# CHEMILUMINESCENCE ELICITATION FROM AN $O_2$ AND/OR HOOH COMPLEX OF THE RIBOFLAVIN-COPPER(I)-CHELATE[a)]

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SUMMARY: Results are presented which indicate that an intermediate compound which accumulates in a riboflavin, copper (II), ascorbic acid, $O_2$ system, and which gives a chemiluminescence upon the addition of HOOH, is an $O_2$ and/or HOOH adduct of the riboflavin-Cu(I)-chelate. Chemiluminescence has been elicited by HOOH addition to this chelate, prepared anaerobically by ligand-ligand transfer of Cu(I) from $Cu(CH_3CN)_4ClO_4^-$ to riboflavin, and from copper (II), ascorbic acid, and riboflavin. Kinetic data are used to substantiate this thesis.

Vorhaben and Steele (1), while studying the chemical generation of electronic excitation states in a redox system containing riboflavin (rf), ascorbic acid ($H_2A$), Cu (II), and HOOH, reported the accumulation of an intermediate which, upon the addition of HOOH, gave a chemiluminescence (CL). The accumulation was observed first (by JEV) in studies on the above system where HOOH additions to the solution containing rf, $H_2A$, and Cu(II)-$SO_4$, in the presence of air (referred to hereafter as the "incubation-system"), were delayed for several minutes; i.e., the longer the time before the injection of HOOH, the greater the intensity of the CL elicited. If $O_2$ was excluded from the system only a nominal CL was elicitable.

These results are illustrated in Fig. 1 where the "summation-curve" (——) represents the build-up and decay with time of the reactive intermediate which

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reacts with HOOH to give a CL (measured as described by Steele, 2). Stone and Steele (3), in a preliminary report, presented evidence that the compound produced was peroxidic in nature. Further, they concluded tentatively that Cu(II)

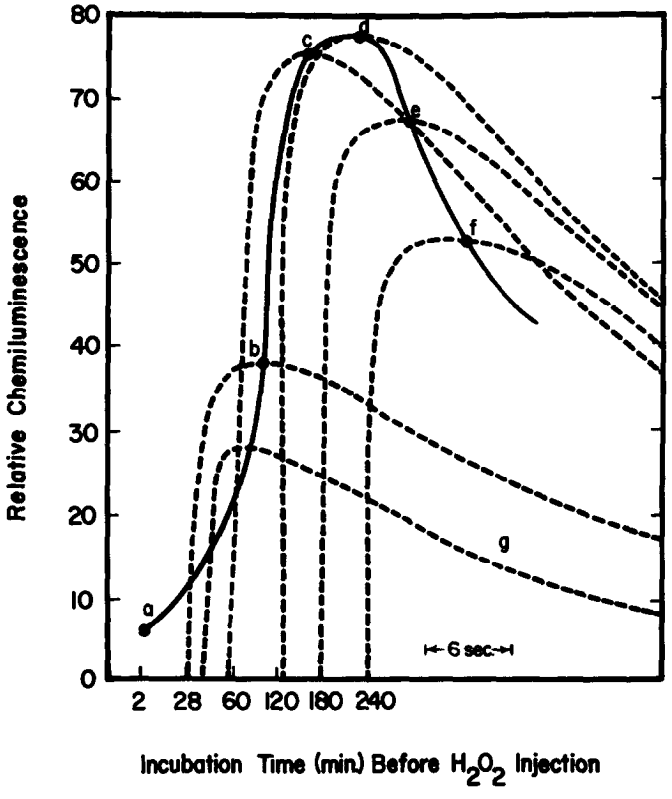


Fig. I. CL elicited from aliquots of an "incubation-system" vs time of incubation, and the rf-Cu(I)-chelate solution by HOOH. "Incubation-system": rf, 5.1 μmoles; $H_2A$, 51 μmoles; Cu(II), 3 μmoles; $PO_4$-buffer, pH 6.2, 1.6 mmoles; incubated under 100% $O_2$; HOOH injected, 5 mmoles; Vol. 15 ml. Rf-Cu(I)-chelate (curve-g) initial concs. in 14.5 ml: rf, 6.1 μmoles; $Cu^+(CH_3CN)_4ClO_4^-$, 6.1 μmoles; $PO_4$-buffer, pH 6.2, 4.1 mmoles; oxygenated for 10 min.; 0.5 ml HOOH, 5 mmoles, injected. Temp. 21°C. 6 sec. = time scale for CL-display for curves b, c, d, e, f, g. CL-curve for point-a not plotted, max peaked off time-scale to right (see Fig. 3).

