The Photochemical Oxidation of Ethylenediaminetetraacetic Acid and Methionine by Riboflavin[1]

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When air-free solutions of riboflavin are irradiated with a mercury arc lamp in the presence of an excess of EDTA, 1 mole of glyoxylic acid is formed per mole of riboflavin reduced. If methionine is substituted for the EDTA, 1 mole of 3-(methylthio)propionaldehyde is obtained. The stoichiometry of the reactions suggests that water is the source of the oxygen for the aldehydic oxidation products. Aerobic irradiation of EDTA–riboflavin solutions gives rise to mixtures of glyoxylic acid and formaldehyde. The photochemical production of the glyoxylic acid and 3-(methylthio)propionaldehyde was confirmed by the isolation of the 2,4-dinitrophenylhydrazones of the aldehydes from aerobically irradiated solutions.

Introduction

When air-free solutions of riboflavin containing ethylenediaminetetraacetic acid (EDTA)[2,3] or methionine[3,4] are exposed to light, the riboflavin is reduced without degradation and the EDTA and methionine are oxidized. The photooxidation products of EDTA in anaerobic solutions are not known, but, on the basis of the work of Frisell, *et al.*,[5] in aerobic solutions they are thought to include formaldehyde. According to Nickerson, *et al.*,[4] the photooxidation product of methionine in anaerobic solutions is methionine sulfoxide. In this investigation EDTA–riboflavin and methionine–riboflavin solutions were irradiated in the presence and absence of air. In the EDTA solutions, depending on experimental conditions, either glyoxylic acid or a mixture of glyoxylic acid and formaldehyde were found. In the methionine solutions 3-(methylthio)propionaldehyde and ammonia were found.

Experimental Section

Materials. Two lots of riboflavin were used, one from Mann Research Laboratories and the other (White Label grade) from Eastman. The values of the absorption maxima and the polarographic $E_{1/2}$ of both lots agreed with those given in the literature and therefore they were used without further purification. The nitrogen for deaerating the riboflavin solutions was freed of oxygen by passing it through a purification train consisting of three flasks containing acidified chromous chloride and amalgamated zinc and two flasks containing distilled water. The following chemicals, obtained from the indicated sources, were used as purchased: glyoxylic acid (reagent grade), K and K Laboratories; methionine sulfoxide, Nutritional Biochemicals; 3-(methylthio)propionaldehyde (White Label grade), Eastman; *dl*-methionine (B.D.H. grade), EDTA (Analar), and inorganic reagents (Analar), British Drug House.

Anaerobic Irradiation. The solutions were irradiated in a Pyrex cylinder, 3.5 cm. in diameter and 8.5 cm. in height and with a wall thickness of 2 mm. A gas dispersion tube was fitted in the wall of the cylinder near the bottom. Nitrogen was passed through the tube prior to irradiation to deaerate the solutions and during irradiation to provide agitation and to keep the solutions air free. The face of the light source, a General Electric H 100-SP4 mercury arc lamp, was placed 32 cm. from the center of the Pyrex cylinder. The solutions were exposed to the lamp until about 90% of the riboflavin was reduced. During the exposure which lasted from 1 to 3 min. the temperature of the solutions tended to increase. However, the temperature of the solutions was adjusted to 25° prior to irradiation and the temperature rise was less than 1°.

Polarographic Analysis. The solutions were analyzed by lowering a dropping mercury electrode and a KCl agar salt bridge into the Pyrex cylinder. With this arrangement it was possible to analyze the solutions which had been anaerobically irradiated without exposing them to air. During the analysis the temperature of the solutions was held at 25 ± 0.1° and the solutions were kept air free by sweeping the surface with a stream of nitrogen. The analyses of the irradiated solutions were made at the pH of irradiation or at 11.7. To raise the pH of the solutions to 11.7 a predetermined volume of 1.5 *N* KOH was added. In the case of the anaerobically irradiated solutions deaerated KOH was used.

