A Photoinduced Chemiluminescence of Riboflavin in Water Containing Hydrogen Peroxide II. Photochemical Aromatic Hydroxylation*

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ABSTRACT: Chemiluminescence can be photoinduced in a buffered riboflavin solution containing HOOH. The chemiluminescence can be inhibited by the addition to the system of free hydroxyl radical trapping agents such as benzene and benzoic acid. The hydroxylation of benzene and benzoate to phenol and salicylate, respectively, has been demonstrated in the photochemical system by chemical, chromatographic, and spectral methods. The hydroxylated products are generated in the riboflavin-HOOH system at rates which fall off in time, owing, presumably, to further quenching of the excited states of riboflavin by the generated products, to additional free radical trapping by the generated products (polyhydroxylation), and to their photochemical destruction.

The influence of the concentrations of the trapping agents, HOOH, oxygen, copper, *p*H, and temperature were evaluated. The data lend support to the postulated production of free hydroxyl radicals in the primary photochemical reaction between riboflavin and HOOH. The data do not distinguish between a homolytic cleavage of the HOOH O—O bond and an electrochemical heterolytic oxidation of HOOH.

In the first paper of this series Steele (1963) presented evidence for the participation of the excited triplet energy term of riboflavin in the primary photochemial event initiating a sequence of chemical reactions which resulted subsequently in chemiluminescence. The finding that the energy of the riboflavin triplet, 47 kcal, equaled the O—O bond energy of hydrogen peroxide (written hereafter as HOOH) in water led to the suggestion that the riboflavin triplet sensitized a homolytic cleavage of the O—O bond of HOOH to give two free hydroxyl radicals. An alternate possible mechanism for the primary photochemical process would be by a heterolytic electrochemical oxidation of HOOH by the excited triplet energy term of riboflavin. These two mechanisms are schematized in Figure 1. As free hydroxyl radicals are produced by both mechanisms we felt that considerable support for a free radical mechanism in the primary photochemical event would be afforded by directly demonstrating their generation. For this purpose we used benzene and benzoate which Stein and Weiss (1948, 1949a,b) and Day and Stein (1949) had demonstrated to be effective in trapping hydroxyl free radicals generated in aqueous solutions by penetrating radiations (γ rays, X rays), and producing, respectively, phenol and salicylate Merz and Waters (1949) and Evans and Uri (1949) have used benzene and benzoate with Fenton's reagent, a free hydroxyl radical generating system, to trap these radicals with the formation of phenol and salicylate, and Konecny (1954) reported the hydroxylation of these same traps by copper sulfate and HOOH with the productions of these same derivatives.

Materials and Methods

Riboflavin was obtained from the Distillation Products Div. of Eastman Kodak and was used without further purification. All riboflavin solutions were kept in actinic ware and refrigerated until used. Working solutions were prepared from more stable stock solutions, and the concentrations were determined with a Beckman DU spectrophotometer. The HOOH used was Baker's AR, 30% (10 M). HOOH concentrations were assayed by permanganate titrations as recommended in *Reagent Chemicals* (American Chemical Society, Washington, D.C., 1955). Salicylic and benzoic acids were Baker's AR grade and their concentrations were established spectrophotometrically. Eastman's Spectrograde benzene was used, and its concentration was established spectrally as described later. Baker's AR grade phenol was used and its concentration was determined iodometrically by the method described in Hawk *et al.* (1954). Phenol and salicylic acid, generated photochemically, were determined quantitatively with the phenol reagent prepared by the W. H. Curtin Co. (New Orleans, La.) according to the

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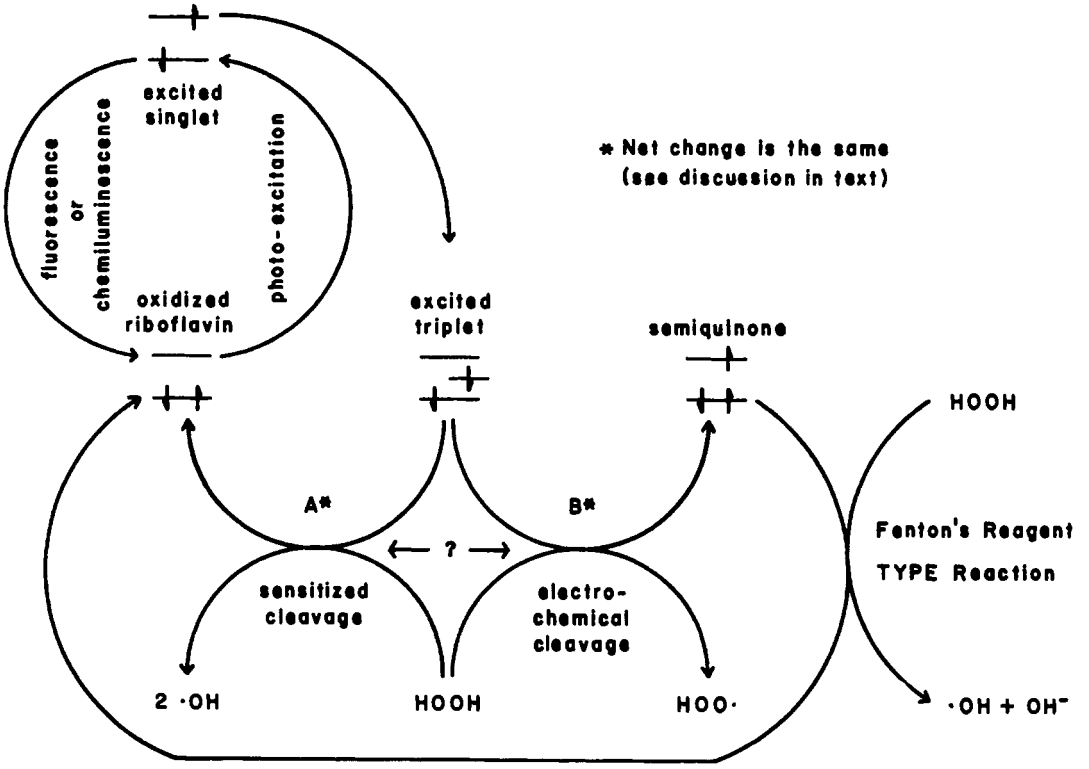

