RIBOFLAVIN AS A PHOTOCATALYST AND HYDROGEN CARRIER IN PHOTOCHEMICAL REDUCTION*

by

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The importance of chlorophyll and carotenoids in photosynthetic reactions and visual excitations has been stressed in many publications. Various biological pigments such as phycobilins, flavones, and anthocyanins have also received attention as participants in various light reactions. Little attention has been directed to the possible importance of riboflavin, another almost universally distributed biological pigment, in photo-biological reduction. Recently we have reported that the photochemical reduction of colourless tetrazolium salts in an aqueous system containing a metal chelating agent is catalyzed by riboflavin (NICKERSON AND MERKEL[1]). The present paper extends our studies on the non-enzymic, photoreduction system, defines some of the limits thereof, and outlines the role played by riboflavin in the reduction. The importance of metal complexing agents in the regulation of biological reactions may be made more evident through studies on the "relatively simple" non-enzymic photochemical reaction which is described.

MATERIALS AND METHODS

For intense illumination a 375 watt, Sylvania flood-light at distances of 10–15 cm was used. To filter out infrared waves the light beam was first passed through 4 cm of an aqueous copper sulfate solution (2%). Generally, however, a heat filter was not used for very short periods of irradiation. Duplicate experiments omitting riboflavin from the system were used to correct for non-specific photolysis and photoreduction found with some of the dyes used. For more controlled irradiations, the reactions were carried out in Pyrex brand, 16 mm test tubes in a Lumetron colorimeter (Model 401) using a 420 mμ filter. The reduction of riboflavin in the photoreactions was estimated spectrophotometrically (Model DU Beckman Spectrophotometer) and by means of a polarograph (Fisher, manually operated Elecdropode). Potential measurements were made with a Beckman, Model G, pH meter with external, shielded electrodes. The reduction of dyes was followed visually. The source of the reagents used in the present studies has been mentioned in the previous paper[1].

EXPERIMENTAL

Light-absorption studies. The time course for the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) in a Lumetron colorimeter with a 420 mμ filter is shown in Curve A of Fig. 1. In this experiment a solution containing 10^{-3} *M* TTC, $4 \cdot 10^{-5}$ *M* riboflavin, $3 \cdot 10^{-3}$ *M* disodium ethylenediamine-tetraacetate (Na_2EDTA) and 1 drop of

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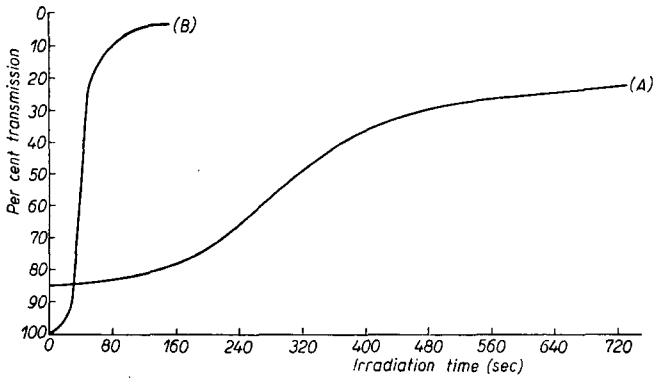

Fig. 1. Photoreduction of 2,3,5-triphenyltetrazolium chloride (TTC). Curve (A): $1 \cdot 10^{-3}$ *M* TTC, $4 \cdot 10^{-5}$ *M* riboflavin, $3 \cdot 10^{-3}$ *M* Na_2EDTA, 1 drop of Tween 80, in a total volume of 6 ml of *M*/15 phosphate buffer at pH 7.38; reduction carried out in a Lumetron photoelectric colorimeter (Model 401) using a 420 mμ filter. Curve (B) obtained with the same solution used for Curve (A), but reduction was achieved with a 375 watt Sylvania flood-light at a distance of 15 cm; TTC reduction measured in the Lumetron using a 530 mμ filter (to avoid additional photoreduction while the measurements were made).

