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A STUDY OF THE PHYSICAL BINDING OF BENZANTHRACENE TO DNA BASES BY OPTICALLY DETECTED MAGNETIC RESONANCE

D. L. MYERS, G. R. BRUNK, G. MOLLER and A. M. NISHIMURA
Department of Chemistry, Wichita State University, Wichita, Kan. 67208 (U.S.A.)
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Summary

Effects on the electronic structure of benzanthracene due to physical binding to bases of nucleic acid were studied by optically pumping the molecular triplet state and monitoring the subsequent decay. Since the triplet electronic states of molecules are sensitive to such perturbations, the observed changes in the total phosphorescence decay rate constants were used as a measure of the extent of molecular interaction. Additionally, changes in the triplet state zero field splitting parameters arising from the anisotropic spin–spin interaction of the triplet electrons were used as another measure of the extent of interaction. Changes in these parameters were observed for base pairs, polynucleotides and methylated adenines.

1. Introduction

Past workers have determined that binding of polycyclic aromatic carcinogens to nucleic acids is necessary before any reaction between the two molecules can occur [1]. Certain hydrophobic and stacking interactions lead to the association of the aromatic hydrocarbon and the purine form of the nucleic acid. Although stacking is not confined to carcinogens, but occurs with similar non-carcinogenic molecules as well, it is clear that such association is important in the *in vivo* microsomal mediated binding leading to covalently bonded complexes [2].

Kasha and Khan [3] have postulated the possible involvement of the excited singlet state of oxygen and the energy transfer from carcinogenic molecules to yield the electronically excited state of the oxygen indirectly which subsequently appears to be involved in the oxidative reaction at the bay region of the carcinogen [4]. In addition, recent observation of bioluminescent and the photochemically induced carcinogencity of certain aromatic hydrocarbons via direct irradiation suggest that the long lived triplet state of these carcinogens might be involved in the induction of cancer [5 - 9]. Essential to such arguments is a good understanding of the nature of the excited triplet states of these polycyclic hydrocarbons.

Within the past decade, a method known as optically detected magnetic resonance (ODMR) has been shown to be a sensitive tool in the study of molecular interaction [10 - 14]. ODMR can yield information regarding the lowest triplet state of molecules. The study reported here is to characterize the nature and properties of the triplet state of the polycyclic aromatic hydrocarbon benzanthracene (BA) via the lowest triplet state zero field splitting energies, phosphorescence decay constants and phosphorescence spectra. Careful observation was made on the effects of different types of solvents on the triplet state parameters of the hydrocarbon.

The effects upon these triplet state parameters were then observed when BA was mixed in solutions containing common bases of nucleic acid, including solutions of complementary base pairs, and polynucleotides. Finally the effects upon the triplet state of BA were observed in solutions containing methyladenines.

2. Materials

The triplet donor molecules were extensively purified to eliminate any impurity emission. Crystalline *p*-dichlorobenzene (DCB) (Fisher Scientific Co.) was recrystallized twice from pentane and chromatographed three times in a silica gel column. After recrystallization, the sample was zone refined for an equivalent of over 200 passes. Purine and guanine were purchased from Sigma Chemical Company while adenine, cytosine and thymine were obtained from Calbiochem. High pressure chromatographic analysis showed impurities to be less than roughly 1% in all cases. 7-Methyladenine (K & K Lab.) and 6-methyladenine (Pfaltz & Bauer, Inc.) and other methylated adenines (Sigma Chemical Co.) were used as supplied. Samples of BA and BA with bases were made such that the BA:base molar ratio was 1:10 with a BA:solvent concentration of approximately 0.01 mol.%.

3. Method

In the triplet electronic state, the two unpaired electrons give rise to a coupling energy known as electron spin–electron spin dipolar interaction. Closed shell electrons do not contribute to the spin–spin coupling and therefore can be neglected. The spin–spin dipolar interaction hamiltonian contributes to the coupling energy in the following form [15]:

$$\mathcal{H}_{s-s} = DS_z^2 + E(S_x^2 - S_y^2)$$

where S_x, S_y and S_z are the triplet state spin operators and the quantities D and E are spatial integrals involving spin–spin dipolar interaction and are called zero field splitting (zfs) parameters, since the interaction is present regardless of an externally applied field. It is important to note that the interaction is anisotropic for a typical molecule. This means that the

dipolar interaction along the three principal axes of the molecule, which is the axis system that diagonalizes the zero field hamiltonian, will depend upon the spatial delocalization and anisotropic interactions of the triplet electrons. A method by which the dipolar interaction energy can be measured is ODMR.