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was only a catalyst for the formation of the intermediate. However, in an occasional experimental control in which all the reagents had been treated with a chelating agent (Bio-Rad Chelex 100, 100-200 mesh) to remove contaminating metals, particularly Cu and Fe, we were unable to detect the build-up of the intermediate for incubation times as long as 27 hours (ordinarily the

intermediate begins to accumulate immediately upon mixing the reactants, and is maximal, at ambient $pO_2$, in 3 to 4 hours; compare Fig. 1). We became suspicious that Cu is an essential component of the intermediate, and have confirmed this prospect recently by demonstrating that the compound has properties similar to those of the $O_2$ and/or HOOH adduct of the rf-Cu(I)-chelate prepared independently. We report at this time preliminary studies supporting our thesis that the intermediate which accumulates in the "incubation-system" is in fact an $O_2$ and/or HOOH adduct of the rf-Cu(I)-chelate.

An aqueous rf-Cu(I)-chelate solution was prepared anaerobically by ligand-

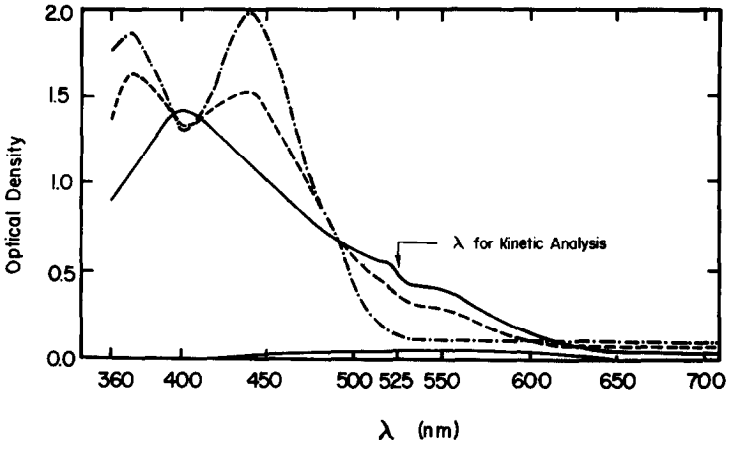


Fig. 2. Absorption spectra (10 mm path) of rf-Cu(I)-chelate prepared in vacuo (——), and exposed successively to $O_2$ (– – –) → (—•—). Initial reactant concs. for formation of rf-Cu(I)-chelate in 5 ml.: rf, 0.7 μmoles; $Cu^{+}(CH_3CN)_4ClO_4^{-}$, 0.7 μmoles; $PO_4$-buffer, pH 6.2, 0.2 mmoles.

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ligand transfer of Cu(I) from an acetonitrile-Cu(I) complex salt to rf to form the rf-Cu(I)-chelate as described by Hemmerich and Sigwart (4); Hemmerich, Müller, and Ehrenberg (5); and Hemmerich and Spence (6). Parenthetically, one of us (MOS) prepared the rf-Cu(I)-chelate in phosphate buffered, pH 6.2, water solution by mixing rf, Cu(II)-$SO_4$, and $H_2A$, or reduced diphosphopyridine-nucleotide (NADH), anaerobically, in a Thunberg tube; i.e., in the anaerobic "incubation-system". This is apparently the same complex reported formed by Baarda and Metzler (7), under slightly different conditions, in a system con-

taining rf, cupric perchlorate, and $H_2A$. The presence of the rf-Cu(I)-chelate was confirmed in both instances titrimetrically, and spectroscopically as described by Hemmerich, Müller, and Ehrenberg (5), and Hemmerich and Spence (6). The spectrum of the rf-Cu(I)-chelate, together with the changes following aeration, are shown in Fig. 2.

Thin layer chromatographic examination (Baker-flex Silica Gel IB plates: solvent, benzene/methanol, 80/20, v/v) of the rf-Cu(I)-chelate prepared anaerobically, and subsequent to 10 minutes oxygenation, showed that the anaerobically prepared sample remained at the origin, while the oxygenated sample displayed a new spot with an $R_f$ of 0.03. Both spots gave a positive test for Cu with the benzoin oxime reagent as described by Feigl (8), and both spots, when scraped from the chromatogram, and "dissolved" in $PO_4$-buffered, pH 6.2, water solution, gave a CL with HOOH, which decreased rapidly with time when monitored with a Nuclear of Chicago Liquid Scintillation Counter. A blank control spot of the chromatogram treated similarly gave no CL. We interpret the new spot on the chromatogram to be an $O_2$ adduct of the rf-Cu(I)-chelate. Support for this view is evident in the enhanced CL and altered kinetics of the oxygenated system relative to the anaerobic system as recorded below. The $O_2$-adduct forms apparently irreversibly for we have been unable to reverse the $O_2$ effect of enhanced CL or altered kinetics, by $N_2$-gassing or evacuation. Spectroscopic and chemical characterization of the $O_2$-adduct must await its isolation.

