Chemical and Photochemical Reductions of Flavin Nucleotides and Analogs*

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The anaerobic photoreduction of riboflavin, FMN, 6,7-dimethyl-9-hydroxyethylisoalloxazone, and lumiflavin in the absence of electron donors is studied. It is shown that lumiflavin, unlike the other three compounds, does not undergo photobleaching. Evidence is presented that the electrons for the reduction are derived from the side chain of the molecule in an intramolecular rearrangement. (The same conclusion was reached by Moore and co-workers on the basis of experiments in D_2O and polarographic analysis, published after this paper had been submitted [Moore, W. M., Spence, J. T., Raymond, F. A., and Colson, S. D., 1963, *J. Am. Chem. Soc.* *85*, 3367].) The effect of metal ions (Ag^+, Cu^{2+}, Ni^{2+}, Mn^{2+}, Na^+ and Mg^{2+}) on the rate of photoreduction of FMN is investigated and interpreted in terms of a mechanism involving the first triplet excited state of FMN. In contrast to previous investigations it is shown by spectroscopic, electron-spin-resonance, and preparative methods that riboflavin in the ground state does not form a stable manganese complex. Using EDTA as the external hydrogen donor, it is shown that the rates of photoreduction of FMN, riboflavin, and lumiflavin are equal but FAD is reduced more slowly. $DPNH_2$ is found to react with FMN in the dark under anaerobic conditions in a second-order process. The Arrhenius activation energy for this reaction is found to be 8.3 kcal/mole. The reaction is not a free-radical process although an electron-spin-resonance signal is observed during the reaction as a result of the FMN + $FMNH_2$ = 2 FMNH· equilibrium (FMNH = semiquinone of reduced riboflavin-5′-phosphate). This latter observation may account for previous spectroscopic and electron-spin-resonance observations attributed to the formation of a charge-transfer complex between FMN and $DPNH_2$. The light-catalyzed reaction of FMN with $DPNH_2$ has an initial quantum yield of 0.25, and is independent of temperature and DPNH concentration. Evidence is presented that the reaction again involves the triplet state of FMN in a complex with the reducing agent.

FMN[1] has been shown to participate in photosynthetic phosphorylation in isolated chloroplast systems (Whatley *et al.*, 1959) yet a detailed quantitative understanding of the oxidation reduction processes of flavin nucleotides and the effect of light on these reactions is lacking.

It has been suggested that water can act as an electron donor in an anaerobic photochemical reaction of riboflavin (Merkel and Nickerson, 1954; Rutter, 1958; Vernon, 1959; Nickerson and Strauss, 1960; Strauss and Nickerson, 1961), but the alternative hypothesis that the side chain of riboflavin is involved in the photoreduction is energetically more likely (Holmström and Oster, 1961). There is disagreement about the reduction of FMN by $DPNH_2$ in the dark (Singer and Kearny, 1950; Uehara *et al.*, 1957; Commoner and Lippincott, 1958), although a recent study on model systems has somewhat clarified this point (Suelter and Metzler, 1960). There are also some suggestions that $DPNH_2$ forms a charge-transfer complex with FMN (Isenberg and Szent-Györgyi, 1959; Isenberg *et al.*, 1961). Finally, only qualitative studies are available on the photoreduction of FMN by $DPNH_2$ (Frisell and Mackenzie, 1959; Vernon, 1959). We have therefore undertaken the study of the anaerobic reduction and photoreduction of FMN and its analogs.

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[1] Abbreviations used in this work: FMN and $FMNH_2$, the oxidized and reduced forms of flavin mononucleotide; FMNH, the semiquinone of reduced flavin mononucleotide; FAD, flavin–adenine dinucleotide; EDTA, ethylenediamine tetraacetic acid; DPN and $DPNH_2$, the oxidized and reduced forms of diphosphopyridine nucleotide; ESR, electron spin resonance.

Materials and Methods

Riboflavin (obtained from California Corp. for Biochemical Research) was purified by recrystallization from water, lumiflavin was kindly supplied by Professor D. E. Metzler of Iowa State University, and 6,7-dimethyl-9-hydroxyethylisoalloxazine was supplied by Dr. H. G. Petering of the Upjohn Company. Riboflavin-5′-phosphate sodium salt, reduced diphosphopyridine nucleotide disodium salt, DPN, D(−)-ribose, and ribitol were California Corp. for Biochemical Research A grade reagents. EDTA dipotassium salt was purchased from Eastman Kodak Co.

The light source used was a 500-w tungsten lamp in combination with suitable lenses, and infrared-glass filter and a glass water bath. The reaction cell was illuminated inside a Cary recording spectrophotometer, Model 14, through a side window. The reaction cell was in a closed circuit with an apparatus with provisions for deoxygenating by bubbling argon through the solutions for 1 hour prior to the experiments, and for mixing the reagents under anaerobic conditions. Both the reaction cuvet and the container in which deoxygenation was carried out were thermostated by circulating water. The temperature inside the reaction cell was measured by an iron-constant thermocouple. (It was shown that the presence of this thermocouple did not affect the reactions.)

Electron paramagnetic resonance measurements were made with a reflection-type spectrometer which employed 3-cm microwaves and 100-kc/sec magnetic field modulation.

Fluorescence spectra were measured with an Aminco-Bowman spectrophotofluorometer.

Quantum yields were measured using a Bausch and Lomb monochromator in conjunction with a 500-w tungsten lamp as the light source and a Weston photovoltaic cell, Model 9950, coupled to a Leeds and Northrup galvanometer for measuring light intensities.

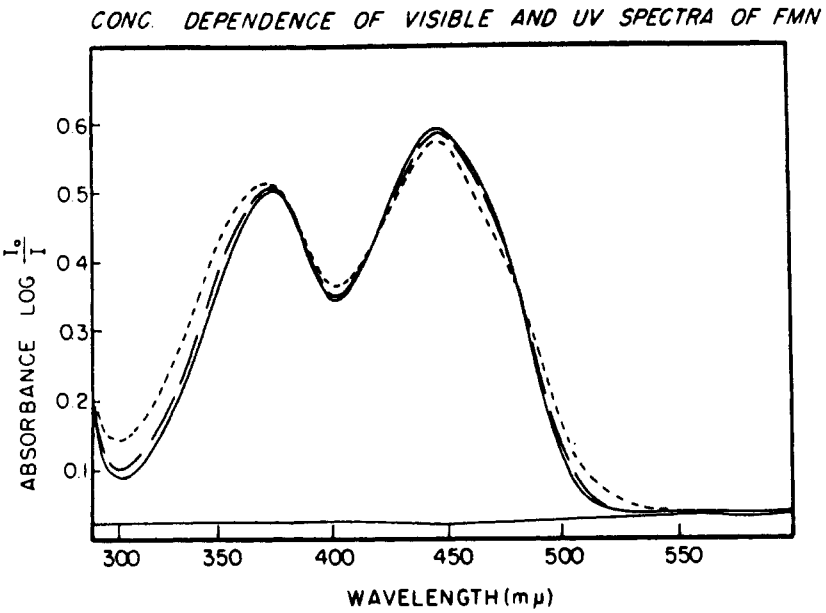

FIG. 1.—Concentration dependence of the visible and ultraviolet spectra of FMN in water, phosphate buffer, *p*H 7. ———, 0.46×10^{-4} M FMN in 1-cm cell; — —, 0.92×10^{-3} M FMN in 0.05-cm cell; - - - -, 0.92×10^{-2} M FMN in 0.005-cm cell.

