Generation and Transfer of Triplet Energy in Enzymatic Systems

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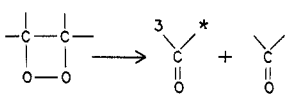
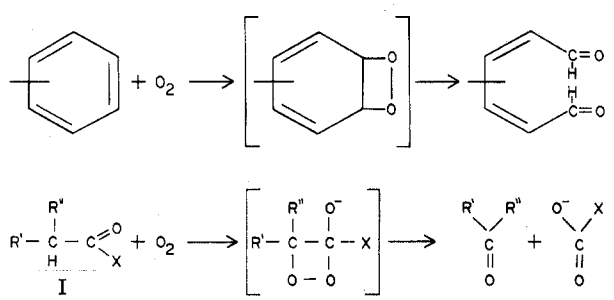
The biochemical generation of electronically excited singlet species is attested to by the well-known phenomenon of bioluminescence.[1] On the other hand, triplet states with their longer intrinsic lifetimes might be expected to have wider biological significance.[2-4] Thus at concentrations prevailing in biological systems, triplet excited species may be much better candidates than singlet excited species in terms of reaction with, or energy transfer to, another species; furthermore, the longer lived triplet excited species can in many cases migrate over longer distances than singlet excited species, enhancing the probability that the excitation energy reaches a reactive site. Broadly speaking, triplet species might, in this manner, act as agents which confer to the cell some of the potentialities of photochemistry in the absence of light.[5] Nonetheless, apart from occasional claims regarding the possible formation of triplet species,[6] a systematic study of the generation of such species in biochemical systems had never been carried out.

Clearly any such systematic study requires, at the outset, the identification of systems in which (at least formally) the generation of triplet excited species might be reasonably postulated to occur. In this regard, an important clue was furnished by the synthesis of dioxetanes[7] and the observation that, when simple dioxetanes cleave, one of the carbonyl derivatives is formed in an electronically excited state,[7,8] principally in the triplet state:[9-14]



We therefore searched for "nonemissive" biochemical reactions which generated products of the type which might be expected from the cleavage of an intermediate dioxetane. Two classes of enzymic reactions formally fit this criterion, the dioxygenases and the internal monooxygenases.[15,16]



Giuseppe Cilento was born in Sorrento, Italy, in 1923, but was raised and educated in Brazil. He received a bachelor's degree in chemistry and the D.Sc. and Privat Dozent from the Universidade de São Paulo, where he is now Professor and Chairman of the Department of Biochemistry.

We soon discovered that horseradish peroxidase (HRP) can act as an internal monooxygenase upon several substrates of the general structure I, leading us to select these systems for more detailed study. The problem was, of course, how to identify triplet carbonyl species which might be eventually formed. This problem is aggravated by the necessity of the presence of oxygen, an efficient quencher of triplet species. Since it was known that triplet carbonyl species formed by thermolysis of dioxetanes transfer energy to 9,10-dibromoanthracene (DBA) to populate the fluorescent state of the latter,[10,17,18] our first approach[19] was to add to the enzymatic system a more water-soluble analogue of DBA, i.e., the sodium salt of 9,10-dibromoanthracene-2-sulfonic acid (DBAS). Striking success was obtained in the case of the HRP-catalyzed oxidation of 2-methylpropanal to acetone and formic acid (I, $R_1 = R_2 = CH_3$; X = H), a reaction[20] particularly appealing in view of the well-known photochemistry of acetone. Indeed, addition of DBAS resulted in a strong acceptor fluorescence.[21,22] In addition, an unexpected

