A Photoinduced Chemiluminescence of Riboflavin in Water Containing Hydrogen Peroxide. I. The Primary Photochemical Phase*

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Chemiluminescence may be considered the converse of a photochemical reaction; in the former the energy of a chemical reaction appears as light, while in the latter the energy of light causes a chemical reaction. Riboflavin (FMN or FAD), peroxide (organic?), and luciferase are thought to react in some manner to produce bioluminescence in the luminous bacteria. By illumination of solutions of riboflavin in water containing hydrogen peroxide a photoinduced chemiluminescence was obtained. Studies of the intensity of the chemiluminescence *versus* the intensity of the exciting light, *p*H, and concentrations of hydrogen peroxide and riboflavin have provided some insight into the over-all mechanism. The action of inhibitors on the photoinducibility indicates that the primary photochemical event initiating the reaction sequence terminating in light emission is mediated by the excited metastable triplet energy term of riboflavin, while the chemiluminescent emission originates from the excited singlet energy term. The phosphorescent spectrum of riboflavin was found to have a maximum at 603 mμ = 47 kcal. per Einstein. This energy is equal to the O—O bond energy for hydrogen peroxide in water and may be significant in the mechanism of the photoinduction.

Electronic excitation states function in photosynthesis and vision. Whether these states, which are widely distributed biologically, have an underlying functional significance in biochemical energetics remains to be determined. Szent-Györgyi (1941, 1947, 1957) has expounded repeatedly over the years that energy states higher than ground state terms may be generally functional in biology; this thesis has been implied, if not stated explicitly, by the emphasis given to the subject in four international symposia in as many years (Symposia: 1958, 1959, 1960, 1961). We have adopted the hypothesis that electronic excitation states may be functional *generally* in some phase(s) of biochemical energetics, and our work has been directed toward demonstrating their existence.

Functional electronic excitation states would be coupled mechanistically in the reaction in which they are functional. Consequently, only when this couple is disrupted should the excited state reveal itself with light emission. It is not unlikely, however, that on a quantum mechanical basis some of the excited states would fail to be coupled and some of the excitation energy would "leak" out as a low-level chemiluminescence which might be detected with sufficiently sensitive instruments. In a sense chemiluminescence is the converse of a photochemical reaction. In the latter a chemical change results from the absorption of light, while in the former a chemical reaction gives rise to light emission. In principle, therefore, it might be envisaged that if the *products* thought to be produced in a chemiluminescent reaction were brought together and exposed to light the chemiluminescent reaction might be reversed, with the transient production of *reactants*. The reciprocal nature of such processes was considered by Strehler and Arnold (1951) in their interpretation of the chemiluminescence of photosynthesizing cells. They associated the chemiluminescent emission with an energy-releasing back-reaction from photochemical products. Operating from these principles we have been searching for chemiluminescence in the ultraviolet and visible spectral regions from redox reactions in rather simple, well-defined, biochemical systems.

While attempting to observe chemiluminescence from rat liver mitochondria we observed instrumentally emission of light upon addition of hydrogen peroxide to the preparation. In studying known components of the mixture for light emission we discovered a photoinducible chemiluminescence of riboflavin in water containing hydrogen peroxide. Strehler and Shoup (1953) reported the discovery of the chemiluminescence of riboflavin in 1953. The chemiluminescence which they studied was also elicited in the presence of hydrogen peroxide, catalytically and thermally, *in the dark*. We have also "dark-induced" riboflavin chemiluminescence by "coupling" the riboflavin–hydrogen peroxide system with other redox components, notably, the reductants reduced diphosphopyridine nucleotide (NADH) and ascorbic acid. The addition of catalase to the preparation also resulted in light emission. The photoinduced chemiluminescence of riboflavin may be studied conveniently in two phases; the primary photochemical phase, which we report in this paper, and a secondary, postillumination phase, to be reported subsequently.

MATERIALS AND METHODS

Riboflavin was obtained from the Distillation Products Division of Eastman Kodak and was used without further purification. All riboflavin solutions were kept in actinic glassware until used. Working solutions were prepared from more stable stock solutions, and the concentrations were determined with a Beckman DU spectrophotometer. The hydrogen peroxide (written hereafter as HOOH) used was Baker Analyzed reagent grade, 30% (10 M). HOOH concentrations were assayed by permanganate titrations as recommended in Reagent Chemicals (American Chemical Society, Washington, D.C., 1955). Reduced diphosphopyridine nucleotide (NADH) was Sigma Grade and was obtained in preweighed vials. Phosphate and acetate buffers were prepared from Baker Analyzed reagent-grade chemicals.

To measure the low-level chemiluminescent emissions with reasonable efficiency we have used two Farrand phototube capsules face to face. One capsule contained an RCA 1P28 photomultiplier tube powered at 875 volts with a Victoreen Model 683 Ultra Stable Power Supply. The signal from the phototube was

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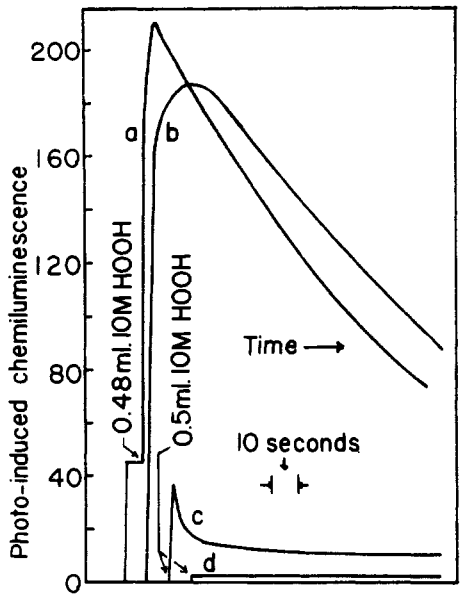

