evaporation and sublimation (130° at 0.05 mm.), white crystals (0.3 mg.) of m.p. 162–163° were obtained. The melting point remained unchanged upon admixture of authentic 1-carbomethoxy-β-carboline. R_f values and ultraviolet and mass spectra were identical with those of 1-carbomethoxy-β-carboline.

Stability Test of Tuboflavine. The alkaloid was dissolved in a mixture of chloroform and methanol; aqueous ammonium hydroxide solution, silicic acid, and alumina (Woelm, neutral) were added. This mixture was allowed to stand at room temperature for 4 weeks with occasional shaking. After this time on separation and esterification, no 1-carbomethoxy-β-carboline could be found.

Photochemical Degradation of Flavins. II. The Mechanism of Alkaline Hydrolysis of 6,7-Dimethyl-9-formylmethylisoalloxazine[1,2]

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The alkaline degradation of 6,7-dimethyl-9-formylmethylisoalloxazine (FMF), an intermediate in the photolysis of riboflavin, was studied kinetically. The reaction followed pseudo-first-order kinetics to give lumiflavin with a less significant competing reaction yielding lumichrome. A mechanism of the carbon–carbon bond cleavage in the alkaline cleavage of FMF has been proposed on the basis of the kinetics and product identification. The significance of the reaction is pointed out in connection with the photolysis of riboflavin.

Introduction

Anaerobic photolysis of a solution of riboflavin with visible light leads to reduction of the isoalloxazine ring with the production of a hypothetical "leuco-deuteroflavin."[3,4] Subsequent aeration causes a return of the yellow color of oxidized flavins and the presumed formation of "deuteroflavin." Recently, Smith and Metzler have isolated from a photobleached and reoxidized riboflavin solution the compound 6,7-dimethyl-9-formylmethylisoalloxazine[1] (9-formylmethylflavin, FMF) which possesses all of the chemical characteristics of the postulated "deuteroflavin," including a faster rate of photobleaching and a rapid conversion to lumiflavin in basic solutions.[5] The present communication is concerned with the mechanism of the alkaline hydrolysis of FMF, an important reaction in the photochemical degradation of riboflavin.

(1) Part I: E. C. Smith and D. E. Metzler, *J. Am. Chem. Soc.*, **85**, 3285 (1963).

(2) Supported by Grant No. G-12339 from the National Science Foundation. Abbreviations used throughout this paper: RF, riboflavin; FMF, 9-formylmethylflavin; LF, lumiflavin; LC, lumichrome.

(3) R. Kuhn, H. Rudy, and T. Wagner-Jauregg, *Ber.*, **66**, 1150 (1933).

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

(5) The term "deuteroflavin" is no longer adequate because the 2'-keto derivative of riboflavin which also fits the description of "deuteroflavin" has been isolated by Terao [M. Terao, *Tohoku Igaku Zassi*, **59**, 441 (1959)].

Experimental Section

Materials. FMF (6,7-dimethyl-9-formylmethylisoalloxazine) was made in this laboratory.[1] All the inorganic compounds used were analytical reagent grade obtained from Mallinckrodt Chemical Co. Silica Gel G for thin-layer chromatography was obtained from E. Merck, A.G., and thin-layer plates were prepared as previously reported.[1]

Kinetic Measurements. The rate of disappearance of FMF in borate buffer and unbuffered solutions in the dark was followed by measuring the decrease in absorbance at 445 mμ under various conditions of pH and ionic strength (μ) using a Cary Model 15 recording spectrophotometer. It must be noted that, since the spectrum of LF shows a marked dependence on temperature, probably due to a complexation equilibrium of LF (unpublished observation), it is important to maintain the same temperature for making spectral measurements of reaction mixture and LF solution. All measurements were made at 25 ± 1°. The pH of reaction mixtures was measured by the Beckman pH meter and the desired pH was obtained with 0.1 or 0.2 N NaOH solution. The amount of NaOH solution to bring about the desired pH was predetermined. The kinetic measurements were then followed immediately after adjusting the pH of the solutinos. The pH of the reaction mixture during the reaction was nearly unchanged both in buffered and unbuffered solutions. The pH of the reaction mixture in unbuffered solution at the end of the reaction was slightly lower.

