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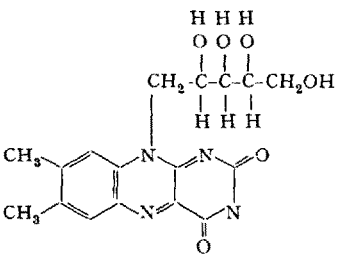
Photochemistry of Riboflavin

By G. OSTER*, JUDITH S. BELLIN*, and B. HOLMSTRÖM**

I. *Introduction.* Riboflavin is of great interest to photochemists first of all because its structure was established by photochemical means[1,2], and secondly, because it has been implicated in many naturally-occurring photochemical processes (see below). Of still further interest is the fact that riboflavin is a unique photochemical agent because it can act as a photosensitizing agent and the light-excited molecule itself can also act as an electron donor[3]. This is in contrast to the interpretation given by other workers in the field[4–6] who claim that light-excited riboflavin can split water by the action of visible light. It is the purpose of the present paper to review the photochemical properties of riboflavin and to examine critically the present status of this problem.

Riboflavin is of practical importance as a photochemical agent because it has been shown to participate in the photo-induced production of off-flavors[7] and the destruction of vitamins[8] in milk as well as in beer[9]. Most botanists[10–12] regard riboflavin as the sensitizer in phototropism (for review see [13]). Riboflavin is also present in the retina of many mammals in high concentrations and even in the crystalline state[14]. Its role in the visual process is still obscure, however. It has been shown that riboflavin enhances bacterial bioluminescence[15] and indeed many workers associate bioluminescence with the fluorescence emission of riboflavin (see, however, [16]). A chemiluminescence accompanying photosynthesis may also be due to riboflavin emission[17].

II. *Structure and Spectral Characteristics.* The structure of riboflavin has been established by KARRER[18] (for review with extensive bibliography see [19]). The ribose is attached *via* its number 1 carbon atom to the number 9 nitrogen in the isoalloxazine ring system (I). This yellow pigment at pH 7 absorbs maximally (Figure 1) in the blue ($\lambda_{max} = 445\,m\mu$, $\varepsilon_{max} = 1.25 \times 10^4$) and in the near ultraviolet ($\lambda_{max} = 373\ m\mu$, $\varepsilon_{max} = 1.05 \times 10^4$) (Figure 1). There are also maxima in the far ultraviolet at 260 and 224 mμ. Riboflavin occurs in biological systems as the 5′ phosphate (FMN) and in combination with adenosine-5′-phosphate as flavin adenine dinucleotide (FAD). As FMN it is the prosthetic group of many flavoprotein enzymes. The presence of a single phosphate group does not appreciably affect the absorption spectrum of riboflavin but considerably enhances its solubility in water. The visible and near ultraviolet peaks of FAD are practically the



I

Riboflavin (6,7-dimethyl-9-(D-1′-ribityl)isoalloxazine)

* Department of Chemistry, Polytechnic Institute of Brooklyn (N.Y., U.S.A.).

** Present address: Institute of Physical Chemistry, University of Uppsala (Sweden).

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same as those of riboflavin but the absorption peak at 260 mμ is greater than that of riboflavin[20].

Riboflavin exhibits a strong green fluorescence when excited by near ultraviolet or by blue light and this property is frequently used for its assay. The luminescence of riboflavin can also be induced by rapid oxidation (chemiluminescence[21]). The ultraviolet-induced fluorescence emission has a maximum at 534 mμ (Figure 1). The quantum yield of fluorescence at pH 7 is 0.26 and the lifetime of the first singlet excited state is 10^{-8} sec. Excitation with blue light or with near ultraviolet yields the same fluorescence. This shows that the absorption maximum at 373 mμ corresponds to transitions to the second singlet excited state which undergoes rapid radiationless internal conversion to the first singlet excited state followed by emission (Figure 2). The fluorescence yield of FMN is the same as that of riboflavin but that of FAD is considerably lower[22], and the reduced flavins are non-fluorescent.

