The Phosphorescent State of Indole. Observations Using Optically Detected Magnetic Resonance

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Abstract: The phosphorescence and optically detected magnetic resonance spectra of indole in various environments are presented and discussed. Emissions from various trap sites of indole in glassy solvents, single crystals, and Shpol'skii matrices are compared. Structureless optical emission bands are seen in some cases to be composites of emissions from several trap sites which may be resolved by optical magnetic resonance. Line-broadening effects of indole in these hosts are examined by optically detected hole-burning experiments.

In previous communications[1,2] we have described the use of optically detected magnetic resonance (odmr) methods in the study of the phosphorescent state of tryptophan and of tryptophan residues in proteins. Since the properties of the triplet state of tryptophan appear to be quite sensitive to environment, odmr is a promising method of site identification and monitoring in proteins. The excited tryptophan molecules of the protein act as intrinsic spin probes. Our progress in this area has been limited, however, by lack of resolution both of the optical emission spectra and the odmr signals from tryptophan residues in distinct protein sites. The lack of resolution persists to the lowest temperatures we have achieved in pumped-helium experiments (~1.15°K). In an effort to understand better the nature of the problem, we have undertaken a detailed investigation of the triplet state of indole, the parent aromatic chromophore of tryptophan, in several environments.

One observation, in particular, which prompted this investigation is that samples of carefully purified indole grown from the melt by the Bridgman method exhibit broad phosphorescence emission bands as well as broad odmr lines at temperatures of *ca.* 1.2°K.[2] More frequently, the low-temperature emission from crystalline samples originates from one or several more or less well-defined traps and consists of narrow lines one to two orders of magnitude narrower than the emission bands which we find in indole.

Experimental Section

Sample Preparation. Indole crystals were grown from melt by the Bridgman method using material which had been extensively zone refined (*ca.* 200 passes). Indole-d_1 was prepared by vigorously mixing molten indole with a 100-fold excess (mole/mole) of D_2O at 60°. After overnight mixing the indole was allowed to solidify, separated from the D_2O, and the process repeated with a fresh batch of D_2O. The solid indole-d_1, after separation from the D_2O, was pumped dry, melted, degassed to remove any traces of D_2O, and sealed in a zone refining tube under a nitrogen atmosphere. The crystals were grown in the same manner as for normal indole.

Indan and indene were purified by spinning band column distillation. *N*-Methylindole was vacuum distilled several times. The structures of all of the compounds discussed here are shown in Figure 1.

Odmr. Quartz tubes containing the samples were inserted in a helical slow-wave structure, which was suspended in a liquid helium cryostat. The samples were excited with a Osram 100-W mercury high-pressure lamp filtered by a 1/4 m Bausch and Lomb monochromator. A rotating sector was used to eliminate the fluorescence and scattered light. The phosphorescence was monitored at right angles to the excitation path with a 1 m McPherson Model 2051 grating monochromator fitted with an EMI Model 9558QA photomultiplier in a cooled housing.

The microwave sources were Hewlett-Packard Models 8614B and 8616B signal generators and a Model 8690B sweep oscillator. A digital multipulse time base synchronized the repetitive microwave sweeps with the sweep of a 1024-channel computer for average transients (CAT).

The applied microwaves were swept through the resonance frequencies between pairs of triplet spin sublevels in times which were long compared with the relaxation times of the phosphorescence emission intensity following a pulse or fast microwave passage. In this way the line shape of the transition is not distorted and the peak frequencies and half-widths can be read directly with high accuracy. It should be pointed out that such a slow-passage odmr technique will not necessarily prove successful in samples where all of the triplet sublevels are radiative, because an increase in the amount of emission from one sublevel is at least partially compensated for by a decrease in the emission from the other sublevel pumped by the microwaves. In the case of indole, where all of the emission originates from only one sublevel,[2] the slow-passage odmr signals have signal-to-noise ratios comparable to fast-passage responses.

