Excited singlet molecular \( \text{O}_2 \) (\(^1\Delta_g\)) is generated enzymatically from excited carbonyls in the dark

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In mammalian tissues, ultraweak chemiluminescence arising from biomolecule oxidation has been attributed to the radiative deactivation of singlet molecular oxygen \( [\text{O}_2 \quad (\Delta_g)] \) and electronically excited triplet carbonyl products involving dioxetane intermediates. Herein, we describe evidence of the generation of \( \text{O}_2 \) (\(^1\Delta_g\)) in aqueous solution via energy transfer from excited triplet acetone. This involves thermolysis of 3,3,4,4-tetramethyl-1,2-dioxetane, a chemical source, and horseradish peroxidase-catalyzed oxidation of 2-methylpropanal, as an enzymatic source. Both sources of excited carbonyls showed characteristic light emission at 1,270 nm, directly indicative of the monomolecular decay of \( \text{O}_2 \) (\(^1\Delta_g\)). Indirect analysis of \( \text{O}_2 \) (\(^1\Delta_g\)) by electron paramagnetic resonance using the chemical trap 2,2,6,6-tetramethylpiperidine showed the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl. Using \([^{18}\text{O}]\)-labeled triplet, ground state molecular oxygen \([^{18}\text{O}_2 \quad (\Sigma_g^-)]\), chemical trapping of \( ^{18}\text{O}_2 \) (\(^1\Delta_g\)) with disodium salt of anthracene-9,10-diyldiethane-2,1-diyl disulfate yielding the corresponding double-\([^{18}\text{O}]\)-labeled 9,10-endoperoxide, was detected through mass spectrometry. This corroborates formation of \( \text{O}_2 \) (\(^1\Delta_g\)).

Altogether, photoemission and chemical trapping studies clearly demonstrate that chemically and enzymatically nascent excited carbonyl generates \( ^{18}\text{O}_2 \) (\(^1\Delta_g\)) by triplet-triplet energy transfer to ground state oxygen \( \text{O}_2 \) (\(^\Sigma_g^\pm\)), and supports the long formulated hypothesis of \( \text{O}_2 \) (\(^1\Delta_g\)) involvement in physiological and pathophysiological events that might take place in tissues in the absence of light.

The generation of excited triplet carbonyls and of singlet molecular oxygen, \( \text{O}_2 \) (\(^1\Delta_g\)), has long been reported to occur in various biological processes, based on the observation of low-level (also called ultraweak) chemiluminescence (CL).\(^{1-11}\)

Triple-carbonyl species can be generated by photoexcitation of carbonyl compounds. Importantly, electronically excited carbonyls can also be generated by chemiexcitation and undergo further typical photochemical processes, i.e. without photoexcitation, which consequently was independently called by G. Cilento (University of São Paulo)\(^{10}\) and by E. H. White (Johns Hopkins University) as “photochemistry in the dark”\(^{11}\). Some examples of such “dark” reactions are the dismutation of alkoxyl radicals\(^{12}\), thermal decomposition of 1,2-dioxetanes\(^{13,14}\), thermolysis of oxetanes (reverse [2+2] Paterno-Büchi reaction)\(^{15}\), and dismutation of alkyl peroxyl radicals, known as the Russell reaction\(^{16,17}\). The quantum yield of excited triplet carbonyl generation may vary from 0.1% up to 60% in these reactions\(^{18}\). Of potential biological interest are triplet carbonyls arising from the annihilation of oxylradical intermediates during lipid peroxidation\(^{18-21}\).

Enzyme-catalyzed peroxidation can also yield excited triplet carbonyls, as in the case of aerobic oxidation of 2-methylpropanal (isobutyraldehyde or isobutanal, IBAL) catalyzed by horseradish peroxidase (HRP), which gives rise to formic acid and triplet acetone\(^{22}\). This reaction is thought to occur by HRP-catalyzed addition of molecular oxygen to the α-carbon of IBAL, yielding a 1,2-dioxetane intermediate whose homolysis renders acetone in the triplet state\(^{22-24}\). Accordingly, the chemiluminescence spectrum matches the phosphorescence spectrum of triplet acetone \((\lambda_{\text{max}} \sim 430 \text{ nm})\). In addition, \( \text{iso-propanol} \) and \( \text{pinacol} \) \((2,3\text{-dihydroxypropane})\) ultimately formed by...
hydrogen abstraction from the carbohydrate portion of HRP by triplet acetone were found in the spent reaction mixtures, thus a process that can be here classified as a source of “photo” chemical products, although formed in the dark.