Production and Isolation of Glyoxylic Acid. Six 500-ml. flasks, each containing 400 ml. of an EDTA–riboflavin solution, were irradiated for 6 to 8 hr. with a General Electric AH-4 100-w. mercury arc lamp. The solutions were 0.266 m*M* in riboflavin and 0.1 *M* in Na_2EDTA with the pH adjusted to about 4.5 with hydrochloric acid. The flasks were placed about the lamp in a circle 30 cm. in radius. During irradiation air was bubbled through the solutions to provide oxygen and agitation. The temperature of the solutions ranged between 25 and 30°.

After irradiation the solutions contained a mixture of glyoxylic acid and formaldehyde in a molar ratio of about 1:1. The contents of the flasks were combined and the pH was lowered to 1.9 by the addition of concentrated HCl to remove the unreacted EDTA by precipitation. After filtration the solution was heated on

(1) Based in part on a dissertation submitted by K. Enns to the University of Toronto in partial fulfillment of the requirements for the Ph.D. degree, May 1963.

(2) (a) J. R. Merkel and W. J. Nickerson, *Biochim. Biophys. Acta*, 14, 303 (1954); (b) B. Holmström and G. Oster, *J. Am. Chem. Soc.*, 83, 1867 (1961).

(3) W. M. Moore, J. T. Spence, F. A. Raymond, and S. D. Colson, *ibid.*, 85, 3367 (1963).

(4) W. J. Nickerson and G. Strauss, *ibid.*, 82, 5007 (1960).

(5) W. R. Frisell, C. W. Chung, and C. G. Mackenzie, *J. Biol. Chem.*, 234, 1297 (1959).

a steam bath under reduced pressure to reduce the volume and distil the formaldehyde. The solution was then cooled in an ice bath and a saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl was added with stirring. The precipitated hydrazone of glyoxylic acid was purified by recrystallization from a 20% methanol solution. A total of six recrystallizations was required before the melting point range was reduced to less than 1°.

Production and Isolation of 3-(Methylthio)propionaldehyde and Detection of Ammonia. A solution which was 0.3 m*M* in riboflavin and 0.05 *M* in *dl*-methionine was exposed in an 1-l. flask to a General Electric H 100 SP4 lamp at a distance of 30 cm. for 3 to 4 hr. During irradiation air was bubbled slowly through the solution and the temperature ranged between 25 and 30°. After irradiation the 3-(methylthio)propionaldehyde was precipitated from the solution as the 2,4-dinitrophenylhydrazone. The hydrazone was purified by two recrystallizations from 95% ethanol.

To demonstrate the formation of ammonia, 1 l. of solution was irradiated in the absence of air until most of the riboflavin was reduced. The solution was then acidified and vacuum distilled to remove the methional. The residue was made alkaline and distilled into a solution of Nessler's reagent which gave a positive test.

Results and Discussion

EDTA Solutions. Figure 1 shows three polarograms of an EDTA–riboflavin solution. Polarogram 1 was obtained before irradiation and polarogram 2 after a 75-sec. exposure to the mercury arc lamp. The polarograms show that the riboflavin is reduced without degradation and that a new, but small wave with an $E_{1/2}$ of −1.34 v. is formed. Polarogram 3 was obtained after raising the pH of the irradiated solution from 7.8 to 11.7. The adjustment in pH produces a pronounced increase in the height of the wave and shifts the $E_{1/2}$ to −1.36 v.

At first it was thought that the new wave was due to formaldehyde. Frisell, *et al.*,[5] investigated the photochemical reaction between riboflavin and a number of amino acids, including EDTA. When they irradiated an EDTA–riboflavin solution in the presence of air, they found that oxygen was consumed and formaldehyde was formed in an approximately 1:1 ratio. However, when formaldehyde was added to our irradiated solution and the solution polarographically analyzed at pH 11.7, the formaldehyde produced a wave with an $E_{1/2}$ of −1.64 v. Although the new wave could not be attributed to formaldehyde, the addition of $NaHSO_3$ or NH_2OH caused a decrease in its height, indicating that it was due to an aldehyde. The presence of acetic acid groups in EDTA suggested that the aldehyde might be glyoxylic acid. Figure 2 shows two polarograms of an irradiated EDTA–riboflavin solution. The first was obtained after adjusting the pH to 11.7 and the second after adding glyoxylic acid. It is seen that the height of the wave is increased without change in the $E_{1/2}$ or the general shape.

