from the ribosome under these conditions.

Finally, in addition to their high protein content, it should be emphasized that the unusually low sedimentation coefficient of these ribosomes remains a major physical-chemical property distinguishing them from other ribosomes, whether of the eukaryotic or prokaryotic variety, including the mitoribosomes of primitive eukaryotes (Kuntzel and Noll, 1967; Chi and Suyama, 1970). In most earlier studies, the sedimentation coefficient of mammalian mitoribosomes was estimated relative to the sedimentation rate of *E. coli* or extramitochondrial ribosomes in sucrose density gradients. In view of the fact that the physical properties of 55S ribosomes differ considerably from those of either of these sedimentation standards, it is interesting that the $s_{20,w}$ of nominal 55S bovine mitoribosomes, 56.3 S, is very close to the relative sedimentation coefficient, 55.4 S, determined earlier for these ribosomes in sucrose gradients (O'Brien, 1971). Thus, while it is no longer appropriate to refer to 55S ribosomes as being "the smallest," they aptly deserve the distinction of being "the slowest."

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An Investigation of the Triplet State of Flavines and Flavoproteins by Optical Detection of Magnetic Resonance†

Thomas A. Moore and Alvin L. Kwiram*

ABSTRACT: The magnetic sublevels of the lowest excited triplet state of riboflavine, deazaflavine, FMN, FAD, and alloxazine, as well as two flavoproteins have been investigated by optical detection of magnetic resonance. Lifetimes, relative steady-state populations, relative radiative rate constants, as well as zero-field splitting parameters are reported for riboflavine and deazaflavine. This double resonance technique has been used to observe and characterize phosphorescence from flavodoxin and yeast hemoglobin and is shown to be a powerful technique for investigating the triplet electronic structure of weakly luminescent biological systems.

The triplet state of riboflavine makes a major contribution to the intra- and intermolecular photochemistry of flavine (Song and Metzler, 1967; Penzer and Radda, 1967; De Kok *et al.*, 1971). Triplet riboflavine also serves as a model system for mechanistic studies of flavoenzyme-catalyzed reactions. Moreover, excited flavine is implicated in a wide range of biological processes including photodynamic action as well as phototropism wherein it may function as the photoreceptor (Foote *et al.*, 1968; Song and Moore, 1968; Thomas, 1965). For these reasons the riboflavine triplet state has been extensively investigated both experimentally,

† From the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received June 24, 1974.* This work was supported by a grant from the National Science Foundation (GP-28183X).

utilizing luminescence spectroscopy, and theoretically (Sun *et al.*, 1972). Zero-field splitting (zfs) parameters have been determined for flavines and pteridine derivatives in glasses at 77°K by electron spin resonance (esr) (Lhoste *et al.*, 1966; Shiga and Piette, 1965).

By contrast, the triplet state of flavoproteins, the usual *in vivo* form of flavines, is largely uncharacterized. The observation of luminescence from a solution of (flavo) proteins is in and of itself not normally instructive in view of the likelihood that numerous independent emission sources (either inherent sources such as amino acid residues, or impurity sources that cannot be easily separated from the protein solution) will obscure a particular emissive component. Moreover, the luminescence of interest may be weak compared to the other sources of luminescence. A weak esr signal, attributed to the FAD[1] triplet state in a D-amino acid oxidase sample, has been reported (Shiga and Piette, 1964). Unfortunately, both the luminescence technique and the esr technique are seriously limited in such studies: the former lacks resolution sufficient to discriminate against competing luminescence centers, whereas the latter lacks the necessary sensitivity. It is therefore of some interest to introduce a method which largely overcomes both limitations.

In this article we report the application of optically detected magnetic resonance (ODMR) (Sharnoff, 1967; Kwiram, 1967, Kwiram, 1972) to the study of flavine moieties. As a necessary prerequisite to the study of flavoproteins we have obtained both the magnetic and kinetic parameters for the triplet state of riboflavine and deazaflavine. Based on these data we demonstrate that the triplet state of flavoproteins can indeed be investigated in detail using ODMR, even though it would be difficult to characterize the phosphorescence from the riboflavine moiety unambiguously using conventional optical measurements. We also find that the magnetic parameters are slightly altered when riboflavine is incorporated in FAD, FMN, or the flavoproteins investigated.