Tween 80 (to keep the reduced TTC in suspension) in a total volume of 5.0 ml of *M*/15 phosphate buffer (pH 7.35) was irradiated in the colorimeter. The tube was shaken between readings. Reduction could be detected within 60 seconds at this light intensity, and was essentially complete after 12 minutes. When a similar system was irradiated with a 375 watt lamp at a distance of 15 cms, reduction of TTC could be detected within 25 seconds and was complete within 60 to 75 seconds (curve B). The apparent "induction" period could be virtually eliminated by bubbling N_2 through the solution for several minutes before irradiation to expel oxygen from the system. Dissolved oxygen not only interfered with the photoreduction of riboflavin, it also protected the riboflavin from destruction by light in the absence of Na_2EDTA.

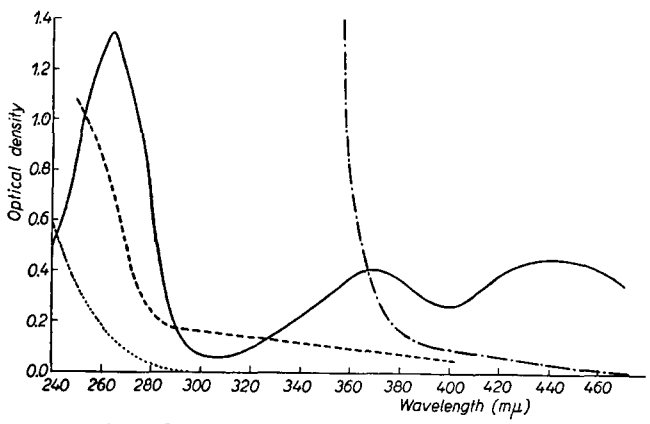

Fig. 2. Absorption spectra of riboflavin and photoreduced riboflavin. ····· $2 \cdot 10^{-3}$ *M* disodium versene (Bersworth Anal., Na_2EDTA); ——— $4 \cdot 10^{-5}$ *M* riboflavin (B_2) or B_2 + $2 \cdot 10^{-3}$ *M* $NaEDTA_2$ (which is the same as riboflavin or riboflavin phosphate alone); – – – – light-reduced riboflavin phosphate in the presence of Na_2EDTA; ·—·—· riboflavin reduced with sodium hydrosulfite. All solutions were made in phosphate buffer, pH 7.0–7.4. The solutions to be irradiated were removed from the spectrophotometer after every third reading and placed under the 375 watt lamp for approximately 30 seconds. Methylene blue (MB) was added to the solutions receiving light to retard the oxidation of photoreduced riboflavin.

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No reduction of TTC was observed in the colorimeter with 490 mμ or 530 mμ filters after 10 minutes of irradiation; reduction proceeds very slowly in the ultraviolet range. The action spectrum for the reduction has not yet been determined, but, with the filters employed, is presumably equivalent to the absorption spectrum of riboflavin in the visible range. It is the 440 and 370 mμ absorption peaks which disappear upon irradiation of a riboflavin-Na_2EDTA solution (Fig. 2).

The reduction of riboflavin to the leuco form could be observed visually when a solution of riboflavin and Na_2EDTA was irradiated. If a dye, such as methylene blue or toluidine blue, is also included in the system one observes first the reduction of the dye upon irradiation, followed by the complete disappearance of the riboflavin colour. When the completely reduced system is removed from the strong light and aerated gently, the yellow colour of the riboflavin reappears first, followed immediately by the reappearance of the colour of the oxidized dye. The air oxidation of photochemically reduced riboflavin was followed in the spectrophotometer; the 370 and 440 mμ peaks reappeared unchanged within 10 minutes*.