Identification of Products. In addition to thin-layer chromatography which was employed as previously described,[1] 1 l. of alkali-treated reaction mixture (about 1 × 10⁻⁴ mole/l. of FMF) was extracted three–five times with about 200 ml. of chloroform, and the chloroform extracts were evaporated. The residue was then dissolved in about 200 ml. of water and extracted with an equal volume of chloroform. The chloroform
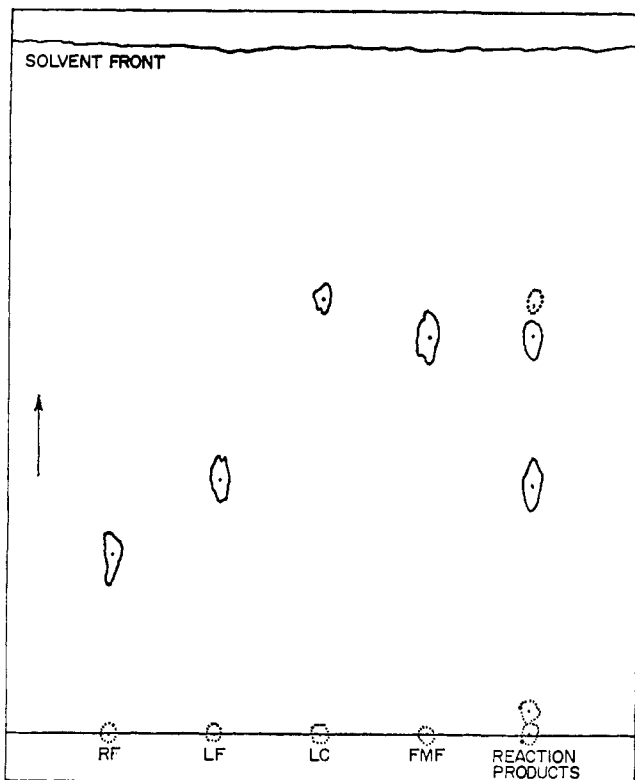
Figure 1. Thin-layer chromatogram of synthetic compounds and of 9-formylmethylflavin after standing 1 hr. in a solution of pH 9.9 (spots on the far right of the chromatogram). Developing solvent: 1-butanol–ethanol–water, 7:2:1.

Figure 2. Absorption spectra of 9-formylmethylflavin (7.9 × 10⁻⁵ mole/l.) as a function of time following addition of 0.1 N NaOH to adjust pH at 11.1. Broken line is the spectrum of the product lumiflavin assuming 100% conversion from FMF.

extract was evaporated to dryness in a rotary evaporator at 50°. The residue was sublimed under vacuum and was then subjected to infrared and n.m.r. spectrometry. Comparison of the infrared spectrum of this sample with that of standard LF in KBr pellets showed them to be identical. N.m.r. spectra of standard LF and of the product sample in CF_3COOH were also identical (n.m.r. measurement at 60 Mc.; peaks at 162 and 170 c.p.s. for 6-, 7-CH_3, 277 c.p.s. for N-CH_3, 493 and 503 c.p.s. for aromatic protons with integrated intensity ratio 3:3:3:1:1 for standard LF; peaks at 161 and 169 c.p.s. for 6-, 7-CH_3, 276 c.p.s. for N-CH_3, 491 and 501 c.p.s. for aromatic protons with intensity ratio 3:3:3:1:1 for the sample).

Attempts were also made to identify the side-chain products from the cleavage of FMF. Using a colorimetric phenylhydrazone method we failed to detect formaldehyde as a possible product. Carbon dioxide also could not be detected using the Warburg apparatus.

