Biological significance of singlet oxygen

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The biological significance of singlet oxygen \( (^{1}O_2) \), an electronically excited species of oxygen, has been realized only in the last two decades. This was mainly due to the lack of proper methodology to generate this reactive oxygen species \( (\text{ROS}) \) in pure form and its reactions with biological molecules. Recent studies, using newly developed detection methods, show that \( ^{1}O_2 \) being generated in many biological systems, can significantly and quite often adversely alter several crucial biomolecules including DNA, proteins and lipids with undesirable consequences including cytotoxicity and/or disease development. The reactions of \( ^{1}O_2 \) with the biological molecules are rather specific, as compared to other ROS. There are various compounds, mainly derived from natural sources that offer protection against damage induced by \( ^{1}O_2 \). Among the antioxidants carotenoids are the most effective singlet oxygen quenchers followed by tocopherols and others. The same reactive species if generated specifically in diseased states such as cancer can lead to the cure of the disease, and this principle is utilized in the newly developing modality of cancer treatment namely photodynamic therapy. Singlet oxygen, in low concentrations can also act as signaling molecule with several biological implications. This review clearly brings out the biological significance of \( ^{1}O_2 \).

People have become more health conscious in recent years. One etiologic agent implicated in diseased state is ‘oxidative stress’ which involves excess generation of prooxidants. These species, in biological systems include excited states, free radicals and other related species mainly derived from oxygen and nitrogen. As such, prooxidants are generated in our body during the normal metabolic processes as well as during exposure to adverse pathophysiological conditions. In a healthy human body the generation of prooxidants in the form of reactive oxygen species \( (\text{ROS}) \) and reactive nitrogen species \( (\text{RNS}) \) are delicately balanced by the antioxidant defenses. Exposure to prooxidants results in oxidative stress that shifts the balance in favour of prooxidants \( ^{1} \text{ROS} \). Of interest, generated during oxidative stress include hydroxyl radical, superoxide, peroxyl radical, hydrogen peroxide and singlet oxygen \( (^{1}O_2) \). The significance of \( ^{1}O_2 \) has been realized only recently due to the development of methods for its generation, free from other contaminants as well as its detection. Singlet oxygen has been considered as a major cytotoxic species to eukaryotic cells, bacteria and viruses. Extra-cellularly generated \( ^{1}O_2 \) has been found to be genotoxic to mammalian cells grown in culture. On several instances, singlet oxygen has been implicated in the induction of tumour by photosensitization and in the metabolic activation of carcinogens. Besides, it has been implicated in several pathological processes like lung-oxidant injury, skin photosensitivity and erythropoietic porphyria. The latter condition has been shown to be due to accumulation of specific pigments below the skin due to a metabolic defect. Some reports also show that \( ^{1}O_2 \) play a significant role in the inactivation of cells or cellular components due to UV-A and near-visible radiation \( ^{15,6} \). This brief review gives a bird’s eye view of the developments in the study of singlet oxygen.

Historical

Even though oxygen has undergone two centuries of investigation, \( ^{1}O_2 \) has been recognized to exist only from 1924. Starting with its accidental discovery by Howard Seliger \(^{2} \) in 1960, due to its 'glowing ability in the dark', this excited species has been a scientific enigma. During the initial 'astrophysical period' (until 1963) \( ^{1}O_2 \) was regarded as a rare species largely of importance in atmospheric physics. The terrestrial significance or its chemical role becomes recognized in the following 'chemical period' during which there was an exponential growth of published literature on this reactive species mainly on its chemical nature and reactions. In the last 3 decades, however, scientists from various disciplines like Physics, Chemistry, Biology and Medicine are attracted towards this enigmatic species and have contributed significantly \( ^{9} \) to the present-day knowledge about \( ^{1}O_2 \). Like many other reactive species, this can be harmful at higher concentrations and at low levels may act as signaling molecule.
Chemical nature

Molecular ground state oxygen, as present in the biological milieu, is kinetically inert. This nature can be explained by its electronic structure. The two unpaired electrons in the outermost orbit have the same quantum number imposing a spin restriction on the reactivity of oxygen. This, however, can be removed by moving one of the unpaired electrons in a way that alleviates the spin restriction. This phenomenon requires an input of energy and generates the singlet states of oxygen. The singlet states of oxygen do not have unpaired electrons and hence do not qualify as a radical. Delta singlet oxygen \((^1\Delta g \text{O}_2)\) is the most important in biological systems and has 22.5 kcal/mole of energy above the ground state. Sigma singlet oxygen \((^\Sigma g \text{O}_2)\) has 37.5 kcal/mole of energy above the ground state and usually decays to the \(^1\Delta g \text{O}_2\) state before it reacts with another matter (half life in aqueous system 10.9 s as compared to 10.6 s for the \(^1\Delta g \text{O}_2\)). Due to their emissions at specific wavelengths, these species can be detected using different photo-detectors

Generation in biological systems

Singlet oxygen can be generated in biological systems by two different routes, — by ‘light reactions’ due to photo-excitation and by ‘dark reactions’ due to chemi-excitation. A major route for the former process is by the type II photosensitization reaction resulting in an energy transfer from triplet state of photosensitizer to ground state molecular oxygen \(^1\text{O}_2\) (see Fig. 1 for various ways for the generation of \(^1\text{O}_2\)).

