Studies on the Generation of Electronic Excitation States in a Riboflavin–Hydrogen Peroxide–Copper–Ascorbate Redox System Leading to Chemiluminescence and/or Aromatic Hydroxylation*

Jean E. Vorhaben† and Richard H. Steele‡

ABSTRACT: Chemiluminescence in the visible spectrum is evidence that exothermic energies of from 45 to 80 kcal have been "coupled" to the generation of electronic excitation states. This paper characterizes a nonenzymatic biochemical redox system consisting of riboflavin–hydrogen peroxide–copper(II)–ascorbic acid which chemiluminesces and/or hydroxylates aromatic substrates. Inhibition studies with free-radical traps, halides, and aromatics indicate that initiating mechanisms proceed *via* free radicals. Copper reacts rather specifically in the light reaction with evidence of a mole for mole stoichiometry with ascorbic acid for optimal emission. The reactions are pH dependent with rates increasing with increasing pH. The chemiluminescent spectrum, elicited with β-mercaptoethanol as a reductant, appears to be a composite of the fluorescent species in the system. Riboflavin peroxides are suggested as intermediates which react with each other or with hydrogen peroxide in the light reaction. Monitoring of low-level chemiluminescence with a scintillation counter has permitted the use of "physiological" concentrations of reactants. Chemiluminescence was obtained also in the copper(II)–hydrogen peroxide system when other dyes and reductants were substituted for riboflavin and ascorbic acid, respectively.

Bioluminescence is a chemiluminescence. When displayed in the ultraviolet or visible spectral regions, it is direct evidence of the collapse of electronic excitation states generated previously by biochemical reactions. The free energies released in these reactions are an order of magnitude larger than the standard free energy of hydrolysis of adenosine triphosphate, *i.e.*, 60–80 (2.6–3.5 ev) *vs.* 7–8 kcal (0.33 ev). These high-energy light reactions are the evidence that biological systems are not restricted to the standard redox potential drop of 1.2 ev between reduced diphosphopyridine nucleotide and oxygen for energy mobilization. The high quantum yields for these light reactions, which can approach one (Seliger and McElroy, 1960), indicate that the reactions have evolved with a high efficiency and are not spurious light leaks from trivial incidental reactions.

The generation of electronic excitation states is postulated increasingly in biological energy transduction phenomena, exclusive of vision, photosynthesis, and bioluminescence. The mechanism for the population of these high-energy terms, if they are indeed functional, should be recognized as one of the basic unsolved problems of bioenergetics. The work reported in this paper is directed to this problem.

We consider here the properties of a redox system consisting of riboflavin, ascorbic acid, copper, and hydrogen peroxide, frequently referred to subsequently as the dark-induced system, which we have found to chemiluminesce under conditions which are milder and more physiological (Steele, 1963; Vorhaben and Steele, 1965) than the analogous system discovered by Strehler and Shoup (1953). This system is particularly suitable for study since we have been able to compare it with a similar photoinducible chemiluminescent system composed of riboflavin, hydrogen peroxide, plus or minus copper being studied in this laboratory (Steele, 1963; Williams and Steele, 1965). Since both of these systems effectively hydroxylate aromatic compounds, an insight into the mechanism of electronic excitation state generation should provide some information on the mechanisms of oxygen activation and aromatic hydroxylation.

Materials and Methods

Riboflavin, hydrogen peroxide, and copper sulfate were obtained and solutions were prepared and assayed as described by Steele (1963) and Vorhaben and Steele (1965). Ascorbic acid (Eastman Kodak) stock solutions were prepared in glass-distilled water which had been deionized previously on ion-exchange beds and stored

* From the Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112. *Received October 18, 1966.* This work has been supported in part by Grants A-2730, GM-10171, and GM-K3-2559 from the National Institutes of Health, U. S. Public Health Service.

† Present address: Department of Biochemistry, Duke University Medical Center, Durham, N. C. 27706.

‡ Research Career Development Awardee of the National Institutes of Health, U. S. Public Health Service. To whom to address inquiries.

in actinic ware under refrigeration. Ascorbic acid concentrations were determined from absorbancy measurements at 244 mμ (Hewitt and Dickes, 1960) and checked daily. Benzoic acid and salicylic acid solution concentrations were determined as described by Williams and Steele (1965). The salicylic acid generated chemically was determined with the Folin–Ciocalteu phenol reagent. The chelating agent used was Bio-Rad Analytical Grade Chelex 100 (100–200 mesh, sodium form). Unless otherwise noted all other solutions were prepared from chemicals of reagent grade quality.

Absorption and emission spectra, pH, and the techniques for the elicitation and measurement of the chemiluminescences were the same as described by Steele (1963). Ambient temperatures were 24–25°.

Chemiluminescent emissions at "low" intensities, which could not be measured by the method described above, were monitored with a Nuclear-Chicago liquid scintillation spectrometer, No. 722. The voltage and channel settings used are presented in Table I. Reactions for "scintillation" counting were begun by adding all reactants to the sample vials, the caps were tightened, and the solutions were mixed by shaking. The vials were lowered immediately into counting position. The counting time was varied, depending upon the emission intensity of the chemiluminescent reaction, from 0.03 to 1.0 min. Since the smallest automatic preset time interval on the instrument is 0.1 min, counting intervals of 0.03 min were obtained by manually stopping the counting after that time had elapsed as monitored on the instrument timer in increments of 0.01 min. Parenthetically, it should be noted that, because of the coincidence circuitry used to lower the background counts, the displayed counts should be multiplied by a factor of *at least two* to obtain the actual number of chemical events giving rise to the emissions. Further, since the counts displayed are a function of the voltage settings, we have made no effort to ascertain the absolute photon flux for the emissions. From crude estimates, however, we estimate that the quantum yields for the chemiluminescent reactions are very low.

