Cleavage of 4,8-Dimethyl-6-methoxy-1,2-dihydronaphthalene (17a).—A mixture of 1.68 g. of the ether **17a**[18] and 18 g. of freshly prepared anhydrous pyridine hydrochloride was maintained at 240° under an atmosphere of nitrogen for 2 hr. Isolation as described above gave 1.35 g. of an amber oil, which was chromatographed on 50 g. of silica gel. Elution with 2.5 l. of 1:1 benzene–hexane gave 0.99 g. (65% yield) of colorless needles which was shown by gas chromatographic analysis to be a 1:1 mixture of 4,8-dimethyl-5,6,7,8-tetrahydro-2-naphthol (**3b**) and 4,8-dimethyl-2-naphthol (**18**).[18] Recrystallization from ether–hexane gave 304 mg. of naphthol **18** as colorless needles, m.p. 152–154°. The phenol **3b** was separated from a portion of the mother liquors by gas chromatography to give colorless compact needle clusters, m.p. 94–95° after final recrystallization from hexane. The melting point was unchanged on admixture with the material obtained from rearrangement of the dienone **1b** in acetic anhydride as described above.

Dehydrogenation of 4,8-Dimethyl-5,6,7,8-tetrahydro-2-naphthol (3b).—Following the general procedure of Wenkert and Dave,[33] 500 mg. of 4,8-dimethyl-5,6,7,8-tetrahydro-2-naphthol obtained from the acetic anhydride–sulfuric acid rearrangement of the dienone **1b**, as described in part A above, was heated under reflux with 50 ml. of *p*-cymene and 200 mg. of 10% palladium-on-charcoal in a nitrogen atmosphere for 76 hr. The solvent was removed by steam distillation and the resulting aqueous residue in the distilling flask (approximately 250 ml.) was passed through a paper filter while still hot. On cooling, the aqueous solution yielded 86 mg. of **4,8-dimethyl-2-naphthol (18)**[18] as colorless fluffy needles, m.p. 153–154°. Recrystallization from benzene–hexane failed to change the melting point. Admixture with a portion of the material obtained from the dihydronaphthyl ether **17a** as described above gave a melting point of 152–153°; reported[18] for **18**: m.p. 151.5–152.5.

Anal. Calcd. for $C_{12}H_{12}O$: C, 83.69; H, 7.02. Found: C, 83.3; H, 7.1.

Acknowledgments.—The author is particularly indebted to Dr. T. J. Flautt for help with the interpretation and calculation of n.m.r. spectra and to Drs. W. F. Erman and C. D. Broaddus for helpful discussions during the course of the work.

(33) E. Wenkert and K. G. Dave, *J. Am. Chem. Soc.*, **84**, 94 (1962).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND BIOPHYSICS, IOWA STATE UNIVERSITY, AMES, IOWA]

The Photochemical Degradation of Riboflavin[1,2]

BY EDDIE C. SMITH[3] AND DAVID E. METZLER

RECEIVED MAY 3, 1963

Anaerobic photobleaching of riboflavin followed by reoxidation with air is shown by means of microbiological assays and thin layer chromatography to produce a mixture of flavins including unchanged riboflavin, lumiflavin, lumichrome, and two additional compounds. The major new product has been identified as 6,7-dimethyl-9-formylmethylisoalloxazine by means of its chromatographic behavior and chemical reactions.

Introduction

When riboflavin is illuminated anaerobically the yellow color fades and the isoalloxazine ring becomes reduced.[4,5] Since riboflavin is known to induce the photooxidation of a variety of compounds, it is reasonable to assume with the early workers in the field that the anaerobic fading of riboflavin results from photooxidation of its own ribityl side chain. Although a considerable body of evidence, recently reviewed by Oster, *et al.*,[6] supports this idea, some workers have proposed that the hydrogen for the reduction of the isoalloxazine ring comes from the cleavage of water.[7–10] With these two contradictory views in mind, we have performed several experiments which help to clarify the nature of the anaerobic photolysis of riboflavin.