The fact that the excitation energy of acetone to its triplet state is about 335 kJ mol⁻¹ 12 whereas that of O₂ (Δg) is 94.2 kJ mol⁻¹ 25,26 makes the triplet-triplet energy transfer process thermodynamically viable. Briviba et al.27 detected monomol light emission of O₂ (Δg) at 1,270 nm in CCl₄ during the thermal decomposition of 3-hydroxy-1,2-dioxetane. Singlet molecular oxygen exhibits a pair of electrons whose opposite spins in the highest occupied molecular orbital gives O₂ (Δg) dienophlic properties, which explains its significant reactivity toward electron-rich organic molecules, particularly with those exhibiting conjugated double bonds28, leading to the formation of allylic hydroperoxides, dioxetanes or endoperoxides.2,5,17,29,30. Singlet molecular oxygen has been shown to be generated in biological systems. As possible biological sources of O₂ (Δg), one can cite (i) enzymatic processes catalyzed by peroxidases or oxygenases; (ii) several reactions that take place in cells, such as annihilation of lipid peroxyl radicals (Russell reaction) 16,17,30,31; (iii) ozone oxidation of amino acids, peptides and proteins;2; (iv) reactions of hydrogen peroxide with hypohorilitre or peroxynitrite33,35; (v) thermolysis of endoperoxides30,35; (vi) in vitro photodynamic processes involving type II photosensitization reactions by suitable dyes35,36; (vii) UV irradiation of aromatic amino acids in proteins and immunoglobulins36,37; and (viii) metal-induced decomposition of a thymine hydroperoxide.38 Production of O₂ (Δg) during phagocytosis in polymorphonuclear leukocytes has also been described32,34 and observed in photodynamic therapy, where the production of this reactive oxygen species (ROS) has been demonstrated using different photosensitizers, including methylene blue, eosin and rose bengal39 or dye-containing nanoparticles40,41. Some endogenous photosensitizers may also lead to the generation of O₂ (Δg) upon exposure to UVA radiation.35,50. Photodynamic therapy has been applied successfully in both antimicrobial and antitumor treatments42,43, including inactivation of viruses in human plasma.42

Thus, there is a potential mechanistic crosstalk between O₂ (Δg) and triplet carbynol in biological environments where both excited species can be produced, either by alkoxyl and alkyleperoxyl radical dismutation or by triplet-triplet energy transfer from excited carbynols. Hence, several hypotheses, such as the production of triplet carbynols from O₂ (Δg)-driven peroxidation of polyunsaturated fatty acids, have been proposed and demonstrated experimentally, although triplet carbynol products have been detected in only a few systems.44

This investigation addresses the question whether electronically excited O₂ (Δg) can unequivocally be produced by energy transfer from excited triplet acetone to triplet molecular oxygen O₂ (Δg) dissolved in aqueous solution. We used the thermolysis of 3,3,4,4-tetramethyl-1,2-dioxetane (TMD)25,26 and the HRP/IBAL/O₂ system as chemical and enzymatic sources of triplet acetone, respectively.27 The generation of O₂ (Δg) was monitored by direct spectroscopic detection and characterization of O₂ (Δg) monomol light emission in the near-infrared region at 1,270 nm. Singlet molecular oxygen was also detected indirectly by electron paramagnetic resonance spectroscopy (EPR) of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) formed by the reaction of the spin trap 2,2,6,6-tetramethylpiperidine (TEMP) with O₂ (Δg). Further, the reaction mechanism was investigated by tracing the energy transfer from triplet excited ketone species to [1⁰²O]-labeled triplet molecular oxygen [¹⁰²O₂ (Δg)] through the detection of [¹⁰²O]-labeled O₂ (Δg) [¹⁰²O₂ (Δg)]. Chemical trapping experiments of [¹⁰²O₂ (Δg)] and [¹⁰²O₂ (Δg)] were performed using the anthracene-9,10-diyldihethane-2,1-diy disulfate disodium salt (EAS) trap by monitoring the corresponding endoperoxide (EAS'O'O, x=16 or 18) with high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS).

**Results**

**Characterization of singlet molecular oxygen generated by energy transfer from triplet acetone to triplet molecular oxygen by CL measurements.** Chemiluminescence produced by a chemical reaction provides useful information about the excited species being generated. Here, the production of O₂ (Δg) in response to the collision of excited triplet acetone with ground state molecular oxygen was investigated by monitoring the near infrared (NIR) light emission at 1,270 nm, which corresponds to the singlet delta state monomol light emission decay of oxygen (Δg → Sg) (Equation 1)25,30. The measurement of ultra-weak light emission or low level CL originating from this radiative transition is an important method for the detection and characterization of O₂ (Δg).

\[
O₂ (Δg) → O₂ (Sg) + hν (λ = 1,270 nm)
\] (1)

