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# ENERGY TRANSFER PROCESSES ACCOMPANYING LASER EXCITATION OF HEMATOPORPHYRIN IN VARIOUS SOLVENTS

Photochemotherapy involving the combined processes of hematoporphyrin injection and subsequent exposure to visible light has proven effective in the treatment of certain cancers at the clinical level. Our research is directed toward understanding the basic physical and chemical processes underlying this method, specifically the role of singlet molecular oxygen.

Results of recent clinical investigations have highlighted the effectiveness of laser-excited hematoporphyrin* both as a diagnostic[1,2] and a therapeutic tool[3,4] in the treatment of a wide variety of tumors. An element common to both is the established fact that not only do cancerous cells tend to retain higher levels of injected hematoporphyrin than does healthy tissue, but they also tend to exhibit higher levels of fluorescence. The process of fluorescence is illustrated in Fig. 1. Here the hematoporphyrin dye is elevated to its first excited singlet electronic state, $S_1$, by means of laser radiation at 5320 angstroms (green). Subsequent deactivation of this excited singlet may occur in three different ways, one of which involves a return to the ground singlet state, $S_0$, with the excess energy being carried away in the form of radiation at a longer wavelength—at 6300 angstroms (red). This rather intense red fluorescence thus serves as an indicator of the presence of localized areas of relatively high hematoporphyrin concentration characterizing tumor formation.

The added effectiveness of laser-excited hematoporphyrin in destroying existing cancerous cells involves a two-step process. First, the excited dye singlet state, $S_1$, is transformed via an intramolecular coupling to the lowest-lying dye triplet state, $T_0$, as indicated in Fig. 1. Subsequent deactivation of this triplet state to the ground singlet, $S_0$, is forbidden by the spin selection rules, resulting in lifetimes of this particular species under anaerobic (oxygen-excluded) conditions as long as one millisecond. This is to be compared with the lifetime of the excited dye singlet, $S_1$, which lies in the range 1 to 10 nanoseconds.

In the presence of oxygen, however, rapid deactivation of the dye triplet to the dye singlet ground state, $S_0$, occurs via an intermolecular spin-conserv-

*See Glossary, page 199.

ing process in which the oxygen molecule is raised from its ground triplet, $^3O_2$, to the first excited electronic singlet state, $^1O_2$, involving an energy of approximately 1 electronvolt. This is possible only because the ground electronic state of molecular oxygen happens to be a triplet, which is not true for most stable molecules.

Recent experimental investigation of the effect of singlet oxygen on cancerous cells shows clearly the lethality of this species.[5] The specific site of singlet oxygen attack has been identified as the cell membrane.[6-12] Significant differences in porphyrin accumulation and membrane damage have been found to occur, depending on the hydrophobic or
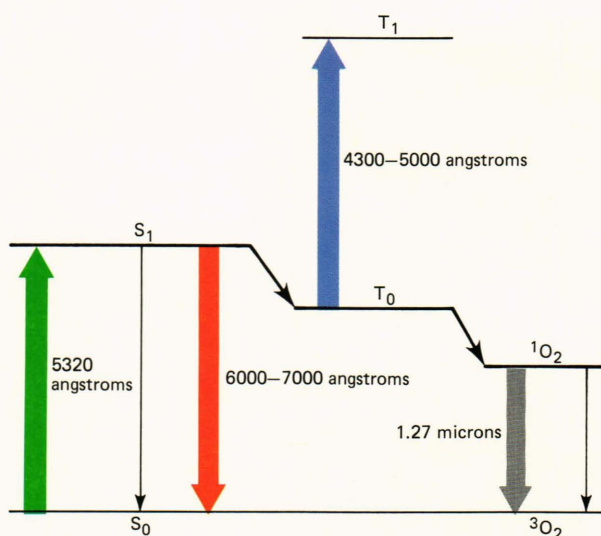


Fig. 1—Schematic representation of energy transfer processes involved in the production of singlet oxygen from laser-excited hematoporphyrin.

hydrophilic nature of a specific membrane site. It has been established, furthermore, that binding of the hematoporphyrin to proteins is an important factor.

