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# CHEMILUMINESCENCE IN THE REACTION OF CYTOCHROME *c* WITH HYDROGEN PEROXIDE

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**(1) Aqueous solutions of 1–10 μM ferricytochrome *c* treated with 100 μM–100 mM $H_2O_2$ at pH 8.0 emit chemiluminescence with quantum yield $\Phi \simeq 10^{-9}$ and absolute maximum intensity $I_{max} \simeq 10^5$ *hν*/s per cm$^3$ (λ = 440), and exhibit exponential decay with a rate constant of 0.15 s$^{-1}$. (2) The emission spectrum of the chemiluminescence covers the range 380–620 nm with the maximum at 460 ± 10 nm. (3) Neither cytochrome *c* nor haemin fluoresce in the spectral region of the chemiluminescence. In the reaction course with $H_2O_2$, a weak fluorescence in the region 400–620 nm with $\lambda_{max}$ = 465–510 nm ($\lambda_{exc}$ = 315–430 nm) gradually arises. This originates from tryptophan oxidation products of the formylkynurenine type or from imidazole derivatives, respectively. (4) Frozen solutions (77 K) of cytochrome *c* exhibit phosphorescence typical of tryptophan ($\lambda_{exc}$ = 280 nm, $\lambda_{em}$ = 450 nm). During the peroxidation, an additional phosphorescence gradually appears in the range 480–620 nm with $\lambda_{max}$ = 530 nm ($\lambda_{exc}$ = 340 nm). This originates from oxidative degradation products of tryptophan. (5) There are no red bands in the chemiluminescence spectra of cytochrome *c* or haemin. This result suggests that singlet molecular oxygen $O_2(^1\Delta_g)$ is not involved in either peroxidation or chemiluminescence. (6) The haem $Fe^{3+}$ group and $H_2O_2$ appear to be crucial for the chemiluminescence. It is suggested that the generation of electronically excited, light-emitting states is coupled to the production of conformational out-of-equilibrium states of peroxy-Fe-protoporphyrin IX compounds.**

## Introduction

Reaction of certain haemoproteins with $H_2O_2$ are accompanied by the generation of electronically excited species, emitting weak chemiluminsecence in the visible range of the spectrum [1–5]. The appearance of chemiluminescence in similar systems, both model [6,7] and enzymatic, such as myeloperoxidase [8], xanthine oxidase [9] and superoxide dismutase [10] has also been reported. It is proposed that these reactions contribute to the biological chemiluminescences of organelles and tissues [8,11], as well as to the so-called 'dark photobiochemistry' [12,13]. Previous studies showed that chemiluminescence of cytochrome *c* or haematin with $H_2O_2$ is mainly due to oxidative degradation of the porphyrin ring [3–5]. while that of catalase, peroxidase [1,2] and haemoglobin [6,7] also involves oxidation of the apoenzyme moiety. However, there is a lack of the fundamental characteristics such as quantum yield, spectral distribution of the chemiluminescence and photoluminescence spectra of reaction products. These data are necessary for the elucidation of the reaction mechanism. Moreover, spectra of chemiluminescence may be an argument for or against $O_2(^1\Delta_g)$ participation.

These important problems led us to determine the quantum yield and the spectral distribution of luminescence in the reaction of ferricytochrome *c* with $H_2O_2$ under physiological conditions in vitro.

## Materials and Methods

Pig heart cytochrome *c*, molecular weight 12 800 (lyophilized solutions of cytochrome *c* in 0.85% NaCl used for injections give slightly different chemiluminescence kinetics with a small second maximum), was a product of Biomed, Cracow, Poland. Crystalline haematin chloride and bovine serum albumin were obtained from BDH. Porphyrin dimethyl ester IX, acetone and ferricyanide were obtained from Aldrich. Methanol for spectroscopy (Merck) and $H_2O_2$ of special purity for semiconductors were employed. Other chemicals were of analytical grade from POCh Gliwice, Poland. They were additionally recrystallized from water + 1 mM EDTA and finally from water twice distilled in an all-glass apparatus.

