Photobiochemistry without light

G. Cilento

Department of Biochemistry, Instituto de Quimica, Universidade de São Paulo, C.P. 20.780, CEP 01498, São Paulo, SP (Brazil)

Summary. Efficient excited state formation – much higher than that hitherto expected – may occur in organelles and in intact cells. Excited triplet states can be enzymatically generated in high yields by different routes. An example is the oxidation of isobutanal to acetone and formic acid, catalyzed by horseradish peroxidase. Other enzymatic systems that generate triplet carbonyls are linear aliphatic aldehydes when oxidized by peroxidase/O_2, or the indole-3-acetic acid/peroxidase/O_2-reaction. The latter is widespread in plants.
This new field – photobiochemistry without light – has led to a growing awareness of the idea that cells may utilize excited states to trigger photochemical processes even in the dark. Such phenomena are of considerable importance, also for the understanding of weak photon emission from biological systems.
Key words. Biological and chemi-excitation; dioxetane/dioxetanone; photochemistry; monooxygenases; chlorophyll; microsomal lipid peroxidation; induced biological light emission.

Introduction

In the unexcited ground state of organic molecules, the electrons are usually paired with opposite spins, and occupy the lower energy orbitals (fig. 1, A). Upon absorption of light of the appropriate frequency, an electron is promoted to an orbital of higher energy. When the electron is promoted to the lowest vacant orbital, we say that the molecule is in the first excited singlet state (S_1) (fig. 1, B). This state has a very short lifetime and its decay or return to the ground state is often accompanied by emission (fluorescence). There is, however, another excited state of lower energy than S_1, namely the first excited triplet state (T_1), in which the two electrons of interest have the same spin (fig. 1, C).

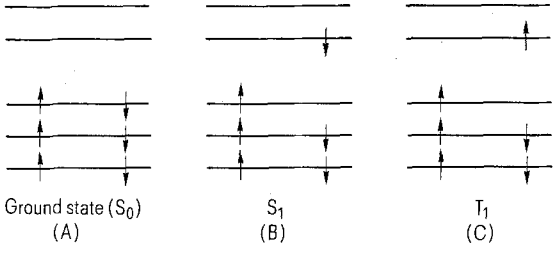


Figure 1.

Since direct promotion of the T_1 state via absorption of light is highly inefficient (the direct absorption process is spin-forbidden), it is usually reached by intersystem crossing (ISC) from the S_1 state. The efficiency of ISC depends on the particular molecule and can vary from unity down to practically zero. The intrinsic lifetime of the T_1 state is much longer than that of the S_1 state. This difference in lifetime is due to the fact that decay of T_1 to the ground state requires a change in spin, a process which is normally forbidden. Emission from T_1, called phosphorescence, is generally observed only in rigid media since in solution other processes, in particular collisions with O_2, quench the triplet state before it can emit.

A qualitative diagram depicting the relationship between the ground state (S_0), S_1 and T_1 appears in figure 2.

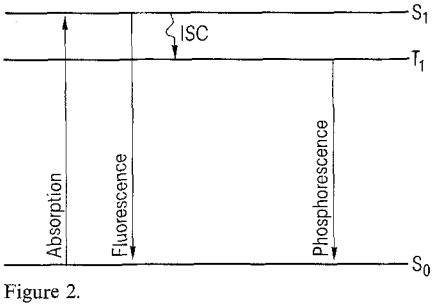
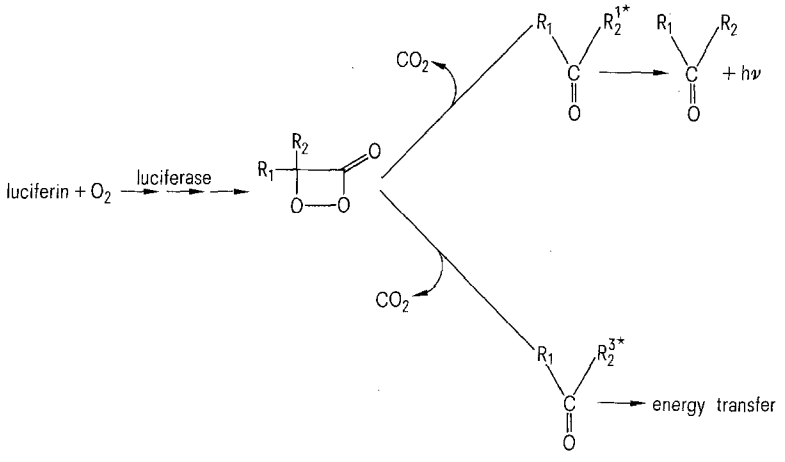


Figure 2.