The photovoltaic cell was calibrated against a Reeder thermopile (Charles M. Reeder Co., Inc., Detroit, Michigan), Model RBL-500, and the wave length and intensity dependence of its response was determined. During these measurements the thermopile was standardized from time to time with a small carbon filament lamp obtained from the National Bureau of Standards. The thermopile output was measured with a Keithley millimicrovoltmeter, Model 149.

RESULTS

In order to define the limitations of the spectroscopic method for following the reduction of FMN and investigating complexing of flavins with metal ions, the absorption spectrum of FMN as a function of concentration was studied by using cells of thickness from 1 to 0.005 cm. The effect of concentration on the visible spectrum of FMN is shown in Figure 1. No further changes were observed on 10-fold dilution of the solution at 0.5×10^{-4} M concentration. The small spectral shifts at higher concentrations are presumably due to aggregation (cf. Gibson *et al.*, 1962), and therefore most experiments were carried out using solutions of about 0.5×10^{-4} M. The spectrum is also affected by metal ions which do not form chelates with FMN. This is shown in Figure 2. These curves were obtained by recording the spectral differences between a solution of FMN (10^{-2} M) in a 1-cm cell and a solution of FMN with added metal salts. Both Ni^{2+} and Mg^{2+} cause an increased absorption at the high end of the FMN spectrum (510–520 mμ). A similar change is produced by Na^{+} ions (again added as the chloride), but to a lesser extent although sodium chloride was added at a concentration that results in the same change in the ionic strength of the solution as was caused by the divalent metal salts.

Three useful conclusions emerge from these observations. First, it is important to study the reactivity and complexing ability of FMN at low concentrations where aggregation is negligible. Second, that specific cations can cause a spectroscopically distinct peak around 520 mμ in the difference spectrum of solutions of FMN at high concentrations. Finally, the effect of divalent metal ions is more pronounced than that of monovalent

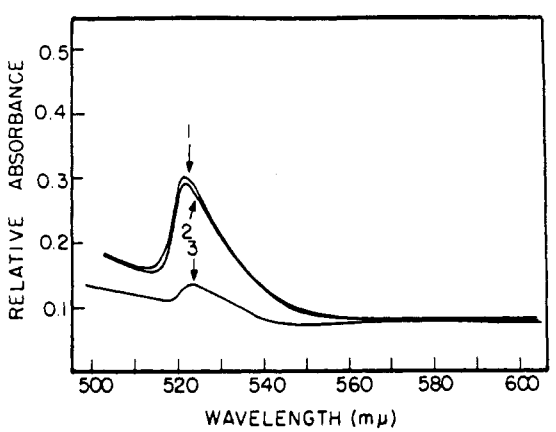

FIG. 2.—Difference spectra of solutions of FMN, with and without metal ions in water (*p*H ~5). Curve 1, 10^{-2} M FMN + 10^{-2} M $MgCl_2$ vs. 10^{-2} M FMN; curve 2, 10^{-2} M FMN + 10^{-2} M $NiCl_2$ vs. 10^{-2} M FMN; curve 3, 10^{-2} M FMN + 3×10^{-2} M NaCl vs. 10^{-2} M FMN.

cations. This may serve as a useful model for studying the effect of metal ions on the denaturation temperature of DNA, where it has been shown that divalent cations are more effective in screening the negative charges of the phosphate groups than monovalent cations and therefore stabilize the ordered structure of DNA more effectively (Zimm and Kallenbach, 1962).

1. Photoreduction without External Reducing Agents

The rates of photoreduction were followed spectrophotometrically. The concentration of riboflavin and of its analogs was chosen so that the optical density was between 0.1 and 0.5 (about 0.4×10^{-4} M). Most experiments were carried out in a phosphate buffer at *p*H 7, with an ionic strength of 0.1. All experiments were carried out under anaerobic conditions unless otherwise stated. The solutions were irradiated with white light, using a Corning glass filter, 3-73, which cuts out light below 400 mμ. The photoreduction of solutions of FMN, riboflavin, lumiflavin, and 6,7-dimethyl-9-hydroxyethylisoalloxazine was investigated. The spectral changes, when observed, were identical to

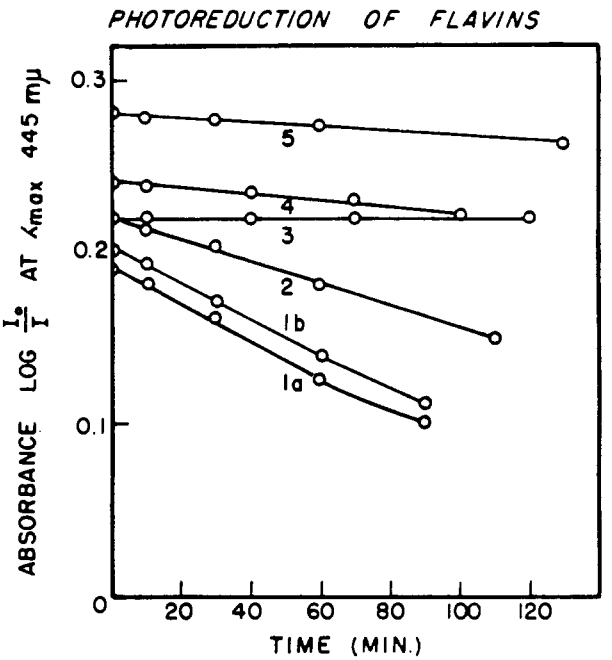

FIG. 3.—Photoreduction of flavins (*p*H 7; phosphate buffer). 1a, riboflavin; 1b, FMN; 2, 6,7-dimethyl-9-hydroxyethylisoalloxazine; 3, lumiflavin; 4, lumiflavin + ribose (10-fold excess); 5, lumiflavin + ethanol (40-fold excess).

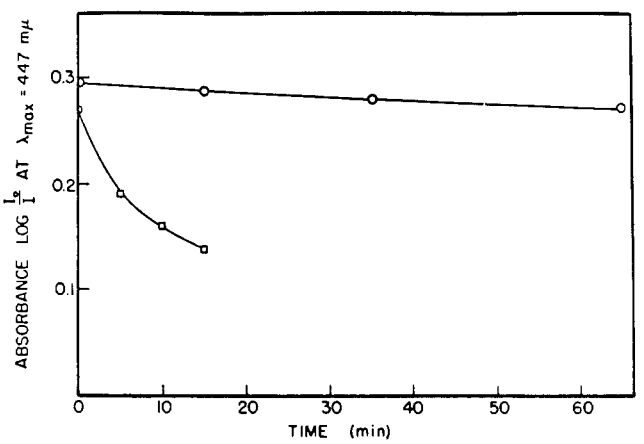

FIG. 4.—Photoreduction of riboflavin (squares) and lumiflavin with ribitol (80-fold excess) (circles).

those brought about by sodium dithionite as the reducing agent. The diminution in the 447-mμ absorption band as a function of illumination time for each compound is recorded in Figure 3. No change can be observed for lumiflavin but riboflavin, FMN, and 6,7-dimethyl-9-hydroxyethylisoalloxazine were photobleached. When air was admitted the original spectra were only partly restored, in agreement with earlier reports (Holmström and Oster, 1961). On addition of 10-fold excess of ribose or 20-fold excess of ethanol to the solution of lumiflavin, a small decrease of the 447-mμ absorption was observed on illumination, and the original spectra were completely restored by oxidation. A quantitative comparison of the rates of photoreduction of riboflavin and that of lumiflavin with added ribitol (80-fold excess) is shown in Figure 4. (The light intensity in these experiments was increased 2-fold compared to those shown in Figure 3.) Figure 5 shows that riboflavin also undergoes photoreduction in dry ethanolic solution.