The CL arising from the thermal decomposition of 10 mM TMD at 70°C in air-equilibrated CCl₄ or acetonitrile (Fig. 1A(b) and 1A(a), respectively) was recorded in the UV-visible region. The CL spectrum of 10 mM TMD in CCl₄ shows a peak at 430 nm (Fig. 1B), which was assigned to the triplet excited acetone.27 Fig. 1C and 1D depict the time course of monomol light emission of O₂ (Δg) at λ = 1,270 nm and the NIR spectrum of O₂ (Δg), respectively. Since the lifetime of O₂ (Δg) in acetonitrile is much lower than in CCl₄ (5.0–8.0 × 10⁻⁷ s and 0.02–0.08 s, respectively), the TMD/O₂ (Sg) NIR light emission in acetonitrile was very low under similar experimental conditions.30,62. For comparison, the time course and spectrum of NIR light emission were recorded during the thermolysis of 1,4-dimethylnaphthalene-1,4-endoperoxide (DMNO₂)36 in methanol (Fig. 1E and 1F).

The rate of triplet ketone produced by TMD concentrations ranging from 2 to 10 mM in CCl₄ was estimated to be 4.89 ± 0.98 nM min⁻¹. The molecular oxygen concentration available in the solvent induces a saturation effect of O₂ (Δg) steady-state concentration. Briviba et al.27 estimated the yield of O₂ (Δg) produced by an analogue of TMD, 3,3,4,4-tetramethyl-1,2-dioxetane to be 0.2%.

Since the concentration of O₂ in solution can limit the generation of O₂ (Δg) by TMD thermolysis, additional luminescence experiments were performed using CCl₄. Ten minutes after starting the reaction, pure O₂ was purged inside the cuvette in an attempt to enhance O₂ (Δg) generation (Fig. 2). As expected, the influx of molecular O₂ into the system decreased the intensity of UV-visible light (Fig. 2A) due to energy transfer of the generated triplet acetone to molecular oxygen, although a slight decrease in NIR monomol light emission of O₂ (Δg) was observed (Fig. 2B). In this respect, we note that, although triplet molecular oxygen is known to be a triplet carbonyl suppressor, McGarvey et al.45 reported an inverse correlation between molecular oxygen quenching of different triplet naphthalenes in benzene and the generation of O₂ (Δg). This finding was then correlated to structural differences in naphthalene, and not to changes in O₂ concentration.

Since the sorbate anion was proposed as a probe for testing the presence or intermediacy and roles of triplet species in biological systems,46 the quenching effect of sorbate on TMD-generated triplet acetone luminescence was also examined (Supplementary Fig. 1). Although 0.5 mM sorbate was able to quench ~25% of the triplet acetone chemiluminescence in CCl₄ (Supplementary Fig. 1A), the NIR light emission generated by O₂ (Δg) did not change significantly (Supplementary Fig. 1B).

Triplet acetone is also produced by O₂-mediated oxidation of IBAL by molecular oxygen, catalyzed by HRP (Fig. 3). The total
Chemiluminescence was recorded in D$_2$O at pH 7.4 in the presence of 5 mM HRP and 10 mM IBAL (Fig. 3A). Low-level O$_2$ (1$\Delta_g$) NIR light emission was also detected at 1,270 nm under similar experimental conditions (Fig. 3B).

Singlet Molecular Oxygen Spectrum in the Near-Infrared Region. The generation of O$_2$ (1$\Delta_g$) by the thermal cleavage of TMD was also confirmed by recording the spectrum of the light emitted in the near-infrared (NIR) region (Fig. 1D). For comparison, the spectrum of O$_2$ (1$\Delta_g$) generated by thermolysis of DMNO$_2$ was also recorded (Fig. 1F). Both spectra showed an emission band with maximum intensity at 1,270 nm, characteristic of the monomolecular decay of singlet oxygen delta state. Additional proof that the light emitted in the TMD reaction corresponds to O$_2$ (1$\Delta_g$) was obtained by testing the effect of solvents. The intensity of light emitted in the reaction performed in CCl$_4$ was higher than in acetonitrile, which is consistent...
Detection of $\text{O}_2$ ($^1\Delta_g$) by EPR. Indirect analysis of $\text{O}_2$ ($^1\Delta_g$) in $\text{D}_2\text{O}$ by electron paramagnetic resonance was performed using 2,2,6,6-tetramethylpiperidine (TEMP) as the spin trap (Supplementary Fig. 2). The lifetime of $\text{O}_2$ ($^1\Delta_g$) in $\text{D}_2\text{O}$ is similar to that observed in acetonitrile ($5.0–6.5 \times 10^{-5}$ s$^{-1}$). The EPR spectrum depicted in Supplementary Fig. 2A (line a) shows a triplet signal ($a_N = 1.60$ mT, $g$-shift $= -0.5$) obtained upon incubation of 30 mM TEMP with 4 mM TMD in normally aerated $\text{D}_2\text{O}$. The pre-addition of 0.4 mM commercial standard 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to the reaction mixture intensified the EPR signal significantly, thus suggesting the generation of $\text{O}_2$ ($^1\Delta_g$) (line b).

