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BIOLUMINESCENCE OF THE FIREFLY. KEY STEPS IN THE FORMATION OF T--ETC(U)
OCT 77 J KOO, S P SCHMIDT, G B SCHUSTER N00014-76-C-0745
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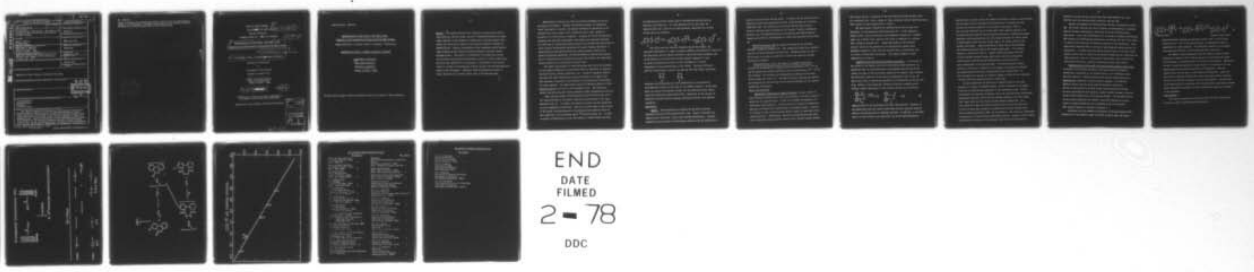

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20. Abstract

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Bioluminescence of the Firefly. Key Steps in the Formation of the Electronically Excited State for Model Systems.

(chemiluminescence / electron transfer / dioxetane / fluorescence)

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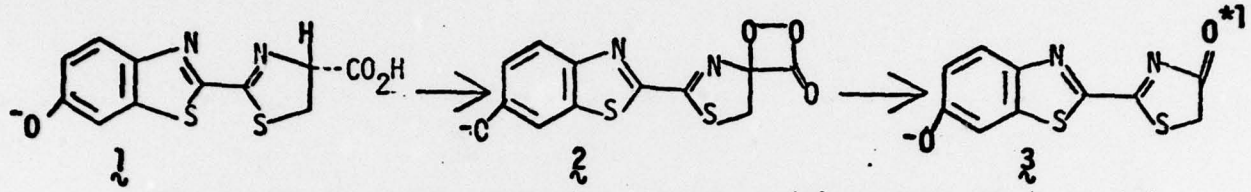
Abstract: The chemical mechanism for formation of electronically excited state molecules from the thermal reaction of dimethyldioxetanone was probed. Light production in the presence of certain easily oxidized aromatic hydrocarbons was found not to conform to the classical mechanistic schemes for chemiexcitation. Detailed investigation of the dioxetanone system revealed light formation by the recently discovered chemically initiated electron exchange process. This result is extrapolated to bioluminescent systems. In particular, the key high energy molecule involved in firefly luminescence, which has been identified as a dioxetanone, is postulated to form excited states as a result of intramolecular electron transfer from the phenoxythiazole moiety to the dioxetanone. Subsequent rapid decarboxylation results in direct formation of an excited singlet state of the emitting amide.

Bioluminescent organisms are widely distributed throughout terrestrial and aquatic environments. Although the biological purpose of luminescence varies from species to species, the chemical mechanism for generation of the electronically excited state, which subsequently emits light, appears to be general in a wide variety of organisms. In nearly all of the bioluminescent prosesses that have been investigated, high-energy cyclic peroxide molecules are implicated as providing the energy necessary for excited state generation (1). In the study of bioluminescent mechanisms the central concerns have been: i) identification of the molecule which has an energy content sufficient to permit excited state generation, ii) characterization of the emitting species, and iii) identification of the molecular process that converts the high-energy content molecule to an electronically excited state.

Our recent investigations of chemiluminescence have led to the discovery of a new general mechanism of excited state formation identified as chemically initiated electron exchange luminescence (2). Studies of exergonic chemical reactions that model bioluminescent systems now permit us to suggest that this mechanism is operative in the formation of electronically excited states in living organisms. The light-forming reaction of the North American Firefly (Photinus pyralis) will serve as the prototypical case. The conclusions reached from this system are readily extended to other bioluminescent reactions.