a. Effect of Metal Ions.—The effect of added metal ions (10^{-3} M), added as chlorides, on the rate of anaero-

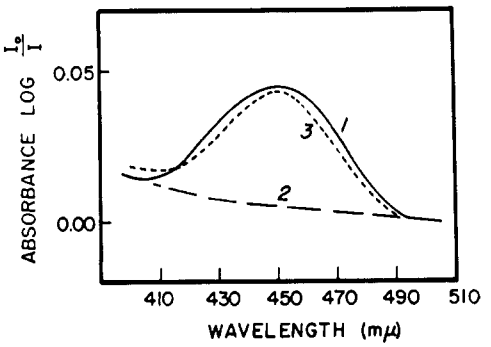

FIG. 5.—Photoreduction of riboflavin in absolute ethanol (saturated solution). 1, riboflavin in ethanol; 2, after 30-minute illumination; 3, after reoxidation.

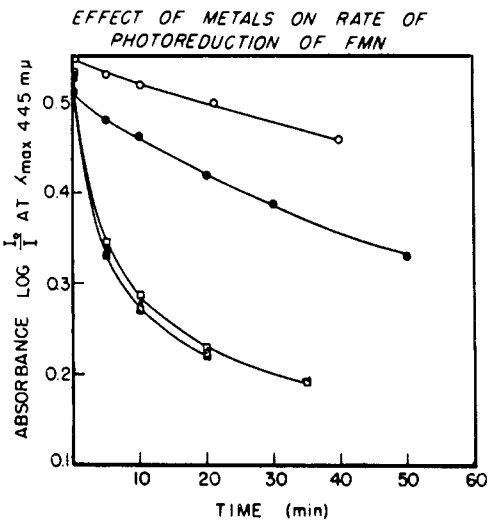

FIG. 6.—Effect of metals on the rate of photoreduction of FMN (0.5×10^{-4} M). Open circles, FMN + 1×10^{-3} M $NiCl_2$, *p*H 5.3; closed circles, FMN + 1×10^{-3} M $MnCl_2$, *p*H 5.3; open squares, FMN + 2×10^{-3} M NaCl, *p*H 5.3; closed squares, FMN at *p*H 5.3; triangles, FMN + 1×10^{-3} M $MgCl_2$.

bic photoreduction of FMN in unbuffered solutions was studied (the *p*H being adjusted to 5.3 in all cases). The results are shown in Figure 6. Mn^{2+} and Ni^{2+} decreased the initial rates to one-third and one-fifth of the original, respectively, while Na^+ and Mg^{2+} ions had no effect on the reaction rates. Ag^+ ions (added as the nitrate) completely inhibited the reaction, although at present it is not possible to say whether this is due to the formation of the flavin silver complex (Weber, 1950) or is a result of the reoxidation of $FMNH_2$ or one of its precursors during the reaction, since the formation of a small amount of colloidal silver can be observed (cf. Holmström and Oster, 1961). Illumination in the presence of cupric ions produced an increase in the optical density at 447 mμ (and at higher wavelengths). A similar increase can be observed in the dark when ascorbic acid is added to the solution (Baarda and Metzler, 1961). The effect of catalase on the rate of photoreduction of riboflavin is shown in Figure 7. Bovine serum albumin has no effect on the rate.

b. Electron Spin Resonance Measurements.—On illumination of a solution of FMN we observed an ESR signal identical in characteristics to that described by Commoner and Lippincott (1958) for the semiquinone of FMN. The time dependence of the appearance of this radical is recorded in Figure 8. After the saturation value has been reached the signal amplitude re-

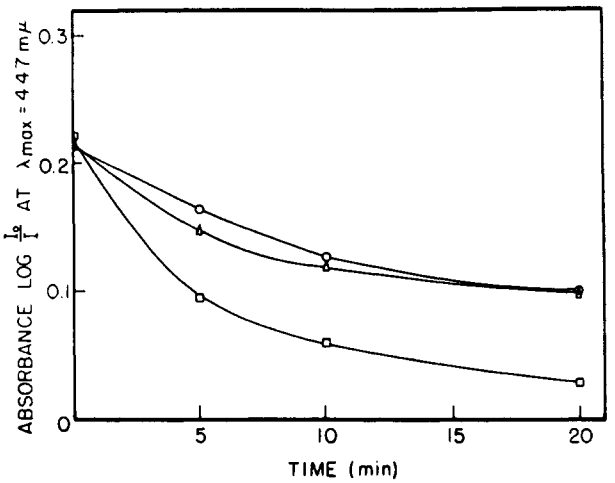

FIG. 7.—Effect of catalase on photoreduction of riboflavin. Circles, riboflavin; triangles, riboflavin + bovine serum albumin (0.02 g/ml); squares, riboflavin + catalase (0.02 g/ml).

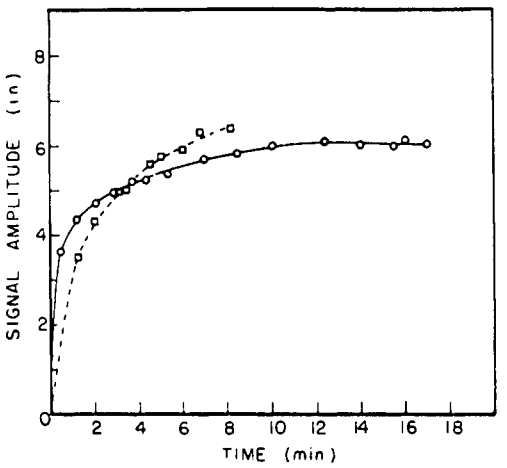

FIG. 8.—Rates of appearance of FMNH on illumination. Circles, FMN (10^{-3} M), *p*H 5.5; squares, FMN (10^{-3} M), *p*H 3.0.

mains constant for at least 1 hour. When the light is turned off at any point we cannot observe a change in the signal in 3 hours. On admittance of air the signal disappears almost instantaneously.

