Photochemically Induced Dynamic Nuclear Polarization in Hydrosol Suspensions of Congo Red[1a]

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Abstract: Visible irradiation of dilute acidic hydrosol suspensions of Congo Red were found to sensitize chemically induced dynamic nuclear polarization of the solvent nmr. Neutral solutions of the dye did not produce the effect. Evidence is presented that the polarization is produced in the hydrosol particles and transferred by chemical exchange to the solvent. A mechanism for the photooxidation of Congo Red is postulated.

Freshly formed products of free radical pairs show emission and enhanced absorption lines in their nmr spectra, a phenomenon which has been named chemical induced dynamic nuclear polarization (CIDNP).[2]

A necessary condition for producing polarized nmr spectra of this sort is a competition between spin-dependent annihilation of the radical pair to form a singlet and spin-independent scattering to form the two separate radicals.[3] In trying to apply this technique to biological systems we are faced with the problem of predicting the behavior, when the participants of the radical pair will be high molecular weight macromolecules.

If the polarized protons are held rigidly to the macromolecule, their line widths, $\Delta\nu$, will be the same as those on unpolarized molecules; a reasonable rule-of-thumb is that $\Delta\nu \sim \text{MW} \times 10^{-3}$ Hz. Thus macromolecules in the range of 50,000–100,000 should yield detectable spectra. If, by contrast, the polarized protons undergo rapid exchange with the solvent, the polarization could be monitored by observation of the relatively narrow solvent nmr lines.[4]

The current concepts of CIDNP have evolved from consideration of simple, homogenous solutions of small molecules.[3] The complexities which may be anticipated for application of CIDNP to biological materials are manifold, but at least two are readily apparent. The first is that of a homogenous solution of macromolecules and the second is the inherent heterogeneity of any but the simplest biochemical systems.

Since the process is bimolecular, the rate constants are diffusion controlled as the relative diffusion coefficients of the constituents determine the lifetimes of the radical pairs. In simple solutions of small molecules this lifetime is shorter than the electron–electron exchange time, *i.e.*, 10^{-9}–10^{-10} *vs.* 10^{-8} sec.

For a simple solution of macromolecules where one or both of the fragments are small, a similar situation might be anticipated. If either fragment is large, however, then diffusion might be restricted, thus reversing the above situation.

Heterogeneous systems contain all of the complexities noted, as well as others, known or unknown. This latter situation is the novel concern of this paper, in which we report that a polarization formed in macroscopic particles is transferred by chemical exchange to the solvent, where it was observed by high resolution nmr.

Experimental Section

All chemicals were commercially available and were used without further purifications. The pH was adjusted using NaOD or DCl. Absorption spectra were taken using a Cary 14 spectrophotometer. The nmr spectra were obtained at 60 MHz using a Varian A-60 spectrometer equipped with a light guide, as described earlier.[5] The epr spectra were obtained using a Varian E-3 spectrometer. Epr spectra in the liquid phase were taken using a flow system driven by a peristaltic pump. For both the nmr and epr experiments, the light source was a Hanovia 1000-W high-pressure mercury–xenon lamp in a Shoeffel housing. The light intensity dependence was measured with a series of calibrated mesh filters.

Results

Absorption Spectra. The absorption spectra of the acidic and neutral forms of dilute solutions of Congo Red (see formula in Scheme I) in H_2O are shown in Figure 1. The neutral form has its absorption maximum at 495 nm, while in the acidic form this maximum shifts to 565 nm. The color of the dye in these two forms changes from red to blue. The pK of the transition is around 4. Another marked difference between the acidic and the neutral form of Congo Red is that in the neutral form it forms a simple red solution, while in the acidic form it coagulates to form a hydrosol suspension. The absorption spectrum of the acidic form, shown in Figure 1, exhibits the effects of light scattering, a manifestation of the coagulation.

Nmr. The nmr spectrum of a 1.0×10^{-4} M acidic suspension of Congo Red in D_2O is shown in Figure 2B. Upon irradiation, the proton line of the 0.4% HOD of the solvent is completely inverted and en-

(1) (a) This work was supported, in part, by the U. S. Atomic Energy Commission; (b) N.I.G.M.S. Postdoctoral Fellow.