TABLE I: Settings for the Liquid Scintillation Counter.

Channel	Mode	Level	Setting
1	L1-00	Data attenuator	1.0
2	L3-00	2	0.0
3	L4-5	3	0.5
		4	9.9
		5	9.9
	Data voltage 1400		
	Gate voltage 1400		

The Nuclear-Chicago scintillator was used also for kinetic studies of chemiluminescent reactions of "low" intensity. The error inherent in a kinetic analysis is fully recognized; however, if the changes in intensity, displayed as counts, are small over the selected time interval, the error is reduced, and the reaction can be followed as a function of time. The spectrometer was operated manually or automatically, depending upon the system under study. The times required for the printout and for the recycling period, when used, were recorded with a stop watch. In general, a good agreement has been observed between the kinetic parameters obtained with the scintillation instrumentation and with the RCA 1P28 photomultiplier instrumentation as used by Steele (1963). Table II contains representative first-order decay rate constants calculated from data obtained using both light-detecting systems to measure identical chemiluminescent systems. The time elapsed during the actual counting was considered when the rate constants were calculated. It can be seen that the agreement is good at the high copper values. At present, we have no explanation for the deviations at the low copper concentrations.[1]

Results

A representative tracing of the chemiluminescent response for the riboflavin–ascorbic acid–copper sulfate–hydrogen peroxide system is shown as curve a of Figure 1. The chemiluminescent signal is characterized by a rapid rise to a maximum intensity followed by a decay to a transient steady-state level after which the signal decreases slowly to zero intensity. The relative intensities at the peak height and at the steady-state level, depend upon the concentrations of the various reactants. We have recorded for comparison (curve b of Figure 1) the chemiluminescent emission signal from

[1] Referee II of our manuscript kindly solved this dilemma for us. The significance of this problem when scintillation counters with coincidence circuitry are used to measure low-level chemiluminescence prompts us to discuss the problem here. Let N_1 and N_2 be the probabilities that independent chemiluminescent events C_1 and C_2 declare themselves, respectively, in phototubes I and II in a given time interval t. Further, consider that both chemiluminescent events have approximately the same probability of declaring themselves in the *other* phototube. The probability, P, that both chemiluminescent events will occur within the same time interval and be *recorded as a count* $= 2N_1N_2t$ or $P \cong 2N^2t$. When the chemiluminescent rate is low the probability that two independent events will declare themselves within the same time interval, t, is low and P follows the approximation, $P \cong 2N^2t$, and many chemiluminescent events occur without being counted. This results in an *apparent* higher rate of *decay* which calculates out to give a higher rate constant than should be obtained. When the chemiluminescent rate is high, and below the saturation limit of the scintillator, the probability that two independent chemiluminescent events will declare themselves within the same time interval, t, approaches 1, *i.e.*, $P \cong 2N^2t \rightarrow 1$, and the counts recorded reflect accurately the true chemiluminescent rate which permits the correct rate constants to be calculated. These constants, with $P \cong 1$, are lower than when $P << 1$.

TABLE II: Comparison of First-Order Rate Constants Evaluated from Nuclear-Chicago Spectrometer and 1P28 Instrumentation Data for Different Concentrations of Copper.[a]

Cu^{2+} (μmoles)	First-Order Rate Constants (sec^{-1} [b])	
	Scintillation Spectrometer	RCA 1P28
0	0.01	0.007
0.0135	0.010	0.008
0.027	0.010	0.009
0.225	0.012	0.013
0.360	0.014	0.014
0.450	0.014	—

[a] All systems: riboflavin, 0.45 μmole; hydrogen peroxide, 5 mmoles; phosphate buffer, 1.5 mmoles, pH 6.1; total volume, 15 ml. Chemiluminescence photoinduced by 30-sec illumination. [b] Averages of three or more runs.

the same system in the absence of ascorbic acid. Chemiluminescence was reported for the latter system by Strehler and Shoup in 1953. By coupling a reductant to this system it is possible, as shown, to stimulate the intensity severalfold. Dehydroascorbic acid, under similar conditions, does not produce a stimulation of the chemiluminescent intensity.

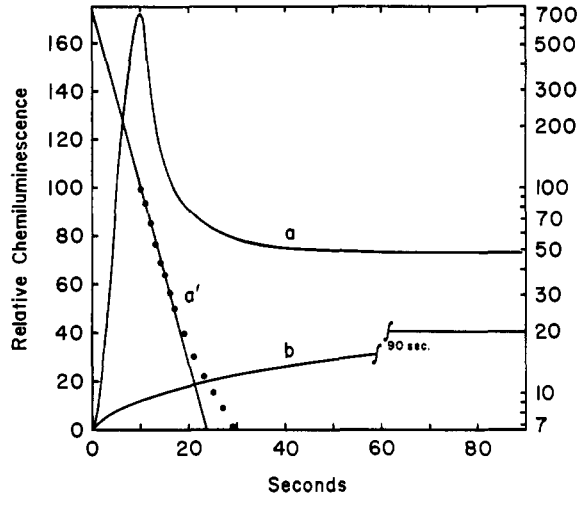

FIGURE 1: Dark-induced chemiluminescence *vs.* time for the riboflavin–hydrogen peroxide system in the absence (curve b) and presence (curve a) of ascorbic acid. The system contained: riboflavin, 0.41 μmole; hydrogen peroxide, 5 mmoles; copper sulfate, 3 μmoles; phosphate buffer, 2 mmoles, pH 6.0; and ascorbic acid, 8.75 μmoles; volume 15 ml. Chemiluminescence for both systems was initiated by injecting the copper sulfate at zero time. The semilog plot (curve a′) was obtained from curve a by subtracting steady-state signal, see text.