2. Interaction of Mn^{2+} with Riboflavin in the Ground State

The possibility of complex formation between manganese and riboflavin was investigated by three methods:

a. Spectrophotometry.—The visible and ultraviolet spectra of 1:1 molar mixtures of riboflavin and $MnCl_2$ (conc 0.5 $\times$ 10^{-4} M) were recorded at a series of *p*H values (*p*H 4–9) and compared with those obtained with riboflavin alone both by difference spectroscopy and by direct measurements. No significant differences were observed at *p*H values below 8, but a very small change was discernible at *p*H 9. This, however, could be attributed to light scattering as a result of the appearance of colloidal manganous hydroxide in the solution. When 2-, 5-, and 10-fold excess of the manganous salt were used no evidence for complex formation was found.

b. Electron Spin Resonance.—The ESR spectrum of divalent manganese consists of six equivalent lines with an overall width of 770 gauss. Complexing agents affect this spectrum in various degrees, resulting in a broadening of the individual hyperfine lines or sometimes even a complete disappearance of the signal. When FMN (10^{-2} M) is added to a solution of manganous chloride (10^{-3} M) at *p*H 6 and 7, no change in the ESR spectrum of the manganous ions can be observed.

c. Isolation of the "Chelate".—The alleged riboflavin manganese complex was prepared by the method of Foye and Lange (1954). This requires the precipitation of the complex from a solution of riboflavin at *p*H 9 by adding to it a solution of $MnCl_2$ simultaneously with 0.1 N NaOH so as to maintain the *p*H of 9. The precipitate thus obtained contained manganese (as shown by qualitative tests using persulfate oxidation to permanganate). Quantitative analysis after purification (Foye and Lange, 1954) gave the following composition: C, 49.64%; H, 5.59%; N, 13.59%; MnO_2, 5.9%. This does not correspond to any stoichiometrically feasible complex. It approximates most closely the formula $riboflavin_2Mn$ in contrast to $riboflavin \cdot Mn_2 2H_2O$ reported by others (Foye and Lange, 1954). In addition, the manganese-to-riboflavin ratio varied in different experiments. The complex prepared in this work also shows a slightly increased absorption relative to riboflavin at 500 mμ both in aqueous solution and dimethylsulfoxide (cf. Mahler *et al.*, 1955). If, however, the solutions were left standing at room temperature for 48 hours, the original riboflavin spectrum was recovered. Similarly the infrared spectrum of the "complex" (taken in Nujol suspension) is different from that of riboflavin in the carbonyl region.

To investigate both these spectral observations, a solution of riboflavin (*p*H 9) was treated the same way as in the preparation of the manganous complex, except that the reprecipitation was brought about by acidifying (*p*H 2) the solution instead of adding a solution of the manganous salt. The spectral characteristics (both visible and infrared) of the precipitate thus obtained were identical to those of the manganese "complex."

The visible spectrum of a half-reduced solution of riboflavin contains a small peak at 570 mμ which has been assigned to the semiquinone radical (Gibson *et al.*, 1962). When manganous ions are added to a degassed solution of half-reduced FMN no change in the spectra can be observed (either by difference or by direct measurements) even on an expanded scale.

3. Rates of Photoreduction with EDTA

The rates of photoreduction of riboflavin, FMN, FAD, and lumiflavin using EDTA as the reducing agent (in 40-fold excess) were compared. In these experiments special care was taken to obtain the same incident light intensity in different experiments (the light intensity did not vary by more than 5% as measured by a Weston light meter) and a blue interference filter (Corning 3-73) was used to cut light out below 400 mμ. The rates of reduction of each compound except that of FAD are identical within the limits of experimental error (Fig. 9). Under these conditions the photobleaching of the solutions without external reducing agent was insignificant compared to the photoreduction with EDTA. In each case reoxidation by air completely restored the original spectra. When the concentration of EDTA was varied (from 1.6 $\times$ 10^{-3} to 1.6 $\times$ 10^{-4} M) no effect on the rate was observed.

4. Reaction of $DPNH_2$ with FMN

a. Complexing.—Since it was reported that $DPNH_2$ and FMN form a charge-transfer complex (Isenberg and Szent-Györgyi, 1959; Isenberg *et al.*, 1961) we investigated this phenomenon first, both by absorption and fluorescence spectroscopy. The visible absorption

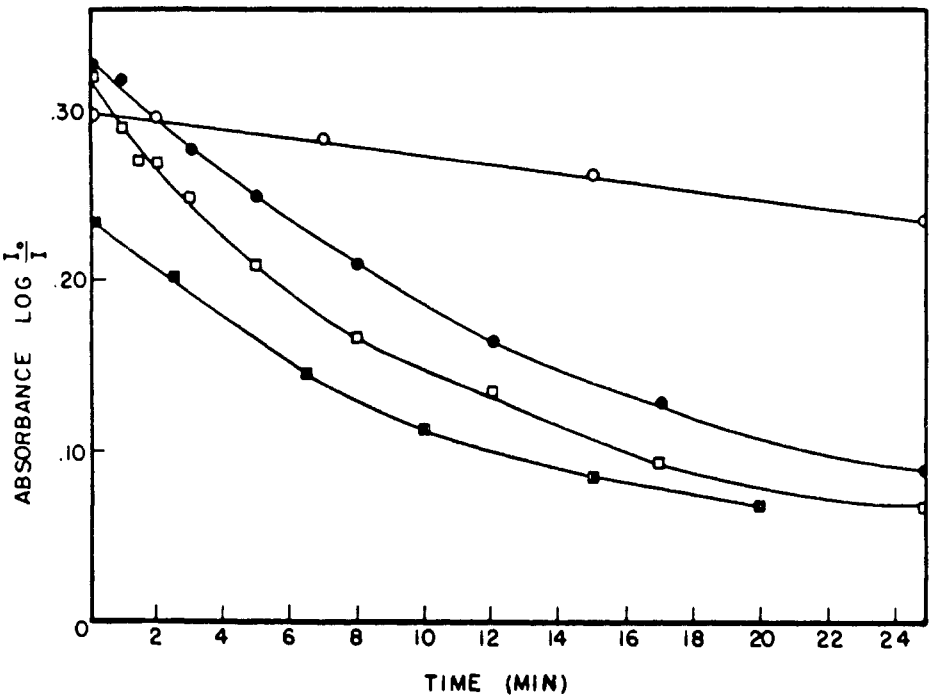

FIG. 9.—Rate of photoreduction of flavins with EDTA (40-fold excess), *p*H 7, phosphate buffer. Open circles, FAD; closed circles, FMN; open squares, riboflavin; closed squares, lumiflavin.

of a solution of FMN (0.5×10^{-4} M) with and without added $DPNH_2$ (5×10^{-3}–0.2×10^{-3} M) was found to be identical under aerobic conditions. In deoxygenated solutions the absorption spectra were identical when extrapolated to zero time. This procedure was necessitated by the reaction described below. The fluorescence excitation spectra of solutions of FMN (under aerobic conditions) (10^{-5} and 10^{-4} M, λ^{Fl}_{max} = 530 mμ, uncorrected, exciting light 450 mμ) were unchanged even when $DPNH_2$ was added to the solutions at high concentrations (0.35×10^{-2} M).

b. Dark Reaction.—When a solution of FMN (0.3×10^{-4} M) is mixed with approximately 10-fold excess of $DPNH_2$ under anaerobic conditions, a slow decrease in the 447-mμ absorption of FMN and in the 340-mμ absorption of $DPNH_2$ is observable. This decrease is considerably enhanced by white light (Fig. 10). That the "dark" reaction is not in fact a photoreduction induced by the monochromatic light of the spectrophotometer is shown by comparing the two curves in Figure 10, as the reaction proceeds at the same rate when the spectrophotometer is turned off.

i. Kinetics of the Dark Reaction.—In these experiments the temperature was kept constant to ±1°. Concentrated solutions of $DPNH_2$ were added after 1 hour (during which time argon was bubbled through the solutions) to the FMN solutions. Excess of $DPNH_2$ was used in all runs and the results (calculated from the decrease of the FMN absorption at 447 mμ) gave good pseudo first-order plots in FMN concentration as a function of time. When the concentration of $DPNH_2$ (still in excess) was varied, a plot of the pseudo first-order rate constants vs. $DPNH_2$ concentration gave a good straight line showing the reaction to be second order (Fig. 11). From these the second-order rate constant at 37° was calculated to be k_2 = 0.159 liter/mole·sec. The temperature dependence of the reaction was measured similarly between 17° and 44°. The Arrhenius activation energy (calculated from Figure 12 by the method of least squares) is 8.3 ± 0.5 kcal/mole. The activation entropy can be obtained from the same graph from the intercept at $1/T = 0$. It is about −7 eu, although it is subject to large errors because of the necessary extent of extrapolation. The rates of $DPNH_2$ reduction of FMN and lumiflavin were found to be equal within 5%.

