PAPER PARTITION CHROMATOGRAPHY OF RIBOFLAVIN DECOMPOSITION PRODUCTS. THE ACTION OF SOME REDUCING AND OXIDIZING AGENTS ON RIBOFLAVIN SOLUTIONS*

S. SVOBODOVÁ-LEBLOVÁ, J. V. KOŠTÍŘ AND I. M. HAIS

Department of Biochemistry, Charles University of Prague and Faculty of Medicine, University of Hradec Krdlové (Czechoslovakia)

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Riboflavin is generally reported to be stable in the presence of oxidizing agents[5]. It does not undergo any changes even under the influence of such strong oxidants as nitrous acid, hydrogen peroxide or bromine, but it is attacked by chromic acid[6]. Treatment with potassium permanganate in an acid medium is very commonly used for specific removal of fluorescent contaminants in many analytical procedures for the fluorimetric estimation of riboflavin[7]. It would thus be of considerable practical interest to show whether riboflavin itself is resistant to this treatment.

Various reducing agents such as hydrosulfite or molecular hydrogen cause the formation of the reversible dihydro-form (leuco-form[8]). Reversible oxidation and reduction form the basis of the biological activity of riboflavin-containing nucleotides. By a more drastic reduction KARRER AND OSTWALD[9] obtained octahydroflavin, which was converted to hexahydroflavin on exposure to air.

Our work[4] was mainly concerned with the paper chromatographic pattern of the photolytic products of riboflavin. For comparative purposes the action of reductants and oxidants was also studied. Some of the spots which appear under the influence of hydrogen peroxide have already been mentioned in a preliminary communication by HAIS AND PECÁKOVÁ[1]. Although our study was mainly descriptive in the present stage, it may perhaps yield some points of departure for further study which would explain our observations.

The system of nomenclature, materials and methods used in the present paper has already been described[4]. The most commonly used solvent system was the butanol–acetic acid–water mixture (4:1:5)[1,4]; the mean R_F values found in this solvent, multiplied by 100, together with the color of the fluorescence of the corresponding spot, form the basis of the nomenclature of the spots[4].

EXPERIMENTAL

In order to investigate photolysis in an alkaline medium, riboflavin was dissolved in 0.1 *N* and 0.2 *N* alkali hydroxide. The study of photolysis in neutral and acid media was carried out by mixing the alkaline solution of riboflavin with an amount of acid necessary to obtain the desired pH. For pH values less than 3, hydrochloric acid was used, for pH 4.7, acetic acid.

* For previous communications in this series, see Refs. 1, 2, 3 and 4.

1. *Treatment with thiosulfate and sulfite*

(a) Without light

On addition of a solution of sodium sulfite or sodium thiosulfate, alkaline, neutral and acid solutions of riboflavin exhibit only a riboflavin spot on paper chromatograms, even if kept in the dark for five days.

(b) With light

Samples containing both sulfite or thiosulfate, in either alkaline or acid medium, were exposed to daylight for 24 h; riboflavin was then accompanied by very faint 12 CX and 16 CX spots. In an alkaline medium, lumichrome was also detectable after five days. In a neutral medium, the simultaneous action of sodium thiosulfate and light led to the disappearance of the riboflavin spot, and no other spot, detectable in either visible or ultraviolet light, appeared.

2. *Treatment with potassium permanganate*

The influence of potassium permanganate on an alkaline, neutral and acid solution of riboflavin was tested, both in presence and absence of visible light.

(a) Without light

When potassium permanganate (0.05 *M*) is mixed with a 1% riboflavin solution (1:1), all fluorescent spots, including riboflavin and some spots with a bluish fluorescence disappear even in the dark. This also occurs in alkaline, neutral and acid solutions of riboflavin.

When a 0.005 *M* solution of potassium permanganate is used, the changes which take place in the dark during the first day of treatment resemble the course of photolysis: acid samples show a weak 27 CX spot[4] and a very weak spot at the position usually occupied by lumichrome (R_F 0.69); in neutral solutions, lumiflavin (50 CX) and lumichrome (69 K) spots are accompanied by a weak 27 CX spot; alkaline samples have a very intense 27 CX spot together with weak lumiflavin (50 CX) and lumichrome (69 K) spots (Fig. 1).

Summarizing, 0.005 *M* $KMnO_4$ without light causes the appearance of a 27 CX spot (most intense in an alkaline medium), a lumiflavin spot (only in alkaline and neutral media) and a lumichrome spot (in all three cases).

No decomposition of riboflavin can be detected with 0.001 *M* permanganate in the dark, irrespective of pH.