The hole-burning[3] and electron–electron double resonance[4] (eedor) experiments were carried out by application of microwaves of fixed frequency together with a slow-passage microwave sweep through a range including the fixed frequency (for hole burning) or through the resonances between another pair of sublevels (for eedor). In contrast with the previously reported procedure,[3,4] our experiments were done by feeding both microwave frequencies down a single transmission line into a single helix. The only precaution which must be taken when this method is employed is that the two microwave sources must be well decoupled in order to avoid spurious effects. A method used to achieve this decoupling is illustrated in Figure 2.

Phosphorescence Spectra. The phosphorescence spectrum of indole-h_1 in ethylene glycol–H_2O (1:1) (EG–H_2O), shown in Figure 3, is basically the same as that of tryptophan but is blue shifted by 200–250 cm^{-1}. The absorption and fluorescence spectra of indole-h_1 are likewise similar to those of tryptophan but also slightly blue shifted.[5]

The phosphorescence spectrum of indole-d_1 single crystal is shown in Figure 4. The phosphorescence of undeuterated indole crystals is similar to that of the deuterated crystals. The origin of the emission from indole crystals lies within 1 nm of that for indole in EG–H_2O.

By varying the wavelength of the exciting light, the relative intensities of the peaks seen in Figure 4 could be changed, excitation to the blue favoring the blue-shifted origins. By examining this effect it became clear that the indole crystal phosphorescence is a composite of emissions from at least three trap sites, each trap

(1) J. Zuclich, D. Schweitzer, and A. H. Maki, *Biochem. Biophys. Res. Commun.*, **46**, 1764 (1972).
(2) J. Zuclich, D. Schweitzer, and A. H. Maki, *Photochem. Photobiol.*, **18**, 161(1973).
(3) M. Leung and M. A. El-Sayed, *Chem. Phys. Lett.*, **16**, 454 (1972).
(4) T. S. Kuan, D. S. Tinti, and M. A. El-Sayed, *Chem. Phys. Lett.*, **4**, 507 (1970).
(5) S. Konev, "Fluorescence and Phosphorescence of Proteins and Nucleic Acids," Plenum Press, New York, N. Y., 1967.

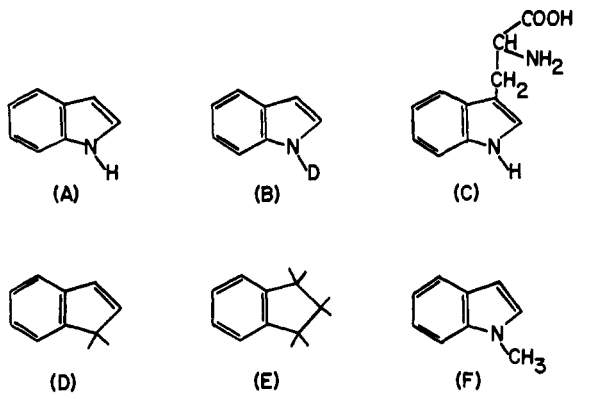

Figure 1. (A) Indole-h_1; (B) indole-d_1; (C) tryptophan; (D) indene; (E) indan; (F) *N*-methylindole.

