ionized sulfhydryl groups, then four –SH groups must be involved. In studies of urease inhibition by thiourea[3] and methylurea[6] a similar dependence of inhibition index on the square of inhibitor concentration was noted.

Although it seems quite reasonable to assume that metal ions inhibit urease by combination with an essential –SH group, the possibility that other important negative groups also act as ligands can not be disregarded. The excellent correspondence between the toxicity sequence and the "natural order" stability sequence noted in the first paragraph of this section is certainly not in conflict with this possibility.

The influence of ionic strength on the inhibition by Cu(II) shown in Fig. 2 is qualitatively in accord with expectations based on the assumption that Cu(II) is combining with a negatively charged group. Quantitative treatment of the data does not seem to be justified. Because of the salt effect, however, pI^* values reported in Table II would be expected to differ from pI^* values obtained at infinite dilution. The difference, in general, should be small, with the greatest deviation expected in the case of Mn(II).

Although the inhibition of urease by metal ions is non-competitive in alkaline solution, it is by no means certain that similar behavior would be observed below pH 7. In the case of thiourea[6] and methylurea[3] a change in the character of inhibition occurs with changing pH. Both of these inhibitions become competitive at pH 6.

Acknowledgment.—It is a pleasure to express our gratitude to the Administration of The University of Texas for the special assignment given to W. H. R. S. that provided time for research and the preparation of this paper.

[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY RUTGERS, THE STATE UNIVERSITY, NEW BRUNSWICK, N. J.]

Photochemical Cleavage of Water by Riboflavin. II. Role of Activators

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The role of ethylene diamine tetraacetate and methionine in promoting the photoreduction of riboflavin and in suppressing other, irreversible, photochemical changes in the flavin was studied by spectrophotometric and chromatographic methods and by measuring the oxidation-reduction potential of riboflavin during photochemical reduction. A special absorption cell, permitting simultaneous measurements of light transmission and potential in deoxygenated solutions, was developed for this purpose. The reoxidation of dihydroriboflavin by oxygen is accompanied by conversion of part of the flavin into lumiflavin and lumichrome. These irreversible changes are inhibited by substances possessing an electronegative nitrogen or sulfur atom. In the presence of such "activators" the oxidation-reduction potentials obtained during photoreduction agree with published values obtained by reductive titration with dithionite throughout the pH range from 4 to 10; without activator, this agreement is limited to pH 4 to 5. It is concluded that, both in the absence and presence of activator, the hydrogen donor is not the ribityl side chain but water which is hydrogen-bonded to the riboflavin. Activators form hydrated complexes with riboflavin. On illumination the complex dissociates, resulting in the formation of dihydroriboflavin and the oxide of the activator. Due to its complexing action, the activator: (1) weakens the O—H bond of complexed water, thus raising the quantum yield of its photodecomposition, (2) serves as acceptor for the oxygen moiety of the water and (3) protects the ribityl group of riboflavin from irreversible degradation by reactive radicals arising during the reoxidation of dihydroriboflavin by oxygen.

Introduction

It has been shown[1] that illumination of an air-free riboflavin solution, in the presence of an "activator," such as methionine, results in the formation of leucoriboflavin and methionine sulfoxide. In addition to its reversible photochemical conversion to the leuco-form, riboflavin may undergo irreversible light-induced changes, *i. e.*, conversion to lumichrome and lumiflavin.[2,3] Thus, photoreduction of riboflavin is accompanied by photodegradation, except in the presence of a relatively high concentration of activator. The results obtained with methionine as activator show this substance to act, not as a hydrogen donor, but as an acceptor for the oxygen moiety derived from water. Other activators, including ethylenediamine tetraacetate (EDTA), appear to function in a similar manner. The role of such activators now has been further characterized by a study of their protective action against irreversible degradation of the flavin and of their stoichiometry in systems containing riboflavin, activator and a third (reducible) substance.

The course of the photoreduction, as distinct from the accompanying photodegradation, was followed by making simultaneous measurements of the potential and of the flavin concentration and by spectral and chromatographic studies before and after the photoreaction.