To confirm the formation of glyoxylic acid, a relatively large amount of the photoproduct was prepared by aerobic irradiation. The photoproduct was isolated

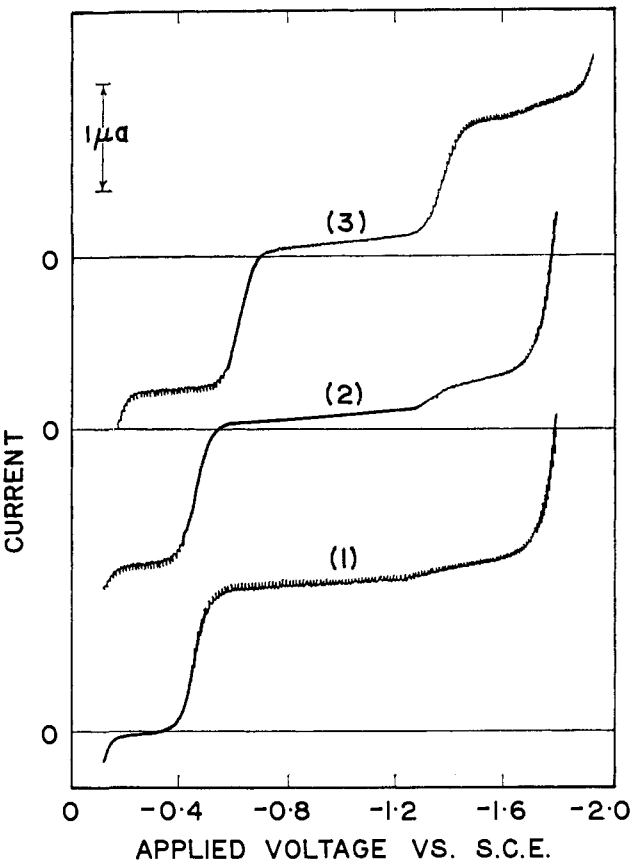

Figure 1. Polarograms of a solution 0.266 m*M* in riboflavin, 5.32 m*M* in EDTA, 0.1 *M* in KCl, 0.08 *M* in phosphate, pH 7.8: (1) before irradiation, (2) after a 75-sec. irradiation, (3) after adjusting pH to 11.7.

as the 2,4-dinitrophenylhydrazone from a solution irradiated at pH 4.5. Table I gives the melting points of the hydrazone of the photoproduct and the authentic glyoxylic acid and of mixtures of the two. Figure 3 compares the infrared absorption spectra of the hydrazones of the photoproduct and of the authentic glyoxylic acid. It is clear that glyoxylic acid is a photooxidation product of EDTA.

Table I. Melting Points of the 2,4-Dinitrophenylhydrazone of the Photoproduct and of Authentic Glyoxylic Acid

	M.p. dec., °C. (uncor.)
Authentic glyoxylic acid	184.1–184.7
Photoproduct	183.6–184.5
Mixtures	
50% glyoxylic acid	183.4–184.5
33% glyoxylic acid	183.4–184.3

Table II shows that in the absence of air and in the presence of an excess of EDTA 1 mole of glyoxylic acid is formed per mole of riboflavin reduced. Under these conditions the over-all reaction appears to be

$$Rf + EDTA + H_2O \longrightarrow RfH_2 + H{-}\overset{O}{\overset{\|}{C}}{-}\overset{O}{\overset{\|}{C}}{-}OH + \underset{\text{EDTA residue}}{}$$

The stoichiometry suggests that water supplies the oxygen for the formation of the glyoxylic acid. There