EPR experiments using TEMP were also conducted with the HRP/IBAL system, as depicted in Supplementary Fig. 2B. The EPR spin-trapping signal obtained also overlaps the TEMPO signal, showing the same coupling constants. This finding provides further evidence of the generation of $\text{O}_2$ ($^1\Delta_g$) by the HRP-treated aldehyde.

When the reaction of TMD was conducted in the presence of 30 mM TEMP and 32 mM sorbate, no significant decrease in TEMPO was observed (data not shown). Although sorbate can reportedly suppress triplet acetone generated from TMD$^{18}$, diene quenching was unable to compete actively with the excitation of oxygen in the presence of TEMP.

When 20 µM HRP and 50 mM IBAL were incubated with 8 mM sorbate, the EPR signal of TEMPO was suppressed (Supplementary Fig. 2B, line e).

Detection of $[^{18}\text{O}]-\text{Labeled Singlet Molecular Oxygen}$ in the Chemical and Enzymatic Reactions. To better characterize the mechanism involved in the generation of $\text{O}_2$ ($^1\Delta_g$) by the thermolysis of TMD or HRP-catalyzed aerobic oxidation of IBAL, $[^{18}\text{O}]-$labeled $\text{O}_2$ ($^2\Sigma_g^-$) was used as a triplet energy acceptor. The generated $[^{18}\text{O}]-$labeled $\text{O}_2$ ($^1\Delta_g$) was trapped with the anthracene derivative, EAS (Fig. 4)$^{5,30}$. The corresponding endoperoxides (EAS $x\text{O}_x\text{O}$, $x = 16$ or 18) were detected by HPLC-ESI-MS/MS.

In the dark, energy transfer from triplet ketone to $[^{18}\text{O}]$ or $[^{18}\text{O}]-$labeled molecular oxygen led to a mixture of mainly two anthracene endoperoxide derivatives, namely, the fully labeled 9,10-endoperoxide (EAS 18O18O) and the related unlabeled endoperoxide (EAS 16O16O), plus a small amount of partially labeled endoperoxide (EAS18O16O).
tion. Analysis of the products by UV absorption at 210 nm showed two peaks corresponding to the endoperoxides EAS 18O18O and EAS 16O16O and to EAS at the time windows 7.2 to 7.9 min and 9.2 to 12.2 min, respectively, for the TMD (Fig. 5A and Supplementary Fig. 3A) and HRP/IBAL systems (Fig. 6A and Supplementary Fig. 4A and 5A). The tandem mass spectrometry detection of EAS 18O18O (m/z 230) and EAS 16O16O (m/z 228) was performed by the Selected Reaction Monitoring (SRM) mode. SRM detection based on the fragmentation of precursor ions at m/z 230 (Fig. 5B and 6B) and 228 (Fig. 5C and 6C), which generated the product ion at m/z 212, shows the presence of EAS 18O18O and EAS 16O16O, respectively. The identity of the precursor ions was confirmed based on an analysis of the mass spectra of product ions derived from each of the endoperoxides (Fig. 5E and 5F, and Fig. 6E and 6F).

Energy transfer from excited triplet acetone generated by thermal cleavage of TMD. The thermolysis of 10 mM TMD in deuterated phosphate buffer (pD 7.4) performed in an 18O2 or 16O2 atmosphere resulted in the generation of the corresponding EAS 9,10-endoperoxides containing the 18O or 16O isotope (EAS'O'O) (Fig. 5). Formation of endoperoxides, which was confirmed by HPLC-ESI-MS/MS analysis, occurred through the mass transition of m/z 230→212 to EAS'O'O and m/z 228→212 to EAS'O'O (Fig. 5B and 5C and Supplementary Fig. 3B and 3C). In the presence of 18O-labeled O2, the amount of EAS'O'O (Fig. 5B) was ten-fold greater than that of EAS'O'O (Fig. 5C). The EAS 16O endoperoxide formed in the presence of the triplet acetone chemical generator system shows an intense [M-2H]2+ ion at m/z 230 corresponding to a molecular weight of 462 (Fig. 5D). This strongly attests to the incorporation of two [18O]-labeled oxygen atoms into the anthracene derivative molecule. This finding also confirms that O2 (1Dg) is produced by energy transfer from TMD-generated triplet acetone, and not through direct oxygen atom transfer from the 1,2-dioxetane, which lacks 18O in its molecular structure. Important to note is the fact that the amount of EAS 18O2 formed in the experiment reached a level of 90%.