Many cellular constituents such as flavins, porphyrins, cytochromes, 4-thiouridine etc. as well as

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Ways, by which singlet oxygen can be generated}
\end{figure}

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Method</th>
<th>Comments</th>
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<tbody>
<tr>
<td>1.</td>
<td>By chemiluminescence from radiative transition of (^1\text{O}_2) to the ground state</td>
<td>Sensitivity low</td>
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<tr>
<td>a.</td>
<td>Dimol emission (2\text{O}_2\rightarrow 3\text{O}_2 + \text{hv}) (634, 703 nm)</td>
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<td></td>
<td>Detection by red sensitive, thermoelectrically cooled photomultiplier</td>
<td></td>
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<tr>
<td>b.</td>
<td>Monomol emission (^1\text{O}_2\rightarrow \text{O}_2 + \text{hv}) (1270 nm)</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>Detection by germanium diode detector</td>
<td>Expensive</td>
</tr>
<tr>
<td>2.</td>
<td>Chemical traps</td>
<td></td>
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<tr>
<td>a)</td>
<td>Diels-Alder reaction of dienes to form endoperoxides</td>
<td></td>
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<tr>
<td>b)</td>
<td>‘Ene’ reactions of alkenes to give allylic hydroperoxides</td>
<td></td>
</tr>
<tr>
<td>c)</td>
<td>With alkynes to form (1-2) - dioxygenates ((2 + 2 \text{ cycloaddition}))</td>
<td></td>
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<tr>
<td>d)</td>
<td>With sulphides to form sulfoxides</td>
<td></td>
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<tr>
<td>e)</td>
<td>With electron rich phenols to form hydroperoxydienones</td>
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<tr>
<td>f)</td>
<td>With deoxyguanosine to form endoperoxide</td>
<td></td>
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<tr>
<td>g)</td>
<td>With tertiary amines to form nitroxy radical, detectable by ESR</td>
<td></td>
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<td>h)</td>
<td>Histidine (\rightarrow) endoperoxide formation and oxidation in the presence of nitrosodimethylaniline</td>
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<tr>
<td>i)</td>
<td>With cholestrol to form 5-e-hydroperoxide derivative</td>
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<td>3.</td>
<td>Quenchers</td>
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<tr>
<td>a)</td>
<td>By energy transfer – carotenoids and nickel complexes with high rate constants ((\sim 10^{10} \text{ M}^{-1}\text{ s}^{-1}))</td>
<td></td>
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<tr>
<td>b)</td>
<td>Electron transfer – DABCO Diazabicyclo ([2, 2, 2]) octane, phenols, sulphides &amp; azides. Lower rate constants ((\sim 10^7) to (10^8 \text{ M}^{-1}\text{ s}^{-1}))</td>
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<td>4.</td>
<td>Use of deuterated solvent</td>
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<td></td>
<td>In (\text{D}_2\text{O}) life time 15-18 times longer than in (\text{H}_2\text{O}). Same with deuterated organic solvents</td>
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Ref. 8
biologically active drugs like tetracycline, chlorpromazine, merbromin, thiazides, psoralens, photosensitizers used in photodynamic therapy (PDT), suspended particles in the polluted atmospheres and fullerenes such as C_{60} and C_{70} have the ability to generate \(^{1}\text{O}_2\) under illumination (Table 1)\(^{13,38}\). During photosensitization these compounds absorb energy, undergo intersystem crossing and transfer energy to the molecular ground state oxygen generating \(^{1}\text{O}_2\). Dyes such as methylene blue, rose bengal etc. also generate \(^{1}\text{O}_2\) on photo-excitation.

The dark reactions that generate \(^{1}\text{O}_2\) include enzymatic reactions catalyzed by dioxygenases, lactoperoxidases, myeloperoxidases, cytochromes, tryptophan pyrrolase and lipoxygenases. There are evidences for \(^{1}\text{O}_2\) production obtained using a number of purified enzyme systems\(^{8,12}\). Cadenas et al.\(^{39}\) obtained evidence for \(^{1}\text{O}_2\) participation in the metabolism of arachidonic acid by prostaglandin-endoperoxidase synthase through the chemiluminescence spectrum at 634 and 703 nm. A similar identical spectrum was obtained using isolated cytochrome P_{550} or microsomal fractions supplemented with hydroperoxide\(^{40}\). Lipid peroxidation in microsomal fractions initiated by hydroperoxides or iron/ascorbate, as well as in isolated hepatocytes has been studied and the available evidence point to the \(^{1}\text{O}_2\) formation via the Russel mechanism. Singlet oxygen is generated either at the

catalytic site of the enzymes or produced by the decomposition of unstable primary oxidation products. It can be produced from peroxyl radicals by Russel mechanism hydrogen peroxide plus hypochlorite, non-enzymatic dismutation of superoxide or dismutation of 1,2-dioxetanes via triplet excited ketones.

Metabolic generation of \(^{1}\text{O}_2\) has been shown to occur in stimulated neutrophils. Some studies show that \(^{1}\text{O}_2\) can be derived from the spontaneous rather than enzymatic decomposition of superoxide. Hence superoxide dismutase indirectly protects from \(^{1}\text{O}_2\). This species has also been found to be generated from other reactions of biological relevance such as lipid peroxidation and reaction of hydroperoxides with peroxynitrite.