Experimental

Materials.—Riboflavin [6,7-dimethyl-9-(D-1'-ribityl)-isoalloxazine] was obtained from Hoffman–LaRoche, Inc. Lumiflavin and lumichrome were formed in solution by exposing a 10^{-4} M solution of riboflavin in water to sunlight for several days. Chromatographically pure solutions of the above three compounds were obtained by column chromatography as described by Whitby[4]; in this procedure, specially purified Whatman paper pulp and water saturated with isoamyl alcohol were used. Recrystallized disodium-EDTA was obtained from the Geigy Chemical Co. DL-Methionine was obtained from the Sigma Chemical Co. and was recrystallized from hot water until chromatographically pure. Lyophilized catalase was obtained from the Worthington Biochemical Corp. Doubly

(1) W. J. Nickerson and G. Strauss, *J. Am. Chem. Soc.*, **82**, 5007 (1960). See this reference for a bibliography of related work.

(2) R. Kuhn, H. Rudy and T. Wagner-Jauregg, *Ber.*, **66**, 1950 (1933); R. Kuhn and H. Rudy, *ibid.*, **67**, 1125 (1934).

(3) B. Holmstrom and G. Oster, Am. Chem. Soc. Meeting, New York, N. Y., September, 1960, p. 72-S.

(4) L. G. Whitby, *Biochem. J.*, **50**, 433 (1952).

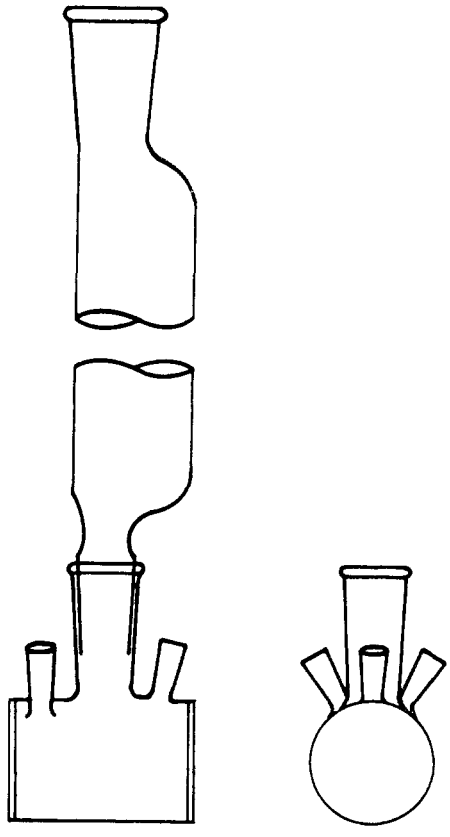

Fig. 1.—Demountable absorption cell employed for photochemical studies; see text for details of operation.

distilled water, which had a specific conductance of 1×10^{-6} ohm^{-1}cm.$^{-1}$ was used. Nitrogen (prepurified, 99.99% pure) was obtained from the Matheson Co.

Paper Chromatography.—Whatman No. 1 paper and water saturated with isoamyl alcohol were used in an ascending system[4] to check the purity of the flavins used as starting materials and to study the appearance of photodecomposition products in illuminated riboflavin solutions.

Spectra.—A 1-cm. Corex cuvette was modified by fusing to it a glass reservoir of 30 mm. diameter and 100 mm. length which carried a standard taper joint at its upper end. To study the irreversible degradation of riboflavin, only the cuvette part of the assembly was filled with solution, which was then deoxygenated by vigorous boiling under vacuum at room temperature and flushing with nitrogen.[5] Illumination was by a 375-watt photoflood incandescent lamp placed 10 cm. from the cuvette, which was cooled under a stream of cold water Spectra of the exposed solutions were determined in a Beckman DU spectrophotometer with photomultiplier attachment.