Many excellent studies of bioluminescence from the firefly have led to the characterization of the enzyme / substrate system involved in the light generation step (3). In summary, the substrate luciferin has been identified as $\underset{\sim}{1}$ and independently synthesized. Reaction of $\underset{\sim}{1}$ with oxygen in the presence of the enzyme luciferase generates a high energy content molecule which has been identified as the dioxetanone ($\underset{\sim}{2}$) by ^{18}O labeling studies (4). In order to produce bioluminescence, $\underset{\sim}{2}$ loses the elements of carbon dioxide and forms

an electronically excited singlet state of the observed emitting amide 3, with very high efficiency. It is the mechanism of this last step, the chemiexcitation step, which has not been previously understood and to which we will direct our attention.



(1)

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The cyclic peroxidic molecules dioxetane (4) and dioxetanones (5) have been investigated in a number of elegant synthetic and mechanistic studies (5). The observation that the thermal reactions of these high energy compounds lead to the formation of electronically excited carbonyl compounds is taken as strong circumstantial evidence for the involvement of a similar intermediate in the bioluminescence of the firefly. The major difficulty with this interpretation, however, has been the fact that simply substituted

4 5

dioxetanes and dioxetanones give rise predominantly to the essentially non-luminescent triplet excited state of the carbonyl compound. On the other hand, the natural bioluminescent systems form the emitting excited singlet state with efficiencies approximating 100%. Application of the concepts of chemically initiated electron exchange luminescence resolves this apparent incongruity.

Experimental

General. Chemiluminescence was detected by the photon counting technique using an EMI 9813 photomultiplier tube. Spectral resolution was achieved with a Jarrel-Ash / Ebert 0.25 m grating monochromator. Emission intensities were corrected for photocathode response using the manufacturer's

spectral sensitivity data and the centers of gravity for the emission spectra. Temperature control was maintained to within ± 0.05° by means of an external temperature bath. All solvents were purified by passing them through a column of activated alumina followed by distillation. Rubrene, naphthacene, perylene, and 9,10-diphenylanthracene were purchased from Aldrich Chemical Company and purified by chromatography on alumina and recrystallization before use.

Dimethyldioxetaneone (8) was prepared according to the literature procedure of Adam and coworkers (6). This compound was found to be remarkably sensitive to minute quantities of impurities. Extreme care must be used in purification of solvents and additives to supress the impurity catalyzed decomposition pathways.

Chemiluminescence of 8 in the Presence of Aromatic Hydrocarbons. Solutions of dioxetaneone 8 in CH_2Cl_2 (typical concentration 10^{-6} - 10^{-5} M) and the appropriate aromatic hydrocarbon (typical concentration 10^{-5} - 10^{-4} M) were prepared. The solutions were deoxygenated by purging with purified, filtered Argon for 3 min at 0°. The chemiluminescent intensity was measured by integrating the light emission at the maximum wavelength for the aromatic hydrocarbon fluorescence.

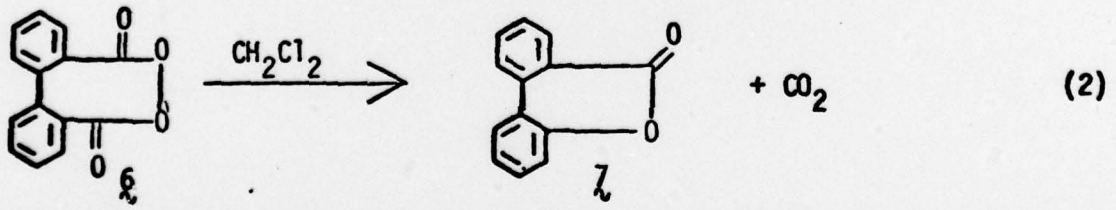
Results and Discussion

Conventional Mechanisms for Chemiluminescence. Previous studies of chemiluminescent phenomena have led to the evolution of two broad classes of mechanisms for chemiexcitation. In the first, thermal rearrangement of a high-energy content molecule, reacting directly or through an intermediate, generates an electronically excited state of a product molecule. Emission of a photon of light from the excited molecule results in observable direct chemiluminescence. Alternatively, the excited product molecule may enter into a bimolecular energy transfer reaction with a suitable energy acceptor.