We present in Fig. 3 pseudo first order kinetic plots of the decreasing CL signals for the results plotted in Fig. 1, together with the CL elicited from the rf-Cu(I)-chelate for a comparison (on a comparable molar basis in rf as used initially in the "incubation-system", and initially to generate the chelate) of its CL intensity and kinetic characteristics with those elicited chronologically from the "incubation-system". From the similarity of the descending rate constants for the CL elicited over a two hour period, compare time base in Fig. 1, it appears that the same reaction is involved in the light reaction in all samples.

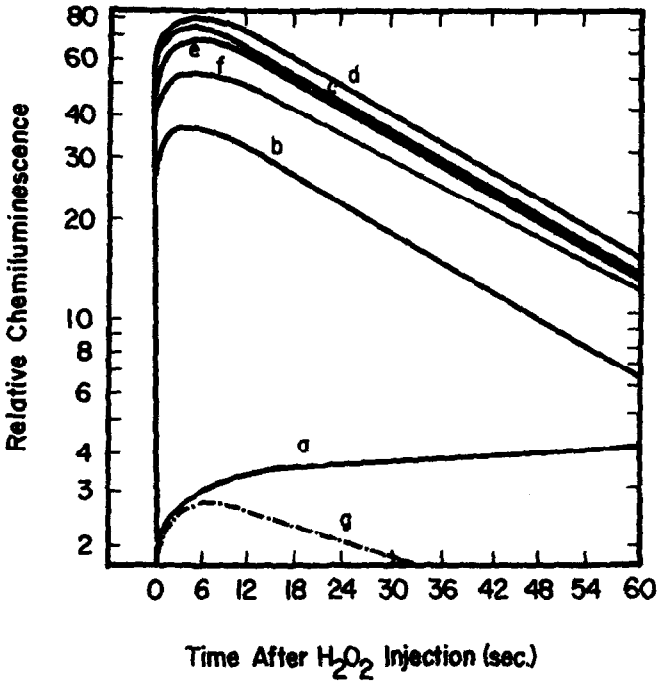


Fig. 3. Kinetic, pseudo first order, plots of the CL-responses presented in Fig. 1. k's in sec.$^{-1}$ for curves-b, -c, -d, -e: 0.033; curve-f: 0.029; curve-g: 0.018.

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In Fig. 4 we present for comparison pseudo first order kinetic plots of the CL responses elicited from: the "incubation-system", curve-a; the rf-Cu(I)-chelate prepared from $Cu^{+}(CH_3CN)_4ClO_4^{-}$ salt described above, oxygenated and unoxygenated (curves-d and -e, respectively); and, the rf-Cu(I)-chelate prepared with Cu(II), $H_2A$, and rf, oxygenated and unoxygenated (curves-b and -c, respectively). The concentrations of rf and Cu were initially the same in both chelate systems. These results reveal three important points:

1) oxygenation enhances markedly the HOOH elicitable CL, compare curve-b with curve-c, and curve-d with curve-e; 2) the similarities in the kinetics for all the oxygenated systems, curves-a, -b, and -d indicate that the same species are involved in the light reaction in all systems; and 3) the kinetic "break" in the anaerobic systems, curves-c and -e, indicates that different species are reacting shortly after the initial decay. We interpret the initial kinetic