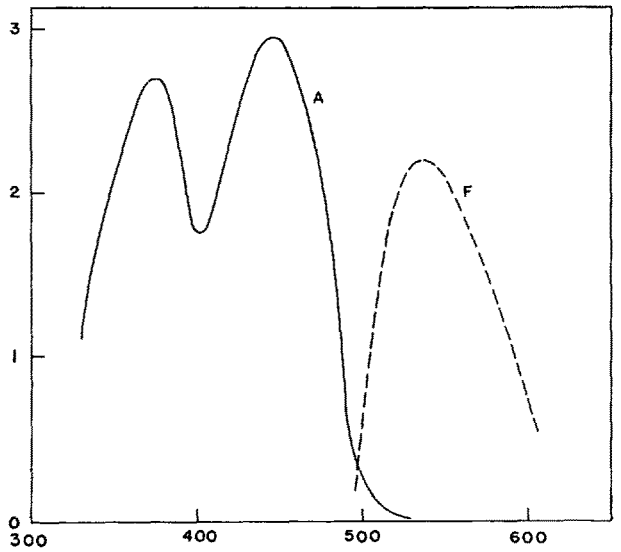

Fig. 1. Spectra of absorption, A, (relative absorbancies) and fluorescence, F, (relative intensities) of riboflavin at pH 7.

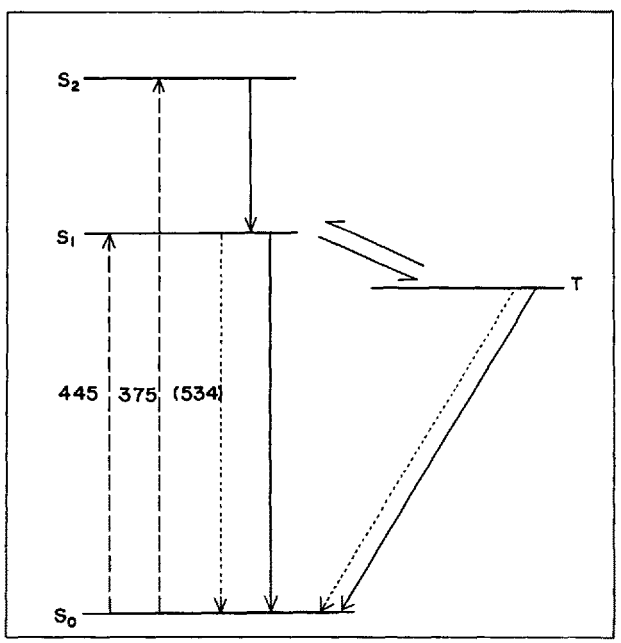

Fig. 2. Diagram of electronic energy levels of riboflavin and possible transitions between levels. Numbers correspond to spectral maxima in mμ. Absorption (omitting the levels corresponding to the 260 and 224 mμ peaks) is indicated by dashed arrows. Dotted arrows indicate luminescence, and unbroken arrows indicate non-radiative transitions. Fluorescence occurs from the first excited singlet (S_1) to the ground level (S_0). Phosphorescence at room temperature (α-phosphorescence) occurs from the metastable state T to S_0 *via* S_1, and at low temperatures (β-phosphorescence) directly from T to S_0. The fluorescence maximum is at longer wavelengths than the absorption maximum (Stokes law[28]).

The fluorescence efficiency of a riboflavin solution is practically independent of pH in the range from neutrality to pH 3. In alkaline media or below pH 3 the fluorescence is weak[23]. The cationic and anionic forms of riboflavin do not fluoresce but the dipolar species present at intermediate pH values does. The non-fluorescent form is in equilibrium with the fluorescent one and this equilibrium is displaced by a change of pH[24]. There is a lack of correspondence between this pH dependence of the fluorescence and the acid-base titration curves of riboflavin[20,25] as well as the changes of absorption spectra with pH[26]. This is reminiscent of the observed discrepancy between the pH dependence of fluorescence and that of absorption of 1-naphthylamine 4-sulphonate[27]. This phenomenon is due to the fact that there is a different dissociation constant for the ground state and for the excited state of the molecule[28]. The light-excited species may be expected to give up its proton during its lifetime in the excited state[28,29].