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(2) S. P. McGlynn, F. J. Smith, and G. Cilento, *Photochem. Photobiol.*, **3**, 269 (1964).
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(4) E. H. White, J. D. Miano, C. J. Watkins, and E. J. Breaux, *Angew. Chem., Int. Ed. Engl.*, **13**, 229 (1974).
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(6) It would be virtually impossible to cite all of the papers in which the formation of a triplet species is either claimed, suggested, or implied. Some of the more recent leading references are the following: (a) A. R. Shoaf and R. H. Steele, *Biochem. Biophys. Res. Commun.*, **61**, 1363 (1974); (b) M. Nakano and K. Sugioka, *Arch. Biochem. Biophys.*, **181**, 371 (1977); (c) Y. Ushijima and M. Nakano, *Biochem. Biophys. Res. Commun.*, **82**, 853 (1978); (d) M. Nakano and K. Sugioka, *Biochim. Biophys. Acta*, **529**, 387 (1978); (e) S. L. Guthans, W. H. Baricos, and R. H. Steele, *ibid.*, **586**, 112 (1979).
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(9) N. J. Turro, H. C. Steinmetzer, and A. Yekta, *J. Am. Chem. Soc.*, **95**, 6468 (1973).
(10) T. Wilson in *Int. Rev. Sci.: Phys. Chem., Ser. Two*, **1975–1976**, 265 (1976).
(11) E. J. H. Bechara, A. L. Baumstark, and T. Wilson, *J. Am. Chem. Soc.*, **98**, 4648 (1976).
(12) W. Adam, *Adv. Heterocycl. Chem.*, **21**, 437 (1977).
(13) K. A. Horn, J.-Y. Koo, S. P. Schmidt, and G. B. Schuster, *Mol. Photochem.*, **9**, 1 (1979).
(14) K. C. Wu and A. M. Trozzolo, *J. Photochem.*, **10**, 407 (1979).
(15) O. Hayaishi, *Rev. Annu. Biochem.*, **38**, 21 (1969).
(16) G. Cilento, *J. Theor. Biol.*, **55**, 471 (1975).
(17) T. Wilson and A. P. Schaap, *J. Am. Chem. Soc.*, **93**, 4126 (1971).
(18) N. J. Turro, P. Lechtken, G. Schuster, J. Orell, H. C. Steinmetzer, and W. Adam, *J. Am. Chem. Soc.*, **96**, 1627 (1974).
(19) N. Durán, O. M. M. Faria Oliveira, M. Haun, and G. Cilento, *J. Chem. Soc., Chem. Commun.*, 442 (1977).
(20) R. H. Kenten, *Biochem. J.*, **55**, 350 (1953).
(21) O. M. M. Faria Oliveira, M. Haun, N. Durán, P. J. O'Brien, C. R. O'Brien, E. J. H. Bechara, and G. Cilento, *J. Biol. Chem.*, **253**, 4707 (1978).

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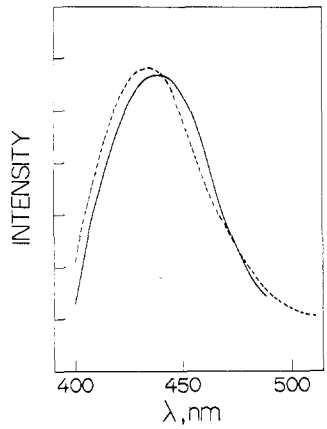

Figure 1. Chemiluminescence spectrum (solid line) from the horseradish peroxidase (2.5 μM) catalyzed aerobic oxidation of 2-methylpropanal (42 mM) in 0.4 M phosphate buffer, pH 7.0, at 35 °C, containing 0.5 M EDTA and 35 mM ethanol. The broken curve is the chemiluminescence spectrum from the thermolysis of 5×10^{-4} M tetramethyldioxetane at 53 °C in deionized water (0.5 M EDTA) under N_2. The chemiluminescence spectrum from the enzyme system has been corrected for the decreasing quenching effect of oxygen during the scanning time.

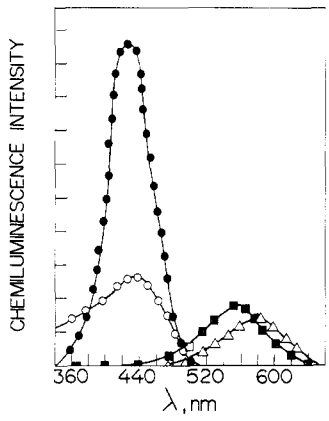

Figure 2. Sensitized chemiluminescence emission spectrum from the horseradish peroxidase (2.0 μM) catalyzed aerobic oxidation of 2-methylpropanal (84.6 mM) in 1.0 M phosphate buffer–0.14 M pyrophosphate buffer (3:2), pH 6.8, containing 0.5 M ethanol: (○) no sensitizer; (●) 10 μM DBAS; (■) 10 μM eosine; (△) 20 μM rose bengal. The ordinates for DBAS-sensitized emission have been contracted by a factor of 3.3.

result was obtained while conditions were being optimized: even in the absence of DBAS, an emission which was essentially indistinguishable from acetone phosphorescence was observed (Figure 1). The triplet nature of the excited species was confirmed by the failure to excite either the anthracene-2-sulfonate or the 9,10-diphenylanthracene-2-sulfonate ions, both of which lack the "heavy" halogen atoms necessary for spin exchange. This result is analogous to that observed in simple chemical systems in which the cleavage of dioxetanes leads to excitation of DBA with much higher efficiency than of anthracene.[10,23] The enzymically excited species was also capable of exciting several other acceptors[21,22,23-29] (Figure 2), among them photoreceptors such as flavins,[24] phytochrome,[26] and chlorophyll;[27] furthermore, the very low concentrations required indicated that the donor species was long-lived, pointing to a triplet species.