The process in which biological damage occurs as the result of optical excitation of a dye in the presence of oxygen is generally referred to as "photodynamic action." Although it has been established that singlet oxygen is involved, a question remained as to whether interaction with the microorganism involved direct attack by singlet oxygen or, alternatively, by chemical reaction with the dye resulting in a toxic compound. Exploratory experiments carried out earlier at APL using laser excitation (1.064 microns) of natural water samples containing high concentrations of dissolved oxygen resulted in significant inactivation of *Escherichia coli* bacteria. The fact that this occurred without the addition of a photosensitizer is significant, emphasizing the unique role of singlet oxygen.
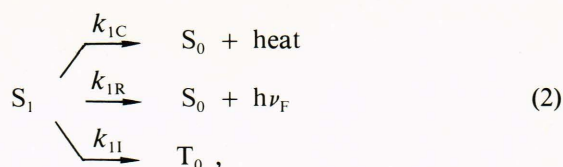
Further research conducted over the past year has been motivated by a need to understand clearly not only the relative role of the different states of excitation involved in photosensitized generation of singlet oxygen but also the rates of energy transfer and how these rates depend on the particular solvent. Although several photosensitizing dyes (i.e., rose bengal, eosin-y, fluorescein, methylene blue) have been used in this research, most of the work has involved free base hematoporphyrin because of the availability of relevant data, as well as our observation that not only is the dye triplet produced in high yield but it is also very durable (i.e., resistant to oxidation).

The overall system kinetics appearing schematically in Fig. 1 may be delineated as follows:

- Optical excitation of the dye from the ground singlet, $S_0$, to the first excited singlet, $S_1$, by means of laser radiation at frequency $\nu_L$ (5320 angstroms)

$$S_0 + h\nu_L \longrightarrow S_1 \; ; \qquad (1)$$
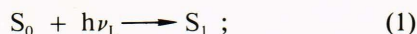
- Deactivation of $S_1$, either collisionally ($k_{IC}$), radiatively ($k_{1R}$), or by intersystem crossing to the dye triplet metastable state ($k_{1I}$)

$$
S_1
\begin{cases}
\xrightarrow{k_{1C}} & S_0 + \text{heat} \\
\xrightarrow{k_{1R}} & S_0 + h\nu_F \\
\xrightarrow{k_{1I}} & T_0 \; ,
\end{cases}
\qquad (2)
$$

where $\nu_F$ is the fluorescence frequency;

- Spin-conserving energy transfer from the dye triplet, $T_0$, to the triplet electronic state of oxygen, $^3O_2$, to form the singlet $^1O_2$,

$$T_0 + {}^3O_2 \xrightarrow{k_2} S_0 + {}^1O_2 \; , \qquad (3)$$

in which quenching by the solvent in the absence of oxygen is assumed to be negligible, as is the radiative process $T_0 \longrightarrow S_0 + h\nu$ (phosphorescence);
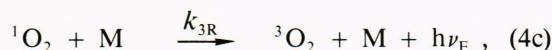
- Deexcitation of the resulting $^1O_2$ occurs primarily by either physical reaction ($k_{3P}$)

$$^1O_2 + M \xrightarrow{k_{3P}} {}^3O_2 + M \; , \qquad (4a)$$
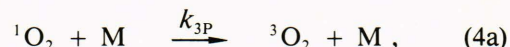
where M is an inert molecule, or by chemical reaction ($k_{3X}$)

$$^1O_2 + R \xrightarrow{k_{3X}} RO_2 \; . \qquad (4b)$$

However, a third possibility involves a relatively weak collisionally induced emission ($k_{3R}$)

$$^1O_2 + M \xrightarrow{k_{3R}} {}^3O_2 + M + h\nu_E \; , \qquad (4c)$$

where $\nu_E$ is the emission frequency (corresponding to a wavelength of 1.27 microns).

The experimental approach used in this research (Fig. 2) involves hematoporphyrin singlet excitation by means of a Q-switched neodymium-doped yttrium-aluminum-garnet laser with pulse energies ranging from 1 to 10 millijoules. The dye triplet, $T_0$, is formed rapidly during the 10-nanosecond pulse duration, with subsequent formation of $^1O_2$ determined by Transition (3). It is thus clear that the quenching of $T_0$ occurs essentially only in one way, while the deactivation of $^1O_2$ may take place in at least three ways. Monitoring the $^1O_2$ decay optically at 1.27 microns (Transition 4c) can provide information about the rate of disappearance of $^1O_2$, while monitoring the triplet gives the $^1O_2$ production rate.