FIG. 1.—Photoinduced chemiluminescence of riboflavin. Final concentrations in all systems: riboflavin, 3×10^{-5} M; phosphate buffer, 0.1 M, *p*H 6.1; HOOH, 0.33 M. Volume, 15 ml. Temperature, 25°. Illumination time (hv), 30 seconds. Chart speed, 10 in. $min.^{-1}$ System: (*a*) 0.02 ml 10 M HOOH present during hv with 0.48 ml 10 M HOOH added subsequently; (*b*) 0.5 ml 10 M HOOH present during hv; (*c*) 0.5 ml 10 M HOOH added after hv; (*d*) 0.5 ml 10 M HOOH added with no hv.

read on a Beckman Micro-microammeter and recorded with a Minneapolis-Honeywell Quarter Second Electronik Strip Chart Recorder (0–50 mv range). The second phototube capsule contained the reaction tube (soft glass, 20 × 105 mm, with side-arm at upper end). The tube was fitted with a rubber collar for snug positioning in the capsule and was wrapped at the upper end with Scotch electrical tape to make it light tight. The tube was closed with a rubber stopper containing two 20-gauge needles. The needles pierced the stopper and were bent at a 90 degree angle externally. By this means it was possible to inject forcefully chemicals into the reaction tube from syringes under light-tight conditions. A useful feature of the Farrand phototube capsules is that they have shutters which may be closed and opened during an experiment to check light tightness and photomultiplier dark current levels.

Light for the studies of the photoinduced chemiluminescence was supplied by a 500-watt TDC Projector. To remove heat the light was filtered through an 8-cm depth of acidified 5% copper sulfate solution contained in a museum jar. The light then traversed a 2-mm 3387 Corning cut-off filter to remove ultraviolet light. This filter absorbed light below 440 mμ and insured that no light capable of being absorbed by HOOH directly (light below 380 mμ) entered the reaction vessel. Only light absorbed by riboflavin was passed. When low-intensity light emissions are being measured caution must be exercised in photochemical work to insure that the glass containers themselves are not excited to emit light. Soft glass and Pyrex are particularly bad in this respect. A few seconds' exposure of these glasses to ultraviolet light can result in an emission, observed instrumentally, lasting 30 seconds or more. The variations in light intensity were effected with Wratten Neutral Density Filters which were checked for calibration under the conditions of the experiment.

A representative procedure used, always in a darkened room, for eliciting the photoinduced chemiluminescence consisted in mixing all the reactants in an actinic ware test tube, with the HOOH additions being made last and immediately prior to photoinduction. The final volume for all studies was 15 ml; this volume was selected as just filling the reaction tube fully illuminated by the exciting light. The reactants were then mixed and transferred to the reaction tube, which was then held in position in front of the lamp and filter arrangement. The lamp was switched on, and exposure times were measured with a stopwatch. Immediately after the illumination period the contents of the reaction vessel were mixed by inversion and the vessel was positioned carefully in the Farrand phototube capsule and the light shutter opened. This technique resulted in the abrupt signal increases seen in Figure 1. The manual positioning of the reaction vessel in the Farrand capsule consistently took from 4 to 6 seconds.

Fluorescence was measured with a Farrand Photoelectric Fluorimeter. The exciting light was isolated from the riboflavin fluorescence emission with the following filter combinations: primary side, Corning band-pass, 7–37; secondary side, Corning ultraviolet cut-off, 3–73, and Wratten band-pass, No. 58.

The chemiluminescent emission spectrum was estimated indirectly because the low intensity of the photoinduced chemiluminescence (signals on the 10^{-8} and 10^{-9} scales of the micromicroammeter) has precluded, to date, direct determination of the spectrum with a monochromator. Estimation was done in two ways: (*a*) "externally" by interposing filters (Wratten 2A, 47, 58, 65, and Corning 3–71) between the 1P28 photomultiplier tube and the chemiluminescing systems induced thermally and by light; and (*b*) "internally" by adding to the system, before and after photoinduction, the chemically inert "pigment" 2,4-dinitrophenol. The suppression of the chemiluminescence by this compound was due to a double internal filter effect resulting from the absorption of both the exciting light and the induced chemiluminescence by the broad absorption band of the conjugate base of 2,4-dinitrophenol. This absorption band, at a *p*H of 6.1, extends from 330 to 450 mμ.

The phosphorescent spectrum of riboflavin was measured with a Farrand UV-Visible grating monochromator and an RCA 1P28 photomultiplier tube which was powered and read as indicated above. The emission was elicited with the sample frozen in Pyrex tubes which were immersed in liquid nitrogen contained in a quartz Dewar flask. The exciting light was isolated from the emitted phosphorescence with a phosphoroscope and filters. The spectrum reported in this paper was corrected for the spectral response of the photomultiplier tube as reported by the manufacturer. We recognize that, owing to variable spectral sensitivities between photomultiplier tubes, some error may be imposed on the data by this corrective procedure. Consequently, we cite in the Results a spectral maximum for this emission which was obtained by independent instrumentation (unpublished data). The value was obtained with a Beckman DK-1 Recording Spectrophotometer. This instrument has also an RCA 1P28 photomultiplier tube as the detector, and corrections for its sensitivity were made as above. Since the differential dispersion of the quartz monochromator in the DK-1 is low in the spectral region of interest (500 to 700 mμ), dispersion corrections were not made.

