Zeitschrift: Archives des sciences [1948-1980]
Herausgeber: Société de Physique et d'Histoire Naturelle de Genève
Band: 13 (1960)
Heft: 9: Colloque Ampère

Artikel: Electron spin resonance spectra of riboflavin and its complexes
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DOI: https://doi.org/10.5169/seals-738584

Electron Spin Resonance Spectra of Riboflavin and its complexes

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Introduction.

Riboflavin and the flavoproteins play an important role in the basic processes of life [1]. The biological activity of these and other substances seems to depend on their ability to undergo various electron transfer processes [2]. There is evidence that in the flavin compounds these processes involve the formation of charge transfer complexes and free radicals, which must therefore be the active forms of the compounds. In this work, the techniques of electron spin resonance were used to study these forms, in the hope of obtaining further clues to their functions in living systems.

Spin resonance spectra of several substances containing riboflavin-5-phosphate were observed before and after irradiation with visible and ultraviolet light. The investigation of these materials was suggested by the observation of Isenberg and Szent-Gyorgyi [1] that dissolved riboflavin-5-phosphate forms a charge transfer complex with tryptophan or other compounds having the basic indole structure. Concentrated solutions of the complex appear red. The visible absorption band of these solutions coincides with that of the riboflavin semiquinone identified by Michaelis and his co-workers [3, 4, 5] as the intermediate product in the two-step reduction of riboflavin, which hitherto could only be stabilized at low temperatures [1], or in very acid solution [3].

It is assumed by Isenberg and Szent-Gyorgyi [1] that the riboflavin molecule takes up one electron from the tryptophan and then combines

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** Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

with it to form the complex. A dynamic equilibrium between the complex and a small number of dissociated free radicals of riboflavin and tryptophan is postulated to explain the steady concentration of free radicals indicated by the spectral evidence. This is in accord with the ideas of Hausser and Murrell [6] on stabilization of free radicals by complex formation. The equilibrium may be represented by the cyclic equation:

$$\begin{aligned} &(a) \quad R + T \rightleftarrows R^- + T^+ \\ &(b) \quad R^- + T^+ \rightleftarrows [R^- T^+] \\ &(c) \quad [R^- T^+] \rightleftarrows R + T \end{aligned} \tag{1}$$

in which $[R^- T^+]$ represents the charge transfer complex. The interesting possibility is raised by these authors [1] that stable riboflavin free radicals formed at neutral *p*H and room temperature play an important role in energy transfer in the living cell.

Isenberg has calculated that a small spin resonance signal should be expected from the complex itself [7]. There is a slight tendency for the donated electron to hop back and forth between the two partners of the complex, as in Equation 1 *(c)*. Thus the donated electron will tend to have its spin antiparallel, so that in first approximation there should be no spin resonance signal at all. However, in second approximation, the donated electron on the riboflavin may flip its spin and become uncoupled from its previous electron partner. This represents the triplet state of the complex. In this state there should be a spin resonance signal. Since each unpaired electron has another unpaired electron about 3.5 Å away [7], there should be tremendous dipole-dipole broadening. Using Van Vleck's formula [8], Isenberg [7] calculated that the half width of the spin resonance line to be expected from the complex is about 400 gauss.

The present study was undertaken in an attempt to observe and study this expected broad signal, and to investigate its dependence on such parameters as light, moisture, and oxygen. The spin resonance signal actually observed did not correspond with that expected from the triplet state of the complex. Although a completely satisfactory interpretation is not yet available, a number of interesting and unexpected results were obtained.

Experimental Methods.

Electron spin resonance spectra were observed for solid samples of riboflavin-5-phosphate, riboflavin-tryptophan complex, and riboflavin-

serotonin complex. The spectrometer employed a transmission cavity and bolometer detection. The derivative signal detected by the spectrometer was fed into an integrator whose output was recorded as an absorption line on a strip chart recorder.

The absorption line of each material consisted of a single broad, unresolved peak, roughly Gaussian in shape. The widths of the lines at half-power and at maximum slope are listed in Table 1. The ratios of these two widths (Table 1) shows that the line shapes are more closely Gaussian than Lorentzian.

TABLE 1.
Line widths.