At room temperature the three spin levels of the triplet state are thermally coupled by the high density of phonon states which are occupied. However, at the temperature of boiling helium (4.2 K) and below, the occupied density of phonon states available for the thermal spin relaxation process is very low and the triplet sublevels effectively become isolated. Owing to the large contribution from one-centered spin–orbit interaction integrals, the selection rule for the intersystem crossing from the singlet to the triplet state picks out one of the triplet sublevels. Since the same spin–orbit interaction will cause the depopulation of the triplet to the ground state resulting in phosphorescence, the steady state population as well as the radiative rate of each triplet sublevel will be different.

The application of microwave energy corresponding to the energies of the spin–spin dipolar interaction results in the redistribution of populations of the two levels which have been connected by the microwave power. Since the total intensity of the phosphorescence emission is a function of the product of the radiative rate and the steady state population, any redistribution of population will affect the total phosphorescence intensity. By optically monitoring the phosphorescence emission, while sweeping the microwave frequency through the zero field resonances, the dipolar interaction energies along the three principal axes can be directly observed.

Optical excitation was accomplished by focusing on the sample a 100 W mercury lamp. Filtering of the undesired emission lines was achieved by passing the light through appropriate filters. Sample emission was detected by placing the lamp at an angle of 90° with a 0.75 m spectrometer. To obtain temperatures as low as 1.4 K, the vapor above the liquid helium was pumped. Microwave measurements were carried out by placing the sample in a slow wave helix at the end of a semirigid 50 Ω coaxial line immersed in a helium-filled quartz optical tip.

Continuous excitation was used for the phosphorescence spectrum. For kinetic experiments, an electronically controlled shutter was placed in front of the lamp. The microwave power used in the double resonance experiments was generated by a sweep generator and amplified with a TWT amplifier. Calibration of the microwave sweep was done with an EIP microwave counter.

The zero field transitions of the triplet sublevels were obtained by a method developed by Schmidt and van der Waals [11] called microwave induced delayed phosphorescence. This procedure is accomplished by continuous excitation of the sample until a steady state population distribution is achieved. The light is then shuttered and the phosphorescence decay is monitored. At some point during the decay, microwave energy corresponding to the transition is swept, causing an instantaneous increase in intensity

and subsequent decay. This process of selective repopulation yields not only the frequency of the zero field transition, but also the total decay rate of the triplet sublevel.

In situations in which the populations of the sublevels of a particular transition were not appreciably different, but the population difference for another transition was sufficient, a method of electron–electron spin double resonance was used [16]. In this case, continuous pumping of the known transition redistributes the populations sufficiently, so that a third sublevel coupled to either of the other two will cause the population difference. This is accomplished by using two microwave generators, one pumping the known transition, while the other is used to sweep the microwave frequency.

For those molecules with short phosphorescence lifetimes, an alternative method developed by Winscom and Maki was used [17]. The microwave power in this experiment was externally frequency modulated by a triangular wave, while the triplet state was continuously pumped.

Analysis of the total phosphorescence decay was accomplished by means of a signal averager. The data points were transferred directly into a minicomputer equipped with a visual analog display of the data points. Successive regressions over selected data points yielded the total decay rate constants.

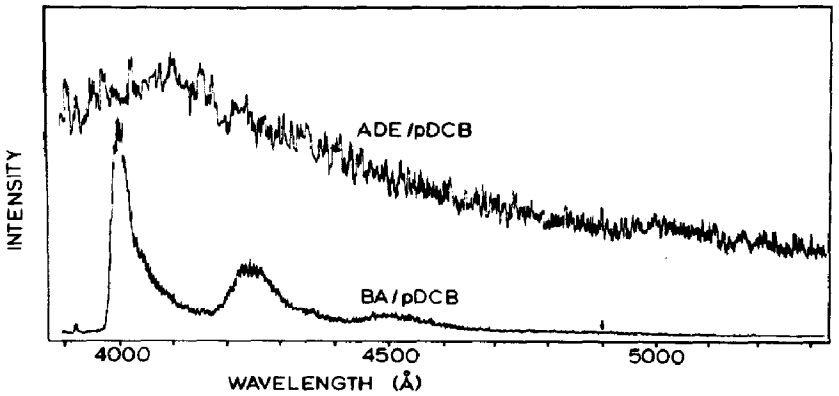

Fig. 1. Total emission spectra for adenine (top) and BA (bottom) in DCB at 1.4 K.