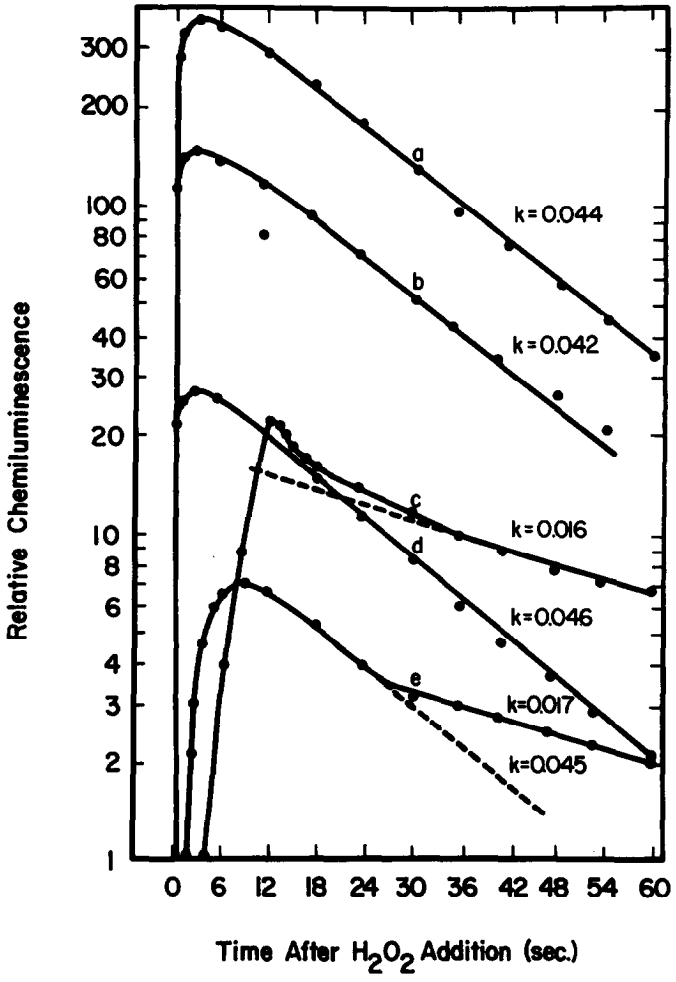


Fig. 4. Kinetic, pseudo first order, plots of the CL elicited by $H_2O_2$ additions to: 1) the "incubation-system", curve-a; concs. in 14.5 ml: rf, 4.78 µmoles; Cu(II), 3 µmoles; $H_2A$, 58 µmoles; $PO_4$-buffer, pH 6.2, 1.6 mmoles; incubated 60 min. under 100% $O_2$; 0.5 ml, 5 mmoles, HOOH injected; 2) the rf-Cu(I)-chelate prepared in vacuo, concs. in 14.5 ml: rf, 5.85 µmoles; Cu(II), 5.85 µmoles; $H_2A$, 16.2 µmoles; $PO_4$-buffer, pH 6.2, 0.4 mmoles; curve-b oxygenated 10 min, curve-c unoxygenated; CL elicited by injection 0.5 ml, 5 mmoles, HOOH into both; 3) the rf-Cu(I)-chelate prepared in vacuo, concs. in 14.5 ml: rf, 5.85 µmoles; $Cu^+(CH_3CN)_4ClO_4^-$, 5.85 µmoles; $PO_4$-buffer, pH 6.2, 0.4 mmoles; curve-d, oxygenated for 10 min., curve-c unoxygenated; CL elicited by injection of 0.5 ml, 5 mmoles, HOOH. Temp. 21°C.

similarity in the anaerobic systems (curves-c and -e) with those in the aerobic systems (curves-a, -b, and -d) as due to rapid partial oxygenation of the chelates by the $O_2$ added with, and/or generated by, the HOOH added to elicit the CL.