ii. ESR Studies of Dark Reaction.—When a solution of FMN (10^{-3} M) is mixed with a solution of $DPNH_2$ (10^{-2} M) under anaerobic conditions, a single symmetrical signal centered around g = 2.00, half width = 18 G, is observed. This signal has been shown to be identical to that obtained in a half-reduced solution of FMN (Isenberg *et al.*, 1961). The time course of the increase and decay of the free radical is shown in Figure 13. Knowing the rate constant of the second-order reaction, the extent of reaction at any given time can be calculated (% marks in Fig. 13). It can be seen that the concentration of the radical is maximal at 50% reaction.

c. Light-catalyzed Reaction.—A blue filter (Corning 3-73) was used in all illumination experiments. The reactions were carried out at 17° (unless otherwise stated) to minimize the dark reaction. The rate of photoreduction of FMN (as measured by the decrease at 447 mμ) parallels the rate of photooxidation of $DPNH_2$ being oxidized for 1 mole of FMN reduced. The rate of photoreduction of FMN is independent of the concentration of $DPNH_2$ within the range 0.8×10^{-4}–1.3×10^{-3} M (two typical curves are shown in Fig. 14). The temperature dependence of the reaction (when corrected for the dark reaction at higher temperatures) is shown in Figure 15. Within the range of 17°–30° the reaction appears to be independent of the temperature, although the rate is somewhat slower at 44°. The buffer evidently has no effect on the reaction, as it proceeds at the same rate in water at the same *p*H, in contrast to the photobleaching of riboflavin without external reducing agents (Holmström and Oster, 1961). Lumiflavin was reduced at the same rate as FMN, and added DPN (10^{-3} M) did not affect the rate of photoreduction of FMN. FAD was again photoreduced at a slower rate (Fig. 16).

The quantum yield of the photoreduction was measured by illuminating the reaction mixture in a narrow wavelength region centered around 447 mμ (half width

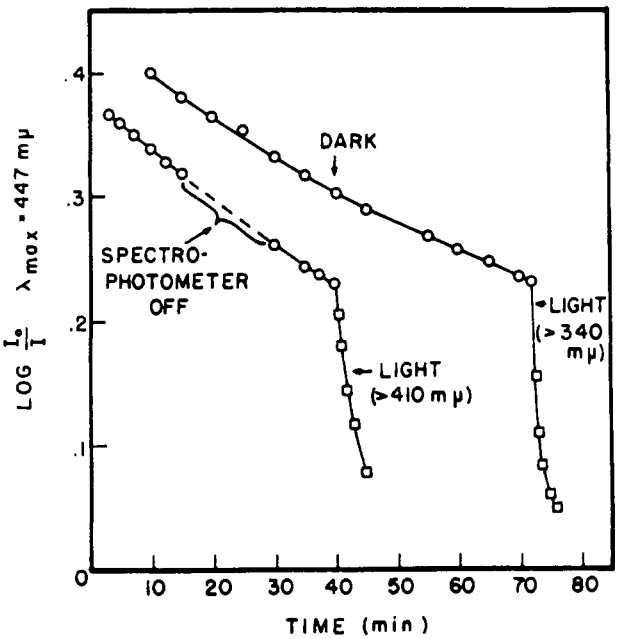

FIG. 10.—Reaction of $DPNH_2$ (0.3×10^{-3} M) with FMN (0.3×10^{-4} M) in dark and light catalyzed; pH = 7, phosphate buffer.

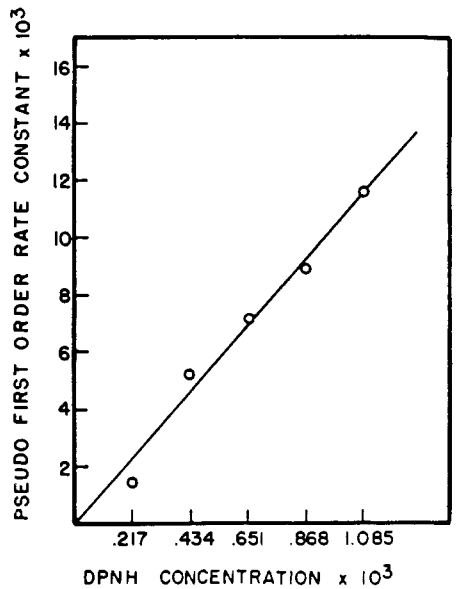

FIG. 11.—Plot of pseudo first-order rate constants of dark reduction of FMN vs. $DPNH_2$ concentration.

= 17.5 mμ). The initial quantum yield was 0.25, and it decreased as the reaction proceeded, as shown in Table I.

DISCUSSION

The results of the photobleaching experiments without added reducing agents clearly indicate that at least a 9-hydroxyethyl side chain on the 6,7-dimethylisoalloxazine nucleus is necessary for the photoreduction. The argument (Strauss and Nickerson, 1961) that water is responsible for the photoreduction seems to us untenable, since then lumiflavin, which is electronically identical to riboflavin, and the hydroxyethyl derivative, as judged by their absorption spectra, should undergo the same reaction. To support this conclusion it was necessary to show that the observed differences are not due to a rate phenomenon perhaps

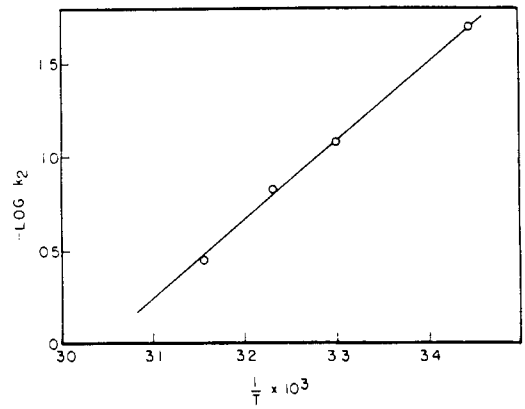

FIG. 12.—Arrhenius plot for FMN + $DPNH_2$ dark reaction.

