The Investigation of Molecular Mechanisms in Photodynamic Action
and Radiobiology with Nanosecond Flash Photolysis and Pulse Radiolysis

ABSTRACT

The mechanism of photodynamic oxidation of aqueous iodide ion sensitized by eosin has been investigated with laser flash photolysis and analog computer analysis. It has been demonstrated that $^1\Delta_g$ singlet oxygen is the principal oxidizing agent leading to the I_2^- intermediate. Evidence for the equilibrium $I_2^- + I^- \rightleftharpoons I_3^{2-}$ was obtained with $K = 1 \times 10^3 \text{ M}^{-1}$. New work on photodynamic action in membranes has been initiated where the spin label ESR technique is employed to probe the local damage. Preliminary results on liquid stearic acid indicate that irradiation with visible light in the presence of eosin alters the local order of the crystalline and liquid phases and that oxygen contributes to the effects. Pulse radiolysis and cobalt-60 studies on splitting of thymine dimer by ionizing radiation have led to a reductive pathway initiated by hydrated electrons and an oxidative pathway from the attack of hydroxyl radicals. The results suggest reasonable mechanisms for photosensitized cleavage of pyrimidine dimers as sensitized by various dyes and aromatic amino acids.

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THE INVESTIGATION OF MOLECULAR MECHANISMS IN PHOTODYNAMIC
ACTION AND RADIOBIOLOGY WITH NANOSECOND FLASH PHOTOLYSIS
AND PULSE RADIOLYSIS

Progress Report

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Construction of Laser Flash Photolysis Apparatus

The laser flash photolysis apparatus described in the previous Progress Report (4/1/72 - 3/31/73) has been improved by replacing the original KD*P first doubler with a temperature-tuned CsDA crystal. The present system provides a 17 nanosec pulse with about 500 millijoules at 530 nm and 50 millijoules at 265 nm. The original spectrophotometric monitoring system has been extended by adding photographic plate detection with a Hilger E498 "medium" quartz prism spectrograph. The spectroflash is obtained with a 10 μ sec xenon flash lamp (Suntron-6A) powered with a Xenon Corp. Model 3 Micropulser and Model C Trigger Module. A block diagram of the present arrangement is given in Fig. 1 .

Oxidation of Aqueous Iodide Ion by Singlet Oxygen

There is ample evidence that singlet oxygen is a major oxidizing intermediary (and frequently the only important one) in a large number of photosensitized processes. In a recent review on the photochemical role of singlet oxygen by Foote (1974), reference is made to published work involving photodynamic effects in amino acids and proteins, nucleic acid derivatives, lipids, polysaccharides, and tocopherols, including medical implications of singlet oxygen in porphyria, neonatal jaundice, and photocarcinogenesis. Although singlet oxygen may be generated by various means, e.g. electrical discharge, chemical reaction, optical pumping, and energy transfer, the last is of most interest in connection with the photodynamic effects. The process for a typical sensitizer S can be represented in the simplest form as:

where competing steps which diminish the quantum yield of ${}^1O_2^*$ have not been included. The minimum sensitizer triplet state energy is 37.5 kcal/mole for

Figure 1. Laser flash photolysis apparatus.

the $^1\Sigma_g^+$ state of $^1O_2^*$ and 22.5 kcal/mole for the $^1\Delta_g$ state. The lifetime of the $^1\Sigma_g^+$ state does not exceed $\sim 10^{-11}$ sec in solution, so that the "singlet oxygen" involved in biological processes and solution chemistry is believed to be the relatively long-lived $^1\Delta_g$ state.