Polarographic studies. Since riboflavin gives a well-defined polarographic reduction wave (BRDIČKA AND KNOBLOCH[2]; LINGANE AND DAVIS[3]) it proved feasible to follow its photoreduction polarographically. At pH 7.3 well-defined waves were obtained for the reduction of riboflavin with an average half-wave potential ($E_{1/2}$) (for a large number of measurements) of —0.450 volts *vs* the saturated calomel electrode (S.C.E.) or a redox potential (KOLTHOFF AND LINGANE[4]) of —0.208 volts *vs* the normal hydrogen electrode (N.H.E.)**. At pH 7.0 the redox potential was calculated to be —0.194 volts. The half-wave potential for the polarographic reduction of riboflavin varied with the pH of the solution and between pH 6.8 to 7.8 the expected shift in $E\frac{1}{2}$ was observed (0.054 volts compared to the theoretical value of 0.059 volts *vs* S.C.E.). At pH values below 7.3 a small "prewave" or "anomalous" wave (BRDIČKA AND KNOBLOCH[2]) appeared in the current-voltage curves. As the pH was lowered the "prewave" became larger and more distinctly separated from the main wave (Fig. 3)***. At pH 2.18 in glycine/HCl buffer two distinct and identical waves appeared in the curves. These waves remained distinct even in the anodic curve for light-reduced riboflavin. The existence of a semiquinone intermediate in the chemical reduction of riboflavin can readily be demonstrated. The conditions necessary to observe semiquinone formation are similar to those required to obtain a separation of the "anomalous" wave from the main wave, *i.e.*, acid conditions. At low pH values a red intermediate is formed in the chemical reduction of yellow riboflavin to the colourless, reduced form (KUHN AND WAGNER-JAUREGG[6]; MICHAELIS

* It appears that this reaction can be used to study the rates of diffusion of O_2 into various solutions. We have been able to use this method to demonstrate the rate of diffusion of O_2 into agar by adding agar (2%) to a solution of riboflavin, Na_2EDTA and methylene blue. The agar is dissolved and solidified in a 16 mm culture tube and strongly irradiated until the colour has completely disappeared. The tube then is immediately placed in an upright position in the dark. The diffusion of O_2 into the agar medium can be followed by the appearance of oxidized methylene blue.

** Using a value for saturated calomel (0.242 v) which, according to LATIMER[5], is generally accepted.

*** According to BRDIČKA AND KNOBLOCH the "anomalous" wave is the result of adsorption of reduced riboflavin to the mercury drops. BRDIČKA AND KNOBLOCH did not observe an increase in the height of the "anomalous" wave with decreasing pH (only a more distinct separation from the main wave), and the occurrence of the double wave was not attributed to semiquinone formation. Further studies of the polarography of the photoreduction of riboflavin in the presence of metal chelating agents are in progress to determine if the reduction process is actually separated into 2 distinct steps (semiquinone formation) or whether these are artefacts due to adsorption.

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et al.,[7]; HAAS[8]). In the system employed in the present study the red intermediate cannot be demonstrated because extremely acid conditions are inhibitory to the photoreduction process; however, the red intermediate has been very clearly demonstrated in the back reaction (reduced riboflavin → riboflavin). If a solution containing riboflavin and Na_2EDTA (pH 3–8) is irradiated until the solution is practically colourless, and concentrated HCl is then added to drop the pH below 1, the solution turns a red-orange colour. On standing the red-orange colour intensifies slightly and gradually returns to the yellow colour of riboflavin starting from the top of the tube and moving to the bottom as oxygen diffuses into the solution. We have been able, with some difficulty, to determine the absorption maximum for the red compound. Our approximate value of 480 mμ compares well with the value of 490 mμ reported by KUHN AND WAGNER-JAUREGG[6].