Energy transfer from enzymatically generated excited triplet acetone. The generation of O2 (1Dg) by energy transfer from HRP-catalyzed production of excited triplet acetone from IBAL oxidation was monitored using water-soluble EAS, which can react with O2 (1Dg), yielding EASO2 as the specific oxidation product (Fig. 4). To this end, EAS was incubated at 37°C with HRP and IBAL in an 16O2 atmosphere. The generation of O2 (1Dg) was monitored using water-soluble EAS, which can react with O2 (1Dg), yielding EASO2 as the specific oxidation product (Fig. 4). To this end, EAS was incubated at 37°C with HRP and IBAL in an 16O2 atmosphere.
or $^{18}$O$_2$ atmosphere. The resulting 9,10-endoperoxides EAS$^{16}$O$^{16}$O and EAS$^{18}$O$^{18}$O were analyzed by HPLC-ESI-MS/MS (Fig. 6 and Supplementary Fig. 4 and 5). As expected in $^{18}$O$_2$ atmosphere, the endoperoxide EAS$^{18}$O$^{18}$O (MW 458) produced in the enzymatic reaction exhibits a [M−2H]$^2^+$ ion at m/z 228 (Supplementary Fig. 5D). Only the SRM chromatogram of EAS$^{18}$O$^{18}$O with the mass transition m/z 228 to 212 can be detected at 7.9 min (Supplementary Fig. 5C). The SRM chromatogram showed no peaks for the EAS$^{16}$O$^{2}$ mass transition (m/z 230 to 212) (Supplementary Fig. 5B).

Conversely, when the HRP-catalyzed reaction was conducted under $^{18}$O$^2$-labeled dioxygen ($^{18}$O$_2$) enriched atmosphere, the fully labeled endoperoxide (EAS$^{18}$O$^{18}$O$^{18}$O) appeared as the most abundant endoperoxide molecule. The signal of the ion corresponding to the unlabeled anthracene endoperoxide at m/z 228 was also detected with a relative abundance of about 50% compared to the fully labeled endoperoxide. Trace amounts of partially labeled endoperoxide (EAS$^{16}$O$^{18}$O$^{18}$O) was also observed (Supplementary Fig. 4C). The detection of unlabeled and partially labeled endoperoxides can be attributed to residual oxygen ($^{16}$O$_2$) present in the reaction media after the freeze-thawing cycles to replace the dissolved $^{18}$O$_2$ with $^{16}$O$_2$ and to subdue the incidence of natural light during sample handling. Because the initial step of HRP-catalyzed IBAL oxidation involves the generation of an IBAL resonant $\pi$-hydroperoxyl/enolyl radical, which ultimately yields the 3-hydroxy-4,4-dimethyldioxetane intermediate – the putative precursor of triplet acetone and formic acid$^{65}$ by thermolysis, O$_2$ ($^{18}$O$_2$) may have arisen from the radical, according to the Russell mechanism$^{16}$. This route can be safely disregarded because TMD alone would not have been able to yield a consistent amount of EAS$^{18}$O$^{18}$O$^{18}$O (Fig. 5) and the radical does not bear a geminal hydrogen, a necessary condition for singlet molecular oxygen generation by the Russell reaction$^{16}$. Sulfur stable isotope distribution in EAS$^{16}$O$_2$ and EAS$^{18}$O$_2$ molecules was also observed by Ultra High Resolution MS, providing further confirmation of the EAS endoperoxide structures (Supplementary Fig. 6 and 7).

When the IBAL/HRP system was investigated under aerated condition (Supplementary Fig. 7), an analysis of the peak corresponding to m/z transition 228 to 212 indicated that 2.0 ± 0.4 μM O$_2$ ($^{18}$O$_2$) is formed. A previous report stated that the HRP-catalyzed oxidation of IBAL generates at least 20% O$_2$ ($^{18}$O$_2$). Much less optimistic, the yield of O$_2$ ($^{18}$O$_2$) measured in our enzymatic experiments points to approximately 0.1%. Nevertheless, one must consider that the HRP-catalyzed reaction consumes the dissolved $^{18}$O$_2$ in a manner that may have arisen from the radical, according to the Russell mechanism$^{16}$. This route can be safely disregarded because TMD alone would not have been able to yield a consistent amount of EAS$^{18}$O$^{18}$O$^{18}$O (Fig. 5) and the radical does not bear a geminal hydrogen, a necessary condition for singlet molecular oxygen generation by the Russell reaction$^{16}$. Sulfur stable isotope distribution in EAS$^{16}$O$_2$ and EAS$^{18}$O$_2$ molecules was also observed by Ultra High Resolution MS, providing further confirmation of the EAS endoperoxide structures (Supplementary Fig. 6 and 7).
efficiency of 5 mM sorbate is lower in the generation of O$_2$ (‘$\Delta g$’) that accompanies the HRP-catalyzed oxidation of IBAL. This is predicted by the fact that the enzymatic system probably produces fewer triplet carbonyls than TMD and that the enzyme structure offers a collisional barrier for triplet acetone quenching produced in the active site$^{36}$. Excited triplet acetone was estimated to be produced at a rate of 0.19 $\mu$M min$^{-1}$ by 10 mM IBAL in the presence of 5 $\mu$M HRP (Fig. 3). A noteworthy fact is that the decay of light emission parallels the oxygen consumption by the enzymatic reaction$^{46}$.