Experimental Section

Riboflavine (7,8-dimethyl-10-D-ribitylisoalloxazine), FMN (flavine monoucleotide), FAD (flavine adenine dinucleotide), and alloxazine (benzo[*g*]pteridine-2,4-(1*H*,3*H*)-dione) were obtained from Sigma Chemical Co. Deazaflavine (3,10-dimethyl-5-deazaisoalloxazine), deuterated flavodoxin (*S. lividus*), and yeast hemoglobin (*C. mycoderma*) were kindly donated by Professor P. S. Song, Dr. J. Norris, and Dr. J. Vanderkooi, respectively. The flavines and derivatives were dissolved in a 1:1 mixture of ethylene glycol–water (at a concentration of less than 10^{-4} M). Approximately 0.1 mg of flavodoxin (dried material from a solution 0.5 M NaCl and 0.01 M phosphate buffer (pH 6.8) containing 6 mg of flavodoxin) was dissolved in 10 ml of 1:1 ethylene glycol–water and the solution kept at 0° until used (~12 hr). 1 ml of yeast hemoglobin (approximately 1 mg/ml, 0.1 M phosphate buffer (pH 7) was added to an equal volume of ethylene glycol and the solution kept overnight at 0° before use. The resulting solutions were degassed and sealed in a quartz tube. The samples were suspended in a slow-wave microwave structure and inserted into a liquid helium dewar. All experiments were carried out at ~1.8°K and in the absence of any externally applied magnetic fields. The phosphorescence, dispersed by a ¼ meter Jarrel-Ash monochrometer, was detected and processed through a photon counter (SSR Instruments Co.) and the usual signal-enhancing instrumentation.

In an ODMR experiment, we look for *changes* in the luminescence intensity while sweeping the microwave frequency. When the microwave frequency satisfies a resonance condition corresponding to one of the energy differences between the sublevels of the lowest triplet state, the transition alters the populations of the sublevels. This in turn affects the phosphorescence intensity, which is given by $I \propto \Sigma k_i^r N_i$, where k_i^r is the radiative rate constant for level i and N_i is the corresponding population.

In all cases the ODMR signals were shown to originate from the flavine of interest by measuring the ODMR signal intensity as a function of both the (optical) excitation and emission wavelengths. Phosphorescence from "impurities" can be effectively discriminated against in this way by excluding those signals that do not simultaneously satisfy the proper optical and microwave requirements.

The magnetic parameters were obtained in steady-state experiments under continuous optical pumping of the $S_0 \rightarrow S_1$ transition. The zero-field splitting (zfs) parameters D and E, reported herein, are relatively low resolution with some uncertainty in the third place. More precise values could be obtained by additional signal averaging.

The kinetic data were obtained from transient ODMR studies similar to those first carried out by van der Waals and coworkers (Schmidt *et al.*, 1969). The method of extracting the rate constants from such measurements is described in detail in several articles (Kwiram, 1972; Schmidt *et al.*, 1969). Deviations from the conventional technique are described below. The transient experiment consists of interrupting the exciting light and then, after a predetermined (though variable) delay (during which time the populations of the triplet sublevels are diminishing), pumping an appropriate microwave transition causing a change in phosphorescence intensity.

In addition to steady state and transient ODMR experiments, a third experimental technique is used in which the exciting light is interrupted and during phosphorescence decay the microwave frequency is swept slowly. In this case both decay time and microwave frequency are resolved along the abscissa. This technique, which we refer to as delayed ODMR, often results in greatly enhanced signal/noise since it generates population differences which may be much greater than under steady-state conditions.