Detection of the $^1O_2$ collisionally induced emission at 1.27 microns was accomplished by means of a system of suitable optical filtering (spectrometer) and lensing combined with the use of a commercially available germanium photodiode coupled to appropriate electronics. Measurement of the $T_0$ population decay rate involved an active approach based on dye triplet-triplet absorption,[13] i.e.,

tions listed in Table 1. (The effect of both deoxygenation and oxygen enrichment on the $^1O_2$ and $T_0$ rates was also investigated.)
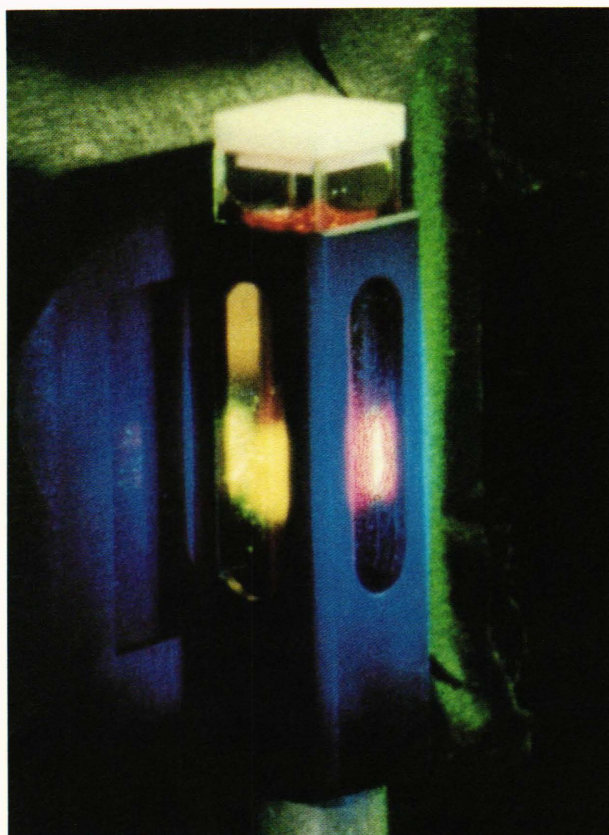


**Fig. 2—Laser excitation of water sample containing a small** amount of dissolved hematoporphyrin. The incident laser beam (travelling from right to left) enters the opening on the right side of the cuvette holder, passes through the sample contained within the cuvette, and emerges through an opening on the left. The violet probe beam emerging from the mercury-xenon lamp in the foreground (not shown) passes through the opening in the front of the cuvette holder and is brought to a focus on the entrance slit of the spectrometer located directly behind the sample. The red light appearing at the center of the cuvette results from fluorescence of the excited dye singlet.

## Table 1

DECAY TIMES FOR AIR-SATURATED SOLUTIONS

| Solvent | $^1O_2$ (microseconds) | $T_0$ (microseconds) |
|---|---|---|
| Methanol | 6 – 8 | 0.3 |
| Ethanol | 10 – 12 | 0.3 |
| Acetone | 40 – 50 | 0.3 |
| Water | not detectable | 2.5 |
| Deuterium oxide | 40 – 50 | 2.5 |
| Freon 113 + 1% ethanol | 1400 | not measured |
| Freon 113 + 10% ethanol | 150 | not measured |

$$T_0 + h\nu_P \xrightarrow{k_4} T_1 , \qquad (5)$$

where $\nu_P$ is the probe beam frequency (corresponding to wavelength $\lambda_P$). $\lambda_P$ is greater than 4300 angstroms so the primary dye singlet absorption (Soret) is weak. Monitoring of the transient probe beam absorption was accomplished by means of a silicon photodiode. On average, $k_4$ falls monotonically with increasing wavelength, becoming rather small for $\lambda_P > 6000$ angstroms. In this work, two probe wavelengths were used: 4350 angstroms obtained by filtering the output of a mercury-xenon lamp and a band centered at 4600 angstroms using the filtered output of a quartz-halogen lamp.