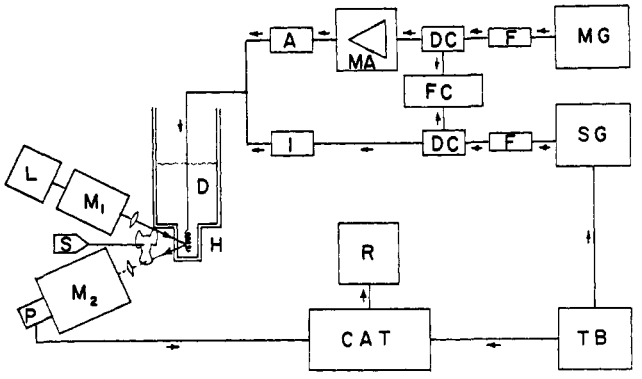

Figure 2. Block diagram of the whole experimental arrangement in the case of hole-burning experiments. During usual slow-passage odmr only the "sweeper path" was used. A = attenuator (−20 D); CAT = signal averaging computer; D = helium dewar; DC = directional coupler; F = filter; FC = frequency counter; H = helix; I = isolator; L = lamp; $M_{1,2}$ = monochromator in the exciting path and in the monitored path, respectively; MA = microwave amplifier; MG = microwave generator; PM = photomultiplier; S = sector; SG = sweep generator; R = recorder; and TB = time base.

emission closely resembling the indole emission shown in Figure 3. The 0–0 bands for these trap emissions are shifted from each other by ~400-cm^{-1} intervals and have half-widths of ~150 cm^{-1} compared with ~300 cm^{-1} for indole in solution. There may be additional traps with weak emissions shifted even further to the red but these cannot be clearly resolved. The resolvable peaks are summarized in Table I.

The fact that the excitation spectra for phosphorescence emission from the indole traps differ from one another implies that energy transfer is inefficient. A reasonable explanation for this observation is the coexistence in the crystals of several solid phases. If the domain size were large in comparison with the distance over which efficient singlet–singlet transfer may occur, the electronic energy would be contained effectively within a domain. On the other hand, if the domains were also small enough that a significant fraction of indole molecules is perturbed by the phase boundaries, the large width of the emission bands (and the odmr signals to be described below) would be a reasonable consequence of trapping in these regions.

At this point it seemed desirable to study isolated indole molecules in a suitable unreactive host matrix. Indole was found to be readily soluble in indan, and polycrystalline samples (10^{-3} mol/mol) of this mixture exhibited a sharp and highly structured indole phosphorescence spectrum at 4°K. On the other hand, indole in indene, which might seem to be another likely Shpol'skii matrix, did not exhibit any guest emission.

The phosphorescence of indole-h_1 in indan again appears to be a composite, this time of four major trap emissions whose origins are shifted by 50–125-cm^{-1} intervals. The half-widths of the 0–0 bands of the four traps (shown in Figure 5) are now only ~12 cm^{-1}. The shifts between these origins are such that all four of them would fall within the broad envelopes of the 0–0 emission

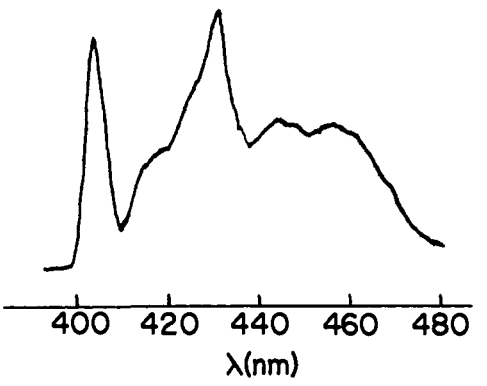

Figure 3. Phosphorescence spectrum of indole-h_1 in ethylene glycol–H_2O (1:1) at 1.25°K with an exciting wavelength of 285 nm.

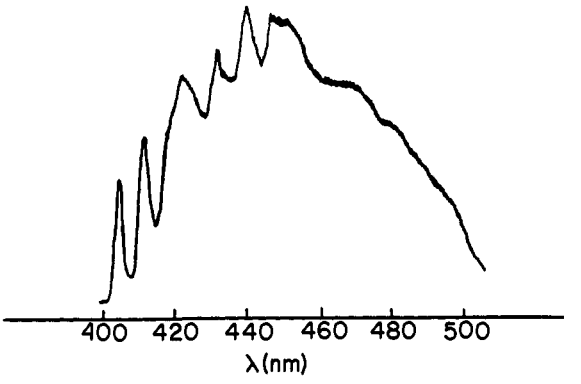

Figure 4. Phosphorescence spectrum of indole-d_1 single crystal at 1.23°K with an exciting wavelength of 285 nm.