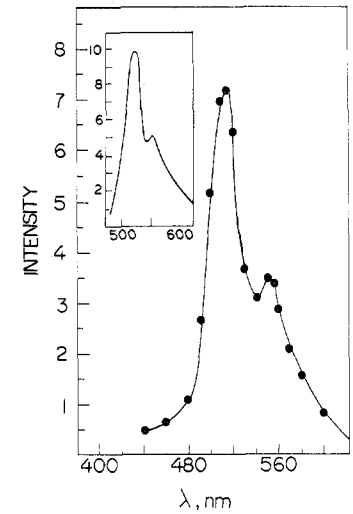

Figure 3. Chemiluminescence spectrum from the horseradish peroxidase (2.5 μM) catalyzed aerobic oxidation of 2-methylpropanal (84 mM) at 35 °C in the presence of biacetyl (2 mM), in 1.0 M phosphate buffer–1.0 M pyrophosphate buffer (2:1), pH 7.4. The insert shows biacetyl phosphorescence spectrum reported by J. T. Dubois and F. Wilkinson (*J. Chem. Phys.*, **38**, 2541 (1963)) corrected for the photomultiplier sensitivity.

The very fact that the species itself emits even in the presence of O_2 indicates that if it is indeed an excited triplet species, it must be generated largely protected from deactivating O_2 collisions, i.e., within, and considerably shielded by, the enzyme. The analogy for an emissive triplet species in the presence of oxygen is provided by some enzymes which have well-buried tryptophan residues that phosphoresce in aerated solutions at room temperature.[30,31] Consistent with the hypothesis of a somewhat protected triplet species, biacetyl phosphorescence (Figure 3) could be readily observed at high acceptor concentration, implying that triplet–triplet energy transfer from the species is occurring.

The important question has been raised by Song[32] with regard to the exact identity of the emissive species. Since the triplet state of formate is essentially degenerate with that of acetone, it is conceivable that the triplet state might be delocalized over two product molecules tightly bound to the enzyme at the time to their formation. Therefore, the observed emission might also be explained in terms of emission from a degenerate triplet exciton or excitation resonance stabilized acetone–formate pair. An answer to this question is provided by the HRP-catalyzed oxidation of propionaldehyde to acetaldehyde and formic acid.[25,33]

(22) E. J. H. Bechara, O. M. M. Faria Oliveira, N. Durán, R. Casadei de Baptista, and G. Cilento, *Photochem. Photobiol.*, **30**, 101 (1979).
(23) W. Adam, G. Cilento, and K. Zinner, *Photochem. Photobiol.*, in press.
(24) M. Haun, N. Durán, and G. Cilento, *Biochem. Biophys. Res. Commun.*, **81**, 779 (1978).
(25) N. Durán, M. Haun, A. Faljoni, and G. Cilento, *Biochem. Biophhys. Res. Commun.*, **81**, 785 (1978).
(26) O. Augusto, G. Cilento, J. Jung, and P.-S. Song, *Biochem. Biophys. Res. Commun.*, **83**, 963 (1978).
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(28) N. Durán and G. Cilento, *Photochem. Photobiol.*, in press.
(29) G. Cilento, N. Durán, K. Zinner, C. C. C. Vidigal, O. M. M. Faria Oliveira, M. Haun, A. Faljoni, O. Augusto, R. Casadei de Baptista, and E. J. H. Bechara, *Photochem. Photobiol.*, **28**, 445 (1978). This review article also reports results of our earlier work.
(30) M. L. Saviotti and W. C. Galley, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4154 (1974).
(31) R. Lumry, *Photochem. Photobiol.*, **27**, 819 (1978).
(32) P.-S. Song, following discussion of the paper in ref. 22.