A number of tests have been devised to diagnose the presence of singlet oxygen as an intermediary in photosensitized processes. They include comparison of the photochemical products with experiments where singlet oxygen is generated chemically or by microwave discharge, demonstration of competitive inhibition by known singlet oxygen acceptors (e.g. β -carotene, 1,3-diphenylisobenzofuran (DPBF), or 1,4-diazabicyclo [2.2.2.] octane (DABCO)) and unique solvent effects on the quantum yields. An example of the solvent effects method particularly useful for biological systems is based on the remarkable finding of Kearns (1972) that the lifetime of singlet oxygen increases from about 2 μ sec in H_2O to about 20 μ sec in D_2O , leading to an isotope effect far in excess of that expected from the H:D mass ratio. This method has been employed to demonstrate that singlet oxygen is responsible for the photodynamic inactivation of the enzymes trypsin and alcohol dehydrogenase as sensitized by methylene blue (Nilsson and Kearns, 1973), and hen lysozyme in the presence of eosin (Kepka and Grossweiner, 1973) and acridine orange (Schmidt and Rosenkranz, 1972).

A major objective in the present work is the application of laser flash photolysis to such enzyme systems in order to study the mechanism of singlet oxygen inactivation reactions. The organic acceptors for singlet oxygen employed in previous work are relatively insoluble in entirely aqueous solvents and it was desired to locate a more appropriate probe for biological systems. Various possibilities were tested by measuring the rate of eosin-sensitized photochemical destruction of the substrate in the presence of oxygen with H_2O and D_2O as the solvents. The OH radical scavengers used as "anion probes"

in enzyme pulse radiolysis were examined first, because it is known that they provide strongly absorbing, characteristic transients e.g. CO_3^- and $(\text{CNS})_2^-$, with negative results. It was then found that the oxidation of aqueous I^- by green light in the presence of eosin (Kepka and Grossweiner, 1971) shows a strong " D_2O effect." In fact, the addition of azide ion as specific quencher for singlet oxygen gives complete protection against photooxidation of I^- . In view of the possible use of I^- as an aqueous probe for singlet oxygen and the historic interest in the mechanism of its photodynamic oxidation extending back at least 70 years (Straub, 1904), this reaction is being investigated with the laser apparatus as a preliminary to the enzyme systems of biological importance.

The rate-determining kinetic parameters can be evaluated from the quantum yields based on the assumed mechanism:

leading to

$$\phi(\text{I}_3^-) = 1/2 \phi_{\text{T}}^{\circ} p^* \frac{\tau^* k_4' [\text{I}^-]}{1 + \tau^* (k_4 + k_4') [\text{I}^-]}, \quad (I)$$

where p^* is the efficiency of reaction-(2), τ^* is the solvent-induced decay lifetime of $^1\text{O}_2^*$ for reaction-(3), and ϕ_{T}° is the eosin intersystem crossing efficiency.

The "double reciprocal" plot of the quantum yield of photosensitized oxidation of air-saturated I^- in the presence of eosin in Fig. 2 follows the predicted linear dependence of (I) with the same intercept for measurements

Figure 2 (below). Quantum yield of eosin-sensitized I^- photooxidation based on (I).

Figure 3 (right). Transient absorption spectrum from laser flash photolysis of I^- at 530 nm; 1 μ sec delay.

Figure 4 (below right). Analog computer fit to I_2^- buildup and decay curves

in H_2O and D_2O . The slope ratio of 9.6 supports the identification of the major oxidizing entity with singlet oxygen, which was confirmed by complete protection in the presence of 0.03M azide ion. The numerical value of the slope leads to $k_4' = 2.4 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ and the slope/intercept ratio gives $(k_4 + k_4') \sim 2 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$, based on $\tau^* = 2 \text{ } \mu\text{sec}$, $\phi_T^0 = 0.71$ (Bowers and Porter, 1967) and $p^* = 0.89$ (Usui, 1973).