FIGURE 1: Proposed mechanisms for the photosensitized cleavage of HOOH by riboflavin. Only the highest filled, the lowest empty, and lowest excited triplet-energy terms of riboflavin are schematized. ↑ and ↓ represent electrons with directed spins.

method of Folin and Ciocalteu (1927). Beef liver catalase, with an activity of 150,000 units/ml, was obtained from the Worthington Biochemical Corp. Baker's AR grade copper sulfate was used to prepare known copper solutions gravimetrically. These latter solutions were quantitated further using the colorimetric chelate method described by Diehl and Smith (1958). The cation-exchange resin, used to free solutions of possible copper and iron contamination, was Chelex 100, obtained as Analytical Grade (100–200 mesh, sodium form) from the Bio-Rad Laboratories (Richmond, Calif.).

The spectrophotometric determinations of benzoic acid, salicylic acid, and benzene necessitated our establishing the molar absorbancy index, A_M, for these compounds as applicable to the conditions of the experiments. For salicylic acid these indexes, at 305 mμ, were 3910 cm²/mole and 3950 cm²/mole, and for benzoic acid at 271.5 mμ they were 831 cm²/mole and 835 cm²/mole as determined with the Beckman DU and Perkin-Elmer spectrophotometers, respectively. Both compounds were in acidified water-saturated diethyl ether. Using these parameters, the concentrations of the saturated aqueous solutions of benzoic and salicylic acids were determined using acidified water-saturated ether extracts which afforded complete removal of both compounds from the aqueous phase. The absorbancies of the ether phases were used, therefore, to calculate the concentrations of the saturated aqueous stock solutions. These concentrations were found to be 2.44×10^{-2} M (2.98 g/liter) for benzoic acid and 7.6×10^{-3} M (0.94 g/liter) for salicylic acid at 24°.

The spectral parameters for benzene were determined on water-ethanol solutions and the mean of triplicate absorbances at 254 mμ gave a molar absorbancy index of 187 cm²/mole. With this value the concentration of the benzene in the benzene-saturated aqueous stock solution, at 24°, was calculated to be 1.99×10^{-2} M (1.55 g/liter).

A representative procedure for the photochemical aromatic hydroxylation of either benzene or benzoate consisted in illuminating 13.5 ml of the reaction solution with the light-filter system described by Steele (1963). The reaction solution consisted, typically, of riboflavin, HOOH, and phosphate buffer (*p*H 6.1) at concentrations, subsequent to the addition of 1.5 ml of a solution of catalase, of 3×10^{-5} M, 0.166 M, and 0.1 M, respectively. Benzene or benzoic acid solutions were introduced into the reaction vessel from burets to give the desired concentrations. Riboflavin was added in the dark, and both the HOOH and the free hydroxyl radical acceptor were added immediately prior to illumination. Illumination times were measured with a stopwatch. Subsequent to the illumination of the 13.5-ml reaction volume, 15 ml of catalase (15,000

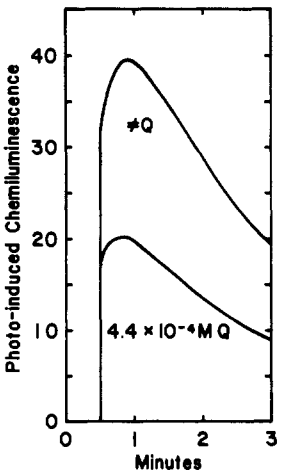

FIGURE 2: Photoinduced chemiluminescence of the riboflavin-HOOH system in the absence and presence of (Q) as a quenching agent (free hydroxyl radical trap). Riboflavin, 3×10^{-5} M; HOOH, 0.33 M; phosphate buffer, 0.13 M, *p*H, 6.1; benzoate (Q), 4.4×10^{-4} M; volume, 15 ml; temperature, 24°; illumination time, 30 seconds.

units/ml) were added to destroy excess HOOH and to terminate the reaction. The amount of the phenolic derivative thus generated by the hydroxylation of the trapping agent was measured as described later. Parenthetically, we should emphasize that the procedure of illuminating 13.5-ml volumes, and then adding 1.5 ml of catalase to make the volumes 15 ml, is the reason we express the molar concentrations in the legends of the figures as "concentrations for 15-ml volumes," rather than as concentrations for 13.5 ml (the volume illuminated in some instances) and then re-expressing them on the basis of the 15-ml volumes analyzed subsequently.

