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

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ABSTRACT

A laser flash photolysis apparatus has been constructed providing outputs of 200 millijoules at 530 nm and 20 millijoules at 265 nm in single 20 nanosecond pulses. The time resolution for transient species absorbing from 220 to 800 nm is 5 nanoseconds for spectrophotometric detection and 10 microseconds with a photographic plate spectrograph. The apparatus is being employed to study the photodynamic and ultraviolet inactivation of enzymes. The pulse radiolysis technique is being used to investigate the radiolytic cleavage of thymine dimer generated by ultraviolet irradiation of thymine. It has been found that the dimer is cleaved efficiently by hydrated electrons, subsequent to the formation of a short-lived intermediate attributed to the dimer electron adduct. The irradiation of thymine dimer in the presence of glucose also induces cleavage. This process is highly selective and has not been observed with alcohols, sucrose, and radical anions including I_2^- , CO_3^- , CO_2^- , and $(CNS)_2^-$. These studies are being extended to dimers generated in oligonucleotides and polynucleotides.

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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 Ultraviolet Flash Photolysis Apparatus

The design and construction of the laser flash photolysis apparatus has been a major activity since the activation of this contract. The preliminary calculations indicated that peak power above 100 megawatts would be required to achieve adequate flash photolysis intensity after two stages of frequency doubling and the decision was made to apply the maximum available equipment funds to the basic laser system. The major item is a Holobeam Model 631 Nd: Glass laser oscillator with Pockel's cell Q-switching rated at 150 megawatts. Measurements on the delivered unit indicate that the laser provides 200 megawatts peak power ($\pm 20\%$) in a 20 nanosec pulse corresponding to about 4 joules at 1060 nm. The first KD*P doubler operates at 5% efficiency, after careful crystal alignment with the autocollimator, providing about 200 millijoules at 530 nm. The 2'nd doubler is a temperature-tuned ADP crystal rated at 10-13% conversion efficiency. The actual available energy at 265 nm is about 20 millijoules as measured with a calibrated photodiode and chemical actinometry.

The green light output of $\sim 6 \times 10^{17}$ quanta is very adequate for the intended flash photolysis experiments on photodynamic systems sensitized by eosin Y and other dyes absorbing at 530 nm, e.g. the flux is 10^3 times higher than required to convert $10 \mu\text{M}$ eosin entirely to the triplet state in an irradiated volume of 0.7 cm^3 . The u.v. output with the same arrangement corresponds to $\sim 4 \times 10^{19}$ einstein/liter at 265 nm. For a transient species generated with 5% quantum efficiency of molar absorbance 1×10^4 liters/mole-cm, the optical density change for flash photolysis would be 0.03 per cm of optical path length. Since the quantum yield of processes of interest may be 1% or lower we propose to improve the output laser power by a factor of about 4 by using a cesium dihydrogen arsenate (CsDA) 1'st doubler with 20% conversion efficiency. In addition, we are planning to improve the measuring sensitivity with a multiple-pass optical monitoring system.

As discussed by Grossweiner (1970) fast transient measurements based on photoelectric detection require high monitoring light intensity in order to collect a sufficient number of quanta compared to the "shot" noise of the photodetector cathode. For example, with a conventional vacuum photomultiplier the minimum detectable optical density change for 10^{-8} sec response at the signal-to-noise factor of 10 is about 0.1. This calculation is based on a cathode current of 0.1 μ amp as might be obtained with a strong constant lamp. However, the use of a flash as the monitoring lamp provides much higher light intensities at a short duty cycle which can be accommodated by the photomultiplier. The present system employs an ILC type 4L2 xenon flash lamp operated at 0.7 kv and 50 joules input. The flash duration is 300 μ sec corresponding to 100 kwatts input, leading to a signal-to-noise gain of 20 compared with a 200 watt arc lamp of similar dimensions and efficiency. The flash lamp is focussed through the irradiation cell (perpendicular to the direction of laser irradiation) into a Jarrell-Ash 0.25 meter grating monochromator with a Hamamatsu HTV R-136 photomultiplier (230-800 nm) at the exit slit. The photoelectric signal is measured with a Tektronix type 7704A oscilloscope and photographed with a Tektronix type C-51 camera, providing usable single-sweep traces at 2 nanosec/div. A timing circuit applies the laser pulse near the flat center region of the monitoring flash, so that transients of lifetime up to 20 μ sec can be measured with this arrangement. Longer-lived species are monitored with a 200 watt mercury-xenon arc (Oriel C-72-20 power supply and lamp holder system) where high flash intensities are not required for good signal-to-noise.