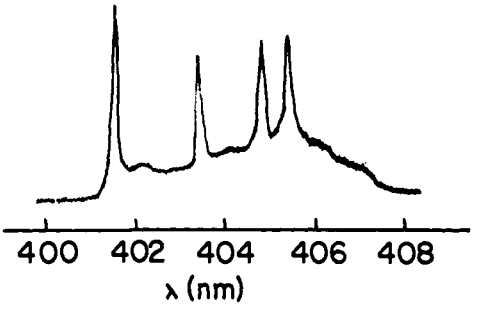

Figure 5. Phosphorescence spectrum of indole-h_1 in indan (10^{-3} mol/mol) at 1.23°K with an exciting wavelength of 285 nm.

Table I. Wavelengths of Prominent Phosphorescence Bands of Indole in Various Environments at 1.25°K

Sample	Phosphorescence peaks, (nm)	Bandwith at half-intensity, cm^{-1}
Indole-h_1 in EG–H_2O (1:1)	403.5, 430.8	300
Indole-h_1 (cryst)	404.2, 411.5, 418.0, 426.5 431.6, 440.2, 447.2, 451.9	150
Indole-d_1 (cryst)	404.3, 411.6, 418.3, 426.0 431.6, 440.2, 447.0	150
Indole-h_1 in indan	401.1, 403.1, 404.5, 405.7	12
Indole-d_1 in indan	401.1, 403.3, 404.7, 405.4	12
N-Methylindole in indan	407.7, 409.4, 410.1	25

Table II. Magnetic Resonance Data for the Optically Resolved Traps in Indole Crystals (T = 1.25°K)

Sample	Indole-h_1	Indole-d_1		
Trap no.	1	1	2	3
Excitation, nm	285	285	298	315
Detection, nm	404.3	404.2	411.3	422.6
D − E signal				
Frequency, GHz[a]	1.682	1.686 1.751	1.590	1.520 1.720
Half-width, MHz	90	60 50	60	120 120
Lifetime, sec[b]	1.39	1.48	1.91	0.94
2E signal				
Frequency, GHz[a]	2.584 2.734	2.557 2.720	2.598	2.570 2.628
Half-width, MHz	130 160	100 120	120	180 190
Lifetime, sec[b]	1.31	1.31	1.74	0.76

[a] Where two frequencies are entered, the odmr response is a doublet. [b] Decay constant of phosphorescence transient following microwave fast passage, ref 2.

Table III. Magnetic Resonance Results for the Optically Resolved Traps of Indole-h_1 in Indan (T = 1.25°K)

	Trap no.			
	1[a]	2	3	4
Excitation, nm	280	280	280	285
Detection, nm	401.1	403.1	404.5	405.7
D − E, GHz	1.730 1.735 1.742	1.822	1.795[b] 1.807	1.797
Half-width, MHz	10.3 10.3	10.7	10.2	11.5
2E, GHz	2.740 2.756 2.772	2.591 2.605[c]	2.609	2.619
Half-width, MHz	13.4	10.0	10.8	10.2

[a] Decay time constant following fast passage was 1.32 sec for the D − E transition and 1.44 sec for the 2E transition at 1.25°K. [b] Weak shoulder. Possibly from trap 4. [c] Weak shoulder. Possibly from trap 3.

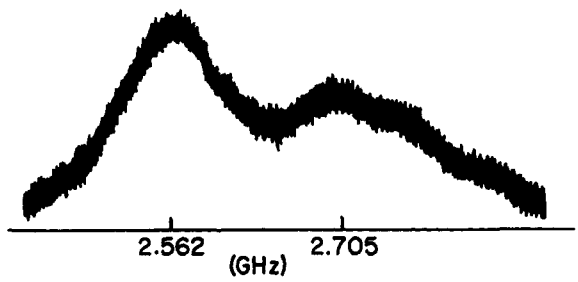

Figure 6. Slow-passage odmr signal (2E transition) of trap 1 of indole-d_1 single crystal at 1.25°K.

