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PHOTOCHEMICAL DEGRADATION OF FLAVINS

V. CHROMATOGRAPHIC STUDIES OF THE PRODUCTS OF PHOTOLYSIS OF RIBOFLAVIN*,**

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SUMMARY

A new solvent system for thin-layer chromatography of flavins has been described which gives improved resolution of some of the products of flavin photolysis. Using this system, two new major products, designated "A" and "C", and numerous minor products, in addition to previously identified products, have been shown to result from the anaerobic photolysis of riboflavin. A study was made of the occurrence of these compounds under conditions of varying pH, wavelength, and time of irradiation. Employing a combination of chromatography, photolysis, and chemical tests, compounds "A" and "C" have tentatively been identified as flavins with a 5-membered side-chain in which one of the hydroxyl groups has been oxidized to a carbonyl group.

INTRODUCTION

An interest in the photochemical degradation of riboflavin has prompted us to study the application of thin-layer chromatography to the separation of flavins. This method was previously employed to identify 9-formylmethylflavin (FMF)*** as a product of photolysis of riboflavin[2]. Subsequently, in addition to FMF, lumiflavin (LF) and lumichrome (LC), at least eight other radioactive products from photolysis of riboflavin (RF) were detected on thin-layer plates[3]. This report describes improved procedures for thin-layer chromatography of flavins which should be of value in the study of naturally occurring flavins as well as of products of photolysis. It also describes a systematic study of the effects of experimental conditions on the photolysis of RF and of FMF and provides new information about the chemistry of several of the unknown products.

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** For Part IV of this series, see SONG AND METZLER[3].

*** Abbreviations used in this paper are as follows: CMF = 6,7-dimethyl-9-(2'-carboxymethyl)-isoalloxazine; FMF = 6,7-dimethyl-9-formylmethyl-isoalloxazine; HEF = 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine; LC = lumichrome; LF = lumiflavin; RF = riboflavin.

EXPERIMENTAL PROCEDURES

Since flavins are extremely sensitive to light, work was done in a windowless room, the only light being that from a 15 W, coral red, fluorescent lamp (General Electric). A solution of riboflavin placed 2 in. from the lamp for 1 h showed no change in absorbance at the 445 mμ peak. Flavins were stored in vacuum desiccators or in a freezer.

Materials

Riboflavin (U.S.P., readily soluble) was obtained from Commercial Solvents Corporation; LF, LC, FMF and HEF were synthesized by previous workers[2]. Nekoflavin[4] was a gift of Dr. K. MATSUI. All chromatographic solvents and chemical reagents were of analytical grade; freshly boiled redistilled water was used.

Photolysis

Flavin solutions of 1×10^{-4} *M* concentration were irradiated. The pH was adjusted, when necessary, with either H_2SO_4 or $Ba(OH)_2$. After irradiation, the solutions were neutralized and the insoluble $BaSO_4$ was removed by centrifugation.

Small samples were photolyzed in modified Thunberg tubes[2] made either of Pyrex glass or silica. The source of light was a 140 W Hanovia mercury lamp (high pressure) made with a fused silica envelope. The output of the lamp spans a spectral range from the green and blue lines of the mercury emission spectrum down to 226 mμ.

Small samples (0.10 to 1.0 ml) of photolyzed flavins were taken to dryness in a spot plate *in vacuo* over KOH. Large samples were frozen immediately after photolysis and lyophilized.

Thin-layer chromatography

Plates (20 × 20 cm) spread with Merck Silica Gels H or G (Brinkmann Instruments, Inc.), 250 μ, were dried for 1 h at 110° immediately before use.

Normally, 6–10 μg of flavins in methanol, or ethanol, were spotted 1.5 cm from the bottom of the plate using capillary tubes. Control samples of known flavins were always spotted on the same plate. The solutions from photolysis were concentrated to such a degree that they were in a state of fine suspension rather than true solution. To prevent variations in sampling, the suspensions were shaken well before spotting. The patterns of spots from these suspensions were the same as those obtained from true solutions.

The chromatographic solvent systems were: *BAW* (upper phase), *n*-butanol–acetic acid–water (4:1:5, v/v); *AAW* (upper phase), *n*-amyl alcohol–acetic acid–water (3:1:3, v/v); *Ketone*, acetic acid–2-butanone–methanol–benzene (5:5:20:70, v/v).

Freshly prepared solvent mixtures were always used. Tanks (12 × 4 × 11 cm) lined with No. 3 Whatman paper were allowed to become saturated before plates were placed in them; all chromatography was done at a constant temperature of about 22°. The solvent front was allowed to move 15.5 cm from the point of spotting. When two-dimensional chromatograms were prepared, the ketone system was always allowed to migrate first, and the plate was dried before migration in the second dimension.

The flavins on the chromatograms were detected by placing them in the light of a long wavelength ultraviolet "transilluminator" (Ultra-violet Products, Inc.). Light from the transilluminator itself is removed by a filter placed between the viewer and the fluorescent substances so that the light observed is that from the fluorescent material.