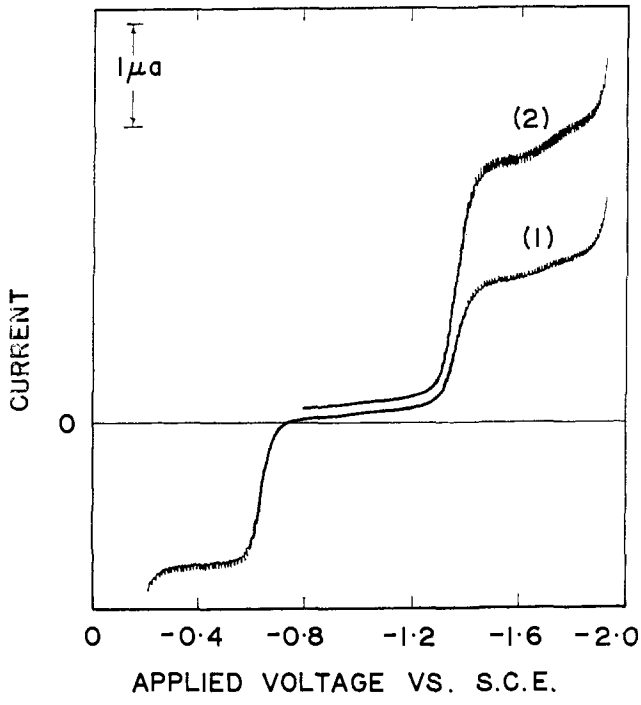

Figure 2. Polarograms of a solution 0.266 mM in riboflavin, 5.32 mM in EDTA, 0.1 M in KCl, 0.08 M in phosphate: (1) after a 75-sec. irradiation at pH 7.8 and adjusting pH to 11.7, (2) after adding authentic glyoxylic acid.

is other evidence that water takes part in reactions of this type. Goodspeed, *et al.*,[6] have studied the photochemical reduction of methylene blue by diethylglycine and found that 1 mole of formaldehyde, carbon dioxide, and diethylamine are formed per mole of methylene blue reduced. They therefore concluded that

Table II. Anaerobically Irradiated[a] EDTA–Riboflavin Solutions[b]

Expt.	EDTA concn., mM	Riboflavin photoreduced,[c] %	Molar ratio glyoxylic acid/reduced riboflavin
1	1.33	95.7	0.97
2	1.86	89.4	1.05
3	3.99	100	0.98
4	5.32	96.5	1.01

[a] Irradiation duration 75 sec.; conditions described in section on Anaerobic Irradiation. [b] Solutions: 0.266 mM in riboflavin, 0.1 M in KCl, 0.08 M in phosphate, pH 7.8. [c] Solutions were analyzed polarographically at pH 11.7. There was no polarographic evidence of any photodegradation of riboflavin. All of the riboflavin was recovered on oxidation with air.

water must be a reactant. It has been reported[7] that the anaerobic photochemical oxidation of N-benzyl-N,N′-dimethylethylenediamine by riboflavin yields benzaldehyde and that water supplies the oxygen.

It is impossible to tell from the stoichiometry whether or not water is involved in the key or rate-determining step. Strauss and Nickerson[4,8] postulate that a riboflavin–H_2O–EDTA complex is broken in the key step with the water donating two hydrogen atoms to the riboflavin and an oxygen atom to the EDTA. However, other workers[3,9,10] who have investigated the photochemistry of the EDTA–riboflavin system are of the opinion that water does not play a role in the primary process. Moore[11] has studied the rate of the photochemical reduction of riboflavin by EDTA in water and in deuterium oxide and found no kinetic isotope effect. This suggests that water is probably not involved in the rate-determining step. Holmström[10] has discovered that semiquinones of riboflavin appear as intermediates in the photochemical reduction and postulates that the primary process is an electron transfer from the EDTA to the riboflavin, producing the semiquinone.