Experimental

Materials.—Riboflavin (6,7-dimethyl-9-(D-1'-ribityl)-isoalloxazine), a gift from Merck & Co., was recrystallized twice from 2 *N* acetic acid and was then extracted with chloroform to remove the lumichrome impurity. Lumiflavin was synthesized by the method of Hemmerich, *et al.*[11] Lumichrome was prepared by the photolysis of riboflavin in 50% methanol–H_2O solution by sunlight.[12] The method of Fall and Petering[13] was used to prepare 6,7-dimethyl-9-formylmethylisoalloxazine and 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine from riboflavin. Riboflavin assay medium was obtained from Difco Laboratories. Silica gel G and silica gel (less than 0.08 mm.) were obtained from E. Merck AG, Darmstadt, Germany. Freshly boiled glass-redistilled water with a specific conductance of 2.2×10^{-6} ohm^{-1} was used.

Apparatus.—All spectral measurements were made on a Beckman DU spectrophotometer which had a special cell compartment cover to accommodate the cells used for anaerobic measurements. These cells consisted of two pieces connected by a 14/20 standard tapered joint. The base piece was made by sealing a 7-cm. length of Pyrex tubing to a standard 1-cm. Pyrex cuvette and the top section was made by sealing a 3-cm. piece of 12-mm. Pyrex tubing containing a medium sintered-glass filter to a stopcock. The function of the sintered-glass filter was to prevent loss of solution through "bumping" during evacuation. The Desaga thin layer chromatography apparatus was used in the preparation of chromatography plates.

Thin Layer Chromatography.—Silica gel G plates (20 cm. by 20 cm.) of 250 μ thickness were used. Normally about 10^{-3} micromole of flavin (10 μl. of 10^{-4} *M* solution) was applied at spots 1 cm. apart on the plate. Two solvent systems were used: butanol (7):ethanol (2):water (1) and water saturated with isoamyl alcohol.[9] The spots were detected after chromatography by their blue or yellow-green fluorescence under an ultraviolet light.

Microbiological Assay.—The procedure of Snell and Strong[14] based on the titration of acid produced during the growth of *Lactobacillus casei* was employed.

Anaerobic Photolysis.—A 10^{-4} *M* solution of riboflavin was placed in a modified Thunberg tube and evacuated, first with a water aspirator for 15 min. and then with a mechanical pump for the same length of time. During the evacuation the tubes were shaken vigorously several times. The solution was illuminated with a 15-watt Sylvania daylight type fluorescent lamp placed approximately 23 cm. from the sample for 16–24 hr. The photoreduction and subsequent oxidation, when oxygen was admitted, were followed spectrophotometrically at 445 mμ. For larger samples up to 2 l. in volume, a special apparatus was constructed which consisted of two 3-l. round-bottom flasks connected with 300 cm. of 1-cm. Pyrex tubing, coiled in such a way that the light source could be placed directly above it. After a 10^{-4} *M* riboflavin solution was placed in flask A, the whole apparatus was evacuated, by means of a mechanical vacuum pump, for 45 min. The solution then was forced through the coils into flask B by 1 atm. of nitrogen (purified by passing over hot copper and stored in a 12-l. reservoir). The desired amount of photobleaching could be accomplished in 2 ways. First, the flow rate of the solution through the coils could be controlled by introducing several sintered glass filters at the end of the coils and, secondly, by

(1) Supported by grant No. G-12339 from the National Science Foundation.

(2) A preliminary report appeared in *Federation Proc.*, **22**, 591 (1963).

(3) Procter and Gamble Fellow, September, 1961–May, 1962.

(4) R. Kuhn, H. Rudy, and Th. Wagner-Jauregg, *Ber.*, **66**, 1950 (1933).

(5) B. Holmström and G. Oster, *J. Am. Chem. Soc.*, **83**, 1867 (1961).

(6) G. Oster, J. S. Bellin, and B. Holmström, *Experientia*, **18**, 249 (1962).

(7) J. R. Merkel and W. J. Nickerson, *Biochim. Biophys. Acta*, **14**, 303 (1954).

(8) L. P. Vernon, *ibid.*, **36**, 177 (1959).

(9) W. J. Nickerson and G. Strauss, *J. Am. Chem. Soc.*, **82**, 5007 (1960).

(10) G. Strauss and W. J. Nickerson, *ibid.*, **83**, 3187 (1961).

(11) P. Hemmerich, S. Fallab, and H. Erlenmeyer, *Helv. Chim. Acta*, **39**, 1242 (1956).

(12) P. Karrer, H. Salomon, K. Schöpp, F. Schlitter, and H. Fritzsche, *ibid.*, **17**, 1010 (1934).