Results and Discussion

Figure 1a shows a typical steady state ODMR spectrum of riboflavine. The decrease in the phosphorescence intensity is a maximum at a microwave frequency of 2.35 GHz. A similar decrease is observed at 1.21 GHz. For reasons that will become apparent below, the third transition at 1.14 GHz is not observed. Following the arbitrary convention that the lowest frequency microwave transition corresponds to the $2|E|$ splitting we calculate $|D| = 0.059$ cm^{-1} and $|E| = 0.019$ cm^{-1} in near agreement with the earlier esr results (Lhoste *et al.*, 1966).

Figure 1b shows a typical transient response that occurs when the 2.35-GHz transition is swept through rapidly (10 msec) 1 sec after the exciting light is interrupted.[2] Refer-

[1] Abbreviations used are: FAD, flavine adenine dinucleotide; FMN, flavine mononucleotide.

[2] The fraction of inversion, f, was found to be approximately 0.8. Saturation ($f = 0.5$) was assured by repetitively sweeping (at 100 Hz) over the entire inhomogeneous line width. Hole-burning experiments established a minimum line width of approximately 20 MHz.

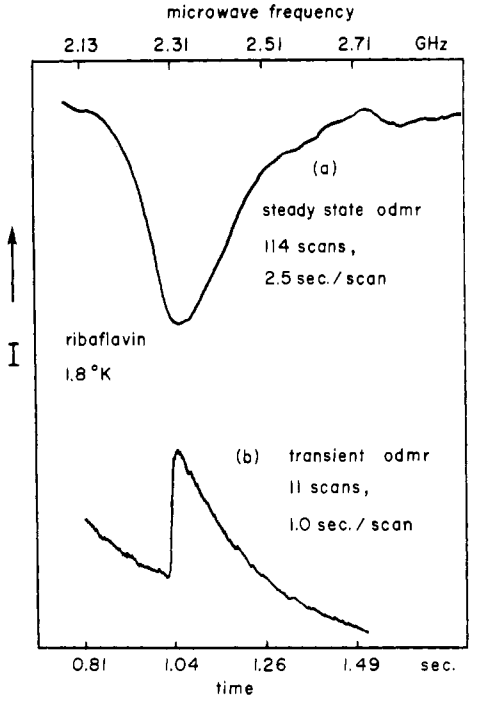

FIGURE 1: Riboflavine ODMR signals under steady state (a) and transient (b) conditions with excitation at 450 nm and emission at 605 nm; ~1.8°K. Upper abscissa refers to (a). Lower abscissa shows time after exciting light is interrupted. The sharp increase in phosphorescence is observed when the 2.0 → 2.6 GHz range is swept rapidly 1 sec after the light is interrupted. Curve (a) is 114 scans, 2.5 sec scan^{-1} and (b) is 11 scans, 1.0 sec scan^{-1}.

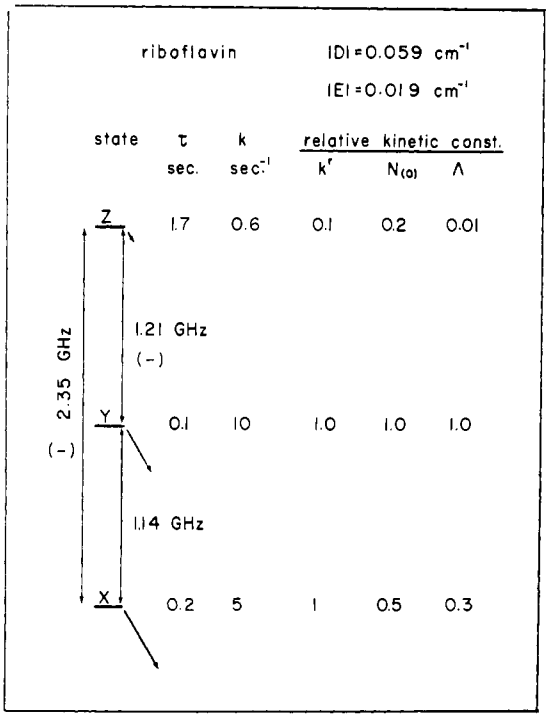

FIGURE 2: Lifetimes, relative kinetic constants, and zfs values for the lowest triplet state of riboflavine. The labels x, y and z are arbitrary. The (−) sign indicates a decrease in phosphorescence intensity when the corresponding transition is saturated in the steady-state experiment. k^r is the relative radiative rate constant, $N(0)$ is the relative steady state population, and Λ is the relative populating rate constant.