A major product from the side chain was found to be formic acid. Identification of this compound was accomplished as follows: FMF solution (5 l., ca. 1 × 10⁻⁴ mole/l.) was adjusted to pH 8 and held for ca. 4 hr. in the dark. After treatment with 30 g. of Norit to remove flavins the filtrate was concentrated to 5 ml. in a rotary evaporator at 30°. In another analysis, 3 l. of Norit-treated reaction mixture was extracted with 500 ml. of ether, and the ether extract was concentrated. Both concentrates were then subjected to (a) a qualitative test for formic acid (reduction of Hg^{2+} by formate) reported by Hopton[6] and (b) chromatography of the hydroxamate derivatives with

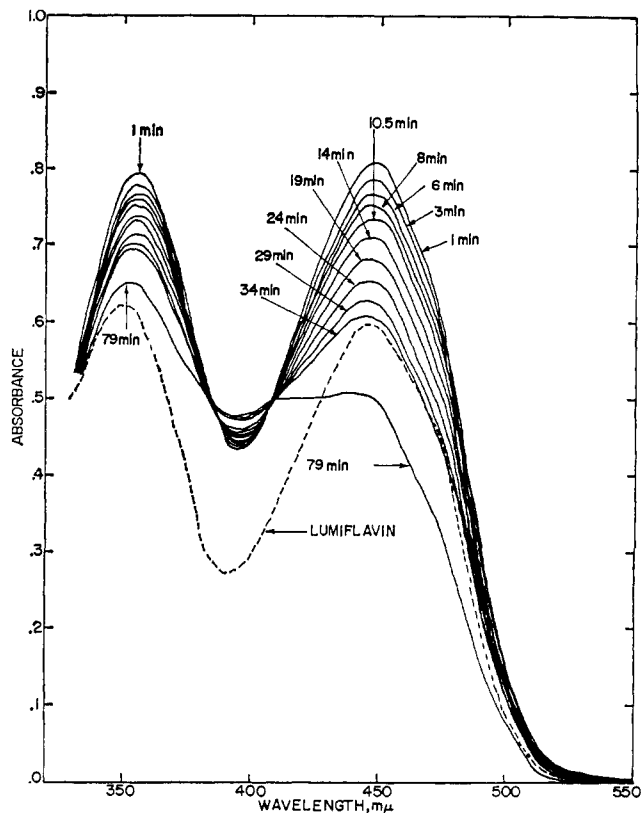(6) J. W. Hopton, Anal. Chim. Acta, 8, 429 (1953).

standard formic acid hydroxamate (R_f 0.28 on Whatman No. 1 paper, 0.458 on Whatman No. 3 in n-amyl alcohol–formic acid–water, 75:25:75, and 0.45 on thin-layer plate in 1-butanol–ethanol–water, 7:2:1). A semiquantitative determination of formic acid hydroxamate from the eluate of the paper chromatograms was spectrophotometrically made by the procedure given by Block, et al.[7] Both standard and sample hydroxamate spots on the paper chromatograms were eluted with 5 ml. of water and their absorbances were read at 500 mμ.

A minor product, glycolaldehyde, was identified as its 2,4-dinitrophenylhydrazone on thin-layer chromatography (R_f 0.718 in 1-butanol–ethanol–water, 7:2:1) according to the procedure of Block, et al.[7]

Results and Discussion

Products Formed by the Action of Base on 9-Formylmethylflavin. It has been previously shown that the action of 2 N sodium hydroxide upon 6,7-dimethyl-9-formylmethylisoalloxazine (FMF) leads to the appearance of a compound which behaves like lumiflavin upon thin-layer chromatography[1] in several solvent systems. We have now shown that under a variety of alkaline conditions this flavin arises as the single major product, and we have proved it to be lumiflavin by isolation and use of n.m.r. and infrared spectroscopy (see Experimental Section). Figure 1 shows the appearance of a thin-layer chromatogram of a solution of FMF

(7) R. J. Block, E. L. Durrum, and G. Zweig, "Paper Chromatography and Paper Electrophoresis," 2nd Ed., Academic Press Inc., New York, N. Y., 1958.

which had been allowed to stand for 1 hr. at pH 9.9 at room temperature. Fluorescent spots were observed corresponding to unreacted FMF, to lumiflavin (LF, the major product), and to lumichrome (LC, a minor product) which has characteristic blue fluorescence. A very small spot was also present near the origin. The chromatographic identity of the products was established by comparing with synthetic lumiflavin and lumichrome. It was also observed, as judged by the intensity of the fluorescent spots on the chromatograms, that the formation of LC decreases with increasing pH.