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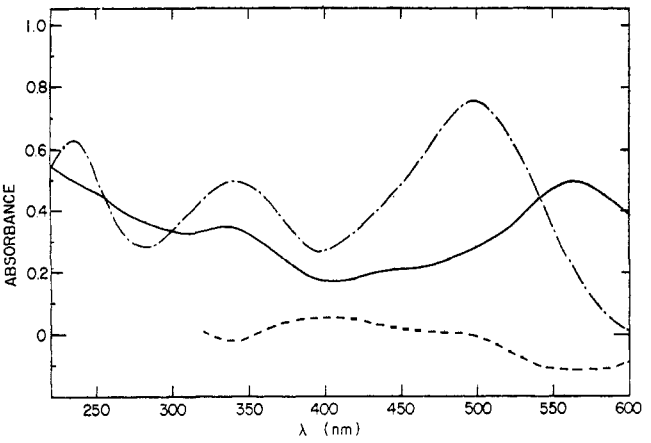


Figure 1. Absorption spectra of Congo Red "solutions:" (- — - —) 3×10^{-5} *M*, pH 5; (———) 3×10^{-5} *M*, pH 2.5; (- - - - - - - - -) 10^{-4} *M*, the difference spectrum between irradiated and nonirradiated "solutions," both at pH 2.5.

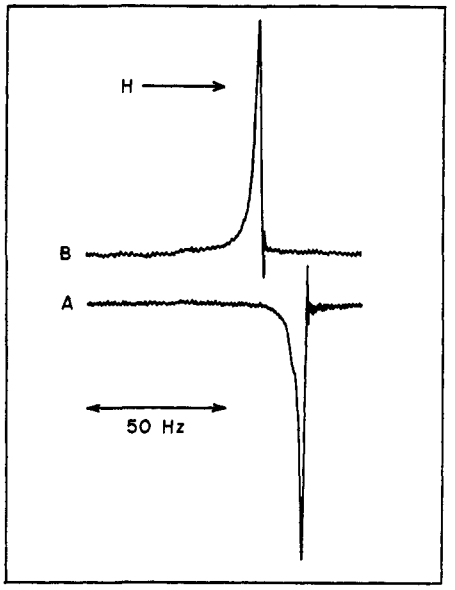


Figure 2. Nmr spectra (60 MHz) of HDO in "solution" of 10^{-4} *M* Congo Red in ~99% D_2O and pD 2.4: (A) during irradiation, (B) after irradiation.

hanced (Figure 2A). D_2O solutions of the same concentration at neutral pD showed no polarization, nor did solutions in H_2O. The pH dependence is discussed in subsequent sections.

Among the many possible reasons for the absence of an observable polarization in the fully protonated solvent, we mention the most important. The steady-state polarization is proportional to the proton T_1,[3f] which is inversely proportional to the proton concentration in H_2O–D_2O mixtures.[6]

We calculated the polarization in the usual manner.[2]

Figure 3 shows how the polarization varies with the light intensity. From the linear dependence we see that the rate of formation of the radical pair is proportional to the light intensity. Consequently, the radical pair must be formed from a single precursor of the radical pair. This is equivalent to stating that the polarization must originate in the initial photochemical act. Owing to the low signal-to-noise, it was impossible to take proper action spectra for producing the polarization. Using Corning glass filters we found that about 50% of the polarization arises from actinic light with wavelength >400 nm.

Epr. The epr of the system was examined to gain some insight into the nature of the radicals. For direct comparison with the nmr data, the first experiments were performed in the liquid phase. No radicals were detected in either the neutral or acidic forms, even after introducing various scavengers such as nitromethane, various aliphatic alcohols, and acetone, which are known to react with small, short-lived radicals to produce relatively long-lived radicals. Next, samples of both acidic and neutral solutions of Congo Red were frozen, irradiated, and observed at 77°K.

Figure 4 gives the epr spectrum of a frozen acidic solution. The neutral solution gave no observable free radicals. This finding confirmed qualitatively the nmr result that a free radical photochemical reaction occurs in the acidic form, while none is observable in the neutral form. Any attempt to analyze the spectrum in Figure 4, using saturation study, partial thermal annihilation, and replacement of H_2O with D_2O as a solvent, failed. Thus, detailed chemical

(6) A. Abragam, "The Principles of Nuclear Magnetism," Clarendon Press, Oxford, 1961, p 398.