ring to the level diagram in Figure 2 it can be seen that both the 1.21- and 2.35-GHz transitions interrogate the T_z level and, indeed, a sequence of interrogations (as a function of time) by either of these transitions yields the lifetime of T_z.[3] The lifetime of T_x is calculated from analysis of the decay curve (for $t > 1$ sec in Figure 1b) generated by pumping the 2.35-GHz transition. The near degeneracy of the $T_x \leftrightarrow T_y$ and $T_y \leftrightarrow T_z$ transitions in riboflavine results in an altered population of T_x whenever the $T_y \leftrightarrow T_z$ frequency is applied. In this case k_y was calculated from the observed rate constant $k = (\frac{1}{3})(k_x + k_y + k_z)$ (Azumi *et al.*, 1966) obtained either under continuous saturation of all three transitions or from measurements at 77°K where spin–lattice relaxation is fast. The relative radiative rate constants, the steady-state population ratio, $N_x(0)/N_z(0)$, and the relative populating rate constants, Λ, are calculated in the usual way. The ratio of $N_y(0)/N_z(0)$ could not be determined in the conventional way since the $T_y \leftrightarrow T_z$ transition overlaps the $T_y \leftrightarrow T_x$ transition. The expression

$$I(t) = c[k_x{}^r N_x(0)e^{-k_x t} + k_y{}^r N_y(0)e^{-k_y t}]$$

describing the initial (first 90% when T_z makes a negligible contribution) phosphorescence decay at 1.8°K was fitted to the observed decay and $N_x(0)/N_y(0)$ extracted.

Figure 2 presents diagrammatically the riboflavine triplet sublevels with their lifetimes, relative rate constants, and steady-state populations. Since the absolute sign of D and E is not established in the coventional zero-field ODMR experiment, the ordering of the levels and the axis assignment is arbitrary to a certain extent. Since the third transition was not observed it could be either 1.14 or 3.56 GHz. We have rejected the latter choice for two reasons; first, it is incompatible with the esr results and, second, all three transitions are detected in deazaflavine and alloxazine, and the sum frequency (3.56) is not consistent with the results on these rather similar compounds.

The transient data summarized in Figure 2 lead to a number of interesting observations. (1) The reason that the $2|E|$ transition was not observed in the ODMR experiment is now apparent. The transition is expected to be very weak since the steady-state populations and radiative rate constants are similar for T_x and T_y. Furthermore, the frequency of the transition is almost coincident with that of the $T_y \leftrightarrow T_z$ transition and is, therefore, overwhelmed by the latter. (2) At 1.8°K T_x and T_y have approximately three and five times greater steady-state populations, respectively, than T_z. This observation, combined with the very low (relative) radiative rate constant, $k_z{}^r$, for the latter, ensures that >99% of the observed phosphorescence is from $T_x + T_y$. At 77°K the levels will be in thermal equilibrium, with resulting percentage contribution to the phosphorescence of T_x ~48%; T_y ~48%; T_z ~4%. Therefore, conventional phosphorescence polarization measurements at 77°K must be interpreted with some care. Phosphorescence polarization studies carried out at 1.8°K, in combination with transient ODMR techniques, should in principle uniquely characterize the polarization of the radiation from the individual sublevels. Such studies now in progress will greatly help in the elucidation of the coupling to the singlet manifold. (3) The lifetimes of the T_x and T_y states are roughly 10 and 20 times shorter, respectively, than the lifetime of the T_z level. Unfortunately, the radiative rates, $k_i{}^r$, are only known to

[3] The analytical technique employed in the extraction of the kinetic parameters assumes that spin–lattice relaxation can be neglected. Recent simulations in our laboratory using the complete rate matrix indicate that even if $k_i/k_{SLR} = 10$, where k_i is the smallest rate constant, the value measured for k_i will be approximately 10% too large.