(13) H. H. Fall and H. G. Petering, *J. Am. Chem. Soc.*, **78**, 377 (1956).

(14) E. E. Snell and F. M. Strong, *Ind. Eng. Chem., Anal. Ed.*, **11**, 346 (1939).

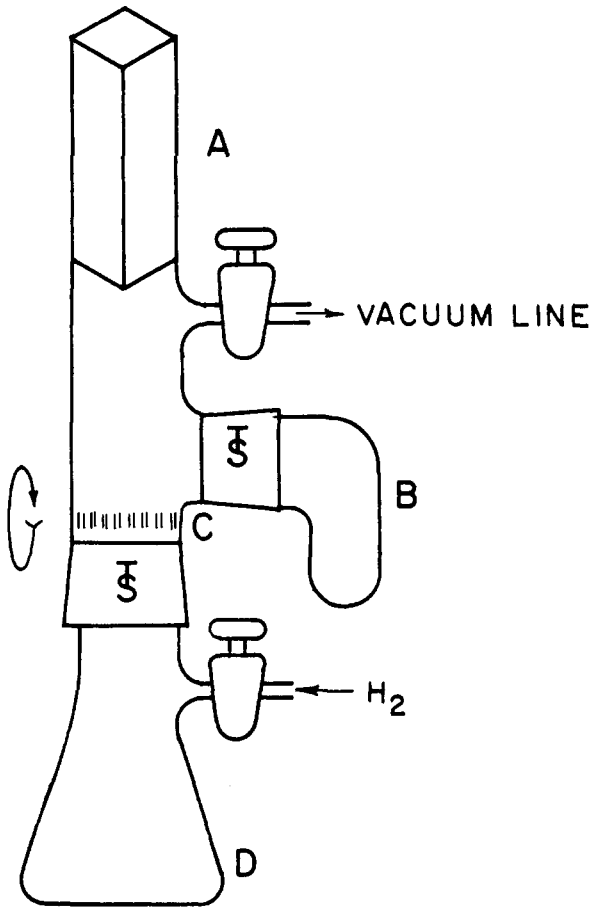


Fig. 1.—Microhydrogenation apparatus for dihydroriboflavin preparation. Riboflavin solution is placed with palladium black in flask D and stirred with magnetic stirrer. After evacuation hydrogen is admitted and rapid hydrogenation occurs. Apparatus is then inverted and solution is filtered through use of vacuum into 1-cm. width spectrophotometer cell A. Palladium black remains on sintered glass filter C. Side arm B permits subsequent addition of other reagents.

placing a stopcock at the end of the coils the bleaching could be accomplished in successive portions. After illumination the flavin solution was evaporated to dryness under vacuum at 50° and prepared for column chromatography. The preparation of the sample and silica gel (less than 0.08 mm.) and the packing of the column was based on the technique described by Dahn and Fuchs[15] except that instead of using a horizontal cellulose tube for the column, a 32 mm. by 300 mm. length of Pyrex tubing held vertically was employed. The eluent was the same butanol–ethanol–water mixture used for the thin layer chromatography. After the first band had moved the length of the column, all the bands were separated mechanically and extracted from the silica gel with water.

Leucoflavin (Dihydroriboflavin) Preparation.—A 2×10^{-5} *M* solution of riboflavin was placed in the flask of a special hydrogenation apparatus (Fig. 1)[16] with a trace of palladium black and a magnetic stirring bar. While stirring the solution, the system was evacuated and then flushed with hydrogen. After hydrogenation the apparatus was inverted and the solution was filtered into the spectrophotometer cell A. That the riboflavin was reduced could be deduced from the decrease in optical density at 445 mμ. This fell from about 0.22 for riboflavin to 0.010 after prolonged hydrogenation. Higher concentrations of riboflavin could not be used since leucoflavin has an extremely low solubility (about 2×10^{-5} *M*) and would be trapped by the filter if present in higher amounts. The lack of complete reoxidation of the hydrogenated riboflavin (Table II) may be the result of further reduction of some of the leucoflavin to forms which are not readily reoxidizable.

Results

Photobleaching Curve.—Figure 2 shows the typical curve obtained when the anaerobic photobleaching of a

(15) W. H. Dahn and H. Fuchs, *Helv. Chim. Acta*, **45**, 261 (1962).
(16) D. O. Carr, Ph.D. Thesis, Iowa State University, 1960.