The intensity of chemiluminescence was measured using an RCA 5596 or an FEU-18A photomultiplier tube with an S-11 photocathode. The signal was recorded with a K-201 integrating Zeiss recorder. Solutions were rapidly injected (0.1 s) into a hemispherical cuvette mounted on the front of the phototube. The standard concentrations of substrates in the reaction mixture were as follows: 10 mM $H_2O_2$, 10 mM phosphate buffer (pH 8.0), 1 μM cytochrome *c* and water up to 10 $cm^3$. The temperature was 22–25°C. The reproducibility of chemiluminescence peak heights was about 15–20% and each measurement was repeated five to eight times.

Spectral distribution of the chemiluminescence was measured combining the cut-off filter method [14] and the single-photon counting mode. An EMI 9558 QB photomultiplier sensitive in the range 180–800 nm (S-20 trialkali photocathode) was cooled to −70°C. Measurements were performed in the flow system (Fig. 1) with a constant delivery of fresh solution to the observation flow cuvette. This allowed maintenance of a constant reaction time throughout the experiments. The efficiency of photon counting was equal to 0.006 ± 0.02 counts per photon. Emission spectra were corrected for the spectral sensitivity of the photomultiplier and filter transparency using the method described in Ref. 14 and additionally with chemiluminescence reactions [15,16].

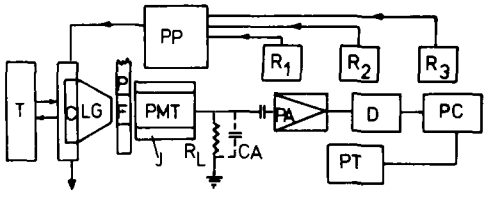


Fig. 1. Block diagram of the system for measurements of chemiluminescence spectra using the single-photon counting method and flow reagent mode. PP, peristaltic pump; $R_1$–$R_3$, reservoirs for solutions; C, flow cell; T, thermostat; LG, light guide; F, cut-off filters; PMT, photomultiplier tube. J, cooling jacket; PA, pulse preamplifier; D, discriminator; PC, pulse counter; PT; preset timer; $R_L$, load resistor; CA, anode circuit capacitance.

Both photoelectric systems were calibrated for the absolute light output in photons (λ = 440 nm) using low-level chemiluminescent standards [15,16]. Linear response of the systems was obtained for luminol concentrations varying from 10 pM to 10 μM that enabled the evaluation of the absolute values of integrated light intensity (light sum), $\Sigma I_a$, and absolute intensity, $I_a$, according to the formulae:

$$\sum I_a = \eta \int_{t=0}^{t=\infty} I(t)\,dt$$

and

$$I_a = \frac{d\left(\sum I_a\right)}{dt}$$

where $I$ is the relative light intensity traced by the recorder and $\eta = (N_{h\nu})_s/\Sigma I_s$. Here $\Sigma I_s$ is the integrated relative intensity equal to the are under the kinetic curve of chemiluminescence, $I = f(t)$, and $(N_{h\nu})_s$ is the absolute number of photons emitted from the standard reaction [15].

All results are expressed as $\bar{x} \pm ts/\sqrt{n_d}$ in which $\bar{x}$ is the mean and $s$ the standard deviation of $n_d$ determinations; $t$ is a factor dependent on $n_d$ and the confidence level. In our studies, $n_d$ varied between 5 and 8, and for the confidence level we chose 95% [17].

Fluorescence and phosphorescence spectra were recorded with a Hitachi MPF-3 spectrofluorimeter. For phosphorescence measurements, samples of cytochrome $c + H_2O_2$ solutions were deoxygenated by bubbling with pure Ar for 40 min and then frozen to 77 K in liquid nitrogen. Methanol/water solutions (8 : 2, v/v) were used.

