esters bind at the same site on the enzyme, as evidenced by the identity of the K_I values measured for the inhibitor AcPhe using either substrate.

A comparison between the k_{cat} and the K_M values observed for the pepsin-catalyzed hydrolysis of BNPS and DPS reveals that the large difference discussed above between the k_{cat}/K_M ratios for the two reactions arises primarily because there is a large difference in the k_{cat} values. In terms of the expression for k_{cat} given in eq 2 we can obtain eq 6 where the primed quantities are those associated with BNPS hydrolysis and unprimed quantities with DPS hydrolysis. It is clear that if $k'_3 \gg k_3$, $k_2 \approx k_3$, and $k'_2 \approx k_2$, the k'_{cat}/k_{cat} ratio would be given by $(k_2 + k_3)/k_3$. Similarly, if $k'_2 \gg k_2$, $k_2 \approx k_3$, and $k'_3 \approx k_3$, $k'_{cat}/k_{cat} = (k_2 + k_3)/k_2$. Since the K_I/K_M ratio obtained in the present study leads to a k_2/k_3 value of 2 the first of these situations would give $k'_{cat}/k_{cat} = 3$, whereas the second would imply that $k'_{cat}/k_{cat} = 1.5$. The fact that the actual value of k'_{cat}/k_{cat} is approximately 4000 means that the presence of the nitro groups in BNPS must cause an acceleration in both the k_2 and k_3 steps of eq 1 relative to the corresponding steps for the hydrolysis of DPS. This is consistent with a mechanism in which 1 mole of *p*-nitrophenol is liberated in the k_2 step of eq 1 when BNPS is hydrolyzed (see ref 9).

$$\frac{k'_{cat}}{k_{cat}} = \frac{(k'_2k'_3)}{(k'_2 + k'_3)}\frac{(k_2 + k_3)}{k_2k_3} \tag{6}$$

As has been mentioned the apparent K_M values for BNPS and DPS differ much less than the k_{cat} values for the hydrolysis of these compounds. If the K_M values are taken to be a measure of relative binding strength,[22] it is evident that the nitro groups of BNPS have only a slight effect on the binding of this ester to the enzyme.

In contrast to this situation it is known that placing two halogen substituents in the Tyr portion of the peptide substrate AcPheTyr markedly decreases the K_M value for the substituted compound as compared to the unsubstituted compound.[23] It is unclear if this decrease in K_M is due to the electron-withdrawing effects of the halogens or to some other factor.[6,23] If the electron withdrawing effects of substituents are indeed important in the binding of substrates to pepsin, they might be expected to be very pronounced for nitro-substituted pepsin substrates. However, the effect of the nitro groups on the binding of BNPS to pepsin is much smaller than that of the bromo substituents in the case of AcPheDiBrTyr.

K_I^c for acetonitrile is estimated as 0.5 *M*, the "corrected" k_{cat}/K_M ratio for DPS in the absence of acetonitrile would be about 200 M^{-1} sec^{-1} which is smaller than that found for BNPS by a factor of 10^3.

[22] The extent to which the dissociation constant for the Michaelis complex, K_s, is reflected in the apparent K_M values for each substrate is determined by the relative magnitudes of the rate constants k_2 and k_3 (eq 1) for the hydrolysis of that substrate ($K_M = K_Sk_3/[k_2 + k_3]$). However, if $k_3 \geq k_2$, the Michaelis constant still provides a good indication of the strength of the binding of the sulfite ester to pepsin.

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Communications to the Editor

Magnetic Field Effects on Anthracene Triplet–Triplet Annihilation in Fluid Solutions[1]

Sir:

Late in 1968, it became clear from two standpoints that investigating the influence of a magnetic field on the fluid-solution triplet–triplet annihilation process would be very useful. The pioneering work of Johnson, *et al.*,[2] on the effect of a magnetic field on the mutual annihilation of triplet excitons in crystalline anthracene had uncovered some of the more intimate details of that process. Later experimental and theoretical reports also aided an understanding of the interaction.[3–5] As a logical extension, a separate study of the fluid-solution triplet–triplet annihilation reaction promised to provide similar information regarding details of that process. Furthermore, such a study was clearly needed to improve the understanding of the recently reported field effects on electrogenerated chemiluminescence (ECL).[6] We have carried out an investigation of field effects on anthracene triplet–triplet annihilation in fluid solutions, and we report here the major results together with some preliminary conclusions.