In chemiluminescent reactions, the excited product is typically formed in the S_1 state and relaxes to the ground state with emission of fluorescence. The corresponding biological process is bioluminescence, which usually involves oxidation of a substrate (luciferin) to an excited product (oxyluciferin) in an enzyme (luciferase)-promoted reaction. Bioluminescence is a widespread phenomenon which is currently under very active investigation. In many cases, the precise functional role of bioluminescence is totally unknown.
The development of dioxetane and dioxetanone (α-peroxylactone) chemistry [1, 2] has led to a deeper understanding of the molecular events which result in excited state formation (chemiexcitation). These four-membered ring cyclic peroxides cleave to give two carbonyl fragments, one of which is generated in the excited state. Typically, excitation occurs to the S_1 state when the peroxide contains substituent(s) with extensive conjugation and to the T_1 state when the substituents contain no conjugation (e.g., alkyl substituents).

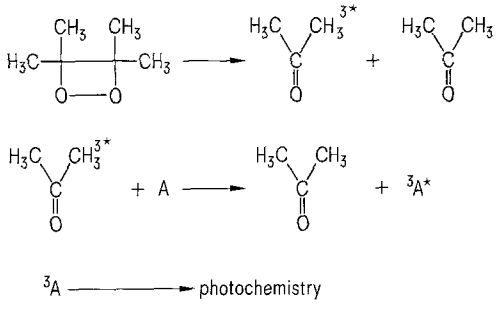
Several bioluminescent processes are now known to proceed through a dioxetanone intermediate.



In most bioluminescent processes, the presence of extensive conjugation favors excitation of the oxyluciferin to the fluorescent S_1 state. In cases where an excited triplet is formed, the energy is subsequently transferred to a fluorescent acceptor.

Enzymatic formation of excited triplet states

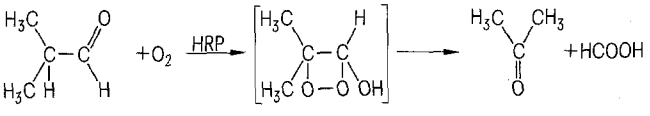
An important extension of dioxetane and dioxetanone chemistry is 'photochemistry without light'[20]; cleavage of a simple dioxetane gives a carbonyl in the T_1 state, which in turn transfers its energy to an appropriate acceptor (A), thereby inducing a photochemical process in the latter:



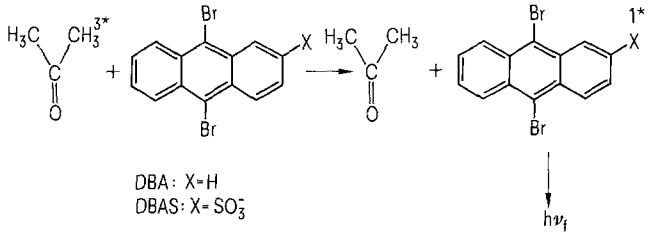
For more than twenty years we have been exploring the possibility that non-emissive excited states might also be formed and play a functional role in vivo. Other workers have also considered this possibility[8, 20].

Since the short intrinsic lifetime of the S_1 state renders the occurrence of photochemical processes induced by chemiexcited singlet states unlikely, the most probable candidate for a functionally important species would be an excited triplet state. Prior to our work, however, no systematic research had been done on biological generation of triplet states. This is not surprising, since excited states are usually detected through direct or sensitized emission, both of which are likely to be inefficient for triplet states in the presence of oxygen. Furthermore, there was no indication whatsoever as to the type of biological reaction which might produce triplet species. A fundamental breakthrough in our search for bioenergized T_1 states came with the development of dioxetane/dioxetanone chemistry.

