from a mixture of ethanol and ether. The final product was obtained as a monohydrate, m.p. 158–160°.

Anal.—Calcd. for $C_{11}H_{22}NIO_3$: C, 38.5; H, 6.41. Found: C, 38.26; H, 6.40.

Pharmacological Testing[5]

Some of the carbamates were screened as follows for cholinesterase inhibitory activity. An 0.1-ml. quantity of inhibitor solution was added to 1.0 ml. of 0.003 *M* acetylcholine bromide in 0.1 *M* NaCl, 0.02 *M* $MgCl_2$, 0.02 *M* phosphate buffer pH 7.0 at 25°. The reaction was started by adding 10 μl. of enzyme solution (electric eel acetylcholinesterase) and stopped 2 min. later with alkaline hydroxylamine. The remaining ester was estimated by the hydroxamic acid formed. The results are as shown in Table VII. Compound *6* exhibited the maximum inhibitory activity.

To see whether carbamylation occurs, 0.1 ml. of 6×10^{-4} *M* inhibitor *6* and 10 μl. of enzyme solution were incubated for 2, 4, and 14 min. before enzyme assay with 1 ml. of solution containing acetylcholine. The inhibition was about 7% in all cases. This value is consistent with reversible inhibition. If it is assumed that the diethyl carbamyl enzyme does not hydrolyze within a few minutes, the above results indicate that little or no carbamylation occurred within 15 min.

[5] The authors are grateful to Professor I. B. Wilson, College of Physicians and Surgeons, Columbia University, New York, N. Y., for the pharmacological screening of some of the compounds.

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Photobinding and Photoreactivity of Riboflavin in the Presence of Macromolecules

By H. B. KOSTENBAUDER, P. P. DELUCA*, and C. R. KOWARSKI

The rate of aerobic photobleaching of riboflavin by visible light is considerably enhanced by the presence of macromolecules such as PVP, polysorbate 80, and sodium decyl sulfate. The catalysis by the macromolecules is attributed in part to a reversible binding of excited riboflavin molecules to macromolecules to produce longer-lived excited species. Methods are described which permit direct determination of enhanced binding of riboflavin to macromolecules during irradiation. Evidence is presented to indicate involvement of a triplet state in both the photobinding and the photodecomposition.

IN THE course of an investigation of binding of dyes to macromolecules in aqueous solution, the macromolecules were observed to have considerable influence on the light stability of the dyes. Although Oster and co-workers (1–6) had previously reported an enhanced rate of photofading for certain dyes in the presence of polymers such as polyacrylic acid, polymethacrylic acid and polyvinylpyrrolidone, and Scott *et al.* (7) reported that the color loss of some FDA certified dyes in aqueous solution at elevated temperatures was accelerated in the presence of nonionic surfactants, the nature of the observed catalysis was not obvious.

The enhanced photosensitivity of dyes in the presence of macromolecules is of considerable

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* Walter G. Karr Fellow, Smith Kline & French Laboratories, Philadelphia, Pa., 1959–1962. Present address: Ciba Pharmaceutical Products, Inc., Summit, N. J.

significance in pharmaceutical formulation, since polymers and surface-active agents are routinely included in liquid dosage forms containing dyes either as coloring agents or as therapeutic entities. Furthermore, any enhanced photoreaction in aggregated states suggests certain similarities between biological systems and the model systems considered in this investigation.

The present study was therefore undertaken in an attempt to elucidate the mechanism by which some photochemical reactions may be catalyzed by macromolecules and to determine what, if any, specificity might exist for such catalysis. Riboflavin was selected as the photosensitive compound for study, although preliminary work indicated that other dyes and drugs such as hydrocortisone would be suitable agents for such study. Macromolecules included in the study were, principally, polyvinylpyrrolidone (PVP), several nonionic surface-active agents, and sodium decyl sulfate (SDS). On the basis of studies of the effect of macromolecules on the kinetics of the photodecomposition, the fluorescence, and the binding of riboflavin in both the ground state and exicted state, a mechanism has been postulated to account for the observed catalysis.

Although the photochemistry of riboflavin has been the subject of extensive investigation since the photosensitivity of the compound was first observed by Warburg and Christian (8), this complex reaction is still incompletely understood. The early work concerning the identification of the products of the photoreaction and the practical importance of riboflavin photochemistry was reviewed recently by Oster *et al.* (9). The recent works of Holmstrom and Oster (10), Smith and Metzler (11), Moore *et al.* (12), and Holmstrom (13) are indicative of the current status of the problem.

Early workers demonstrated that when riboflavin [6,7-dimethyl-9-(D-1′-ribityl)isoalloxazine] is illuminated with visible light under aerobic conditions, the principal products recovered are lumiflavin (6,7,9-trimethylisoalloxazine), major product of reaction in an alkaline medium, and lumichrome (6,7-dimethylalloxazine), major product of reaction in neutral or acid media. It has recently been shown that when the photoreaction occurs under anaerobic conditions and in the presence of an excess of a readily oxidizable substrate, such as ethylenediaminetetraacetate or other amines (14), the photoproduct is reduced riboflavin (dihydroriboflavin or leucoflavin). The reduced riboflavin is readily reoxidized to riboflavin upon aeration. In the absence of an excess of readily oxidizable substrate, the photoreduction of the isoalloxazine nucleus is accomplished *via* an intramolecular hydrogen transfer from a side chain hydroxyl to the 1-nitrogen, with oxidation of the ribityl side chain and irreversible decomposition of riboflavin to photoproducts such as lumiflavin and lumichrome (11–13).[1]

Halwer (15) observed general acid and general base catalysis of the photofading of riboflavin, but Holmstrom (13) has presented evidence that buffers may change the ratio of the several photoproducts without affecting the rate of riboflavin disappearance. Oster and Holmstrom (10) demonstrated that riboflavin photolysis involves a long-lived excited state (triplet), and Moore *et al.* (12) have recently discussed possible mechanisms involving participation of a triplet excited state.