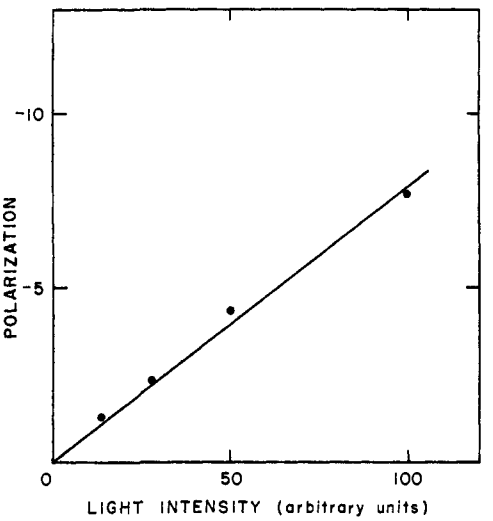


Figure 3. The light intensity dependence of the nmr polarization for a "solution" of 10^{-4} *M* Congo Red in 99.8% D_2O at pD 2.4.

information about the nature of the radicals involved is not yet available.

Functional Group Analysis. An alternative way to get information about the nature of the intermediates involved was to test different compounds with the same functional groups. The compounds checked were aniline, 3,5-diaminobenzoic acid, *p*-aminoacetophenone, 3-amino-2,7-naphthalenedisulfonic acid, benzeneazo-α-naphthylamine, and Methyl Red. These compounds cover the possible functional groups in Congo Red (see Scheme I for formulas), and all were checked over a large pH range. The last two in the list are also dyes which can serve as indicators, with a similar pH range. We observed no nmr polarization in any of these compounds. The only obvious difference between these compounds and Congo Red was that in the range of optical absorption involved (OD ≈ 2) all these compounds gave a true solution over the pH range studied, while Congo Red at acidic pH was in a co-

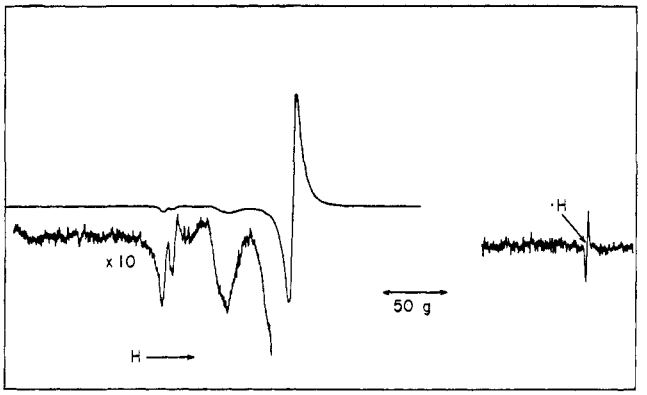


Figure 4. Epr spectra of an irradiated "solution" of 10^{-4} *M* Congo Red in H_2O, pH 2. The irradiation and the spectra were taken at 77°K. H· is the high field component of the hydrogen atom signal created in the glass by irradiation.

agulated form. The last observation gave us good reason to suspect that the nmr polarization and the photochemistry might be a solid-state phenomena.

State of Aggregation. To check the last possibility, an acidic suspension was centrifugated at 20,000 rpm. The supernatant appeared clear and gave no polarized signals while the resuspended pellet produced polarized signals equal to those observed before the centrifugation. An alternative way to explain the last result is that upon irradiation the Congo Red gives some soluble photochemical product which absorbs the light and gives the polarization. We will return to this point later when dealing with the time evolution of the polarization. To check this possibility, the sample was irradiated outside the spectrometer and aliquots taken at various times. The supernatant and the washed pellet were centrifugated and checked. In all cases the supernatant gave no polarization, while the resuspended washed pellet exhibited its full polarization upon reillumination. To check further, the difference spectrum between identical fresh and 1-hr irradiated samples was measured. The spectrum is shown in Figure 1. Less than 10% of the Congo Red disappeared during this time, while traces of some new compound with a maximum absorption at 400 nm appeared. (The light intensity during the irradiations outside the nmr probe is much higher than that during the polarization experiments.) The observed polarization is greater than could be accounted for by the optical density of the new compound. Besides, as mentioned before, about 50% of the polarization appeared with actinic light of $\lambda > 400$ nm. As a final proof for demonstrating that the polarization is indeed a solid-state phenomena, the red neutral solution of Congo Red, which gave no polarization, was salted out with 5% NaCl and heated to boiling for 1 hr. (The same result could be achieved without heating, but would require a waiting period of a few days.) The supernatant from this solution was again clear. The precipitate was resuspended and the observed polarization was of the same order of magnitude as the original acidic solution.