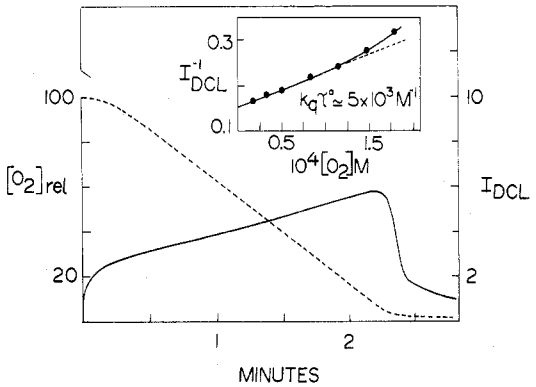


Figure 4. Chemiluminescence intensity during the horseradish peroxidase (2.5 μM) catalyzed aerobic oxidation of 2-methylpropanal (42 mM) in 0.4 M phosphate buffer, pH 7.4, at 30 °C in the presence of 1.7 mM EDTA. The sharp drop in intensity coincides with O_2 depletion; oxygen consumption is linear along the reaction. The insert shows the plot of reciprocal I_{DCL} vs. concentration of O_2 assuming the initial concentration of O_2 to be 0.21 mM. (Reprinted with permission from ref 22. Copyright 1979, Pergamon Press.)

Although this system is practically nonemissive, an excited species is clearly generated, the sensitized emission behavior in the presence of appropriate acceptors being similar to that of the 2-methylpropanal/HRP/O_2 system. The fact that the propionaldehyde system is not emissive implies that formate is not the emitter in the 2-methylpropanal/HRP/O_2 system and that the radiative properties of a triplet pair, if present, are determined in large part by the carbonyl fragment.

Protection of triplet acetone by the enzyme is not complete because both the nonsensitized and the DBAS-sensitized emissions increase somewhat during O_2 depletion. From this fact and the demonstration that the rate of the enzymatic reaction is zero order with respect to the oxygen concentration[22] a Stern–Volmer analysis was possible (Figure 4); $k_q\tau^0$ values for O_2 quenching are in the range of (2–5) × 10^3 M^{-1}.[22] Obviously the relative contributions of the effect of the microenvironment on τ^0 and k_q cannot be unambiguously separated. In line with the known quenching of triplet carbonyls by dienes,[34-36] sodium sorbate had a quenching effect upon the phosphorescence from enzyme-generated triplet acetone.[25] Since the K_{sv} value (6 × 10^3 M^{-1}) did not change with temperature, the barrier to quencher penetration is probably more entropic rather than enthalpic.[37] Stern–Volmer constants for the effect of several agents upon the enzymically generated phosphorescence are presented in Table I, along with $k_{ET}\tau^0$ values obtained from the Y-intercept/slope ratios of double-reciprocal plots of the effect of the acceptor concentration upon the intensity of sensitized emission.[10] Lower values are observed for quenchers such as oxygen, the sorbate ion, and biacetyl, which can only act collisionally; therefore, transfer to the other acceptors in Table I appears to be a long-range process (vide infra).

Table I
Quenching of Enzymically Generated Triplet Acetone[a]

quencher	$k_q\tau$,[b] M^{-1}	$k_{ET}\tau$,[c] M^{-1}
oxygen	5 × 10^3	
sorbate ion[d]	6 × 10^3	
biacetyl	6 × 10^2	
DBAS		2 × 10^5
fluorescein	2.3 × 10^5	9.8 × 10^4
eosine	1.8 × 10^4	3.3 × 10^4
Rose Bengal	4.2 × 10^4	6.7 × 10^4
riboflavin	1.9 × 10^4	2 × 10^4
FMN	2.4 × 10^4	4.8 × 10^4
FAD	4.7 × 10^4	
chlorpromazine	2.9 × 10^4	
chlorophyll	4.8 × 10^4	2.4 × 10^4
2-methyl-1,4-naphthoquinone	1 × 10^5	

[a] In 0.6 M phosphate–0.04 M pyrophosphate buffer, pH 7.4, at 40 °C. [b] Stern–Volmer quenching constant. [c] Intercept/slope ratio of double-reciprocal plot of the effect of emissive acceptor concentration upon the intensity of the acceptor emission. [d] At 18, 31, and 47 °C.