The formation of \(^{1}\text{O}_2\) by these mechanisms is likely to be increased under the influence of certain xenobiotics capable of inducing oxidative stress. In the mammalian tissues, one of the main candidates for \(^{1}\text{O}_2\) production is the activated polymorphonuclear leukocytes. The primary function of these cells is to destroy invading microbes. In response to such stimuli, the cells generate superoxide, hydrogen peroxide and hypohalous acids during a process known as ‘respiratory burst’. Fairly large and toxic quantities of \(^{1}\text{O}_2\) are produced during respiratory burst via reactions catalyzed by the lysosomal myeloperoxidase. The generation of \(^{1}\text{O}_2\) by polymorphonuclear neutrophils

<table>
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<tr>
<th>Category</th>
<th>Chemicals/drugs</th>
<th>Refs.</th>
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<tbody>
<tr>
<td>Cellular constituents</td>
<td>flavins, porphyrins, cytochromes, 4-thiouridine</td>
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</tr>
<tr>
<td>Drugs</td>
<td>tetracycline, chlorpromazine, merbromin, thiazides, quinine, chloroquine, primaquine, quinacrine, mefloquine</td>
<td>13,14</td>
</tr>
<tr>
<td>Photosensizers for PDT</td>
<td>haematoporphyrin, haematoporphyrin derivative, chlorins, bacteriochlorins, phthalocyanines, hyporeEllins, hypercin, meso tetraakis(4-(carboxymethyl)enoxy) phenylporphyrin, meta (tetrahydroxyphenyl)chlorin, lutetium bis-ethyltetraazaporphyrin, Merocyanine-540, tin ethyl etiopurpurin-I, tin octaethybenzochlorin, nitrophenyl ether, 5,10,15,20-tetakis(4-N-Methylpyridyl)porphyrin 5,10,15,20-tetraarylthiaporphyrinatozincl thiopyrylium, selenopyrylium, telluropyrylium</td>
<td>15-17,18-19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37</td>
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<td>Plant and bacterial pigments</td>
<td>thespone, thespesone, mansonone-D, mansonone-H, antiarauquinone, barleriaquinone-I</td>
<td>29,30,31</td>
</tr>
<tr>
<td>Other xenobiotics</td>
<td>naphtalolines, silica, diperoxovanadate, fullerenes, titanium dioxide, p-phenilene vinelene, biphenyl derivatives, azo-dye Orange II, rose Bengal, aloxazines, isoalloxazines</td>
<td>32,33,34,35,36,37</td>
</tr>
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</table>
or myeloperoxidase during bactericidal action, photosensitization and during lactoperoxidase activity was proved. This provided evidence showing microbactericidal activity is related to \( \cdot O_2 \) generation. Human saliva, in presence of low amounts of hydrogen peroxide can also generate \( \cdot O_2 \) (ref. 12).

**Detection and quantification of \( \cdot O_2 \)**

Several techniques have been developed for the detection and quantification of \( \cdot O_2 \) (Table 2). Among these techniques the highly specific ones are expensive. The more practical ones are the ones that use 'traps' and measurement of specific products formation. A variety of spectroscopic techniques have been developed to evaluate the \( \cdot O_2 \)-quenching capacity of antioxidants. Infra-red photoemission accompanies the spontaneous decay of \( \cdot O_2 \) quenching. A group of quenching assays is based on a pulse of \( \cdot O_2 \) generated with a photochemical source. The lifetime of the photoemission in absence and presence of carotenoid is measured by time-resolved spectroscopy. The generation of \( \cdot O_2 \) can also be monitored continuously either chemically or photochemically and the effect of antioxidant on the steady state level of photoemission is evaluated.

**Reaction with biological molecules**

Due to its relatively long half-life (in the range of 1-50 \( \mu s \) in aqueous systems), \( \cdot O_2 \) can travel appreciable distances in the cellular environment and is capable of damaging various biomolecules. In human plasma, which is rich in antioxidants, the life-time of \( \cdot O_2 \) is calculated to be 1 \( \mu s \). It can move freely across water-lipid interfaces. It can behave like a strong electrophile in solution and reacts with biomolecules possessing regions of high electron density (for instance guanine in DNA). Oxidative damage in biomolecules mediated by \( \cdot O_2 \) is rather frequent. Lipids, proteins and DNA are all at risk.

**Lipids**

Cellular biomolecules like lipids are the most susceptible to oxidative damage. Reaction of ROS with lipids leads to the highly damaging reaction, lipid peroxidation. Singlet oxygen reacts with unsaturated fatty acids and forms lipid hydroperoxides that break down to several products of lipid peroxidation. Lipid peroxidation induced by \( \cdot O_2 \) has been implicated in the haemolysis of erythrocytes, damage to cardiomyocytes and degeneration of cellular membranes in different tissues. The other processes of biological interest initiated by \( \cdot O_2 \) include rancidity of oils, spoilage of milk and coloured foodstuff exposed to light.

Lipid hydroperoxides (LOOH) are prominent non-radical intermediates of lipid peroxidation whose identification can often provide valuable mechanistic information, e.g. whether a primary reaction is mediated by \( \cdot O_2 \) or oxyradicals. Certain cholesterol-derived hydroperoxides (ChOOHs) have been used effectively in this regard, both in model systems and cells. Being more polar than parent lipids, LOOHs perturb membrane structure/function and can be deleterious to cells. However, LOOHs can also participate in redox reactions, the nature and magnitude of which often determines whether the resulting peroxidative injury is enhanced or prevented. Enhancement may result from iron-catalyzed one-electron reduction of LOOHs, leading to free radical-mediated chain elongation, whereas prevention may reflect selenoperoxidase-catalyzed two-electron reduction of LOOHs to relatively non-toxic alcohols. An aspect of related research that is under intensive investigation is lipid peroxidation/LOOH-mediated stress signalling that may eventually lead to induction of antioxidant enzymes and apoptotic cell death.