## Results

*The effects of components, concentration and pH*

Some components of cytochrome *c* and related compounds were tested with $H_2O_2$ for their ability to give chemiluminescence (Table I). It can be seen that neither tryptophan, bovine serum albumin nor Fe salts, e.g., $Fe(CN)_6^{3-}$, give chemiluminescence comparable with that of haemin or haemoproteins. Thus, the haem group and $H_2O_2$ are the necessary and satisfactory components for the production of excited states. However, the protein moiety clearly influences the values of $I_{max}$ and $\Sigma I$ as well as the kinetic parameters of chemiluminescence (cf. Table III). The optimum concentration of cytochrome *c* with respect to $I_{max}$ and $\Sigma I$ is about 12 $\mu$M at 10 mM $H_2O_2$ and pH 8.0. The effect of substrate concentration on the shape of the kinetic curve $I = f(t)$ is small. From the function $I_{max}$ $(\Sigma I) = f([H_2O_2])$ it is found that the maximum light output is obtained for a large excess of $H_2O_2$ equal to $4 \cdot 10^3$. Such a high value implies that some products are formed from $H_2O_2$ which are critical for chemiluminescence. As can be seen from Table II, the light output increases with the increase of pH. This is also valid for Fe salts without a protein moiety. Therefore, not only the ionization state of the protein part, but also that of $H_2O_2$ ($pK_a = 10.8$ for $H_2O_2 \rightleftharpoons HO_2^- + H^+$) contribute to this effect. Changes of pH also differentiate between the effects of the protein moiety on parameters of chemiluminescence kinetics, as is clearly seen for cytochrome *c* but not for haemin (Table III). The interpretation of these data needs further study.

*Quantum yield of chemiluminescence*

The total quantum yield depends on the chemical yield of the reaction, $\Phi_{ch}$, on the excitation yield, $\Phi_{exc}$, i.e., the ratio of the number of excited molecules to that of all molecules formed in the reaction and on the quantum yield of luminescence: $\Phi = \Phi_{ch}\Phi_{exc}\Phi_l$. Having the absolutely calibrated system it was possible to evaluate $\Phi$ from the formula:

$$\Phi = \frac{\left(\sum I_a\right)_{1\,min}}{VcN}$$

where $V$ is the total volume of the light-emitting solution in the observation cell, $c$ is the concentration of cytochrome *c* and $N$ is Avogadro's number. The appropriate value of $\Phi$ is found to be $(8 \pm 3) \cdot 10^{-10}$ photons per molecule of cytochrome *c* for the integration time 1 min. The small value of $\Phi$ results from two factors: (1) a low value of $\Phi_l$, since the fluorescence from the reaction solution is extremely weak, and (2) some fraction of photons emitted is reabsorbed because of the chemiluminescence and the

TABLE I

CHEMILUMINESCENCE FROM CYTOCHROME *c* AND RELATED COMPOUNDS IN THE REACTION WITH $H_2O_2$

10 ml of the reaction solution contained 20 mH $H_2O_2$, 10 mM phosphate buffer, pH 8.0, and the tested compound as indicated. Each result represents the average of five to eight measurements ± S.D.

| Compound | Maximum light intensity, $I_{max}$ (arbitrary units) | Integrated intensity, $\Sigma I_{1\,min}$ (arbitrary units) |
|---|---|---|
| 1.7 $\mu$M ferricytochrome *c* | 163 ± 12 | 136 ± 9 |
| 10 mM $K_3Fe(CN)_6$, pH 9.0 | 8.0 ± 0.8 | 6.5 ± 0.9 |
| 1.7 $\mu$M haemin hydrochloride | 283 ± 17 | 217 ± 14 |
| 1.7 $\mu$M porphyrin IX dimethylester in 3.3 M acetone | 0.5 ± 0.1 | 0.5 ± 0.1 |
| 0.1 mM tryptophan, pH 9.0 | 1.0 ± 0.2 | 1.3 ± 0.4 |
| 1–10 $\mu$M bovine serum albumin | 0.5 ± 0.1 | 0.5 ± 0.1 |
| 10 $\mu$M haemoglobin [a] | 240 | 820 |
| 10 $\mu$M peroxidase [a] | 200 | 500 |
| 10 $\mu$M catalase [a] | 58 | 400 |

[a] Reaction conditions: 20 mM carbonate buffer, pH 8.5, 10 mM $H_2O_2$; temperature 30°C [5].

TABLE II

EFFECT OF pH ON CHEMILUMINESCENCE OF FERRICOMPOUNDS AND HAEMOPROTEINS

4 $\mu$M Fe compound, 2 mM $H_2O_2$ and 8 mM acetate buffer. Each entry represents the average of four to six experiments ± S.D.