4. Results and discussion

The lower part of Fig. 1 shows the total emission spectrum for BA in DCB and the upper part is the spectrum for adenine in the same solvent taken at 1.4 K. The onset of the phosphorescence for BA is about 490 nm (indicated by an arrow) but is at least two orders of magnitude less intense than the fluorescence. The zfs and total phosphorescence decay constants for BA in the hosts DCB and phenanthrene are shown in Table 1. The significant differences in the zfs and rate constants are primarily due to the external heavy atom effect and the crystals fields of the two hosts, both of

TABLE 1

Zero field resonance frequencies and total phosphorescence rate constants for BA in DCB and phenanthrene at 1.4 K

		Host	
		DCB	Phenanthrene
Zero field transitions (MHz ± 10 MHz)		3357	3688
		2344	2073
		1013	1036
Decay constants (s^{-1} ± 10%)	k_3	100	10
	k_2	65	3.2
	k_1	9.9	1.8

which in turn affect the zero field energy of the triplet electrons of the BA. Such results are well documented in our previous studies [18].

It was found that, when a sample of adenine in DCB was prepared, the emission spectrum was found to be that of adenine. When a very small amount of BA was added to the same sample, the spectrum of BA alone was observed even when the sensitivity of the spectral recording instrument was not changed from that when the spectrum of adenine in DCB was taken. A careful concentration study showed that such results were obtainable at an adenine:BA molar ratio of 300:1 when the concentration of adenine was 0.075 mol.% in DCB. Below this concentration, spectra of *both* adenine and BA are simultaneously observed. However, when naphthalene was substituted for BA at a similar concentration, a composite spectrum was obtained when the ratio of adenine to naphthalene was 4:1 in which the concentration of adenine was 0.31 mol.% in DCB. Aqueous solvent instead of DCB yielded similar results. Simultaneous spectra were obtained until the ratio of adenine:BA was 100:1 when the concentration of adenine was 0.0052 mol.% in water.

These results seem to indicate that energy transfer occurs from adenine when the ratio of adenine to BA molecules exceeds 300:1. The intermolecular distance between two BA molecules was calculated to be roughly 350 Å, assuming them to be spatially equidistant. Since energy transfer via dipolar interaction between BA and adenine will probably occur at distances of about several tens of angstroms and since the concentration of BA was about 2.5×10^{-4} mol.%, the model which can be proposed is that most of the adenine molecules are clustered around a single BA molecule. Thus there appears to be a strong affinity for adenine by BA which is essentially solvent independent.

The slight increase in the molar concentration of adenine:BA in aqueous solvent can be explained by the hydrophobic interactions of the adenine and to a larger extent BA, which would cause such clustering even more than in a non-polar solvent such as DCB. Thus DCB would represent a

TABLE 2

Zero field resonances and total phosphorescence rate constants for BA with several bases in DCB at 1.4 K

		Base			
		Adenine	Guanine	Cytosine	Thymine
Zero field transitions (MHz ± 10 MHz)		3387	3071	3010	3064
		2473	2175	2191	2162
		914	896	819	902
Decay constants (s^{-1} ± 10%)	k_1	47	384	63	365
	k_2	8.2	95	10	87
	k_3	0.98	0.98	1.3	3.0

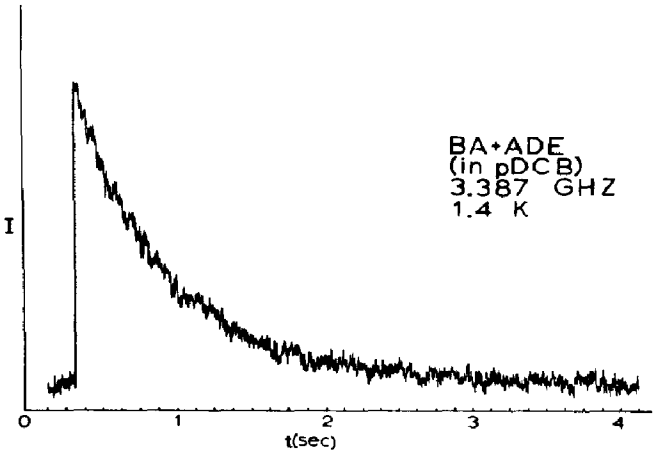

Fig. 2. Typical rapid passage ODMR signals for BA and adenine in DCB. The transition was at 3.387 GHz.

less extreme solvent in observing physical binding of adenine to BA such that any results reported here involving DCB solvent would have to be modified with larger interactive effects in aqueous solvent.