The chromatograms on the transilluminator were photographed through the U.V. filter using Ektachrome EH (Kodak ASA 160) 35 mm color film (2 sec exposure at f 3.5, 55 cm plate to film distance, magnification = 1/10). For the purpose of determining R_F values, a grid of white nylon thread (size No. 50) sandwiched between 2 glass plates was sometimes placed over the chromatogram before photography. The photographic technique was as sensitive as the eye in detecting 10^{-3} μg of RF on a spot about 4 mm^2 in area. When color prints were made from the transparencies, much of this sensitivity was lost. Color reproduction was good if more than 0.1 μg of flavin was on a spot.

A report on the use of other color emulsions and filters for the recording of the fluorescence of thin-layer chromatographed compounds has been given by JACKSON[5]. More recently, the recording of U.V. excited fluorescence using Polacolor has been described[6].

Preparative thin-layer chromatography

Approximately 35 g of Silica Gel H in 90 ml of water were spread using an adjustable spreader at maximum opening to cover two 20 × 20 cm plates. The final thickness was about 500–750 μ. The plates were dried on the spreading board for 4 h, then in an oven at 110° for 1 h. Flavin samples were spotted heavily at 1 cm intervals using capillaries. Large quantities were applied as a band with a small brush. After migration, the desired bands were scraped from the plate and the compounds were eluted by washing several times on a sintered glass filter with redistilled water or ethanol.

Chemical tests on flavins

RF and FMF were treated with an excess of potassium permanganate in neutral solution and in 2.5×10^{-4} M NaOH. They were allowed to stand in 0.3% hydrogen peroxide. Excess peroxide was destroyed with manganese dioxide. When acidic or basic conditions were required, either formic acid or tetramethylammonium hydroxide was used, since their salts interfered less than others in the chromatography or H_2SO_4 or $Ba(OH)_2$ were used so that insoluble $BaSO_4$ could be removed after neutralization. The solutions were always neutralized before spotting.

Photolysis and chemical tests on plates

The low yield of some of the photolysis products made difficult their rapid isolation for further study. For this reason, many tests were done on the chromatographic plates. Small samples (0.25 ml) of freshly photolyzed solutions were rapidly dried over KOH, spotted on chromatograms, and run in one dimension in the ketone solvent. The chromatograms were dried, sprayed with test reagent, or photolyzed in front of the mercury lamp (5–15 min at 35 cm) and rerun in the second dimension in the same solvent or in BAW. When the ketone solvent was used to develop in the second dimension, reacting products were observed by their departure from the

diagonal; and when BAW was used, reacting products were observed by comparing with the product distribution of the untreated photolysis sample.

The spray reagents were all saturated methanolic solutions. Great care had to be taken to spray with the correct amount of reagent, and this had to be established by trial-and-error. Although many unclear results were obtained, enough extremely clear plates were obtained to fully establish the results reported.

RESULTS

Thin-layer chromatography

The conditions affecting chromatographic separations were studied systematically, and a procedure was devised that gives sharp separations and reproducible R_F values. (See EXPERIMENTAL for the solvent compositions.)

A solution of riboflavin that had been incompletely photolyzed anaerobically was used as a test solution. Fig. 1A shows the separation achieved with three of the best solvent systems. In the BAW system, riboflavin (RF, R_F = 0.37) lumichrome (LC, R_F = 0.68) and formylmethylflavin (FMF, R_F = 0.60) separate well. The unknown compounds, "A" and "C" are crowded together along with lumiflavin (LF). Chromatography is slow (about 2.5–3 h) but separations are sharp and consistent. Formic acid was substituted for acetic acid with no improvement.

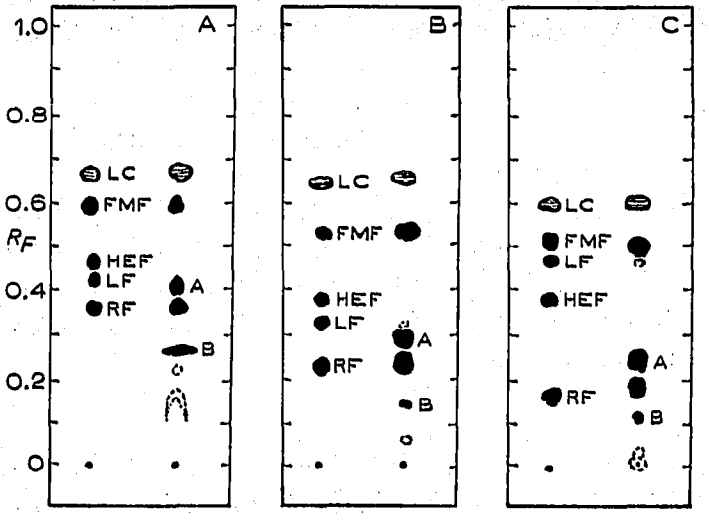


Fig. 1. Thin-layer chromatograms of a mixture of photolysis products from riboflavin. (A) BAW solvent; (B) AAW solvent; (C) "Ketone" solvent. In all three cases the left-hand column of spots (labeled) are known standards, while the photolysis mixture is in the right-hand column.

In the system AAW, the R_F value of riboflavin is decreased to about 0.20 (Fig. 1B). Some of the minor spots below RF are not as well separated, but all of the faster-moving components are separated better.