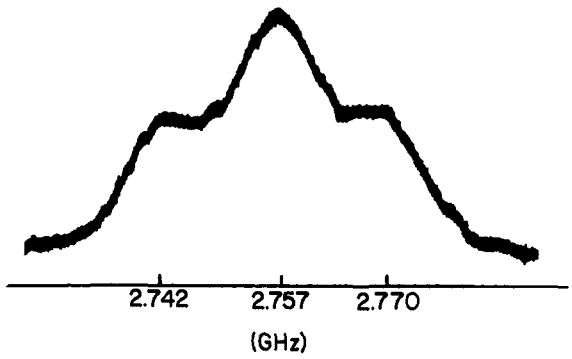

Figure 7. Slow-passage odmr signal (2E transition) of trap 1 of indole-d_1 in indan (10^{-3} mol/mol) at 1.25°K.

band of indole in EG–H_2O or the most blue-shifted origin band of indole crystals.

Indole-d_1 in indan has a nearly identical phosphorescence spectrum as undeuterated indole in the same host. Small isotope shifts of the origins are observed and given in Table I. Again, the relative intensities of the four trap emissions of both indole-h_1 and indole-d_1 in indan exhibit a dependence on exciting wavelength.

The behavior of *N*-methylindole is similar to that of indole. The polycrystalline sample when excited at ~300 nm shows 0–0 bands which peak at 416.3 and at 427.5 nm; they are broad (300 cm^{-1}) at 4.2°K. The phosphorescence at these wavelengths decays with a time constant of τ = 4.65 sec. A strong impurity phosphorescence is present to the red (510 nm) and decays with a time constant of τ = 1.07 sec. When dissolved in crystalline indan, a Shpol'skii effect is evident. Three origins are found with half-widths of 25 cm^{-1} at 1.2°K. The luminescence data are given in Table I.

Odmr Measurements. Slow-passage microwave frequency sweeps led to the observation of odmr signals from indole in EG–H_2O at 1.806 and 2.570 GHz. Each response is an increase in light intensity. The half-widths are 125 and 220 MHz, respectively. These results are similar to those from tryptophan in the same solvent[2] and correspond to the D − E and 2E transitions of the indole triplet state, respectively. As in tryptophan, the D + E signal is not observed optically.

In order to observe the odmr signals from the optically resolved traps in indole single crystals, slow-passage measurements were made while monitoring optically the various origins. The resulting data for the three prominent traps of indole-d_1, as well as the most blue-shifted trap of indole-h_1, are given in Table II. In each case, the wavelength of the exciting light was chosen to minimize interference from the emission of the other traps. It is apparent that each optical origin (except trap 2 of indole-d_1) represents the emission of at least two distinct traps because of the doublet structure of the odmr signals. A typical slow-passage signal, the 2E transition region of trap 1 of indole-d_1, is shown in Figure 6. The half-widths of the odmr signals are somewhat less than in the EG–H_2O solvent, but they are nonetheless much broader than would be expected for unique, well-defined traps in single crystals. Also presented in Table II are the decay lifetimes of the transient responses following a microwave fast passage through the triplet sublevel transitions. It is seen that the lifetimes vary significantly from trap to trap indicating differences in the sublevel decay constants or in the spin–lattice relaxation probabilities, more likely the latter.[6]

Results of similar measurements on the trap origins of indole-h_1 dissolved in indan are given in Table III, while those for indole-d_1 in indan are presented in Table IV. There is a great similarity between the measurements of undeuterated indole and for indole-d_1 except that there is in some cases a difference in the number of odmr lines for a given trap. For example, the odmr response is a symmetrical triplet for the 2E transitions of trap 1 of indole-d_1 (Figure 7), whereas it appears as a doublet for normal indole

(6) J. Zuclich, J. U. von Schütz, and A. H. Maki, *Mol. Phys.*, in press.