This process results in formation of the electronically excited acceptor which, in a subsequent step, emits a photon of light, producing indirect chemiluminescence. This sequence is shown schematically in Figure 1.

The second general mechanism for chemiexcitation that has been well documented is electrogenerated chemiluminescence (7). In this approach a radical cation, usually generated by electrochemical oxidation at a suitable anode, and a radical anion, typically the result of reduction at a cathode, form a diffusive encounter pair. Charge annihilation in the encounter ion pair results in the generation of an electronically excited state. Subsequent emission of light from this excited state, or one derived from it, results in detectable luminescence. This general pathway is shown schematically in Figure 2.

Chemically Initiated Electron Exchange Luminescence. In the course of our studies of the thermal reactions of high-energy content molecules, we established that the chemiluminescence from diphenoyl peroxide (6) did not conform to either of the conventional mechanisms for chemical light formation. The thermolytic conversion of peroxide 6 to benzocoumarin (7), in ca. 70% yield occurs with no significant generation of an electronically excited state of 7. However, it was found that inclusion of any one of several easily oxidized aromatic hydrocarbons in the reaction solution led to efficient



(2)

6 7

chemiexcitation of the hydrocarbon and bright light emission. Moreover, it was demonstrated that the aromatic hydrocarbon exerted a powerful catalytic effect on the decomposition of diphenoyl peroxide. In addition, it was shown that it is this catalytic path that gives rise to the chemiluminescence.

Significantly, the rate at which the aromatic hydrocarbon catalyzes chemiexcitation, and the actual yield of electronically excited states, correlates with the one-electron oxidation potential of the aromatic hydrocarbon, Figure 3. Thus, we have identified the initiating step of this reaction sequence as an activated electron transfer from the aromatic hydrocarbon to diphenoyl peroxide. Subsequent rapid decarboxylation and ring closure generates the radical anion of benzocoumarin within the same solvent cage as the radical cation of the aromatic hydrocarbon. Charge annihilation of these species then generates the luminescing excited state of the aromatic hydrocarbon. This reaction sequence is detailed in Figure 4. Further convincing evidence that this reaction proceeds through a charge annihilation involving benzocoumarin radical anion is derived from the observation of exciplex emission when triphenylamine is the electron donor. The chemiluminescent exciplex emission in this case is identical in all respects to the emission generated by photoexcitation of mixtures of benzocoumarin and triphenylamine. We have designated this sequence of reactions leading to light emission chemically initiated electron exchange luminescence (CIEEL) (2).

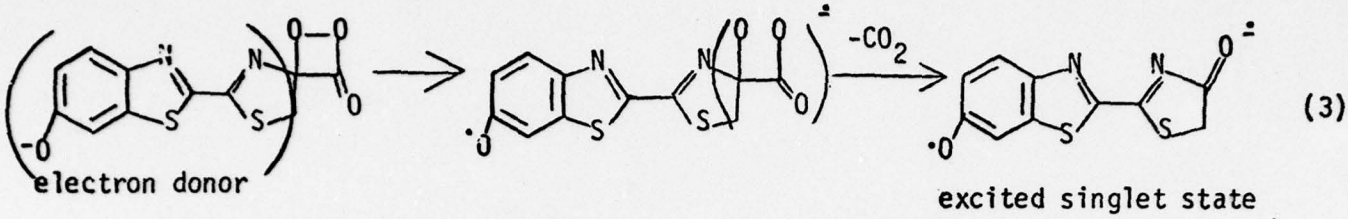
We have demonstrated the generality of chemiexcitation by the CIEEL mechanism in a number of different systems. Of most relevance to the present discussion of bioluminescent mechanisms is the dioxetanone system. Initial investigation of dioxetanone thermolysis led to the observation that unimolecular decomposition generates triplet excited carbonyl compounds in at least twenty times higher yield than the singlet excited carbonyl compound (8). In a later report (9), Adam noted that the overall yield of light was greater by a factor of twenty when rubrene was included in the reaction solution than when 9,10-diphenylanthracene (DPA) was the additive. This result was interpreted as being due to a triplet-triplet annihilation reaction. However, triplet-triplet annihilation as the cause of the increased light yield is inconsistent on

theoretical grounds and has recently been shown experimentally not to be possible under the chemiluminescent conditions reported (10).