Potential Measurements.—A special cell was constructed[6] for the simultaneous measurement of light transmission, oxidation-reduction potential and *p*H during the photochemical reaction. The demountable cell (Fig. 1) consisted of a Pyrex body with quartz end windows, and had an optical path length of 60 mm. The volume of the cell was 60 ml. A platinum electrode, a glass electrode (Beckman No. 39024) and a saturated calomel electrode (Beckman No. 39270) were fitted with rubber sleeves and inserted into the cell (but not into the path of the light) through vacuum-tight side arms. The obstruction-free cross section of the cell was 33 mm. in diameter. The cell carried a reservoir of 120 ml. capacity through the top of which it could be connected either to a vacuum system or to a source of nitrogen. The cell was illuminated by a mercury vapor lamp (Hanovia SH), the light of which was collimated by a system of quartz lenses upon 2.5 cm.^{2} of the end window. During illumination, the solution contained in the cell was stirred magnetically. The 436 mμ mercury line, which is near one of the absorption maxima of riboflavin (445 mμ), was isolated by a combination of Corning 3–73 and 5–57 band filters. The rate of illumination was of the order of 4×10^{-9} einsteins per second as determined by actinometry using uranyl sulfate. The transmitted light was passed through a quartz condensing lens placed immediately behind the cell. A circular bismuth-silver thermopile (Eppley) was placed behind this lens so that the light beam exactly filled its window of $^3/_8$ inch diameter. The output of the thermopile was read on a Leeds and Northrup No. 2284B galvanometer whose sensitivity could be varied by means of a shunt system.

In operation, the cell was filled with solution, then turned on its side so that the solution flowed into the reservoir where it presented a large surface. The solution was boiled vigorously under vacuum at room temperature to remove dissolved air; purified nitrogen was then cautiously admitted until the contents of the cell were again at atmospheric pressure. An open-end mercury manometer, connected to the cell, was used to follow these pressure changes. This procedure was repeated three times before the cell was returned to an upright position. By keeping the contents at atmospheric pressure, except for relatively brief periods, electrolyte seepage from the calomel electrode was minimized.

The potential between the platinum and calomel electrodes was measured with a Beckman Model G *p*H meter. During a run, the potential and the intensity of transmitted light were read simultaneously every 1–2 minutes for 30 minutes or so. The transmittance readings were converted to optical densities by referring to the transmittance of the cell when filled with water. The latter was checked before and after each experiment. The lamp was constant to within 1–2% during a given run. At the low light intensities employed, the reaction rate was sufficiently slow so as to allow the platinum electrode to become, and remain, equilibrated with the solution.

Results

Reaction Products.—Deoxygenated solutions containing 2.5×10^{-5} *M* riboflavin and 0.05 *M* acetate buffer at *p*H 4.8, in water or in 1×10^{-3} *M* methionine solution, were examined chromatographically and spectrally before illumination, and after illumination for 1 to 2 minutes followed by aeration. Paper chromatographs of such solutions are reproduced in Fig. 2, together with those of chromatographically pure samples of riboflavin, lumiflavin and lumichrome. The spots were identified by their R_f values and their characteristic fluorescence when examined by ultraviolet light. In the system used, riboflavin and lumiflavin had R_f values of 0.45 and 0.27, respectively, and gave a greenish-yellow fluorescence. Lumichrome, with an R_f of 0.13, had a light blue fluorescence. The chromatographs show that riboflavin, when photoreduced anaerobically and subsequently aerated, is converted in part to lumiflavin and lumichrome. Repeated "cycling" in this manner results in further production of lumichrome, at the expense of riboflavin and lumiflavin, both of which are gradually destroyed. No other fluorescent spots were detected. On repeated cycling, the fading became progressively more rapid but less complete, as also noted by Holmstrom and Oster.[3]

The spectra of these same solutions, shown in Fig. 3, also indicate the presence of lumichrome in reoxidized solutions. Curve B, representing the spectrum of a riboflavin solution after photore-

(5) Bubbling nitrogen through the solution, without evacuation, also reduced the oxygen content to negligible levels. Mere evacuation, however, without vigorous movement of the solution (produced by boiling or mechanical stirring), was quite inefficient.

(6) We are indebted to the Fischer and Porter Co., Warminster, Pa., for valuable assistance in the design and construction of this cell.