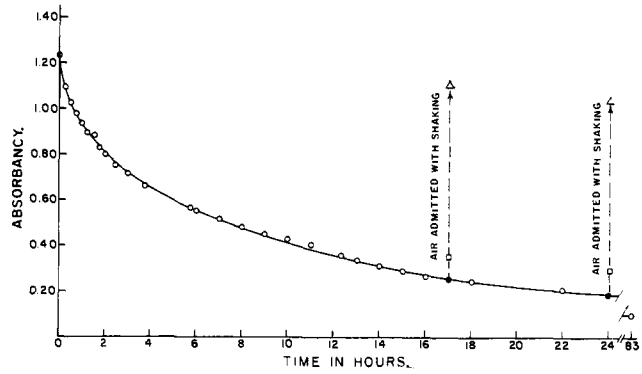


Fig. 2.—Graph of absorbancy at 445 mμ *vs.* time during anaerobic photolysis of a 10^{-4} *M* solution of riboflavin (○). Samples (●) upon reoxidation by shaking in air regained most of the original absorbancy (△). However, microbiological assay showed that actual effective riboflavin concentration (□) was much lower.

riboflavin solution is followed at 445 mμ. This curve agrees fully with that shown by Holmström and Oster[5] and with the descriptions of Strauss and Nickerson.[9] When air was admitted to the partially bleached solution, reoxidation of the isoalloxazine ring occurred as was indicated by the partial return of the yellow color. The amount of color restored depended on the time of illumination, as is also indicated in Fig. 2. After 83 hr. the optical density at 445 mμ had dropped to 0.046. Upon longer illumination the optical density did not change appreciably, and it was assumed that after 83 hr. photoreduction was complete. When samples were removed after 17 and 24 hr., respectively, 89% and 84% of the color was restored upon admission of air.[17]

Lumiflavin, 6,7,9-trimethylisoalloxazine, on the other hand, is very slowly photobleached, to the extent of only 9.0% in 17 hr. (in contrast to 81% for riboflavin) and about 13% in 24 hr. Admission of air to a sample bleached for 24 hr. resulted in no recovery of color. When illuminated in the presence of 1 *M* ethanol or glycerol, however, lumiflavin was rapidly bleached.[16]

Microbiological Activity.—On the 17- and 24-hr. samples of photobleached riboflavin, microbiological assays were run in order to determine whether or not riboflavin was re-formed when the photoreduced samples were oxidized (Table I). The amount of

TABLE I
MICROBIOLOGICAL ASSAY OF REOXIDIZED PHOTOREDUCED RIBOFLAVIN

	Per cent[a]	
	17-hr. sample	24-hr. sample
Riboflavin photoreduced[b]	81	87
Original flavin color restored	89	84
Microbiological activity expected[c]	89	84
Riboflavin found by microbiological assay	29 ± 5	24 ± 4

[a] Percentages are based on a value of 100% for the original unbleached riboflavin solution. [b] From Fig. 2, assuming that after 83 hr. photoreduction was complete. [c] Assuming that all the color restored represents riboflavin.

growth-promoting activity clearly indicated that most of the yellow color restored upon reoxidation did not represent riboflavin. This finding is in agreement with the experiments of Theorell[18] in which reoxidation of

(17) An entirely similar photobleaching and reoxidation was obtained using more intense light sources and shorter exposure times. For example, irradiation of a sample in a water-cooled cuvet with a General Electric No. 2 photoflood incandescent bulb at a distance of 7–15 cm. produced a 50–66% bleaching of the flavin in 5 min. Admission of oxygen restored the 445 mμ absorption to 78–86% of the original. The product distribution observed with thin layer chromatography was identical with that reported here for samples irradiated with a fluorescent lamp.
(18) H. Theorell, *Biochem. Z.*, **279**, 186 (1935).