(b) With light

When alkaline, neutral or acid solutions of riboflavin with 0.05 *M* potassium permanganate are exposed to light under the conditions described previously[4], the riboflavin spot disappears during the first four hours. The only fluorescent spots are 39 KI (a bluish purple spot, which is also formed by the action of this concentration of $KMnO_4$ on lumichrome[2]) and small, very weak yellowish spots in a position with an R_F value less than that of riboflavin.

0.005 *M* and 0.05 *M* concentrations of potassium permanganate do not modify appreciably the course of photolysis of riboflavin in neutral and acid samples during the first hours. In an alkaline medium considerably more 27 CX and less lumiflavin (50 CX) are formed than without the acid ion of permanganate.

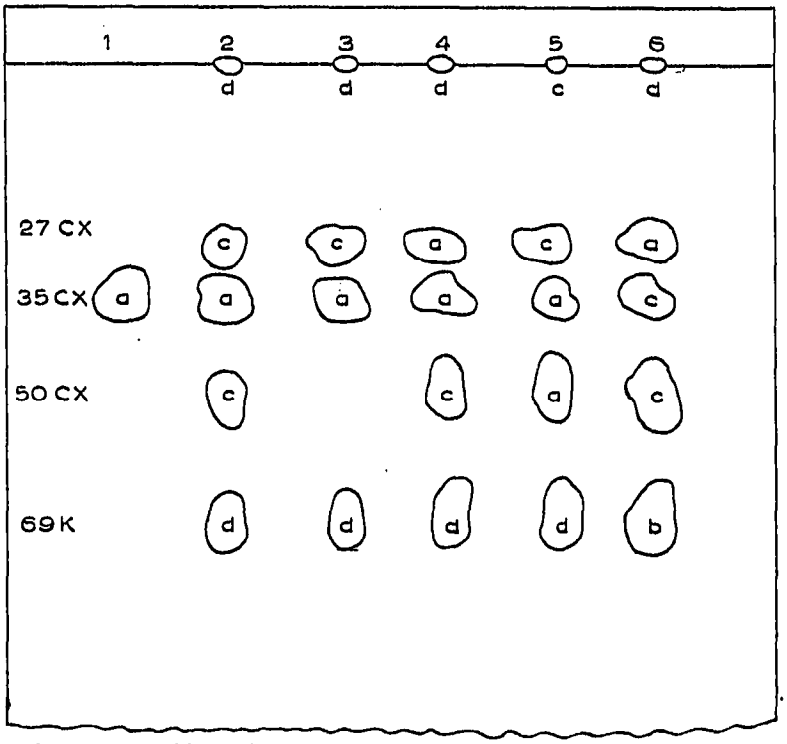

Fig. 1. The influence of 0.05 *M* $KMnO_4$ on a neutral, acid or alkaline solution of riboflavin. In absence of visible light: 1. a neutral, acid or alkaline solution of riboflavin; 2. a neutral solution of riboflavin with $KMnO_4$; 3. an acid solution of riboflavin with $KMnO_4$; 4. an alkaline solution of riboflavin with $KMnO_4$. In presence of light: 5. an alkaline solution of riboflavin; 6. an alkaline solution of riboflavin with $KMnO_4$. The spot fluorescence: a yellow, b blue, c weak yellow, d weak blue.

3. *Treatment with hydrogen peroxide*

Hydrogen peroxide causes the appearance of a large number of spots which are very difficult to define. Many conditions, which are difficult to reproduce, affect the picture, *e.g.*, the influence of light, good or bad fit of the stopper, slight variations in the concentration of hydroxide and perhaps some other factors.

(*a*) *Acid medium*

No concentration of hydrogen peroxide which was added to a riboflavin solution of pH 3 was found to cause any change without the influence of light. When these acid solutions are irradiated, a series of spots with a blue and yellow fluorescence is formed, with an R_F value of 0.15–0.25 in butanol–acetic acid mixture. It will be referred to as the group 15–25. These spots with very irregular R_F values have various colors of fluorescence and we therefore omit in their names the letters which we use to indicate the color of fluorescence. Some blue and orange spots have already been mentioned in the preliminary communication.

The spot of riboflavin is here accompanied by a weak 43 CX spot, the usual spot of acid photolysis.

(*b*) *Alkaline medium*

Alkaline solutions which are kept in darkness are appreciably attacked by 15 % hydrogen peroxide only after 24 h. Group 15–25 and riboflavin (35 CX) spots are

accompanied by the 54 KI spot. The decomposition of alkaline solutions, under the influence of both hydrogen peroxide and light, yields a rich display of 15–25, 54 KI and lumichrome (69 K) spots (Fig. 2).