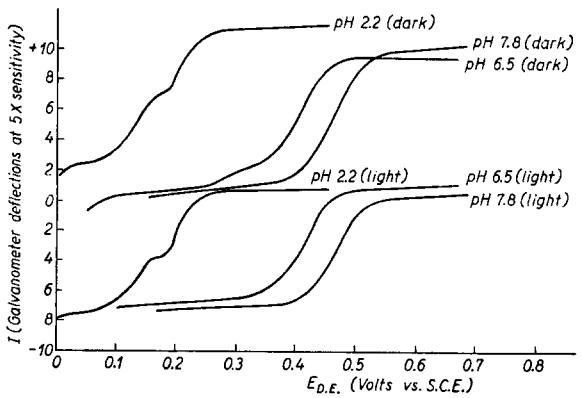

Fig. 3. Polarography of riboflavin and reduced riboflavin (several representative curves). Each solution contained 10^{-4} *M* riboflavin and 0.013 *M* Na_2EDTA in either phosphate buffer (pH 6.5) or glycine buffer (pH 2.2 and 7.8). Deoxygenated solutions of riboflavin were irradiated for 30 seconds with a 375 watt lamp, and a 100 watt Bausch and Lomb microscope lamp was shown on the solutions while the polarograms were being obtained. All determinations were made at 25° C with the realization that irradiation usually caused the temperature of the solutions to rise to 28–30° C by the end of a run. One drop of methyl red (0.5 %) was used as a maximum suppressor, and oxygen-free nitrogen was used to deoxygenate the solutions. Sat'd KCl/Hg_2Cl_2 reference electrode used.

From Fig. 3 it is clear that the polarographic reduction of riboflavin is reversible. Similar cathodic curves were obtained for solutions of riboflavin and riboflavin + Na_2EDTA. Irradiation of the solutions containing only riboflavin did not affect the polarographic wave, however, irradiation of a solution containing riboflavin and Na_2EDTA caused reduction of the riboflavin, and an anodic wave was obtained which had a wave height almost equal to that obtained for oxidized riboflavin (there is some loss of riboflavin during irradiation under these conditions because nitrogen must be bubbled through the solutions). The half-wave potential remained essentially the same (Fig. 3).

Plots of log $(i/i_d - i)$ *vs* $E_{d.e.}$* (KOLTHOFF AND LINGANE[9]) as tests for reversibility were made for most of the current-voltage curves obtained. The average of all the slopes was 0.071 volts with extremes of 0.045 and 0.095 volts (0.059 volts is the theoretical value for a one electron reduction). The values obtained indicate that the reaction is probably proceeding stepwise in one electron transfer. The variation obtained can probably be attributed to the same factors which cause the strange type of "prewave" observed in the current-voltage curves.

KAYE AND STONEHILL[10] have studied the polarographic reduction of some natural hydrogen carriers, including riboflavin. They reported that the polarographic reduction of $4 \cdot 10^{-4}$ *M* riboflavin in phosphate buffer at pH 7.38 gave a single wave with an index potential equivalent to a one electron transfer. Utilizing as the diffusion coefficient (*D*)

* i = current flowing at a particular applied potential ($E_{d.e.}$); i_d = limiting current or wave height. For an explanation of the symbols generally used in polarography see KOLTHOFF AND LINGANE[4,9].

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the value obtained by BREYER *et al.*[11] for acridine in borate/HCl buffer at pH 9 in the Ilkovic equation[12] ($i_d = 607nCD^{1/2}m^{2/3}t^{1/6}$), these authors obtained $n = 2$. Therefore, the reaction was assumed to proceed by 2 overlapping one electron-transfer steps. Using the value for $D^{1/2}$ ($2.75 \cdot 10^{-3}$) utilized by KAYE AND STONEHILL we obtain values for n ranging from 1.5–1.7. There is no assurance that the diffusion coefficient for acridine in borate buffer can reasonably be substituted for riboflavin in phosphate buffer with a substance (Na_2EDTA) which may be chelated with it. The value of n to the closest whole number is 2.