**Discussion**

It is well established that electronically excited triplet carbonyl products are produced chemically or enzymatically via the thermolysis of dioxetane intermediates$^{1,3,4,8}$.

Carbonyls in the triplet excited state are known to undergo unimolecular reactions (e.g., isomerization, $\alpha$- and $\beta$-cleavage) and bimolecular processes (e.g., hydrogen abstraction, (2+2) cycloadditions), or to act as an electronic energy donor to a wide spectrum of biomolecules, thus triggering typically photochemical reactions. This inspired Cilento$^{5,6}$ and White$^{1}$, in the mid-1970s, to postulate independently that chemically or enzymatically generated triplet species in cells may drive physiological and/or pathological processes in the dark, a phenomenon they coined as “photochemistry and photobiology without light,” or “photochemistry in the dark.” The isomerization of natural products (e.g., colchicine, santonin), initiation of polyunsaturated fatty acid peroxidation, generation of the plant hormone ethylene, formation of cyclobutane thymine dimers, and several other biological processes, have been predicted and some of them have been shown to occur in the dark via triplet carbonyl intermediates$^{14}$.

Our results show for the first time that singlet molecular oxygen is produced enzymatically. This paper described the generation of O$_2$ (‘$\Delta g$’) via energy transfer from excited triplet acetone from both the thermolysis of TMD and the aerobic oxidation of HRP-catalyzed IBAL.

The chemiluminescent catalytic activity of hemeproteins such as cytochrome $c$ acting on the peroxidation of fatty acids$^{4,29}$, and soybean lipooxygenase$^{58}$ or myeloperoxidase$^{59}$ inducing the oxidation of IBAL, were also accounted for by enzymatic sources of triplet excited species. The generation of methylglyoxal and diacetyl, putatively in the triplet state, by the oxidation of myoglobin-catalyzed aerobic oxidation of acetoacetate and 2-methylacetoacetate, respectively, was reported more recently$^{60}$.

From the biological viewpoint, it is worth mentioning that the generation of electronically excited triplet carbonyls in biological systems has been shown to cause oxidative injury to biologically important molecules such as DNA$^{71}$ and proteins, to trigger lipid peroxidation$^{72}$, and to induce phosphate-mediated permeabilization of isolated rat liver mitochondria$^{73}$.

In this work the formation of O$_2$ (‘$\Delta g$’) in chemical and enzymatic reactions was clearly demonstrated by direct detection of the O$_2$ (‘$\Delta g$’) monomol light emission at 1,270 nm using a photomultiplier coupled to a monochromator (Fig. 1C, 2B and 3B); and the observation of the effect of D$_2$O on the acquisition of the spectrum of the light emitted in the near infrared region showing an emission with maximum intensity at 1,270 nm (Fig. 1D).

Another evidence supporting the involvement of this mechanism was obtained by the direct detection of radicals TEMPO in the incubation reaction of TMD or HRP/IBAL with TEMP (Supplementary Fig. 2). The observed EPR spectrum suggests the presence of O$_2$ (‘$\Delta g$’) in the reaction mixture due to a mechanism involving energy transfer from the excited triplet acetone generated to molecular oxygen.

Finally, the transfer mechanism involved in the generation of O$_2$ (‘$\Delta g$’) was studied using [$^{18}$O]-labeled molecular oxygen. Experiments conducted with [$^{18}$O]$_2$ in the presence of EAS (Fig. 4), showed that TMD thermolysis and the enzymatic HRP/IBAL generation of excited triplet acetone yields a mixture of endoperoxides containing [O] and/or [O] atoms namely EAS$^{18}$O$^{18}$O, EAS$^{16}$O$^{16}$O (Fig. 5 and 6 and Supplementary Fig. 3 to 7), EAS$^{18}$O$^{16}$O (Supplementary Fig. 4). Comparison of the relative amounts of EAS$^{18}$O$^{18}$O:EAS$^{18}$O$^{16}$O: EAS$^{16}$O$^{16}$O detected before and after removal of molecular oxygen showed a significant increase in the amount of EAS$^{18}$O$^{16}$O and a decrease in the amount of both EAS$^{18}$O$^{18}$O (Fig. 5 and 6). These results indicate that the reactions yield mainly O$_2$ (‘$\Delta g$’). The differences observed with and without [$^{18}$O]-labeled molecular oxygen shows that the [$^{18}$O]-oxygen molecule present in the reaction mixture decreases the amount of detected O$_2$ (‘$\Delta g$’).