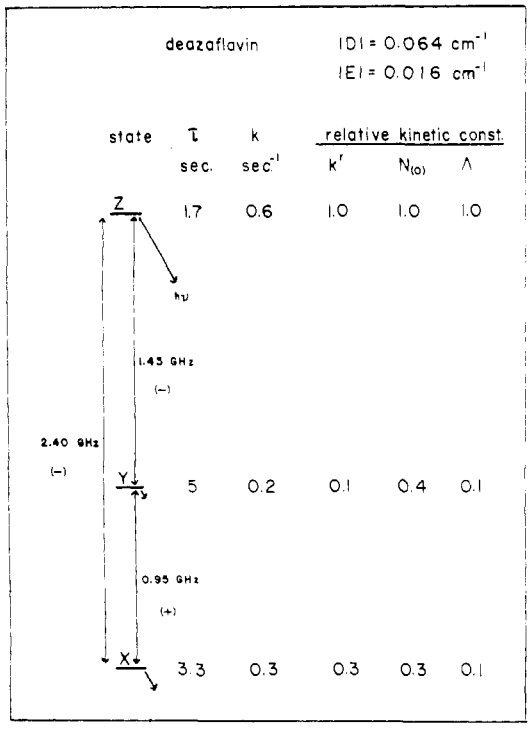

FIGURE 3: Lifetimes, relative kinetic constants, and zfs values for the lowest triplet state of deazaflavine. See Figure 2 for a definition of the symbols.

TABLE I: Zero-Field Splitting Parameters of Flavines.[a]

Flavine	D (cm^{-1})	E (cm^{-1})
Riboflavine	0.059	0.019
Alloxazine	0.079	0.019
FMN	0.058	0.019
FAD	0.059	0.019
Deazaflavine	0.065	0.015

[a] These values must be compared with caution since the level correlation is not known.

within an arbitrary scale factor γ. However, the quantum yields for intersystem crossing, Φ_{isc}, and for phosphorescence, Φ_p, have been reported as >0.5 (Song and Moore, 1968) and 10^{-3} (Sun *et al.*, 1972), respectively. If one uses these values and assumes that they are independent of temperature, one can show that $\gamma \sim 10^{-3}$. Therefore, the contribution of the radiative rate constant to the total rate constant is negligible ($k_i = k_i^r + k_i^{nr} \approx k_i^{nr}$). This conclusion is somewhat unsatisfactory since it requires unusually long radiative lifetimes for these states. Our results suggest that Φ_{isc} should be evaluated at a suitably low temperature in order to determine whether the intersystem crossing process is temperature dependent (Song, 1971). (4) The steady-state population ratio N_i/N_j was measured as a function of exciting wavelength from 300 to 485 nm and found to be constant. This result suggests that wavelength dependent intersystem crossing does not occur in this region.

In order to confirm independently the choice of 1.14 GHz for the $2|E|$ transition in riboflavine, we investigated a molecule that was expected to have similar zfs parameters but somewhat different kinetic parameters. Deazaflavine, in which N(5) is replaced by a carbon atom, yielded all three

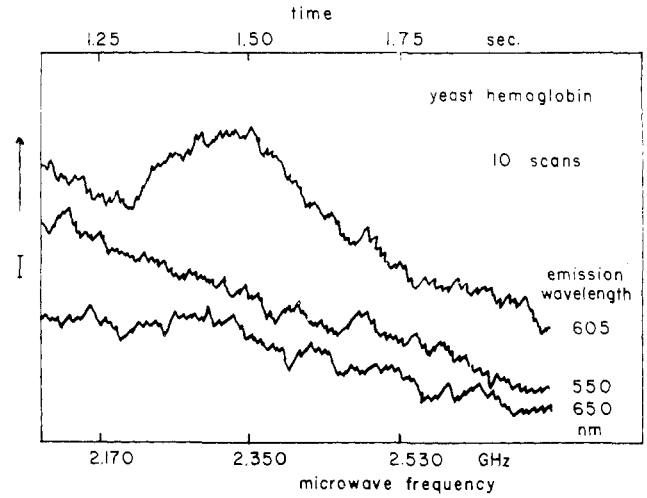

FIGURE 4: Delay ODMR of yeast hemoglobin. Upper abscissa refers to time after exciting light is interrupted, and lower abscissa to microwave frequency. The observed light intensity is shown at three different emission wavelengths. The excitation wavelength is 436 nm. The spectra shown are from 10 scans at 1 sec scan^{-1}.