To the 15 ml of test solution was added 0.75 ml of a 1:2 dilution of the Folin-Ciocalteu reagent, followed by 3 ml of a 20% sodium carbonate solution. After mixing for 20 seconds the solution was placed in a boiling-water bath for 1 minute and then cooled under the tap. The resulting blue color was read with the Beckman DU spectrophotometer at 610 mμ and the absorbancies were compared with a standard curve, for either phenol or salicylic acid, to determine the concentrations. The finding that the HOOH remaining at the end of the photohydroxylation reaction interfered with the development of the blue color in the test necessitated the incorporation of the peroxide-destroying step in the method. The use of either sulfite or hydrazine to destroy excess HOOH proved unsatisfactory owing to false positive color tests produced with the Folin-Ciocalteu reagent by excess reductant. We found that catalase could be used successfully for this purpose. The reaction of the phenolic groups in the amino acid residues of the catalase enzyme with the phenol reagent proved slight enough to be compensated readily with a catalase blank in a nonilluminated solution.

Ascending paper chromatograms were run using Whatman No. 1 filter paper in a conventional chromatography tank using 1-butanol-NH_4OH, 4:1 (v/v) as the solvent system. Ultraviolet light was used for spot identification on the paper. Chemiluminescent and fluorescent emissions were measured using the methods and instrumentation described by Steele (1963).

The water used in preparing the reagent solutions used for most of this work was distilled from a Barnstead still and transported to a ceramic reservoir through copper tubing. When it became apparent subsequently (J. E. Vorhaben, paper in preparation) that copper enhanced both the photoinduced chemiluminescence and the photochemical aromatic hydroxylations, we began using glass-distilled water which had been previously deionized on ion-exchange columns. Analyses of water from the Barnstead still showed that the copper content of 15-ml aliquots varied from 0.021 to 0.027 μmole. Unless otherwise indicated, therefore, this amount of copper must be regarded as present as a minimum concentration in the 15-ml volumes used in this study; reagent contaminations may have increased this slightly. Unless otherwise indicated the concentrations of the components in the systems described are presented in the legend of the appropriate figure.

Results

In Figure 2 we present representative curves of the photoinduced chemiluminescent intensities in the riboflavin HOOH system in the absence and presence of benzoic acid as a free hydroxyl radical trap (Q). The concentration of benzoate (4.4×10^{-4} M) is that which produced a 50% depression of the photoinducible chemiluminescence in a study of the influence of benzoate on the photoinduced chemiluminescence from the same system for which the results are presented in Figure 3. Similar results were obtained when benzene was used as the trapping agent. The finding made, important in terms of mechanism, was that in order to depress the photoinducible chemiluminescence the aromatic trapping species had to be present during the illumination period. If the traps were added subsequent to photoinduction they exerted no depressive action on the photoinducible chemiluminescence.

In order to demonstrate that the depression of the photoinducible chemiluminescence was actually caused by the removal of hydroxyl radicals, which had been postulated as being generated in the primary photochemical event, we undertook to demonstrate the production of hydroxylated derivatives from benzene and benzoate incorporated into the riboflavin-HOOH system as hydroxyl traps. These hydroxylated derivatives were identified by three independent methods: chemical, chromatographic, and spectroscopic.

Chemical. In Figure 4 we present representative data illustrating the photoproduction versus time of phenol and salicylate in the riboflavin-HOOH system from

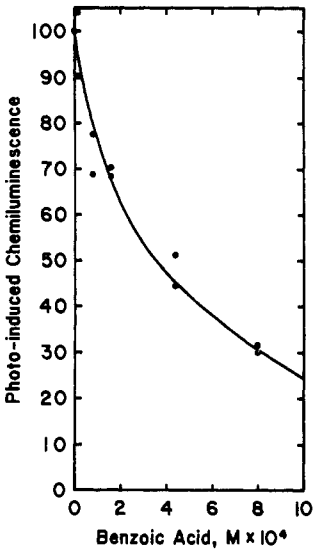

FIGURE 3: Photoinduced chemiluminescence of the riboflavin-HOOH system versus benzoic acid concentration. Riboflavin, 3×10^{-5} M; HOOH, 0.33 M; phosphate buffer, 0.13 M, *p*H, 6.1; volume, 15 ml; temperature, 24°; illumination time, 30 seconds.