The "ketone" system gives a rapid separation (45 min) with LF moving well ahead of "A" and "C" (Fig. 1C). Compounds below RF are crowded.

The composition of the "ketone" system was varied systematically to find an optimum. All the flavins are moderately soluble in methanol, and increasing the content of this component leads to higher R_F values. Riboflavin is almost insoluble in 2-butanone, but LC is slightly soluble. The most important effect of the presence of

2-butanone in the solvent s to increase the separation of LC and FMF by increasing the R_F of LC. The inclusion of acetic acid is necessary to obtain good separation of the lower spots. However, the acid may be omitted if one is concerned only with the upper spots: the composition 2-butanone–methanol–benzene (5:20:75) is suitable. None of the major products is soluble in benzene, but a rather high fraction is desirable and appears to lead to sharper spots.

Sometimes the simpler mixture, methanol–benzene (20:80) is useful, but it provides little separation between LC and FMF. A basic solvent was also devised consisting of pyridine–2-butanone–methanol–benzene (5:5:20:70). In this system, compound "B" and certain minor products (spots 4 and 5 of Fig. 3), presumably acidic in nature, remain at the origin.

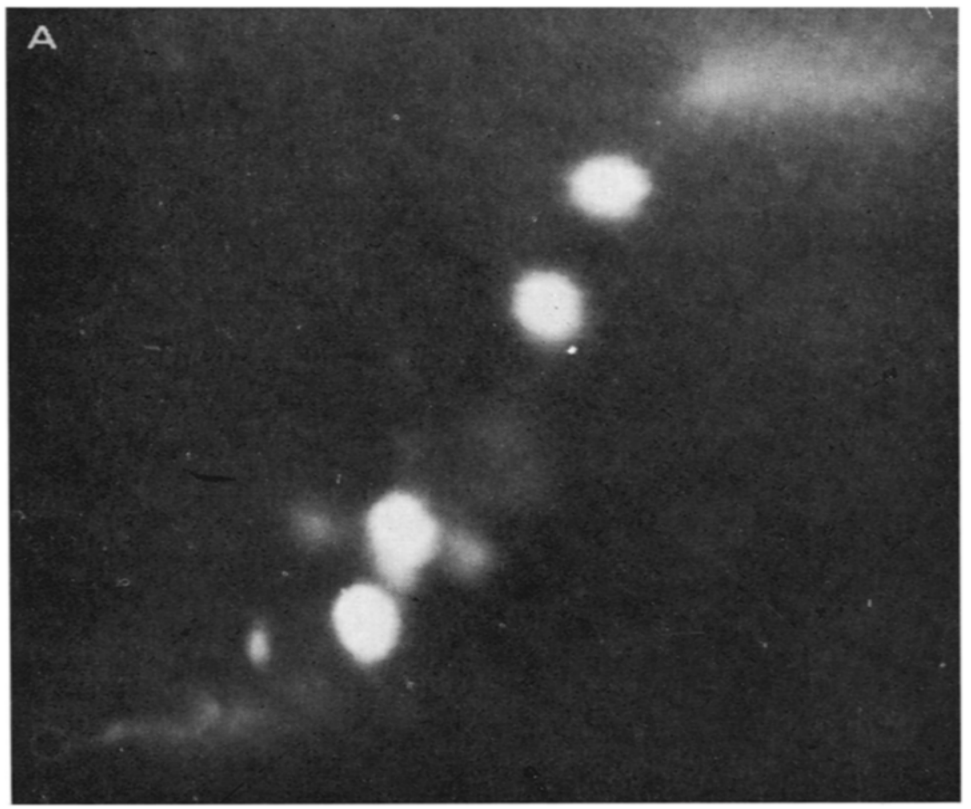
The degree of saturation of the air in the chromatography chamber is of critical importance. Sharp separations and reproducible R_F values were obtained when the plate was placed quickly in a tank after the atmosphere of the tank with the liner was saturated until vapor condensed on the inner surface of the cover. This procedure was adopted as standard and was used for all experiments. When the paper tank liner was omitted or when the atmosphere was not allowed to equilibrate completely, markedly inferior separations were observed. Suspension of the plate, by a string, in the solvent vapors for 6 h prior to lowering into the solvent did not significantly improve the separations over those obtained by the standard procedure.

The method of application of the spot was also very important. Usually, direct spotting of aqueous solutions was unsatisfactory and led to poor resolution. Spotting from anhydrous methanol suspensions of vacuum-dried samples (large samples were lyophilized) was satisfactory. Salts interfere; therefore in experiments requiring addition of acid or base, we used sulfuric acid and barium hydroxide and neutralized and centrifuged out the barium sulfate before spotting.

Other variables considered were the size of the spots (always under 5 mm diameter), the quantity of flavin spotted (between 10^{-3} and 10 μg per spot), and thickness of the layer of silica gel. These were found to be of relatively minor importance. As little as 10^{-3} μg of RF could be detected after migration. Amounts greater than 10 μg of RF resulted in streaking and poor resolution.