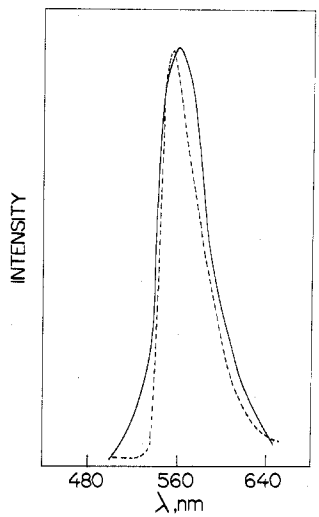
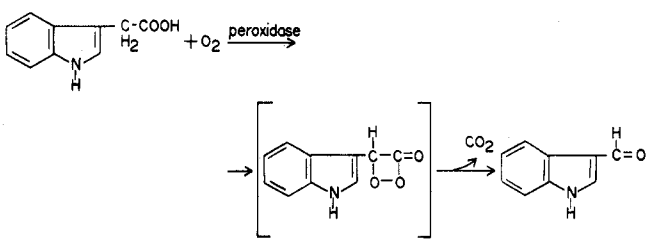


Figure 5. Chemiluminescence spectrum (solid line) from the horseradish peroxidase (2.2 μM) catalyzed aerobic oxidation of indole-3-acetate (1.5 mM) in the presence of 0.2 mM eosine–50 mM acetate buffer, pH 5.6, containing 0.28 M ethanol. The broken curve is the fluorescence spectrum of eosine. (Reprinted with permission from ref 39. Copyright 1979, Pergamon Press.)

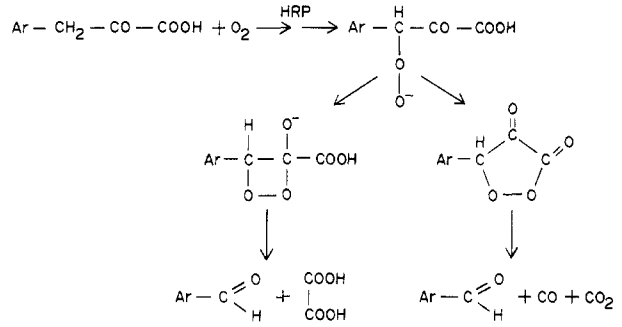
Another exceptionally interesting system which generates an excited species is the HRP-catalyzed oxidation of the plant hormone indole-3-acetic acid.[38] Under the proper conditions, indole-3-carboxaldehyde and CO_2 are generated. This system, like the 2-methylpropanal/



HRP/O_2 system, can excite eosine even when this acceptor is present in very low concentrations[39] (Figure 5).

(33) M. Haun, N. Durân, O. Augusto, and G. Cilento, *Arch. Biochem. Biophys.*, **200**, 245 (1980).
(34) P. J. Wagner, *J. Am. Chem. Soc.*, **89**, 5715 (1967).
(35) T. Wilson, D. E. Golan, M. S. Harris, and A. L. Baumstark, *J. Am. Chem. Soc.*, **98**, 1086 (1976).
(36) H.-C. Steinmetzer, P. Lechtken, and N. J. Turro, *Justus Liebigs Ann. Chem.*, 1984 (1973).
(37) M. R. Eftink and C. A. Ghiron, *Biochemistry*, **16**, 5546 (1977).
(38) J. Ricard and D. Job, *Eur. J. Biochem.*, **44**, 359 (1974).
(39) C. C. C. Vidigal, A. Faljoni-Alário, N. Durân, K. Zinner, Y. Shimizu, and G. Cilento, *Photochem. Photobiol.*, **30**, 145 (1979).

Scheme I



Additional Criteria for the Detection of a Dioxetane-type Intermediate and of Triplet Species

The fundamental tool that we have exploited in the search for triplet species is sensitized emission, partly because no other systematic methods of investigation are presently available. Once such species have been found one can exploit a variety of additional data. Three relevant examples of such data which serve to corroborate the inference of a dioxetane-like intermediate and/or formation of a triplet species are delineated below.

First, the formation of known photochemical products may, as in simple chemical systems, provide presumptive evidence for an excited precursor. Thus, the formation of excited acetone in the 2-methylpropanal/HRP/O_2 system is accompanied by substantial amounts of 2-propanol.[19,21,22] All readily conceivable controls indicate that 2-propanol must have been produced by reduction of excited acetone. The ultimate H donor must be the EtOH present in the medium because acetaldehyde is also formed.

Second, in the case of the HRP-catalyzed aerobic oxidation of aromatic pyruvates, one can detect CO and CO_2 in addition to the aromatic aldehyde, oxalate, and a weak light emission.[40] This indicates the intermediate formation of a five-membered ring α-keto-β-peroxy lactone; therefore, one may infer that formation of the four-membered ring, i.e., the dioxetane, should also be competitive (Scheme I).