*p*H values were determined with a Beckman "Zeromatic" glass electrode *p*H meter.

RESULTS

Figure 1 illustrates several characteristic features of

the photoinduced chemiluminescence of riboflavin. The zero emission intensity represents the dark current reading for the RCA 1P28 photomultiplier tube and usually read between 0.6 and 0.9 $\times 10^{-9}$ amp when the tube was powered at 875 v at room temperature (25°). The apparent abrupt increases in emission intensity above the zero base line were due either to the opening of the capsule shutter subsequent to photoinduction or to the injection of reagents into the systems during the course of a reaction with the capsule shutter open. Curve *a* shows a typical response for the photoinduced chemiluminescence and depicts three important features of the reaction. First, the intensity of the postillumination chemiluminescence (before the injection of additional HOOH) increased when either the time of photoinduction or the concentration of HOOH present during the illumination period was increased. Second, the intensity of the chemiluminescence obtained subsequent to the injection of additional HOOH depended on the time elapsed subsequent to illumination before the addition was made as well as upon the amount of HOOH injected, the prior illumination time, and the amount of HOOH present initially during the period of illumination. The intensity was decreased as the concentration of HOOH present during the photoinduction period or added subsequently was decreased. Prolonging the time between photoinduction and the subsequent addition of HOOH resulted also in decreased emission intensities. Third, curve *a* illustrates the fact that the capacity for light emission may be potentiated with much lower concentrations present (0.013 M in this case) during the illumination period than the 0.33 M present in the system from which curve *b* was obtained. Curve *b* represents a typical response for the system which we have used for most of the studies reported in this paper. Curve *c* shows the chemiluminescent response obtained when HOOH was not present during the illumination period but was added subsequently. This curve illustrates the requirement for HOOH for the characteristic photoinduced response. Curve *d* depicts the low-level emission occasionally obtained thermally, *i.e.*, in the absence of photoinduction. Only infrequently, under the conditions of our experiments, did this thermally induced emission exceed our dark current levels. The intensity of the thermally induced chemiluminescence can be increased markedly by modest temperature increases.

We have found that the chemiluminescence of riboflavin in a chemically induced reaction could be seen with the eye after 30 minutes of dark adaptation. The color appeared white. Indirect estimates of the chemiluminescent spectrum for our system by filter interposition techniques (see Methods), where the emission was induced thermally and by light, indicated (*a*) that the thermally induced and photoinduced spectra were similar and probably identical, and (*b*) that the spectra seemed somewhat blue shifted relative to the fluorescence spectrum for riboflavin. We are aware of the crudeness of this approach, and until the spectrum can be measured directly we are inclined tentatively to locate the chemiluminescent emission maximum induced thermally and by light, in the vicinity of 520 mμ. This result was corroborated by the injection of 2,4-dinitrophenol as an internal filter (absorption band extends from 330 to 450 mμ) into the photoinduced chemiluminescing system. This compound suppressed the chemiluminescence by 25% without affecting the first-order decay rate constant for the reaction. When the same concentration of the 2,4-dinitrophenolate anion was present in the system during the photoinduction period the intensity of chemiluminescence was suppressed 39% relative to the intensity obtained in the absence of the pigment. The percentage suppressions differ in the two instances because in the former instance only the chemiluminescent light is being absorbed, while in the latter instance both the photoinducing light and the chemiluminescent light are being absorbed. The lack of an effect upon the rate constant indicated that the compound suppressed the chemiluminescence by filter-absorption and not by chemical interaction.

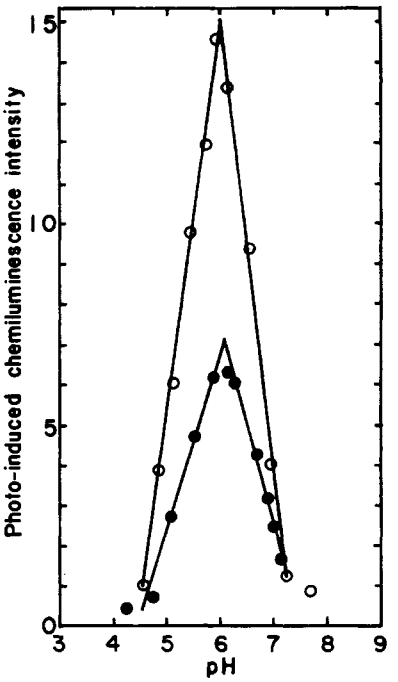

FIG. 2.—Photoinduced chemiluminescence of riboflavin *vs.* *p*H. ○, Phosphate buffer (0.1 M), riboflavin, 2.2 $\times 10^{-5}$ M. ●, Acetate buffer (0.1 M), riboflavin, 3.1 $\times 10^{-5}$ M. HOOH, 0.66 M; volume, 15 ml; illumination time, 30 seconds.

The photoinducibility of the chemiluminescence was found to be markedly dependent on *p*H and on the type of buffer (Fig. 2). The photoinduced chemiluminescence was most intense at *p*H 6.1 and was less than one half as intense in acetate buffer as in phosphate buffer of the same molarity. The *p*H optimum was found to be independent of the degree of inhibition.