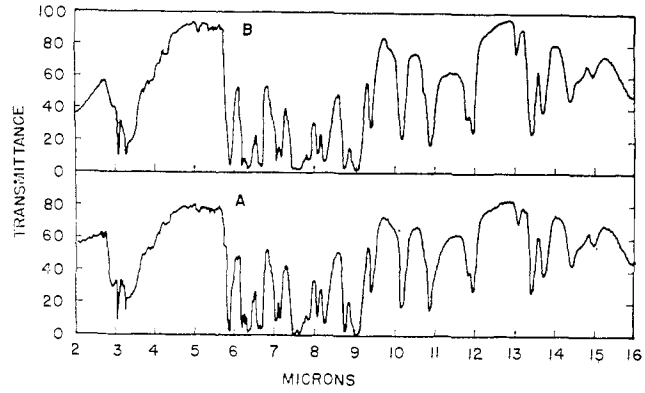

Figure 3. Infrared absorption spectra in KBr of the 2,4-dinitrophenylhydrazone of authentic glyoxylic acid (A) and of the photoproduct (B).

Goodspeed, *et al.*,[6] have proposed a mechanism to explain the photochemical reduction of methylene blue by diethylglycine and EDTA. The first step consists of the transfer of an electron from OH^- to the dye. The resulting dye semiquinone abstracts hydrogen from the α-carbon atom of the amino acid which in turn undergoes decarboxylation to produce an amine radical. The amine radical is then oxidized by OH or H_2O_2. This mechanism has the virtue of fitting the established stoichiometry of the diethylglycine–methylene blue reaction and accounts for the fact that the rate of the EDTA–methylene blue reaction in water and in deuterium oxide is essentially the same.[6] With a slight modification the mechanism could be applied to the EDTA–riboflavin reaction. However, the evidence that H_2O_2[12] is involved in reactions of this type is not clear-cut,[9] and as Goodspeed, *et al.*,[6] state it is not certain that the first step is energetically favorable.

In their investigation of the oxidation products of the EDTA–methylene blue reaction, Goodspeed, *et al.*,[6] found formaldehyde, carbon dioxide, and a nonvolatile aldehyde. Enns[13] has obtained polarographic evidence that glyoxylic acid is an oxidation product in this photochemical reaction. It is possible that the nonvolatile aldehyde is glyoxylic acid.

Table III shows that prolonged irradiation of EDTA–riboflavin solutions in the presence of air yields mix-

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(7) K. Enns and W. H. Burgess, *J. Am. Chem. Soc.*, **87**, 1822 (1965).
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(11) W. M. Moore, Utah State University, personal communication.
(12) L. P. Vernon, *Biochim. Biophys. Acta*, **36**, 177 (1959).
(13) K. Enns, Ph.D. Thesis, University of Toronto, May 1963.

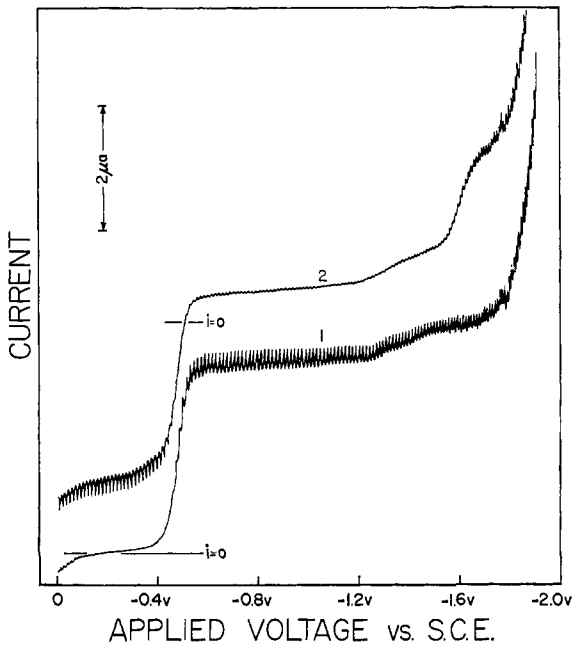

Figure. 4. Polarograms of a solution 0.3 m*M* in riboflavin, 6.0 m*M* in *dl*-methionine, 0.1 *M* in KCl, 0.1 *M* in phosphate, pH 7.8: (1) before irradiation, (2) after a 3-min. irradiation.

tures of formaldehyde and glyoxylic acid and that the accumulation of glyoxylic acid decreases markedly with increasing pH. These puzzling results were obtained when a preliminary investigation was made to determine the optimum pH for the production of glyoxylic acid by aerobic irradiation. The reason for the production of the formaldehyde is not known. However, it may be due in part to reactions between riboflavin and EDTA residues which presumably include secondary amines containing acetic acid groups. There is evidence that the photochemical oxidation of such secondary amines yields formaldehyde. Using carbon-14 Frisell, *et al.*,[5] made a careful study of the photochemical oxidation of sarcosine by riboflavin phosphate in the presence of air. They observed that oxygen and sarcosine were consumed and methylamine, formaldehyde, and carbon dioxide were produced on an approximately mole for mole basis.