Participation of \( \cdot O_2 \) in lipid peroxidation reactions can be established by analyzing the product of cholesterol oxidation. The hydroperoxides 3-beta-hydroxy-6-alpha-cholest-6-ene-5-hydroperoxide (5-alpha-OOH), 3-beta-hydroxycholest-4-ene-6-alpha-hydroperoxide (6-alpha-OOH) and 3-beta-hydroxycholest-4-ene-6-beta-hydroperoxide (6-beta-OOH) are derived specifically from \( \cdot O_2 \) addition. During the photodynamic process, 5-alpha LOOH is photogenerated at a much greater initial rate and it also decays much more slowly during GSH/PHGPX treatment and hence more toxic to cells. The ratio of 7-LOOH/5-alpha LOOH or 7-LOOH/6-LOOH can be used as a highly sensitive index of singlet oxygen vs free radical dominance in photodynamically stressed cells. 5-Alpha LOOH has also been identified as a product in skin of rats treated with oral doses of pheophorbide and subsequent visible irradiation that have been shown to induce photosensitive diseases in animals and humans. This shows the evidence for involvement of \( \cdot O_2 \) in vivo in the etiology of disease.

Various studies have also shown the involvement of \( \cdot O_2 \) in lipid peroxidation induced by different photosensitizers: that includes (1) in mitochondria of Sarcoma 180 ascites tumour exposed to the porphyrin derivative meso-tetraakis (4-carboxymethyleneoxy)
phenyl] porphyrin\textsuperscript{47,48}; (2) melanotic M6 cell line exposed to bis(tri-n-hexylsiloxy)silicon phthalocyanine\textsuperscript{49}; (3) photodynamic treatment of promyelocytic K562 cells in the presence of monoglucosylporphyrin or hematoporphyrin\textsuperscript{50}; (4) fullerene C\textsubscript{60} exposed to UV or visible light\textsuperscript{51} in rat liver microsomes; (5) murine L1210 cells exposed to merocyanine 540\textsuperscript{52}; (6) rat brain mitochondria exposed to 5,10,15,20-tetrakis[4-(carboxymethylenoxy)] phenyl] porphyrin\textsuperscript{53}; and (7) liposomes exposed to hypocrellin A\textsuperscript{54} and other compounds\textsuperscript{55,56}.

Lipid peroxidation can have both direct and indirect consequences. Normally cellular membranes are selectively permeable, hence allow only certain solutes to pass through. This ability is lost due to lipid peroxidation whose products modify the physical characteristics of biological membranes. Incorporation of \textit{LOOH} changes the physical structure of the membrane by decreasing the fluidity and increasing permeability. When free fatty acids get damaged membrane confirmation is lost and may lead to 'gaps' in the membrane. It can also cause cross-links between two fatty acids, fatty acid and proteins etc. This can eventually lead to change in membrane properties and loss of its bound enzymes. Lipid peroxidation can also result in formation of several toxic byproducts that can attack other cellular targets, including DNA, away from the site of generation. They can also alter cell signaling or act as 'toxic second messengers' that amplify damage. Such byproducts include 4-hydroxynonenal, malonaldehyde etc induce apoptosis. They form adduct with DNA and induce mutagenicity and carcinogenicity and induction of apoptosis\textsuperscript{57}.

During lipid peroxidation the products formed such as \textit{LOOH} can alter the physical characteristics of the membrane. Thus, the removal of the lipid peroxidation products from the membrane is necessary to repair its damage and is accomplished by two separate enzymatic systems: the sequential action of phospholipase A\textsubscript{2} with glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase. Phospholipase A\textsubscript{2} catalyzes the hydrolysis of the phospholipid hydroperoxides to the hydroperoxy fatty acids. Once released, the fatty acid hydroperoxides may undergo a reaction with glutathione peroxidase to form stable, reduced hydroxy products. A second enzymatic system eliminates phospholipid hydroperoxides from lipid membranes through the direct reaction of phospholipid hydroperoxide glutathione peroxidase with the esterified phospholipid hydroperoxides\textsuperscript{58}.

**Proteins**

Reaction of \textit{\textsuperscript{1}O\textsubscript{2}} with proteins is more selective yielding specific products. Among the amino acids histidine, tryptophan, methionine and tyrosine are more reactive towards this ROS. Oxidation of these amino acids results in sulphoxides and short-lived endoperoxides that may be toxic to other cells. If ROS is generated in solution, it may lead to non-specific (global) protein damage, while if it is 'site-specifically generated' can lead to site-specific or localized damage. Global damage can be measured by estimating protein carbonyls. In the localized damage, the defensive action of scavengers to remove ROS decreases dramatically, since they are unable to access the microenvironment. The study of oxidation of proteins has gained momentum in recent years and has been linked to various diseases states and the process of ageing. Lipofuscin, an aggregate of peroxidized lipid and proteins, accumulates in lysosomes of aged cells, brain cells of patients with Alzheimer's disease and in iron-overloaded hepatocytes. The carbonyl content of protein in rat hepatocytes increases with age. Oxidative inactivation of several enzymes has been associated with ageing and in pathological states like ischemia-reperfusion. Singlet oxygen can inactivate proteins as exemplified by enzymes of citric acid cycle exposed to intracellularly generated \textit{\textsuperscript{1}O\textsubscript{2}} and crystallin proteins of the eye exposed to sunlight. Such oxidation of crystallins leads to formation of high molecular weight cross-links that may eventually result in cataract\textsuperscript{12,59,60}.