Our first step was to select biochemical reactions that generate products of the type expected from the cleavage of a hypothetical dioxetane/dioxetanone intermediate. One of the first reactions selected for investigation was the oxidation of isobutanal to acetone and formic acid catalyzed by horseradish peroxidase (HRP)[4, 8, 9]:



a reaction which had been described by Kenten[11]. The rationale behind this choice was the fact that the photochemistry of both optically and chemiexcited (dioxetane generated) triplet acetone was rather well-known, facilitating the identification of this species. Thus, the fact that 9,10-dibromoanthracene (DBA) had been used to quantify triplet acetone generated in the thermolysis of dioxetanes led us to prepare the more water-soluble 9,10-dibromoanthracene-2-sulfonate (DBAS, as the sodium

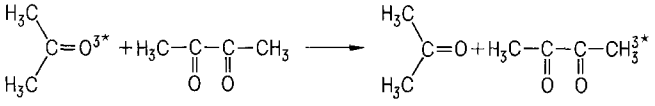


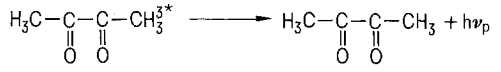
salt) and test it in the isobutanal/HRP/O_2 system. To our delight, strong DBAS fluorescence could be readily observed. As expected for triplet acetone, no sensitized fluorescence emission was observed with anthracene-2-sulfonate. When conditions were optimized, the enzymatic reaction itself turned out to be strongly emissive even in the absence of DBAS. The emission spectrum under

these conditions corresponds to that of acetone phosphorescence, providing direct confirmation of the enzymatic formation of triplet acetone. Recently, the details of the enzymatic mechanism have been largely elucidated[5]. Of particular importance is the fact that the reactive species is not the aldehyde, but rather its enolic tautomer[3]. Indeed, when precursors which generate the enol are used, the acetone phosphorescence can be observed with the dark-adapted eye and, when DBAS is added, the emission can be photographed[4].

The observation of strong acetone phosphorescence is in itself intriguing since phosphorescence is usually not detectable in the presence of oxygen. If triplet acetone were generated within the enzyme, it might, however, be largely protected from deactivating oxygen collisions. This possibility is strongly supported by the finding that D- and L-tryptophan quench the enzyme-generated acetone phosphorescence with widely different efficiencies; this chiral discrimination implies that the quencher encounters triplet acetone in an asymmetric environment, i.e., in the enzyme[5, 9]. Further support for a protective role of the enzyme comes from the fact that the intensity of the phosphorescent emission increases only 2–3-fold even after extensive oxygen depletion.

Despite protection, enzyme-generated triplet acetone can be readily quenched by several species by mechanisms not yet fully understood. Addition of flavins results in flavin fluorescence, indicating energy transfer, presumably triplet-singlet transfer of the Förster type. When biacetyl is added its phosphorescence is observed, indicating triplet-triplet energy transfer:





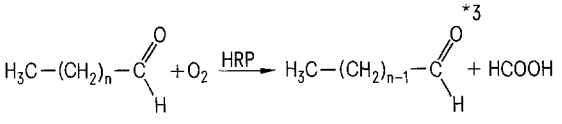
As a result of energy transfer, chlorophyll is readily excited to the S_1 state, even in chloroplasts[9]. Sensitized chlorophyll fluorescence is also elicited by several other enzymatic systems that generate excited triplet carbonyls (see below). Chlorophyll is now being used to detect triplet carbonyls in biological systems.

Photobiochemistry without light

As expected, enzyme-generated triplet acetone promotes photochemistry in appropriate acceptors. Examples include the $P_r \rightleftarrows P_{fr}$ phytochrome transformations, the covalent addition of riboflavin to lysozyme, and, of special interest in view of their biological implications, the conversion of colchicine into lumicolchicines[9] and the fission of the β-ring in provitamins D (unpublished results). Although the colchicine-lumicolchicine transformation is thermally forbidden, it occurs in the plant *Colchicum autumnale L.*, even in parts of the plant not exposed to light. Likewise, fission of the β-ring in provitamins almost always requires light in vivo. Our results indicate that enzyme-generated triplet species may also be capable of promoting these conversions in vivo. We have also succeeded in promoting photochemical-like destruction of tryptophan(s) in proteins and breaks in DNA[9]. More broadly, photobiochemistry without light may provide a general rationale for the biological occurrence of photochemical-like processes in the dark. Several of these processes have been enumerated by White et al.[20].