EXPERIMENTAL

Materials.—Riboflavin was recrystallized from hot water, m.p. 276–278° dec. Lumichrome was prepared in the manner described by Strauss and Nickerson (16), m.p. above 300°. Acetylriboflavin was prepared according to the procedure described by Kuhn and Wagner-Jauregg (17), m.p. 235–236° dec.

Polysorbate 80[2] was a commercial sample used as obtained from the manufacturer. Sodium decyl sulfate was prepared by the method of Dreger *et al.* (18) and was purified by recrystallization from 50% ethanol and extraction with petroleum ether in a Soxhlet apparatus for 36 hr. (19). Polyvinylpyrrolidone[3] was also Soxhlet extracted with petroleum ether for 36 hr. prior to use.

The ion exchange resin, Amberlite No. 200 sodium form,[4] was sieved and only those beads were used which failed to pass through a No. 20 mesh sieve.

Paraffin wax beads were obtained by pouring molten wax[5] into a Waring Blendor containing water at approximately 5°. Only those congealed particles were employed which were retained by a No. 20 mesh sieve.

Other materials employed in the study were methylcellulose,[6] polyethylene glycols,[7] yeast nucleic acid,[8] D-sorbitol, Pluronic F-68,[9] and Polybrene.[10]

Photodegradation Studies.—The light source employed was a 500- or 1000-w. tungsten lamp in a Delineascope projector fitted with a heat absorbing filter. A combination of Corning 3-73 and 5-57 filters were used to isolate those wavelengths near

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[1] Holmstrom (13) has suggested that the term *photoreduction* be reserved for the reversible reaction occurring in presence of excess oxidizable substrate and the term *photobleaching* (or photofading) be applied to the irreversible destruction of riboflavin which occurs in absence of external oxidizable substrate.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

[3] Marketed as Plasdone C by Antara Chemicals Division of General Aniline and Film, New York, N. Y.

[4] Rohm and Haas Co., Philadelphia, Pa.

[5] Gulf Wax. Gulf Oil Corp., Pittsburgh, Pa.

[6] Methocel, 15 cps. Dow Chemical Co., Midland, Mich.

[7] Calbiochem Co., Los Angeles, Calif.

[8] Nutritional Biochemicals Co., Cleveland, Ohio.

[9] Polyoxyethylene-polyoxypropylene surfactant. Wyandotte Chemicals Corp., Wyandotte, Mich.

[10] Cationic polymer of *N,N,N′,N′*-tetramethylhexamethylenediamine and trimethylene bromide. Abbott Laboratories, North Chicago, Ill.

the absorption maximum of riboflavin, 445 mμ. The light was focused on a 1 × 5 × 5 cm. reaction cell and transmitted light was passed through an Aminco grating monochromator and the light of 445 mμ was monitored with either a Photovolt electronic photometer model 501A equipped with a type B phototube or with an Aminco photomultiplier microphotometer equipped with a type 1P21 phototube. The intensity of the light falling on the reaction cell was determined by means of a potassium ferrioxalate actinometer (20).

Photodecomposition studies were conducted at a temperature of 27 ± 1°, with the exception of one set of studies designed to examine the temperature dependency of the reaction. Rate of photobleaching was determined for solutions in equilibrium with air and also for solutions which were flushed continuously with either oxygen or nitrogen during the photolysis and at least 10 min. prior to photolysis. The tendency of surfactant solutions to foam during the purging was overcome by placing horizontally above the surface of the solution a baffle perforated with 2 mm. holes. Fresh solutions of the dye were prepared in 0.05 *M* phosphate buffer of pH 6.8, and 20 ml. of solution was placed in the sample cell. During the course of the photolysis, samples were removed by means of a hypodermic syringe and absorption spectra were determined with a Bausch & Lomb Spectronic 505 recording spectrophotometer. Samples were immediately returned to the reaction cell.

Fluorescense.—Fluorescence measurements were made on each of the solutions subjected to photodegradation. An Aminco-Bowman spectrophotofluorometer was employed for these measurements. The activating wavelength for riboflavin was set at 445 mμ, and the emitted intensity was measured at 520 mμ. Polarization of fluorescence was determined with a Wollaston polarizer attachment.

Interaction Studies in the Ground State.—Solubility studies and equilibrium dialysis studies were utilized to determine any interaction of riboflavin in the ground state with the macromolecules. The solubility of riboflavin in presence of the macromolecules was studied by placing 50 mg. of riboflavin in each of a series of 125-ml. bottles containing 100 ml. of surfactant or polymer solution of varying concentrations. Dialysis studies were carried out by placing 20 ml. of riboflavin solution into a bag fashioned from Visking cellulose casing and immersing the bag in 80 ml. of polymer solution in a 125-ml. wide-mouth bottle. The bottles for both solubility and dialysis studies were rotated at 9 r.p.m. in a constant-temperature bath at 30.0° until equilibrium was attained.