ODMR results in solutions of BA with the bases thymine, adenine, guanine and cytosine in DCB are shown in Table 2. As can be clearly observed, the triplet state parameters, zfs and phosphorescence decay constants were changed from that for BA alone in DCB. As a check, zfs and total phosphorescence rate constants for a non-carcinogen, phenanthrene, were observed in DCB. These parameters did not vary within 5% when adenine was also added to the solution of phenanthrene in DCB.

Table 3 shows zfs and decay constants for BA with benzimidazole and with indole. This series was done to observe whether there was some functional group essential for physical binding. Although not all of the transitions could be observed, it appears that even in the simple case of indole where only one nitrogen is substituted into the indene ring the zero

TABLE 3

Zero field transitions and total phosphorescence decay constants for BA with several bases in DCB at 1.4 K

		Benzimidazole	Indole	Guanine + cytosine	Adenine + thymine
Zero field resonances		—	—	—	—
(MHz ± 10 MHz)		2180	2171	2400	2415
		—	—	—	—
Decay constants	k_3	380	97	150	81
(s^{-1} ± 10%)	k_2	17	14	1.9	3.7
	k_1	1.7	0.43	0.72	0.75

TABLE 4

Total phosphorescence decay constants for BA with polynucleotides in ethanol–water solvent at 1.4 K

	k_3	k_2	k_1
Polyadenine	3.6	0.95	0.43
Polyguanine	7.5	1.1	0.30
Polycytosine	110	9.9	0.60
Polyuracil	160	—	0.20
Polyadenine + polyuracil	—	2.6	0.55
Polyguanine + polycytosine	300	—	0.85

field resonance does not compare with BA alone in DCB, suggesting BA to indole interaction. To observe any effects of physical binding upon base pairs, BA was added to solutions of guanine and cytosine in DCB and of adenine and thymine (*cf.* Table 3). Although only one transition for BA could be observed, it does not compare with BA in pure DCB, again suggesting interaction. Finally BA was added to the nucleotide polymers in water–ethanol solvent and the data are shown in Table 4.

As a further study of physical binding of BA to bases several methyl-modified adenines were observed. Preliminary to the addition of BA to solutions of methyladenine, their zfs and total phosphorescence rate constants in DCB were observed. The results are shown in Table 5. Upon methylation, increases in the total phosphorescence rate constants for adenine were observed. Methylation at positions 1, 2 and 7 had equal effect on the total rate constant. Methylation of the amine at position 6 decreased the rate of the decay, probably because of the amine nitrogen and the larger distance of the perturbing methyl group from the ring π cloud.

The zero field splittings for the methyladenines are shown in Table 5, along with the relative differences in the D values with respect to the parent compound, adenine. The data are in good agreement with earlier work by

TABLE 5

Zero field splitting parameters and total phosphorescence rate constants for methylated adenines in DCB crystal observed at 1.4 K

	$\|D\|$ (cm^{-1})	$\Delta\|D\|$[a] (%)	$\|E\|$ (cm^{-1})	k_3 (s^{-1} ± 10%)	k_2 (s^{-1} ± 10%)	k_1 (s^{-1} ± 10%)
Purine	0.1013		0.0571	1.8	0.48	0.18
Adenine	0.1034	2.1	0.0582	6.9	1.3	0.32
1-Methyladenine	0.1098	6.2	0.0542	19	4.4	0.92
2-Methyladenine	0.1060	2.5	0.0538	17	1.6	0.47
N^6-Methyladenine	0.0982	−5.1	0.0610	8.3	2.0	0.40
N^6-Dimethyladenine	0.0996	−3.7	0.0598	16	1.8	0.59
7-Methyladenine	0.0974	−5.8	0.0562	19	1.8	0.39

[a]Percent change in $|D|$. For adenine the change is relative to purine. For the methyladenines, the change is relative to adenine. The axis system was extrapolated from ref. 19.