Assuming that $n = 2$ for the reduction, and with the following experimentally determined values: $m^{2/3} = 2.16$; $t^{1/6} = 1.2$; $C = 0.1$ mM/l; $i_d = 0.8$ microamps; $T = 25°$ C; 2.0 ml $M/15$ Na_2HPO_4–KH_2PO_4; pH 7.3; 2.0 ml of 0.05 M Na_2EDTA; total volume of 6.0 ml with distilled water, 1 drop methyl red as maximum suppression in 3.0 ml soln., we obtain the value $D^{1/2} = 2.58 \cdot 10^{-3}$.

With the evidence at hand we feel reasonably certain that the reduction of riboflavin is actually proceeding by 2 simultaneous one-electron reduction steps. These steps can only be separated at very low pH values where the life of the semiquinone is sufficiently long to give a detectable intermediate. At higher pH values the life of the semiquinone must be extremely short.

Metal-riboflavin complex. Neither the polarographic data nor the spectrophotometric data gave any indication of complex formation between riboflavin and Na_2EDTA, but since we have not been able to activate the photoreduction by first extracting the riboflavin and the dyes with 8-hydroxyquinoline and *o*-phenanthrolene in CCl_4 and $CHCl_3$, we are not yet ready to dismiss the possibility of complex formation. In addition to being inhibited by various metals (Fe^{+2}, Cu^{+2}, Zn^{+2}), the light reaction can be inhibited with 2,4-dinitrophenol, *p*-nitrobenzaldehyde or sodium azide. Inhibition by the latter can be reversed by increasing the amount of riboflavin. It seems likely that riboflavin may already be associated with a metal and a mixed chelate, riboflavin-metal-Na_2EDTA, is necessary for the photoreduction. This point will be clarified when metal-free riboflavin is obtained for starting material.

Photoreduction potentials. It is clear that riboflavin has two functions in the photochemical reactions described—one, absorbing light and, two, acting as an intermediate carrier of hydrogen and electrons for the reduction of dyes or other reducible substances placed in the system. To establish the limitations of riboflavin in this system, a series of dyes with different redox potentials was employed as hydrogen acceptors. Reduction was observed visually with adequate controls to insure that the disappearance of dye colour was not due to photolysis of the dye. Other hydrogen- or electron-acceptors which were found to be reduced by the light reaction included iodine, $HgCl_2$, and *o*-dinitrobenzene. A potential limit in the photoreduction with the riboflavin-Na_2EDTA system is met slightly below the redox potential of rosinduline 2B ($E_0' = -0.122$ v) which is reduced rather slowly. Phenosafranin ($E_0' = -0.252$ v) was not visibly reduced after 5 minutes irradiation with the 375 watt light. The limiting potential, as suspected, is close to the reduction potential of riboflavin.

Potentiometric measurements of the photoreduction reaction were made to define the limits of the reduction. The data shown in Fig. 4 were obtained on irradiating 50 ml of the given solution in Fisher titration jars; in some runs the system was deoxygenated by continuous flushing with oxygen-free nitrogen. Temperature corrections were made as the reaction proceeded. In the absence of Na_2EDTA and oxygen, riboflavin solutions

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irradiated under the above conditions produced a potential; however, under these conditions a large part of the riboflavin was irreversibly destroyed. Solutions containing riboflavin and Na_2EDTA which had not been deoxygenated developed the same potential as found in solutions which had been flushed with nitrogen. Aerated solutions containing only riboflavin did not develop a reduction potential when irradiated. This finding indicates that Na_2EDTA may combine with a light activated form of riboflavin thereby preventing loss of the activated compound by oxidation or dimerization.

In the absence of Na_2EDTA the spectrum of riboflavin phosphate has been found to change slightly (Fig. 5) upon irradiation. A closer study of this change may supply additional evidence for the formation of light activated riboflavin intermediates and the possible function of Na_2EDTA in extending the life of such active intermediates.