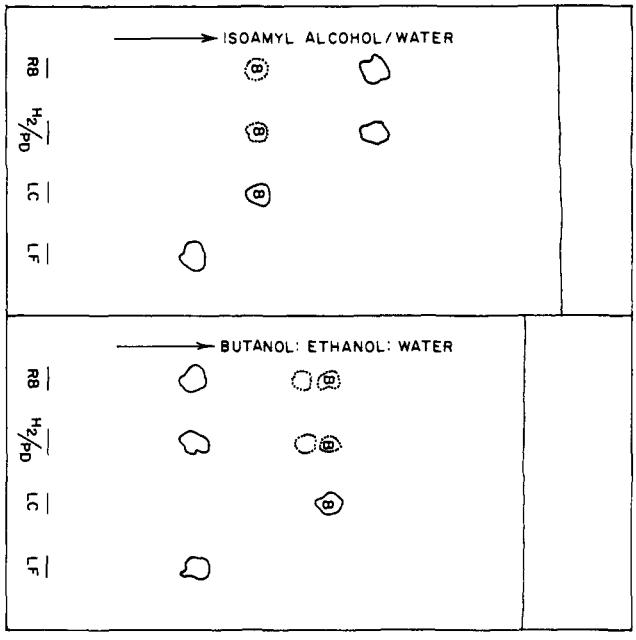


Fig. 3.—Thin layer chromatography of reoxidized leucoflavin prepared by catalytic hydrogenation; solvents: on the left, water saturated with isoamyl alcohol and on the right, butanol (7):ethanol (2):water (1); Rb = riboflavin standard; H_2/Pd = reoxidized leucoflavin prepared by catalytic hydrogenation; Lc = lumichrome; Lf = lumiflavin; B = blue fluorescence. The very faint spots (◌) appeared only when the chromatography plate was spotted much more heavily than usual.

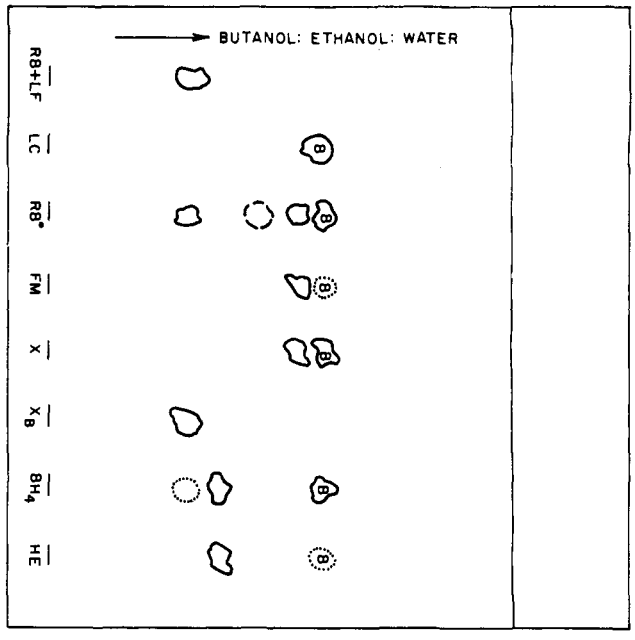


Fig. 4.—Thin layer chromatography showing presence of 6,7-dimethyl-9-formylmethylisoalloxazine (FM) as an anaerobic photolysis product of riboflavin (Rb); solvent = butanol (7):ethanol (2):water (1); Lf = lumiflavin; Lc = lumichrome; Rb' = anaerobically photobleached and reoxidized Rb; FM = 6,7-dimethyl-9-formylmethylisoalloxazine standard. X = unknown compound isolated by column chromatography; X_B = X treated with 2 *N* NaOH; BH_4 = X after $NaBH_4$ treatment; HE = 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine; B = blue fluorescence (other spots fluoresce yellow-green). The (◌), (◌), and (○) indicate increasing fluorescence in that order.

photoreduced riboflavin phosphate produced a flavin which was no longer active as a coenzyme for "old yellow enzyme."

Microbiological assays were also run on air-oxidized leucoflavin prepared by catalytic hydrogenation (Table II). These results verified that leucoflavin is reoxidized to microbiologically active riboflavin and that the photoreduced flavin is for the most part not leucoflavin.

TABLE II

MICROBIOLOGICAL ASSAY OF OXIDIZED LEUCOFLAVIN, PREPARED CHEMICALLY

	Per cent[a]	
	Sample 1	Sample 2
Riboflavin reduced by H_2/Pd[b]	76	80
Original color restored	74	69
Microbiological activity expected[c]	74	69
Riboflavin by microbiological activity	70 ± 8	61 ± 10

[a] Percentages are based on a value of 100% for the original unbleached riboflavin solution. [b] Assuming an optical density for leucoflavin of 0.010. [c] Assuming that all the color restored is caused by riboflavin.