The monitoring geometry is improved by focussing the laser beam with a cylindrical lens to give a horizontal strip image 0.95 cm long by 2 mm high perpendicular to the monitoring beam, while the latter is restricted to a

Figure 1. Laser flash photolysis of 16 μM Eosin(Oxygen-Saturated). The trace shows the bleaching of the initial dye at 517 nm within 20 nanosec followed by recovery of the bleached dye from the fast reaction of triplet Eosin with oxygen. The 530 nm laser flash was attenuated about 100 times.

2 mm region inwards from the irradiated face with a 2 mm vertical slit. This arrangement confines the optical path length to 0.95 ± 0.01 cm and ensures that the monitoring beam passes through the irradiated volume of known dimensions. The response of the system is illustrated with the trace in Fig.1, in which oxygen-saturated 16 μ M eosin was irradiated with a greatly attenuated 530 nm light flash and monitored at the peak of the dye absorption (517 nm). The trace shows that about half of the initial dye was bleached within 20 nanosec, followed by a recovery of about 200 nanosec lifetime resulting from the fast reaction of triplet eosin with oxygen. (A high yield of $^1\Delta_g \text{O}_2^*$ is generated by this process).

Previous work with xenon flash systems has shown that spectroscopic detection with a plate spectrograph is very useful for preliminary experiments, particularly with expensive biologicals. This method requires synchronizing a broad-band spectroflash after the irradiation flash of duration comparable to the desired time resolution. As an interim measure, we have an auxiliary arrangement using a Xenon Corp. Suntron-6A lamp providing a 10 μ sec spectroflash at 50 joules input. The lamp is powered with a Xenon Corp. Model B Micropulser and triggered with the Model C Trigger Module. The spectra are obtained on 3 $\frac{1}{4}$ " x 4 $\frac{1}{4}$ " plates with a Hilger 518 "small" glass prism spectrograph for the visible region and a small Gaertner quartz prism spectrograph for the u.v. range. We are planning to extend the time resolution for this mode from the present 10 μ sec to about 10 nanosec by using part of the laser beam to excite the fluorescence of a dye mixture. The dye emission pulse will be focussed through the irradiation cell into the spectrograph delayed by the optical path. The more efficient CsDA 1'st doubler will make it possible to split the u.v. laser pulse for this purpose. Another planned future improvement is the acquisition of a ruby laser rod which will provide strong pulses at 694 nm and 347 nm. The use of these wavelengths with dye conversion should make it poss-

Figure 2. Laser Flash Photolysis Apparatus. The dashed lines indicate the auxiliary monitoring systems.

ible to obtain a wide range of visible and near-u.v. wavelengths for the flash photolysis experiments.

The experimental arrangement is shown schematically in Fig.2 .The solid lines indicate the optical path for fast spectrophotometric detection with the monitoring flash lamp. The dashed lines show the optional arrangements for constant lamp photoelectric monitoring and photographic detection with the plate spectrograph and delayed spectroflash lamp. The apparatus is being used to investigate the fast photochemical processes involved in the inactivation of enzymes by u.v. and visible light in the presence of sensitizing dyes, as described in the accompanying Renewal Proposal.