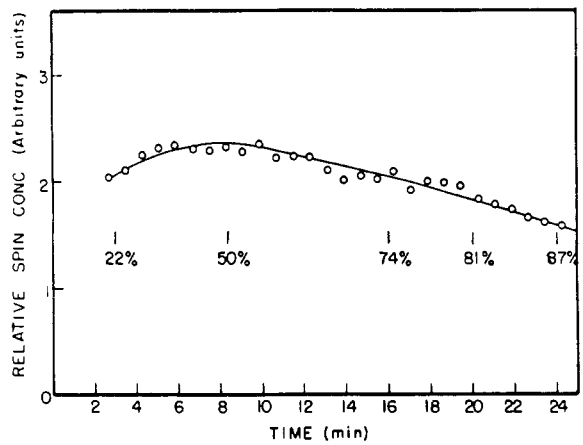

FIG. 13.—Spin concentration as a function of reaction time in $DPNH_2$ + FMN dark reaction (10^{-3} M FMN + 10^{-2} M $DPNH_2$). Per cent marks (%) are the calculated extent of reaction as determined in the optical spectrophotometer.

associated with a property of the molecules not manifest in the absorption spectra. For instance, there has been a suggestion (Tether and Turnbull, 1962) that the side chain of riboflavin promotes the transition from the first excited singlet to the triplet, since the rate of photosensitized dimerization of ergosterol is faster when riboflavin is the sensitizer than in the presence of lumiflavin. We therefore compared the rates of photoreduction of riboflavin, FMN, FAD, and lumiflavin using an external reducing agent (EDTA). The results shown in Figure 9 can be better expressed if we assume the following mechanism for the photoreduction:

$$F + h\nu \xrightarrow{k_1} F^* \quad (1)$$

$$F^* + S \xrightarrow{k_2} F \quad (2)$$

$$F^* + B \xrightarrow{k_3} \text{Products} \quad (3)$$

F = flavin, S = solvent, B = EDTA

Equation (1) represents the light-induced excitation of the flavin to a reactive state (either to the first singlet

TABLE I
QUANTUM YIELD OF FMN AND $DPNH_2$ REACTION

Reaction (%)	Quantum Yield
0–10	0.25
10–30	0.17
30–60	0.13
60–70	0.10

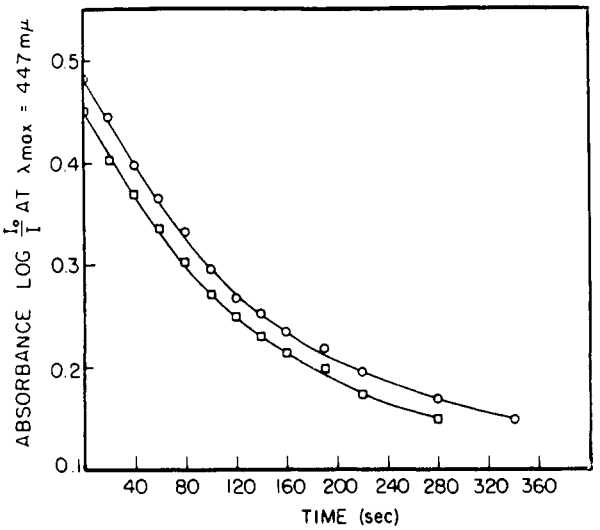

FIG. 14.—Effect of $DPNH_2$ concentration on rates of photoreduction of FMN. Circles, 0.54×10^{-4} M $DPNH_2$; squares, 0.87×10^{-3} M $DPNH_2$.

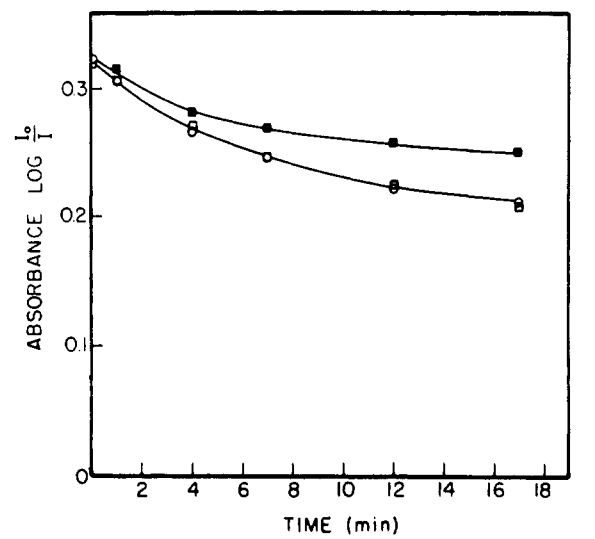

FIG. 15.—Temperature dependence of light reaction (corrected for dark reaction). Circles, 17°; open squares, 30°; closed squares, 44°.

or indirectly the triplet), equation (2) represents the solvent quenching of the reactive state, and equation (3) represents the reduction step. Since S and B are in large excess and solvent quenching experimentally does not compete with reduction by EDTA, their concentration terms in the rate expressions can be included in constants and the reduction rates should be independent of the concentration of B. The rate of quanta absorbed: $k_1 I_{abs} = k_1(I_0 - I)$; and $I = I_0 e^{-\epsilon[F]l}$. Using a steady-state treatment, the following rate expression is obtained:

$$\frac{-d[F]}{dt} = \frac{k_1 \cdot k_3 \cdot I_0(1 - e^{-\epsilon \cdot l[F]})[B]}{k_2[S] + k_3[B]} \qquad (4)$$

and on integration

$$-\left[\overbrace{[F] + \frac{1}{\epsilon \cdot l} \log (1 - e^{-\epsilon \cdot l[F]})}^{\Phi}\right]_{F_1}^{F_2} = \frac{k_1 k_3 \cdot I_0[B]}{k_2[S] + k_3[B]}(t_2 - t_1) \qquad (5)$$

Knowing the extinction coefficients, the concentrations can be calculated. The plots for Φ vs. time for the four reactions are shown in Figure 17. The slopes of the lines (the constants of the right hand side of the equation 5) are equal for all compounds save FAD. Since one can assume that the solvent quenching and the rate of quanta absorbed are identical for all three compounds with the parallel plots (their absorption spectra are identical), the rates of reduction must also be equal. The rate expressions above are, of course, oversimplified, since if a triplet is involved a term for the singlet triplet conversion ought to be included (and terms for self-quenching and product quenching should also be considered) (cf. Holmström, 1962). These, however, do not affect the overall form of the expression in equation (5) (cf. equation 12), and in any case the conclusion drawn does not depend on the correctness of the detailed mechanism. The slower rate of the photoreduction of FAD may be due to self-quenching of the excited state through the formation of an intramolecular complex (perhaps of the charge-transfer type) between the adenine and flavin parts. It has long been known that the formation of such an intramolecular complex is responsible for the lower fluorescence efficiency of FAD than that of FMN (Weber, 1950).

The conclusion that oxidation of the side chain accompanies the photoreduction of riboflavin is also supported by the observation (also noted by Holmström and Oster, 1961) that when no external reducing agent is present reoxidation does not restore the original spectra completely while complete reversal can be obtained when the reducing agent is EDTA. Lumiflavin, lumichrome, and fragments of the side chain have been identified as oxidation product of a photoreduced solution of riboflavin, while no such fragmentation was observed on oxidation of a solution of riboflavin photoreduced in the presence of EDTA (Smith and Metzler, 1963).

We can now consider the evidence that has been reported in favor of the "water-splitting" mechanism, namely, that (1) hydrogen peroxide is produced during the anaerobic photoreductions (Vernon, 1959; Strauss and Nickerson, 1959); (2) catalase has an inhibitory effect on the rates of photoreduction (Strauss and Nickerson, 1961); (3) methionine sulfoxide is produced when methionine is added to the solution in the anaerobic photoreduction of riboflavin (Nickerson and Strauss, 1960); and (4) the reaction does not occur in nonaqueous solvents such as ethanol (Vernon, 1959). Evidence for hydrogen peroxide was deduced from the apparent oxidation of $DPNH_2$ (followed spectroscopically at 340 mμ) when the photobleached solutions were added under anaerobic conditions to a system containing ethanol, $DPNH_2$ and alcohol dehydrogenase (Vernon, 1959). Since, however, the nature of the oxidation products of EDTA (used in the photobleaching) or of the side chain without external reductant are unknown, it is not impossible that they may be responsible for the oxidation of $DPNH_2$ if, in fact, the change in OD at 340 mμ represents this oxidation. Strauss and Nickerson (1961) cite the effect of catalase on the rate of photoreduction as additional evidence for hydrogen peroxide formation, the other evidence having been mentioned briefly (without experimental details) earlier by them (Strauss and Nickerson, 1959). Under our conditions, catalase, in fact, has the opposite effect (as also observed by Rutter, 1958), which may be attributed to the addition of some reductant present in the catalase preparation. The fact that methionine sulfoxide is formed when riboflavin solutions are photobleached by methionine and reoxidized electrochemically probably means that methionine is the reducing agent and that its oxidation products react with water directly.