The major product arising from the side chain of FMF was identified as formic acid. In one experiment formic acid was detected in about 45% yield after 1 hr. at pH 9.9. This analysis was conducted on the same solution used for the chromatograms shown in Figure 1. No carbon dioxide production could be detected, and the reaction was shown not to depend upon the presence of oxygen.

A small amount of glycolaldehyde was detected. It seems likely that this compound arises concomitantly with the small amount of lumichrome which is revealed on the chromatograms.

Kinetic Studies. When FMF was placed in an alkaline solution, a rapid change in the absorption spectrum took place with a decrease in the peak height at 445 mμ (Figure 2). We assume that the initial decrease in absorbance at 445 mμ results mainly from the conversion of FMF to LF, the latter having lower extinction coefficient (7.82×10^3) than the former (1.05×10^4) at pH 11.1, although minor products other than LF, such as LC, were undoubtedly formed in lesser amounts during later stages. It is apparent from Figure 2 that the spectrum of the reaction mixture after 2 hr. resembles qualitatively that of LF except in the region between 370 and 430 mμ. This is also the region in which LC absorbs strongly. Thin-layer chromatography also showed both LF and LC, but with a predominant quantity of the former. It is to be noted that alkaline degradation of the isoalloxazine ring system may also occur to some extent at this later stage of the reaction.

Plots of decrease in absorbance *vs.* time showed that the 445 mμ absorption decreased by a first-order process, although some deviation from linearity was observed in the later portion of the plot. Therefore, rate constants were calculated from the slope of the linear portion.

It is found that the rate of disappearance of FMF is first order with respect to (OH$^-$), up to pH 12, as shown by linearity of the plot of log k *vs.* pH. It turns out also (see Figure 3) that the reaction is not general base catalyzed but is specific lyate ion catalyzed, since the effect of changing the buffer concentration was merely that of the ionic strength effect at higher μ. In accordance with the extended Debye–Hückel theory the rate appears to depend upon the activity coefficient of a reacting species with neutral (FMF) and negative (OH$^-$) charges (the reaction was not affected by μ at much lower concentrations of ions).[8] The over-all reaction was kinetically irreversible.

From the above results we can describe the major reaction by means of

(8) K. J. Laidler, "Chemical Kinetics," McGraw Hill Book Co., Inc., New York, N. Y., 1950. p. 131.
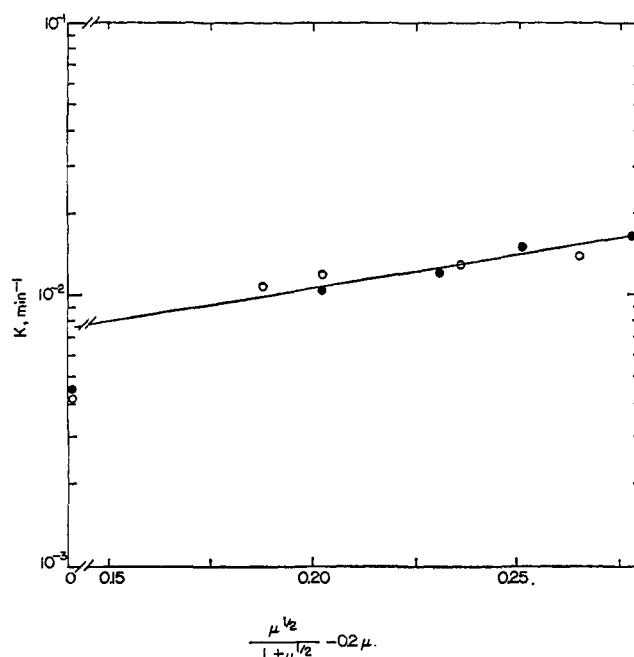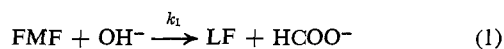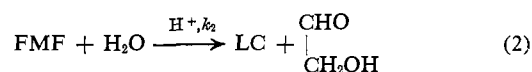

$$\frac{\mu^{1/2}}{1+\mu^{1/2}} - 0.2\mu.$$

Figure 3. Plot of the observed rate constant k against [$\sqrt{\mu}/(1 + \sqrt{\mu})] - 0.2\mu$ according to the extended Debye–Hückel equation. (●), μ calculated from concentration of Clark's borate buffer at pH 10.8; (O), μ calculated from KCl added to unbuffered solution at pH 10.9.