Factors influencing the photolysis of riboflavin

Photolysis under various conditions was compared with that observed for the following "standard" experiment. A 10^{-4} M solution of RF in water at pH 7.2, and under anaerobic conditions, was irradiated in a Silica Thunberg tube with the full spectrum of the Hanovia lamp at a distance of 5 cm until the absorbance of the solution at 450 mμ dropped to 46–50% that of the unphotolyzed solution (from about 1.2 to 0.6). The products of this standard photolysis are shown in the photograph in Fig. 2A. Fig. 2B is a diagram of the same chromatogram on which the major components, as well as those such as "A", to which special attention is given in the text, are labelled for convenience in discussion. Spots 10, 12, 14, 15 and 26 are present in the chromatogram of Fig. 2A and are clearly visible in the original color transparency, but are present in such small amounts that they cannot be seen clearly in the reproduction of this photograph. In agreement with earlier findings[2,3], we observe that LC and FMF are always major products but that LF is formed in trace amounts by neutral photolysis. A third major product, present in quantity, is com-



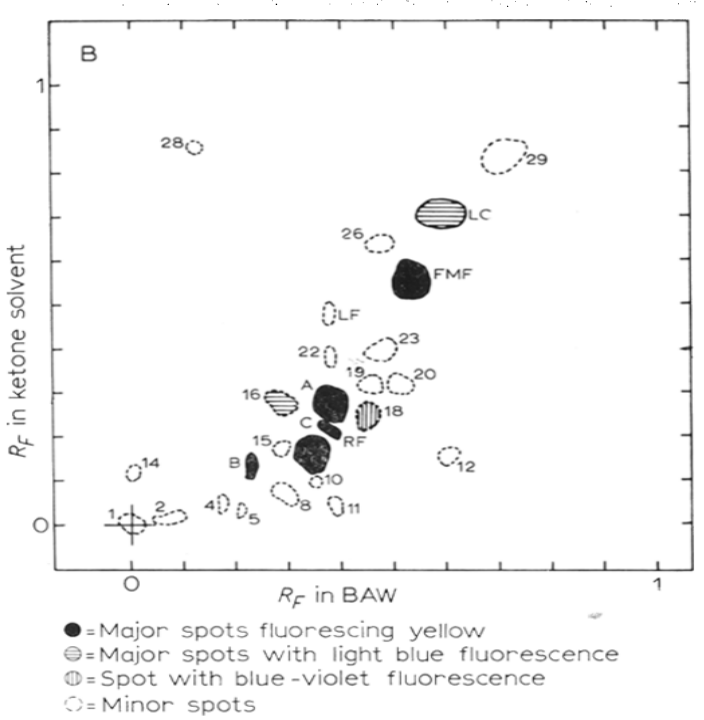
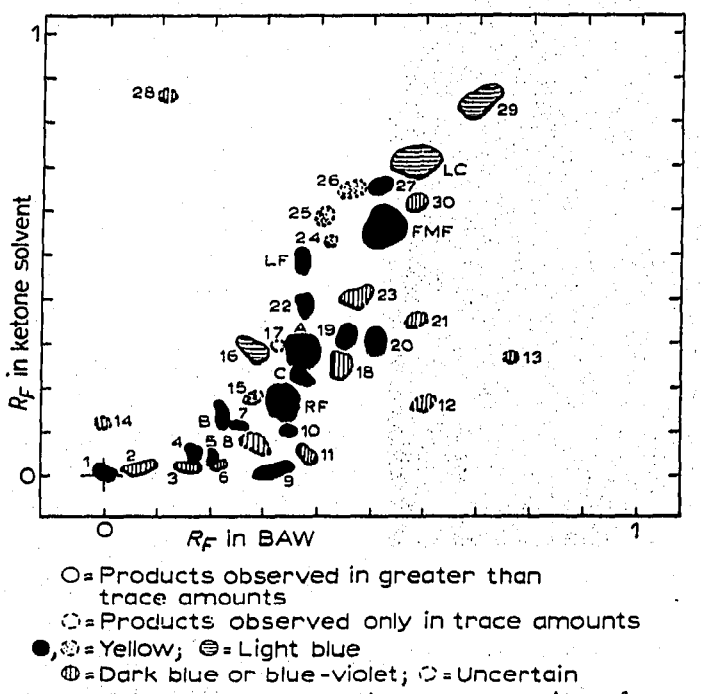


Fig. 2. (A) Photograph of two-dimensional chromatogram of the products of a "standard" photolysis of riboflavin. Riboflavin, 1×10^{-4} *M* at pH 7.2, was irradiated with a mercury lamp anaerobically until 50% bleached (at 445 mμ, 4 min). About 10 μg of the resulting mixture of flavins was used for the chromatogram. The photograph was made from an Ektachrome color transparency. (B) Diagram of spots visible on the thin-layer plate from which Fig. 2A was prepared.

pound "A". The next most abundant is labelled "B". Our attention was later drawn to another yellow spot which we have called "C". This compound is a major spot which largely replaces "A" in alkaline photolysates. Other minor spots have been numbered.

Fig. 3 is another diagram which represents a composite of chromatograms run under many conditions, and which shows the position of various minor components whose formation was variable.



○ = Products observed in greater than trace amounts
◌ = Products observed only in trace amounts
●, ◎ = Yellow; ⊜ = Light blue
◍ = Dark blue or blue-violet; ○ = Uncertain

Fig. 3. Diagram representing a composite of many chromatograms of photolysis products from riboflavin.