The decrease in the amount of O$_2$ (‘$\Delta g$’) detected in the presence of oxygen may be explained by an energy transfer mechanism between O$_2$ (‘$\Delta g$’) and O$_2$ (‘$\Sigma_g^-$’), yielding O$_2$ (‘$\Delta g$’) and O$_2$ (‘$\Sigma_g^-$’) as recently demonstrated for aqueous system by Martinez et al.$^{44}$. The chemiluminescence, EPR and chemical trapping of O$_2$ (‘$\Delta g$’) experiments were also performed in the presence of sorbate, showing a triplet carbonyl quenching effect.

Quenching of triplet carbonyls by the addition of conjugated dienes such as hexa-2,4-dienoates (sorbates)$^{48}$ or even by the presence of ground state, triplet molecular oxygen can abate the level of chemical damage promoted by triplets to studied targets, either biomolecules or cell organelles$^{44,29}$.

Considering the enzymatic reactions that give rise to O$_2$ (‘$\Delta g$’) through a dioxetane intermediate involving a peroxyl radical. An alternative mechanism by which the formation of O$_2$ (‘$\Delta g$’) could be explained is the Russell mechanism$^{45}$. This requires the generation of [O]-labeled IBAL, peroxyl radicals that recombine to form a hypothetical tetroxide intermediate, which then decomposes to generate O$_2$ (‘$\Delta g$’). However, this mechanism can be disregarded because it requires the presence of a geminal hydrogen in the IBAL-derived hydroperoxy radical for the formation of O$_2$ (‘$\Delta g$’) and the detection of O$_2$ (‘$\Delta g$’) containing a mixture of [O] and [O] atoms.

**Conclusion**

The present study unequivocally demonstrates that singlet molecular oxygen is generated by energy transfer from chemically and enzymatically produced excited triplet acetone to ground state triplet molecular oxygen in aqueous solution (Fig. 7).

This was substantiated by ultrawide CL studies in the near IR region at 1,270 nm with both chemical and enzymatic sources of triplet acetone, which is characteristic of the singlet delta state monomolecular decay of excited molecular oxygen. Indirect analysis based on mass spectrometry and EPR measurements strongly supports the formation of O$_2$ (‘$\Delta g$’)$.^{46}$ Moreover, the use of [O]-labeled molecular oxygen in association with HPLC-ESI-MS/MS analysis is a highly suitable way to gain relevant mechanistic insights into the formation of singlet molecular oxygen and the decomposition pathways of initially generated peroxide compounds such as dioxetanes and subsequently excited ketones.

The quantum yield of singlet molecular oxygen was found to be higher in aqueous medium than previously demonstrated in organic solvents. This work proposes that enzymatically generated triplet carbonyl may be a contributing source of O$_2$ (‘$\Delta g$’) in non-illuminated biological systems such as root and liver tissues, as earlier proposed independently by Cilento$^{58}$ and White$^{1}$.

**Biological implication** - Taking into consideration that (i) molecular oxygen is c.a. ten times more soluble in membranes that in aqueous medium, (ii) membrane peroxidation involves the intermediacy of alkoxyl and alkylperoxyl radicals derived from polyunsaturated fatty acids, whose dismutation affords triplet carbonyls$^{18}$, and (iii) phosphate-induced and sorbate-inhibited deleterious permeabilization of mitochondrial membranes via amplification of triplet pro-
It is of utmost interest to investigate the participation of singlet molecular oxygen in membrane damage induced by pro-oxidants. In addition, membrane cholesterol and proteins could also be victimized by singlet molecular oxygen formed from triplet carbonyls leading to loss or gain of biological functions. In this regard, noteworthy are the findings by several groups that cholesterol secoaldehyde formed by addition of ozone or singlet molecular oxygen to cholesterol may be implicated in atherosclerosis, Alzheimer disease, and apoptosis involving signaling pathways.

**Methods**

**Materials Used.** Peroxidase from horseradish (HRP) type VI, K_2HPO_4, KH_2PO_4, NH_4HCO_3, 2,2,6,6-tetramethylpiperidine (TEMP), 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and hexa-2,4-dienoic acid (sorbic acid) were purchased from Sigma (St. Louis, MO). 2-Methylpropanal (isobutyraldehyde or isobutanal, IBAL), D_2O and CCl_4 were purchased from Aldrich (Steinheim, Germany). HPLC grade solvents were obtained from Merck (Darmstadt, Germany). IBAL was distilled before use.