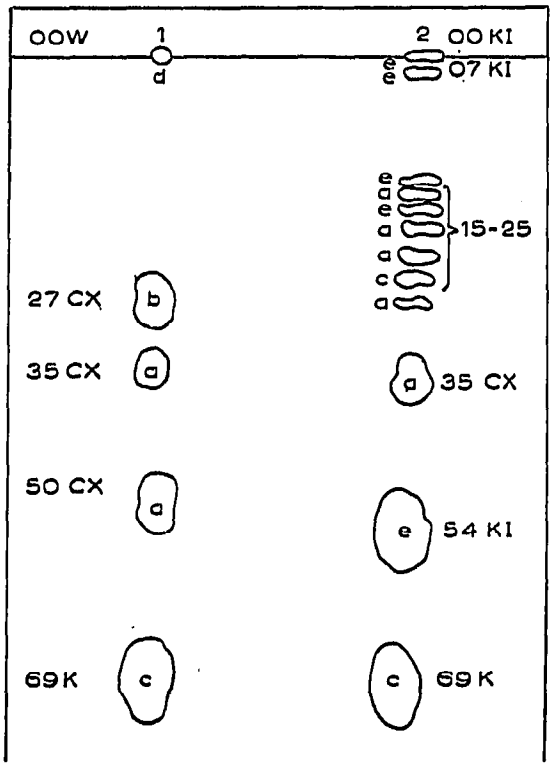

Fig. 2. The influence of hydrogen peroxide on an alkaline solution of riboflavin in presence of visible light: 1. an alkaline solution of riboflavin; 2. an alkaline solution of riboflavin with hydrogen peroxide. The spot fluorescence: a yellow, b weak yellow, c weak blue, d white, e violet-blue.

Lumiflavin (50 CX) is thus mostly missing in the products of alkaline photolysis with hydrogen peroxide, whereas it is present when photolysis takes place without hydrogen peroxide. When 30% hydrogen peroxide was added in a ratio of 1:1 to already photolyzed alkaline samples, the complex 15–25 spots appeared after two days of exposure to daylight, but the intensity of the lumiflavin spot did not diminish appreciably. It follows that lumiflavin (50 CX) is not formed in an alkaline medium through photolysis in the presence of hydrogen peroxide, but that, if already formed, it is not decomposed by hydrogen peroxide.

DISCUSSION

Our results show, in accordance with previous authors, that riboflavin is protected against photolysis by reducing agents. If the sample is applied to the paper in the presence of air, the leuco-form, which is believed to be resistant to photolysis, is oxidized during the handling of the sample for chromatography and unchanged riboflavin is then found on the chromatogram.

The disappearance of any fluorescent spot by the action of sodium thiosulfate and light in a neutral medium is very surprising and merits further investigation.

We could not substantiate the common opinion that riboflavin is not attacked

by oxidants; potassium permanganate causes very drastic decomposition, even in darkness. There can, nevertheless, be no objection to treating analytical samples with permanganate in an acid medium in the light of our results, as we have found that the concentrations used in common fluorimetric analytical procedures do not cause significant losses of riboflavin in acid solution.

It is interesting to note that permanganate itself causes similar qualitative changes to those encountered after irradiation.

The appearance of the intermediate 27 CX seems to be favored by oxidation, as it is encountered even in an acid medium, and its spot is stronger in alkaline medium than without permanganate. Whitby's flavin, whose chromatographic behavior resembles that of 27 CX, is also formed under conditions which suggest oxidation.

Hydrogen peroxide produces some very complicated pictures. Various yellow and bluish purple spots appear, with very irregular R_F values.

54 KI is regularly found in alkaline solutions. After irradiation in an alkaline solution it is interesting to note the absence of lumiflavin (50 CX) and of 27 CX, which is a common precursor of both lumiflavin and lumichrome[4]. Both these substances, nevertheless, are resistant to the action of hydrogen peroxide[3,4], and would not be decomposed if they had been formed.

SUMMARY

The influence of some reducing and oxidizing agents on riboflavin solutions was studied, both in the dark and after irradiation. Sodium sulfite and sodium thiosulfate (except sodium thiosulfate in a neutral medium) seem to protect riboflavin against photolytic decomposition. Riboflavin is attacked by permanganate. The products formed resembled those formed on photolysis. The formation of 27 CX seems to be favored. With hydrogen peroxide, a 54 KI spot and a series of 15–25 spots, with a yellow or bluish-purple fluorescence, are formed. The formation of 27 CX and lumiflavin by alkaline photolysis is inhibited by the presence of hydrogen peroxide.

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