**DNA**

Reaction of \textit{\textsuperscript{1}O\textsubscript{2}} with DNA can lead to strand breaks and formation of altered bases. \textit{\textsuperscript{1}O\textsubscript{2}} was generated by 3 different methods namely i) microwave discharge, ii) photosensitization with rose bengal immobilized on a glass plate, and iii) chemical generation using the thermal decomposition of the endoperoxide of naphthalene dipropionate\textsuperscript{3,57,60,62}. Exposure of single-stranded bacteriophage M13 DNA to \textit{\textsuperscript{1}O\textsubscript{2}} led to a decrease in transforming activity. Loss of such activity was doubled following replacement of H\textsubscript{2}O in the buffer by D\textsubscript{2}O, increasing the life time and consequently, the diffusion path-length of the \textit{\textsuperscript{1}O\textsubscript{2}} generated. Single-stranded DNA was more susceptible than double-stranded DNA. Later studies have shown that reaction of \textit{\textsuperscript{1}O\textsubscript{2}} with bases like guanosine, the most susceptible base, produced through cycloaddition mechanism showed a 7-fold higher reactivity with single-stranded as compared to duplex 8-hydroxyde-
oxyguanosine. Recent results demonstrated that $^{1}O_{2}$, when released within cells, is able to oxidize cellular DNA directly.

Devasagayam et al. have used several simple biological systems for studying the effect of photosensitization/$^{1}O_{2}$ and its possible prevention by natural and/or dietary compounds that function as antioxidants. One of the model systems used for studying the mechanisms and modulation of DNA damage caused by photosensitization is plasmid DNA. After exposure in presence and absence of different modifiers the DNA was subjected to agarose gel electrophoresis. Form II that results from single-strand breaks. Damaged as Form I (supercoiled form) of DNA. When the resulting photo-negative is scanned in a scanning densitometer. In control DNA most of DNA gets separating effect of in presence and absence of different modifiers the DNA gets exposed to photosensitization, for example in presence of methylene blue plus visible light, there is a significant increase in the relative amount of Form II that results from single-strand breaks. Damaging effect of $^{1}O_{2}$, generated by thermal decomposition of naphthylidine dipropionate on DNA and its protection by several natural antioxidants has also been studied.

Studies showing the ability to induce strand breaks was performed with plasmid pBR322 for the formation of single-strand formation, a second-order mechanism was suggested, as the rate of single-strand breaks is proportional to the square of the $^{1}O_{2}$ production. Studies using the combination of scavengers of ROS and D$_{2}$O have conclusively proved that $^{1}O_{2}$ was indeed the species responsible for strand break formation during the thermal decomposition of the endoperoxide of naphthalene dipropionate.

Singlet oxygen induced strand-breaks occur specifically at guanine residues and there was no selectivity among these bases. Such strand-break formation was also accompanied by formation of 8-hydroxydeoxyguanosine. These reactive species react with guanine moiety in nucleosides and DNA. The oxidation products include 8-hydroxydeoxyguanosine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua). Presence of 8-hydroxydeoxyguanosine in DNA can have serious biological consequences. One such event is random termination of DNA replication occurring at the position of the modified guanine residue and at its neighbouring bases resulting in misreading by DNA polymerase. These errors in DNA replication can eventually result in mutagenesis and carcinogenesis. Singlet oxygen can also causes alkali-labile sites and single-strand breaks in DNA. The biological consequences associated with $^{1}O_{2}$ induced DNA damage include loss of transforming ability in plasmids and bacteriophages, mutagenicity and genotoxicity.

The favoured oxidation of guanine within DNA may be explained by its lower oxidation potential with respect to that of other bases. DNA strand breaks induced by $^{1}O_{2}$ seem to be initiated by its reaction with guanine which is hydroxylated at the 8-position, leading to endoperoxide formation. Recently, Chanon et al. have proposed that $^{1}O_{2}$ reacting with guanosine or deoxyguanosine part of nucleotides does not, by itself, cause DNA cleavage. The strand break originates at the endoperoxide stage whenever this link evolves into an O-centered radical. This radical is then in a good spatial position to abstract an hydrogen intramolecularly from the ribose or deoxyribose part of the nucleotide. The carbon-centered radical thus formed on the sugar part may lead to strand break either by a p-scission mechanism or by a homolytically induced lysis.

Singlet oxygen has been shown to be the mediator of DNA damage induced by UVA (320-400 nm) and induce the formation of 8-hydroxydeoxyguanosine. Similar participation of $^{1}O_{2}$ was shown during reaction of peroxytrinitro acid and hydrogen peroxide. Such role for $^{1}O_{2}$ also has been assigned during photosensitization induced by photosensitizers such as methylene blue, rose bengal, meso-tetrakis [4-carboxymethyleneoxy] phenyl] porphyrin, meso-tetrakis [3-carboxymethylenyloxy] phenyl] porphyrin; meso-tetrakis [3,4-bis(carboxymethyleneoxy)phenyl] porphyrin; meso-tetrakis [4-(N-methylpyridyl)] porphyrin; meso-tetra(4-sulphonaphenyl)porphyrin; cationic tetrauthenated porphyrin and the fullerene C$_{60}$.