It is evident from curve a of Figure 1 that the kinetic features of the light reaction are complex. Although we have not determined a rate equation describing the complete course of the light reaction, a partial kinetic analysis of the first stages of the chemiluminescent emission has been obtained. The initial increase in the signal is linear and indicates that the reaction is probably pseudo-zero order. The signal decay appears to follow first-order kinetics as indicated by curve a′ of Figure 1 which represents a semilogarithmic plot of the values obtained by subtracting the steady-state intensity from the original decay curve a. The plot is linear through more than 1 half-life.

The order of addition of the reactants to the system has a marked effect on the chemiluminescent response. When riboflavin was injected as the *final* reactant to the system containing ascorbic acid, hydrogen peroxide, and copper the results shown in Figure 2 were obtained. It is seen that the chemiluminescent intensities at the maxima and the "steady-state" levels decrease progressively as the time elapsed before the injection of riboflavin is increased. Curve a, for which riboflavin was present when chemiluminescence was elicited by the injection of copper sulfate, represents the zero-time response. With a delay of 10 sec before the addi-

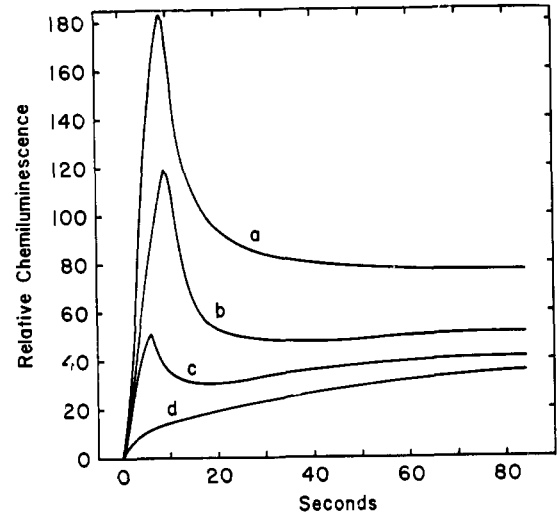

Figure 2: Chemiluminescence *vs.* time for the system ascorbic acid–copper(II)–hydrogen peroxide–riboflavin. The effect of delaying the addition of riboflavin to the system. All systems contained: riboflavin, 0.41 μmole; hydrogen peroxide, 5 mmoles; copper sulfate, 3 μmoles; ascorbic acid, 8.75 μmoles; and phosphate buffer, 2 mmoles, pH 6.0; volume 15 ml. (curve a) copper sulfate was the final addition to the system containing riboflavin = zero-time response. (curve b) riboflavin was injected approximately 2 sec after the addition of copper sulfate to the system containing the other reactants. (curve c) riboflavin was injected after 5 sec. (curve d) riboflavin was injected after 10 sec.

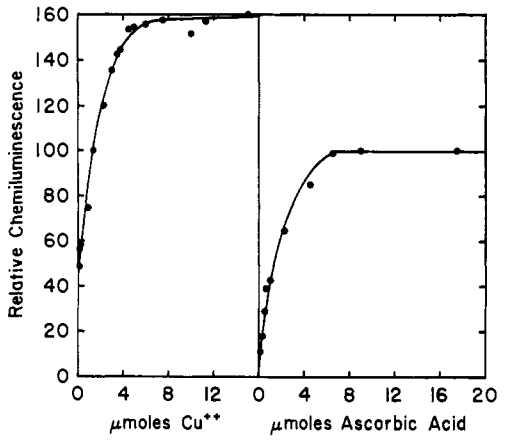

FIGURE 3: Chemiluminescence at the maximum *vs.* copper concentration (left system) and ascorbic acid concentration (right system) for a riboflavin–hydrogen peroxide system. (left system) riboflavin, 0.45 μmole; ascorbic acid, 4.5 μmoles; hydrogen peroxide, 215 mmoles; and phosphate buffer, 2 mmoles, pH 6.0. (right system) riboflavin, 0.45 μmole; hydrogen peroxide, 5 mmoles; phosphate buffer, 2 mmoles, pH 5.8; and copper sulfate, 7.5 μmoles; all volumes 15 ml. Chemiluminescence initiated by injecting copper sulfate into the reaction vessels containing the other reactants.