Radiolytic Cleavage of Thymine Dimer

It is well known that thymine and thymine derivatives can be photodimerized via cycloaddition at the 5,6-double bond. This reaction is promoted by u.v. irradiation at 260 nm in fluid or frozen solutions and may be sensitized with molecules of higher triplet energy including acetone, acetophenone and benzophenone. Cyclobutane type dimers have been produced with other natural pyrimidines (uracil, cytosine, cytosine-thymine, uracil-thymine) and in nucleotides such as TpT, dUpU, CpC, poly rU, poly dT, poly dI:dC. They have been generated in bacteriophage DNA (e.g. T4, ϕ X174), bacterial DNA (*E. coli* strains) and in a number of vertebrate cells. The repair of pyrimidine dimers can be effected with short-wavelength irradiation (which cleaves the dimers preferentially compared to the rate of photodimerization), by enzymic photoreactivation with visible light, and in a dark repair replication process requiring a specific enzyme UV-endonuclease. (It has been demonstrated that human skin fibroblasts are capable of repair replication after u.v. irradiation, while patients with the hereditary skin disease Xeroderma pigmentosum (XP), who frequently develop fatal skin cancers upon exposure to sunlight, are deficient towards this mode of repair; Cleaver, 1968). Evidence is accumulating to show that pyrimidine dimers are the major type of lethal lesion in DNA generated by u.v. irradiation; e.g. Setlow (1967) estimated that they are the most prevalent photoproduct by a factor of 10 in T2 phage and *E. coli*(B/r) at the mean lethal dose. The dark repair mechanisms may have broader implications than for u.v. damage and may be involved in repair of DNA damage induced by chemical agents (e.g. nitrogen mustard) and ionizing radiations.

The cleavage of thymine dimer by ionizing radiation was first reported by Lachmann (1963) and by Schmidt et al. (1963). Deering and Snipes (1968)

found that Co-60 irradiation of thymine dimer in aqueous solutions led to efficient cleavage, recovering 1.6-2.0 thymines per dimer. Subsequently Hélène and Charlier (1971) reported that tryptophan and other indole derivatives photosensitize the splitting of thymine and orotic acid dimers and proposed that electrons ejected from the excited indole are responsible, either as hydrated electrons or within a molecular complex. This work was followed by a cooperative pulse radiolysis investigation of this group with Dr. Hélène (Santus et al., 1972), where the hydrated electrons can be generated directly from the water without involving photooxidation of tryptophan. It was found that e_{aq}^- reacts rapidly with the dimer: $k(e_{aq}^- + \hat{T}T) = 1.5 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$ at pH 8.0, 0.02M phosphate buffer. A transient absorption band near 440 nm is attributed to the electron adduct of $\hat{T}T$, and the permanent cleavage yield was found to be $G(\hat{T}T \rightarrow 2T) = 2.2$.

The initial studies have been continued as part of this new program using the pulse radiolysis system at Michael Reese Hospital, providing 1 μsec pulses of 40 MeV electrons at a maximum dose of 2 krad in 20 ml of irradiated volume. The 440 nm transient spectrum has been observed from pH 8 to pH 12 except when e_{aq}^- scavengers such as H^+ or N_2O are present. On the other hand, OH scavengers such as formate or glucose do not quench this band, supporting the identification with the $\hat{T}T$ electron adduct. (The corresponding thymine electron adduct has been attributed to a transient band at 340 nm by Hayon (1969) and Greenstock et al. (1970)). Kinetics studies analyzed with the analog computer show that the 440 nm absorption disappears by a 1st order process with $k(-\hat{T}T) = 2.2 \times 10^5 \text{ sec}^{-1}$, independent of the initial dimer concentration and the dose.

A search is being made for radicals other than e_{aq}^- that cleave thymine dimer. Negative results have been obtained with the anion radicals: CO_2^- , I_2^- , $(ONS)_2^-$ and CO_3^- . The radiosensitizer NEM (N-ethyl maleimide) was

found to act as a non-cleaving electron acceptor:

This reaction parallels the electron transfer reported by Greenstock et al. (1970) for aqueous thymine with $k(T^- + NEM \longrightarrow T + NEM^-) = 8 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$ at pH 11.5 .