Table III. Aerobically Irradiated[a] EDTA–Riboflavin Solutions

pH[b]	Glyoxylic acid,[c] m*M*	Formaldehyde,[c] m*M*
4.5	0.92	0.73
6.0	0.59	0.79
7.8	0.37	1.62
9.5	0.08	2.14

[a] Irradiation conditions are described in section on Production and Isolation of Glyoxylic Acid. [b] Buffer 0.08 *M*, Britton and Robinson (O. H. Müller, "The Polarographic Method of Analysis," 2nd Ed., Chemical Education Publishing Co., Easton, Pa., 1951, p. 194). [c] Polarographically analyzed at pH 11.7.

The data in Table III are not a reliable measure of the absolute or relative amounts of the two aldehydes produced at the various pH values. Air saturated with water was bubbled through the solutions to provide oxygen and as a result an unknown quantity

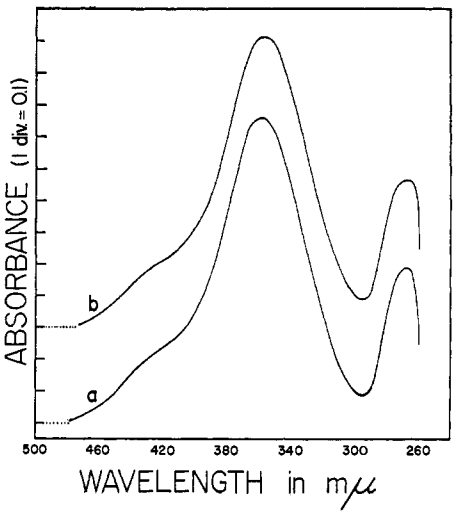

Figure 5. Ultraviolet absorption spectra of the 2,4-dinitrophenylhydrazone of authentic methional (a) and of the photoproduct (b) in 1,1,2-trichloroethane: concentration, 0.0150 g./cc.; light path, 1 cm.

of formaldehyde was swept away. The low accumulation of glyoxylic acid at high pH values may be due to low production, but it may also be due to its destruction by hydrogen peroxide. Hydrogen peroxide is produced by the reaction between reduced riboflavin and air[5] and is capable of oxidizing glyoxylic acid in EDTA–riboflavin solutions.[13] Crude kinetic data[14] obtained with the polarograph indicate that the rate of this oxidation increases rapidly with pH.

Methionine Solutions. Figure 4 shows polarograms of a methionine–riboflavin solution before and after irradiation in the absence of air. It is seen that about 90% of the riboflavin is reduced without photodegradation and that a new wave with an $E_{1/2}$ of -1.60 v. is formed. It was first assumed that the new wave was due to methionine sulfoxide, the reported oxidation product of methionine.[4] However, when methionine sulfoxide was added to an unirradiated methionine–riboflavin solution, no new wave was obtained. Patton[15] in a paper overlooked by workers in the field of riboflavin photochemistry reported the production of 3-(methylthio)propionaldehyde (methional) in methionine–riboflavin solutions which had been exposed to sunlight in the presence of air. Hence methional was added to an anaerobically irradiated EDTA–methionine solution. It was discovered that the height of the new wave was increased without changing the $E_{1/2}$ or shape.

To verify the production of methional a relatively large quantity of the photoproduct was prepared and isolated as the 2,4-dinitrophenylhydrazone. Figure 5 compares the ultraviolet absorption spectra of the hydrazones of the photoproduct and authentic methional. The infrared absorption spectra of the two hydrazones also match closely. Analysis of the polarograms of anaerobically irradiated solutions (Table IV) indicates that 1 mole of methional is formed per mole of riboflavin reduced. The presence of ammonia was qualitatively demonstrated. Therefore the over-all equation for the photochemical oxidation of methio-

(14) K. Enns, unpublished data.
(15) S. Patton, *J. Dairy Sci.*, 37, 446 (1954).