The transient spectrum in Fig. 3 was obtained with the laser flash photolysis system by irradiating O_2 -saturated 200 mM I^- in the presence of 300 μM eosin (pH 6.2). The overall transient at 1 μsec delay is identified with the I_2^- band at 380 nm (Grossweiner and Matheson, 1957) with a shoulder attributed to product I_3^- and has been resolved into these components. (The I_2^- spectrum in ethanol reported by Dobson and Grossweiner (1964) was used for this fit.) The kinetics analysis is complicated by the overlap of the I_2^- and I_3^- absorptions as well as the decay of I_2^- during its formation by reaction-(4'). The reaction system was simulated on the analog computer (EAI TR-20) with variable parameters τ^* , k_4 , k_4' , $k_5(2\text{I}_2^- \longrightarrow \text{I}_3^- + \text{I}^-)$, and fit to the observed build-up and decay curves. (The concentrations were related to measured optical densities by taking as peak molar absorbance values $\epsilon_{\text{I}_2^-} = 15,600$ (Grossweiner and Matheson, 1957) and $\epsilon_{\text{I}_3^-} = 26,400$ (Awtrey and Connick, 1951)). A preliminary fit at 365 nm and two initial I^- concentrations in Fig. 4 gives $k_4 = 2.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$ and $k_4' = 6.9 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$, indicating that 1 of 5 interactions of $^1\text{O}_2^*$ with I^- leads to electron transfer. These results are in reasonable agreement with the quantum yield data, except that it was necessary to use $\tau^* = 4 \text{ } \mu\text{sec}$ in order to obtain a satisfactory fit. However, the value of k_5 was much smaller than $1.5 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$ reported by Grossweiner and Matheson (1957) for uv flash photolysis of aqueous I^- and appears to vary with the I^- concentration.

The resolution of this discrepancy is suggested by the pulse radiolysis study of Zehavi and Rabani (1972) on aqueous Br^- , where it was necessary to postulate the equilibrium between Br_2^- and Br^- to explain their data. The extension of this result to the present work indicates that the apparent bimolecular decay rate constant of I_2^- should follow:

$$k_5(\text{app}) = k_5 / (1 + K[\text{I}^-]) \quad (\text{II})$$

where $K = [\text{I}_3^{2-}] / [\text{I}_2^-][\text{I}^-]$. The results in Fig.1 were calculated with $K = 1 \times 10^3 \text{ M}^{-1}$ and provide a satisfactory fit to the decays at 100 μM and 200 μM I^- . This work is being extended to other monitoring wavelengths and a broader range of I^- concentrations.

Singlet oxygen reactions generally involve 1,4-addition to dienes and heterocyclics, ene reaction with olefins, 1,2-addition to electron-rich olefins, (enamines or vinyl ethers), or oxidation of sulfides (to sulfoxides) or phenols (Foote, 1964). Although electron transfer has been postulated in some instances, particularly for the last class, reaction-(4') can only be explained by electron transfer from I^- to $^1\text{O}_2^*$. The energetics of this process can be estimated with published oxidation potentials for the reaction

with $\Delta F'(\text{pH } 7) = + 31.5 \text{ kcal}$, based on $E^0(\text{I}_3^-, \text{I}^-) = - 0.535 \text{ volts}$ and $E'(\text{O}_2, \text{O}_2^-) = + 0.15 \text{ volts}$ (Rao and Hayon, 1973). Taking 22.5 kcal for the excitation energy of singlet oxygen gives $\Delta F'' = - 7 \text{ kcal/mole I}^-$ for the overall one-electron process

(The reaction is more exothermic by about 1.5 kcal under flash photolysis conditions because of the high I^- concentration.) Foote has suggested (private communication) that the actual mechanism is

in parallel with the oxidation of sulfides, in which the "persulfoxide" intermediate has been postulated (Foote and Peters, 1971)

A similar mechanism might apply for the quenching of singlet oxygen by azide ion.

Splitting of Thymine Dimer by Ionizing Radiations

The central problem in molecular radiation biology is the identification of the primary lesions produced by exposure of cells to radiation and relating them to observable biological endpoints. It is generally accepted that chromosomal DNA is the principal molecular target of ionizing radiations and ultraviolet, leading to breaking of the polynucleotide chains, alterations of the base and sugar moieties, intramolecular cross linking, and other damage types. The formation of cyclobutane dimers between adjacent pyrimidine bases on a polynucleotide chain has been shown to be one of the most important products of ultraviolet irradiation in vitro and in vivo. This lesion is repaired by a dark process leading to excision of the dimer and enzymatic photoreactivation in which dimer splitting takes place. Both processes have been observed in a wide variety of cell types, ranging from unicellular organisms to vertebrate cells, suggesting that the recognition and repair of other base alterations may have similar or parallel steps.