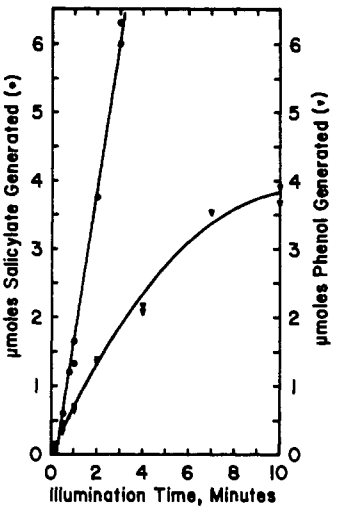

FIGURE 4: The generation of salicylate (●) and phenol (▾), from benzoate and benzene, respectively, in the riboflavin-HOOH system versus illumination time. The reactants were illuminated in a volume of 13.5 ml and then made to 15 ml with the addition of 1.5 ml of catalase. The concentrations for 15-ml volumes are: riboflavin, 3×10^{-5} M; HOOH, 0.165 M; phosphate buffer, 0.13 M, *p*H, 6.1; benzoate trap, 2.2×10^{-3} M; benzene trap, 1.6×10^{-3} M; temperature, 24°.

benzene and benzoate respectively. From studies of the intensity of the photoinducible chemiluminescence versus time (Steele, 1963), and the concentration of hydroxylated product produced versus time we were able to obtain evidence indicative of a direct correlation between the generation of free hydroxyl radicals in the primary photochemical event and the intensity of chemiluminescence. By the elimination of the common time axis from the data provided by these two studies the results presented in Figure 5 were obtained.

By alternately eliminating various components from the hydroxylating system the reactants necessary for hydroxylation were established to be riboflavin, HOOH, and benzene (or any other free radical trapping agent). The efficiency of the photochemical aromatic hydroxylating system was found to be a function of the light intensity, illumination time, *p*H, and the concentrations of HOOH, aromatic trap, copper, and oxygen. The influence of these parameters is described.

Chromatographic. Two systems containing riboflavin, HOOH, benzoate, and buffer in the same concentrations as used in the hydroxylation versus time studies (Figure 4) were prepared. One system was subjected to illumination for a prolonged time and the other was set aside in the dark for the same time interval. At the end of the time interval catalase was added to each tube to destroy the excess HOOH and to terminate the reaction. Acidified ether extracts were spotted on chromatographic paper. Salicylic acid was identified as a definite spot in the illuminated sample but was absent in the nonilluminated control. Benzoic acid spots were present in the chromatograms for both systems. The results are listed in Table I.

TABLE I: Chromatographic Identification (R_F values) of the Photochemical Generation of Salicylate in the Riboflavin-HOOH System.[a]

Substance		R_F Value(s)	Identification
Benzoic acid		0.44	
Salicylic acid		0.53	
Nonilluminated control	Spot 1	0.21	Unknown
	Spot 2	0.43	Benzoic acid
Illuminated sample	Spot 1	0.20	Unknown
	Spot 2	0.42	Benzoic acid
	Spot 3	0.55	Salicylic acid

[a] Concentrations as in Figure 4. Solvent system: 1-butanol-NH_4OH, 4:1 (v/v).

Spectroscopic. For a more quantitative demonstration of phenolic group generation by independent means we used spectrophotometry. Spectra were taken on ether extracts of illuminated and nonilluminated samples of the riboflavin-HOOH-buffer-benzoate system of the same composition as used for the hydroxylation versus time study (Figure 4). The ether extracts were kept at constant volume during the experiment. In Figure 6 we present tracings of a set of spectra ob-

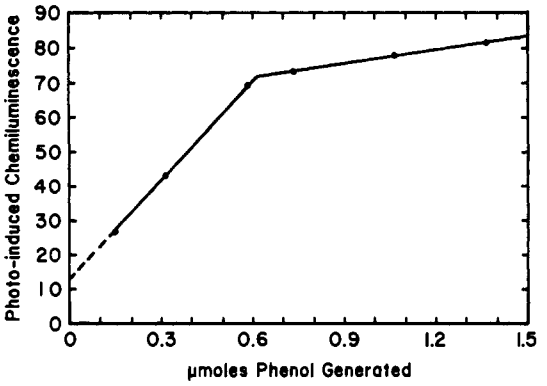

FIGURE 5: Data showing that the intensity of the chemiluminescence photoinduced in the riboflavin-HOOH system is directly proportional to the concentration of the free hydroxyl radicals generated in the system. The data were obtained by the elimination of a common time axis between independent experiments, where (a) the chemiluminescent intensity was determined versus illumination time, and (b) the degree of benzene hydroxylation (as phenol) was determined versus illumination time. The concentrations for 15-ml volumes are: riboflavin, 3×10^{-5} M; HOOH (for experiment a), 0.33 M; HOOH (for experiment b), 0.165 M; phosphate buffer, 0.1 M, *p*H, 6.1; benzene trap (experiment b), 6.6×10^{-3} M; temperature, 24°.