	(a) Width at Half Power (gauss)	*(b)* Width at Maximum Slope (gauss)	*(c)* Ratio *(a)/(b)*
Riboflavin	23.4 ± 0.4	17.4 ± 0.2	1.34
Riboflavin-Tryptophan . .	23.7 ± 0.5	16.3 ± 0.5	1.45
Riboflavin-Serotonin . . .	25.3 ± 0.4	18.5 ± 1.0	1.37

Ratio for a Gaussian curve = 1.18.
Ratio for a Lorentzian curve = 1.73.

A value of 2.0040 ± 0.0001 was obtained for the *g*-value of the riboflavin-5-phosphate free radical, the only *g*-value measured. The value is based on the displacement of the resonance maximum from that of diphenyl picryl hydrazyl.

To obtain the number of unpaired spins in a given sample, the area of the absorption line was compared with that of a standard sample of diphenyl picryl hydrazyl. The smallest signal observable with this apparatus corresponded to about 10^{14} spins, or about one spin per 10^6 molecules of a 100 mg sample containing about 10^{20} molecules.

Since the sample cavity did not permit the use of water, only dry samples were used. The riboflavin-tryptophan complex was prepared by dissolving equimolecular quantities of the constituents in triple distilled water, and evaporating to dryness in air, at room temperature, under ordinary room light. This complex crystallizes in large red plates. X-ray

analysis shows that its crystal structure differs from that of either riboflavin or tryptophan [7]. The riboflavin-serotonin complex was prepared similarly. To determine the effects due to separate constituents, or to possible impurities, separate samples of riboflavin-5-phosphate and of tryptophan were prepared by dissolving each material, and evaporating to a dry solid. Measurements were also made on solid stock samples of riboflavin-5-phosphate and tryptophan.

The concentration of unpaired electron spins in each sample is given in Table 2. No signal was obtained from tryptophan. However, the stock sample of riboflavin-5-phosphate contained about the same number of unpaired spins as either of the air-dried complexes. Thus, riboflavin-5-phosphate itself gives a resonance signal. It is also striking that the air-dried riboflavin-5-phosphate contained many more unpaired spins than either complex. It appears that wetting and drying the material influences the formation of free radicals.

TABLE 2.

Spins per 10^6 molecules, before and after ultraviolet exposure.

Material	Before U.V.	After U.V.
Tryptophan (stock)	less than 1	not tried
Tryptophan (air dried)	less than 1	not tried
Riboflavin (stock)	6	14
Riboflavin (air dried)	100	250
Riboflavin-Tryptophan (air dried) . . .	5	50
Riboflavin-Serotonin (air dried)	8	45

All of these samples except tryptophan were then exposed to ultraviolet radiation in the range of 3000-4000Å, with an intensity of 170 ergs mm^{-2} sec^{-1} for 5 hours. As shown in Table 2, additional free radicals were formed during this exposure.

The work of Commoner and Lippincott [9] has shown that oxygen influences free radical formation in solution of FMN (flavin mononucleotide), a compound having the flavin structure in common with riboflavin. Subsequent experiments were therefore directed toward investigating more carefully the influence of three factors, air, light, and water, on free radical formation in riboflavin and its complexes.

Measurements were made on solid samples of riboflavin-5-phosphate, riboflavin-tryptophan, and riboflavin-serotonin, prepared by evaporating their solutions to dryness under four different sets of conditions: (1) in vacuum, exposed to room light, (2) in vacuum in the dark, (3) in air, in the room light, and (4) in air in the dark. During vacuum evaporations, the pressure was reduced to 1 micron. All samples were then exposed to air, and tested in the spectrometer. The results are summarized in Table 3, and discussed below.

Effects of visible light on free radical formation.

The highest concentration of electron spins was found in the two solids dried in the room light in vacuum. Thus free radical production during drying of these materials depends strongly on light exposure. In accord with the preliminary results of Table 2, dried riboflavin-5-phosphate was considerably more sensitive to light than the riboflavin-tryptophan complex.

The color changes associated with light exposure in vacuum-dried samples (Table 3), illustrate the usual shift in optical absorption toward the red when free radicals are formed. In samples dried in air in the room light, the color was not uniform. The portions which showed a shift in color were those in surface layers, which received a stronger light exposure during drying. These also contained more free radicals.

The smallest concentrations of unpaired spins were observed in the two solids dried in the dark in vacuum. The production of free radicals during drying must therefore require the presence of either light or air. Few radicals are formed when both are absent.