Detection of Binding in the Excited State.—Photobinding of the excited state of riboflavin and acetylriboflavin was detected by both precipitation and resin desorption techniques. Polysorbate 80 can be precipitated from aqueous solution by heating the solution to 90–95°. When riboflavin was present in the aqueous polysorbate system, it was possible to demonstrate changes in distribution of riboflavin between the separated phases when the precipitation was effected in the presence of and in the absence of light. A 40-ml. 2.5 × 10 cm. cell containing a solution of riboflavin in aqueous polysorbate 80 was suspended in a water bath at a distance of 75 cm. from a 500-w. light source. The conditions were such that minimal photodegradation occurred during the experiment. The solution was heated to approximately 90–95° to cause precipitation of the polysorbate. Both the aqueous and the polysorbate phases were sampled, and the concentration of riboflavin in each phase was determined after precipitation effected in both presence and absence of light.

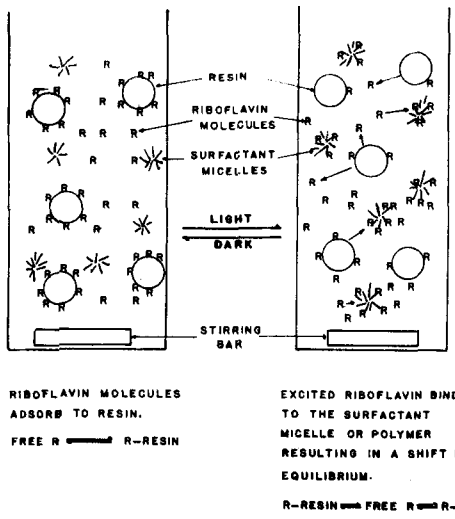

Fig. 1.—Schematic diagram for the detection of photoinduced binding of riboflavin using the resin desorption method.

The second method employed was the effect of visible light on the distribution of riboflavin between an ion exchange resin, or beads of paraffin wax, and an aqueous polymer solution. The resin, Amberlite No. 200, was washed with 1.0 *M* hydrochloric acid and rinsed with distilled water until washings were neutral to litmus. Approximately 1.5 to 2.0 Gm. of resin was transferred to a reaction vessel, and 50 ml. of a riboflavin solution containing the desired concentration of polymer was introduced. The suspension was stirred until riboflavin was equilibrated between resin and solution. At equilibrium, approximately 50% of the riboflavin present was adsorbed to the resin. By means of a hypodermic needle and syringe, samples of the solution phase were removed for analysis of riboflavin concentration before irradiation, during irradiation, and after irradiation. Figure 1 is a schematic diagram of the system. The photobinding of acetylriboflavin to SDS was studied in a similar manner, with the exception that paraffin wax beads were employed in place of the ion exchange resin.

RESULTS

Kinetics of Riboflavin Photobleaching.—The characteristic spectral changes which occur when a solution of riboflavin is irradiated with visible light under the conditions imposed in the present study have been presented by Holmstrom and Oster (10) and by Holmstrom (13). The photobleaching of the solutions is expressed as the rate of change of absorbance at 445 mμ. The absorbance at this wavelength is primarily, but apparently not solely, due to intact riboflavin. Although lumichrome,

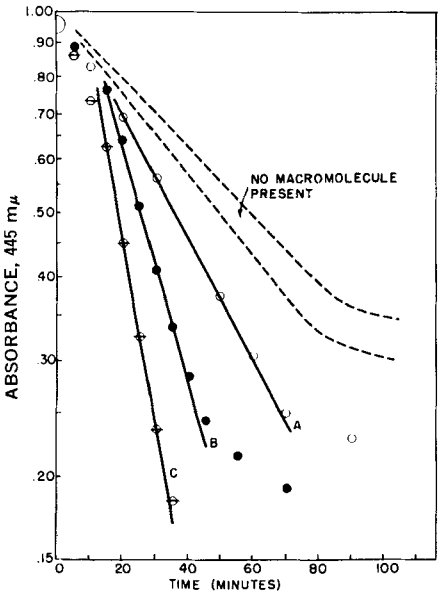

Fig. 2.—Rate of aerobic photofading for solutions of riboflavin in aqueous polysorbate 80. Polysorbate concentrations: A, 0.25%; B, 0.50%; C, 1.0%.

the principal product recovered after exhaustive photolysis under conditions of this study, is not produced in a quantity sufficient to invalidate the use of loss of absorbance at 445 mμ as an indication of rate of riboflavin disappearance, other flavin-type and unknown products or intermediates may produce interference (13).

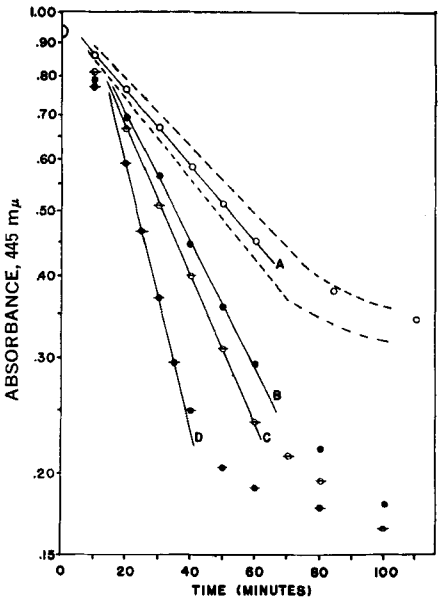

Fig. 3.—Rate of aerobic photofading of riboflavin in aqueous solutions containing macromolecules or surface-active agent. Key: A, SDS, 0.01 *M;* B, SDS, 0.05 *M;* C, Pluronic F-68, 1.0%; D, PVP, 1%.

In agreement with the findings of others (10, 12), the quantum yield for the photobleaching is not constant, but decreases as the reaction proceeds. Such a result might be expected if a photoproduct or an intermediate quenches excited molecules (10) or if a product of the reaction or an intermediate absorbs incident light. Under these conditions the photobleaching data expressed as decrease in absorbance at 445 mμ fit a pseudo first-order plot of logarithm absorbance *versus* time. A short induction period is evident in some of the plots, and there is deviation from the first-order plot as the reaction approaches completion.