We have investigated the chemiluminescent reaction of dimethyldioxetanone (8). Our results show that the rate of reaction of dioxetanone 8 is dependent upon the presence, concentration, and nature of easily oxidized aromatic hydrocarbons. Also, most significantly, for the series of aromatic hydrocarbons, DPA, perylene, 9,10-diphenylethynylanthracene and rubrene, the total light yield is directly correlated with the oxidation potential of the hydrocarbon. These observations implicate CIEEL as the major light producing reaction pathway for dioxetanones. If simple electronic energy transfer from the initially formed singlet excited state of acetone to the aromatic hydrocarbon was responsible for light production, then all of the aromatic hydrocarbons, when corrected for fluorescence efficiency, should have generated the same photon yield.

Bioluminescence by Chemically Initiated Electron Exchange Luminescence. In considering the options available to the high energy bioluminescent intermediate, dioxetanone 3, for chemiexcitation, it is immediately clear that this molecule is ideally constituted for excited state production by intramolecular chemically initiated electron exchange. In particular, electron transfer from the easily oxidized polycyclic heterocycle portion of the molecule to the high energy dioxetanone moiety followed by rapid decarboxylation will generate a charge transfer resonance structure of the excited singlet state of the observed emitter. The intramolecularity of this process prejudices the reaction toward the formation of excited states of singlet multiplicity, as is observed in the living system. This reaction sequence is outlined in equation 3.

Consistent with this mechanistic postulate, it has been observed that methylation of the phenolic oxygen of firefly luciferin makes the system



(3)

non-bioluminescent although the methylated ketone itself is an efficient fluorescer (11). This is the expected result if intramolecular CIEEL is responsible for light production. The methylated substrate is anticipated to be much more difficult to oxidize than the phenol anion (12).

In summary, we have demonstrated that our recently discovered CIEEL mechanism for chemical light formation operates in the dioxetanone system. The dioxetanone is a reasonable model for firefly bioluminescence. Application of CIEEL to these bioluminescent systems results in an explanation for the observed high singlet yield and the effect of methylation. Moreover, chemiexcitation by this path is quite efficient biologically. Nature has built both an effective electron donor and a high energy electron acceptor, as well as an efficient fluorescer, into the same molecule. Certainly this is an effective way to insure high yield light formation. Experiments directed toward the detailed elucidation of the bioluminescent pathway are underway in our laboratory.

Acknowledgment

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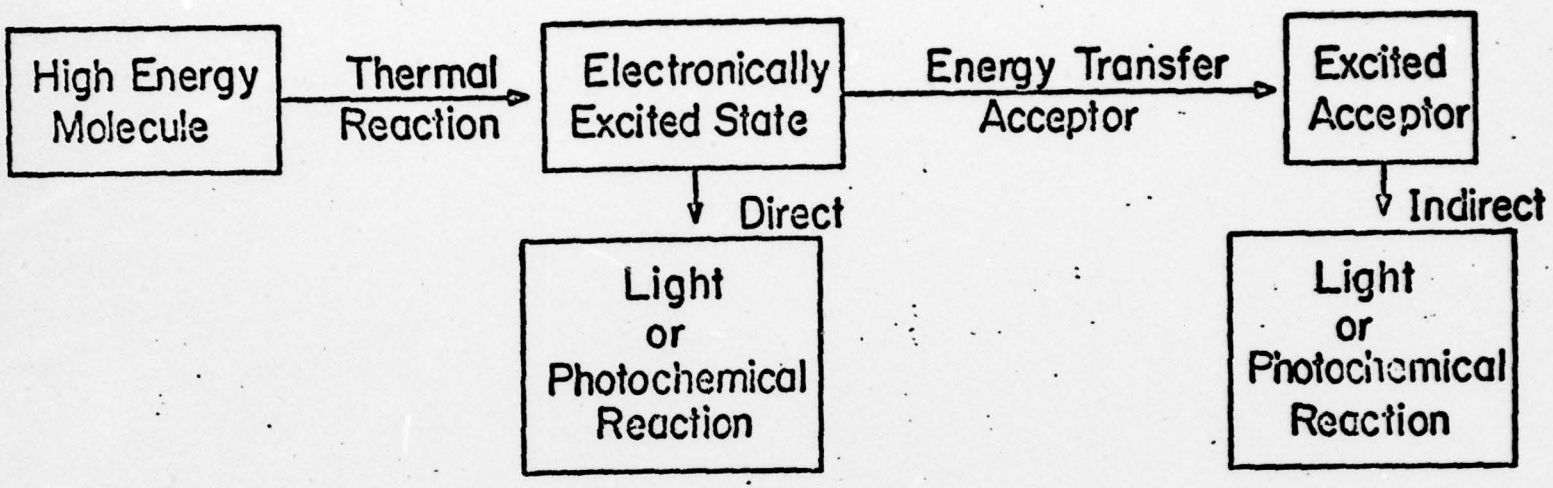
Conventional Chemiluminescence of Organic Molecules