Wavelength range of light

When a yellow filter with a sharp cut-off at 400 mμ was placed between the mercury lamp and the sample, the time required to obtain a desired degree of bleaching under anaerobic conditions was increased by a factor of approximately 5 in both acidic and basic as well as neutral solutions.

The changes in photolysis products observed by insertion of the filter, when bleaching is carried to the same stage (50%), are relatively minor and are clearest in neutral solution. Since the fluorescence of the major spots is intense, it is impossible to say whether significant changes in the relative amounts of these products occur with filtered *vs.* unfiltered light; none can be seen. Of the minor products, the dark blue spots, 2, 18 and 23 definitely decrease with filtered light, and a small decrease in yellow spots 19 and 20 and in light blue-fluorescing spot, 16, is observed. The amounts of LF, of compound "B" and of yellow spots, 1, 4 and 5 appear to increase noticeably. In acidic solutions (pH 4) the differences are less noticeable, and surprisingly, are mostly in the opposite direction to those observed at pH 7.2. Thus, while the production of several blue fluorescent spots is decreased by use of a filter, at pH 7.2, it is increased at pH 4. At pH 10 the changes are also very slight, the most characteristic being that blue spots, 12 and 13, completely disappear when the filter is used.

Time of irradiation

The influence of exposure time on anaerobic photolysis patterns was studied using filtered light, of wavelength greater than 400 mμ, at pH 7.2. Exposures of 10, 20, and 50 min, corresponding to decrease in absorbance (ΔA) of 0.30, 0.60 and 1.0 (to about 75, 50 and 16% of the original absorbance) were compared. The results were judged visually by the fluorescent intensity of the spots on the chromatograms. The following changes with time in the relative concentrations of products were seen:

(1) The concentrations of LC, LF, spot 16 and several faint blue spots increased progressively.

(2) Compounds "A", "B" and "C" appeared rapidly and later decreased gradually, "C" disappearing the most rapidly.

(3) FMF also appeared quickly but its concentration seemed to stay relatively constant at later times.

Variation of pH

Anaerobic photolysis products at pH 3.9, 7.2 and 10.0 were compared by bleaching 50% using the full spectrum of the mercury lamp. Solutions were maintained at alkaline pH's no longer than 30 min. Only minor differences were seen between pH 3.9 and 7.2, but several important changes in the product distribution at pH 10 were observed.

(1) Compound "A" was completely lacking at pH 10.

(2) Compounds "B" and "C" were more abundant at pH 10 than at pH 7.2. They were also somewhat more abundant at pH 3.9.

(3) LF, as expected, was present in large amounts at pH 10 but in very small amounts at lower values of pH.

(4) Spot 1 was much decreased at pH 10 whereas spot 27 was increased.

The time required to obtain a given degree of photobleaching at pH 3.9 was slightly longer than that at pH 7.2, but at pH 10.0, it was only 1/12 that at pH 7.2.

Effect of oxygen on photolysis

In a series of experiments, oxygen was bubbled through the solution continuously during photolysis. The reaction was carried to an arbitrary degree of bleaching. It is important to recognize that this bleaching is irreversible, (unlike that in anaerobic conditions) and that a greater degree of degradation of the riboflavin is observed when aerobic bleaching is carried to the same degree as with anaerobic bleaching.

At pH 7.2, aerobic photolysis with 28% bleaching gives a product distribution similar to that of an anaerobic photolysis at the same pH carried to 50% bleaching except that the amount of FMF is much decreased, the other yellow products FMF, LF, "A" and "B" are also decreased, and the dark blue-fluorescing products 2, 3, 6, 11, 18 and 23, the light blue-fluorescing spot 16 and the yellow-fluorescing compounds 20 and 22 are all increased.

When the aerobic photolysis at pH 7.2 is carried to 50%, the yellow spots are further decreased in amount and LC and the blue-fluorescing spots continue to increase. Compounds "A" and "C" are the major yellow compounds. If filtered light is used, the minor blue-fluorescing products are also largely absent. Similar results are seen at pH 3.9 except that spot 30 appears, spots 15 and 20 increase and 16 decreases. At pH 10 aerobic photolysis (50% bleaching, unfiltered light) gave large

amounts of both LF and LC, somewhat less of "B" and still less FMF and "C". Blue-fluorescing products 2, 3, 6 and 11 were also formed. Compound "A" could not be detected.

Photolysis of other flavins

Formylmethylflavin, FMF, is very sensitive to light and is rapidly bleached. When illuminated anaerobically with the full spectrum of the mercury lamp at pH 7.3 until the absorbance had decreased by 50% (from an initial 1.23), the products shown in Fig. 4 were formed (the spots are numbered arbitrarily and without reference to the numbering in Fig. 3). The major product is LC, in agreement with the report of McBRIDE AND METZLER[10] but small amounts of "B" and LF are also formed together with some dark blue-fluorescing materials and a number of other substances in trace amounts. As with photolysis of RF, use of a filter decreased the amount of dark blue-fluorescing materials. Spot 12 was found at short photolysis times and decreased with longer irradiation. At pH 4.1 similar results were observed, but yellow trace products 6 and 10 increased somewhat and LF and spot 13 decreased. At pH 8.5 the products were almost exclusively LC, LF and "B". The latter two compounds were present in larger amounts than at pH 7.2 but their production (especially of LF) was decreased by insertion of the yellow filter. During the time required to carry out the photolysis and neutralize the solution, negligible decomposition of FMF occurred in a control held in the dark. The rapid decomposition at pH 10 prevented studies of photolysis at this pH[7].