The effect of macromolecules on the rate of photobleaching was expressed by determining the apparent first-order rate constant, k, according to

$$2.303 \log (A_2/A_1) = -k(t_2 - t_1) \quad \text{(Eq. 1)}$$

For every rate study with a macromolecule, a

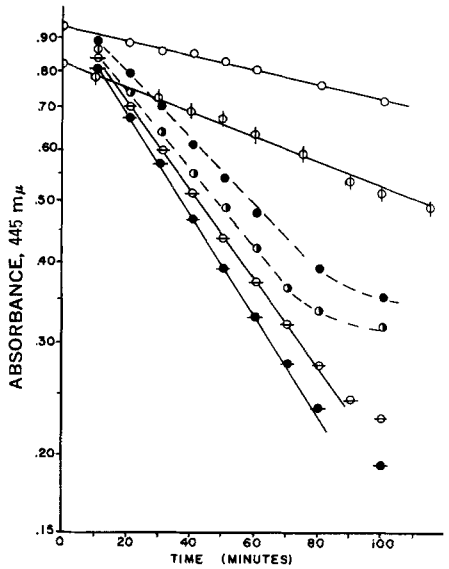

Fig. 4.—Rate of aerobic photofading of riboflavin in aqueous solutions containing macromolecules and other additives. Key: ϕ, 0.5% nucleic acid; ○, 1.0% Polybrene; ◑, 0.5% methylcellulose; ●, 0.5% sorbitol; ⊖ 1.0% PEG 6000; ◉, 1.0% PEG 4000.

control study was run without macromolecule and the ratio of the apparent first-order rate constants k/k_{H_2O} was determined.[11] Consequently, considerable data were obtained in absence of macromolecule, and where many experiments were performed under identical light intensities, these data are presented in some of the figures as a pair of broken lines to represent maximum variation in rate among the controls. With the exception of those solutions containing methylcellulose or polyethylene glycols, all riboflavin solutions were stable when stored in the dark.

Figures 2–4 illustrate the effect of several macromolecules on the rate of photobleaching of riboflavin solutions in equilibrium with air. As illus-

[11] All reactions were run in 0.05 *M* phosphate buffer, and the rate constant k_{H_2O} therefore contains a term for the buffer catalysis.

trated in Fig. 3, SDS enhanced the aerobic photobleaching of riboflavin only at concentrations above the critical micelle concentration of SDS. The ethylene oxide polymers, polyethylene glycol 4000 and polyethylene glycol 6000, enhanced the photobleaching only slightly, and *d*-sorbitol and methylcellulose had no significant effect on the rate of photobleaching. Nucleic acid and the cationic polymer, Polybrene, considerably inhibited the fading of riboflavin. The effect of macromolecules on the rate of photofading of riboflavin solutions in equilibrium with air is summarized in Table I.

Data for studies in solutions saturated with oxygen and in solutions purged with nitrogen are presented in Table II. Although both PVP and polysorbate 80 enhanced the rate of photobleaching of riboflavin in anaerobic systems, the data did not conform to a first-order plot, and results for these studies are not presented.

The retarding effect of iodide ion in the photobleaching of riboflavin is illustrated in Fig. 5. Potassium iodide at a concentration below that necessary to cause fluorescent quenching retards photobleaching of riboflavin in both the presence and absence of polysorbate 80.

The temperature dependency of the photobleaching of riboflavin in solutions in equilibrium with air is illustrated in Fig. 6. The apparent heat of activation calculated from the slopes of the Arrhenius plots of apparent first-order rate constants is 13.2 Kcal. mole^{-1} in absence of surfactant and 18.8 Kcal. mole^{-1} in presence of 1% polysorbate 80.

Kinetics of Acetylriboflavin Photodecomposition.—Figure 7 shows the rate of fading of acetylriboflavin in phosphate buffer at pH 6.8 in the presence and absence of some macromolecules. In absence of macromolecule, the rate of aerobic photobleaching of acetylriboflavin is $^1/_{75}$th that of riboflavin.[12] In the presence of polysorbate 80 or PVP, however, acetylriboflavin fades at a rate only slightly less than that of riboflavin under similar conditions. The addition of 1% polysorbate 80 increased the rate of bleaching acetylriboflavin by a factor of 200 and 1% PVP produced a sixtyfold increase.

There was no increase in the rate of photobleaching of acetylriboflavin in micellar SDS unless an oxidizable substrate such as ascorbic acid was present. As shown in Fig. 8, however, ascorbic acid had relatively little influence on the photodecomposition of acetylriboflavin in absence of micellar SDS. The effect of macromolecules on the photobleaching of acetylriboflavin is summarized in Table III.

Fluorescence Studies.—As shown in Table I, the fluorescence of riboflavin was not altered by most macromolecules. Quenching of fluorescence was observed in solutions containing mucleic acid or Polybrene.