Thin Layer Chromatography.—Chromatography on silica gel plates further verified the conclusions drawn from the microbiological assays. Figure 3 shows typical chromatograms which establish that reoxidized leucoflavin solutions contain no compounds other than riboflavin and trace impurities (lumichrome plus one unknown flavin) which are present in the original riboflavin solution as well.

On the other hand, chromatography of the anaerobically photobleached and reoxidized riboflavin in the butanol:ethanol:water solvent showed four spots (Fig. 4). Two of these corresponded both in the color of their fluorescence and in R_f values to lumichrome and to lumiflavin and/or riboflavin. The other two spots represent unknown photolysis products, the major product (R_f value 0.54) being designated X and the minor one (R_f 0.45), Y. This solvent system did not separate lumiflavin from riboflavin, but chromatography in water saturated with isoamyl alcohol showed that both compounds were present. With this latter solvent system chromatograms contained three spots corresponding to lumiflavin, lumichrome, and riboflavin. Unknown spot X moved with the same R_f value as lumichrome. Strauss and Nickerson[9] obtained similar results using the isoamyl alcohol–water solvent system for paper chromatography with one minor difference. On paper lumiflavin moves faster than lumichrome while the reverse is true on thin layer plates. It was observed that the unknown compound X had the same R_f value as 6,7-dimethyl-9-formylmethylisoalloxazine in both solvent systems.

Separation and Identification of Photolysis Products.—In order to verify the thin layer chromatography results, larger samples (up to 2 l. of 10^{-4} *M* solutions) were illuminated and then chromatographed on silica gel columns with the butanol:ethanol:water solvent. While separation was not as good as on thin layer plates, four bands appeared corresponding to the four spots of the thin layer chromatograms. Attention was focused on unknown compounds X and Y. From 75 mg. of photolyzed flavin placed on a column, about 10 molar % estimated spectrophotometrically of compound X, contaminated with a small amount of lumichrome and about 1 molar % of compound Y, was recovered.

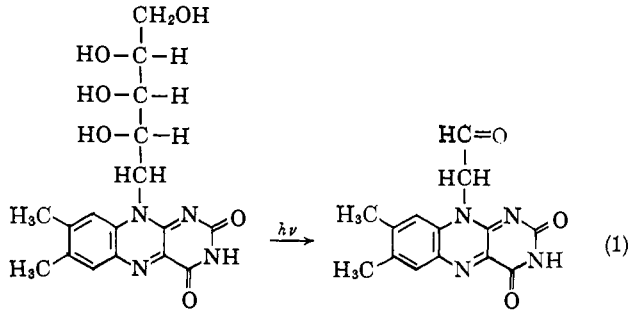
Despite persistent attempts it has not yet been possible to obtain a pure, crystalline sample of compound X. However, the following experiments indicate that compound X is 6,7-dimethyl-9-formylmethylisoalloxazine. In addition to possessing the correct R_f values in two chromatography solvents, as previously mentioned, when reduced with sodium borohydride it was almost quantitatively converted to a flavin which corresponded in R_f value to 6,7-dimethyl-9-(2'-hydroxyethyl)-iso-

alloxazine (Fig. 4). Upon treatment (in the dark) with 2 *N* NaOH both compound X and authentic 6,7-dimethyl-9-formylmethylisoalloxazine are converted to a compound corresponding to lumiflavin in R_f value (Fig. 4). Furthermore, both compound X and the authentic 9-formylmethyl flavin undergo a similar anaerobic photobleaching which is much more rapid than that of riboflavin. The reoxidation product from this photobleaching is exclusively lumichrome (in acid or neutral aqueous solution).

Protonation of the isoalloxazine ring of flavins results in a distinct spectral change by means of which the pK_a of the conjugate acid maybe determined. The pK_a value for authentic 6,7-dimethyl-9-formylmethylisoalloxazine is 3.5, three pK units higher than those of other flavins.[19] We have determined the pK_a for compound X spectrophotometrically as 3.46 ± 0.08, providing further proof of its identity to 6,7-dimethyl-9-formylmethylisoalloxazine.