The experimental studies are based entirely upon the field dependence of the P-type delayed fluorescence intensity. All measurements were carried out using a phosphorimeter which was constructed especially to operate with the same immersed in a magnetic field.[7] The delayed fluorescence component was isolated by the conventional technique which involves out-of-phase chopping of the excitation and emission beams.[8] The instrument used a C.S. 7-54 glass filter in the excitation beam. Since Pyrex cuvettes were used for all measurements, the sample was excited by light derived mostly from the 365-nm line of the mercury arc employed as an excitation source. The emission beam was filtered by a C.S. 5-57 glass filter. Intensity measurements were recorded using a phase-sensitive detection system. Tests of instrument performance showed it to be completely independent of the magnetic field below 8000 G.

The delayed fluorescence intensity registered from each sample was proportional to the square of the incident excitation intensity, indicating that the emission

[1] This research was supported by the Robert A. Welch Foundation and the National Science Foundation (GP6688X).

[2] R. C. Johnson, R. E. Merrifield, P. Avakian, and R. B. Flippen, *Phys. Rev. Letters*, 19, 285 (1967).

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[8] C. A. Parker, "Photoluminescence of Solutions," Elsevier Publishing Co., Amsterdam, 1968.

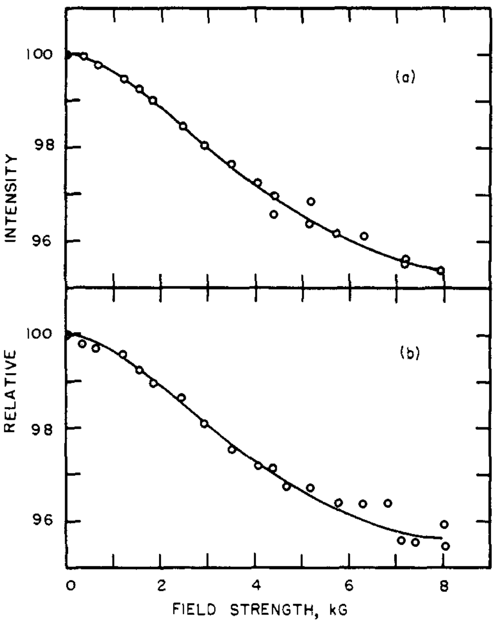

Figure 1. Magnetic field effect on delayed fluorescence from anthracene solutions in DMF: (a) 5 × 10^{-4} *M*, delayed fluorescence lifetime 2.1 msec; (b) 7 × 10^{-5} *M*, delayed fluorescence lifetime 6.3 msec. The triplet lifetime is twice the delayed fluorescence lifetime (see ref 8).

intensity was proportional to the annihilation rate constant.[8] The intensity recorded from a degassed sample was always 50 to 1000 times greater than from an aerated blank; hence cell phosphorescence was negligible.

Figure 1 shows the variation of the delayed fluorescence intensity with field strength for two different concentrations of anthracene in N,N-dimethylformamide (DMF). The intensity in both cases declines with increasing field strengths, but it levels off at higher fields. There is no indication of a low-field enhancement of the intensity like that observed with the solid. In addition, the magnitude of the field effect is not as great as that measured even in a randomly oriented polycrystalline sample.[7]

Parker[8] has derived the following equation for the intensity of directly excited delayed fluorescence under steady-state illumination

$$I_{\mathrm{DF}} = (1/2)\phi_f k_a (I_a \phi_t \tau)^2$$

where ϕ_f is the fluorescence efficiency, k_a is the annihilation rate constant, I_a is the rate of light absorption, ϕ_t is the triplet formation efficiency, and τ is the triplet lifetime. This equation shows five quantities which could be field dependent, giving rise to the observed effect on delayed fluorescence. Three of the quantities, I_a, ϕ_f, and ϕ_t, are related to physical properties of a diamagnetic species and would not be expected to be grossly field dependent. Indeed, the recent observation that magnetic fields do not affect the intensity of prompt fluorescence from crystalline anthracene indicates that I_a and ϕ_f are probably both field independent.[9] Of the three remaining possibilities, none can be measured accurately enough to be tested directly, and one must rely on indirect evidence to advance further.