There are some natural barriers designed to protect the genome from radical attack. These include compartmentalization of the sensitive target molecules and shielding of nonreplicating DNA by histones and polyanines. If protection of DNA is not successful, cellular regulatory mechanisms such as induction of apoptosis and inhibition of cell cycle progression may prevent transfer of damaged DNA to the offspring. Alternatively, DNA repair processes can correct the damage. Excision repair is performed by enzymes such as DNA glycosylases and AP endonucleases that occur before replication, while postreplication repair provides a method for repairing lesions during or after replication.

Major repair occurs by excision of the oxidized deoxyguanosine moieties by Fpg protein (formamidopyrimidine-DNA glycosylase), preventing mismatch
of 8-hydroxydeoxyguanosine with dA, which would generate G:C to T:A transversions. More recent studies have shown that the repair of \( \text{O}_2 \)-induced DNA lesions requires several enzymes of the nucleotide and base excision repair pathways, including exonuclease III and endonuclease IV that are known apurinic/apyrimidinic endonucleases in \textit{Escherichia coli}. The other types of mutation induced by \( \text{O}_2 \) can be G:C to C:G transversions. Exonuclease III may act on the repair of \( \text{O}_2 \)-induced lesions altering the DNA repair sequence specificity. Biological protection against such damage in the form of natural antioxidants is afforded by compounds like lipoate, carotenoids, flavonoids, curcumin, tocopherols and the food-flavouring agent vanillin.

**Cellular defenses against \( \text{O}_2 \) and damage induced**

The defenses to counteract the potentially hazardous reactions initiated by \( \text{O}_2 \) include all levels of protection namely, prevention, interception and repair. Prevention mainly deals with the alteration of reactions that result in \( \text{O}_2 \) generation involving both enzymatic and non-enzymatic reactions. This can occur in various ways such as reduction and availability in the amount/availability of endogenous sensitizers and/or substrates for photosensitizing/enzymatic reactions that generate such reactive species. Apart from this and the repair enzymes that can take care of the \( \text{O}_2 \)-induced lipid, protein and DNA damage mentioned earlier, tissues also contain other lines of defenses in the form of antioxidants capable of quenching \( \text{O}_2 \).

There are two types of quenchers based on the mechanism of action: 1) compounds such as carotenoids and nickel complexes quench \( \text{O}_2 \) by energy transfer with high rate constants, generally in the region of \( 10^6 - 10^8 \text{ M}^{-1}\text{s}^{-1} \); 2) compounds like DABCO (diazabicyclo(2.2.2)octane), phenols, sulphides and azides are known to quench \( \text{O}_2 \) by electron transfer (or charge transfer) mechanisms with lower rate constants, generally in the region of \( 10^6-10^8 \text{ M}^{-1}\text{s}^{-1} \). Almost all quenchers of \( \text{O}_2 \) are compounds with low oxidation potential and will certainly react with other strong oxidants in the system. Hence there may not be "specific \( \text{O}_2 \) quenchers" to 'characterize' the involvement of \( \text{O}_2 \) reactions, especially in biological systems.

There is an increasing interest in the role of diet nutrition in pathogenesis and possible prevention of cancer. The question has been raised whether \( \beta \)-carotene may have anticarcinogenic properties independent of its provitamin activity. An inverse relationship between \( \beta \)-carotene intake and the incidence of certain types of cancer has been observed. Animal experiments have revealed anticarcinogenic properties of carotenoids. The anticancer ability of carotenoids have been attributed to physical quenching capacity of \( \text{O}_2 \) was first described by Foote and Denny. However, oxidation products of \( \beta \)-carotene can behave as prooxidants under certain conditions and promote carcinogenesis.

Potential cellular and plasma antioxidants that protect against \( \text{O}_2 \) include carotenoids, tocopherols, thiols and small molecular compounds such as carnosine, bilirubin etc. Among the biological compounds (Table 3), carotenoids are the most efficient quenchers. Though the quenching abilities of the tested carotenoids are close to the limit of diffusion control, there are considerable differences. Lycopene (present in tomato), the biologically occurring open-chain isomer of \( \beta \)-carotene, shows the greatest quenching ability. Capsorubin, present in chillies show a very high amount of quenching.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rate constant (-10^6 \text{M}^{-1}\text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene</td>
<td>9,000</td>
</tr>
<tr>
<td>( \gamma )-Carotene</td>
<td>7,300</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>6,100</td>
</tr>
<tr>
<td>( \beta )-Carotene</td>
<td>4,100</td>
</tr>
<tr>
<td>Methylbixin</td>
<td>3,000</td>
</tr>
<tr>
<td>Lutein</td>
<td>2,300</td>
</tr>
<tr>
<td>Norbixin</td>
<td>2,300</td>
</tr>
<tr>
<td>Bixin</td>
<td>1,800</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>1,500</td>
</tr>
<tr>
<td>Uric acid</td>
<td>360</td>
</tr>
<tr>
<td>Azide</td>
<td>200</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>180</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>160</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>158</td>
</tr>
<tr>
<td>( \beta )-Tocopherol</td>
<td>153</td>
</tr>
<tr>
<td>( \gamma )-Tocopherol</td>
<td>138</td>
</tr>
<tr>
<td>( \alpha )-Tocopherol</td>
<td>130</td>
</tr>
<tr>
<td>Lipoate</td>
<td>60</td>
</tr>
<tr>
<td>Vanillin</td>
<td>60</td>
</tr>
<tr>
<td>( \delta )-Tocopherol</td>
<td>53</td>
</tr>
<tr>
<td>Imidazole</td>
<td>40</td>
</tr>
<tr>
<td>Histidine</td>
<td>31</td>
</tr>
<tr>
<td>Caffeine</td>
<td>29</td>
</tr>
<tr>
<td>Histamine</td>
<td>28</td>
</tr>
<tr>
<td>Methionine</td>
<td>9</td>
</tr>
<tr>
<td>Cysteine</td>
<td>8</td>
</tr>
<tr>
<td>Thiourea</td>
<td>4</td>
</tr>
<tr>
<td>Glutathione</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3 — Singlet oxygen quenching ability of various antioxidants
biologically occurring carotenoids like γ-carotene, α-carotene, astaxanthin, zeaxanthin, lutein and cryptoxanthin also showed a high extent of quenching\(^{2,4,5,6}\). The quenching by carotenoids depends largely on physical quenching and to a lesser extent by chemical reaction.