Table IV. Magnetic Resonance of Optically Resolved Traps of Indole-d_1 in Indan (T = 1.25°K)

	Trap no. 1	Trap no. 2	Trap no. 3	Trap no. 4
Excitation, nm	280	280	280	285
Detection, nm	401.5	403.3	404.8	405.3
D − E signal				
Frequency, GHz[a]	1.733 1.743	1.824 1.828	1.804[c]	1.796
Half-width, MHz	12 12	9 9	13.5	11
Lifetime, sec[b]	1.36	1.50	1.76	1.72
2E signal				
Frequency, GHz[a]	2.742 2.757 2.770	2.590 2.600	2.613[c]	2.619 2.630
Half-width, MHz	13 13 13	11 11	18	13 13
Lifetime, sec[b]	1.37	1.74	1.75	1.85

[a] Multiple entries indicate resolved components. [b] Decay time constant following fast passage. [c] Possibly a poorly resolved doublet.

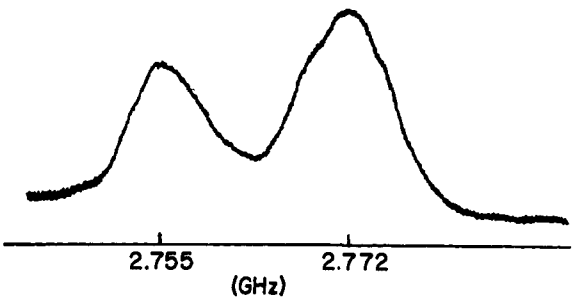

Figure 8. Slow-passage odmr signal (2E transition) of trap 1 of indole-h_1 in indan (10^{-3} mol/mol) at 1.25°K.

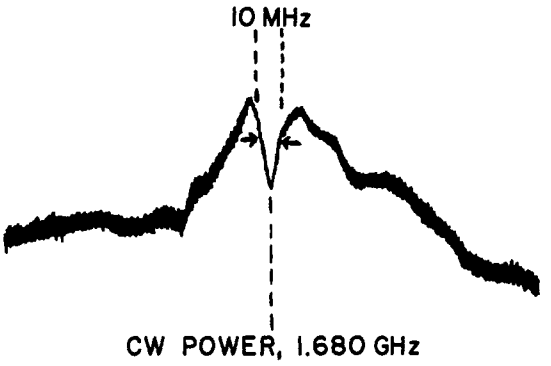

Figure 9. Hole burning in indole-h_1 single crystal at 1.24°K.

(Figure 8).[7] The multiplet splittings are too large roughly by an order of magnitude to be ascribed to hyperfine and quadrupole interactions. They are due to a multiplicity of sites with the same optical origin but varying zero-field splittings. We have demonstrated this using the eedor method. On the other hand, the widths of the individual components which appear in Figures 7 and 8 are consistent with an envelope of unresolved hyperfine structure, principally from ^{14}N as a result of its quadrupole interaction.[8] The odmr signals shown in Figure 8 contain a clear indication of substructure which may be due to these interactions.

The magnetic resonance data for the triplet of *N*-methylindole dissolved in crystalline indan are presented in Table V. Due to the somewhat larger optical bandwidths, the odmr signals of traps 2 and 3 could not be well resolved. In contrast with the results from indole, there is little evidence for multiplicity of sites having the same optical origin in the *N*-methylindole system. As might be expected, a small change in zero-field splitting results from the methyl substitution, but generally speaking, *N*-methylindole exhibits very similar optical and magnetic resonance behavior as indole and indole-d_1.