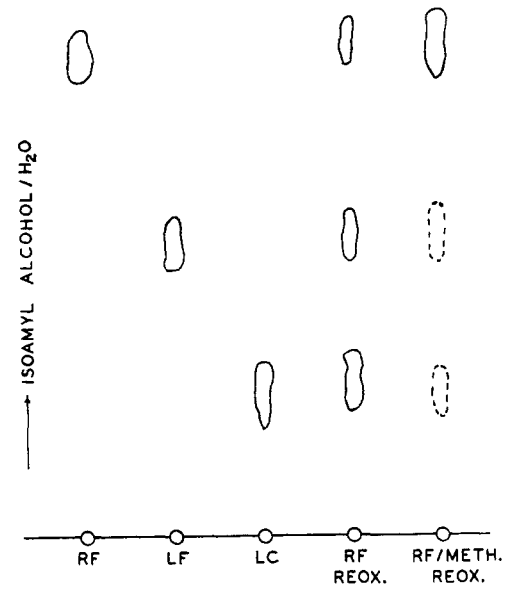

Fig. 2.—Products from illumination of riboflavin in presence or absence of activator. Paper chromatography on Whatman No. 1 paper; solvent: water saturated with isoamyl alcohol, ascending. RF = riboflavin; LF = lumiflavin; LC = lumichrome; all samples chromatographically pure. RF REOX. = riboflavin, illuminated anaerobically and oxidized by air; RF/METH. REOX. = riboflavin with methionine (activator) illuminated anaerobically, and oxidized by air.

duction and aeration, could be almost exactly duplicated by mixing riboflavin and lumichrome in a molar ratio of 1:0.3. The spectrum of pure lumichrome in neutral solution is also shown in the figure as a matter of general interest. The only published spectra of lumichrome[7] are those in 0.4 *N* HCl and in 0.4 *N* NaOH. At neutrality, the spectrum differs from both of these, although it resembles the spectrum in acid solution. Lumiflavin could not be distinguished from riboflavin in reaction mixtures, due to the close spectral similarity of these compounds.

Spectral changes observed during the cycling of riboflavin solutions are summarized in Table I, which expresses optical densities at 445 mμ—where riboflavin and lumichrome show maximal differences—as % of the initial optical density. Substantial recoveries in color, in excess of 50%, were found when riboflavin was cycled in the absence of activator.

TABLE I

SPECTRAL CHARACTERISTICS OF PHOTOREDUCED AND REOXIDIZED RIBOFLAVIN (2×10^{-5} *M*)

Solvent	*p*H	O.D. at 445 mμ, as % of original: 1st illumin.	1st aeration	2nd illumin.	2nd aeration
Water	*ca.* 6	35	88	32	65
0.05 *M* phosphate	7.0	12	78	21	47
0.05 *M* phosphate + 0.001 *M* Na_2EDTA	7.0	12	100	18	100

In the presence of methionine, the formation of lumiflavin and lumichrome on illumination and

(7) W. S. McNutt, *J. Biol. Chem.*, **210**, 511 (1954).

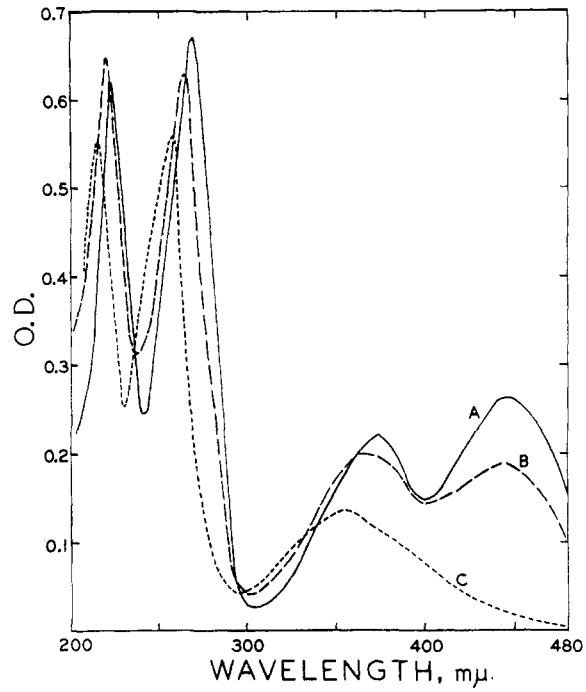

Fig. 3.—(A) Absorption spectra of chromatographically pure riboflavin in water; (B) same as (A), illuminated anaerobically and oxidized by air; (C) chromatographically pure lumichrome, in neutral solution. All concentrations approx. 2.5×10^{-5} *M*.

aeration was almost completely inhibited; the original riboflavin spectrum was restored, and only faint spots of the degradation products appeared on the chromatograph.