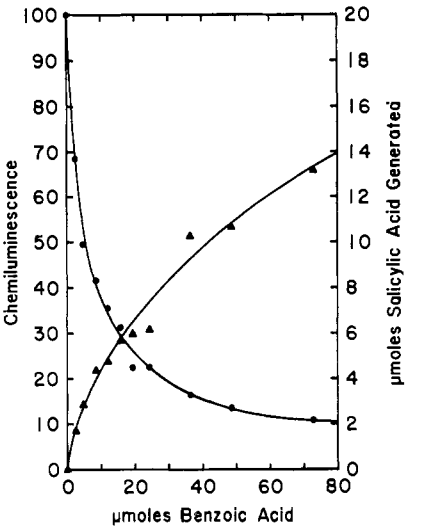

FIGURE 4: Chemiluminescence (●) at the maximum, and concentration of salicylic acid (▲) generated *vs.* benzoic acid concentration. The system contained: riboflavin, 0.45 μmole; hydrogen peroxide, 5 mmoles; copper sulfate, 3 μmoles, ascorbic acid, 9.6 μmoles; and phosphate buffer, 2 mmoles, pH 6.0; volume 15 ml; reaction time, 30 sec.

tion of riboflavin (curve d), the system responded as if no ascorbic acid were present (compare with curve b of Figure 1). Indeed, by following the rate of ascorbic acid oxidation spectrophotometrically in the presence, only, of copper sulfate and hydrogen peroxide under similar conditions, it was determined that the oxidation had been completed within 10 sec. Consequently, no enhancement of the chemiluminescent signal, above that obtained for the riboflavin–copper sulfate–hydrogen peroxide system alone, was observed.

Similar, if not identical chemiluminescent responses, were obtained when either copper sulfate or hydrogen peroxide were made the final reactants added to the otherwise complete system. Delays of up to 3 min before the addition of the final reactant caused no observable differences in the chemiluminescent responses. However, as will be shown in a subsequent paper, delays longer than 3 min in the addition of hydrogen peroxide, had a marked effect on the chemiluminescent reaction. Curves a of Figures 1 and 2 are representative of the chemiluminescent emissions elicited when either copper sulfate or hydrogen peroxide are made the final reactants added to the otherwise complete system. The addition of ascorbic acid as the last reactant produces essentially the same type of response as hydrogen peroxide or copper sulfate, the only difference being the low-level chemiluminescence displayed by the riboflavin–hydrogen peroxide–copper sulfate system *alone* prior to the addition of the reductant. Subsequent additional injections of ascorbic acid into the chemiluminescing system yielded the characteristic rapid increase in the light intensity with each injection. Little or no changes in the chemiluminescent signals were noted when second additions of either riboflavin, copper sulfate, or hydrogen peroxide were injected into a chemiluminescing system at the steady-state level. These reactants, in contrast to ascorbic acid, were apparently not limiting in concentration at the time of the injection.

Representative ascorbic acid oxidations, for our system, as measured spectrophotometrically, follow first-order kinetics with rate constants dependent, apparently, upon the metal concentration. For a 15-ml volume containing ascorbic acid (8.75 μmoles) and chelex-treated phosphate buffer (2 mmoles) at pH 6.0 the first-order decay rate constant, $k = 7.8 \times 10^{-4}$ sec^{-1}. When 5 mmoles of chelex-treated hydrogen peroxide was added to this system the rate constant rose to 175×10^{-4} sec^{-1} compared with 241×10^{-4} sec^{-1} when 5 mmoles of nonchelex-treated hydrogen peroxide was added to the system. When 3 μmoles of copper sulfate was added to the ascorbic acid–buffer system, a rate constant $k = 164 \times 10^{-4}$ sec^{-1} was obtained. As indicated above, in the presence of both copper sulfate and hydrogen peroxide, the oxidation of ascorbic acid was too rapid to be followed by our instrumentation, being complete within 10 sec.

The influence of reactant concentrations on the relative chemiluminescent intensities was investigated in a series of studies in which the concentration of each reactant was varied while holding the others constant. These intensities, *measured at the peak* heights, were plotted against the reactant concentrations. The chemi-

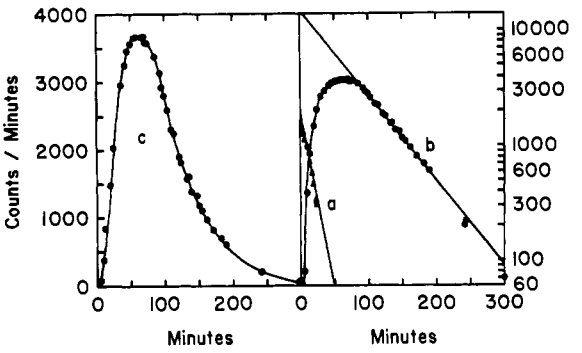

FIGURE 5. Chemiluminescence measured on the scintillation counter, plotted linearly (curve c) and semilogarithmically (curves a and b), *vs.* time. The system contained: riboflavin, 0.26 μmole; hydrogen peroxide, 1.0 μmole; copper sulfate, 1.5 μmoles; ascorbic acid, 0.63 μmole; and phosphate buffer, 2.5 mmoles, pH 5.8; volume 15 ml. Chemiluminescence was initiated by adding copper sulfate to a mixture of the other components in the counting vial. First-order decay rate constants: (a) k_1 = 0.033 min^{-1}; (b) k_2 = 0.017 min^{-1}.

luminescent signals for the riboflavin and hydrogen peroxide studies rose monotonously while the signals for the copper and ascorbic acid systems, depicted in Figure 3, peaked to a plateau in both instances at a concentration of copper equivalent to that of the ascorbic acid. The latter finding suggests a requisite mole for mole stoichiometry for optimal chemiluminescence.