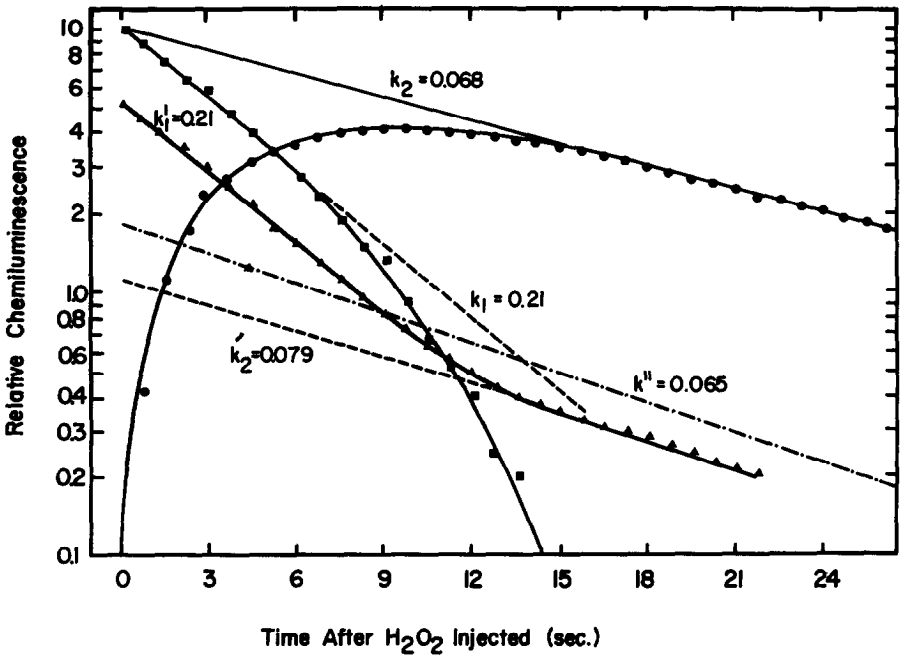


Fig. 5. Combined kinetic analysis, plotted semilog. of the CL response (—●—), and the absorbancy change (—▲—) at 525 nm, elicited from different aliquots of the same rf-Cu(I)-chelate solution, prepared anaerobically, by HOOH injection. Initial concs. in 17.5 ml: rf, 3.5 μmoles; $Cu^{+}(CH_3CN)_4ClO_4^{-}$, 3.5 μmoles $PO_4$-buffer, pH 6.2, 0.48 mmoles; HOOH after injection, 2.9 mmoles. Temp. 21°C. See text for explanation. (—•—•—) = CL response, elicited by HOOH injection, from a sample of an "incubation-system" purified by silicic acid column chromatography, solvent, benzene/methanol 80/20, v/v and plotted for comparison; Reactant concs. in 15 ml: rf, 7 μmoles; $H_2A$, 21 μmoles; $PO_4$-buffer, 0.4 mmoles; Cu(II), 1.5 μmoles; $H_2O_2$, 0.5 ml, 5 mmoles.

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We present in Fig. 5 a combined kinetic analysis, as described by Steele and Breckenridge (9), plotted semilogarithmically, of the CL response —●—, and the absorbance changes, —▲— , monitored at 525 nm, elicited from different aliquots of the same rf-Cu(I)-chelate solution by the injection of HOOH.

It is apparent that the pseudo first order decay constant, $-k_1^1$, for the disappearance of the chelate is equal to the formation constant, $k_1$, for the intermediate reactant ($O_2$ and/or HOOH adduct of the chelate) which reacts then with excess HOOH at the rate defined by $k_2$ to give light. This mechanism may be schematized as:

$$\text{rf-Cu(I)-chelate} \underset{k_1}{\overset{-k_1'}{\overset{O_2/HOOH}{\longrightarrow}}} \text{Adduct} \xrightarrow[k_2]{HOOH} \text{light} + \text{products.}$$

We cannot at this time rule out unambiguously the possibility that the "adduct" may be a peroxide or perhydroxyl radical of rf. The Cl-spectrum for this reaction, to be reported elswhere, extends from 400 to 650 nm, and peaks at 520 nm. This spectrum delimits the minimum exothermicity of the reaction as requiring 75 kcal. (3.1 electron volts), and is greater than that displayed in rf fluorescence which has its 0-0 transition at approximately 495 nm (58 kcal.). The CL cannot therefore be arising only from rf fluorescence. One intriguing possibility is that the reaction yields, in one concerted reaction or in a sequential reaction, electronically excited singlet sigma oxygen species, which can then pool their energy and transfer it by the Khan-Kasha mechanism (10) to fluorescent species in the system which may have their excited singlet states blue shifted relative to rf and which then decay as normal fluorescence, which event constitutes the CL. Included also in Fig. 5 is the kinetic plot k" of the CL obtained from a partially purified sample (by silicic acid column chromatography, see Fig. 5) of the intermediate from the "incubation-system", elicited by HOOH, and measured on the Scintillation Counter (see above, and compare Vorhaben and Steele, 1).

We recognize in retrospect that the elicitation of CL photochemically from the rf-HOOH-Cu system (Steele, 2), and chemically from the rf-HOOH-Cu-$H_2A$ system (Vorhaben and Steele, 1) probably both proceed via the rf-Cu(I)-chelate as that intermediate common precursor which then reacts to form an $O_2$-activated compound which can then react with HOOH to give light. This intermediate was,in fact, proposed by Vorhaben and Steele (11) as one of several possible mechanisms by which Cu enhanced the CL in the photoinduced rf-HOOH system.

To our knowledge the report by Hercules and Lytle (12) of CL from Ru-chelates is the only other work which has shown the participation of chelates in a CL reaction. These authors cite unpublished work of J. P. Paris as having previously also obtained CL from Ru-chelates.

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