Table V. Magnetic Resonance Data of Optically Resolved Traps of *N*-Methylindole in Indan (T = 1.20°K)

	Trap no. 1	Trap no. 2 + 3
Excitation, nm	290	295
Detection, nm	407.7	409.4
		410.1[c]
D − E signal		
Frequency, GHz	1.733	1.631 1.702[a]
Half-width, MHz	14	11 13
Lifetime, sec[b]	0.93	
2E signal		
Frequency, GHz	2.516	2.540 2.561[a]
Half-width, MHz	15	10 10
Lifetime, sec[b]	1.18	

[a] The doublet is due to the overlapping of trap 2 + 3. [b] Decay following fast passage. [c] Not well resolved.

Hole-Burning Measurements. In order to investigate the nature of the line-broadening effects for indole in various environments, the method of odmr hole burning, first reported by Leung and El-Sayed,[3] was applied. Fixed frequency microwave power was applied continuously at a frequency near the center of an observed odmr signal, while a second microwave source was swept through the region of the odmr line. In the case of indole-h_1 or indole-d_1 in indan, no hole was found, but rather the entire odmr line diminished in intensity with the application of the hole-burning signal. Thus, the observed lines (10 MHz wide) in these cases behave like homogeneously broadened lines. We found it possible, however, to burn holes in the odmr lines of indole single crystals, as well as indole, and tryptophan in EG–H_2O glass. The odmr lines in these cases are much broader, of course, than the corresponding lines in the indan solvent, which exhibits a Shpol'skii effect. With the application of *ca.* 20 mW of microwave power at a fixed frequency at the center of the D − E transition of the trap 1 line of crystalline indole, a hole with a half-width of 9 MHz is observed as seen in Figure 9. Similar results were found for the D − E and 2E transitions of each trap. The holes could be made deeper (but broader) by increasing the continuous wave power, and 9 MHz represents the narrowest observable hole. We are again led to the conclusion that the "homogeneous" line width of the indole triplet state in zero field is approximately 10 MHz. The additional broadening in indole crystals and in frozen solutions is thus caused by a distribution of intermolecular interactions between each emitting indole site and the environment. We are observing, in effect, an inhomogeneous distribution of sites. Hole-burning experiments on the odmr signals of the tryptophan triplets in HLAD similarly reveal that the sites in this enzyme are inhomogeneously broadened.[9]

(7) Three frequencies are listed for each of the regions, D − E and 2E, for trap 1 in Table III, although only two signals are apparent in the 2E region in Figure 7. Actually, the third odmr line is observed weakly at the low-frequency end of the range. In other samples, it is the high-frequency signal which is very weak relative to the other two. There are thus, apparently, three distinguishable sites contributing to the trap 1 emission in indole-h_1; their relative intensity depends in some manner upon sample preparation.

(8) I. Y. Chan, J. Schmidt, and J. H. van der Waals, *Chem. Phys. Lett.*, **4**, 269 (1969); C. B. Harris, D. S. Tinti, M. A. El-Sayed, and A. H. Maki, *ibid.*, **4**, 409 (1969); M. J. Buckley, C. B. Harris, and A. H. Maki, *ibid.*, **4**, 591 (1970).

(9) J. U. von Schütz, J. Zuclich, and A. H. Maki, *J. Amer. Chem. Soc.*, **96**, 714 (1974).

Eedor Measurements. We showed above that a number of odmr lines may be associated with a given trap emission, in general. There is thus ambiguity in the assignment of D and E values to a particular triplet site, since it is not known which line of the multiplet appearing in the D − E region originates from the same triplet as a particular line in the 2E region. This ambiguity may be resolved in this case by use of the eedor optical method.[4] Microwave power was applied at the fixed frequency of the center of an odmr line in either the D − E or 2E region as in the hole-burning experiment, while a second microwave source was swept slowly through the D + E frequency region. In each case, a signal was observed in the D + E region in the presence of the fixed frequency power, whereas nothing was observed in its absence. An accurate measurement of the frequency of the eedor response in the D + E region allows the assignment of D and E values to the triplet being pumped by the continuous wave microwave power. In this manner it was found, for example, that the odmr responses observed for trap 1 emission of indole-h_1 in indan originate from three distinguishable triplets with ($|D|$, $|E|$) cm^{-1} values of (0.10379, 0.04570), (0.10383, 0.04596), and (0.10394, 0.04624).