The fluorescence of a solution of FMN is quenched by the addition of purines or adenosine[24]. This probably explains the fact that the fluorescence of FAD is much weaker than that of FMN. Complexing either FMN or FAD with a protein, as in most flavoproteins abolishes the fluorescence. This type of fluorescence quenching is not necessarily associated with changes in absorption spectra, and arises because the complex formed is non-fluorescent. Heating the solution dissociates the complex, and the fluorescence therefore increases with temperature[24]. Fluorescence quenching of riboflavin can also occur by a diffusional encounter between a

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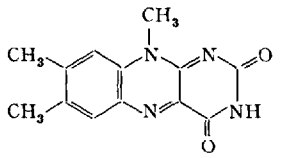
riboflavin molecule in the first singlet excited state and metal ions (e.g., mercuric, ferric, ferrous, etc.[30]) or iodide ion. For this collisional quenching an increase in temperature results in a decreased fluorescence, since encounters are more frequent at the higher temperatures[24,31].

Complex formation of FMN with indoles (tryptophane and serotonin) on the other hand, is manifested by a large shift in absorption spectra[32,33], possibly due to the formation of charge transfer complexes.

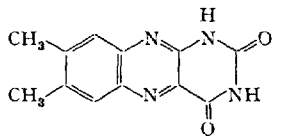
Riboflavin, like many other fluorescent substances, exhibits a phosphorescence in rigid media[34]. At room temperature the phosphorescence has the same spectral characteristics as the fluorescence but has a duration of several seconds (α-phosphorescence, Figure 2). At low temperatures (below about 100°C) where the thermal activation to the first excited singlet cannot occur, an orange phosphorescence (β-phosphorescence) is observed. An orange emission is also observed in ice at 0°C[35]. This phenomenon has been attributed to the particular ability of ice to induce singlet-triplet transitions. Similar phenomena in ice are also observed with other dyes[35] including auramine O, a diphenyl methane dye capable of internal rotation[36]. We interpret the phenomena as being due to decreased internal rotational mobility when the dye is adsorbed to the ice crystals (compare [37]).

Removal of the ribose side chain by methods described below yields lumiflavin, Lf (II), or lumichrome, Lc (III). The solubility of these substances differ



II

Lumiflavin (6,7,9-trimethylisoalloxazine)



III

Lumichrome (6,7-dimethyl alloxazine)

markedly from that of riboflavin. The yellow colour of the flavine is retained by Lf since it is an isoalloxazine derivative. With Lc (an alloxazine derivative), however, the colour is pale yellow. In chloroform lumiflavin has absorption maxima at 445, 385, and 265 mμ[1] and lumichrome has absorption maxima at 385, 350, and 260 mμ[2]. This latter compound has a blue fluorescence. By fusing alloxazine with alkylating agents it was demonstrated that the spectra of the resulting compounds were those characteristic of flavins[38]. An altered isoalloxazine, namely, deuteroflavin, has been postulated ([1], compare [39] and [40]). There is spectral evidence of the existence of such a species absorbing maximally 400 mμ[3].

III. *Photochemical Reactions.* Riboflavin is notoriously unstable under illumination with visible light. Under anaerobic conditions most dyes are comparatively much more light stable. Many dyes can undergo rapid photoreduction in the presence of a mild reducing agent such as ascorbic acid, or glutathione (for review see [41]). Riboflavin is unique, however, in that it will undergo photoreduction even in the absence of an added electron donor.

In the presence of oxygen riboflavin (Rf) is irreversibly decomposed by light to give lumiflavin (Lf) and lumichrome (Lc)[1,3,5], as well as fragments of the ribytyl side chain[31]. Riboflavin when anaerobically photobleached and subsequently aerated is converted in part to Lf and Lc. Repeated 'cycling' in this manner results in the production of progressively more Lc at the expense of Rf and Lf[5]. It was further noticed that such a cycled solution photobleaches more rapidly than did the original solution[3]. This new species which is more light sensitive than the original riboflavin is considered to be deuteroflavin (Df) ([3], compare [1]). Deuteroflavin could arise from the oxidation of leuco-deuteroflavin (DfH_2), a compound formed by buffer catalysis from light-excited riboflavin. The rate of fading of riboflavin is increased with the square of the buffer concentration. This occurs both in the presence[42] and in the absence[3] of oxygen, and at acid[42] or neutral[3] pH. Of further interest is the fact that the quantum yield of photoreduction of riboflavin increases with increasing initial riboflavin concentration.

If an anaerobically-photobleached solution of riboflavin is allowed to stand in the dark for several days Lf is formed. This dark reaction is accelerated if the solution is rendered alkaline.