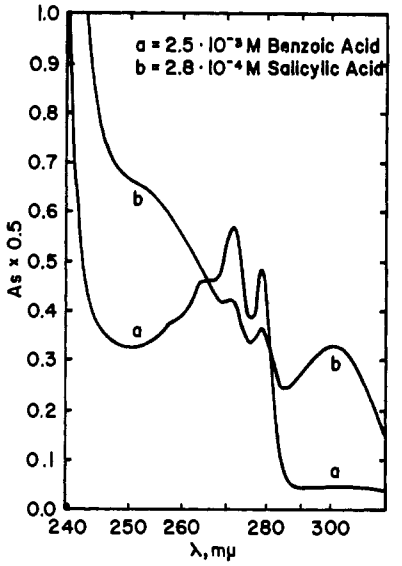

FIGURE 6: Absorption spectra determined on extracts of nonilluminated and illuminated riboflavin-HOOH systems containing benzoic acid as the free hydroxyl radical trap. Curve a, spectrum for the nonilluminated control system; curve b, spectrum for the test system illuminated for 10 minutes. The concentrations for 15-ml volumes are: riboflavin, 3×10^{-5} M; HOOH, 0.165 M; phosphate buffer, 0.13 M, *p*H, 6.1; benzoate trap, 2.5×10^{-3} M. Volumes, 15 ml; temperature, 24°.

tained in this manner. Curve (a) represents the spectrum run on the ether extract of the nonilluminated control, and shows only the presence of benzoic acid (λ_{max} at 271.5 mμ). Curve (b) represents the spectrum run on the sample illuminated for 10 minutes, and demonstrates the generation of 3.8 μmoles of salicylic acid in the system together with a concurrent decrease in the concentration of benzoate. On a molar basis the decrease in benzoate was greater than the corresponding increase in the salicylate generated. In order to study the mechanisms of aromatic hydroxylation more extensively we varied other parameters in the system.

Benzoate Concentration. In Figure 7 we present the results of benzoate variation on the concentration of salicylate generated in the riboflavin-HOOH system illuminated for constant 3-minute periods. As the concentration of the benzoate was increased, a corresponding increase was obtained in the amount of salicylate generated with a ratio, which is probably a function of the illumination time, of salicylate to benzoate of 1:4. The linear increase in salicylate with increasing benzoate concentration continues, at constant illumination time, until a point is reached at which the 3-minute illumination time, rather than the benzoate concentration, becomes the factor limiting the production of salicylate. Beyond this concentration of benzoate no further increase in salicylate generation was obtained.

HOOH *Concentration.* In an analogous manner we studied the effects of varying the HOOH concentration upon the photogeneration of salicylate in the riboflavin-HOOH system using benzoate as the hydroxyl trap. In these experiments the benzoate was kept constant at a suboptimal concentration of 5.6 μM (see Figure 7) in order that the effect of benzoate limitation might be demonstrated at excessive HOOH concentrations. The results showed that, with this concentration of benzoate, the HOOH concentration can be varied between 2.85 μM and 2.85 mM with a corresponding increase in salicylate generation. A further increase in the concentration of HOOH added to the system under these conditions failed to yield additional salicylate owing to the limiting concentration of benzoate.

*p*H. In our study of the *p*H dependency of the hydroxylating system we used benzene as the trap to eliminate *p*H effects on the trapping species per se. For this study the illumination time was kept constant at 2.5 minutes. Because of the variability of catalase activity over the *p*H range studied, the solutions were adjusted to *p*H 6–6.5 following the illumination and prior to the addition of catalase to destroy the excess HOOH and terminate the reaction. Since some hydroxylation appeared to proceed up to 2 minutes postillumination (the absorbancy reading obtained upon developing the blue Folin-reagent color at different times subsequent to illumination increased as much as 12%), we adopted the procedure of adding the catalase to the preparations 2 minutes after the illumination and after adjusting the *p*H to within the 6–6.5 range. The *p*H

optimum for photochemical aromatic hydroxylation, with benzene as the trap, was found to be 5.

Oxygen Concentration. Some preliminary studies were made on the effect of oxygen concentration on salicylate generation. Using a suboptimal concentration of benzoate, 5.6 μM, three systems, in duplicate, at 0, 20, and 100% oxygen saturation, were given 3 minutes' illumination. Salicylate (1.1, 1.7, and 2.6 μM) was generated at 100, 20, and 0% oxygen saturation, respectively, and revealed an inverse relation between oxygen content and photochemical aromatic hydroxylation using benzoate as the trap substrate.

Temperature. Steele (1963) reported that chemiluminescence from the riboflavin-HOOH system could be thermally induced. If this chemiluminescence is also mediated by free hydroxyl radicals these radicals should be trapped by one of the trapping agents used in this work. We therefore made a study of the thermal cleavage of the O—O bond of HOOH *in the absence of riboflavin.* For this study we brought solutions of benzene-water to 73–75° and injected HOOH in amounts to give a final HOOH concentration of 0.2 M. These solutions were allowed to remain at this temperature for various times before cooling and adding catalase. Determinations of the amounts of phenol produced in this manner indicated an essentially direct relationship between the amount of phenol generated and the period of time at the elevated temperature. Heating for a 10-minute period with an initial benzene concentration of about 0.02 M gave 1.5 μM of phenol. This indicated to us that some degree of cleavage of the O—O HOOH bond could be achieved thermally in the absence of riboflavin as well as photochemically in the presence of riboflavin.