Early observations that ionizing radiation cleaves isolated thymine dimer demonstrate that small free radicals can affect the reactions as well (Lachmann, 1963; Schmidt et al., 1963; Deering and Snipes, 1968). This section reports a study of the initial steps based on pulse radiolysis and ^{60}Co G values, with results relevant to the photosensitized dimer splitting reactions.

Thymine dimer was prepared by 254 nm irradiation of aqueous thymine solutions at -80°C followed by warming and recrystallization two times. This procedure gives the cis-syn isomer (Blackburn and Davies, 1966) as confirmed by a single spot on TLC plates at the reported R_f (Smith, 1963). (Macherey-Nagel TLC Polygram Cel 300 PEI/UV₂₅₄ pre-coated plates were employed with ^{14}C labeled thymine dimer and the solvents: n-butanol-water-acetic acid (80:30:12) and n-butanol-water (86:14)). The pulse radiolysis was carried out with the

Table 1. G values for thymine formation by cobalt-60 irradiation of thymine dimer

<u>Condition</u>	<u>G(T)</u>	
	<u>Optical Absorption</u> [*]	<u>TLC</u> [#]
N ₂ -Sat	6.2±0.2	6.4±0.6
N ₂ O-Sat	3.0±0.2	3.0±0.2
O ₂ -Sat	1.4±0.2	1.4±0.2
N ₂ O-Sat + 0.5M <u>t</u> -butanol	0.0	
N ₂ -Sat + 0.5M <u>t</u> -butanol	6.5±0.3	8.6±0.4

^{*} 100 μM thymine dimer (no buffer, pH 6.1); 3.9 krads.

[#] 1.0 mM thymine dimer (no buffer, pH 6.1); 6.5 krads.

apparatus at Michael Reese Medical Center described by Masuda, et al. (1971) in which the samples were irradiated with 1 μ sec pulses of 30 MeV electrons (700 rads/pulse) and the transient spectra measured with spectrophotometric detection. The G values were determined by irradiating with ^{60}Co at a dose rate of 1.3 krad/min. Thymine formation was measured by the optical absorption increase at 265 nm and TLC with labeled dimer.

The results in Table 1 show that $G(T)$ increases by about 2 times in going from oxygen-saturated solutions to nitrous oxide saturation. The yields indicate that each reacted $\text{OH}\cdot$ leads to about 1/2 thymine. The inactivity of $\text{H}\cdot$ is supported by the negative results in nitrous oxide containing 0.5M t-butanol to scavenge $\text{OH}\cdot$. $G(T)$ in nitrogen-saturated solutions indicates that each reacted e_{aq}^- generates about 1-1/2 thymines, assuming that each $\text{OH}\cdot$ contributes $\frac{1}{2}$ thymine. The high thymine yield with nitrogen-saturated solutions containing 0.5M t-butanol is unexpected, because the negative result with nitrous oxide saturation plus t-butanol indicates that the $\dot{\text{C}}\text{H}_2\text{C}(\text{CH}_3)_2\text{OH}$ radical does not cleave thymine dimer directly.

The initial pulse radiolysis transient detected in nitrogen-saturated solutions with or without 0.5M t-butanol is a short-lived species with absorption bands at 465 nm and 565 nm (Fig. 5a). It corresponds with the thymine dimer electron adduct reported by Santus, et al. (1972), who did not observe the longer wavelength band because of overlap with the e_{aq}^- absorption. The decay of these bands in about 0.2 μ sec leads to a longer-lived band peaking at 325 nm (Fig. 5b). This absorption corresponds with the thymine anion reported by Theard, et al. (1971) formed by e_{aq}^- reaction with thymine. These results lead to the following proposed mechanism:

Reaction-(11) generates 1 thymine per e_{aq}^- , suggesting that some subsequent reaction of T^- or $\text{TH}\cdot$ gives the additional 1/2 thymine deduced from the $G(T)$