If this polarization is a solid-state phenomena, can it occur on a solid support? To analyze for this possibility, we attached the Congo Red to 0.1% of bovine serum albumin which was lyophilized from

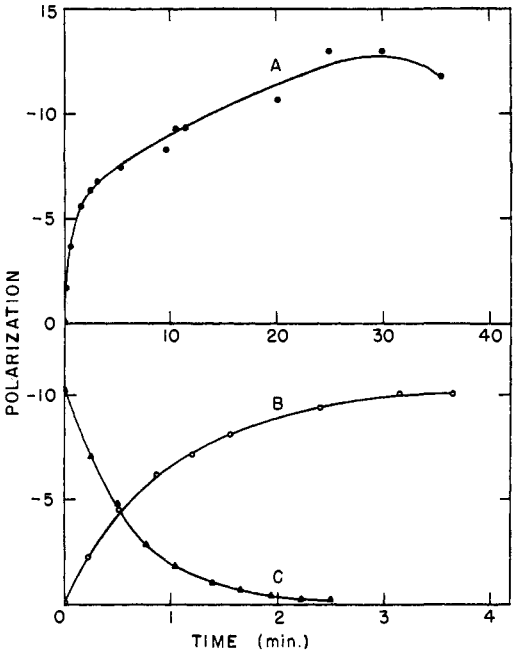


Figure 5. Time evolution of the polarization of 10^{-4} *M* Congo Red in 99.8% D_2O, pD 2.4: (A) rise of the polarization of a fresh "solution" upon illumination, (B) rise of the polarization of a preilluminated sample after equilibration in the dark to zero polarization, (C) decay of the polarization following extinction of the light. Note the different time scales for the upper and lower traces.

D_2O. The nmr spectra in the pH range of 3–7 showed no detectable polarization. That the dye was actually attached to the protein was verified by the fact that the p*K* of the dye changed by approximately 1 pH unit, and the scattering at pH 3 was negligible in the protein solution.

Cotton was used as another support. The cotton was stained by boiling it for 1 hr in an aqueous 5% NaCl solution of 1 m*M* Congo Red.[7] After cooling, the cotton was removed, washed with running distilled water, and dried under vacuum. The stained cotton was suspended in D_2O at various pD values, and the resulting suspension was checked for nmr polarization. No polarization was observed. It appears from the foregoing that a solid-state support is not sufficient for observation of the polarization, but some type of close aggregation between the Congo Red molecules is needed. Another possibility might be that the aggregation must be ionic in character.

Time Evolution of the Nmr Signal. Figure 5A shows the time evolution of the polarization upon illumination of a freshly prepared solution, and Figure 5C shows the decay of the polarization upon removal of the light. The decay is exponential with a time constant of some 37 sec, a value reasonable for the proton T_1 of this system.[6] It is independent of the illumination time. Figure 5B presents the time evolution of the polarization for a solution which had been preirradiated for some 40 min inside the nmr probe and then allowed to relax in the dark for several minutes to zero polarization.

The form of curve B in Figure 5 results from the diffusion of the polarized protons from the light guide solution interface to the sensitive volume of the rf coil. We have determined quantitatively the param-

(7) H. A. Lubs, Ed., "The Chemistry of Synthetic Dyes and Pigments," Hafner Publishing Co., New York, N. Y., 1965, p 112.

eters of the diffusion equation. The details are omitted, as they serve no other purpose than to verify the origins of the curve.

The shape of curve A, Figure 5, is more difficult to rationalize since it is dependent upon sample history, the spinning rate, agitation after preirradiation, and temperature. The possibility of formation of another chemical compound, which in turn is responsible for the polarization, was ruled out on the basis of arguments given above and the fact that samples which were preirradiated outside the probe and then reintroduced gave the same results as freshly prepared samples irradiated within the nmr probe. Additionally, the shape of type A curves depended on the sample spinning rate, as did the maximum polarization. The polarizations of a freshly prepared sample and a preirradiated sample were the same at 80°.

Based on these observations, we infer that the difference between curves A and B is determined, mainly, by diffusion of the hydrosol particles toward the light guide interface; the conditions for maximizing the polarization are achieved when the particles form a thin layer beneath the light guide. In addition, the actinic light has a marked effect on the size of the hydrosol particles.