Table IV. Anaerobically Irradiated[a] Methionine–Riboflavin Solutions[b]

Expt.	Riboflavin photoreduced,[c] %	Molar ratio methional/ reduced riboflavin
1	88.3	1.02
2	89.3	1.06
3	87.7	1.01
4	90.0	1.04

[a] Irradiation duration 3 min.; conditions described in section on Anaerobic Irradiation. [b] Solutions: 0.3 m*M* in riboflavin, 6.0 m*M* in methionine, 0.1 *M* in KCl, 0.1 *M* in phosphate, pH 7.8. [c] Solutions were analyzed polarographically at pH 7.8. There was no polarographic evidence of any photodegradation of riboflavin. All of the riboflavin was recovered on oxidation with air.

nine by riboflavin in air-free solutions appears to be

$$Rf + CH_3SCH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCO_2H + H_2O \longrightarrow RfH_2 + CH_3SCH_2CH_2CHO + NH_3 + CO_2$$

As in the case of the EDTA–riboflavin reaction the stoichiometry suggests that water is the source of the oxygen for the aldehyde formation.

It is not known why Patton[15] and we appear to obtain methional and Nickerson and Strauss[4] methionine sulfoxide. Since the polarographic technique does not detect methionine sulfoxide in the methionine–riboflavin solutions, there is no positive evidence that it was not produced. However, under our experimental conditions where about 1 mole of methional is obtained per mole of riboflavin reduced, it seems that very little, if any, methionine sulfoxide is formed.

It is interesting to note that the behavior of methionine is unusual in that it is easily photooxidized by riboflavin, whereas primary amines in general are not.[5] The unusual behavior is probably due to the presence of the sulfur atom, but its role in this reaction is not understood.

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The Role of Substituents in the Hydrophobic Bonding of Phenols by Serum and Mitochondrial Proteins

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The adsorption of 19 phenols by bovine serum albumin was studied. It was found that binding depends on the lipophilic character of the substituent and that a linear free-energy relationship exists between the logarithm of the binding constants and substituent π. It was also shown that the hydrophobic binding constant π can be used to study the mechanism of the uncoupling of phosphorylation in mitochondria.

Introduction

The binding of organic compounds by proteins and, in particular, serum albumin has received extensive attention from theoretical as well as practical points of view.[1-3] In our own work[4] on the analysis of the activity of a series of penicillin derivatives we came to the conclusion that the adsorption of the penicillins to serum albumin could be rationalized using substituent constants. In particular, it was shown that using a substituent constant, π, to estimate the hydrophobic bonding of functions such as Cl, NO_2, etc., the activity of the penicillins could be explained in terms of their binding capacity for serum albumin. We have defined[5,6] π as $\pi = \log P_X/P_H$, where P_H is the partition coefficient of a parent compound between octanol and water and P_X is that of the derivative X. Thus, π is the logarithm of the partition coefficient of a function and, as such, represents the free energy of transfer of the substituent from an aqueous phase to a lipophilic phase.

More recently we have found[7] that using π and the Hammett constant σ it is possible to rationalize the highly specific binding of a congeneric series of substrates to enzymes. In fact, π appears to be an extremely useful parameter for many biochemical and pharmacological problems.[8]

The purpose of the work in this report was to investigate a simpler system than the above mentioned in which more careful control of the variables would be possible. For this purpose we chose to study the adsorption of phenols by bovine serum albumin (BSA). Phenols were chosen because of their well-known tendency to bind to protein, because of their ease of spectrophotometric determination, and because an extensive series of π constants are available.[5] The adsorption of 19 monosubstituted phenols by BSA was measured using the equilibrium dialysis technique of Klotz.[9]

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(9) D. Glick, *Methods Biochem. Analy.*, 3, 265 (1956).