Figure 5. Pulse radiolysis transient spectra of 300 μM thymine dimer (pH 6.1)

results. The enhanced thymine yield with t-butanol present can be explained by postulating that the alcohol radical oxidizes $T^{\cdot -}$ or $TH^{\cdot}$ leading to a predicted $G(T) \sim 6$. (A similar reaction in which the t-butanol radical oxidizes the cysteinyl residue was suggested by Adams and Redpath (1974) to explain the enhanced yield of papain inactivation in nitrous oxide-saturated solutions containing t-butanol.) A spot corresponding with dihydrothymine was observed on the TLC plates for nitrogen-saturated solutions, but the yield was only about 2% of thymine.

A transient band at 380 nm was the only absorption observed after 0.1 μ sec by pulse radiolysis of thymine dimer in nitrous oxide or oxygen-saturated solutions (Fig. 5c). It corresponds with the absorption attributed to the thymine OH adduct (Theard, et al., 1971) and decays by a 2nd order process with $k/\epsilon = 1.0 \times 10^6$ cm/sec, in good agreement with the value reported by Hayon and Simic (1973). The formation of this product can be explained by oxidation of thymine dimer followed by hydrolytic splitting:

The postulated dimer cation $(\widehat{T}T)^+$ was not observed. The G values indicate that only about half of the initial $OH^{\cdot}$ reacts via this pathway and the remainder generates permanent products with R_f much smaller than thymine. It is not likely that $TOH^{\cdot}$ generates additional thymine by disproportionation because $G(T)$ in nitrous oxide is close to twice $G(T)$ in oxygen (Table 1) and it is known that oxygen reacts rapidly with $TOH^{\cdot}$ (Theard, et al., 1968; Willson, 1970). However, the secondary reaction of $TOH^{\cdot}$ with $TH^{\cdot}$ can account for the additional contribution of e_{aq}^- to $G(T)$ as well as the low yield for dihydrothymine.

The cleavage of thymine dimer by an oxidative and a reductive process

has an interesting implication for the photosensitized splitting reactions investigated in connection with the molecular mechanism of photoreactivation. It has been found that a surprising variety of sensitizers are active, including uranyl oxalate (Wacker, et al., 1964), anthraquinone derivatives (Ben-Hur and Rosenthal, 1970), chloranil (Rosenthal and Elad, 1968), naphtho-imidazoles and arylsulfonic acids, such as 3-benzophenonesulfonic acid, (Lamola, 1966), and indole derivatives (Hélène and Charlier, 1971). The active indole derivatives such as tryptophan are known to be electron donating in the excited state and may cleave by a process paralleling e_{aq}^- in reaction (1). On the other hand, molecules with the $n-\pi^*$ triplet as the lowest excited state such as benzophenone are good H atom abstractors and can follow the pathway proposed by $OH^\cdot$ in reaction-(2).

Photodynamic Damage to Model Membrane Systems

Preliminary experiments have been carried out on photodynamic damage to stearic acid using the ESR spin label technique. The measurements are being made with a Varian 4500-10A EPR Spectrometer located in the IIT Department of Chemistry. The spin label "2N19" (below) was synthesized from 2-ketononadecane following the methods of Keana et al. (1967) and mixed with stearic acid (no solvent) in ~0.1% (mole). The mixture was heated to 100°C in a water bath, drawn into a capillary, sealed and placed in the temperature-controlled ESR cavity.

N₂ Bubbled
30 Minute Irradiation

Figure 7

O₂ Bubbled
15 Minute Irradiation

— Slow Cooling
- - - Quenched

$10^{10} \tau_c$
sec

10^2
 10^1
 10^0
sec

0.1

1

O₂ Bubbled
Unirradiated

Spin Label ESR of Photodynamically Damaged Stearic Acid

82°C

40°C

5°C

2.80

3.20

3.60

$10^3/T^\circ K^{-1}$

The measurements were made by recording the first derivative of the absorption spectrum. The rotational correlation time of the spin label (τ_c) was calculated from the line height ratios (peak-to-peak of the mid-field and high-field lines) with the analysis of Kivelson (1960).