Examples:

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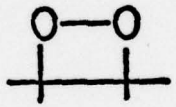
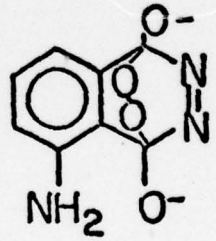
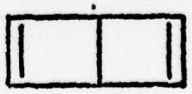


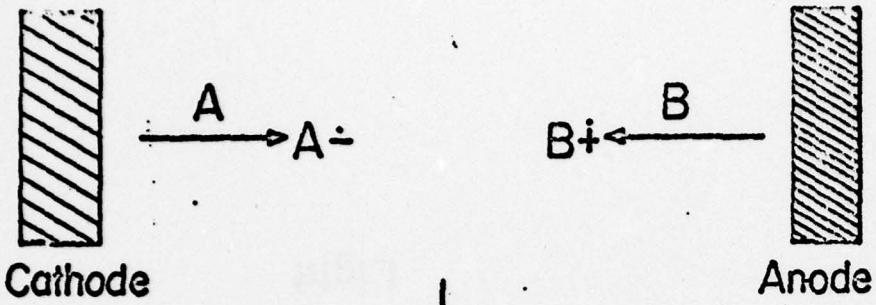
b) Luminol



c) Dewar Benzene



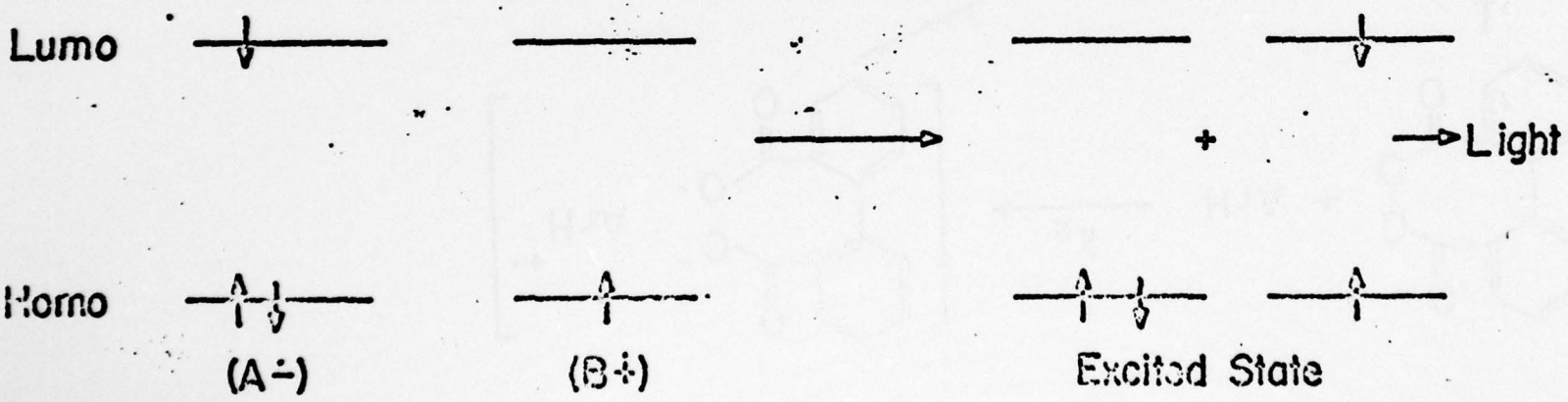
Electrogenerated Chemiluminescence (ECL)