Fortunately, the two curves shown in Figure 1 furnish some very useful information concerning possible field effects on τ. In solutions having an anthracene concentration greater than about 10^{-4} *M*, anthracene triplet lifetimes are drastically shortened by either self- or impurity quenching. At concentrations below 10^{-4} *M* the lifetime is essentially constant. Any field effect on the lifetime would probably be concentration dependent in the range where the quenching term begins to be important toward determining τ. Thus the magnitude of a field effect on delayed fluorescence arising from this source would also be concentration dependent in the same range. Since experimentally the field variation is independent of concentration in the range where the lifetime begins to change markedly, one is persuaded that the lifetime is not significantly field dependent.

The results of an experiment dealing with sensitized anthracene delayed fluorescence provided a useful key to the question of a field-influenced ϕ_t. In this experiment, a DMF solution 7 × 10^{-4} *M* in phenanthrene and 5 × 10^{-5} *M* in anthracene was illuminated frontally. The field effect on anthracene delayed fluorescence generated in this way was identical with that shown in Figure 1. Since the anthracene triplet is populated here by energy transfer rather than by intersystem crossing from the anthracene singlet, ϕ_t is not a factor governing the delayed fluorescence intensity. Since the field dependence of emission in this experiment was identical with that for directly excited delayed fluorescence, one has strong evidence for a field-independent anthracene ϕ_t.

Eliminating these factors leaves only a field-influenced annihilation rate constant to account for the magnetic field effect. Thus the effect reported here is apparently the solution-phase analog to the field-dependent annihilation rate observed in earlier solid-state studies.[2]

This conclusion infers several others concerning the fluid-solution triplet–triplet annihilation process. First, the fact that one observes a field effect at all indicates that the encounter reaction probability for populating the first excited singlet state of anthracene is less than unity, and it is field dependent. This conclusion is in agreement with the findings of Parker and his coworkers in their delayed fluorescence studies.[8,10] A second implication is that the fluid-solution process differs somewhat from the solid-state interaction, especially in its behavior toward low fields. Furthermore, it seems very clear that one cannot account for the field effects on ECL in terms of triplet–triplet annihilation alone. Since the ECL intensity from the two energy-deficient systems which have been studied increases with field, one must conclude that the rate of triplet–triplet annihilation is not the only major element governing the ECL intensity from these systems. The results do not, however, eliminate the annihilation from the mechanism of ECL emission from energy-deficient systems, a conclusion which is reinforced by the results described in the following communication.[11]

Still another interesting aspect of these studies is the observation that there is a solvent effect on the intensity–field strength curve. Additional studies carried out in methylene chloride[11] and in cyclohexane have shown

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(10) C. A. Parker in "The Triplet State," A. B. Zahlan, Ed., Cambridge University Press, London, 1967, Chapter 6 and references contained therein.

(11) L. R. Faulkner and A. J. Bard, *J. Am. Chem. Soc.*, **91**, 6497 (1969).

that the solvent markedly affects both the shape of the curve and the magnitude of the effect. Heretofore, the solvent has not been regarded as an important factor in the annihilation process. However, these results imply that the solvent plays a more significant role than has been previously supposed.

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Larry R. Faulkner,[12] Allen J. Bard
Department of Chemistry
University of Texas at Austin, Austin, Texas 78712
Received August 8, 1969

Wurster's Blue Cation as an Anthracene Triplet Quencher in Fluid Solution and the Effect of Magnetic Field on This Interaction[1]

Sir:

Hoytink[2] has suggested that ion radicals might be effective triplet quenchers in fluid solution. To date, however, the only experimental support for the idea has come from some solid-state studies which showed that radicals do quench triplet excitons effectively.[3] Aside from its interest to investigators working with triplet reactions, and to theories of the influence of paramagnetic molecules on singlet–triplet transitions,[4] the question is important to those concerned with electrogenerated chemiluminescence (ECL), because any triplets generated in an ECL experiment are certainly generated in a region cooccupied by ion radicals. Thus, if triplets are intermediates in the emission scheme for ECL from some systems, and if the radicals are effective as quenchers, there is no doubt that radical quenching is intimately linked to the efficiency of ECL emission. We report here a confirmation that Wurster's blue cation is an effective quencher of anthracene triplets in methylene chloride, and we describe a study of the effect of a magnetic field on the rate of that process.