| Compound | pH 6.0 | | pH 10.0 | |
|---|---|---|---|---|
| | $I_{max}$ | $\Sigma I_{1\ min}$ | $I_{max}$ | $\Sigma I_{1\ min}$ |
| $FeCl_3$ | 0 | 0 | 3.0 ± 0.4 | 1.6 ± 0.5 |
| $K_3Fe(CN)_6$ | 0 | 0 | 4.6 ± 0.7 | 1.3 ± 0.8 |
| Haemin | 0.8 ± 0.2 | 1.1 ± 0.3 | 6 200 ± 550 | 2015 ± 690 |
| Cytochrome *c* | 10.7 ± 0.3 | 3.0 ± 0.1 | 2000 ± 470 | 1 130 ± 117 |
| Haemoglobin | 4.9 ± 0.6 | 5.7 ± 0.3 | 6 630 ± 180 | 8 130 ± 860 |

TABLE III

PARAMETERS OF THE KINETICS OF CHEMILUMINESCENCE AT VARIOUS pH VALUES

Experimental conditions as in Table II. Subscripts 1 and 2 refer to the first and second maximum in kinetic curves of chemiluminescence.

| Compound | pH 6.0 | | pH 10.0 | | | | |
|---|---|---|---|---|---|---|---|
| | $t_{max}$ (s) | $k$ ($s^{-1}$) | $t_{max\ 1}$ (s) | $t_{max\ 2}$ (s) | $k_1$ ($s^{-1}$) | $k_2$ ($s^{-1}$) | $I_{max\ 1}/I_{max\ 2}$ |
| Haemin | 1.0 ± 0.3 | 0.07 ± 0.003 | 0.1 | – | 0.6 ± 0.1 | – | – |
| Cytochrome *c* | 2.6 ± 0.3 | 0.38 ± 0.07 | 0.1 | 31 ± 6 | 0.5 ± 0.08 | $3.6 \pm 0.5 \times 10^{-2}$ | 9.9 |

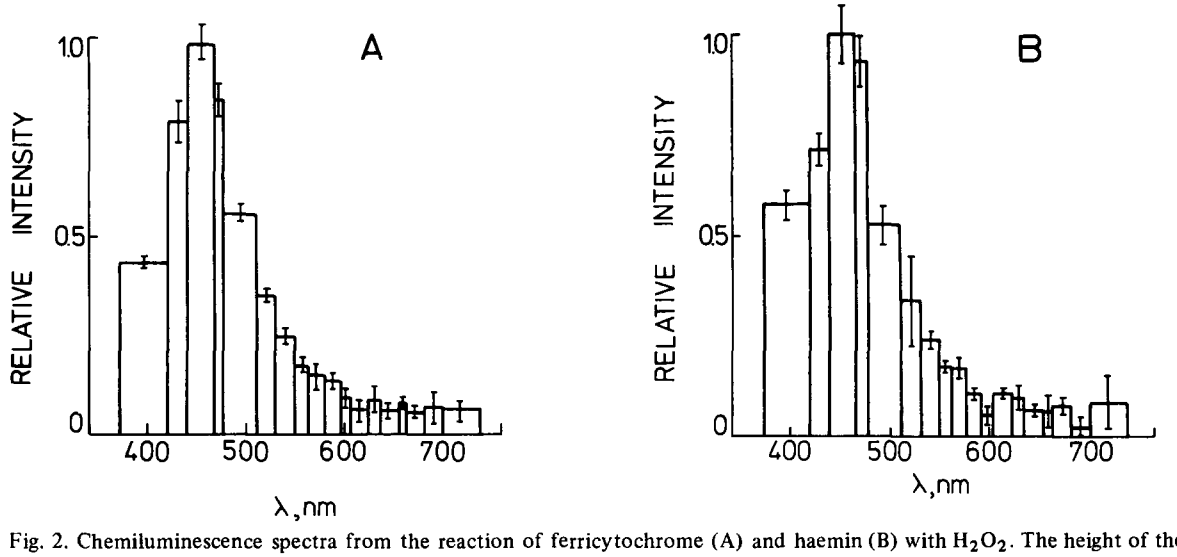


Fig. 2. Chemiluminescence spectra from the reaction of ferricytochrome (A) and haemin (B) with $H_2O_2$. The height of the rectangles is equal to the mean value of the relative spectral intensity computed from six to ten parallel measurements. The length of the vertical segments is equal to the maximum experimental error. The width of rectangles corresponds to the difference in wavelength between two successive cut-off filters. All spectra are corrected for the spectral response of the phototube. The dead time between mixing and observation is 3 ± 0.3 s, concentration of cytochrome *c* or haemin 5 $\mu$M, 10 mM $H_2O_2$, 20 mM phosphate buffer, pH 8.1, temperature 35°C.