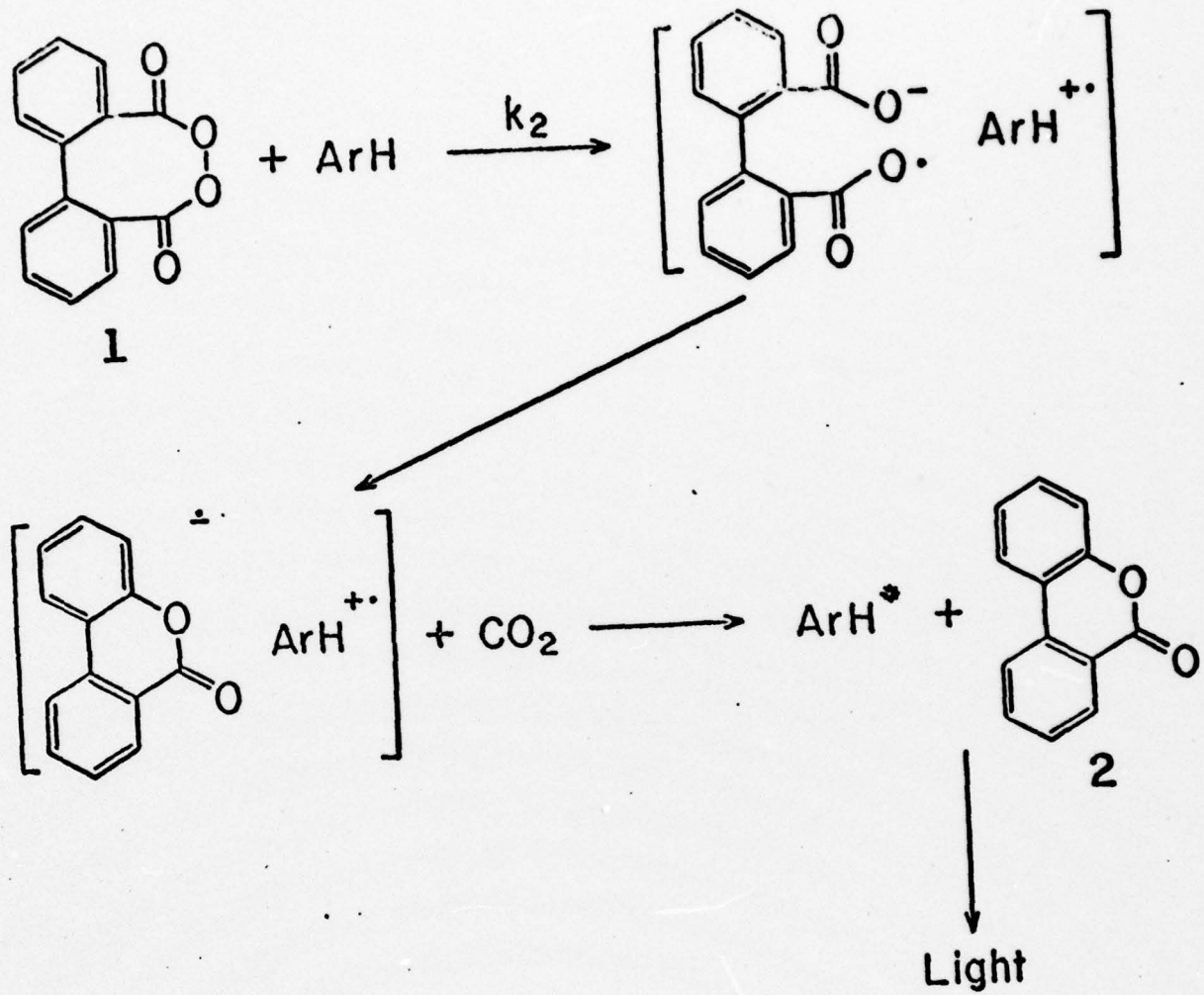


Annihilation

$(A\ B)^{*}$ electronically excited (A, B, or Exciplex)