Figures 3, 4, and 5 show the influence upon the intensity of the photoinduced chemiluminescence of variations respectively in the concentrations of riboflavin and HOOH, and in the intensity of the exciting light. At *p*H 6.1, the *p*H at which these results were obtained, the chemiluminescent signal after photoinduction rose to a maximum and then decayed with first-order kinetics (curve *b*, Fig. 1). The relative intensity data recorded in Figures 2, 3, 4, and 5 were measured at the maxima of the chemiluminescent emission intensity curves. Figure 3 shows that the intensity of photoinduced chemiluminescence increased linearly with increasing riboflavin concentrations. The intensity of the photoinduced chemiluminescence could be increased by increasing the illumination time. Use was made of this property in the riboflavin study (Fig. 3), where the photoinduction period was increased to 45 seconds to permit attainment of reasonable chemiluminescent signals at low riboflavin concentrations. We observed that the maximal intensity of photoinduced chemiluminescence gave a straight line when plotted against the square root of the illumination time. This observation prompted our study of the influence of the intensity of the exciting light upon the intensity of the chem-

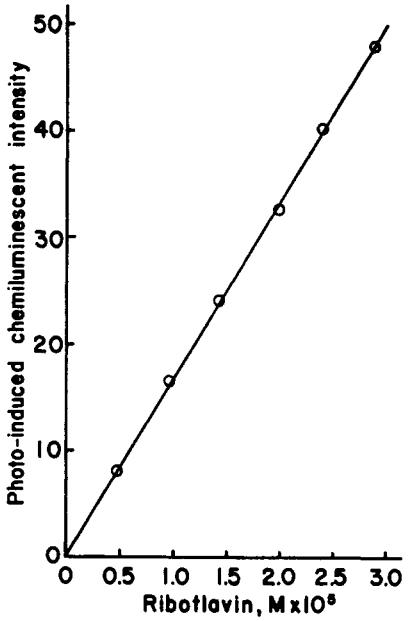

FIG. 3.—Photoinduced chemiluminescence *vs.* riboflavin concentration. Phosphate buffer, 0.13 M, *p*H 6.1; HOOH, 0.33 M; volume, 15 ml; illumination time, 45 seconds.

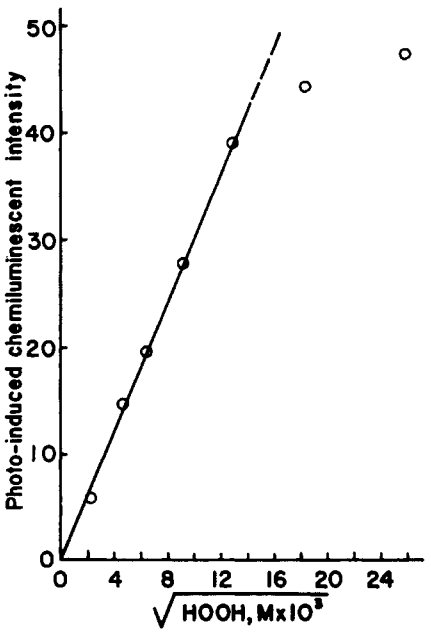

FIG. 4.—Photoinduced chemiluminescence of riboflavin *vs.* HOOH concentration. System: riboflavin, 3×10^{-5} M; phosphate buffer, 0.13 M, *p*H 6.1. Volume, 15 ml; illumination time, 30 seconds.

iluminescence. Figures 4 and 5 show that the chemiluminescent intensities are linear when plotted against the square roots, respectively, of the HOOH concentration and the light intensity. The results presented in Figures 3, 4, and 5 have kinetic significance and will be shown to provide some insight into the *over-all* mechanisms of the reactions involved in the light reaction.

The photoinducibility of the chemiluminescence was found to be inhibited by a wide variety of compounds (Table I).

In an effort to determine which excited electronic state of riboflavin, singlet or triplet, effects the photosensitization act which results eventually in chemiluminescence, we studied the influence of inhibitors on both the fluorescence intensity (fluorescence quenching) and the photoinduced chemiluminescence intensity

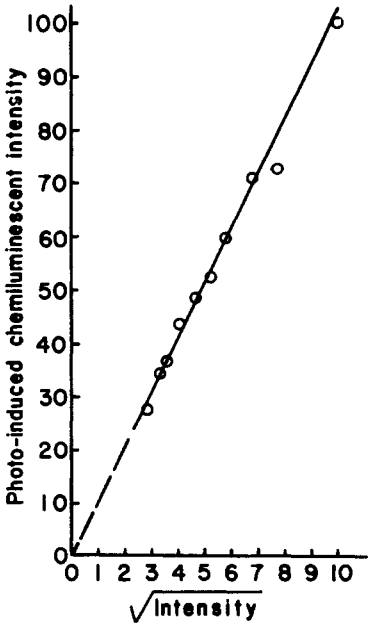

FIG. 5.—Photoinduced chemiluminescence of riboflavin *vs.* intensity of exciting light. Riboflavin, 2.95×10^{-5} M; phosphate buffer, 0.1 M, *p*H 6.1; HOOH, 0.33 M. Volume, 15 ml; illumination time, 30 seconds.

under the same experimental conditions. The results of this study, with KI and KCl used as inhibitors, are presented in Figure 6. In this figure note that the concentration scale (abscissa) differs by a factor of 100 for the two halides, the KI concentrations being the lower. These scales apply to both the photoinduced chemiluminescence emissions and the fluorescence emissions. For both halides the development of the intensity of photoinduced chemiluminescence was inhibited markedly at concentrations below those required to quench the fluorescence. For example, the halide concentrations producing 10% fluorescence quenching and 10% inhibition of the photoinduced chemiluminescence were, respectively, 325×10^{-3} M and 4.5×10^{-3} M for chloride and 2.8×10^{-3} M and 0.015×10^{-3} M for iodide. The photoinduced chemiluminescence of riboflavin was inhibited 50% by 0.11×10^{-3} M KI, whereas Weber (1950) found that 21×10^{-3} M KI was required to quench the fluorescence by