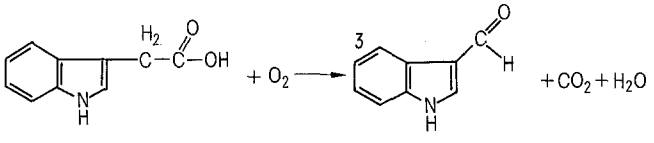
Other enzymatic systems that generate triplet carbonyls

Like isobutanal, linear aliphatic aldehydes are also efficient generators of triplet carbonyls when oxidized by peroxidase/O_2:

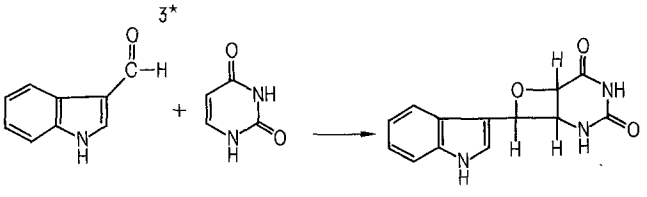


Obviously, while isobutanal leads to an excited (triplet) acetone, the product with linear aldehydes is an excited aldehyde. The aldehyde emits only weakly, one important reason being self-quenching by the starting substrate. Sensitized emission can, however, be readily observed in the presence of chlorophyll or xanthene dyes. Strong aldehyde phosphorescence emission can also be observed when the enol of the aldehyde is employed as the substrate[3].

Another system which has been extensively investigated is the indole-3-acetic acid/peroxidase/O_2 reaction[9]. At low pH, a major reaction path is



Indole-3-aldehyde is generated in the triplet state, as shown by the fact that it reacts with uridine to give the same product(s) as that formed in the photochemical reaction (Paternó-Büchi reaction):

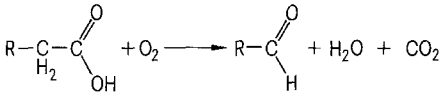


Other bases do not substitute for uridine. Triplet indole-3-aldehyde also reacts analogously with uridine groups in t-RNA. The formation of these adducts suggests that triplet indole-3-aldehyde is generated in the bulk solution. The indole-3-acetic acid/HRP/O_2 system elicits sensitized emission from the thiouridine group in t-RNA^{Phe},

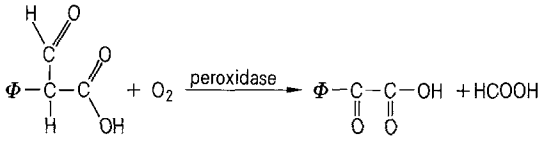
presumably as the result of energy transfer. Xanthene dyes and chlorophyll are also excited when added to the reacting system.

The indole-3-acetic acid/HRP/O_2 system is of special interest because it is widespread in plants. In view of our results, one might hypothesize that the generation of excited triplet indole-3-aldehyde is involved in the hormone (auxin) activity of the acid.

Other natural systems that generate excited states are the conversion of long chain fatty acids to the lower aldehyde [13]:



and the conversion of α-formylphenylacetic acid into benzoyl formic acid [9]:

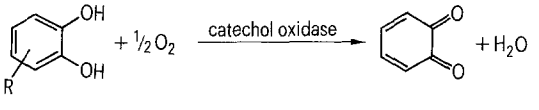


The first of these two systems is quite widespread; the latter occurs in the roots of the plant *Datura innoxia*.

Although all of the systems described above are related to the internal monooxygenases, lipoxygenase systems are also efficient generators of excited species [15].

Excited state generation by electron transfer

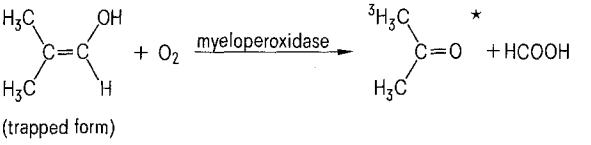
Efficient enzymatic generation of electronic energy is not restricted to the dioxetane/dioxetanone route, but can also result from electron transfer. Recently, we [19] have presented strong evidence (detection of chlorophyll sensitized fluorescence) that the oxidation of catechols by catechol oxidase – a reaction which proceeds by two electron transfer – produces electronically excited states. Both the chemiexcitation and the transfer steps are efficient.



Excited state generation in more complex systems

Using enhancers, it has now been shown that quite efficient excited state formation – indeed, much higher than was hitherto suspected – may occur in organelles and in intact cells. Thus, the very weak emission that accompanies microsomal lipid peroxidation can be greatly enhanced by binding chlorophyll to the microsomes, the sensitized emission being identical to chlorophyll fluorescence [6].