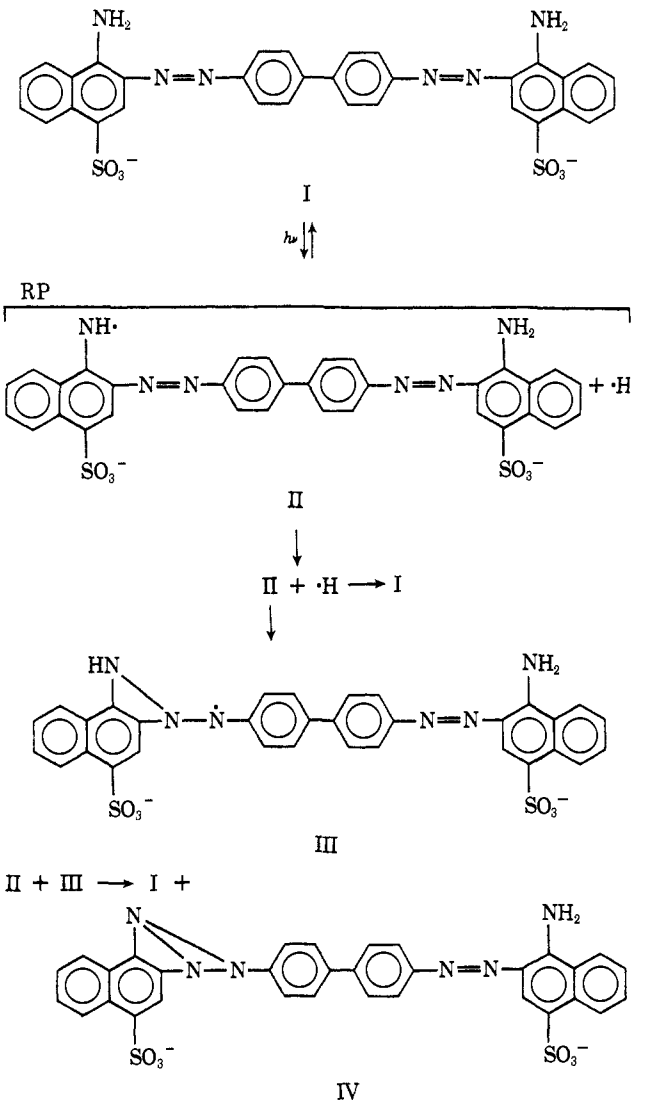
In sum, we believe that curve B shows the rise time for the steady state of polarized protons due to water diffusion, while curve A is a superposition of that phenomenon together with the rise time to achieve steady state in the macroscopic physical characteristics of the system.

Discussion

A few conclusions can be drawn from this work. The first is the finding that CIDNP signals could be observed in acidic suspensions of Congo Red. The observation of a net polarization is consistent with the fact that a radical pair, composed of two different radicals (different g values), was produced. The acidity of the solution was found not to be an essential factor in observing the polarization, but the physical characteristics of the solutions were found to be of primary importance. Hydrosol formation was found to be essential for the observation of the polarization. The hydrosol state was formed either by working in acidic solutions or by salting out from neutral solutions. The nmr polarization was found to be proportional to the light intensity, which is indicative of formation of the polarization in the initial photochemical act. The polarized protons were observed in the solvent, indicating that they must be part of an exchangeable group. The only exchangeable protons in Congo Red (at least in the neutral form) are the amino group protons.

Scheme I is a suggestion of a mechanism which will account for some of these observations. The initial photochemical step proposed is the photoejection of a hydrogen atom from one of the amino groups of Congo Red (I). The resulting radical (II) forms a radical pair with the hydrogen atom. These two radicals can recombine to give back I, but now, with polarized hydrogens on the amino group. The polarized protons will exchange with the solvent to cause the solvent polarization. The hydrogen atoms will easily escape from the cage and can finally recombine with

Scheme I



radical II to produce another cage *via* direct or indirect processes. The final result of these processes is no net decomposition of the dye. We believe that these are the major events occurring, and they account for the very low quantum yield for decomposition. However, some of the hydrogen atoms may not reach II and may dissipate by recombination or by some other process. II in turn may disproportionate by reacting with its resonance form III to form I and IV. IV has been identified as the major photochemical product of the photolysis of Congo Red.[8] This mechanism can also account for the observation that the polarization could not be observed in solution. The reason is, probably, the very short lifetime of the resultant radical pair because of the very high mobility of the hydrogen atoms. This would also account for the observation that, although most of the aromatic amines are known to photoeject hydrogen atoms[9] and thus become photooxidized, no polarization has been observed for any of them.