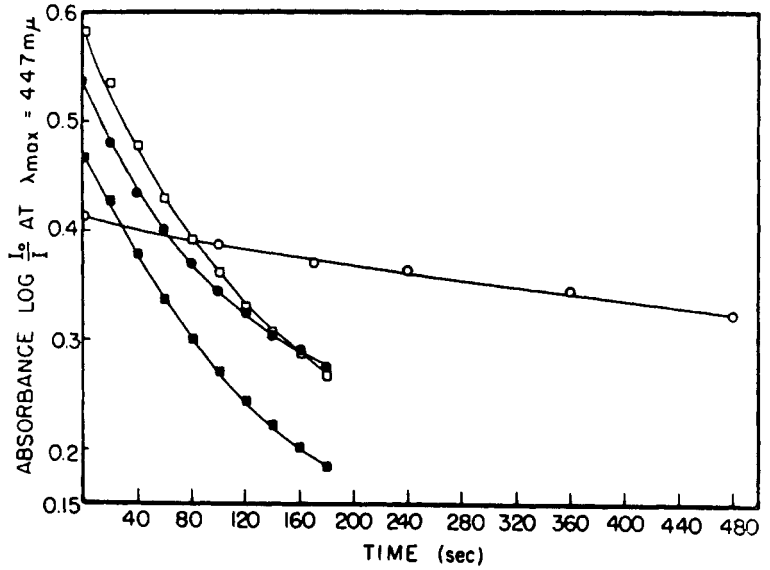

FIG. 16.—Photoreduction of flavins by $DPNH_2$. Open squares, FMN + $DPNH_2$ ($DPNH_2$ 0.217 × 10^{-3} M); closed circles, lumiflavin + $DPNH_2$; closed squares, FMN + $DPNH_2$ in presence of DPN (2 × 10^{-3} M); open circles, FAD + $DPNH_2$.

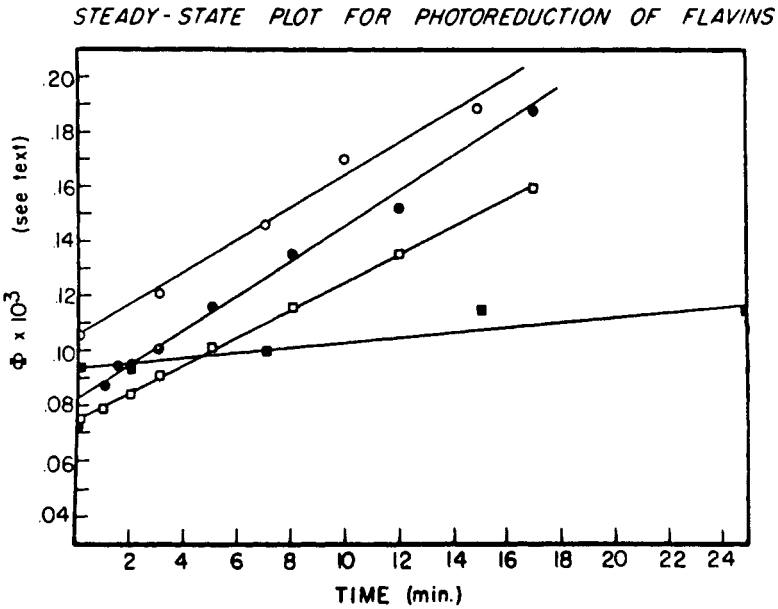

FIG. 17.—Steady-state plot for photoreduction of flavins by EDTA. Open circles, lumiflavin; closed circles, riboflavin; open squares, FMN; closed squares, FAD.

Finally, we find that riboflavin is photoreduced in dry alcoholic solutions, and also that illuminated lumiflavin reacts with ethanol.

Our results therefore are in agreement with the suggestion that the side chain is involved in the photoreduction without external reductant (Holmström and Oster, 1961), and, furthermore, the finding that the photoreduction of lumiflavin with ribitol is considerably slower than the reduction of riboflavin suggests that the process follows an intramolecular path.[2]

The fact that Mn^{2+} and Ni^{2+} ions decreased the initial rates of photoreduction of FMN to one-third and one-fifth, respectively, while Na^+ and Mg^{2+} ions had no effect on the reaction, suggests that the paramagnetism of the two transition metals is responsible for the decreased rates and that therefore the activated state may involve a triplet (Berends and Posthuma, 1962). This seems particularly attractive, since manganese does not form a chelate with riboflavin in the ground state. Similar conclusions were reached on the basis of inhibition experiments by potassium iodide (Holmström and Oster, 1961). The apparent inhibition of the reaction with Cu^{2+} ions is due to their reduction by $FMNH_2$, while the effect of Ag^+ may be a combination of the photoreduction to metallic silver, as noted by Holmström and Oster (1961), and the formation of a strong silver-riboflavin complex (Baarda and Metzler, 1961; Bamberg and Hemmerich, 1961). The fact that we were unable to detect free radical intermediates by ESR when photoreduction was carried out in the presence of silver suggests that the second effect (complex formation) may be more important.

Reaction of $DPNH_2$ with FMN.—Our results show conclusively that there is a second-order reaction between $DPNH_2$ and FMN under anaerobic conditions. The second-order rate constant is in qualitative agree-

[2] Exactly the same conclusion was reached by Moore *et al.* (1963) on the basis of experiments in D_2O and polarographic analysis, published after this paper had been submitted.

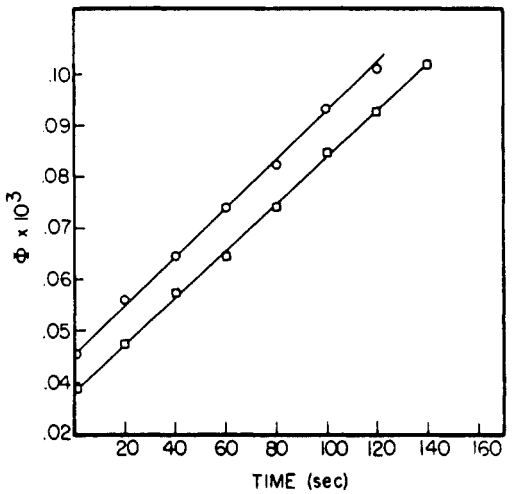

FIG. 18.—Steady-state plot for photoreduction of FMN by $DPNH_2$. Circles, FMN + 0.87 × 10^{-3} M $DPNH_2$; squares, FMN + 0.54 × 10^{-4} M $DPNH_2$.

ment with that obtained by Suelter and Metzler (1960) under aerobic conditions. The quantitative difference may be a result of the different buffer systems used by us, especially since the rate of the reaction depends on the ionic strength of the solution (e.g., the reaction in pure water is slower) (see under Results, and also Suelter and Metzler, 1960). It is interesting to compare the activation energy of our reaction (8.3 kcal/mole) with that for an analogous enzymic process, namely, the microsomal cytochrome b_5 reductase system (Strittmatter, 1962). We calculate the activation energy for the enzymic process as about 4.0 kcal/mole from the data given by Strittmatter. Since in the enzymic system it was shown that the rate-determining step involved the reduction of the flavoprotein by $DPNH_2$, this indicates that the differences in rates of the enzymic and nonenzymic reactions (which we estimate to be of the order of 10^8–10^9 from our rate data and those given by Strittmatter) cannot be accounted for in terms of activation energies alone. While the difference in activation energies may represent a rate difference of about 100-fold only, the remaining factor of 10^6–10^7 can be understood in terms of proximity and orientation effects as described by Koshland (1960).