The degree of polarization of fluorescence, p, was calculated from the expression

$$p = (F_{||} - F_{\perp})/(F_{||} + F_{\perp})$$

where $F_{||}$ is the intensity of the fluorescence with

TABLE I.—COMPOSITE PHOTOCHEMICAL RESULTS FOR RIBOFLAVIN IN SOLUTIONS CONTAINING MACROMOLECULES AND IN EQUILIBRIUM WITH AIR

Macromolecule	pH[a]	$k^b \times 10^2$	k/k_{H_2O}	RFI[c]
None	6.8	1.35	1.00	12.0
0.25% Polysorbate 80	6.8	2.10	1.55	11.9
0.5% Polysorbate 80	6.8	4.00	2.96	12.0
1.0% Polysorbate 80	6.7	6.30	4.67	11.9
1.0% PVP	6.8	4.70	3.48	12.0
1.0% Pluronic F-68	6.7	2.60	1.93	11.7
0.01 *M* SDS	6.8	1.35	1.00	12.0
0.05 *M* SDS	6.8	2.40	1.78	12.3
0.5% Nucleic acid	6.2	0.44	0.33	7.5
0.5% Methylcellulose	6.7	1.45	1.07	11.7
1.0% PEG 4000	6.8	1.60	1.18	11.7
1.0% PEG 6000	6.8	1.63	1.21	11.9
1.0% D-Sorbitol	6.8	1.30	0.96	11.6
1.0% Polybrene	6.7	0.25	0.19	5.4

[a] pH adjusted with 0.05 *M* phosphate buffer. [b] Pseudo first-order rate constant in min.$^{-1}$. [c] Relative fluorescent intensity.

TABLE II.—EFFECT OF OXYGEN ON RATE OF PHOTOBLEACHING OF RIBOFLAVIN IN THE PRESENCE OF MACROMOLECULES[a]

Macromolecule	$k^b \times 10^2$	k/k_{H_2O}
Solutions Saturated with Oxygen During Reaction (1000-w. Lamp)		
None	.74	1.00
0.02 *M* SDS	.74	1.00
0.03 *M* SDS	.80	1.08
0.04 *M* SDS	1.01	1.36
0.05 *M* SDS	1.09	1.47
0.065 *M* SDS	1.19	1.61
1% PVP	.84	1.14
1% Polysorbate 80	.97	1.16
Solutions Purged with Nitrogen During Reaction (500-w. Lamp)		
None	2.404	1.00
0.065 *M* SDS	0.648	.27

[a] All solutions in pH 6.8, 0.05 *M* phosphate buffer. [b] Pseudo first-order rate constant in min.$^{-1}$.

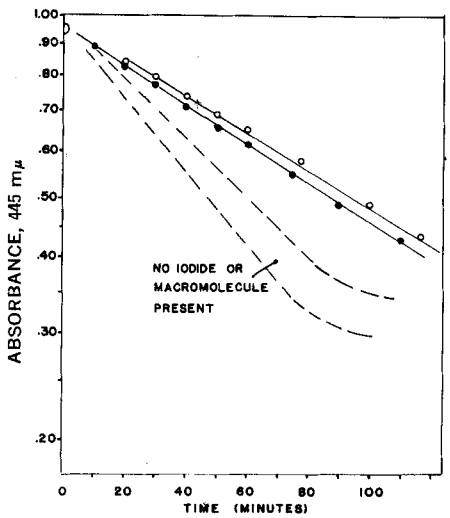

Fig. 5.—Effect of iodide ion on the rate of aerobic photofading of riboflavin. Key: ○, 10^{-4} *M* KI; ●, 10^{-4} *M* KI and 1% polysorbate 80.

[12] The photolytic reaction may not be the rate-determining step in the fading of acetylriboflavin. A rate-determining hydrolysis of acetate ester to produce a free side chain hydroxyl may precede the photolytic reaction.

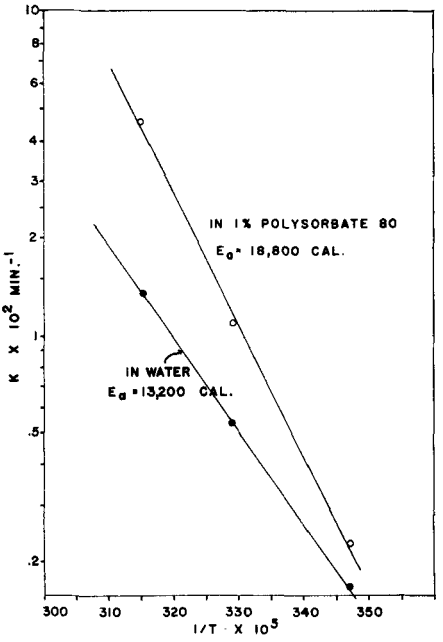

Fig. 6.—Temperature dependency for aerobic photofading of riboflavin in presence and absence of polysorbate 80.

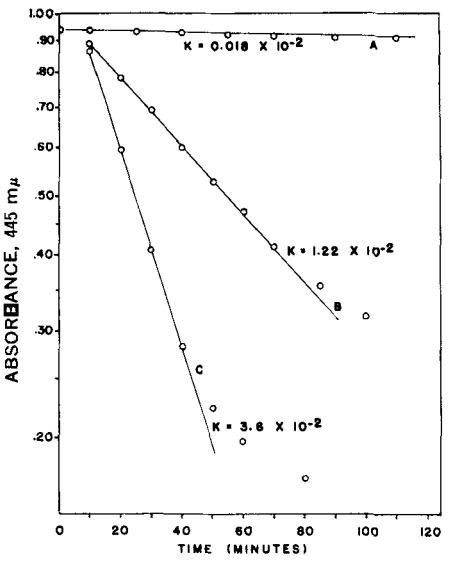

Fig. 7.—Influence of PVP and polysorbate 80 on rate of aerobic photofading of acetylriboflavin. Key: A, acetylriboflavin in water; B, acetylriboflavin in 1% PVP; C, acetylriboflavin in 1% polysorbate 80.

electric vector parallel to that of the exciting light, and $F_\perp$ is the intensity of fluorescence with electric vector normal to that of the exciting light (21). The ratio of the lifetime of the excited state in water to that in solutions containing macromolecule can be deduced from the expression

$$t_0/t = (1/p_i - 1/p_0)/(1/p - 1/p_0)$$

where p_i is the initial polarization in water, p_0 is the maximum polarization in a rigid medium, and p is the polarization in presence of macromolecule (22). The maximum value of p_0 in a completely rigid system is 0.5. Since only relative values were required, the value of p_0 was taken as 0.5. There was no increase in the lifetime of the excited state associated with riboflavin fluorescence in systems containing up to 5% polysorbate 80, nor in systems containing 0.065 *M* SDS.