Soret absorption band (415 nm) overlapping. The probability of the excited state generation $\Phi_{exc}$, roughly estimated from the above equation, should not exceed $10^{-5}$.

*Chemiluminescence spectra*

The spectral distribution of cytochrome *c* and haemin chemiluminescences (Fig. 2A and B) covers a broad range from 360 to 620 nm with the maximmum at about 465 nm. The chemiluminescence intensity of haemin in the red is slightly higher than that of cytochrome *c*. A resolution of the order of 20 nm is achievable, depending on the pair of cut-off filters. It should be pointed out that the low-intensity chemiluminescence can give rise to spectral 'ghosts' anywhere in the spectra. Particularly, the emission observed in the red might be due to artifacts because of the following factors: (1) decreasing quantum efficiency of photocathodes with increasing wavelength, (2) attenuation of the photo flux by cut-off filters, and (3) some amount of the scattered and reflected light from the chemiluminescence reaction itself as well as from the low-level emission of materials, e.g., filters, shutter and walls of the light-tight camera. The energy of photons emitted varies from 1.8 to 3.1 eV. Therefore, the enthalpy $-\Delta H$ of an elementary exergonic reaction has to be at least 300 kJ · mol$^{-1}$, since $h\nu \leqslant \Delta H$. The spectral halfwidth $\Delta_{1/2}$ of emission bands is 4 300 ± 480 and 3 900 ± 410 cm$^{-1}$ for cytochrome *c* and haemin, respectively. These values are compared to those of photoluminescence and discussed further.

*Photoluminescnce spectra*

Cytochrome *c* in the 25 $\mu$M solution at pH 8.0–9.0, excited at $\lambda$ = 400 nm, exhibits only a spurious fluorescence (Fig. 3). The same situation occurs with haematin. During the peroxidation of cytochrome *c*, a blue fluorescence appears and its intensity gradually increases. The emission spectrum covers the range from 400 to 640 nm ($\lambda_{exc}$ = 315–400 nm) with the maximum at about 465 nm. Values of $\Delta_{1/2}$ vary from 4 950 to 9 600 cm$^{-1}$ depending on $\lambda_{exc}$, and do not change with the reaction time. The reaction system haemin + $H_2O_2$ also shows fluorescence (Fig. 3, insert) in the range 380–600 nm ($\lambda_{exc}$ = 300–430 nm) with the maximum at 505 nm. Using the maximum sensitivity of the spectrofluorimeter, it was not possible to detect any fluorescence from cytochrome *c* or haemin in the raction with $H_2O_2$ in the range

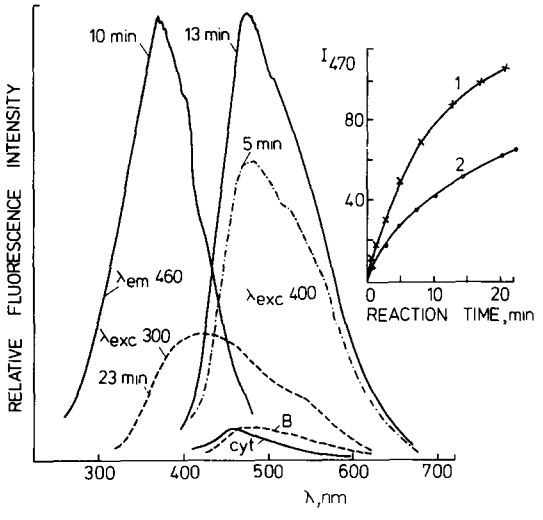


Fig. 3. Fluorescence excitation and emission spectra from ferricytochrome *c*, peroxide solutions. B, fluorescence from buffer and peroxide without cytochrome; cyt, emission from cytochrome alone. Insert: kinetics of the fluorescence from cytochrome (1) and haemin (2) vs. the reaction time with $H_2O_2$. $\lambda_{exc}$ = 400 nm, $\lambda_{em}$ = 470 nm, 2.5 $\mu$M cytochrome or haemin, 15 mM $H_2O_2$, 25°C.