Photolysis on plates

A valuable procedure for study of flavins was introduced by SVOBODOVÁ, HAIS AND KOŠTÍŘ[8] who chromatographed flavin mixtures on a square sheet of paper, first

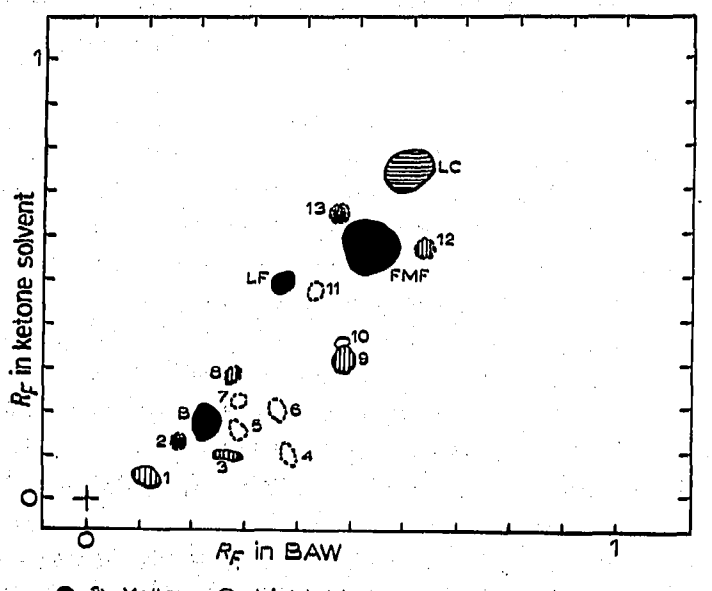


Fig. 4. Diagram of spots visible on thin-layer chromatogram of photolysis products from 9-formylmethyl-6,7-dimethyl-isoalloxazine (FMF). An anaerobic solution (1×10^{-4} *M*) of FMF, pH 6.8, was bleached 50% by the full spectrum of the mercury lamp. About 10 μg of flavins were used for the chromatogram.

along one edge as for a conventional two-dimensional chromatogram. They then irradiated the flavins on the paper and chromatographed in the second direction using the same solvent system as initially. All flavins unmodified by the irradiation lie on the diagonal on such a plate; spots falling off the diagonal are photolysis products, whose origin can be traced to a particular spot in the one-dimensional chromatogram prior to photolysis. We have employed the same technique with thin-layer chromatography.

Photolysis of RF on a silica gel plate resembles that in solution at neutral pH, but the yields of FMF, "A" and "C" are much reduced and a large amount of a yellow-fluorescing spot lying above FMF in the Ketone solvent is formed. The presence of acetic acid strongly promotes formation of this substance, which we will call "S". Compound "S" evidently arises from FMF, for it is the major product of photolysis of FMF on plates in the presence of acetic acid. Photolysis of "A" on plates gives rise to most of the series of products obtained from RF, including "S", but neither "C" nor its special photoproduct (see below) is formed. Compound "C" is less sensitive to light, but on prolonged irradiation gives LC and "S" as well as smaller amounts of FMF and "B". At shorter times of irradiation, however, it is converted mostly into a faster-moving, yellow spot, possibly 20 (Fig. 3).

Oxidation

Oxidation of riboflavin with excess, neutral potassium permanganate in a test tube gave FMF and, as judged by chromatographic mobility, compound "B" (CMF). A yellow-fluorescing spot at the upper edge of the origin and traces of LC were also formed. A large amount of unoxidized RF remained. Under the same conditions, FMF was oxidized almost completely to "B" (under alkaline conditions LC was formed also).

Treatment of RF with acidic hydrogen peroxide gave a small amount of "B" and traces of LC and a bluish-white spot at the origin. FMF also gave "B" and LC. With alkaline peroxide RF gave compound "B", traces of LC and FMF, a large yellowish-white spot and a blue-white spot at the origin. Under the same conditions, FMF also gave "B" and LC as well as a small amount of LF, a spot between LC and FMF, a faint spot above the origin, a spot at the origin, and trace amounts of other compounds which were obscured by streaking.

Photolysis of RF in alkaline solutions containing hydrogen peroxide gave large amounts of LC and "B" as well as FMF, a small yellow spot directly below LC, a blue streak below "B" and traces of several yellowish-white compounds.

Oxidation of "A" by periodate (on a plate) yields FMF as nearly the sole product, just as does RF. Compound "C", on the other hand, is converted largely to "B".