Since we think it probable that the dark-induced chemiluminescent system (riboflavin–ascorbic acid–hydrogen peroxide–copper(II)) and the photoinduced system (riboflavin–hydrogen peroxide–copper(II)) studied previously share common steps in the reaction mechanism we made comparative studies of the two systems. As Williams and Steele (1965) and Steele (1963) had demonstrated a marked inhibition of the photoinducible chemiluminescence by benzoic acid and halides, we studied the influence of these compounds on the chemiluminescence for the dark-induced system. The results for the benzoic acid study are presented in Figure 4. The chemiluminescent intensity was found to decrease progressively and the aromatic hydroxylation, expressed as the amount of salicylic acid generated, to increase with increasing concentrations of benzoic acid. The benzoic acid was added to the reaction vessel prior to the elicitation of the chemiluminescence by the injection of copper sulfate. The addition of the benzoic acid to the system subsequent to the initiation of the light reaction by copper sulfate addition had no impact on the course of the reaction. Salicylic and gentisic acids were identified in the reaction mixture by ascending paper chromatography (Williams and Steele, 1965; Block *et al.*, 1958). It should be noted that riboflavin is not necessary for the hydroxylation of the benzoic acid in this system, which is comparable to the hydroxylating system consisting of ascorbic acid, ferrous iron, Versene, and molecular oxygen or hydrogen peroxide described by Udenfriend *et al.* (1954) and Brodie *et al.* (1954).

Halide inhibition of the chemiluminescence from the dark-induced system provides an additional feature for comparison with the emission photoinduced from the riboflavin–hydrogen peroxide–copper(II) system. The halides markedly inhibited the chemiluminescences from both systems with the iodide inhibition being two orders of magnitude greater than that of chloride; *e.g.*, the concentrations producing 50% inhibition were, for the dark-induced system, KI = 1.5 μmoles and KCl = 750 μmoles, and for the photoinduced system, KI = 1.5 μmoles and KCl = 175 μmoles. The inhibitions by iodide for both systems were the same. The chloride inhibition, particularly at low concentrations, was considerably more effective in the photoinduced system but approached the inhibition in the "dark" system at higher chloride concentrations. In these experiments the halides were added to the systems prior to the elicitation of the chemiluminescence by copper sulfate addition, for the dark system, or light exposure, for the photoinduced system. When the halides were added to *either* system subsequent to the initiation of the chemiluminescence, essentially no effect on the course of the chemiluminescence was observed.

Several compounds shown previously to inhibit the photoinduced chemiluminescence (Steele, 1963; Vorhaben and Steele, 1965) have also been shown to inhibit light emission in the dark-induced system. Histidine, Versene, pyrophosphate, and 8-hydroxyquinoline exerted an inhibitory effect, presumably by chelating the copper. Ethanol and dimethyl sulfoxide also suppressed the chemiluminescence when added prior to the elicitation of the chemiluminescence; however, ethanol caused a slight enhancement of the chemiluminescent intensity, with an accompanying increased first-order decay rate constant, when injected into a "glowing" system.

Use of the liquid scintillation counter for measuring chemiluminescence made it possible to follow the light reactions in the dark-induced system at low reactant, physiological, concentration, especially for low concentrations of hydrogen peroxide. In Figure 5 we present the data obtained from a system containing only 1 μmole of added hydrogen peroxide in contrast to the 5000 μmoles used routinely to produce the higher chemiluminescent intensities described previously. The data are plotted linearly (curve c) and semilogarithmically (curves a and b) *vs.* time. The decays appear to be first order as observed for the systems containing high concentrations of hydrogen peroxide (compare Figure 1) although no transient steady-state intensity of the emission was observed for the low hydrogen peroxide system. The slow course of this reaction (60 min to reach maximum intensity) enabled us to examine the possible relationship between the rate of ascorbic acid oxidation and the appearance of the chemiluminescence. Ascorbic acid oxidation for this system, in the absence of riboflavin only, was followed spectrophoto-

metrically. No correlation between the rate of ascorbic acid oxidation and the rate of increase of the chemiluminescent intensity was found; however, the effect of riboflavin on the rate of ascorbic acid was not studied.

Using the scintillation counter, we have detected a chemiluminescence from a riboflavin–ascorbic acid–hydrogen peroxide system in the absence of copper. Counts of the order of 2×10^5 min^{-1} were obtained at the onset of the reaction, compared with 6×10^6 cpm obtained in the same system containing 1.5 μmoles of copper. No light was detected in the system in the absence of both ascorbic acid and copper, *i.e.*, from a system containing only riboflavin and hydrogen peroxide.

In an analogous manner, using the scintillation counter, we have detected a low-level chemiluminescence of about 500 cpm from a system containing riboflavin, ascorbic acid, copper, and no *added hydrogen peroxide*. This system, however, must remain in contact with ambient pO_2 for 6–8 hr for these counts to be obtained. Higher counts are obtained in a shorter time (5 min) if pure oxygen is bubbled through the system. These results were anticipated, for Lyman *et al.* (1937) and Hand and Greisen (1942) had demonstrated the *generation of hydrogen peroxide* in a system containing ascorbic acid, copper, and oxygen. Controls with riboflavin alone, riboflavin and ascorbate, riboflavin and copper(II), ascorbate and copper(II), and N_2 gave no counts. No light was detected unless O_2 was added to the system and unless ascorbic acid was present during the O_2 addition. The chemiluminescent response in the presence of O_2 appears similar to that observed with hydrogen peroxide; a steady-state intensity is reached and maintained for several minutes before falling to low levels. By subtracting the steady-state intensities from the first part of the curve, a semilogarithmic first-order decay was obtained. The decay following the steady-state emission did not follow first-order kinetics. It is conceivable that the decrease in intensity is the result of a depleted O_2 supply.