Copper. After the completion of most of the work reported in this paper attempts to repeat some of our experiments in another laboratory where deionized water was supplied routinely led us to suspect that at least some of the kinetic aspects of our studies were dependent upon copper concentration. The influence of copper concentration on the chemiluminescent intensity obtained subsequent to the photoinduction of the riboflavin-HOOH system led to the discovery (J. E. Vorhaben, paper in preparation) of a marked enhancement in the chemiluminescence which appeared to be highly specific for copper. Iron was specifically eliminated as having an influence on the photoinducible chemiluminescence. After the removal of all detectable copper ion from our reagents and water with Chelex 100, we repeated photochemical hydroxylation studies in the presence and absence of copper, and compared the results with the data obtained previously. Samples of the reagents used in our earlier studies were also analyzed for copper (see Methods) and were found to contain approximately 0.02 μM copper per 13.5 ml. Table II lists the influence upon salicylate generation of copper and illumination time.

The data show the marked enhancement of hydroxylation produced by the incorporation of copper into the system. It should be noted however that the copper effect is one of enhancement and not an obligatory requirement, for hydroxylation occurs in the system in the absence of added copper. The same results were obtained for the photoinduced chemiluminescence (J. E. Vorhaben, paper in preparation).

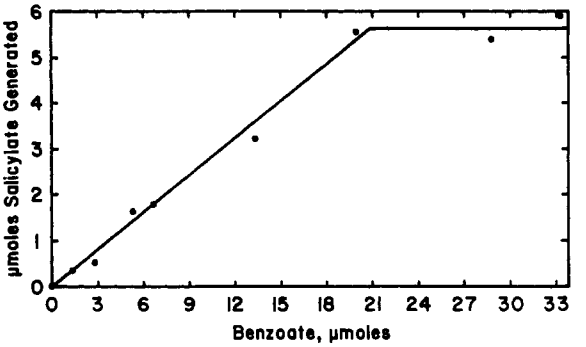

FIGURE 7: Benzoate trap concentrations versus salicylate concentrations generated photochemically in the riboflavin-HOOH system. The concentrations for 15-ml volumes are: riboflavin, 3×10^{-5} M; HOOH, 0.165 M; phosphate buffer, 0.1 M, *p*H, 6.1. Volumes, 15 ml; illumination time, 3 minutes; temperature, 24°.

TABLE II: Influence of Copper and Illumination Time on the Photochemical Hydroxylation of Benzoate to Yield Salicylate in the Riboflavin-HOOH System.[a]

Salicylate (μM/13.5 ml) At Illumination Time of:			Copper content
30 sec	60 sec	120 sec	(μM/13.5 ml)
0.3	0.71	1.3	0
0.53	1.5	3.8	0.022
1.5	2.8	9.0	0.080

[a] Concentrations for 15-ml volumes: riboflavin, 3×10^{-5} M; HOOH, 0.165 M; chelexed phosphate buffer, 0.1 M, *p*H 6.1; benzoate, 6.3×10^{-4} M.

Other Factors Affecting the Yields of Hydroxylated Products. Additional factors which we considered must be important as limiting the yields of phenol and salicylate from benzene and benzoate, respectively, in the photochemistry were, on the one hand, the repression of the hydroxylation by the generated products per se, and, on the other hand, their continued photochemical destruction during the course of the illumination period. The evidence for the repression of the hydroxylation by the generated phenol, owing probably to polyhydroxylation, is presented in Figure 8. Similar results were obtained with salicylate. The photoinduced chemiluminescence, itself dependent upon apparent free hydroxyl radical generation, as illustrated in Figure 5, was found to be suppressed where the systems were photoinduced in the presence of increasing concentrations of phenol and salicylate. In an experiment de-

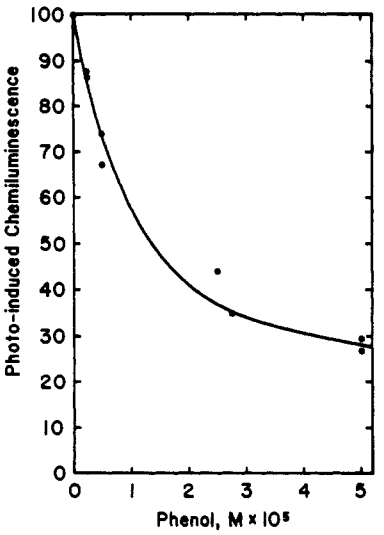

FIGURE 8: Photoinduced chemiluminescence of the riboflavin-HOOH system versus phenol concentrations. The concentrations for 15-ml volumes are: riboflavin, 3×10^{-5} M; HOOH, 0.165 M; phosphate buffer, 0.1 M, *p*H, 6.1. Volumes, 15 ml; illumination time, 30 seconds; temperature, 24°.

signed to show the photodestruction of generated hydroxylated product we measured the destruction of phenol in the riboflavin (0.45 μM)-HOOH (5 mM) system, containing 0.09 μM of copper, versus illumination time. The system was buffered with phosphate (0.1 M) at *p*H 6.1 and contained 1 μM of phenol. Phenol loss began at 2 minutes' illumination and increased linearly with time to a loss of 30% in 10 minutes. This experiment is analogous, with the exception of the light, to that reported by Wieland (1924) for the oxidation of benzoate and other organic compounds to carbon dioxide and water by HOOH and copper.