Interaction in the Ground State.—The presence of concentrations of up to 3% polysorbate 80, PVP, polyethylene glycol 6000, and Pluronic F-68 failed to increase the water solubility of riboflavin in absence of light. A dialysis study also indicated that riboflavin did not interact with PVP in the ground state. SDS, at a concentration of 0.065 *M*, increased the solubility of riboflavin by a factor of 2.7 at 30°.

Detection of Binding in the Excited State.—It was possible to show that several polymers which showed no interaction with riboflavin in the dark bound riboflavin during irradiation with visible light. Upon removal of the light, the binding was rapidly reversible. Figure 9 shows typical data for binding of riboflavin to polysorbate 80 during heat precipitation of polysorbate 80 from aqueous solution in the presence and absence of light. Acetylriboflavin also exhibited photobinding to polysorbate 80.

The resin desorption method was employed to demonstrate photobinding of riboflavin to PVP and to permit determination of photobinding to polysorbate 80 at room temperature. Typical data are presented in Table IV. Acetylriboflavin was found to exhibit similar photobinding tendencies, as indicated by the effect of light on the distribution of acetylriboflavin between paraffin wax beads and aqueous SDS. The photobinding of riboflavin to these polymers is the first demonstration of such an interaction in solution (23).

It was not possible to demonstrate any photobinding of riboflavin when temperature was reduced to 15°. The presence of 1×10^{-4} *M* potassium iodide also eliminated photobinding as determined by either resin desorption or heat precipitation.

DISCUSSION

The enhanced rate of photobleaching of riboflavin in the presence of polysorbate 80 cannot be attributed solely to the presence of functional groups such as hydroxy or ethylene oxide in the surfactant molecule. The insignificant effects of molecules such as methylcellulose, D-sorbitol, and the low molecular weight polyethylene glycols (PEG 4000 and 6000) indicates that the colloidal nature of the macromolecules is of considerable importance.

The observation that micellar SDS, but not nonmicellar SDS, enhances the aerobic photobleaching of riboflavin serves as additional evidence for the significance of the colloidal state and also indicates that apparently many kinds of micellar aggregates or colloidal materials can provide a favorable environment for the photobleaching of riboflavin.

The importance of the ribityl side chain as a hydrogen donor is illustrated in the studies with acetylriboflavin. Replacement of the hydrogens

of the hydroxyl side chain of riboflavin provides a relatively light-stable molecule, for which the rate of photofading is decreased by a factor of at least 75. The fact that polysorbate 80 and PVP can greatly accelerate the photobleaching of acetylriboflavin indicates that these macromolecules are capable of providing oxidizable substrate. SDS, which is in a highly oxidized state, has little effect on the photobleaching of acetylriboflavin unless oxidizable substrate is added to the system.

The system acetylriboflavin–SDS–ascorbic acid provides an excellent example of the influence of an inert colloidal material on the photobleaching. Acetylriboflavin is relatively stable to light in aqueous solution or in aqueous solution containing only SDS or only ascorbic acid. However, in an aqueous solution containing both SDS and ascorbic acid the rate of photobleaching is greatly accelerated. This is a striking demonstration of significant enhancement of a photoreaction involving a biologically important substance in a remarkably simple model system.

For these reactions where an enhanced rate of fading was observed, the rate plots show a lag time typical of free radical mediated reactions. The lag time might also be attributed to the establishment of an equilibrium between ground state molecules, excited state molecules, and bound excited molecules.

The decrease in rate of photobleaching of riboflavin and the quenching of fluorescence in presence of nucleic acid is attributed to a dark reaction (24) between ground state dye molecules and the adenine moieties of nucleic acid. Presumably this quenching of the singlet state is a consequence of charge–transfer complexation. Polybrene, a cationic polymer, also retards the fading and quenches the fluorescence of riboflavin. Although micellar SDS

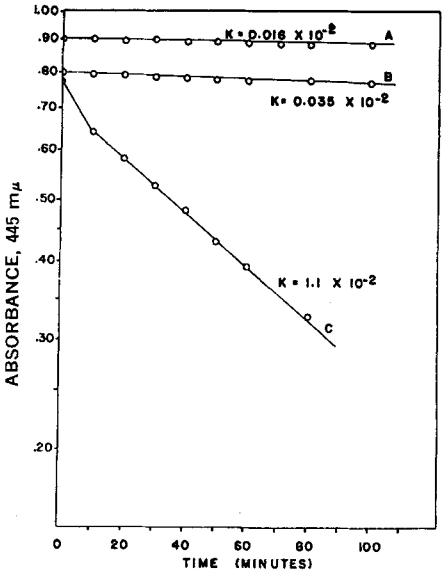

Fig. 8.—Effect of ascorbic acid on the rate of aerobic photofading of acetylriboflavin in the presence of sodium decyl sulfate. K is the pseudo first-order rate constant in min.$^{-1}$. Key: A, acetylriboflavin in 0.07 M SDS; B, acetylriboflavin in 0.01 M ascorbic acid; C, acetylriboflavin in 0.07 M SDS and 0.01 M ascorbic acid.