TABLE I

INHIBITION OF THE PHOTOINDUCIBILITY OF THE CHEMILUMINESCENCE OF RIBOFLAVIN[a]

Compound	Concentration (M)	% Inhibition
None	—	0
Iodide	0.0001	50
NADH	0.00018	50
Benzene	0.00074	50
Nitrobenzene	0.0013	64
Pyruvate	0.0033	60
Glucose	0.0033	53
Succinate	0.0033	50
Glyceraldehyde	0.0033	48
Lactate	0.0033	25
Ethanol	0.006	60
Chloride	0.032	50
n-Dodecylaldehyde	?, H_2O-saturated	30
Acetate	0.1	55

[a] System: riboflavin, 3×10^{-5} M; HOOH, 0.33 M; phosphate buffer, 0.1 M, *p*H 6.1; total volume, 15 ml, illumination time, 30 seconds.

50%. These results indicate that the energy terms responsible for the fluorescence and the photosensitization event are different and that the long-lived metastable triplet energy term of riboflavin is presumably effecting the photosensitization act. These results may be expressed more effectively by what may be called a "photochemical quenching constant." for which we propose the term P_q. This constant is similar to the fluorescence quenching constant, K, in

$$KQ = \frac{F_0}{F} - 1, \text{ or } K = \frac{\frac{F_0}{F} - 1}{Q}.$$

the Stern-Volmer equation, where F_0 and F are the fluorescence intensities in the absence and presence, respectively, of a quencher at concentration Q. This equation describes the influence upon the fluorescence yield (F/F_0) of the competition between fluorescence and quenching by an inhibitor. The quenching of the fluorescence by an inhibitor results from the collisional inactivation of the excited singlet state from which the fluorescence originates. We reasoned that the Stern-Volmer equation for quenching of the excited singlet state should apply equally well for describing the quenching of the excited triplet state should the latter be responsible for the photosensitization event giving rise to the chemiluminescence. We rewrite the Stern-Volmer equation as

$$P_q = \frac{\frac{P_0}{P} - 1}{Q}$$

where P_q is the photochemical quenching constant and P_0 and P are, respectively, the intensities of the photoinduced chemiluminescence in the absence and presence of a quencher at concentration Q. Since the excited triplet state has usually a longer life than the excited singlet state, a molecule in the excited triplet state would be subjected to many more potentially inactivating collisions during its life than a molecule in the excited singlet state. In other words, quenching should be effected at much lower quencher concentrations for the excited triplet state than for the excited singlet state. This fact should be observed experimentally in considerably larger values for the photochemical quenching constants, P_q, than for the fluorescence quenching constants, K. We suggest that the extension of the Stern-Volmer equation to include collisional inactivation of the excited triplet state, where a reaction thought to be mediated by that state can be monitered, should provide a useful parameter in the form of the photochemical quenching constant, P_q, by which the participation of the excited singlet or triplet energy terms for an excited species may be quantitatively distinguished. Bowen and Wokes (1953) have discussed the kinetics of fluorescence quenching processes in solution in terms of the Stern-Volmer equation and pointed out that fluorescence quenching constants in excess of 300 are not to be expected. In Figure 7 we present Stern-Volmer plots (the slopes of the lines give the constants) for the photoinduced inhibition and for fluorescence quenching by the inhibitors KCl and KI. It is evident that the Stern-Volmer equation describes the inhibition of the photoinduced chemiluminescence as effectively as it does the quenching of fluorescence. The fluorescence quenching constants obtained were 0.34 for KCl and 45 for KI. The photochemical quenching constants were 32 for KCl and 9750 for KI. The photochemical quenching constants exceed the fluorescence quenching constants by factors of 94 for KCl and 217 for KI. These differences, by two

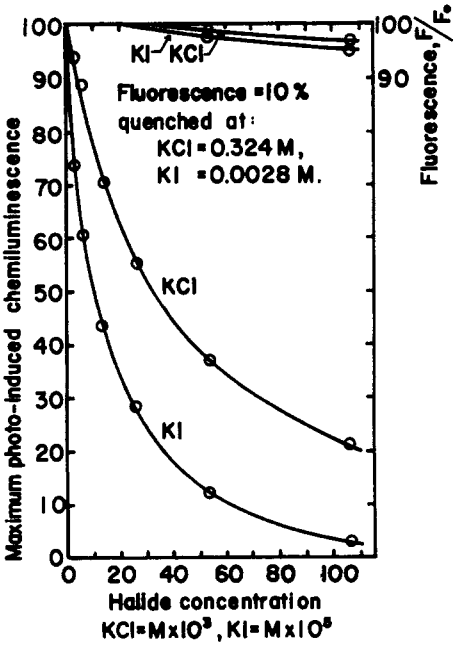

FIG. 6.—Fluorescence and photoinduced chemiluminescence of riboflavin *vs.* halide concentration. KCl systems: riboflavin, 3.1×10^{-5} M; HOOH, 0.33 M; phosphate buffer, 0.13 M, *p*H 6.1; illumination time, 45 seconds. KI systems: riboflavin, 3.0×10^{-5} M; HOOH, 0.33 M; phosphate buffer, 0.1 M, *p*H 6.2; illumination time, 30 seconds. Volumes, 15 ml; temperature, 24°.