The experimental work rests on the measurement of the intensities and the lifetimes of delayed fluorescence arising from methylene chloride solutions of anthracene and Wurster's blue perchlorate. All measurements were carried out using the instrument described in the preceding communication.[5] Experimentally the problem is difficult because the radicals are not stable at room temperature at the low concentrations used for the period required for the measurements. The solutions in methylene chloride are not entirely stable, but they do decay slowly enough to permit some useful measurements.

That Wurster's blue cation is an effective quencher of anthracene triplets is shown by the shortened lifetimes in the presence of the radical. As an example, a methylene chloride solution containing initially 1.8×10^{-7} *M* Wurster's blue perchlorate and 8×10^{-5} *M* anthracene showed a delayed fluorescence lifetime of 1.4 msec, thus a triplet lifetime of 2.8 msec. This figure is much shorter than the 6.4-msec triplet lifetime recorded for a solution containing the same anthracene concentration without Wurster's blue cation. The radical decay makes accurate measurement of the quenching rate constant virtually impossible. Nevertheless, one can calculate a lower limit to the quenching rate from the lifetime data by using the initial radical concentration as an upper limit. The triplet lifetime in the presence of quencher concentration [Q] is given by[6]

$$1/\tau = 1/\tau_0 + k_q[Q]$$

where τ is the lifetime in the presence of the quencher, τ_0 is the lifetime measured in the absence of quencher, and k_q is the quenching rate constant. Using the triplet lifetimes mentioned above together with a maximum radical concentration of 1.8×10^{-7} *M*, one calculates a lower limit quenching rate constant of 2×10^9 l./(mole sec). This value is comparable to the quenching rate constant calculated from triplet–triplet energy-transfer data in solvents of similar viscosity. Thus, one must conclude that Wurster's blue cation is a very effective quencher of anthracene triplets, a fact which lends strong support to Hoytink's hypothesis regarding radicals in general.

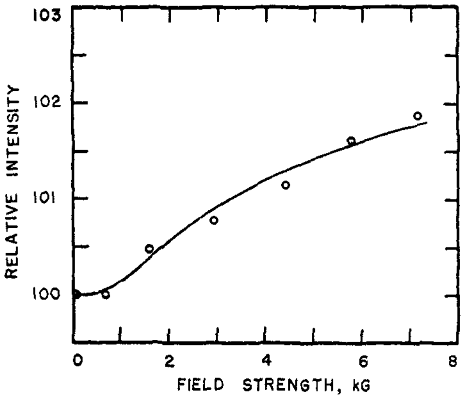

Figure 1. Magnetic field effect on delayed fluorescence from a methylene chloride solution 1.8×10^{-7} *M* in Wurster's blue perchlorate and 8×10^{-5} *M* in anthracene.

The results of a study of magnetic field effects on delayed fluorescence from the solution discussed above are shown in Figure 1. One notes there that the field enhances delayed fluorescence. When one compares these results to those obtained with methylene chloride solutions containing only anthracene (Figure 2), one concludes that the magnetic field inhibits radical quenching of anthracene triplets. Moreover, the longer lifetimes which result from this situation more than compensate for the field effect on the annihilation rate; thus there is a positive change in the intensity as the field is applied. These qualitative conclusions about the direction of the field effect are similar to those reached by Ern and Merrifield[3b] in their solid-state studies.

It is worth noting here that the existence of the field effect on the quenching rate probably indicates that the rate is not entirely diffusion controlled. This observation enables one to place a tentative upper limit on

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[1] This research was supported by the Robert A. Welch Foundation and the National Science Foundation (GP 6688X).

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