Base treatment

When 1 μl of 2% tetramethylammonium hydroxide is added to 1 ml of neutral photolysate of RF and the solution taken to dryness, and chromatographed, most of the spots are unchanged except for FMF, which has now been partially converted to LF? and "A" and "C", which have been mostly converted to two yellow-fluorescing compounds, apparently the same as spots 4 and 5 (Fig. 3). Spot 4 arises from "A" and spot 5 from "C". Unlike "A" and "C", these "base products" are relatively stable to the action of light, but with prolonged irradiation on plates, they, too, are converted largely into compound "S".

Other chemical tests on plates

Reduction with sodium borohydride converts "A" largely to a spot with the same R_F as riboflavin. This result, together with the photolysis of "A" on plates, to yield the whole array of spots arising from RF, suggests that the ribityl chain in compound "A" may be intact, but that it may have been oxidized to a compound (aldehyde or ketone?) which can be reduced back to riboflavin (or an isomer). During the borohydride reduction of "A" variable amounts of a second compound of lower R_F are also formed. Reduction of "C" under the same conditions is less clear, but again a material migrating like RF is formed. When a semicarbazide spray was used, both compounds "A" and "C", as well as FMF, were converted extensively to slower-moving spots. Similar but less clear-cut results were obtained with hydroxylamine.

Nekoflavin

The isolation of a new flavin from the eyes and liver of cats, nekoflavin, by MATSUI[4] prompted us to ask whether this flavin corresponds in structure to any of the photolysis products. Nekoflavin, in a two-dimensional chromatogram such as that of Fig. 3 occupies a position very close to that of compound "B", but migration of nekoflavin in a pyridine containing solvent is not retarded, as is that of "B". We conclude that nekoflavin does not correspond to any of the photolysis products. Furthermore, nekoflavin by photolysis on the plate is converted largely to the same spots as is RF including compounds "A" and "S", confirming a recent report of MATSUI[9].

DISCUSSION

Paper chromatography of flavins has been used widely, but few reports of the use of thin-layer chromatography have appeared. The method has been employed extensively by Dr. P. HEMMERICH *et al.* in the Institute of Inorganic Chemistry of the University of Basel. We were introduced to the technique through a visit to Dr. HEMMERICH's laboratory. In our laboratory, it has been used for separation of products of photolysis of flavins[2,3,10]. By careful attention to the details of technique, we have been able to significantly improve reproducibility and resolution. Thin-layer chromatography, in our opinion, is an outstanding method for detection of flavins, both photoproducts and naturally-occurring flavins. The most critical experimental factors appear to be the initial dryness of the plates (all through the time required for spotting of samples) and the completeness of equilibration of the solvent with the atmosphere in the chamber. Plates of Silica Gels H and G give excellent separation, but several pre-prepared plates and flexible TLC media (which must be completely free of fluorescence) proved to be far inferior to plates spread in the laboratory.

The use of a high-intensity ultraviolet light source below the plate and a contrast filter for viewing are of great importance in providing high sensitivity. It is gratifying that the chromatograms can be easily photographed and that the presence of even the weakest spots visible to the eye can be reliably recorded on colored transparencies. The utility of thin-layer chromatography for study of naturally-occurring minor flavins is being demonstrated in our laboratory. An example is the comparison of the migration of nekoflavin[4] with that of RF photoproducts described here. We verified the lack of identity of nekoflavin with these photoproducts and observed the pattern

of photolysis products of nekoflavin on plates. Photolysis on plates can be of real value in characterization of new flavins.

Photolysis of riboflavin leads to a surprising number of photoproducts, most of which remain unidentified. Most are minor and are produced in variable amounts, but their formation is indicative of the complexity of the photodegradation process. We have attempted to correlate our findings with those of others, who have mostly used paper chromatography and aerobic photolysis conditions.

We believe that compound "B" is 9-carboxymethylflavin (CMF), on the basis of its ease of formation by action of oxidizing agents on 9-formylmethylflavin (FMF). CMF has been proposed by FUKAMACHI AND SAKURAI[11] as a photolysis product, and it has been isolated by these authors as methyl, ethyl and propyl esters. Larger amounts were obtained by photolysis in alkaline solutions containing hydrogen peroxide just as we have observed for compound "B". FUJISAWA[12] provided strong evidence for the formation of the same compound by action of permanganate on riboflavin in acidic solutions. The synthesis of CMF was reported by KUHN AND RUDY[13], but HEMMERICH *et al.*[14] reported that he could not obtain the compound.

A series of paper chromatographic studies of flavin photolysis were made by HAIS and associates[8,15,16]. The compound designated by them "27CX" is probably CMF. In addition, they observed a transient intermediate, "43CX" ((possibly FMF) lying between RF ("35CX") and LC (69K). (The numerical designation indicates the $R_F \times 100$ on paper in BAW and the letters, the color of fluorescence.) Other minor products with low R_F values were also formed and in acidic solution, a yellow green-fluorescing compound "69CX" was a major product, convertible into LC (69K) by irradiation on paper. Lumichrome could be converted slowly by irradiation into compound "50K" and LF into "29CX", "39CX" and "72X" (yellow)[17]. It is difficult to correlate any of these with spots we have observed but it should be noted that we probably did not irradiate long enough to cause significant conversion of LC and LF into secondary products. The observation of BERENDS[18] that LC is converted anaerobically by irradiation in acidic solution to a ring-hydroxylated derivative is pertinent.