Tocopherols are less efficient than carotenoids, whereas thiols and related sulphur-compounds are the least effective. The concentration of latter group of compounds in tissues however, is several fold higher than carotenoids. Baltschun _et al._\(^7\) have determined the bimolecular rate constants of 27 natural and novel synthetic carotenoids. Among these compounds, an empirical correlation between ππ* excitation energy and the structure of the carotenoid was found. The quenching abilities depend on the excitation energy of their transition at long wavelengths in a characteristic way showing as limiting factors either the thermal Arrhenius activation or the diffusion-controlled rate.

Carotenoids are abundant in many fruits and vegetables and they play diverse roles in photobiology, photochemistry and medicine. They react with \(^1\)O\(_2\) as well as other ROS of biological significance. They can also interact with other antioxidants and under certain conditions behave as prooxidants\(^8\). The radiomodifier buthionine sulfoximine quenches \(^1\)O\(_2\) with a rate constant of \(4 \times 10^4\) M\(^{-1}\)s\(^{-1}\) (ref.99). There are also several other compounds that quench \(^1\)O\(_2\). These include probucol (10⁶ M\(^{-1}\)s\(^{-1}\)), phenolic, nitrogenous and sulphur-containing compounds\(^9\), squalene\(^10,12\) and lipoic acid\(^103-105\).

Several cellular antioxidants are capable of preventing damage caused by \(^1\)O\(_2\) to biological molecules. Carotenoids and tocopherols are able to prevent/delay the peroxidation of lipids induced by \(^1\)O\(_2\) in cellular membranes. Several natural antioxidants play key roles in the preservation of membrane integrity. Roles of vitamins A, C and E in this aspect are well documented. Very little information is known about the function of Vit B3, nicotinamide against oxidative damage. Kamat and Devasagayam\(^104\) demonstrated for the first time that nicotinamide, an endogenous antioxidant exhibits excellent antioxidant ability against photosensitization-induced \(^1\)O\(_2\) with the rate constant of \(1.8 \times 10^6\) M\(^{-1}\)s\(^{-1}\).

They devised a simple set-up for exposing biological samples to photosensitization. The tissue sample is kept in a 'trap' maintained at 37°C. Oxygen is bubbled through and a visible light source is placed at about 15 cm from the trap. Using this the component requirement for lipid peroxidation whose product was estimated as thiobarbituric acid reactive substances (TBARS) was studied. Lipid peroxidation in absence of \(O_2\), light or methylene blue was negligible. Whereas when all the three components are present, the peroxidation is very high. Another point to be noted is if \(O_2 + \) light is given there was considerable peroxidation. This indicates that there are certain endogenous compounds present in microsomes that can act as sensitizer\(^104\). In these studies they have examined the antioxidant ability of certain natural and dietary components as protectors of membranes against oxidative damage induced by \(^1\)O\(_2\). Among the compounds examined chlorophyllin, caffeine, nicotinamide, vanillin and tocotrienols from palm oil were found to be effective. Chlorophyllin, the sodium-copper salt and water-soluble analogue of the ubiquitous plant pigment chlorophyll has been attributed to have several beneficial properties. It is highly effective in protecting rat liver mitochondria against photosensitization even at low concentrations. It also has a fairly high rate constant with \(^1\)O\(_2\) in the order of \(1.3 \times 10^8\) M\(^{-1}\)s\(^{-1}\) (ref.105). Similarly caffeine present in coffee, tea and cola-based soft-drinks also protects rat liver microsomal membranes against \(^1\)O\(_2\) and also reacts with this reactive species (rate constant of \(7.3 \times 10^7\) M\(^{-1}\)s\(^{-1}\)). Vanillin, the commonly used food flavouring agent also protected subcellular membranes, in the form of rat liver mitochondria against damage induced by photosensitization (rate constant \(6 \times 10^7\) M\(^{-1}\)s\(^{-1}\)). Nicotinamide (vitamin B\(_3\)) is an effective protector of both rat liver microsomes and rat brain mitochondria against photosensitization\(^104,106\). In addition to this property nicotinamide also has other beneficial effects such as like chemoprevention and induction of drug metabolizing enzymes\(^107,108\). Tocotrienols from palm oil that are vitamin E derivatives also show membrane protective properties in rat brain microsomes and rat liver microsomes\(^109-111\). Biological antioxidants such as lipoate, methionine, flavonoids, related polyphenols, β-carotene, α-tocopherol and curcumin from turmeric also prevent DNA damage induced by \(^1\)O\(_2\).

There are several biologically useful effects and potential applications of \(^1\)O\(_2\) (Table 4).