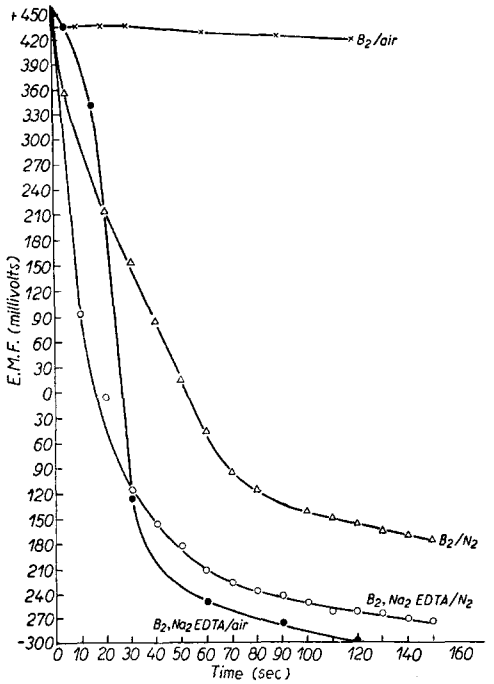

Fig. 4. Development of reduction potentials in irradiated systems. Measurements were made on 50 ml of solution containing $4 \cdot 10^{-5}$ *M* riboflavin in Fisher titration jars. The solutions were magnetically stirred. Those marked N_2 were flushed with oxygen-free nitrogen before and during the irradiation period; the other solutions were not deoxygenated.

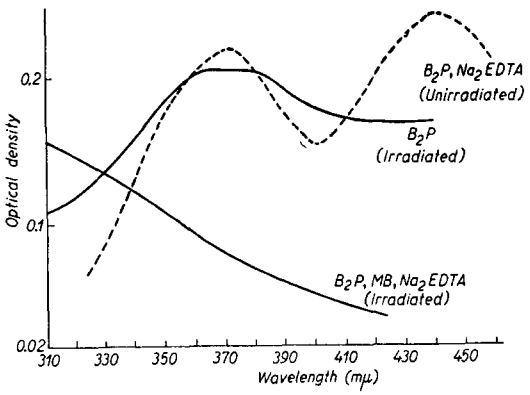

Fig. 5. Absorption spectra of riboflavin-5-phosphate and photoreduced riboflavin-5-phosphate.

Photocatalytic activity of riboflavin. The true capacity of riboflavin as a hydrogen carrier in the photoreduction system has not been determined. After one minute of irradiation with the 375 watt lamp, values for the amount of reduction per mole riboflavin have varied from 2:1 to 15:1, and in some instances higher ratios were obtained. Fig. 6 shows the rate of methylene blue reduction with a given amount of riboflavin and Na_2EDTA. TTC and $HgCl_2$ have been reduced in this light system but quantitative determinations were made difficult by the production of insoluble lightscattering particles upon reduction, and by the further breakdown of the reduced products.

In all the photoreductions investigated in the present study we were not able to demonstrate the production of gas, however, in the reductions involving hydrogen transfer, a small increase in the pH of the reaction medium was always noted. Studies are now being carried out to ascertain our belief that water is split in the photoreduction process.

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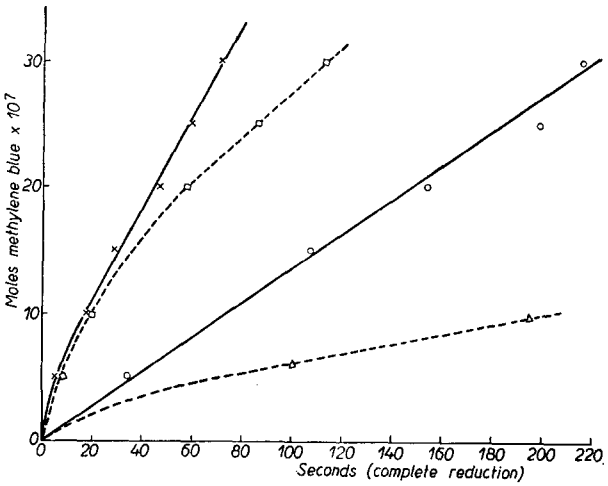