Effects of air on free radical formation.

The effects of exposure to air during drying of the samples may be determined by reference to Table 3. A small number of free radicals are formed when air is present during drying of samples in the dark. A color change is also associated with the presence of air during drying in the dark. However, the observed effect is small, and may therefore be due to stray light to which these materials are very sensitive.

For samples dried in the light, the presence of air during drying inhibits or reverses the formation of light-induced free radicals. For

instance, the free radical concentration in riboflavin-5-phosphate decreases from 1100 per 10^6 molecules for the sample dried in vacuum in the light to 100 per 10^6 molecules for the sample dried in air in the light (Table 3). The quenching effect of air is even stronger in riboflavin-tryptophan.

TABLE 3.

Spins per 10^6 molecules, and colors of solids dried under various conditions.

	Riboflavin	Riboflavin-Tryptophan	Riboflavin-Serotonin
Dried in vacuum, room light	1100 (Brown)	150 (Blood-Red)	not tried
Dried in vacuum, dark	8 (Orange)	3 (Rust)	not tried
Dried in air, room light	100 (Mixed) 270 (Brown Portion) 18 (Orange Portion)	5 (Mixed) 6 (Blood-Red Portion) 2 (Rust Portion)	8 (Yellow-Brown)
Dried in air, dark	18 (Mixed)	9 (Mixed)	4 (Yellow-Brown)

All measurements described above, including those on vacuum-dried solids, were made while the samples were in air. Since the concentrations of free radicals in vacuum-dried solids and air-dried solids were very different, although both were measured in air, it is apparent that the air introduced after drying does little to reverse the effects produced by drying the samples in vacuum.

The effects of removing the air from samples containing many light-induced radicals were studied. Solids dried in air and light were first exposed to ultraviolet for fifteen hours. A large number of free radicals were formed (Table 4). The sample containers were then evacuated down to a pressure of one micron. No change was observed in the number of free radicals (Table 4), or in the color.

In summary: The presence of air during drying in the dark, has little effect in producing free radicals. Air introduced after drying the sample in vacuum has little effect. Removal of the air above a dried sample

does not affect free radicals already present. When a drying sample is exposed to light and air simultaneously, fewer free radicals are formed than under light alone.

TABLE 4.

Effect of water, and air on light-induced free radicals
(spins per 10^6 molecules).

	Riboflavin (Stock)	Riboflavin (Dried in Air)	Riboflavin-Tryptophan	Riboflavin-Serotonin
U.V. 5 hours	14	250	50	45
H_2O and U.V. 5 hours .	170	150	20	not tried
U.V. 15 hours	not tried	700	90	70
U.V. 15 hours, then wet and dried in dark . .	not tried	8	5	4
U.V. 15 hours, then evacuated to 1 micron .	not tried	700	90	70

EFFECTS OF WATER ON FREE RADICAL FORMATION.

The influence of water on light-induced spin resonance signals was studied for *(a)* water present before the light exposure, *(b)* water added after the light exposure, and *(c)* water present during the exposure. The discussion of *(b)* is essential to understanding *(c)*.

(a) In the preliminary experiments, it was found that a stock sample of riboflavin-5-phosphate forms a small number of free radicals under light exposure, and that the action of light is strongly enhanced by wetting and drying the solid before or during light exposure (Table 2). Thus wetting and drying the riboflavin-5-phosphate sample increases the number of unpaired spins produced by the light exposure.

A comparable experiment could not be performed with the two complexes, because they do not form if the constituents are mixed mechanically without water.

(b) Solid samples of the three materials, riboflavin-5-phosphate, riboflavin-tryptophan, and riboflavin-serotonin, all dried in air in the light, were irradiated with ultraviolet for 15 hours. They were then dissolved in triple distilled water, and dried in air in the dark. The free

radical concentration in each solid before and after the addition of water is given in Table 4. It is clear that in the presence of water the free radicals formed by a previous light exposure decay rapidly.