Third, in the HRP-catalyzed oxidation of indole-3-acetic acid, total light emission and indole-3-carboxaldehyde formation correlate with each other; in contrast, oxygen consumption is much faster (Figure 6). We take this to be evidence for the accumulation of a hydroperoxide or dioxetane intermediate.[22]

Simple stoichiometric considerations (at low pH, O_2 consumed $\approx$ indole-3-carboxaldehyde formed $\gg$ enzyme present) imply that the intermediate has time to leave the enzyme active site, in which case it is probable that most of the excited species will be generated in the bulk solution. Our observations are only compatible with indole-3-carboxaldehyde being the excited species generated in the chemiexcitation step.

Mechanism of Generation of the Excited Species

The reaction which leads to triplet acetone must occur within the enzyme because the excited species is considerably shielded from deactivating collisions. The fundamental reaction is an oxygen insertion reaction, which cannot, however, occur by the usual chain mechanism. It is the enzyme that catalyzes oxygen insertion, shuttling between two states differing by one redox equivalent. Since absorption spectra of the reacting system indicate the presence of peroxidase compound II and there is additional evidence, including circular dichroism data, that compound I is also present, the mechanism shown in Scheme II can be envisioned.[22]

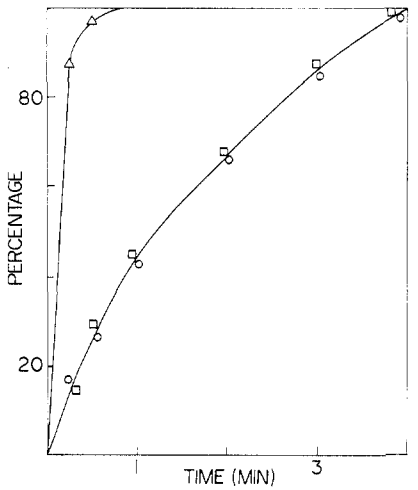
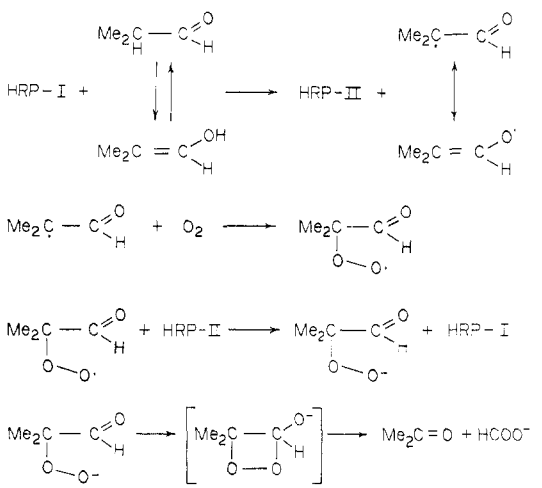


Figure 6. Attempted correlation between O_2 uptake (△), total light emission (□) and indole-3-carboxaldehyde formation (○) in the system indole-3-acetic acid (0.20 mM)–horseradish peroxidase (2.0 μM)–EDTA (30 μM)–O_2; 50 mM acetate buffer, pH 3.8, containing 39 mM ethanol. (Reprinted with permission from ref 39. Copyright 1979, Pergamon Press.)

Scheme II



Mechanism of Energy Transfer

Trivial emission–reabsorption transfer processes are immediately ruled out, either because the system is not emissive in the absence of enhancers (e.g., oxidation of propionaldehyde) or because the population of the S_1 state of the acceptor can exceed that expected from simple reabsorption of the phosphorescent emission. Even at the low acceptor concentration in Figure 2, this conclusion is apparent if one takes into account the quantum yields of fluorescence[41] for eosine (ϕ_F = 0.19) and Rose Bengal (ϕ_F = 0.02).

(40) K. Zinner, C. Vidigal-Martinelli, N. Durán, A. J. Marsaioli, and G. Cilento, *Biochem. Biophys. Res. Commun.*, **92**, 32 (1980).

(41) J. D. Spikes in "The Science of Photobiology", K. C. Smith, Plenum Rosetta Ed., New York, 1977, Ed., pp 87–112.