zf ODMR transitions at 2.40, 1.45, and 0.95 GHz. This result tends to support our 1.14 GHz choice for the $2|E|$ transition in riboflavine rather than the 3.56 GHz alternative. The level splittings and the kinetic data are presented in Figure 3. It must be emphasized that since the level correlation is not known it is not meaningful to compare, level for level, Figures 2 and 3. The deazaflavine kinetic parameters are different than those of riboflavine as one might expect in view of the fact that deazaflavine lacks the (n,π^*) state contributed by N(5). The rate constants are different for all three levels; T_x and T_y are approximately three and ten times less radiative, respectively, than T_z and the total lifetimes of the states are substantially increased.

In order to determine the sensitivity of the riboflavine zfs parameters to environment we studied alloxazine, FMN, and FAD as well. The zfs parameters for these species are included in Table I.

Having thus characterized the riboflavine moiety, we initiated a search for the corresponding signals in flavoproteins. The flavoprotein samples chosen (flavodoxin and yeast hemoglobin) were weakly luminescent, although no distinguishing structure was observed. As shown in Figure 4, upon application of microwave power the characteristic flavine ODMR transition at 2.3 GHz was detected. Also shown on Figure 4 are traces obtained at different emission wavelengths. By systematically observing the ODMR intensity as a function of emission (excitation) wavelength a complete emission (excitation) spectrum is obtained. In this way the flavine spectrum is extracted from competing luminescence of comparable or greater intensity. We showed that the optimization of the ODMR signal is consistent with the known excitation maximum at 450 nm and emission maximum at 605 nm. The ODMR line widths, ~150 MHz, are similar to those one normally observes in such protein systems. A contribution to the observed luminescence from trace amounts of free flavine cannot at this point be absolutely ruled out. The absence of free flavine in similar flavodoxin preparations is indicated since the rotational correlation times are characteristic of protein bound FMN (Norris and Crespi, 1972). The yeast hemoglobin samples were purfied (Oshino *et al.*, 1973) and transported in frozen buffer solution in order to avoid conditions which

result in the release of flavine. (Free flavine is released from yeast hemoglobin upon extraction with 5% trichloroacetic acid or urea denaturation followed by gel filtration (Oshino *et al.*, 1973).) As this technique is extended to other flavoproteins, especially those with covalently linked flavines, it may be possible to determine whether free flavine makes a detectable contribution to the ODMR signal of the flavoprotein.

In the case of yeast hemoglobin, it has been suggested that FAD and protoheme are bound on a single peptide chain in sufficiently close proximity to promote the redox reactions between these moieties (Oshino *et al.*, 1973). Although the presence of paramagnetic iron (ferrous, deoxy) might be expected to alter the lifetimes of the FAD triplet sublevels, no gross changes were observed. Work is in progress to determine in greater detail the dependence of the ODMR signals on the nature of the protoheme. This should allow us to make some specific comments regarding the microenvironment of the riboflavine in the flavoprotein (as well as the proximity of the protoheme) based on the fact that the zfs, when measured at high resolution (to four significant figures), is a sensitive function of the "solvent" molecules.

In conclusion, we have used the ODMR technique to characterize in detail the magnetic and optical parameters for the triplet state of riboflavine. In addition we have observed ODMR signals from the corresponding states in flavoproteins. Thus we have demonstrated that the ODMR technique is potentially a powerful tool for investigating systems that exhibit extremely weak luminescence from multiple sources. Preliminary results suggest that the detection of fully reduced flavine is feasible using this approach. It is worth noting that the sensitivity of the method approaches that typical of optical emission spectroscopy whereas the resolution approaches that normally expected for magnetic resonance investigations.

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