Discussion

The results of microbiological assays and thin layer chromatography verify that anaerobically photobleached riboflavin is not dihydroriboflavin (leucoflavin) but a mixture of flavins including riboflavin itself, lumichrome, and two unknown compounds. The major unknown component which accounts for about 10% of the total flavin has been identified as 6,7-dimethyl-9-formylmethylisoalloxazine (eq. 1).



riboflavin (1)

This finding is in agreement with the conclusions of Kocent[20] that glyceraldehyde and glycolaldehyde are the products of side-chain cleavage. He also postulated the existence of the 9-formylmethyl flavin as an intermediate giving rise to glycolaldehyde and lumichrome during flavin photolysis. This compound meets the description of "deuteroflavin," the riboflavin oxidation product postulated by Kuhn as the precursor of lumiflavin in basic solution.

The very slow bleaching of lumiflavin alone and its rapidity in the presence of alcohol together with the microbiological assays and chromatographic evidence lend credence to the idea that the initial photochemical reaction of riboflavin consists of the oxidation of one of the alcohol groups of the ribityl side chain. Whether or not this results in a single step in cleavage to yield glyceraldehyde and 6,7-dimethyl-9-formylmethylisoalloxazine remains uncertain. These results do not offer any support for the proposal that water is cleaved during the photoreduction.

The presence of a certain amount of genuine dihydroriboflavin in the photobleached riboflavin solution is evident from both the microbiological assays and the chromatography. This can be understood if we assume that any reduced flavin with modified side chain, $F'H_2$, could react rapidly by an exchange reaction with riboflavin, F.

$$F'H_2 + F \longrightarrow F' + FH_2 \qquad (2)$$

Dihydroriboflavin so produced is "trapped" in the reduced state and is no longer as sensitive to light. Depending upon the equilibrium constant for the exchange reaction, a greater or lesser amount of dihydroriboflavin could be trapped in the reduced state.

Work is in progress in an attempt to identify the second minor photolysis product and to elucidate the mechanisms of the photolysis reactions.

Acknowledgments.—We gratefully acknowledge the able assistance of Martha L. McDonald.

(19) C. H. Suelter and D. E. Metzler, *Biochim. Biophys. Acta*, **44**, 23 (1960).

(20) A. Kocent, *Chem. Listy*, **47**, 195 (1953).

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO., KALAMAZOO, MICH.]

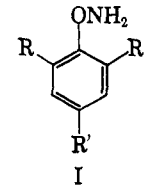
Dihydroazepinone Chemistry. II.[1a] Mechanistic Considerations of the Formation and Acid Hydrolysis of the 1,3-Dihydro-2H-azepin-2-ones

BY LEO A. PAQUETTE[1b]

RECEIVED MAY 3, 1963

The one-step preparation of 1,3-dihydro-2H-azepin-2-ones by ring expansion of appropriate sodiophenoxides with chloramine and methylchloramine is discussed. Evidence is presented to suggest that the ring enlargement proceeds by initial C-alkylation of the ambident phenoxide ion to give an amino dienone which undergoes thermal rearrangement to give the observed products. Acid hydrolysis of the dihydroazepinones affords novel dihydro-2(3H)-furanones. The mechanisms of these reactions are discussed.

Because of our recent activity in various aspects of hydroxylamine chemistry, including the preparation and rearrangement of aminooxy compounds,[2] we noted with more than casual interest the recently published communications of Theilacker and co-workers[3-5] describing the preparation of O-arylhydroxylamines (I) *via* the reaction of sodio-2,6- and 2,4,6-alkyl-substituted phenoxides with chloramine. The resulting O-arylhydroxyamines (I) were described as colorless, stable, highly crystalline solids, capable of distillation without change,



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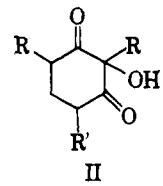

II

but incapable of condensation with aromatic aldehydes; in addition, they were not acetylatable and, in the

(1) (a) For a preliminary account of this work see L. A. Paquette, *J. Am. Chem. Soc.*, **84**, 4987 (1962); (b) Department of Chemistry, The Ohio State University, Columbus 10, O.

(2) L. A. Paquette, *Tetrahedron Letters*, No. **11**, 485 (1962), and other papers in this series to be published.

(3) W. Theilacker and E. Wegner, *Angew. Chem.*, **72**, 127 (1960); for a correction of their earlier structural assignments, see W. Theilacker, K. Ebke, L. Seidl, and S. Schwerin, *ibid.*, **75**, 208 (1963).

(4) W. Theilacker, *ibid.*, **72**, 498 (1960).

(5) K. Ebke, Ph.D. Dissertation, University of Hannover, Germany, 1959.