Polymorphonuclear leukocytes are rich in mieloperoxidase and, when they are challenged with the enol of isobutanal, triplet acetone is formed intracellularly; the emission is dramatically enhanced by DBAS taken up by the cells [12]:



This constitutes the first case of induced biological light emission (apart from bioluminescence) in which the identity of the enzymatic reaction leading to chemiexcitation is known.

Conclusions and comments

Excited triplet states can be enzymatically generated in high yields and by different routes. These species can efficiently transfer their energy and, as a consequence, promote sensitized emission and photochemical processes. This new field – photobiochemistry without light – has led to a growing awareness that the potential of photochemistry is available to the cell and that there is a concrete basis for the biological occurrence of what appear to be typical photochemical processes in the absence of light. In this context, a significant recent finding is that the photoactivable enzyme urocanase can be activated in the dark by the system indole-3-acetic acid/HRP/O_2 [18], which, as mentioned above is a very efficient generator of excited states. Applied to molecules with informational value (e.g. DNA) which are prone to undergo photochemical alterations, the concept of in vivo generation of excited states may lead to a better understanding of the molecular chemical events that result in deleterious processes (mutation, aging). In fact, endogenous excited species appear to be responsible for certain spontaneous mutations [14, 17]. In conclusion, the biological formation of excited states is not limited to bioluminescence. Excited state formation appears to be a widespread phenomenon in living systems, a view supported by biological chemiluminescence [7, 16]. Evidence that these species may perform important functional and/or deleterious roles is growing.

Acknowledgments. It is a pleasure to acknowledge the fundamental contributions of my colleagues and students. I reiterate my thanks to Prof. Frank H. Quina for his continuous, generous, and valuable theoretical assistance. Our work has been supported by FINEP (Rio de Janeiro), FAPESP (São Paulo), CNPq (Brasilia) and the Volkswagen Foundation (Hannover).

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Physical aspects of biophotons

F.-A. Popp[a], K. H. Li[b], W. P. Mei[c], M. Galle[d] and R. Neurohr[d]

[a]*Institute of Biophysical Cell Research, Technology Center, Opelstraße 10, D-6750 Kaiserslautern 25 (Federal Republic of Germany),* [b]*Institute of Physics, The Chinese Academy of Sciences, Beijing (People's Republic of China),* [c]*Physics Department, The Suzhou University, Suzhou (People's Republic of China), and* [d]*Zoologisches Institut, Universität Saarbrücken (Federal Republic of Germany)*

Summary. By comparing the theoretically expected results of photon emission from a chaotic (thermal) field and those of an ordered (fully coherent) field with the actual experimental data, one finds ample indications for the hypothesis that 'biophotons' originate from a coherent field occurring within living tissues. A direct proof may be seen in the hyperbolic relaxation dynamics of spectral delayed luminescence under ergodic conditions.
A possible mechanism has to be founded on Einstein's balance equation and, under stationary conditions, on energy conservation including a photochemical potential. It is shown that the considered equations deliver, besides the thermal equilibrium, a conditionally stable region far away from equilibrium, which can help to describe both 'biophoton emission' and biological regulation.
Key words. Photobiology; bio-communication; thermal radiation; spontaneous chemiluminescence; coherent radiation fields; exponential and hyperbolic relaxation; photochemical potential; phase transition phenomena; Bose condensation.

Introduction

Although the mechanisms of bioluminescence are still not completely known, there are ample indications that this intermittent light emission of at least 10^8 photons/s has some informational significance[1, 20, 32].
As well as in this more or less curious phenomenon of common 'bioluminescence' which seems to be confined to evolutionarily underdeveloped systems, photons play a fundamental role in a variety of important biological functions, namely photosynthesis[9, 23, 33], phototaxis and phototropism[21, 22, 59], photoperiodicity[3, 7, 57], photoreactivation[14, 19, 30] and, last but not least, seeing[6, 18, 36]. More and more the interrelations between all these photobiological fields are becoming evident[24, 25, 51, 58, 63].
The very existence of these phenomena obliges us to give thoughtful consideration to the biological role of 'low-level luminescence' which, as the topic of this multi-author review, is discussed here from several points of view.
It is the very low intensity, ranging from a few photons/$(s \cdot cm^2)$ up to some hundreds that provokes the prevalent opinion that this 'ultraweak photon emission from living tissues' (PE, which is actually a quasi-continuous