The conclusion that hydrogen ejection can take place with illumination with visible light should not be too surprising in view of the observation that Congo Red

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(9) E. J. Land and G. Porter, *Trans. Faraday Soc.*, **59**, 2027 (1963).

can be bleached through its triplet state by exciting a sensitizer with light around 700 nm.[10]

The observation that upon cooling to 77°K radicals form only from the hydrosol suspension cannot be explained solely in terms of reducing the macroscopic diffusion coefficients. It seems that a special microcrystalline arrangement is essential to block the back reaction. This microcrystalline arrangement is provided by the hydrosol formation but not by cooling or by attaching the dye to a support like cotton wool or a protein.

(10) J. S. Bellin and C. A. Gergel, *Photochem. Photobiol.*, 10, 427 (1969).

Conclusion

It was established in this work that CIDNP can be produced in macroscopic particles. The essential diffusional equilibrium is provided here by the relatively high mobility of the hydrogen atoms, while the observation of the polarization was made possible because of sufficiently fast exchange with the solvent compared to the spin lattice relaxation time.

Viscosity of the Hydrocarbon Region of Micelles. Measurement by Excimer Fluorescence[1]

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Abstract: The relative intensities of excimer and monomer fluorescence of pyrene are shown to be a simple linear function of the viscosity of a homologous series of solvents. This relationship may be represented by $\eta = kC'_h$, where C'_h is the pyrene concentration at which half the fluorescence intensity appears in both the excimer and monomer form. The constant k includes both instrumental and theoretical parameters and is determined empirically. These results have been used to measure the viscosity of the hydrocarbon region of micelles labeled with pyrene. The viscosities of alkyltrimethylammonium bromides with alkyl chain lengths from C_{10} to C_{18} were in the range of 125 to 200 cP.

Interest in the structure and function of biological membranes has been greatly intensified with the advent of probe systems for study of these systems. For example, spin-labeled compounds provide information about the rates of lateral diffusion of molecules in membranes, inside–outside transitions of phospholipid bilayers, and about strictures on molecular motion of the membrane components.[3-9] Changes in the spectral characteristics of fluorescent probes as the result of noncovalent interaction with macromolecules are frequently used to detect hydrophobic regions in proteins and membranes.[10-16] More recently, Shinitzky, *et al.*,[17] have employed fluorescent aromatic hydrocarbons to determine the viscosity of the hydrocarbon regions of synthetic micelles. In this method the rate of molecular rotation, but not fluorescence lifetime, is a function of the viscosity of the probe environment so that fluorescence depolarization as the result of rotational diffusion can be used to determine microviscosity.

In this report we describe a new probe technique which is based on the rate of translational diffusion of the fluorescent aromatic hydrocarbon, pyrene, measured by the relative intensities of monomer and excimer fluorescence at defined hydrocarbon concentrations. Pyrene can undergo the processes in Chart I.[18]

Chart I

	Rate	Process
$P + h\nu \rightarrow P^*$	I_a	Absorption, where I_a is the intensity of absorbed light
$P^* \rightarrow P + h\nu'$	$k_f P^*$	Monomer fluorescence
$P^* \rightarrow P$	$k_{r1} P^*$	Monomer radiationless transition
$P^* + P \rightarrow P^*_2$	$k_a(P)(P^*)$	Excimer formation
$P^*_2 \rightarrow P^* + P$	$k_d(P^*_2)$	Excimer decomposition
$P^*_2 \rightarrow P_2 + h\nu''$	$k'_f(P^*_2)$	Excimer fluorescence
$P^*_2 \rightarrow P_2$	$k'_{r1}(P_2^*)$	Excimer radiationless transition
$P_2 \rightarrow 2P$	$k'_d(P_2)$	Dimer decomposition

(1) Supported in part by Damon Runyon Memorial Fund Grant DRG-1162, The Robert A. Welch Foundation Grant Q-343, the American Heart Association, Texas Affiliate, and NHLI-SCOR 14194-01.

(2) Established Investigator of the American Heart Association, 72-172.

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