The overall scheme for these complicated series of reactions can be represented as follows[3]

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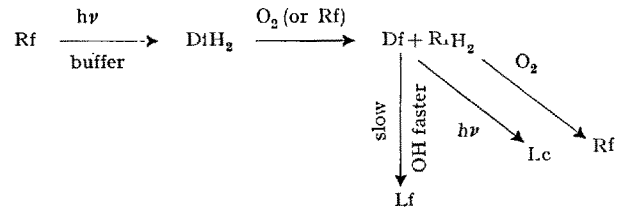

Trace amounts (of the order of $10^{-6}M$) of potassium iodide appreciably decrease the quantum yield of the photobleaching of riboflavin. Such low concentrations of KI do not affect the fluorescence of riboflavin, in fact, about one hundred fold greater concentration of KI is required to quench the fluorescence. The lifetime of the fluorescing species (S_1 of Figure 2) is 10^{-8} so that a diffusing quencher molecule (iodide ion) could encounter this excited species only if its concentration were greater than about $0.1M$. Since micromolar concentrations of KI are sufficient to retard the photobleaching we calculate that the chemically reactive species (T of Figure 2) is long-lived with a lifetime of about 10^{-3} sec. Further indications that T but not S_1 is the reacting species is the fact that increasing buffer concentration increases the rate of photofading although it does not affect the fluorescence intensity of the solution. The reaction between buffer and T may consist of a series of intermediate steps (see [42]).

Nowhere in the above reaction scheme was it necessary to postulate the direct participation of water in the photo-decomposition of riboflavin. In contrast, some other workers[4–6] have postulated the decomposition of water in this reaction. Such a hypothesis has previously been put forth to explain the production of hydrogen peroxide in the illuminated dye solutions under allegedly oxygen-free conditions[43]. The splitting of water by this means has been vigorously contested on energetic grounds[44] although the workers with riboflavin consider water to be complexed with the pigment in a special way[4–6]. Even if 'bound' water were to have less stability than ordinary water molecules, in order for the reaction to continuously proceed the additional energy of binding would have to be overcome.

In anaerobic solutions riboflavin (but not lumiflavin) sensitizes the oxidation of methionine and by regenerating the dye electrolytically, comparatively large amounts of methionine are consumed[4,5]. It is presumed[4,5] that electrons for this reaction are supplied by water. We, however, have found that methionine can act as the electron donor for the photoreduction of methylene blue, among other dyes. This is most easily demonstrated by dye-sensitized photopolymerization of vinyl monomers[45]. In this reaction methionine acts just as does ethylene diaminetetraacetic acid (EDTA)[46] and other tertiary amines[47,48]. The amine is consumed in the reaction[47] to give formaldehyde[49,50] among other products.

When an anaerobic solution of riboflavin in which a platinum electrode is immersed is illuminated with visible light, a potential of about 700 mV is produced. With a high resistance measuring device (e.g., a vacuum voltmeter) the potential remains constant while the system is illuminated since very little current is drawn. If, however, the measuring device draws current of the order of milliamperes the potential rapidly falls. This means that riboflavin is destroyed in the photochemical process and cannot be regenerated in the absence of an added electron donor. If the experiment (in which large currents are drawn) is carried out in the presence of an added electron donor (such as EDTA) the potential is maintained and no riboflavin is consumed until all the electron donor is consumed.

In contrast to those workers[4–6] who suppose that light-excited riboflavin obtains hydrogen from water we feel that the hydrogen atoms (and/or electrons) are obtained from its ribityl side chain. An equivalent amount of riboflavin is thereby destroyed in such photochemical reactions. The fact that this is so was demonstrated in the photopotential experiments but also is seen in reductions photosensitized by riboflavin. For example[3] (see also [30]) riboflavin will sensitize the photoreduction of silver ion or of 2,6-dichlorophenolindophenol in amounts proportional to the amount of riboflavin initially present. In the presence of an added electron donor, however, the amount of material reduced is equivalent to the amount of added electron donor. In this latter case riboflavin acts as a true photocatalyst since it is not consumed in the overall process. Here as with other dyes[51], the light-excited dye abstracts electrons from the added donor and subsequently (in a dark step) reduces the substrate (e.g. silver ion) to give regenerated dye.