(c) Solid samples of riboflavin-5-phosphate from stock, and air-dried solid samples of riboflavin-5-phosphate, riboflavin-tryptophan, and riboflavin-serotonin, were exposed to ultraviolet for five hours. Other samples of these materials received the same ultraviolet exposure after being mixed with water to form a paste. The latter samples dried during the exposure. The concentrations of free radicals in the eight samples are recorded in Table 4. Riboflavin-5-phosphate from stock showed a strong increase in free radical concentration when exposed in an initially wet state. As shown above wetting and drying this material sensitizes it to light-induced formation of free radicals. The other solids showed a smaller concentration of unpaired spins when exposed in the wet, as compared to the dry state. This is because water decreases the lifetimes of the light-induced free radicals. Some radicals were produced by light after the samples were dry.

Decay of light-induced free radicals.

The decay of free radicals was observed in air-dried samples of riboflavin-5-phosphate and riboflavin-tryptophan after exposure to visible and ultraviolet light. The ultraviolet intensity was the same as that previously used. A visible light intensity of 1400 ergs mm^{-2} sec^{-1} from a 60 watt tungsten source was used in these exposures.

The decay curves for riboflavin-5-phosphate are depicted in Figure 1. A half-life of about a week is observed for the light-induced free radicals when stored in the dark. After four weeks, 7% to 11% of the radicals remain. The long lifetime accounts for the fact that stock samples of riboflavin-5-phosphate contain free radicals after being kept in the dark for some time (Table 2).

The decay curves for the riboflavin-tryptophan complex (fig. 2) show that the complex is less sensitive to visible light than to ultraviolet. The number of free radicals produced by visible light is only five times the minimum number found in unexposed samples vacuum-dried in the dark (Table 5).

The ultraviolet-induced free radicals have a half life of about 4-½ hours (Curve B). The decay becomes imperceptibly slow after two days with

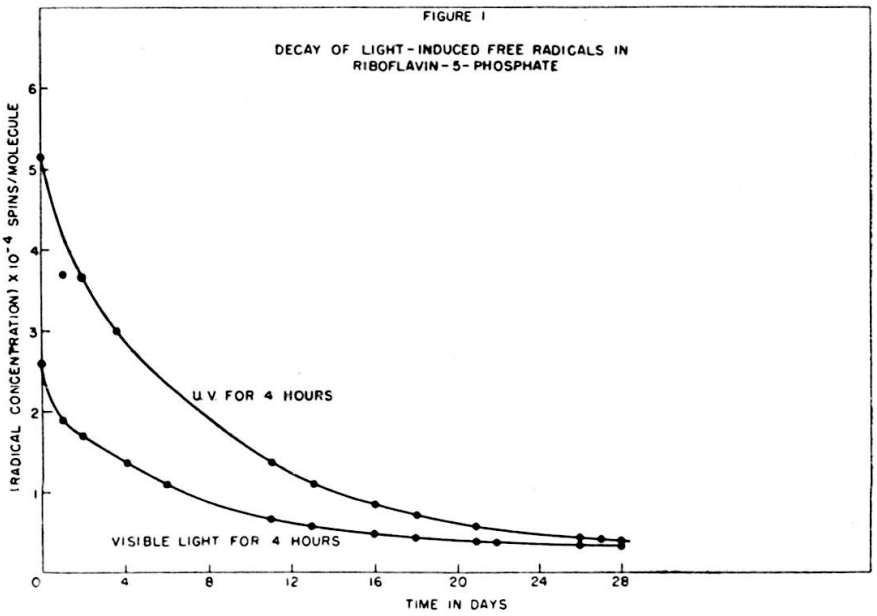

Fig. 1.