In the presence of catalase and absence of activators, the rate of photoreduction of riboflavin was only 20–40% of that found in controls. This provides confirmatory evidence[1] that hydrogen peroxide is formed when riboflavin is photoreduced anaerobically in the absence of activators. The action of catalase on flavin-bound peroxide will be reported in detail in a subsequent communication.

With a glass electrode immersed in the photochemical cell, it was found that no significant *p*H changes occurred during illumination of flavin in the presence or absence of activator.

Riboflavin as Photocatalyst.—EDTA, in common with other activators, permits riboflavin to act as catalyst for the photoreduction of other reducible substances, such as oxygen and various dyes.[8] The reaction was studied quantitatively as follows: A solution of 4×10^{-5} *M* riboflavin and 2×10^{-4} *M* EDTA was deoxygenated by bubbling nitrogen through it and illuminated. During the illumination a standardized methylene blue solution (8.6×10^{-3} *M*) was added dropwise. Decoloration of the dye was at first rapid, became progressively slower and finally ceased. At this point the concentration of leuco-methylene blue in the reaction mixture was 3.9×10^{-4} *M*. In this experiment, and in others carried out in water and in phosphate buffers at *p*H 6 to 8, the amount of dye reduced

(8) W. J. Nickerson and J. R. Merkel, *Proc. Natl. Acad. Sci. U. S.*, **39**, 901 (1953); J. R. Merkel and W. J. Nickerson, *Biochim. et Biophys. Acta*, **14**, 303 (1954).

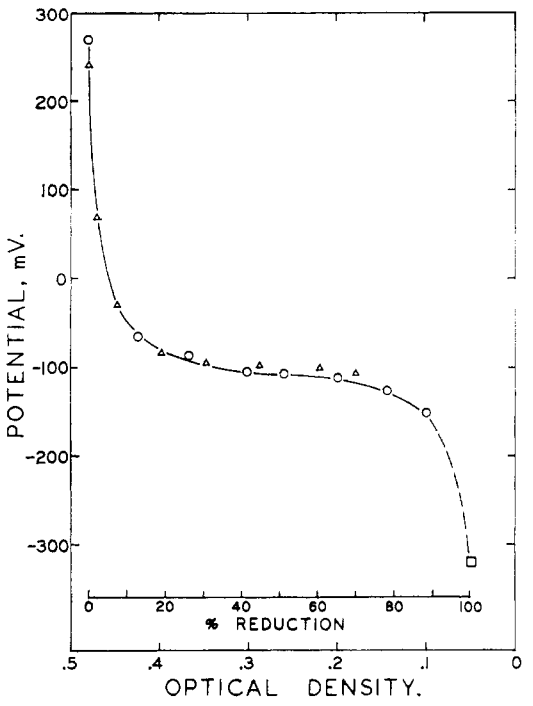

Fig. 4.—Change of potential (*vs.* normal hydrogen electrode) and of optical density (at 436 mμ) accompanying anaerobic illumination of 2.5×10^{-5} *M* riboflavin in 0.05 *M* acetate buffer at *p*H 4.8. Per cent. reduction is calculated from change in O. D. Δ, without activator; O, with 10^{-3} *M* methionine; □, after addition of excess $Na_2S_2O_4$.

was always close to twice that of EDTA. Dye in amounts up to 100 times that of the riboflavin could be photoreduced, provided that sufficient EDTA was present. It was essential to add the dye slowly, so as to keep the concentration of oxidized dye low at all times.