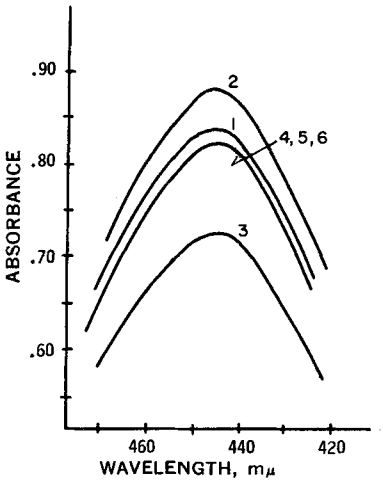

Fig. 9.—Detection of riboflavin photobinding by heat precipitation of polysorbate 80. Key: 1, homogeneous solution prior to irradiation and precipitation; 2, polysorbate 80 phase precipitated during irradiation; 3, aqueous phase during irradiation; 4, 5, 6, redispersed solution after irradiation, polysorbate 80 phase precipitated in dark, and aqueous phase remaining after precipitation of polysorbate phase in dark.

TABLE III.—RATE OF PHOTOBLEACHING OF ACETYLRIBOFLAVIN IN SOLUTIONS OF MACROMOLECULES IN EQUILIBRIUM WITH AIR

Macromolecule	pH[a]	$k^b \times 10^2$	k/k_{H_2O}	RFI[c]
None	6.8	0.018	1.00	4.50
1% Polysorbate 80	6.8	3.60	200	4.40
1% PVP	6.8	1.22	67.7	4.50
0.07 M SDS	6.8	0.016	0.90	4.30
0.07 M SDS + 0.01 M ascorbic acid	6.5	1.10	61.1	
None + 0.01 M ascorbic acid	6.5	0.035	1.94	

[a] pH adjusted with 0.05 M phosphate buffer. [b] Pseudo first-order rate constant in min.$^{-1}$. [c] Relative fluorescent intensity.

TABLE IV.—INFLUENCE OF VISIBLE LIGHT ON DISTRIBUTION OF RIBOFLAVIN BETWEEN AQUEOUS POLYMER SOLUTIONS AND AN ION EXCHANGE RESIN (AMBERLITE 200)

	Riboflavin in Aqueous Phase Absorbance, 445 mμ
PVP, 2.0 Gm./100 ml.	
Before irradiation	0.584
Light on (3 min.)	0.613
Light off	0.562
Polysorbate 80, 1.5 Gm./100 ml.	
Before irradiation	0.317
Light on (5 min.)	0.329
Light off	0.310

has been found to interact with riboflavin in the dark, inability to demonstrate quenching of fluorescence in the presence of SDS suggests that the mechanism of this interaction differs from that for interaction of riboflavin with nucleic acid or Polybrene.

Other workers (1–7, 25–28) have shown that the photodecomposition of some dyes in aqueous solution can be facilitated by the presence of macromolecules, and in some cases the enhanced rate of photodecomposition has been related to binding of the dye in the ground state. The present study of catalysis of riboflavin photobleaching implicates binding of an excited state of riboflavin to the macromolecule, rather than binding of riboflavin in the ground state. Inability to demonstrate interaction in the absence of light between riboflavin and polysorbate 80 or PVP by dialysis or solubility studies excludes the possibility of attributing the enhanced rate of photobleaching to interaction in the ground state. Demonstration of reversible photobinding of riboflavin to all polymers catalyzing aerobic photobleaching provides evidence for interaction of an excited riboflavin molecule with the polymer. Suppression of the photobinding with trace amounts of potassium iodide suggests not only that a long-lived excited species is involved in the binding, but that the excited state involved in the binding is the same as that undergoing photobleaching and that the species is either a triplet or a reactive species resulting directly from the triplet.

The influence of increasing concentrations of SDS on the apparent first-order rate constant for riboflavin photobleaching in solutions saturated with oxygen was examined by calculating the rate constant for the alternative assumptions that (*a*) rate is proportional to total riboflavin (Eq. 2) and (*b*) rate is proportional to free riboflavin (Eq. 4). If ground-state binding has no effect on the photoreactivity of riboflavin, the influence of SDS concentration on rate of photobleaching might be formulated as

$$-d(R_f)/dt = [k_{H_2O} + k'(\text{SDS})]\ T \quad \text{(Eq. 2)}$$

$$k_{obs} = k_{H_2O} + k'\,(\text{SDS}) \quad \text{(Eq. 3)}$$

In the above equations, $d(R_f)/dt$ is considered to be the rate of loss of absorbance at 445 mμ, T represents total riboflavin in solution, k_{H_2O} is the uncatalyzed reaction, k' refers to an SDS-catalyzed reaction of both free and adsorbed riboflavin, and k_{obs} is the observed apparent first-order rate constant. A plot of k_{obs}/k_{H_2O} *versus* (SDS) should be linear.

If riboflavin bound in the ground state is unreactive, but the reaction of excited free dye molecules to form products is catalyzed by SDS, the rate at a constant SDS concentration might be formulated as

$$-d(R_f)/dt = [k_{H_2O} + k''\,(\text{SDS})]T/R \quad \text{(Eq. 4)}$$

$$k_{obs} = [k_{H_2O} + k''(\text{SDS})]/R \quad \text{(Eq. 5)}$$

The value R is the ratio of total/free riboflavin as determined by the influence of SDS on riboflavin solubility (28). The validity of representing data for binding of drugs to surfactants by this transformation of the Langmuir adsorption isotherm has been illustrated for several systems (30, 31). A plot of Rk_{obs}/k_{H_2O} *versus* (SDS) should be linear.[13]

As illustrated in Fig. 10, when the rate of aerobic photobleaching is calculated as a function of total riboflavin present, there does not appear to be a direct proportionality between rate constant and SDS concentration. However, when rates are calculated on the assumption that riboflavin bound in the ground state is unreactive, the calculated rate constant for fading is directly proportional to micellar SDS concentration, as illustrated in curve B of Fig. 10.