The effect of the 9,10-dibromo atoms of DBAS in promoting the transfer in our systems cannot be easily explained by spin–orbital modulation of a direct transfer process—the process being noncollisional, yet very fast, and resulting in net excitation of the acceptor from the S_0 to the S_1 state. Any triplet–triplet transfer process, such as to form the second triplet of DBAS followed by ISC to the fluorescent state,[42] is untenable because it would require collision. The effect of the 9,10-dibromo atoms of the acceptor in the biochemical system is indeed dramatic because no transfer whatsoever occurs to anthracene-2-sulfonate or to 9,10-diphenylanthracene-2-sulfonate, despite the fact that some excitation of these acceptors (as unsulfonated hydrocarbons) occurs in the simple chemical systems.[23]

According to Kasha,[43,44] a triplet–triplet exciton transfer, which would be long range and Z-dependent, could explain our results. Kasha postulated an exact resonance interaction between the enhanced $T_{n,\pi} \leftarrow S_0$ transition of acetone and the spin–orbitally perturbed $T_2 \leftarrow S_0$ transition of DBAS, the states most likely to be involved in the triplet–triplet exciton interaction. Subsequently, the S_1 state of DBAS would be populated by intersystem crossing from the T_2 state with consequent fluorescence of DBAS. Kasha infers that such transfer could be operative at donor–acceptor distances of 10–20 Å.

The transfer to xanthene dyes is also noncollisional (Table I). Although the rates of transfer do not differ markedly and are Z-independent, the relative populations of the S_1 state are 1:15:100 for fluorescein, eosine, and Rose Bengal.[28] This excludes a Förster-type T–S transfer because the best overlap between acetone phosphorescence and dye absorption occurs in the case of fluorescein. A CIEEL mechanism[13] also appears unlikely; indeed, an intermolecular electron transfer would probably require contact between the donor and the dioxetane, a situation not readily conceivable when the dioxetane is generated within the enzyme. In any event simple dioxetanes, such as the one which precedes the generation of triplet acetone, do not appear to be particularly prone to decomposition via a CIEEL mechanism. An upper triplet of the xanthene dye may be initially populated, with the heavy-atom perturbation playing a role in the efficiency of crossing to the S_1 state.

In the case of transfer to flavins, a Förster mechanism is feasible because flavins absorb strongly in the region of acetone phosphorescence. Moreover, the transfer might be connected with the fact that the lowest singlet excited state of the acceptor should have some n,π^* character; therefore, spin–orbit coupling may be enhanced even though flavins contain no heavy atoms.[45] It is interesting to note that transfer to flavins in our systems bears some resemblance to *Latia* bioluminescence, in which a ketone is formed in the chemiexcitation step and a flavin is excited by energy transfer.[46] Transfer to flavins also suggests the possibility (vide infra) that photoreceptors can be excited in the absence of light.[5]

Work in progress shows that enzyme-generated triplet acetone is also able to transfer its energy very efficiently to quinones. The acetone phosphorescence is quenched with initial Stern–Volmer constants of 10^5–10^6 M^{-1}. The quinones undergo alterations which, as in the case of 2-methylnaphthoquinone, are spectrally very similar to those observed upon direct irradiation of the quinone with UV light.[47] The alteration can be considerably reduced in the enzymic system by 3,5-diiodotyrosine, which is both a quencher of enzyme-generated triplet acetone[48] and an inhibitor of the light-induced alteration of 2-methyl-1,4-naphthoquinone.[49] With some of the quinones, extensive, if not complete, quenching can be observed even when the quinone concentration is much smaller than the enzyme concentration. This indicates that (at least in the case of quinones) the acceptor need not be bound to the enzyme.

The mechanism of energy transfer from enzyme-generated triplet acetone to various acceptors is still under active investigation in this laboratory. Moreover as pointed out by Kasha,[43,44] our results for transfer to DBAS show that the well-known physical principle of electronic excitation during chemical reaction and subsequent energy transfer may require modification in order to understand the biological or enzymic processes which utilize the same substrate.

Photochemical Processes Induced with Enzyme-Generated Triplet Species

As noted in the case of flavins, the very fact that the energy can be transferred to appropriate acceptors indicates that the enzymic system may drive photoprocesses in the absence of light. However, except for the transfer to biacetyl, we have considered up to now only processes which resulted in sensitized fluorescence, presumably involving long-range transfer to upper triplets. Even in these cases there should be competition in the acceptor between nonradiative transitions to the S_1 and the T_1 states. It is feasible, however, that an appropiate acceptor can be directly excited to the T_1 state, resulting in the quenching of the donor phosphorescence. Participation of acceptors in photochemical processes involving their triplet state is thus quite conceivable. Indeed we have had some success along these lines with the following systems.