The Oxidation Reduction Potential of riboflavin is *p*H-dependent; therefore, the solutions for potential studies were buffered with 0.05 *M* acetate, phosphate or carbonate, respectively, in the *p*H ranges 4 to 5, 6 to 8 and 9 to 10. The changes in potential and optical density accompanying illumination are shown in Fig. 4, wherein the scale labeled "per cent. reduction" was calculated directly from the decrease in optical density. In agreement with Beinert,[9] it was established by titration with dithionite that the optical density at 436 mμ is almost exactly proportional to the concentration of oxidized flavin (up to 10^{-4} *M*). Since full reduction could not be achieved photochemically, the optical density in Fig. 4 corresponding to fully reduced flavin was obtained by adding a few drops of dithionite solution at the end of each experiment.

The potential curves obtained during the photoreduction of riboflavin (Fig. 4) closely resembled those obtained by potentiometric titration.[10,11] An initial sharp drop in potential upon exposure to light was followed by a leveling off; the plots have an inflection point at an optical density corresponding to 50% reduction. In these respects the plots obtained for photoreduction of riboflavin in the presence or absence of activator are essentially identical. From this it may be concluded that the course of the reduction of riboflavin effected either by chemical means or by the photoprocess (either with or without activator) is the same. Therefore, the degradation products which are found only in photoreduced and reoxidized solutions of riboflavin without activator must arise in the reoxidation stage, subsequent to the photoreduction.[12]

Effect of *p*H on Potential.—The oxidation-reduction potentials, *i.e.*, the potentials at 50% photochemical reduction, denoted by E_m, were measured over the *p*H range from 4 to 10 (Fig. 5). Each value represents the mean of two or more experiments. Included for comparison are potentials obtained by reductive titration of riboflavin[10] and of its 5′-phosphate (FMN).[11] In the presence of EDTA or methionine, the potentials measured during photoreductions agreed well with those derived by chemical titrations. As discussed by Clark,[13] the change in slope of a plot of E_m *vs.* *p*H is a consequence of the dissociation of the reduced species. The photochemical data indicate $pK_r = 6.0$; this may be compared to Michaelis' value of 6.1,[10]

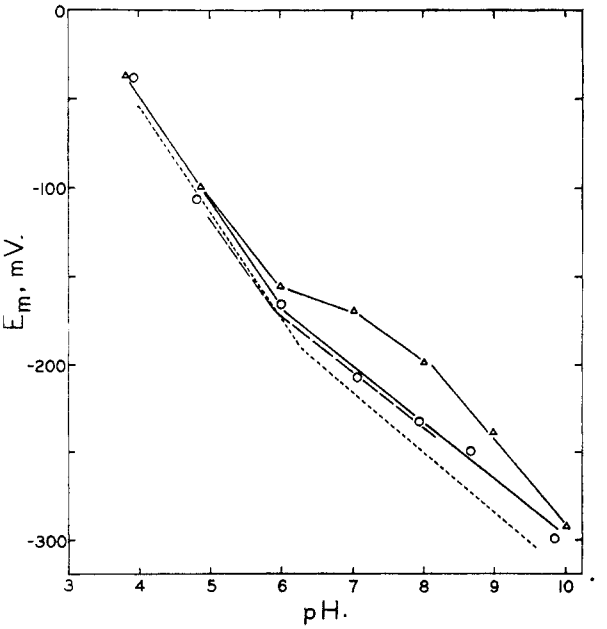

Fig. 5.—Effect of *p*H on the oxidation-reduction potential, (E_m) of riboflavin. Photochemical reduction: Δ, 2.5×10^{-5} *M* riboflavin in 0.05 *M* buffer; O, same with 10^{-3} *M* methionine (activator). Reductive titration with dithionite: — — —, riboflavin[10]; -----, FMN.[11]

obtained by chemical titration. In the absence of activator, there was agreement only at *p*H 4 and 5

(9) H. Beinert, *J. Am. Chem. Soc.*, **78**, 5323 (1956).

(10) L. Michaelis, M. P. Schubert and C. V. Smythe, *J. Biol. Chem.*, **116**, 587 (1936).

(11) H. J. Lowe and W. M. Clark, *ibid.*, **221**, 983 (1956).

(12) A sensitive test for the absence of oxygen was provided by the general shape of the potential curves, since any oxygen, when present, caused a delay in the fall of potential and distorted the initial part of the curve.