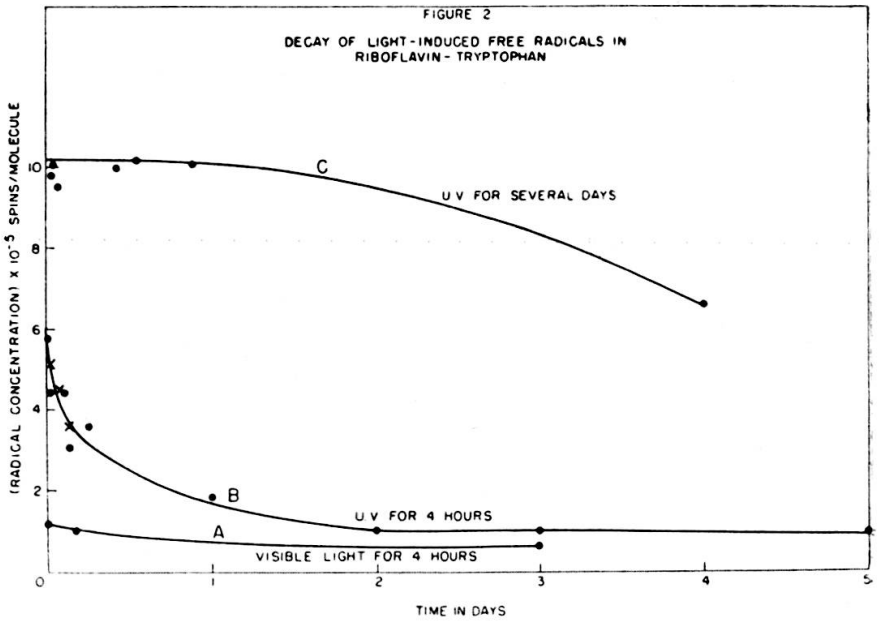

Fig. 2.

about 16% of the free radicals remaining. Therefore, samples of riboflavin-tryptophan complex kept in the dark for some time after light exposure will still have free radicals.

Curve C represents the decay of free radicals after an ultraviolet exposure lasting several days. The shape of this curve indicates that the radicals formed by the extended exposure represent a different, more stable species.

Discussion.

The low broad signal to be expected from the triplet state of the riboflavin-tryptophan charge transfer complex was not observed. Instead, the signal obtained from this complex had a half-width of 25 gauss, and sufficient intensity to obscure the other signal if it had been present. Similar signals were obtained from air-dried riboflavin, and from riboflavin-serotonin complex. All of these appeared to be light-induced.

Although the observed light-induced signals cannot be due to the triplet state of the complex, they might arise from the free radicals formed by dissociation of the complex. Thus, light may shift the equilibrium represented by Eq. 1, to favor an increased concentration of such free radicals. The shift toward red in the optical absorption of these materials after exposure to light gives evidence of increased electron mobility in the molecule, which one would expect when a free radical is formed. The observed long lifetime of the light-induced radicals during dark storage indicates that a very long dark time may be required before the original equilibrium condition is restored after light exposure.

Riboflavin may form a self-complex. Pullman and Pullman [10] have calculated the energy levels of the highest occupied and lowest empty molecular orbitals of riboflavin. Their results indicate this molecule should be a moderate electron donor, and a good electron acceptor, so that self-complex formation is possible. In addition, self-complexes of the related compound FMN have been experimentally observed [11]. Thus, one may assume the reactions of Eq. 1 to occur, with T representing a riboflavin molecule.

It was found that the light-induced spin resonance signal was much stronger for riboflavin than for its complexes with tryptophan and serotonin. Thus complex formation with an unlike molecule may serve to stabilize riboflavin against formation of free radicals under light.

The molecular structure of riboflavin-5-phosphate is shown in figure 3. It incorporates the isoalloxazine structure which consists of the unsubstituted system of aromatic rings. The π electron system of riboflavin is identical with that of isoalloxazine.

Pullman and Pullman [12] have calculated the distribution of the unpaired electron in the riboflavin free radical involved in complex formation. They found the electron to be partly localized on the 10-position nitrogen, and partly distributed throughout the rest of the isoalloxazine ring structure. If one assumes that exposure to light shifts the equilibrium of Eq. 1, then the observed spectral line is due to the same free radical described by these authors.

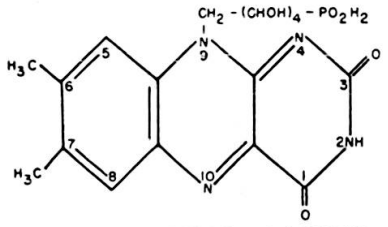

Fig. 3.

The Gaussian shape of the spectral line shows that anisotropic broadening due to magnetic dipole-dipole interaction must occur. This may indicate interaction of the unpaired electron spin with that of a nitrogen nucleus on which it is partly localized. There is some evidence of exchange narrowing, as the line is not perfectly Gaussian.

The photodecomposition products of riboflavin include unsubstituted flavin (6, 7 dimethyl isoalloxazine), and 9-methyl flavin [3]. Thus, under light exposure, the bond between the flavin and the sugar side chain is ruptured. The observed spectrum may be due to an unpaired electron introduced somewhere in the isoalloxazine structure during photodecomposition. The free radical thus formed may or may not have the same distribution of unpaired electron density as that described above.