We have varied the prototype riboflavin–ascorbic acid–hydrogen peroxide–copper(II) system by substituting different compounds for some of the reactants. Chemiluminescent responses, elicited from the system when the dyes Rhodamine B and Pyronine B were substituted for riboflavin, were compared with the emission from the riboflavin system at equal molar concentrations (3×10^{-5}). Although we have not studied these dye systems systematically, they appear not to exhibit the transient steady-state phenomenon as exhibited by riboflavin (compare Figure 1) nor riboflavin's capacity for photoinduced chemiluminescence. The emission intensities, however, are comparable or greater than with riboflavin.

We have detected chemiluminescence from the ascorbic acid–hydrogen peroxide–copper(II) system when the amino acids tyrosine and tryptophan were substituted for riboflavin. The emissions were several orders of magnitude lower in intensity than for riboflavin, and thus far have been measured only with the scintillation spectrometer.

In contrast to an apparent lack of anion specificity, the nature of the cation used in the riboflavin–ascorbic acid–hydrogen peroxide–metal system has a marked influence on the elicitable chemiluminescence response. The maximum intensities measured for a number of different cations are compared with that for the cupric ion in Table III. With the exception of copper and cobalt, which had intensities at the maximum of 66 and 23 units, respectively, only low-level emissions were observed with the other cations.

TABLE III: Relative Effects of Cations in Enhancing Chemiluminescence in the Dark-Induced System.[a]

Metal Ion (3 μmoles)	Max Intensity (rel chemiluminescent units)
Cu^{2+}	66.3
Co^{2+}	23.3
Zn^{2+}	9.9
Fe^{2+}	8.5
Fe^{3+}	8.1
Ca^{2+}	7.9
Mg^{2+}	7.9
Cd^{2+}	7.8
Ag^{+}	7.2
Mn^{2+}	5.4
Ni^{2+}	5.2

[a] System: riboflavin, 0.45 μmole; hydrogen peroxide, 5 mmoles; ascorbic acid, 5 μmoles; and phosphate buffer, 2 mmoles, pH 6.0; volume, 15 ml. Chemiluminescence initiated by injecting the metal (3 μmoles) into the reaction vessel containing the other reactants.

In Figure 6 we present the chemiluminescent responses observed when ascorbic acid, in the riboflavin–ascorbic acid–hydrogen peroxide–copper(II) system, was replaced by cysteine (curve c) and, interestingly, by cystine (curve b). The ascorbic acid induced emission (curve a) has been included for comparison. No transient steady-state emission was observed with these amino acids. The decay rates for the emissions were much slower as compared to that for ascorbic acid having first-order rates constants, for the systems depicted, of 0.002, 0.004, and 0.15 sec^{-1} for cysteine, cystine, and ascorbic acid, respectively.

Until recently we have not been able to obtain a chemiluminescence from any riboflavin system of sufficient intensity to measure the emission spectrum directly. Steele (1963), using an admittedly crude filter-interposition technique, had estimated that the chemiluminescent spectra from photoinduced and chemically dark-induced riboflavin systems were identical and probably somewhat blue shifted relative to the fluorescent spectrum for riboflavin. Using the reductant β-mercaptoethanol in place of ascorbic acid (curve d of

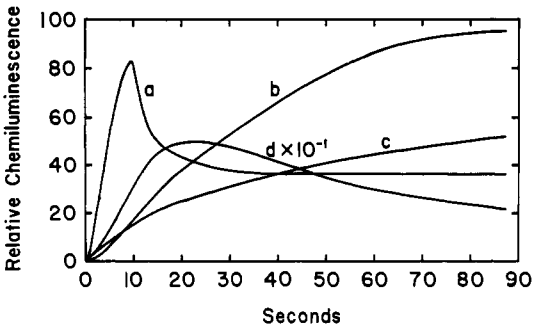

FIGURE 6: Chemiluminescence *vs.* time for the dark-induced system using reductants other than ascorbic acid, namely, cystine, cysteine, and β-mercaptoethanol. All systems contain: riboflavin, 0.45 μmole; hydrogen peroxide, 5 mmoles; and phosphate buffer, 2 mmoles, pH 6.0; total volume, 15 ml. (curve a) 10 μmoles of ascorbic acid was added, then 3 μmoles of copper sulfate was injected to initiate chemiluminescence. (curve b) as for a except 2 μmoles of cystine was added in place of ascorbate. (curve c) as for a except 10 μmoles of cystine was added in place of ascorbate. (curve d) 20 μmoles of β-mercaptoethanol was added, then 5 μmoles of copper sulfate was injected to initiate the chemiluminescence, signals were divided by 10 for comparison with the other data.