Measurements of $^1O_2$ and $T_0$ quenching rates using hematoporphyrin concentrations for the most part in the range of $10^{-5}$ to $10^{-4}$ mole per liter have yielded the various decay times for air-saturated solu-

For the solvents in Table 1, the $^1O_2$ quenching process was entirely physical, with no significant loss of oxygen through chemical reaction observed. In the case of water, it was not possible to detect spectroscopically the presence of $^1O_2$ under the same conditions for which clear indications were obtained in deuterium oxide ($D_2O$). This no doubt results in part from the rather long $^1O_2$ quenching time in deuterium oxide; for water, the expectation is that the times should be shorter because of the presence of infrared active bands that overlap those of $^1O_2$.

An estimate of the quenching time of $^1O_2$ in water, based on extrapolation of data for methanol-water mixtures, has yielded[14] a value of 2 microseconds. The fact that this number is very close to the dye triplet quenching time given in Table 1 opens the possibility that for water as solvent, dye triplet quenching is the rate-limiting step and the $^1O_2$ quenching time is much shorter than that. If this is true, the implication is that for biological systems, membrane attack by $^1O_2$ generated in an external aqueous medium should be relatively unimportant.

Comparison of dye triplet lifetimes appearing in Table 1 with the solubility of oxygen in the respective solvents suggests that the observed differences may be explained solely in terms of dissolved oxygen content, with solvent quenching unimportant. Thus, for air saturation (room temperature) the dissolved oxygen concentration in water is $2.6 \times 10^{-4}$ mole per liter increasing to approximately $2.0 \times 10^{-3}$ mole per liter for the ethanol, methanol, and acetone group, or roughly a factor of eight, which is quantitatively consistent with the observed decrease in the corresponding dye triplet lifetimes. Thus, in a hydrophobic environment (e.g., Freon 113) characterized by still higher oxygen solubilities, the dye trip-

## GLOSSARY

**Hematoporphyrin** — Porphyrin derived from red blood cells.
**Hydrophobic** — The tendency to repel water.
**Hydrophilic** — The tendency to attract water.
**Intersystem crossing** — An internal molecular process in which transition from singlet to triplet state occurs.
$^1O_2$ — The molecular oxygen singlet state.
$^3O_2$ — The molecular oxygen triplet state.
**Singlet state** — Molecular electronic state in which all electron spins are paired.
**Spin conservation** — The process in which spin characteristics of individual components may change but the total system spin character remains unchanged.
**Triplet state** — The molecular electronic condition in which the electrons exist in unpaired states.
**Vascularization** — The development of a system to provide blood flow.

let lifetimes should be rather short. Singlet oxygen lifetimes for such solvents, on the other hand, tend to become quite long as evidenced by the experimental data.

Investigation of solvents containing significant levels of protein and also individual amino acids clearly showed evidence of oxygen depletion, an effect that can be explained most directly in terms of the chemical reaction of $^1O_2$ with those components at a rate that exceeds the rate of oxygen diffusion in the solvent. The disappearance of oxygen is manifested in dramatic increases of the dye triplet quenching times. Thus, for a solution containing the amino acid histidine ($10^{-4}$ mole per liter ) in water (pH = 7.2), the quenching time lengthened to approximately 65 microseconds. For a sample of cell-growth medium containing 10 to 15% fetal calf serum, times longer than 1 millisecond were recorded, depending on the time of sample exposure. Similar effects have been observed in the case of zinc- and magnesium-substituted hemoglobin.[15]

Whether oxygen depletion is an important factor in the photosensitized destruction of tumors, either directly or indirectly, is an open question. Active tumor development is accompanied by vascularization of the affected area; thus, an oxygen input is provided that, in terms of total flow rate, exceeds that existing in bulk solution, which is diffusion-limited. The increased solubility of oxygen in hydrophobic areas of the cell may also be an important factor. It is hoped that future research will provide a deeper insight into these matters and thus elucidate more clearly the specific role of singlet oxygen in biological systems.

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