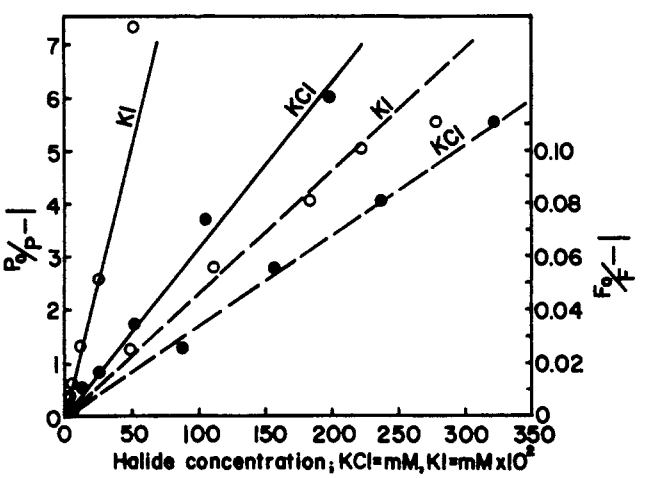

FIG. 7.—Stern-Volmer plots for halide quenching of the photoinduced chemiluminescence, P (solid lines), and fluorescence, F, (broken lines), of riboflavin. Systems: riboflavin, 3×10^{-5} M; HOOH, 0.33 M; phosphate buffer, 0.1 M, *p*H 6.1. Volumes, 15 ml. Illumination time for photoinduced systems: for KCl, 45 seconds; for KI, 30 seconds.

orders of magnitude, indicate that different energy terms are responsible for the photochemistry and the fluorescence. The excited metastable triplet energy term effects, we conclude, the photosensitization event which initiates the sequence of chemical events which result in chemiluminescence, while the fluorescence originates from the excited singlet energy term. It should be noted, with caution, that it is not the excited triplet energy term which decays as the chemiluminescence; for, as we have indicated, the chemiluminescence emission *per se* originates from the excited singlet state in a manner analogous to the fluorescence. Direct evidence that the chemiluminescence does not originate from the excited triplet energy term of riboflavin was provided by our finding that the chloride and iodide inhi-

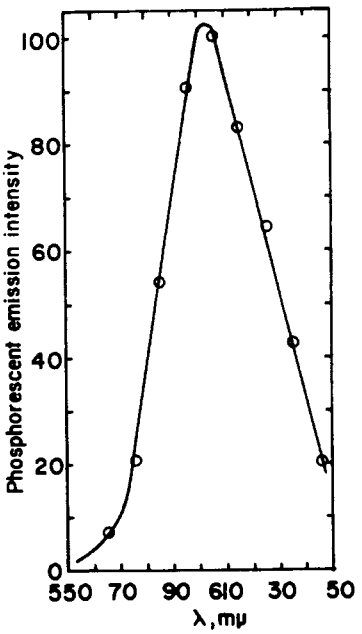

FIG. 8—Phosphorescent spectrum of riboflavin. Temperature, 77°K. Riboflavin, 8 × 10^{-5} M in oxygen-saturated ethyl alcohol. Corrected for spectral response of RCA-1P28 photomultiplier tube. Maximum at 603 mμ.

bitions were obtained only if they were present in the system *during* the photoinduction period, *i.e.*, they blocked the photosensitization event. When the halides were added to the riboflavin-HOOH system *subsequent* to illumination the chemiluminescent emissions were suppressed only to the extent of their known capacity for the quenching of the fluorescence. That is, the suppressions were much less than would have been obtained if the chemiluminescence had originated from the excited triplet state. This fact excludes the excited triplet state, therefore, as the energy term from which the chemiluminescence arises.

As indicated in Table I, NADH was found to be an effective inhibitor (50% inhibition at a concentration of 1.8 × 10^{-4} M) of the photoinduced chemiluminescence of the riboflavin-HOOH system. We found this inhibition to obey the Stern-Volmer quenching equation and obtained a photochemical quenching constant for NADH of 5300.

The evidence implicating the excited triplet energy term of riboflavin as the term responsible for the photosensitization event which initiates the chemiluminescence of riboflavin in the photoinduced system prompted us to measure the phosphorescent emission spectrum of riboflavin. Dhere and Castelli (1938) observed the long-lived emission of riboflavin and estimated its maximum to be at approximately 600 mμ. They designated it as a phosphorescence. We present the phosphorescent emission spectrum of riboflavin in Figure 8. The emission has a maximum at 603 mμ. The energy content of this light is equal to 47 kcal per Einstein at the maximum. By independent instrumentation (see Methods) we determined the phosphorescent emission maximum to be at 595 mμ, which is equivalent to 48 kcal per Einstein. These values are equal to the O–O bond energy, 46.7 kcal per mole, of HOOH *in water* as reported by Evans *et al.* (1952). We will consider the possible significance of this energy correlation in the discussion.

Preliminary studies showed a considerable oxygen effect on the photoinducibility of the chemiluminescence from the riboflavin-HOOH system. The decay rate for the chemiluminescent intensity increased with increasing oxygen tension and goes apparently through a maximum below a tension of 20%. The presence of oxygen was necessary for the phosphorescence of riboflavin to be observed visually.

DISCUSSION

Strehler and Shoup (1953) determined the chemiluminescent spectrum for riboflavin in their system to have a maximum at 550 mμ and thus to be similar to the fluorescence spectrum (Beinert, 1960). Our estimate that both the photoinduced and the thermally induced chemiluminescent spectra, which by our methods appear identical, lie in this same general region, with a maximum in the vicinity of 520 mμ, together with the results which preclude the emission as originating from the excited triplet state, indicates that the photoinduced chemiluminescence arises from the excited singlet energy term. This means that the chemical reaction providing the energy for the generation of the electronic excitation state, which decays with chemiluminescence, must be exothermic at least in the energy content of the shortest wave length of light emitted (Strehler, 1955). In this instance this energy must exceed 55 kcal per mole. The apparently identical spectra for the chemiluminescent emissions induced thermally and by light implies that there is a similar type of emissive act for the two processes, which differ only in their initiating mechanisms. This fact must be considered in any mechanism worked out for generating the excited species from which the chemiluminescence originates.