(13) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," The Williams and Wilkins Co., Baltimore, Md., 1960, Chapt. 4.

in the E_m values obtained by the two methods; in more alkaline solutions the values diverged.

Discussion

The evidence presented in this paper supports the concept that water is decomposed photochemically by riboflavin, not only in the presence of methionine (and other activators) as previously postulated, but also in the absence of such activators.

The results indicate that the following reactions occur during the *anaerobic* photoreduction

$$R + 2H_2O \longrightarrow R{\cdot}2H_2O \xrightarrow{h\nu} RH_2 + H_2O_2 \quad (1)$$

$$R + M + H_2O \longrightarrow R{\cdot}H_2O{\cdot}M \xrightarrow{h\nu} RH_2 + MO \quad (2)$$

where R and RH_2 stand for riboflavin and its dihydro-form, M represents methionine and MO its sulfoxide. The reduction reactions with and without activator closely resemble each other in that a complex consisting of riboflavin, water and activator (when present) is formed.

As judged by their effect of raising the quantum yield for the photoreduction of riboflavin,[8] activators evidently facilitate the cleavage of the O–H bond of water. They (1) provide sites for hydrogen bonding within a complex consisting of riboflavin, water and activator and (2) promote the reaction by accepting the oxygen moiety from water. The extremely low quantum yield for the photoreduction of lumiflavin without activator suggests that the ribityl group also functions in some manner in lowering the O–H bond strength of water; however, in contrast with external activators which are irreversibly consumed, the side chain remains "available" during successive photoreductions.

The shapes of the potential *vs.* optical density plots obtained both with and without activator indicate that equilibria between the oxidized and reduced species are established at the platinum electrode in both these reactions. It has been shown that the irreversible degradation of riboflavin, observed only in the absence of activator, occurs during the reoxidation step. According to Weiss,[14] the oxidation of leucodyes by oxygen results in HO_2 and OH radicals, leading to the formation of hydrogen peroxide. Further support for the appearance of reactive radicals only during the oxidative step is provided by Oster's[15] finding that dye-sensitized photopolymerizations require the presence of oxygen. It is probable, therefore, that the degradation of the ribityl group is due to reactive radicals derived from oxygen, according to

$$RH_2 + O_2 \longrightarrow R + 2OH \quad (3)$$

$$OH + R \longrightarrow \text{degradation to lumiflavin and lumichrome} \quad (4)$$

$$OH + R{\cdot}H_2O{\cdot}M \longrightarrow \text{no reaction} \quad (5)$$

$$2OH \longrightarrow H_2O_2 \quad (6)$$

The OH radical is produced only in presence of oxygen, as a result of reaction 3, since degradation does not occur anaerobically. Our experience shows that conversion of riboflavin to lumiflavin and lumichrome (reaction 4) is possible only under conditions which allow the production of OH.

The additional role of activators in suppressing the formation of lumiflavin and lumichrome (reaction 5) suggests that the side chain is involved in the riboflavin–water–activator complex and thus is "protected" during the reoxidation step. The detailed structure of the complex is unknown; however, the 2′-OH of the ribityl group may be involved, in view of the reaction scheme postulated by Shimizu[16] for the degradation of riboflavin, which includes 2′-ketoriboflavin as an intermediate.

The supposition that the ribityl group can function as hydrogen donor during the reductive step is refuted by the following results: (1) riboflavin can repeatedly undergo photoreduction, (2) substantial recovery of color is obtained on repeated cycling and (3) migration of hydrogen atoms from a side chain to the isoalloxazine ring would not be expected to be reversible at the electrode. Therefore, the hydrogen source must be water, as was previously concluded for the corresponding reaction in the presence of methionine.

Acknowledgments.—This work was supported in part by grants from the National Institutes of Health, U. S. Public Health Service, and from the National Science Foundation.

(14) J. Weiss, *Naturwissenschaften*, **23**, 64 (1935).

(15) G. Oster, *Nature*, **173**, 300 (1954).

(16) S. Shimizu, *Vitamins (Japan)*, **6**, 775, 763 (1953).