The G values for radiolytic cleavage of thymine dimer are summarized in Table 1. The low yield of 0.33-0.35 obtained with N_2O -saturated solutions (or with inactive additives) is due to photolytic cleavage induced by Cerenkov radiation trapped in the cell and was subtracted in analyzing the contributions due to radicals. The results without additives lead to $f_e = 0.89$ in triply-distilled water and $f'_e = 0.73$ in 0.5M phosphate buffer (based on $g_e = 2.6$) , where f_e is the probability that e^-_{aq} reacts to cleave $\hat{T}T$. The corresponding value for non-buffered solutions with D-glucose is $f_{glu} = 0.35$ (based on $g_{OH} = 2.6$) , i.e. the probability that the OH oxidation product of glucose subsequently reacts to cleave $\hat{T}T$. However, the results obtained with glucose in the buffer give $f'_{glu} = 1.6$, a conversion yield in excess of $(g_e + g_{OH})$ by 60% . The pulse radiolysis experiments of Davies et al. (1965) show that the attack of OH on D-glucose leads to a transient species absorbing near 260-270 nm, also characteristic of other sugars xylose, α -methyl glucoside, and inositol. Although the yield of this transient saturates at 0.005M glucose, the permanent loss G(-glu) increases with solute concentration attaining 3.5 in oxygenated solutions at $\sim 0.05M$. These findings suggest that the reaction of the initial 260 nm radical with glucose generates additional species which can cleave thymine dimer. The role of the buffer in promoting this process is the subject of current work. The negative results obtained with ethanol, i-propanol, and sucrose show that the oxidized hydroxyl group is not sufficient and suggest that steric effects are important in the cleavage reaction.

<u>Additive</u>	<u>Solvent</u>	<u>pH</u>	<u>Atmosphere</u>	<u>-G(T⁺T)</u>
none	H ₂ O	7	N ₂	2.6
none	buffer*	8.3	N ₂	2.2
none	buffer	8.3	N ₂ O	0.33
0.01M formate	buffer	8.3	N ₂	2.2
0.01M sucrose	H ₂ O	7	N ₂	2.6
0.01M sucrose	H ₂ O	7	N ₂ O	0.33
0.01M glucose	H ₂ O	7	N ₂	3.5
0.01M glucose	H ₂ O	7	N ₂ O	1.9
0.01M glucose	buffer	8.3	N ₂	6.4
0.01M glucose	buffer	8.3	N ₂ O	8.6
25μM KI, KCNS, K ₂ CO ₃	buffer	8.3	N ₂ O	0.34
0.1M C ₂ H ₅ OH, i-C ₃ H ₇ OH	H ₂ O	7	N ₂ O	0.34

* 0.5M phosphate buffer

Table 1. G values for radiolytic cleavage of thymine dimer by 40 MeV electrons.

Project Activity

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

Scientific Salaries

Dr. L. I. Grossweiner (Professor): 1 month Summer 1972 and 20% of time 1972-73 academic year (through 3/31/73)

Dr. A. G. Kepka (Visiting Assistant Professor): 2 months Summer 1972 and 50% of time 1972-73 academic year (through 3/31/73)

Dr. J. A. Vigil (Research Associate): 69% of time (9/1/72-3/31/73)

Mr. W. A. Wiatrowski (Research Assistant): 50% of time 1972-73 academic year-supported by IIT

Major Equipment Purchased

Holobeam Model 631 Nd:Glass, Pockel's Cell Q-Switched Laser Oscillator; KD*P doubler; ADP doubler, autocollimator - \$16,115

Xenon Corporation Model-B Spectroscopic Micropulser; Trigger Module C; FH-1282 Flashtube Housing (2) - \$2,021

Tektronix type 7704A Oscilloscope; type 7A16 single-trace amplifier; type 7B70 time base; type 7B71 delaying time base; type C-51-R camera - \$5,610 (provided by IIT)

Foreign Travel

Dr. L. I. Grossweiner attended the IV'th International Biophysics Congress in Moscow, USSR, August 7-14, 1972 - \$300 (AEC)

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