The detailed quenching data presented for KCl and KI in Figures 6 and 7 reveal that the excited triplet energy term mediates the photosensitization event which initiates the reaction sequence leading to chemiluminescence. The influence of KI on the triplet emission (phosphorescence) of riboflavin has been dramatically presented by Szent-Györgyi (1957) with color photographs. He found the triplet emission to be quenched by 10^{-3} M KI; the fluorescence emission however, remained visible and bright. Holmstrom and Oster (1961) have concluded, also from photochemical studies with riboflavin involving inhibitions with iodide, that the triplet energy term was responsible for the photosensitization reactions. The finding that the free energy of the riboflavin triplet energy term is equal to the O–O bond energy of HOOH in water appears to us to be more than coincidence, especially since HOOH is a normal product of leucoflavin oxidations in many biochemical reactions. The participation of the triplet energy term in photochemical oxidations appears increasingly to be a quite general phenomenon. As indicated in Table I, NADH inhibited markedly the photoinducible chemiluminescence from the riboflavin-HOOH system and gave a photochemical quenching constant, P_q, of 5300. This result prompts us to raise the possibility that this excited triplet energy term of riboflavin is the functional electronic state mediating the "dark-oxidations" of NADH by flavoprotein-linked NADH dehydrogenases.

In the primary photochemical event, which initiates the reaction sequence leading to chemiluminescence, we propose, tentatively, a photosensitized homolytic cleavage of HOOH, mediated by the excited triplet energy term of riboflavin, to produce either two free hydroxyl radicals or some activated riboflavin derivatives (*e.g.*, *rfx*, see below) which are precursor reactants in the chemiluminescent reaction. The inhibitions of the photoinducible chemiluminescence by halides, acetate, nitrobenzene, and benzene, evidenced in Table I, which have been shown to "trap" or "inactivate" free hydroxyl radicals, supports this thesis. Merz and

Waters (1947, 1949) and Kolthoff and Medalia (1949) have interpreted, for example, the acetate and chloride inhibitions of oxidations by Fenton's reagent as due to their interference with free hydroxyls which are generated by this reagent. Parenthetically, R. J. Williams, in this laboratory (manuscript in preparation), has recently "trapped" hydroxyls in the riboflavin-HOOH-light system by using benzene and benzoic acid as "traps" and identifying the formation of phenol and salicylic acid, respectively, as products.

Although free radicals are characterized by high reactivity, water is the *milieu par excellence* for stabilizing the hydroxyl radical, for, as Waters (1948) has emphasized, hydroxyl reactions with water would result only in a continuous regeneration of hydroxyl radicals:

$$\mathbf{HO}\cdot + \mathrm{HOH} \longrightarrow \mathbf{HOH} + \cdot\mathrm{OH}$$

They disappear, of course, when two hydroxyls combine to regenerate HOOH. In the HOOH concentrations used in most of this work, however, Kroh *et al.* (1961) have shown that the hydroxyl free radical reacts rapidly with HOOH to give water and the perhydroxyl free radical:

$$\cdot\mathbf{OH} + \mathrm{HOOH} \longrightarrow \mathbf{HOH} + \cdot\mathrm{OOH}$$

Since the perhydroxyl free radical is a reasonably strong acid, $pK_a = 2$ (Evans *et al.*, 1952), it would be present only as the perhydroxyl free radical anion throughout the *p*H range used in our experiments. Uri (1952) points out that whereas ·OOH is an oxidant, $\cdot OO^-$ is a reductant. Riboflavin may be reduced by this species to the semiquinone or to a hydroperoxide radical (*rfx*, ?, see below). Oxygen could react with the semiquinone to produce transiently a riboflavin perhydroxy radical.

Kinetically, the reaction sequence, initiated by light and leading to chemiluminescence, appears to be a series pseudo-first-order reaction, *i.e.*, of the type

$$A \xrightarrow{k_a} B \xrightarrow{k_b} C$$

where the curves of relative fluorescent intensity reflect the buildup and decay of intermediate *B*. This property of the reaction is evident in curve *b* of Figure 1. The *p*H dependency of the rate constants, k_a and k_b, currently being studied, accounts in part for the sharp maximum observed for the intensities of photoinduced chemiluminescence shown in Figure 2. It should be emphasized that the well-known *p*H dependency of the fluorescence emission intensity of riboflavin, as determined by Kuhn and Moruzzi (1934), does not explain this phenomenon. The fluorescence intensity curve, when plotted against *p*H, is almost flat, and its maximum intensity extends from *p*H 4 to 8, which is the *p*H region of maximum change for the intensity of the photoinduced chemiluminescence. Further, there is no sigmoid character evident in the *p*H *versus* chemiluminescent intensity plot, which should, however, be manifest if a dissociable group alone were being "titrated." The mechanism worked out for the reaction sequence must account for the *p*H dependency of these rate constants.