Fig. 6. Photoreduction of methylene blue. △ Methylene blue plus 0.005 *M* Na_2EDTA irradiated (375 watt) with a 4 cm $CuSO_4$ solution (2 %) interposed between the sample and the light source (a). ○ Methylene blue plus 0.005 *M* Na_2EDTA irradiated without a filter (b). ◇ (a) plus $4 \cdot 10^{-5}$ *M* riboflavin. × (b) plus $4 \cdot 10^{-5}$ *M* riboflavin. All solutions were irradiated in 16 mm Pyrex test tubes with oxygen-free nitrogen bubbled through the solution 1 minute prior to irradiation and during irradiation. Concentrations of methylene blue assume 100 % purity of the reagent (Merck, U.S.P., zinc-free).

DISCUSSION

Riboflavin apparently plays a dual role in the photoreduction system. Riboflavin absorbs light in the range 370–450 mμ and, in the presence of a suitable metal-chelating agent, is reduced to the leuco form. From the polarographic analyses, this reduction occurs in two one-electron steps. The reduced riboflavin then transfers its hydrogen and/or electrons to an oxidizing agent which lies within its potential range. The exact function of the metal-chelating agent in the photoreduction system has not yet been determined. By virtue of the strong metal-chelating tendencies of such substances as Na_2EDTA one might assume that a metal is involved in the photochemical reaction, or in the inhibition of the reaction. Exactly which metal and the function of the metal and metal-chelating agent are yet to be determined. Na_2EDTA can be substituted by other chelating agents such as cysteine, ethylenediamine, mercaptosuccinic (thiomalic)* and carboxymethylmercaptosuccinic acids (Evanacid 3CS)*. Considerable specificity is required of the metal chelator and our inability to activate riboflavin by first extracting with metal-complexing agents suggests that the chelator is involved directly in the reaction and does not function merely by removing a metal from riboflavin. The inhibitor studies also lend some substance to the argument that Na_2EDTA must combine with riboflavin (through a metal?) to produce a complex molecule that can be activated by light, or alternatively, Na_2EDTA might combine with a light-activated riboflavin molecule (metal present) allowing the activated molecule to be reduced. The results obtained for the photolysis of riboflavin and the inhibition of this destruction of riboflavin by Na_2EDTA would support the latter view. The relatively large amounts of Na_2EDTA

* Samples kindly supplied by Evans Chemetics, Inc., Waterloo, New York.

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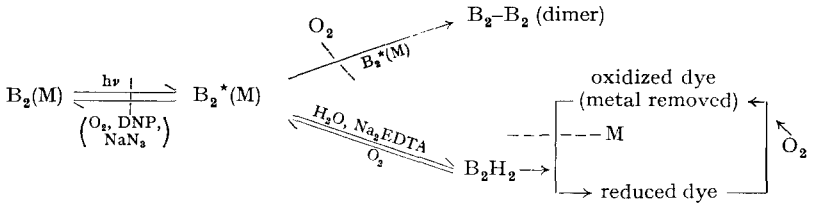
necessary for maximum reduction probably mean that some metals are also associated with the dyes and must be removed to permit maximum reduction.

To summarize these relationships we tentatively propose the following "working" scheme:



(B_2 = riboflavin, B_2^* = active intermediate, M = metal ion, DNP = 2,4-dinitrophenol)

Just what significance this reaction may have in biological systems remains to be shown. Certainly it seems that riboflavin should not be ignored when considering photobiological reductions. The overall abundance of riboflavin in biological substances, and the presence of many natural metal chelators which could serve to control these reactions, speaks in favour of riboflavin playing some role in light-activated reactions.

SUMMARY

1. In the presence of certain metal-chelating agents riboflavin was reversibly reduced by visible light. This reduction has been followed visually, spectrophotometrically and polarographically.