TABLE 6

Zero field energies and total phosphorescence rate constants for BA with methylated adenines in DCB

	zfs (MHz ± 10 MHz)	Rate constants (s^{-1} ± 10%)
1-Methyladenine	3045	85
	2184	—
	861	0.21
2-Methyladenine	—	46
	—	3.1
	—	0.70
N^6-Methyladenine	3045	—
	2189	14
	856	2.2
N^6-Dimethyladenine	2939	53
	2203	9.5
	736	0.56
7-Methyladenine	2679	30
	2186	5.1
	493	0.36

Harrigan and Hirota [19] in which most of the purine-type molecules showed $3|E| > D$. The zero field splittings and the total phosphorescence decay rate constants of indole in *p*-dibromobenzene showed reproducibility of our data to those of Harrigan and Hirota to within 10% and 0.5%, respectively [19].

Both 1- and 2-methyladenines showed significant increase in D, with 1-methyladenine having about twice the effect as methylation at the 2

position. Since the most stable form of 1-methyladenine is the imino form [20] the increased D value upon methylation at the 1 position is somewhat unexpected. The effect of the loss of double bond character with methylation at the 7 position is reflected in the large negative change in the D parameter (*cf.* Table 5).

It is particularly interesting that N^6-methyl- and N^6-dimethyladenine, which are common *in vivo*, showed a negative change in D value. Since the base modification at the N^6 position causes an electron shift, the acidity of the amine protons in the monomethylated adenine may become increased owing to the inductive effect. N^6-dimethyladenine would form a weaker hydrogen bonded base pair, because of the absence of the amine proton and because of the steric hindrance of the two methyl groups. Thus modification in N^6-methyl and in N^6-dimethyladenine could form stronger and weaker Watson–Crick base pairs, respectively.

The net effect due to electron density shifts in modified bases could cause structural modifications of ribonucleic acids and could explain the differences in the functions of normal and cancer ribonucleic acids [21]. Although the data presented here do not allow a complete explanation of how specific modifications relate to actual structural changes between normal and cancer ribonucleic acids, it is evident that shifts in electron densities such as those observed here could alter the interaction energies of hydrogen bonded base pairs.

Table 6 shows ODMR data for BA with several of the modified bases in DCB. The effect upon the zfs is much more enhanced with N^6-dimethyladenine and 7-methyladenine. The phosphorescence decay constants are larger than for BA in DCB alone.

The observed increases in the total phosphorescence decay rate constants of all of the solutions described are good indicators of interactions, although the actual direction of the interaction is difficult to ascertain. In a previous study, the directionality of the interaction and its effects upon the rate constants were found to be predictable by the atomic structure of the molecule [18]. The random effect upon the decay constants found in this study could be due to either the larger uncertainties in the measured constants because of the weaker phosphorescence signals or the non-specific distribution of the base cluster around the BA molecule.

Assuming that the zero field states do not vary drastically from that of the parent molecule anthracene, we have observed that when BA is mixed in a solution containing a base, the energy $|E|$ was consistently smaller by as much as 19%. In addition when the host was changed, the $|E|$ value of BA alone was observed to decrease from phenanthrene to DCB. Such a decrease in $|E|$ has been observed in other studies of ${}^3\pi, \pi^*$ states in which the solvent imposes a large interaction upon the solute via external heavy atoms [18]. Considering the low symmetry of BA, the decrease in $|E|$ may be attributed to the less anisotropic crystal fields arising from the interaction with the base.

The $|D|$ value decreased when BA was mixed in solutions containing the bases, except for the solution containing adenine. The difference in $|D|$ values could be attributed either to a distortion of BA when it interacts with a base or an effect upon the zfs due to spin–orbit contribution from the increased interaction with the base. Our study here does not preclude either interpretation.

In summary, the results of the study here have indicated that ODMR technique can yield information regarding intermolecular interactions by physical binding of BA to bases of nucleic acid. BA seems to interact with all common bases, as well as with base pairs and polynucleotides. Interaction of BA with methyladenines appear to be strong in N^6-dimethyl- and 7-methyladenines. Energy transfer studies showed an unusually large affinity of BA to adenine. Substituents or the existence of a heterocyclic ring do not seem to affect the affinity of BA to the base. Such non-specific binding might be the primary step prior to the formation of the reactive metabolite in carcinogenesis.

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