Discussion

Steele (1963) postulated that the chemiluminescence photoinduced in a riboflavin-HOOH system was initiated in the primary photochemical event as a triplet-sensitized homolytic cleavage of the O—O bond of HOOH with the production of two free hydroxyl radicals. The experiments reported in this paper were done to test the proposed mechanism by trapping the free hydroxyl radicals generated. The data appear to indicate that the hydroxylation of the benzene and benzoate, incorporated into the riboflavin-HOOH system, ostensibly as free hydroxyl radical traps, has been effected photochemically. The hydroxylation of these compounds has been interpreted as proceeding by mechanisms involving the free hydroxyl radical (Stein and Weiss, 1948, 1949a,b; Day and Stein, 1949; Merz and Waters, 1949). In terms of mechanism, however, and as we indicated in our introductory remarks, the photoinduced production of hydroxylated products in the riboflavin-HOOH system, while indicating strongly that free hydroxyl radicals have been generated, does not differentiate between the homolytic and heterolytic mechanisms of HOOH rupture depicted as paths A and B, respectively, in Figure 1. We consider the reason for this now.

While the mechanism of free hydroxyl radical generation via path A (Figure 1) is evident, the mechanism via path B warrants comment. Kroh *et al.* (1961) have shown that for HOOH concentrations greater than approximately 0.001 M any free hydroxyl radicals generated in the system react rapidly with HOOH to give water and the perhydroxyl free radical:

$$\cdot OH + HOOH \rightarrow HOH + \cdot OOH$$

Since the perhydroxyl free radical, as indicated by Evans *et al.* (1952), has a pK_a of 2 it would be present only as the perhydroxyl free radical anion throughout the *p*H range used in our experiments. The importance of these facts, as emphasized by George and Griffith (1959), lies in the widely different redox characteristics of the undissociated and dissociated free perhydroxyl radical species, ·OOH and $\cdot OO^-$, respectively. While the ·OOH radical is a strong oxidant the $\cdot OO^-$ radical is a strong reductant with its "standard" potential lying below that of the hydrogen electrode at all *p*H values. This knowledge, coupled with the fact emphasized by Waters (1948) that the O—O bond of HOOH is sufficiently weak to be broken by any one electron reductant with a sufficiently low redox potential, provides the basis for the generation of free hydroxyl radicals via path B (Figure 1), namely:

$$\cdot OOH \xrightarrow{pK_a = 2} \cdot OO^- + H^+$$

$$\cdot OO^- + HOOH \rightarrow OH^- + O_2 + \cdot OH$$

$$\cdot OH + HOOH \rightarrow HOH + \cdot OOH$$

in a continuing cycle.

The cyclic process depicted, coupled with the free hydroxyl radical regenerating process,

$$HO\cdot + HOH \rightarrow HOH + \cdot OH$$

provides the means for stabilizing these highly reactive species until they can react with each other or with free radical trapping agents incorporated in the medium. These free radical trapping agents have reacted with generated free radicals to give hydroxylated products in quite high yield. In Figure 5, e.g., where benzoate

was used as the radical trap, it is shown that 6 μmoles of salicylate were produced in 3 minutes, or at the rate of 0.033 μmole/second. This means that at least 2×10^{16} quanta/second were effective in producing HOOH rupture. This is a reasonable quantum flux for the 500-w exciting projector when it is realized that only a rather narrow spectral region in the blue, the long-wavelength absorption band of riboflavin (445 mμ), is photochemically active. Further, since the yield of salicylate in 3 minutes (6 μmoles) was 13 times the molar concentration of the photosensitizing species (riboflavin, 0.45 μmole), it is evident that riboflavin is cycled in the system; and, what is more important, to the extent that it is cycling, that riboflavin so involved has not been destroyed. Any molecular species in such a highly reactive environment must be destroyed eventually, and this is true for the riboflavin present as well as for the the added trapping agents, and the products generated therefrom.

The highly reactive redox environment of different free radical species accounts adequately for the marked decrease in the yield of hydroxylated products with continued illumination. The decreased yields of hydroxylated derivatives are caused by further hydroxylations (polyhydroxylation), and the subsequent degradative oxidation of the hydroxylated products. Our results on the oxidation of phenol in the riboflavin-HOOH photochemical system points to the extreme susceptibility of the hydroxylated products to further hydroxylation and destruction. We are led to concur with the observation of Merz and Waters (1949) that these extensive degradations of the hydroxylated derivatives render the direct oxidation of aromatic ring systems by free hydroxyl radicals a process, unfortunately, at present, of no preparative value. An important additional feature is that the riboflavin in the photochemical system is converted to derivatives which, in reactions subsequent to illumination, react to give chemiluminescence with an accompanying, partial destruction of the riboflavin (J. E. Vorhaben, paper in preparation). Other characteristics are that riboflavin tied up as some derivative is presumably, and transiently, nonfunctional in producing free radicals, and the longer the illumination time the greater the amount of riboflavin made inactive and the lower the yield of hydroxylated products.