2. Riboflavin not only absorbs the light but is reduced in the process, and in turn can transfer its electrons to suitable hydrogen acceptors added to the system.

3. Polarographic data were used to establish the reversibility of the photoreduction system. Semiquinone formation was strongly suggested.

4. From the half-wave potential ($E_{1/2}$) for the polarographic reduction of riboflavin, the reduction potential at pH 7.3 was calculated to be —0.208 volts, and —0.194 volts at pH 7.0.

5. Oxygen inhibited the photoreduction of riboflavin and also protected the riboflavin from photolysis. Na_2EDTA not only protected riboflavin, but also promoted the photoreduction.

6. Certain metal ions, dinitrophenol, *p*-nitrobenzaldehyde, and sodium azide inhibited the photoreduction of riboflavin. This inhibition could be reversed by excess riboflavin.

7. Evidence points to a metal-mediated complex between riboflavin or light-activated riboflavin and the metal-chelating agent.

RÉSUMÉ

1. En présence de certains agents complexant les métaux par chélation, la riboflavine est réduite réversiblement par la lumière visible. Cette réduction est suivie par examen direct, par spectrophotométrie et par polarographie.

2. La riboflavine absorbe la lumière, se trouve réduite de ce fait et peut à son tour transférer ses électrons à des accepteurs d'hydrogène convenables ajoutés au système.

3. Les données polarographiques ont permis d'établir la réversibilité de la photoréduction. Il est vraisemblable qu'il se forme une semiquinone.

4. La valeur du potentiel de réduction, calculée à partir du potentiel ($E_{1/2}$) observé au cours de la réduction polarographique de la riboflavine, est de —0.208 volt à pH 7.3 et de —0.194 volt à pH 7.0.

5. L'oxygène inhibe la photoréduction de la riboflavine et protège en même temps la riboflavine contre la photolyse. Na_2EDTA protège la riboflavine et, en outre, provoque la photoréduction.

6. Certains ions métalliques, le dinitrophénol, la *p*-nitrobenzaldéhyde et l'azoture de sodium inhibent la photoréduction de la riboflavine. Cette inhibition peut être supprimée en présence d'un excès de riboflavine.

7. Les résultats obtenus permettent de supposer l'existence d'un complexe entre la riboflavine ou la riboflavine activée par la lumière et l'agent complexant, formé par l'intermédiaire du métal.

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ZUSAMMENFASSUNG

1. In Anwesenheit von gewissen Metall-Komplex-Bildern wird Riboflavin durch sichtbares Licht reversibel reduziert. Diese Reduktion wurde visuell spektrophotometrisch und polarographisch verfolgt.

2. Riboflavin absorbiert nicht nur Licht sondern wird auch gleichzeitig reduziert und umgekehrt kann es seinen Elektronen auf passende Wasserstoff-Acceptoren, die dem System zugesetzt werden, überführen.

3. Die Reversibilität des Photoreduktionssystems wurde polarographisch festgestellt. Die Bildung von Semichinon wurde als naheliegend erwogen.

4. Aus dem Halbwellenpotential ($E_{1/2}$) der polarographischen Reduktion des Riboflavin wurde das Reduktionspotential bei pH 7.3 zu —0.208 Volt und bei pH 7.0 zu —0.194 Volt berechnet.

5. Sauerstoff hemmt die Photoreduktion des Riboflavin und schützt auch das Riboflavin vor der Photolyse. Na_2EDTA schützt nicht nur Riboflavin aber fördert auch die Photoreduktion.

6. Gewisse Metall-Ionen, Dinitrophenol, *p*-Nitrobenzaldehyd und Natriumazid hemmen die Photoreduktion von Riboflavin. Diese Hemmung kann durch einen Überschuss von Riboflavin aufgehoben werden.

7. Anzeichen deuten auf einen Metall vermittelnden Komplex zwischen Riboflavin oder Licht aktiviertem Riboflavin und Metall-Komplex-bildner hin.

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