Deuterated phosphate buffer at pD 7.4 (equivalent to pH 7.0) was prepared by mixing D_2O stock solutions of KH_2PO_4 and K_2HPO_4. 3,3,4,4-Tetramethyl-1,2-dioxetane (TMD) was prepared as previously described by Kopecky et al. Standard anthracene-9,10-diylthioether 2,1-diyl disulfate disodium salt (EAS) endoperoxide (EASO_2) was prepared by methylene blue photosensitization in aerated deuterium water containing 8 mM EAS, and was subsequently quantified spectrophotometrically. 1,4-Dimethylnaphthalene (DMN) endoperoxide (DMNO_2) was also prepared by UVA irradiation of DMN/methylene blue at 50°C.

**Low level luminescence emission of excited triplet acetone produced by thermal cleavage of TMD or oxidation of IBAL by HRP/H_2O_2 and NIR detection of the monomol light emission of O_2 (^1A_g).** TMD dissolved in CCl_4 at concentrations ranging from 2 to 10 mM was transferred from ice to a cuvette holder set at a temperature of 70°C. The light emission was immediately recorded by a FLSP 920 photon counter (Edinburgh Instruments, Edinburgh, UK) consisting of two UV-Visible Hamamatsu detectors R9110, maintained at −20°C by a CO1 thermostatic cooler also purchased from Edinburgh Instruments. The detector used to measure the steady-state light emission from TMD thermal cleavage was not preceded by any monochromator; therefore, light was recorded directly from the cuvette source. To trace the TMD-elicited chemiluminescence, a second detector was used and its wavelength was determined using a monochromator. The chemical yield of 10 mM TMD-generated triplet acetone was confirmed in acetonitrile, reportedly evaluated as approximately 30%.

**HPLC-ESI-MS detection of EASO_2.** HPLC-ESI-MS/MS analyses of the anthracene endoperoxide EASO_2 were conducted by injecting 25 μL of the sample in a Shimadzu HPLC system (Tokyo, Japan) coupled to a mass spectrometer Quattro II triple quadrapole (Micromass, Manchester, UK). Endoperoxide EASO_2 was separated using a Luna C18 reverse phase column, 250 × 4.6 mm, 5 μm particle size (Phenomenex, Torance, CA) that was kept at 25°C. The liquid phase consisted of 80% acetonitrile and 20% water containing 0.1% formic acid. The elution time for EASO_2 was confirmed in acetonitrile, reportedly evaluated as approximately 10 min.
25 mM ammonium formate (solvent A) and acetonitrile: methanol 7:3, v/v (solvent B) with linear gradient of 25% B during 15 min, 25 to 70% B for 1 min, 70% B until 25 min, 70 to 25% B during 1 min and 25% B until 30 min. The eluent was monitored at 210 nm with a flow rate of 0.8 mL/min. First 5 min of run gradient was discarded and 10% of flow rate was directed to the mass spectrometer. Ionization of the sample was obtained by electrospray ion source (ESI) in the negative ion mode using the following parameters: source temperature, 120 °C, desolvation temperature, 200 °C; cone voltage, 15 V; collision energy, 10 eV. The endoperoxides EASO₂ were detected by the loss of the oxygen molecule, in the Selected Reaction Monitoring mode (SRM). The transitions recorded were m/z 228→212 for EASO₂O⁻, m/z 229→212 for EASO₂O⁻ and m/z 230→212 for EASO₂O⁻.

UHR-ESI-Q-TOF detection of EASO₂. High resolution mass spectrometry analysis of EASO₂O endoperoxides were performed in an UHPLC Agilent coupled to UHR-ESI-Q-TOF Bruker Daltonics MaXis 3G mass spectrometer with CaptiveSpray source in the negative mode. The UHPLC mobile phase consisted of ammonium formate (solvent A) and acetonitrile: methanol 7:3, v/v (solvent B) with the following linear gradient: 25% B during 15 min, 25 to 70% B for 1 min, 70% B until 25 min, 70 to 25% B during 1 min and 25% B until 30 min. Endoperoxides was separated on a Luna C18 reverse phase column, 250 × 4.6 mm, 5 μm particle size (Phenomenex, Torance, CA) and monitored at 210 nm. The flow rate was 0.8 mL/min. Reverse phase column was kept at 30 °C. The ES conditions were: capillary, 4.0 kV; dry heater, 180 °C; dry gas, 8.0 l/min; end plate, −450 V. Nitrogen was used as collision gas and the CID (collision-induced dissociation) energy was 10 eV. The instrument was externally calibrated using an ESI low concentration tuning mix over the m/z range of 100 to 200. The Bruker Data Analysis software (version 4.0) was employed for data acquisition and processing.
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Author contributions

C.M.M., E.J.H.B., F.M.P. & P.D.M. contributed equally to this work. P.D.M., E.J.H.B., S.M., G.R.M., G.E.R., J.C., H.S. & M.H.G.M. developed the concept of the experiments and the analyses. P.D.M., E.J.H.B., S.M. & T.M. conducted the experiments. All the authors made significant contributions to the discussion and writing of this manuscript.

Additional information

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