The data reported in this paper provide no insight into the mechanism of aromatic hydroxylation in the riboflavin-HOOH photochemical system. Regardless of whether the free radicals are produced via paths A or B (Figure 1), at the *p*H values used in these studies the ·OH, ·OOH, and ·OO^- radicals are probably present transiently as long as the exciting light is on. Though there was some suggestive evidence that some hydroxylation proceeded after the light was off, this does not mean necessarily that these radicals were still present or being generated. The fact that free radical traps repressed the chemiluminescence yield (Figures 3 and 4) only when present during photoinduction argues against the persistence of free radicals in the system after the light is off. In the active free radical environment of the riboflavin-HOOH light system we are inclined to favor direct hydroxylation mechanisms such as reactions (1-2), (1-3-4), or (1-3-5).

$$\text{Benzene} + \begin{bmatrix} \cdot\text{OOH} \\ \text{or} \\ \cdot\text{OH} \end{bmatrix} \longrightarrow \varphi\cdot + \begin{bmatrix} \text{HOOH} \\ \text{or} \\ \text{HOH} \end{bmatrix} \quad (1)$$

$$\varphi\cdot + \cdot\text{OH} \longrightarrow \text{phenol, or} \quad (2)$$

$$\varphi\cdot + \cdot\text{OOH} \longrightarrow \varphi\text{-OOH} \quad (3)$$

$$\varphi\text{-OOH} + \text{HOOH} \longrightarrow \text{phenol} + O_2 + H_2O \quad (4)$$

$$\varphi\text{-OOH} + \varphi\text{-OOH} \longrightarrow 2\varphi\text{-OH} + O_2 \quad (5)$$

$$\varphi\cdot + \varphi\cdot \longrightarrow \text{diphenyl} \quad (6)$$

We have not analyzed the riboflavin-HOOH light system for diphenyl which is probably formed in the system when benzene is used as the trap (reaction 6). The data presented in Figure 7, however, indicate that four benzoate molecules are necessary to produce one salicylate molecule. While this ratio of trap to product changes with the illumination time (Figure 4), the fact that its value is consistently greater than unit permits the likelihood of diphenyl formation.

There are three features of reactions (4) and (5) which are significant. The reactions of hydroperoxides are metal and base catalyzed, and are highly exothermic. These are features which we have found to be characteristic of those reactions of the riboflavin-HOOH system when it is induced to chemiluminescence either by light (Steele, 1963) or in a dark reaction by the addition of cupric ions plus ascorbic acid (J. E. Vorhaben, paper in preparation). Reactions (4) and (5) may serve as prototype reactions for microsomal hydroxylations which appear to be mediated by organic peroxides instead of HOOH.

A subtle feature of the aromatic hydroxylation reactions which we should like to point out, and which has been alluded to briefly by Kaufman (1962), is that of energetics. Whereas the electrons of the reduced pyridine nucleotides usually flow to oxygen via the flavin-cytochrome redox continuum with the generation of at least three molecules of ATP,[1] in the biological hydroxylation reactions the electrons of the reduced pyridine nucleotides flow within the enzyme-metal complex to oxygen directly with the liberation, and presumably the utilization, of the energy of at least three ATP molecules in the one tightly coupled reaction. The high-energy feature of these reactions is revealed in the riboflavin-HOOH light system where in the absence of a free radical trap some of the energy of the reaction is dissipated in the light of the chemilu-

[1] Abbreviation used in this work: ATP, adenosine triphosphate; NADPH, reduced nicotinamide-adenine dinucleotide phosphate.

minescence, whereas in the presence of the free radical traps the energy dissipated is not large enough to excite chemiluminescence from the trap species per se. These reactions may be formulated tentatively as:

$$\text{riboflavin-OOH} + \text{HOOH} \longrightarrow \text{X (X–OH?)} + H_2O + O_2 + \text{light}$$

$$\text{riboflavin-OOH} + \text{riboflavin-OOH} \longrightarrow 2\text{X (X–OH?)} + O_2 + \text{light}$$

$$\varphi\text{-OOH} + \text{HOOH} \longrightarrow \varphi\text{-OH} + H_2O + O_2 + \text{heat}$$

where X is an unknown riboflavin derivative. The high-energy feature of the hydroxylation reaction is apparent also in the work of Anderson (1947) who was able to elicit chemiluminescence from several aromatic hydrocarbons with the Milas reagent, a redox hydroxylating system (Milas and Sussman, 1936), with an accompanying hydroxylation of the hydrocarbons. But perhaps the most revealing insight into the energetics of NADPH-O_2-metal hydroxylating systems is provided in the recognition that they are essentially the reverse of the photosynthetic reactions which yield NADPH + O_2 + energy (ATP) instead of consuming them.

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