It is not certain whether light irradiation of riboflavin produces oxidation or reduction. In this connection, Commoner and Lippincott [9] have observed that free radicals are formed in solutions of FMN exposed to light under anaerobic conditions. The observed line shape is identical with that obtained on chemical reduction of these solutions. Since riboflavin and FMN have the flavin structure in common, with different side chains substituted in the 9-position (fig. 3), these results indicate that our signal is probably due to a reduction product of riboflavin.

One must also consider the possibility that the observed resonance signal is not due to a free radical, but rather to some other configuration

involving an unpaired electron spin. Since riboflavin is a photoconductor, we may be observing electrons excited into a conduction band by light absorption. Ingram and Allen [13] attribute the spin resonance signals obtained from some organic molecules after ultraviolet irradiation to such a photoconductive effect. However, in Isenberg's view [7], the high electrical resistance of riboflavin even under light indicates a short mean free path for the conduction electrons, so that the signals would be broadened beyond detection.

The effects of exposure to air on free radical formation are due to one or more constituents of the atmosphere, possibly water or oxygen. There is some evidence that oxygen influences this reaction. The work of Commoner and Lippincott [9] has shown that the reduction of FMN solutions by light under anaerobic conditions is instantly reversed when oxygen is introduced into the system. The reduction does not proceed at all if oxygen or air is present. The reaction may be represented by the equation.

$$\text{FMN} \underset{O_2}{\overset{\text{light}}{\rightleftarrows}} \text{FMN free radical} \qquad (2)$$

in which the forward reaction represents a reduction, and the reverse reaction oxidation. These results may be correlated with our observation that air inhibits the formation of light-induced free radicals in solid riboflavin. However, the quenching action of air is far less effective for the riboflavin solids than it is for the FMN solutions. This may be attributed to the higher mobility of molecules in the liquid, as compared with the solid phase.

The observed lifetime of light-induced FMN free radicals in anaerobic solution is about 50 minutes [9]. This is much shorter than the lifetime of light-induced free radicals in solid riboflavin. Again, this may be attributed to differences in mobility of the molecules.

Since water assists both free radical formation and free radical quenching, by increasing the mobility of the riboflavin molecules, perhaps the effects due to air can be attributed to the presence of water, rather than oxygen. It is also possible that light-induced formation of free radicals during drying depends on the crystal form, which is in turn influenced by the rate of evaporation of the water. In that case, the pressure of air, rather than its constituents, would inhibit radical formation.

Although in general water accelerates the decay of free radicals, light-induced radical formation in riboflavin is more rapid during or after drying than it is in stock samples of riboflavin which have not been wet. This effect is not well understood. Perhaps bound water plays a role in free radical formation. It is also possible that the water contains a reducing agent even after triple distillation.

The author wishes to thank Drs. Irvin Isenberg and Albert Szent-Gyorgyi for suggesting the problem. The assistance and cooperation of Drs. John S. Kirby-Smith, Malcolm Randolph, John Jagger, Mr. Oakley Crawford, and many other colleagues at Oak Ridge National Laboratory is gratefully acknowledged. The author is also indebted to Drs. Saul Gurevitz, J. Warren Blaker, and Neil Wotherspoon for valuable discussions on this problem.

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Résumé.

Nous avons observé le spectre de résonance électronique du riboflavin-5-phosphate et celui de ses complexes avec le tryptophan et le serotonin. En gros, les spectres étaient de forme gaussienne, d'une largeur de raie d'environ 25 gauss, le facteur g valant 2,0040 $\pm$ 0,0001. Il est apparu que les signaux étaient induits par la lumière avec des intensités qui dépendaient de la présence d'air et d'eau. Durant la période dans l'obscurité suivant l'irradiation lumineuse, les signaux

diminuaient lentement jusqu'à environ 10% de la valeur initiale au bout de quatre semaines.

Nous avons montré que le comportement de ces systèmes en présence d'air est semblable à celui des radicaux libres FMN en solution en présence d'oxygène.

Les signaux observés ne peuvent être attribués à l'état de triplet des complexes du riboflavin, puisqu'on s'attend à ce que ces derniers donnent lieu à de larges signaux d'environ 400 gauss.

Deux autres possibilités sont envisagées, à savoir que le spectre soit dû à des radicaux libres ou à des effets de photoconductivité.