$$\text{FMF} + \text{OH}^- \xrightarrow{k_1} \text{LF} + \text{HCOO}^- \tag{1}$$

and competing reactions by

$$\text{FMF} + \text{H}_2\text{O} \xrightarrow{\text{H}^+,k_2} \text{LC} + \begin{array}{l} \text{CHO} \\ | \\ \text{CH}_2\text{OH} \end{array} \tag{2}$$

The rate of the disappearance of FMF in an alkaline solution can be expressed by

$$\frac{-\text{d(FMF)}}{\text{d}t} = \frac{k_1 K_w}{(\text{H}^+)}(\text{FMF}) + \frac{\text{d(LC)}}{\text{d}t} \cong k(\text{FMF}) \tag{3}$$

where K_w is the dissociation constant of water.

Since the rate of lumichrome production, d(LC)/dt, is low, and since its dependence upon pH has not been studied quantitatively, we have neglected this term in order to arrive at a value of k_1.[9] From dependence of k on pH, k_1 is estimated to be 6.9×10^{-2} mole^{-1} sec.$^{-1}$ at pH 10.9 and $\mu \simeq 0$.
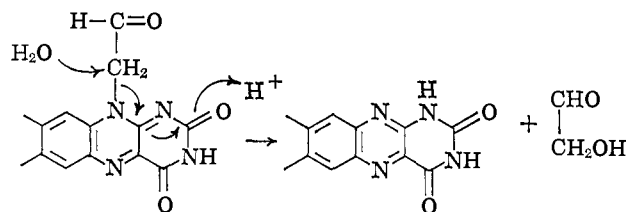
The mechanism of the reactions may be visualized as

+HCOO$^-$
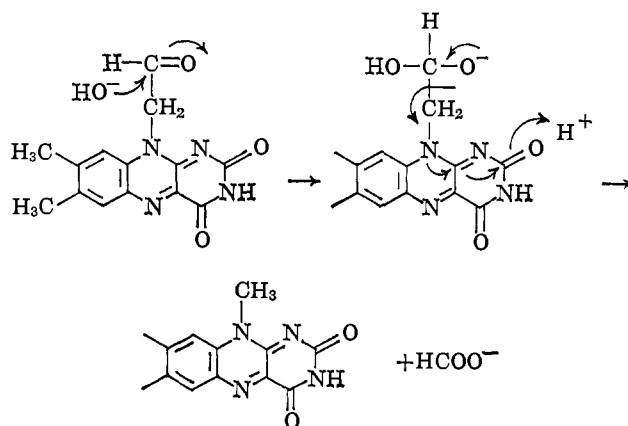
(9) See Appendix.

This alkaline cleavage reaction is quite analogous to the alkaline hydrolysis of β-diketones reported by Pearson and co-workers.[10,11]

The second and quantitatively less important reaction is the production of lumichrome according to eq. 2:

In a preliminary work, we found formic acid as a product in the photolysis of FMF at pH 7.4 and 8.0. However, it remains to be established to what extent the alkaline hydrolysis contributes to the rate of photolysis of both RF and FMF, which is an intermediate in the photolysis of the former. No triplet-state species (thermochromic) of isoalloxazine ring was involved in the alkaline hydrolysis of FMF, as revealed by unaffected rate in the presence of 1.2×10^{-4} M KI.

In conclusion, it is significant to note that the nonphotochemical formation of LF in the alkaline photolysis of RF has been established for the reaction path in which FMF is the confirmed intermediate, as studied previously in our laboratory. Studies on the mechanism of the photochemical formation of LF from FMF are to be published elsewhere.