The relationships between the intensities of the chemiluminescence and variations in the concentrations of riboflavin (Fig. 3) and hydrogen peroxide (Fig. 4) and the intensity of the exciting light (Fig. 5) are significant. The results indicate that the intensity of the chemiluminescence increases linearly with changes in the riboflavin concentration but gives a linear relationship only when plotted against the square root of the HOOH concentration and the intensity of the exciting light, *i.e.*,

$$\text{chemiluminescent signal} \propto \text{riboflavin concentration}$$

$$\text{chemiluminescent signal} \propto \sqrt{\mathrm{HOOH} \times I}$$

Chapman (1926) considered this type of situation and pointed out that "where the rate of disappearance of an active substance, or of activity, is proportional to the square of its concentration, then the rate of chemical change should be proportional to the square root of the intensity of the light, provided that the rate of the chemical change is proportional to the concentration of the active substance." These considerations provide a framework within which we hope to construct the details of the intermediary mechanisms as clarified by our developing kinetic analysis. The general kinetic picture must take apparently some *over-all* form as shown in the scheme, where *rfx* represents the con-

$$\mathrm{HOOH} + I + 2rf \xrightarrow{k_1} 2rfx$$

$$\frac{+\,d\,rfx}{dt} = k_1 \times \mathrm{HOOH} \times I \times rf^2$$

$$rfx + rfx \xrightarrow{k_2} 2rf$$

$$\frac{-d\,rfx}{dt} = k_2 \times (rfx)^2$$

In a steady state:

$$k_2 \times (rfx)^2 = k_1 \times \mathrm{HOOH} \times I \times rf^2$$

$$\text{chemiluminescent signal} \propto rfx = \sqrt{\frac{k_1}{k_2}} \times \mathrm{HOOH} \times I \times rf^2$$

$$\text{chemiluminescent signal} \propto rfx = k \times rf\sqrt{\mathrm{HOOH} \times I}$$

centration of the "active" substance, of presently unknown composition, to which the intensity of the chemiluminescence is proportional, and *I* is the intensity of the exciting light. This scheme follows essentially the mechanistic stoichiometry for chemiluminescence proposed by Weiss (1939) and adopted subsequently by Johnson *et al.* (1945). It leaves open the question of the influence of oxygen and *p*H. Johnson *et al.*, however, consider mechanisms whereby bimolecular reactions can give rise to pseudo-first-order kinetics and discuss the possible impact of *p*H upon the reactive species. We find it difficult to believe that a dismutation of semiquinone radicals, *per se*, can provide the requisite exothermic energy to generate the excited singlet state of riboflavin. Our results indicate that HOOH must be present for chemiluminescence to be elicited (curve *a*, Fig. 1); however, we have not tried other oxidants. We have observed, for example, that when NADH was added to solutions of phenazine methosulfate an intense green color was produced which is characteristic of the semiquinone of phenazine methosulfate. We have not observed light emission from this reaction. When, however, the NADH additions were made to the phenazine methosulfate solutions in the presence of HOOH, the reaction was accompanied, instrumentally, by a marked flash of light. Parenthetically, we would emphasize that this chemiluminescent reaction does not require photoinduction. Oxygen is important and may exercise a multiple role. Apparently it accelerates the population of the excited triplet state from the excited singlet state of riboflavin as well as the decay from the excited triplet state to the ground singlet state. Oxygen must certainly destroy some of any semiquinone generated, although this reaction may be slower than expected (see above).

Several features of the riboflavin-HOOH system appear sufficiently similar to those of some biolumi-

nescent preparations to merit emphasis. Strehler and Cormier (1953) studied the effect of ultraviolet light (365 mμ Hg line) on the luminescence of bacterial extracts. Interestingly, exposure times up to about 3 minutes' duration resulted in marked increases (3-fold) in the luminescence. Apparently this observation has not been pursued.

The chemiluminescent "flash" produced upon the addition of HOOH to the riboflavin-HOOH preparation photoinduced in the presence of a suboptimal concentration of HOOH (curve *a*, Fig. 1) resembles the "HOOH-flash" observed by Dure and Cormier (1961) in extracts from a luminous Balanoglossid species. This HOOH-effect, in the riboflavin-HOOH system, may be related to the rate constant, k_a, in the series first-order reaction which we are currently studying (note, *e.g.*, curve *a* of Fig. 1).

An interesting feature of the riboflavin-HOOH chemiluminescent system, which appears similar to the luminous bacterial preparations, is the stoichiometry. On kinetic grounds we conclude tentatively that two riboflavin species must interact in the light reaction. McElroy and Green (1955) concluded from a kinetic analysis of the requirement for reduced flavine mononucleotide for light emission from bacterial preparations that two molecules were required and that the luciferase molecule has two flavin sites. This conclusion was supported by Totter and Cormier (1955) and by Cormier *et al.* (1956). This stoichiometry has been discussed also by Strehler (1961). It is somewhat surprising that we have obtained indications for the same stoichiometry in free solution, *i.e.*, in the absence of enzyme; however, if the mechanism for the photoinduced chemiluminescence has any feature in common with the bioluminescent reactions, this would explain partially the low emission intensities we have found.

Strehler (1961) discusses also the possibility that the observed aldehyde oxidation by the luminous bacterial preparations is incidental to the chemical reaction producing the light. Aldehyde is not required in the reactions giving the chemiluminescence in the riboflavin-HOOH system. In fact, the presence of dodecyl aldehyde inhibited partially the photoinducibility of the chemiluminescence.

If the electronic excitation states of riboflavin have functional significance and the two-flavin stoichiometry is a requirement in the generative mechanism, then flavoproteins with two flavin prosthetic groups per mole of enzyme might be profitably examined from this point of view. This is not to imply that other generative mechanisms may not be operative, for the presence of the protein enzyme, metals, and other flavin derivatives, not yet investigated in our study, certainly exert profound effects upon mechanisms not currently recognized.

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