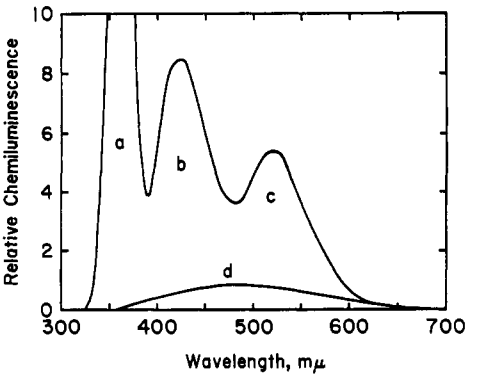

FIGURE 7: Chemiluminescence emission spectrum (curve d), and the fluorescence spectrum (curves b and c), with the exciting light (curve a) (not the activation spectrum) for a dark-induced system. The system contained: riboflavin, 1.2 μmoles; hydrogen peroxide, 7.5 mmoles; β-mercaptoethanol, 40 μmoles; and phosphate buffer, 1.5 mmoles, pH 6.0; chemiluminescence initiated by injecting 1.5 μmoles of copper sulfate into the reaction mixture positioned in the fluorometer; volume, 15 ml. Fluorescence spectrum was measured on the system subsequent to the measurement of the chemiluminescent spectrum.

Figure 6), we have been able to elicit a chemiluminescence from the riboflavin–hydrogen peroxide–copper(II) system of intensity sufficient to traverse the grating monochromator of the Aminco-Bowman spectrofluorometer for a direct estimate of its spectral distribution. This result, together with the fluorescent emission (uncorrected for phototube sensitivity) displayed by the same system subsequent to the elicitation of the chemiluminescence, is shown in Figure 7. The violet 425-mμ fluorescence appears in those systems from which chemiluminescence has been elicited chemically, as in this study, or photoinduced. The long-wavelength emission (520 mμ) represents riboflavin fluorescence. The chemiluminescent emission spectrum appears to be a composite of these two fluorescent emissions. Parenthetically, we should emphasize that the compound responsible for the 425-mμ fluorescent emission (Figure 7) is not an impurity in the system, but a product which accumulates when chemiluminescence is elicited chemically or photochemically.

Discussion

In all of the aspects studied the processes of chemiluminescence elicited from the dark- and photoinduced systems appear to differ only in the display of a transient steady state in the latter and in the mechanism of induction. The emissions from both systems are enhanced rather specifically by copper and inhibited by halides and benzoic acid, the latter compound yielding salicylic and gentisic acids. The inhibitors are effective only when present prior to the elicitation of the chemiluminescence with no appreciable effect when added subsequent to the initiation and are thought to react with free hydroxyl radicals (Kolthoff and Medalia, 1949; Merz and Waters, 1949; Williams and Steele, 1965). We interpret this as tentative evidence that both initiating mechanisms proceed *via* free-radical species (however, see below for a possible alternate interpretation) while the subsequent reactions producing the chemiluminescence are thought to proceed by non-free-radical species generated by the free-radical reactions.

The parallel effectiveness of the halide inhibition of the chemiluminescence from the dark- and photoinduced systems invalidates the inhibition mechanism involving halide perturbation of the riboflavin triplet energy term as proposed by Steele (1963) since this energy term would not be populated in the dark-induced reaction. It seems more probable to consider the halide inhibitions as due to an interaction with free-radical species, possibly the free hydroxyl radical.

In the absence of any knowledge of the identity of any intermediates leading to chemiluminescence and/or hydroxylation, any proposed mechanisms must be in part speculative. There are, however, several features of the results which any proposed mechanism must include and which, in turn, limit the possibilities. The reaction sequence, on the basis of the inhibition studies, appears to partition naturally into three phases: the initiating phase, intermediate phase, and chemiluminescence phase. We will consider these phases separately.

Initiating "Free-Radical" Phase. A discussion of this phase must account for the initiating mechanisms

leading to chemiluminescence which have been observed for the following systems:

$$Rf + Cu^{2+} + HOOH \longrightarrow \text{light} \quad (1)$$

$$Rf + HOOH \xrightarrow[\pm Cu^{2+}]{h\nu} \text{light} \quad (2)$$

$$Rf + H_2AA + HOOH \longrightarrow \text{light} \quad (3)$$

$$Rf + H_2AA + Cu^{2+} \xrightarrow{O_2} \text{light} \quad (4)$$

$$Rf + HOOH + RH_2 + Cu^{2+} \longrightarrow \text{light} \quad (5)$$

where Rf, H_2AA, and RH_2 represent riboflavin, ascorbic acid, and reductants, respectively.

All of these possible initiating mechanisms can be written with common free-radical species, particularly the $\cdot OH$, $\cdot O_2H$, and $\cdot O_2^-$ radicals which could react with "traps" to inhibit the reaction sequence (see, for example, earlier papers on this study). Further, since the free-radical reactions and interactions would be expected to be very rapid, their concentration would be essentially zero once the generating processes are terminated. Generation termination would consist of turning off the exciting light for the photoinduced initiation, and the exhaustion of reductant, usually ascorbic acid, for the dark-induced systems. It should be recalled that the inhibitors had essentially no influence on the reaction sequence, light intensity or kinetics, if added to the *emitting* system subsequent to the initiating phase.