Conclusions

Odmr, eedor, and hole-burning measurements have been made on the triplet state of indole in various environments. It is concluded that the triplet energy (origin of the phosphorescence spectrum) as well as the zero-field splitting is very sensitive to the environment. The emission from pure crystalline indole and indole dissolved in EG–H_2O glasses is broad and originates from an inhomogeneous distribution of traps. Indole dissolved in crystalline indan exhibits a Shpol'skii effect and at least four optically resolvable origins are present. The optical origins themselves originate from distinguishable sites as evidenced by multiplet structure in the odmr lines and optical eedor measurements which show that the multiplicity is due to a system of triplets with a distribution of zero-field splittings. It is probable that the width of the 0–0 bands of indole in indan ($\sim$12 cm^{-1}) contains a contribution due to the discrete trapping sites which we are able to resolve by odmr. The behavior of the odmr lines of indole in the pure crystal as well as dissolved in indan upon hole burning point to an apparent "homogeneous" line width of about 10 MHz. It is important to note that *in zero field* the major contribution to the odmr line width of ^{14}N-containing triplet states is due to "forbidden" satellite transitions involving simultaneous ^{14}N-electron spin flips.[8] These are split out from the "allowed" transitions by the ^{14}N quadrupole splitting frequencies, or typically from ± 1 to ± 4 MHz in azaaromatics. Thus, the line is not inhomogeneously broadened over its entire width but only over a width represented by *second-order hyperfine splittings*, typically $\sim$0.5 MHz. Consequently, spin diffusion need only be effective over the latter frequency band to allow saturation of the entire 10-MHz width by monochromatic microwave power. It is also possible that frequency instability of our signal source could result in saturation over a 0.5-MHz bandwidth. The situation in zero field is thus fundamentally different from that encountered in a strong magnetic field in which first-order hyperfine interactions are responsible for the major portion of the line width and monochromatic microwave power burns a hole in a portion of the nuclear spin population.

Comparison of the results for indole-h_1 with those for indole-d_1 and *N*-methylindole reveal a great similarity. Consequently, we conclude that the sensitivity of the indole triplet state optical and magnetic properties to the environment does not involve direct interactions of the N–H bond.

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Resolution of Tryptophan Phosphorescence from Multiple Sites in Proteins Using Optical Detection of Magnetic Resonance

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Abstract: A new method is presented which makes it possible to detect heterogeneity in the phosphorescence emission of tryptophan in different protein sites. By observing optically detected magnetic resonance (odmr) signals through a monochromator with a narrow optical window, multiple sites are revealed by discontinuities in a plot of zero-field splitting parameters *vs.* monitored wavelength. It is shown that the phosphorescence emission of lysozyme and of the lysozyme-tri-*N*-acetylglucosamine complex at 1.2°K originates from two distinct types of tryptophan sites, although the 0–0 origins of the sites are not resolved as separate optical bands. Odmr hole-burning experiments and optical eedor experiments are presented which show that the homogeneous magnetic resonance line width in the horse liver alcohol dehydrogenase triplet state is $\sim$10 MHz in zero field (in accord with measurements on the isolated tryptophan triplet), whereas the homogeneous magnetic resonance line width of the principal lysozyme triplet is $\sim$100 MHz under the same conditions. The latter result is probably due to a motional averaging process present at 1.2°K, the nature of which is not presently understood.

The aromatic amino acids, tryptophan and tyrosine, which are components of many proteins, can be optically excited to give reasonably strong fluorescence and phosphorescence emission. These spectra have been studied extensively in the past with a view toward obtaining information about the interactions of the