MO – Picture

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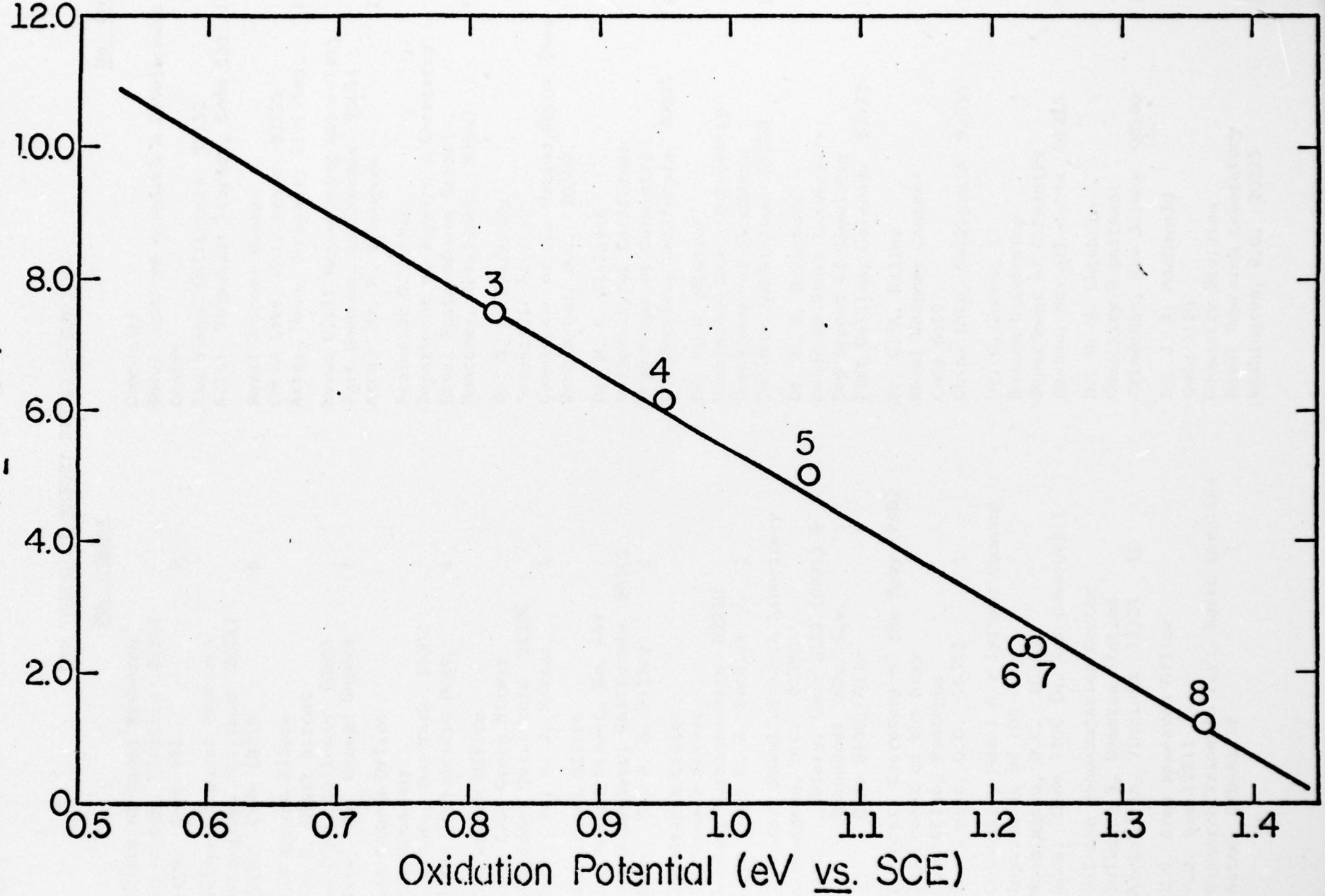
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