(10) R. G. Pearson and E. A. Mayer, *J. Am. Chem. Soc.*, **73**, 926 (1951).
(11) R. G. Pearson and A. C. Sandy, *ibid.*, **73**, 931 (1951).

Appendix

If we assume reaction 2 is a reasonable approximation to the mechanism of LC formation, equation (3) can be written as

$$-\frac{d(FMF)}{dt} = \frac{k_1 K_w}{(H^+)}(FMF) + \frac{d(LC)}{dt} =$$

$$\left[\frac{k_1}{(H^+)} + \frac{k_2}{(OH^-)}\right] K_w(FMF) \quad (3)$$

$$\simeq k \,(FMF)$$

Integrating the last expression, we get

$$(FMF) = (FMF)_0 e^{-kt}$$

Thus, eq. 1 can be expressed as

$$\frac{d(LF)}{dt} = \frac{k_1 K_w}{(H^+)} (FMF)_0 e^{-kt}$$

with the integrated relation

$$(LF) = (LF)_0 + \frac{\frac{k_1 K_w}{(H^+)} (FMF)_0}{k}(1 - e^{-kt})$$

Similarly, for eq. 2

$$(LC) = (LC)_0 + \frac{\frac{k_2 K_w}{(OH^-)} (FMF)_0}{k}(1 - e^{-kt})$$

Plots of (LF) and (LC) against $(1 - e^{-kt})$ would then yield k_1 and k_2 if accurate kinetic measurements of LF and LC were possible.

Studies on Nicotine Biosynthesis[1]

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Contribution from the Department of Biochemistry, Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma. Received March 19, 1965

Quinolinic acid was found to be an efficient precursor of nicotine in whole tobacco plants (Nicotiana tabacum L.). A method for the stepwise degradation of the pyridine ring of nicotine has been developed which involves the oxidation of nicotine to nicotinic acid, the biological conversion of nicotinic acid to ricinine, and the chemical degradation of ricinine. Using this degradation method it has been shown that glycerol is incorporated without randomization into carbons 4, 5, and 6 of the pyridine ring of nicotine. This degradation method has also been used to confirm the mechanism of fusion of the pyridine ring of nicotinic acid with the pyrrolidine ring during nicotine biosynthesis, previously proposed by Dawson, et al.[2a]

Nicotine and several other pyridine alkaloids have been utilized in studies of the biosynthetic pathway leading to nicotinic acid. Most of the available evidence[2b-8] supports the view that in higher plants and bacteria nicotinic acid is formed by the condensation of glycerol (or a closely related compound) and a four-carbon dicarboxylic acid. This suggests that pyridine-2,3-dicarboxylic acid, quinolinic acid, may be the first aromatization product resulting from this condensation. In fact it has been found that quinolinic acid is converted into nicotinic acid in *E. coli*[9] and plants.[10]

(1) A preliminary report of a portion of this work has been presented: R. K. Gholson, J. L. R. Chandler, K. S. Yang, and G. R. Waller, *Federation Proc.*, **23**, 528 (1964).

(2) (a) R. F. Dawson, D. R. Christman, A. F. D'Adamo, M. L. Solt, and A. P. Wolf, *J. Am. Chem. Soc.*, **82**, 2629 (1960); (b) M. V. Ortega and G. M. Brown, *J. Biol. Chem.*, **235**, 2939 (1960).
(3) D. Gross, H. R. Schütte, G. Hubner, and K. Mothes, *Tetrahedron Letters*, No. 9, 541 (1963).
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(6) P. F. Juby and L. Marion, *ibid.*, **5**, 461 (1916).
(7) P. F. Juby and L. Marion, *Can. J. Chem.*, **41**, 117 (1963).
(8) J. M. Essery, P. F. Juby, L. Marion, and E. Trumbell, *ibid.*, **41**, 1142 (1962).
(9) A. J. Andreoli, M. Ikeda, Y. Nishizuka, and O. Hayaishi, *Biochem. Biophys. Res. Commun.*, **12**, 92 (1963).