Chlorpromazine, when added to the triplet acetone generating system, quenches the acetone phosphorescence very efficiently. Concomitantly, chlorpromazine is oxidized to the sulfoxide and cation radical,[25] as occurs upon irradiation.[50] The oxidation is faster than that promoted by peroxidase/H_2O_2 and is inhibited by DBAS, which competes for the electronic energy.

It is well-known that the photomorphogenic receptor phytochrome exerts its function through the reversible light-mediated conversion:[51]

$$P_r \underset{\text{far red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{fr}$$

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In the dark, enzymatically generated triplet acetone can promote the conversion of P_r into P_{fr} and of P_{fr} into P_r.[28] The efficiency with which phytochrome quenches the chemiphosphorescence of triplet acetone excludes a trivial energy-transfer process and indicates that the rate constant of the energy transfer is of the order of 10^{12} M^{-1} s^{-1}. We do not know the mechanism of energy transfer, although conditions for efficient T–S transfer of the Förster type are satisfied: the emission spectrum of the long-lived acetone (λ_{max} = 435 nm; $\tau \sim 10^{-5}$ s) overlaps the absorption spectrum of P_r and of P_{fr} in the blue region (λ_{max} = 380 and 400 nm, respectively).

Enzyme-generated triplet acetone induces single-strand breaks in DNA.[52] The alteration leads to changes in the CD spectrum[53] which are similar to those induced photochemically and is partially inhibited by DBAS. All the readily conceivable nonphotochemical mechanisms of alteration could be experimentally ruled out. In other studies it has been shown that enzyme-generated triplet acetone will induce binding of riboflavin to lysozyme; the adduct has been isolated by gel filtration and appears to be similar to, if not identical with, that induced by light.[54] In addition, a photohemolysis effect has recently been achieved with enzyme-generated triplet acetone.[55] In view or these results we can now be more confident that certain biochemical transformations, which superficially appear to be photochemical alterations,[4] may indeed be accomplished through enzyme-generated excited species.

Quantum Yield of Chemiexcitation

Independent calculation of the chemiexcitation yields from DBAS emission requires a knowledge of the precise mechanism of energy transfer. On the basis of the Kasha[44] hypothesis, we find $\phi_{chemiexc} = 6 \times 10^{-3}/\phi_{T\text{-}T_2}\phi_{ISC}$), where $\phi_{T\text{-}T2}$ is the (unknown) limiting efficiency of population of the T_2 state of DBAS by transfer from triplet acetone at infinite DBAS concentration and ϕ_{ISC} is the (unknown) efficiency of ^{1}DBAS* formation from ^{3}DBAS(T_2). When triplet acetone is enzymically generated, there is a population (7% of the total) whose only fate is reduction to 2-propanol;[19,21,22] from this yield of 2-propanol, the value of $\phi_{chemiexc}$ must be high. Thus there is no possibility that the excited species is formed in a minor side reaction; suffice it to say that acetone and 2-propanol account for a total yield of 92–100%.

The inference of very high yield chemiexcitation explains the high efficiency with which enzyme-generated triplet acetone drives photochemical processes in the dark. In the case of chlorpromazine[25] there is an almost quantitative formation of "photoproducts".

Concluding Remarks

Long-lived triplet carbonyl species can be generated enzymically in high yields. As indicated by the results for the triplet acetone generating system, some of these species are generated largely, though not completely, protected from collisional deactivation. Long-range energy transfer can occur efficiently from these excited species, whether shielded or not, to appropriate acceptors. In some cases, triplet–triplet exciton transfer, which appears to be modulated by spin–orbital coupling, may occur. As a result of these processes, photoreceptors can be excited in the absence of light. As already attested to by several "in vitro" experiments described in this Account, "photobiochemistry in the dark" is a real possibility.[56]

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(56) Although we have presented the case for a predominant role of triplet species in "photobiochemistry in the dark", it is not our intention to rule out participation by excited singlets. Indeed some possibility of their participation is suggested by the recent finding that certain luciferase systems may generate nonemissive excited singlets which can be detected only by energy transfer (R. C. Hart, J. C. Matthews, K. Hori, and M. J. Cormier, *Biochemistry*, 18, 2204 (1979).