None of the compounds reported by HAIS *et al.* seem to correspond to our compounds "A" or "C", but this is not surprising since these compounds may have R_F values very close to or identical to those of RF. On the other hand, KOZIOŁ[19] shows paper chromatograms of photolyzed riboflavin with a small spot immediately above RF and very possibly representing compound "A".

SONG AND METZLER[3], using somewhat cruder techniques of chromatography, prepared several radioautograms of thin-layer chromatograms prepared from photolysis of 2-^{14}C-riboflavin. They described the formation of the new, major unknown, "A", but we now know that their spot "A" also contained compound "C", especially at high pH. Of the other unknown spots reported by SONG AND METZLER, spot 7 in BAW (and 9 in AAW) is probably largely "B", but it is hard to make exact correlations of their other spots with the numbered unknowns of Fig. 3. Many of their spots are doubtless mixtures.

KURTIN, LATINO AND SONG[20] have shown that photolysis of RF in pyridine gives a distribution of products, including "A", that is remarkably similar to that found for photolysis in water.

The oxidation of the ribityl side-chain to a ketone has often been assumed a

feature of the photodegradation of RF, the 2′-keto compound being postulated most frequently[21–23]. Isolation of the 2′-keto derivative has been claimed by TERAO[24] and by HOTTA[25], but the proof of structure is equivocal.

Compound "C" possesses several of the properties expected of the 2′-ketone: (1) It is reduced by sodium borohydride to a product similar in chromatographic mobility to RF. (2) It reacts with carbonyl reagents. (3) It is oxidized with periodic acid to compound "B" (CMF). Compound "A" also meets the first two of these criteria, but it is oxidized by periodic acid to FMF rather than CMF. It may, therefore, have a carbonyl group in the 3′, 4′ or 5′ position.

The reason for the lability of "A" and "C" in weakly basic solutions is not clear. The low R_F values of the "base products" from these compounds (spots 4 and 5, Fig. 3), in ketone solvent suggests that these products may be acids, or perhaps higher molecular weight products arising through base-catalyzed condensation. Work is now underway to form appropriate derivatives for characterization of "A", "C" and their "base products".

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REFERENCES

1 G. E. TREADWELL, *M. S. Thesis*, Iowa State University, 1967.
2 E. C. SMITH AND D. E. METZLER, *J. Am. Chem. Soc.*, 85 (1963) 3285.
3 P.-S. SONG AND D. E. METZLER, *Photochem. Photobiol.*, 6 (1967) 691.
4 K. MATSUI, *J. Biochem. (Tokyo)*, 57 (1965) 201.
5 R. JACKSON, *J. Chromatog.*, 20 (1965) 410.
6 I. D. JONES, L. S. BENNETT AND R. C. WHITE, *J. Chromatog.*, 30 (1967) 622.
7 P.-S. SONG, E. C. SMITH AND D. E. METZLER, *J. Am. Chem. Soc.*, 87 (1965) 4181.
8 S. SVOBODOVÁ, I. M. HAIS AND J. V. KOŠTÍŘ, *Chem. Listy*, 47 (1953) 205.
9 K. MATSUI, *Abstr. 38th Gen. Mtg. Japanese Biochem. Soc.*, (1967).
10 M. MCBRIDE AND D. E. METZLER, *Photochem. Photobiol.*, 6 (1967) 113.
11 C. FUKAMACHI AND Y. SAKURAI, *Bitamin*, 7 (1954) 939, 1040; *J. Vitaminol. (Kyoto)*, 1 (1955) 217.
12 T. FUJISAWA, *Nagoya J. Med. Sci.*, 21 (1958) 69.
13 R. KUHN AND H. RUDY, *Ber.*, 68 (1935) 300.
14 P. HEMMERICH, C. VEEGER AND H. C. S. WOOD, *Angew. Chem. (Intern. Ed.)*, 4 (1965) 671.
15 I. M. HAIS AND L. PECÁKOVÁ, *Nature*, 163 (1949) 768.
16 S. SVOBODOVÁ-LEBLOVÁ, J. V. KOŠTÍŘ AND I. M. HAIS, *J. Chromatog.*, 14 (1964) 451.
17 S. SVOBODOVÁ, *Chem. Listy*, 45 (1951) 225.
18 W. BERENDS, *Photochem. Photobiol.*, 6 (1967) 491.
19 J. KOZIOŁ, *Photochem. Photobiol.*, 5 (1966) 55.
20 W. E. KURTIN, M. A. LATINO AND P.-S. SONG, *Photochem. Photobiol.*, 6 (1967) 691.
21 R. BRDIČKA, *Chem. Listy*, 36 (1942) 286, 299.
22 W. M. MOORE, J. T. SPENCE, F. A. RAYMOND AND S. D. COLSON, *J. Am. Chem. Soc.*, 85 (1963) 3367.
23 I. ISHIGURO, Y. KATO AND M. SUGIURA, *Ann. Proc. Gifu Coll. Pharm.*, 8 (1958) 49.
24 M. TERAO, *Tohoku Med. J.*, 59 (1959) 441.
25 K. HOTTA, *Bitamin*, 8 (1955) 248.