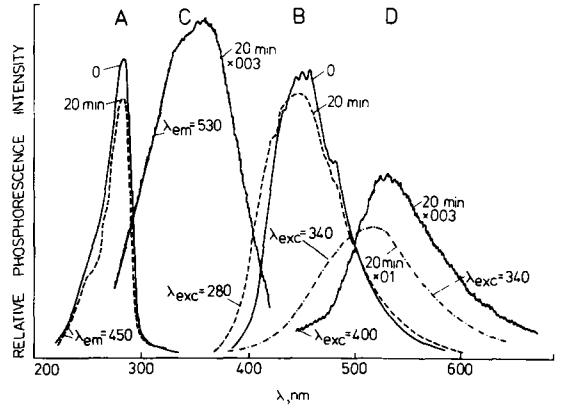


Fig. 4. Phosphorescence excitation and emission spectra of cytochrome *c* with $H_2O_2$. A and B, excitation and emission spectra, respectively, of cytochrome *c* alone (0) and with $H_2O_2$ after 20 min of peroxidation; C and D, excitation and emission spectra from reaction solution after 20 min. Sensitivity factors 0.1 and 0.03 denote that these spectra were recorded with sensitivities 10- and 100-times, higher, respectively, than those without a number.

600–750 nm for $\lambda_{exc}$ = 450–650 nm.

Cytochrome *c* in the methanol/water solution at 77 K exhibits only tryptophan phosphorescence ($\lambda_{exc}$ = 280, $\lambda_{em}$ = 348 nm). During the peroxidation with $H_2O_2$ a new band appears within the range 420–650 nm with the maximum at 525 nm ($\lambda_{exc}$ = 340 nm). As can be seen from Fig. 4, the intensity of tryptophan phosphorescence slowly decreases, while that at 525–530 nm increases. This result indicates a slow destructive oxidation of the Trp-59 residue and the gradual accumulation of formylkynurenine derivatives [18,19]. Under the experimental conditions, the absolute rate $-d[Trp]/dt \simeq 1.6 \cdot 10^{-10}$ M · s$^{-1}$ and the pseudo first-order rate constant is about $8 \cdot 10^{-5}$ s$^{-1}$. Thus, the rate of tryptophan destruction does not correlate with the chemiluminescence, which is about 1 000-times faster than the former. Values of $\Delta_{1/2}$ depend on $\lambda_{exc}$ and range from 2 600 ($\lambda_{exc}$ = 400 nm) to 3 600 cm$^{-1}$ ($\lambda_{exc}$ = 340 nm).

## Discussion

Results of this and previous works (2–7] have demonstrated that chemiluminescnce in the reaction of several haemoproteins with $H_2O_2$ reveals similar kinetics and requirements with respect to reaction conditions and substrates. The rate of the light-producing reaction is of the order of milliseconds or less, the rate constant of the chemiluminescnce decay 0.15 ± 0.02 s$^{-1}$, the absolute rate intensity $10^5$–$10^6$ photons/s per cm$^3$ and the quantum yield of the order of $10^{-9}$. Since maximum light output requires a large excess ($10^2$–$10^4$) of $H_2O_2$, the reaction is pseudo first order and consumes $H_2O_2$ at a rate of 80 μmol/min [3]. Values of $I_{max}$ and $\Sigma I$ increase with pH, suggesting that ionization of $H_2O_2$ and the protonation state of -COOH groups in pyrrole rings of the haem, and perhaps amino acids in the protein moiety, control the chemiluminescence process.

Several points are raised by the spectra data: (1) chemiluminescence spectra of cytochrome *c* and haemin clearly indicate that there are no emission bands at 634 and 704 nm characteristics of the transitions in singlet bimolecular species:

$$(O_2)_2(^1\Delta_g{}^1\Sigma_g^+) \rightarrow 2\,O_2(^3\Sigma_g^-) + h\nu$$

This conclusion is in agreement with the result of thermodynamic calculations on the ferricytochrome *c* + $O_2^-$ system [20] and our preliminary experiments [21]. (2) Chemiluminescnce bands cover the fluorescence emission from oxidation products of cytochrome *c* or haemin. It is known that the oxidative degradation of tryptophan produces derivatives of formylkynurenine, kynurenic, xanthurenic and anthranilic acids which fluoresce in the spectral region 380–560 nm [18,19]. Products of haemin peroxidation also show a weak fluorescence in this region, although fluorescers cannot be identified. Values of $\Delta_{1/2}$ are smaller for chemiluminescence than for fluorescence, since the distribution of the chemiexcitation energy among reaction products is more selective than that of photoexcitation. Phosphorescence spectra of cytochrome *c* + $H_2O_2$, originating from products of the Trp-59 peroxidation, also cover chemiluminescence. Although phosphorescence, i.e., $^1T^* \rightarrow S_0$ transitions are forbidden under the experimental conditions, they might be observed in chemiluminescence due to a very high sensitivity of the single-photon counting method. For all these reasons, the observed chemiluminescence could be formally interpreted as $^1S^* \rightarrow S_0$ and $^1T^* \rightarrow S_0$ transitions from excited reaction products.