**Beneficial effects of \(^1\)O\(_2\)—the photodynamic therapy**

If \(^1\)O\(_2\) can be selectively generated in diseased tissue, such as tumor, it can behave as a 'therapeutic agent', in controlling the disease. Photodynamic therapy (PDT) is the combination of light and light sensi-
Table 4 — Potential applications of singlet oxygen

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Defense mechanisms: phagocytosis and degradation of endogenous hallucinogens</td>
</tr>
<tr>
<td>2</td>
<td>Hormonal activity of prostaglandins</td>
</tr>
<tr>
<td>3</td>
<td>Photodynamic therapy of cancer, atherosclerosis, skin diseases etc.</td>
</tr>
<tr>
<td>4</td>
<td>Inactivation of pathogens like viruses, bacteria etc. in blood</td>
</tr>
<tr>
<td>5</td>
<td>As a disinfectant</td>
</tr>
<tr>
<td>6</td>
<td>Involved in cell signaling</td>
</tr>
<tr>
<td>7</td>
<td>As a bleaching agent</td>
</tr>
<tr>
<td>8</td>
<td>For water treatment</td>
</tr>
<tr>
<td>9</td>
<td>For pest control using photoactive pesticides</td>
</tr>
<tr>
<td>10</td>
<td>For synthetic reactions</td>
</tr>
<tr>
<td>11</td>
<td>In PUVA therapy for psoriasis</td>
</tr>
<tr>
<td>12</td>
<td>Chemiluminescence of specific biocompounds</td>
</tr>
</tbody>
</table>

A typical PDT session involves (i) intravenous injection (i.v.) or topical application of a photosensitizing agent such as a porphyrin; (ii) permit time for systemic porphyrins (i.v. injection) to be cleared from normal tissues and be preferentially retained by rapidly growing tissues (e.g., cancer or psoriasis), or for topical porphyrins to be absorbed by the skin; (iii) application of light to provide the catalyst for chemical reactions; (iv) generation of toxic oxygen species in illuminated tissues and (v) tissue damage usually resulting from damage to vasculature giving rise to regression of diseased tissue like cancer. Photodynamic therapy (PDT) is not only just an application of technology, but may be considered as a completely new concept. A variety of different types of tumors also respond to PDT. Photodynamic effect has also been used in sterilizing blood and blood products, as antiviral agents and in the control of insect pests. Furocumarin derivatives (psoraleens) were used as photosensitizers during UV treatment of psoriasis and other types of skin diseases. This reactive species generated in these studies, may be a key component in effecting the treatment.

The mechanisms involved in cell killing in PDT may be related to damage to mitochondria and induction of apoptosis. It is believed that the generation of singlet oxygen helps in inducing cell death. At higher concentrations singlet oxygen and other ROS may induce death by necrosis, while at lower doses apoptosis predominates. The former is the result of overwhelming incident stress, while the latter involves the cell’s systemic self-destruction without affecting the surrounding tissue.

Signalling effects of singlet oxygen

Recent studies showed that \( {O_2}^* \) is involved in cell signaling. This species generated during PDT or exposure to UVA, was shown to induce a series of

tive agents (such as porphyrins) in an oxygen-rich environment. Porphyrins, a component of hemoglobin can absorb energy from photons and transfer this energy to surrounding oxygen molecules. Toxic oxygen species such as singlet oxygen and free radicals are thus formed. These species are very reactive and can damage proteins, lipids, nucleic acids and other cellular components. Porphyrins utilize energy from light to produce toxic oxygen species.

Modern PDT originated at the turn of the century in Germany. Researchers experimenting with self-injection of porphyrins noted sunburns due to photodynamic reactions in their skin. Derived from animal hemoglobin, two forms of porphyrin are well known: hematoporphyrin derivative (HPD) and porfimer sodium, (Photofrin), which is in Phase III clinical trials has received approval in Canada for use with bladder carcinoma where treatment with BCG vaccine has failed. Photofrin has been approved in other countries for treatment of esophageal cancer and lung cancer. These first generation photosensitizers display prolonged and generalized photosensitivity of the skin as their primary side effect. Second generation photosensitizers exhibit far less photosensitization and are now in early clinical trials (one example is BPD verteporfin). Which was recently in Phase I/II clinical trials for primary skin carcinoma, cutaneous lesions where cancer has metastasized to the skin, and chronic stable plaque psoriasis.

Lasers are the primary light source for activation of porphyrins because laser light is monochromatic (exactly one colour), coherent (light waves are parallel permitting precise focusing), and intense (allowing for shorter treatment times). Light Emitting Diodes (LEDs) and florescent light sources are now being used as alternative light sources as they are more convenient than lasers but do result in longer treatment times.
genes involved in signaling cascade such as JNK and p38 MAP kinase and NF-κB. Both may contribute to induction of enzymes involved in signaling pathways. Three pathways have been shown to be induced by \( ^1O_2 \), the AP-1, NF-κB, and AP-2 pathway. The cell membrane appears to play a role as one of the primary targets for \( ^1O_2 \).

**Conclusion**

Though the biological relevance of \( ^1O_2 \) has been realized only recently a large number of studies show that \( ^1O_2 \) is being generated by a number of biologically important reactions with significant implications in disease development. Damage caused by \( ^1O_2 \) can be prevented by many natural antioxidants. This species also has several potential applications including in cancer therapy. At low levels \( ^1O_2 \) can be a signaling molecule. Newer approaches to the study of \( ^1O_2 \) can yield rich dividends.

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