The fact that ground-state binding of riboflavin to SDS does not enhance the rate of photobleaching in absence of oxygen further suggests that it is not the transition from bound ground state to bound excited state which is facilitated. This observation also suggests that the observed catalytic effect of polymers in aerobic systems might arise because bound excited riboflavin species (triplet) may be protected from oxygen quenching. Oxygen is recognized as an efficient quencher of the triplet state.

Binding of the acetylated form of riboflavin by polysorbate 80 upon irradiation tends to direct attention to the isoalloxazine nucleus as the binding site. If binding involved the ribityl side chain, then certainly more dramatic changes in the binding should occur upon acetylation. The photobinding effect does not involve photoexcitation of the macromolecules, since these agents are transparent in the visible region.

Fluorescence measurements and polarization of fluorescence measurements show that it is not the excited singlet state that is undergoing photochemical change. No quenching of fluorescence was observed in those systems where enhanced fading occurred. Also, polarization of fluorescence measurements indicated quite clearly that the singlet state is not involved in the photodegradation of photobinding since the lifetime of the singlet state is not changed by the addition of polysorbate 80 or PVP.

Weber (24) showed for riboflavin that an increase in the polarization of fluoresence upon increase in concentration of potassium iodide was due to collisions in which the electronic excitation energy is transformed into kinetic energy. If the concentration of iodide is such that it will collide with the excited fluorescent state (singlet) in less than 10^{-8} sec., then the excited state will revert to the ground state *via* radiationless transition. In the present investigation it was found that at a concentration of 1×10^{-4} *M* KI, the photochemical degradation is significantly inhibited while the fluorescence is not altered. Iodide ion and dye molecules cannot collide in 10^{-8} sec., the lifetime of the fluorescent state, unless the concentration of electrolyte is 10^{-2} *M* for a 10^{-4} to 10^{-5} *M* dye solution (32–35). Therefore, below 10^{-2} *M* KI, the inhibiting effect

[13] This model is, of course, kinetically indistinguishable from that for a mechanism involving excitation of adsorbed ground state riboflavin. If both free and bound dye are photobleached, but at different rates, the expression would be

$$-d(R_f)/dt = [k_{H_2O} + k'''\,(R-1)]T/R \quad \text{(Eq. 6)}$$

$$k_{obs} = [k_{H_2O} + k'''\,(R-1)]/R \quad \text{(Eq. 7)}$$

Since R can often be represented (30) as $R = 1 +$ (constant) (SDS)

$$Rk_{obs} = k_{H_2O} + k'''\,(\text{constant})\,(\text{SDS}) \quad \text{(Eq. 8)}$$

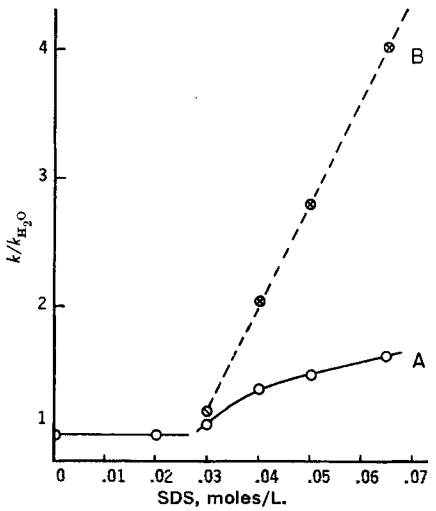

Fig. 10.—Influence of SDS on photofading of riboflavin in solutions saturated with oxygen. Key: A, pseudo first-order rate constant calculated using total riboflavin in solution; B, rate calculated, assuming riboflavin bound in the ground state is unreactive.

of the iodide ion must be due to collision with a longer-lived excited species.

On the basis of studies of the kinetics of photobleaching, the photobinding and the fluorescence of riboflavin, it is proposed that the catalytic effect of macromolecules on the photobleaching of riboflavin may arise from interaction of dye in the triplet state, or some reactive species resulting from the triplet, with the macromolecule to provide a longer-lived excited state. Although both polysorbate 80 and PVP apparently are capable of serving as oxidizable substrates for excited riboflavin molecules, evidence indicates that this property of these molecules does not represent the only factor in the catalysis. Molecules such as ascorbic acid can also serve as oxidizable substrate, yet the photobleaching of acetylriboflavin in the presence of ascorbic acid is markedly enhanced only upon addition of a substance such as micellar SDS. The SDS cannot serve as oxidizable substrate, and therefore its effect must be due to its colloidal nature.

As noted previously, in absence of oxygen SDS does not catalyze but actually stabilizes riboflavin to photofading. Although photobinding of an excited triplet has been shown to occur in anaerobic systems (29), there is no evidence for a catalytic effect in anaerobic systems. This observation is not in conflict with the proposed mechanism. Since there is no oxygen quenching in anaerobic systems, adsorption to the polymer would not necessarily increase the reactivity of the excited state. The observation that the degree of stabilization produced by SDS in anaerobic systems is greater than can be accounted for by assuming bound ground state molecules to be unreactive might even suggest that in anaerobic systems the bound triplet species show a lesser rate of breakdown than the free triplet species.

A rate law illustrating dependence of initial quantum yield on polymer concentration and including a steady-state condition for ground state, excited state, and binding of an excited triplet state to polymer might be formulated. However, present knowledge of the complex photochemical reactions of riboflavin is such that any rate law formulated would have to be a considerable oversimplification.

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