However, there are two contradictory facts with respect to point 2, namely: (a) the light emission occurs instantaneously and chemiluminescence decay is also fast. Thus, chemiluminescence kinetics do not fit to those of fluorescence or phosphorescence; (b) the protein moiety of the cytochrome *c* macromolecule is not necessary for chemiluminescence, since light emission is observed from haemin or even Fe(CN)$_6^{3-}$ + $H_2O_2$.

These facts imply that the chemiexcitation and light emission processes are coupled to the rapid reaction between $H_2O_2$ (or $O_2H^-$) and $Fe^{3+}$ protoporphyrin IX.

In this respect, the redox potentials $E_0'$ of the $O_2^-$/$H_2O_2$ and ferri/ferrocytochrome *c* couples are important. They are 0.98 and 0.26 V, respectively. It is evident that $E_0'$ of $O_2^-/H_2O_2$ is too high to be reasonably involved in $Fe^{3+}$ reduction as it is in the case of, e.g., $MnO_4^-$. In the case of two-electron donation, the product will be $O_2$. This redox couple has $E_0'$ = 0.27 V near to the $Fe^{3+}/Fe^{2+}$ of cytochrome *c* and Fe(CN)$_6^{3-}$/$^{4-}$ [20,22]. On the other hand, it is evident that two-electron donation by $H_2O_2$ per Fe ion implies the presence of another one-electron acceptor

of sufficiently high $E'_0$ in the porphyrin ring.

It is well established that catalase, peroxidase and superoxide dismutase produce with $H_2O_2$ a primary peroxide compound [10,23,24]. This process is accompanied by a broad diminution in the Soret band intensity. The same is observed in our chemiluminescent systems with cytochrome *c* or haemin [3,21]. Therefore, one may imply the formation of the ferryl peroxide complex:

$$Fe^{3+} + HO_2^- \rightarrow [Fe^{2+}O_2H] \rightarrow [Fe^{2+}O_2^-] \rightleftharpoons [Fe^{3+}O_2^{2-}] + H^+$$

being responsible for the one-equivalent oxidation. The second oxidizing equivalent might be located at the γ-methene bond of the porphyrin moiety. This center has the lowest π-electron density [25] and is susceptible to the nucleophilic attack of $HO_2^-$, $O_2^-$ or electron transfer. The ferryl peroxide complex is most likely produced in the conformational out-of-equilibrium state [26]. As a result, a reorganization of the haem ligands occurs which perturbs the rhombic symmetry of the complex and brings about changes in levels of the electronic energy. These states might be electronically excited and their radiative deactivation would manifest as the observed chemiluminescence. However, it is necessary to assume that the ground state of the product is chemically different from that of the complex, since the amount of free energy $-\Delta G'_0 = nF\Delta E'_0$ is about 150 kJ/mol (for $n = 2$), i.e., not enough to stimulate the light emission with $\lambda = 465$ nm (236 kJ/mol).

Conformational out-of-equilibrium states of peroxide complexes are expected to be not only powerful biological oxidizing agents which initiate microsomal lipid peroxidation, hydroxylations and peroxide breakdown [27], but also factors enabling performance of the so-called dark photobiochemistry [12,13].

Perhaps the cytochrome *c* or haemin + $H_2O_2$ chemiluminescence might be a simple, adequate model of biological chemiluminescence, since it inheres the most fundamental features of this phenomenon, namely: (1) the generation of electronic excited states by chemienergization, (2) the requirement for oxidative agents and their activation, and (3) the involvement of the electron-transporting metaloproteins.

Moreover, the data obtained are pertinent to mechanisms of the generation of highly reactive cytotoxic oxygenated species involved in cell damage, inactivation of DNA and carcinogenesis [8,28–30].

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