The object of the initial experiments was to determine whether irradiation of liquid stearic acid with visible light in the presence of 0.01%(mole) eosin leads to damage as reflected in the local order of the liquid and solid as evaluated from the tumbling motion of the label. The irradiations were carried out at 80°C with a 150-watt xenon arc and Corning C.S. 3-74 filter ($\lambda > 400$ nm) prior to introducing the spin label. One set of runs was made by slowly cooling from 80°C in the spectrometer and another set involved fast quenching in dry ice-acetone followed by slow warming from 0°C in the spectrometer. Typical results showing the dependence of τ_c on temperature for irradiations in nitrogen and oxygen are given in Fig.6 and Fig.7 .

Slow cooling of the unirradiated sample (Fig.6 lower; solid line) shows a discontinuity near 50°C leading to a smaller correlation time (faster tumbling) followed by a decrease in the tumbling rate at lower temperatures with activation energy of about 5 kcal/mole. Warming of the quenched sample (Fig.6 lower; dashed line) leads to an opposite discontinuity near 50°C , with similar activation energies in the solid and liquid phases. The unexpected direction of the discontinuity observed on slow cooling may be due to aggregation of the spin label leading to liquid pools in the solid matrix. On the other hand, fast quenching should freeze a random distribution of the label with slower tumbling below the transition temperature. The comparison with samples irradiated in nitrogen (Fig.6 upper) indicates that the slowly cooled sample (solid line) has a "normal" discontinuity suggesting that pooling in the solid phase is less significant. The most striking change, however, is the occurrence of much faster tumbling in the quenched sample

(dashed line) above the transition temperature. A possible explanation is that photodynamic damage to the stearic acid promotes a higher degree of disorder in the liquid. The results for irradiations in oxygen are shown in Fig.7 . The data show evidence of a new discontinuity near 40°C and significantly different activation energies in the high and low temperature regions.

These experiments are being continued with a view towards relating the ESR results to chemical reactions of the substrate and their influence on the structure. Work is being initiated on linolenic acid, a conjugated derivative of stearic acid which should be more reactive towards singlet oxygen. The extension to liposomes and yeast membranes is discussed in the Renewal Proposal.

Reporting of Research

Publications

"Thymine Dimer Splitting by Ionizing Radiation" (COO-2217-3)
L.I.Grossweiner, A.G.Kepka, R.Santus, and J.A.Vigil,
International Journal of Radiation Biology (in press)

"Oxidation of Iodide Ion by Singlet Oxygen" (COO-2217-4)
F.Dudley Bryant, A.G.Kepka, and L.I.Grossweiner
Proceedings Eleventh Informal Conference on Photochemistry
Nashville, Tennessee, June 16-20, 1974

Meetings

VII International Conference of Photochemistry,
Jerusalem, August 29 - September 4, 1973.

"Photochemical Inactivation of Lysozyme", L.I.Grossweiner

"Photochemical Oxidation of Aqueous Iodide by Singlet Oxygen",
A.G.Kepka and L.I.Grossweiner

Project Activity

The expenditure of scientific effort and funds was consistent with the technical program in the Renewal Proposal and the current budget.

Scientific Salaries

Dr. Leonard I. Grossweiner (Professor): 8% time 4/1/7 - 5/30/73;
10% time 1973-74 academic year; 1 month Summer 1973

Dr. Alan G. Kepka (Visiting Assistant Professor): 50% time 4/1/73 - 5/30/73.

Dr. Gerald E. Cohn (Assistant Professor): 45% time 1973-74 academic year.

Dr. Jerome A. Vigil (Research Associate): 100% time 4/1/73 - 5/30/73;
25% time 9/1/73 - 12/31/73.

Dr. Arun G. Kaluskar (Research Associate): 100% time 1/1/74 - 6/30/74.

Major Equipment Purchased

Holobeam Inc. Glass Laser Rod \$986.00

Foreign Travel

Dr. L. I. Grossweiner: VII International Conference of Photochemistry,
Jerusalem, Israel, August 29 - September 4, 1973\$500.00 (AEC)

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