The fact that there is a reaction between $DPNH_2$ and FMN, coupled with our failure to observe formation of a complex between these two reagents at room temperature by absorption and fluorescence spectroscopy, seems to cast doubt on the interpretation that there is a charge-transfer complex formation between $DPNH_2$ and FMN at room temperature as deduced from spectroscopic observations at low temperature (Isenberg and Szent-Györgyi, 1959) and ESR measurements at room temperature (Isenberg, *et al.*, 1961), as both these observations may be due to the formation of the semiquinone of FMN as a result of the reaction between $FMNH_2$ and FMN (Gibson *et al.*, 1962). The fact that the concentration of the radical measured in our ESR experiments is maximal at 50% reaction is strong support for this interpretation.

The Light-catalyzed Reaction.—The rates of photoreduction of FMN by $DPNH_2$ can be plotted in the same form as that derived above for the reaction with EDTA. If, however, we now write a somewhat modified overall mechanism as follows:

$$F + h\nu \xrightarrow{k_1} F^* \qquad (6)$$

$$F^* + S \xrightarrow{k_2} F \qquad (7)$$

$$F^* \xrightarrow{k_3} F' \qquad (8)$$

$$F' + S \xrightarrow{k_4} F \qquad (9)$$

$$F' + DPNH_2 \xrightarrow{k_5} FH_2 + DPN \qquad (10)$$

$$F' + DPNH_2 \xrightarrow{k_6} F + DPNH_2 \qquad (11)$$

where equation (8) represents the radiationless transition of the first excited singlet to the triplet, equation (9) represents the solvent quenching of the triplet, equation (10) shows the reaction of the triplet with $DPNH_2$ yielding the products, and equation (11) represents the quenching of the triplet by $DPNH_2$, the integrated form will be:

$$-\Big[\underbrace{[F]+\frac{1}{\epsilon\cdot l}\log(1-e^{-\epsilon\cdot l[F]})}_{\Phi}\Big]_{F_1}^{F_2} = \frac{k_1k_3k_5I_0[DPNH_2]}{(k_2[S]+k_3)(k_4[S]+k_5[DPNH_2]+k_6[DPNH_2])}(t_2-t_1) \qquad (12)$$

The corresponding plots for two runs with different concentrations of $DPNH_2$ are shown in Figure 18, and the slopes of the lines of Figure 18, together with those from a series of runs with different concentrations of $DPNH_2$, are tabulated in Table II. Good linear plots are obtained up to about 50% of the reaction only, indicating that the reaction products have a quenching effect (presumably on the triplet). The decrease in quantum yield as the reaction proceeds may be a manifestation of the same phenomenon. Since DPN itself does not affect the rates of photoreduction, the observed product inhibition must be attributed to the quenching effect of $FMNH_2$ or FMNH.

It is important to note that our kinetics alone are not sufficient to distinguish between a mechanism involving two intermediates (equations 6–10) or only one intermediate (equations 1–3). We can, however, calculate a minimum lifetime for the excited state responsible for the reaction from the rate of the reaction, measured during the determination of the quantum yield, and the number of quanta absorbed. Using simple collision theory, the calculation results in a lifetime of 10^{-4}–10^{-5} seconds, which is consistent with a triplet's being responsible for the reaction. This is supported by the fact that $DPNH_2$ does not quench the fluorescence of FMN as one would expect if the first singlet-excited state were responsible for the photoreaction.

An interesting feature of the photoreduction is the independence of the rate on the concentration of $DPNH_2$ (and also EDTA). This, together with the fact that the rates of the two reactions (i.e., with $DPNH_2$ and EDTA) differ by about a factor of 5, can only be explained if we assume that a complex is formed

TABLE II
EFFECT OF $DPNH_2$ CONCENTRATION ON RATES OF PHOTOREDUCTION OF FMN

$DPNH_2$ Concentration (mole/liter)	Slope of Steady-State Plot (Φ/time; mole/liter·sec)
0.54 × 10^{-4}	4.55 × 10^{-7}
1.08 × 10^{-4}	4.40 × 10^{-7}
2.20 × 10^{-4}	4.10 × 10^{-7}
4.30 × 10^{-4}	3.90 × 10^{-7}
6.50 × 10^{-4}	4.60 × 10^{-7}
8.70 × 10^{-4}	4.77 × 10^{-7}
13.00 × 10^{-4}	4.70 × 10^{-7}

between FMN in the excited triplet state and the reactant, and that the relative efficiencies of the conversion of this complex to products and to the ground state without reaction are inherent to the reducing agent, i.e., that k_5/k_6 ratio (equations 10 and 11) is constant for a given reductant. The fact that the rates are the same with lumiflavin and FMN shows that the ribitol side chain is not involved in the formation of this complex. This interpretation is consistent with the observation that the reaction is independent of the temperature, or possibly even slower at high temperatures, since complex formation would be less favored at higher temperatures. The possibility that the slower rate observed at 44° is due to the necessity of large corrections for the dark reaction cannot entirely be excluded. These observations and conclusions are similar to those of Huennekens and Calvin (1949) for the photooxidation of zinc tetraphenyl chlorin with a series of quinones.

The reaction of $DPNH_2$ with FMN is a thermodynamically downhill process, the oxidation-reduction potentials of $DPNH_2$ and FMN being −0.320 v (Burton and Wilson, 1953) and −0.219 v (Lowe and Clark, 1956), respectively. The light-catalyzed reaction therefore is not an energy storing process—the only function of light is to provide a reaction path with a lower activation energy.

When these two coenzymes are bound to proteins, in general a positive shift in their oxidation-reduction potentials is observed. For instance, Theorell and Bonnichsen (1951) found a shift of potential, +0.064 v, in liver alcohol dehydrogenase relative to the free DPN-$DPNH_2$ system. Vestling (1955) estimates a shift of +0.063 v in the old yellow enzyme compared to free FMN. In some metalloflavoproteins the changes are even larger (e.g., butyryl-coenzyme A dehydrogenase with Cu has an E'_0 value of +0.187 v) (Green *et al.*, 1954). Xanthine oxidase seems to be an exception to this trend, as $E'_0 = -0.45$ v (Mackler *et al.*, 1954) for the bound FAD—that is, this seems to be an enzyme which in its reduced form is capable of reducing DPN to $DPNH_2$. This reaction has in fact been reported (Mackler *et al.*, 1954).

It is thus apparent that the oxidation potential of the flavin chromophore is relatively sensitive to its environment (protein, metal, *p*H, etc.). We may therefore expect to find it at several different potential levels in the electron transport chain.

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