Another unique property of riboflavin is its ability to sensitize polymerization in the absence of added electron donor[52]. Here again riboflavin acts as its own photoreducing agent. Riboflavin is irreversibly consumed in this reaction and the resulting polymer is of extremely high molecular weight, showing that very few initiating free radicals were produced. In this connection it is interesting to note that illuminated anaerobic solutions of riboflavin phosphate exhibit an electron spin resonance spectrum characteristic of free radicals[53].

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Riboflavin like many other dyes[54] can photosensitize the oxidization of a great variety of substrates in the presence of oxygen[55]. Such action can manifest itself as inactivation of microorganisms[55] hemolysis of red cells[55], inactivation of transforming principle[56], inactivation of tumor cells[57], and inactivation of a fungicide[58]. Of particular interest to botanists is the riboflavin-sensitized photoxidation of indole acetic acid[10]. It has been suggested that such destruction of this growth hormone is the origin of phototropism[10]. Another suggestion is that the substrate for photoxidation is the enzyme which produces indole acetic acid rather than the hormone itself[11]. Since the action spectrum of phototropism resembles that of riboflavin in the visible region but not in the ultraviolet region, it has been questioned whether riboflavin is the sensitizer for phototropism. However, light scattering by cellular material at shorter wavelengths could obscure the 375 mμ peak of riboflavin[12].

Résumé. Le spectre d'absorption, les caractéristiques luminescentes et photochimiques de la riboflavine sont présentés. L'interprétation des données expérimentales d'autres auteurs sur la décomposition photochimique de l'eau sensibilisée par la riboflavine est critiquée. Il faut distinguer deux cas: dans les réactions photochimiques qui ont lieu en l'absence de donneurs d'électrons ajoutés, la portion ribosique de la riboflavine se comporte en donneuse d'électrons et elle est détruite. Par contre, en présence de donneurs d'électrons ajoutés, la riboflavine agit comme véritable photosensibilisateur et n'est pas consumée dans l'ensemble de la réaction photochimique.

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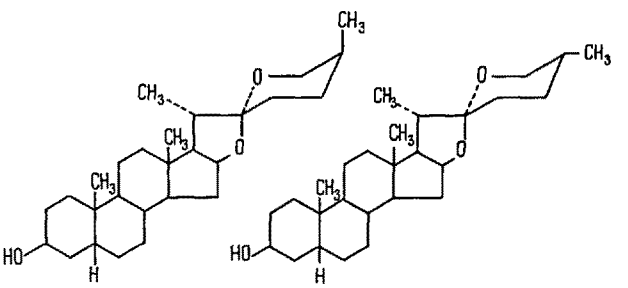
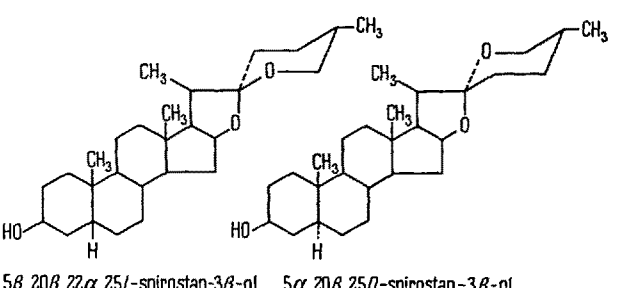
A Mnemonic for Configurations of Steroidal Sapogenins

The spiroketal side chain of steroidal sapogenins presents nomenclature and formula writing problems which remain difficult even though structural and conformational aspects are now reasonably secure. The latter have been summarized by FIESER and FIESER[1], whose clarity of presentation conceals the confusing variety of names and multiplicity of prefixes in the original literature. Resolution of the chemical problems has removed most of the naming conflicts but has left unsolved the selection of a concise systematic nomenclature for these compounds.

If we adopt the spirostan nomenclature set forth in IUPAC Definitive Rule 3.8[2], keeping in mind what we now know about structure, we may use the *D* and *L* convention for carbon 25[3].





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[2] *Definitive Rules for the Nomenclature of Amino Acids, Steroids, Vitamins, and Carotenoids*, J. Amer. Chem. Soc. *82*, 5575 (1960).
[3] This suggestion was first introduced in an unpublished manuscript by Dr. B. RIEGEL and the author, which came to the attention of Dr. M. E. WALL. He graciously recognized and adopted our convention and first published the use of *D* and *L* in this connection (see Exper. *11*, 340 (1955)).