Intermediate Phase. Any consideration of the mechanism of the reaction sequence leading to chemiluminescence must take into account the high-energy requirements associated with the chemiluminescent reaction. These can be appreciated by a study of the chemiluminescent emission spectrum (Figure 7). The liberation of light in the 400-mμ range requires at a minimum 71.5 kcal of energy in the exothermicities of the reactions generating the electronic excitation states. For this reason we have invoked the participation of organic peroxides, probably of riboflavin and/or some derivative thereof. Vassil'ev (1965), *e.g.*, reports the energy release for the reaction

$$2C_6H_5\underset{\displaystyle OO\cdot}{\underset{|}{C}}HCH_3 \longrightarrow C_6H_5\underset{\displaystyle O}{\underset{\|}{C}}CH_3 + C_6H_5\underset{\displaystyle OH}{\underset{|}{C}}HCH_3 + O_2$$

to be about 110–120 kcal. In the absence of any knowledge as to what this intermediate peroxide may be or how it is formed, we simply depict it as being formed by the interaction of riboflavin or its semiquinone with the perhydroxyl radical or anion, possibly also with copper, as follows:

$$Rf + \cdot O_2^- \longrightarrow Rf\cdot + O_2$$

$$Rf\cdot + \cdot O_2^- \longrightarrow Rf\text{-}OO^-$$

$$Rf\text{-}OO^- + H^+ \longrightarrow Rf\text{-}OOH$$

The evidence for the production of an intermediate "stable" reactant rests on the demonstrated accumulation with time of a compound in the riboflavin–ascorbic acid–copper–oxygen system which upon the addition of hydrogen peroxide gives a chemiluminescence of intensity sufficient to measure the spectrum. We will discuss the details of this system in a subsequent paper.

Chemiluminescent Phase. We envisage the terminating chemiluminescent reaction proceeding as previously depicted by Williams and Steele (1965), namely

$$Rf\text{-}OOH + HOOH \longrightarrow X(XOH) + H_2O + O_2 + \text{light}$$

or

$$Rf\text{-}OOH + Rf\text{-}OOH \longrightarrow 2X(XOH) + O_2 + \text{light}$$

where X may be the compound fluorescing at 425 mμ detected in the chemiluminescent reaction mixture.

In the course of this work we have become increasingly impressed with the reciprocal relation between the elicitable chemiluminescence and aromatic hydroxylation obtained in the presence of suitable substrates. The two processes appear to compete for a common intermediate. It is for this reason that we wish to allude to the production of molecular oxygen in the initiating and chemiluminescent reactions as a common product. We emphasize this feature since the exothermicities of these oxygen-yielding reactions may be sufficient to promote molecular oxygen to its electronically excited states, which, may in turn be important in chemiluminescent and aromatic hydroxylation reactions. Stauff and Schmidkunz (1962), Stauff (1965), and Khan and Kasha (1966) have discussed those mechanisms whereby excited molecular oxygen species, by concerted action, can pool their energies to yield chemiluminescence in the visible and near-ultraviolet electromagnetic spectrum. Arnold *et al.* (1965) have discussed those features of excited singlet oxygen, such as its 1-hr half-life and its high affinity for olefinic groups which may make it a likely candidate for the primary reactant leading to aromatic hydroxylation. Steele and Cusachs (1967) have discussed the possibility that excited molecular oxygen species, *via* their concerted action, may provide a general mechanism in biological energetics for the mobilization of energies an order of magnitude larger than the standard free energy of hydrolysis of adenosine triphosphate. The data of this paper are as yet insufficient to warrant serious consideration or rejection of excited molecular oxygen as responsible for the observed effects

References

Arnold, J. S., Browne, R. J., and Ogryzlo, E. A. (1965), Symposium on Chemiluminescence, Durham, N. C.,

Advanced Research Projects Agency Office of Naval Research, U. S. Army Research Office, p 35.
Block, R. J., Durrum, E. L., and Zweig, O. (1958), A Manual of Paper Chromatography and Paper Electrophoresis, New York, N. Y., Academic, pp 306–308.
Brodie, B. B., Axelrod, J., Shore, P. A., and Udenfriend, S. (1954), *J. Biol. Chem. 208*, 741.
Hand, D. B., and Greisen, E. C. (1942), *J. Am. Chem. Soc. 64*, 358.
Hewitt, E. J., and Dickes, G. J. (1960), *Biochem. J. 78*, 384.
Khan, A. U., and Kasha, M. (1966), *J. Am. Chem. Soc. 88*, 1574.
Kolthoff, I. M., and Medalia, A. I. (1949), *J. Am. Chem. Soc. 71*, 3777.
Lyman, C. M., Schultze, M. O., and King, C. G. (1937), *J. Biol. Chem. 118*, 757.
Merz, J. H., and Waters, W. A. (1949), *J. Chem. Soc.*, 2427.
Seliger, H. H., and McElroy, W. D. (1960), *Arch. Biochem. Biophys. 88*, 136.
Stauff, J. (1965), Symposium on Chemiluminescence, Durham, N. C., Advanced Research Projects Agency Office of Naval Research, U. S. Army Research Office, p 389.
Stauff, J., and Schmidkunz, H. (1962), *Z. Phys. Chem. 35*, 295.
Steele, R. H. (1963), *Biochemistry 2*, 529.
Steele, R. H., and Cusachs, L. C. (1967), *Nature 213*, 800.
Strehler, B. L., and Shoup, C. S. (1953), *Arch. Biochem. Biophys. 47*, 8.
Udenfriend, S., Clark, C., Axelrod, J., and Brodie, B. B. (1954), *J. Biol. Chem. 208*, 731.
Vassil'ev, R. F. (1965), Symposium on Chemiluminescence, Durham, N. C., Advanced Research Projects Agency Office of Naval Research, U. S. Army Research Office, p 261.
Vorhaben, J. E., and Steele, R. H. (1965), *Biochem. Biophys. Res. Commun. 19*, 187.
Williams, J. R., Jr., and Steele, R. H. (1965), *Biochemistry 4*, 814.