentrations of remain substrates: 0.5 mM CoCl_2 , 10 mM H_2O_2 , 1 mM dopa and dopamine. All measurements were performed in universal buffer at pH 10.8. Temperature of solutions was 310 K.

and $^1\text{O}_2$ has been postulated as the emitting species [18].

The interaction of DPBF with particular reactants were also investigated spectroscopically. They appeared to be negligible under our experimental conditions, then the obtained results suggest that this effect is caused by $^1\text{O}_2$.

Discussion

The overall results of this investigation is that $^1\text{O}_2$ appear to be produced during dopa and dopamine peroxidation and this species acts as a major emitter of the CL. This conclusion is based on the observation that CL is diminished by the $^1\text{O}_2$ -quenchers (Table II) and is strongly enhanced in solvents in which $\text{O}_2(^1\Delta_g)$ has longer lifetime than in water (Table I, Fig. 1 and Fig. 2). Anterior measurements of fluorescence of catecholamines oxidation products [4, 5] show the emission in the 440–630 nm region, then the fluorescence spectrum does not match the red CL.

The light emission from the present systems was also quenched by free radical inhibitors, such as $\text{O}_2^{\cdot-}$, HO^{\cdot} and catalase [4, 5]. The maximum intensity as well as the quantum yield increased rapidly for higher values of H_2O_2 concentrations as well as for higher

Table II. Effect of $^1\text{O}_2$ -quenchers on chemiluminescence from dopa and dopamine peroxidation. Reaction mixtures contained: 1 mM dopa, 0.5 mM CoCl_2 , 10 mM H_2O_2 , universal buffer according to Britton pH 11.2. Reaction conditions for the dopamine, were following: 2 mM dopamine, 0.5 mM CoCl_2 , 20 mM H_2O_2 , 0.5 mM KOH.

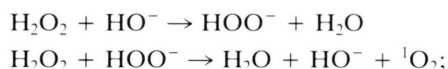
Quencher was added to the reaction mixture before the addition of H_2O_2 . K_q is the total bimolecular rate constant for deactivation of $^1\text{O}_2$. Temperature of solutions was 310 K.

Quenchers	Quenching constant $k_q \times 10^{-8}$ [M $^{-1}$ · s $^{-1}$ · I]	
	Dopa	Dopamine
Methionine	2.1 ± 0.3	4.1 ± 0.2
Histidine	2.8 ± 0.3	9.3 ± 0.3
Sodium azide	0.9 ± 0.2	0.5 ± 0.1
5,5-dimethylcyclohexandione-1,3	1.17 ± 0.1	0.9 ± 0.2
Biliverdine	10.8 ± 1.0	9.1 ± 0.3

values of pH. These results would provide reasonable evidence for the presence of the $^1\text{O}_2$.

$^1\text{O}_2$ would be formed in these reactions in a secondary order, as a product of the interaction of the various intermediates of oxygen reduction. We list below several hypothetical reactions which can involve $^1\text{O}_2$ during the peroxidation of dopa and dopamine.

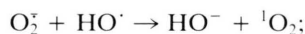
(1) The base catalyzed disproportionation of H_2O_2 [19]



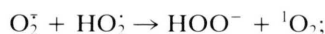
(2) the interaction of O_2^- and H_2O_2 in the presence of transition metal ion (M^{n+}) through the modified Haber-Weiss reaction [20, 21]



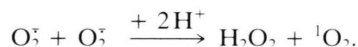
(3) the interaction of O_2^- and HO^\cdot [22]



(4) the interaction of O_2^- and HO_2^\cdot [23]

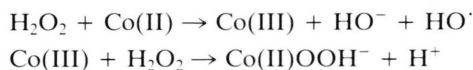


(5) the spontaneous and catalyzed disproportionation of O_2^- [24, 25]

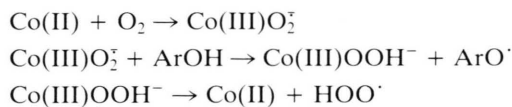


The formation of the abovementioned oxygen species (HOO^- , O_2^- , HO^\cdot) during polyphenols oxidation is well established [26, 27]. It is known that when the light emission from a chemical reaction is induced by O_2^- and H_2O_2 the inhibition of CL by superoxide dismutase, catalase and HO^\cdot -inhibitors as well as its stimulation by H_2O_2 has been observed.

In our experiments there are two independent possibilities for the generation of the HOO^- ions, namely, the decomposition of the H_2O_2 molecules [19, 28],



and a sequence of the following reactions [29]:



where Co denotes cobalt ion, ArOH = dopa or dopamine.

The finding of this work seems to support the idea that there exists the involvement of $^1\text{O}_2$ in this emission. One can expect that the same mechanism takes place *in vivo* on nonenzymatic pathway of catecholamines metabolism.

Some doubt may arise from the fact that not all criteria for the presence of $^1\text{O}_2$ were applied to the investigated systems. The detection of $^1\text{O}_2$ production by observation of monomol CL at 1268 nm and by the ESR which rely on the generation of stable nitroxide radicals upon reaction of $^1\text{O}_2$ with sterically hindered amines provide further proofs of this [30, 31]. These techniques are very sensitive tests for $^1\text{O}_2$ production especially in biochemical systems. Infrared CL around 1268 nm provides unambiguous identification of $^1\text{O}_2$ because of the absence of interference from other CL emitters generated in biological oxidation reactions. In spite of the fact that our model systems are very simple in comparison to biochemical ones, the detection of $^1\text{O}_2$ by the ESR is actually under study, and measurements of CL at 1268 nm is being planned.

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