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

Progress Report

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

ABSTRACT

Initial mechanisms in the UV photooxidation of aromatic amino acids are being investigated with laser flash photolysis at 265 nm in connection with enzyme inactivation. Aqueous tryptophan (Trp) is photoionized by an efficient monophotonic process, followed by a hitherto unreported pseudo-first order recombination competing with bimolecular e_{aq}^- decay and electron scavengers. Measurements of the photoionization quantum efficiency, the aromatic radical extinction coefficients, and the electron decay kinetics are reported. The flash photolysis of N-formylkynurenine (FK) has been studied in connection with its role in "internal" photodynamic action in bovine carbonic anhydrase (BCA). The triplet state of FK oxidizes Trp to the radical formed also by UV photolysis, leading to the FK semiquinone which reacts with oxygen to produce O_2^- . The same FK semiquinone species is formed by radiolytic reduction by e_{aq}^- and CO_2^- . A parallel radiolysis study on BCA using radical anions as probes of specific residues has shown that the zinc atom protects against the inactivating attack of e_{aq}^- and CO_2^- . Evidence for sensitive aromatic residues in BCA has been found with this technique. Photodynamic damage to biological membranes is being studied with spin label ESR methods. New work is reported on damage to unsaturated lipids sensitized by Eosin based on changes in the temperature-dependence of the spin label rotational correlation time. Preliminary results with diploid yeast membranes (Saccharomyces cerevisiae) show a loosening of the structure accompanying photodynamic inactivation.

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Laser Flash Photolysis of Aromatic Amino Acids

The aromatic amino acids tyrosine (Tyr) and tryptophan (Trp) account for essentially all light absorption in most proteins at $\lambda > 280$ nm and 65% to 95% of the absorption at 254 nm. Flash photolysis of Tyr generates a phenoxyl-type radical with characteristic absorption bands at 390 nm and 410 nm (Grossweiner, 1956) and Trp gives a broad band near 500 nm (Grossweiner, 1956) subsequently identified with the neutral radical product of indole N-H bond photolysis (Santus and Grossweiner, 1972; Pailthorpe and Nicholls, 1971). The identification of the hydrated electron as a co-product of these reactions (Grossweiner et al., 1963) indicates that photoionization is a major (but not necessarily the only) photolysis step. The tyrosine radical $\dot{\text{Tyr}}$ was observed by flash photolysis of several proteins without tryptophan including RNase A (Volkert and Grossweiner, 1973) and histone (Kaluskar, 1973) as exemplified in Fig.1. Flash photolysis of proteins containing tryptophan led to the $\dot{\text{Trp}}$ band in lysozyme (Grossweiner and Usui, 1971), serum albumin (Kaluskar, 1973), trypsin (Kaluskar and Grossweiner, 1974), papain (Baugher and Grossweiner, 1975), and bovine carbonic anhydrase (Kaluskar, 1973); Fig.2. These spectra show also the disulfide bond electron adduct band at 420 nm ($-\dot{\text{S}}\text{S}-$) and the red e_{aq}^- band at $\text{pH} > 4$. Comparisons with flash spectra from mixtures of the chromophoric amino acids led to estimates of the number of aromatic residues per molecule capable of photoionization in the enzyme. The results in Table 1 show that this number is comparable to the "exposed" residues as evaluated by conventional procedures. The initial reactions were related to the biological endpoint by considering inactivation quantum yields, action spectra, and permanent residue destruction in connection with the molecular structure of the enzyme. In general, photolysis of essential aromatic residues (e.g. in lysozyme and papain) or aromatic residues adjacent to essential histidyl residues (e.g. in trypsin) or cystyl residues (e.g. in trypsin and RNase A)

Figure 1. Flash photolysis transient spectra from proteins with no tryptophan.

Figure 2. Flash photolysis transient spectra from proteins with tryptophan.

<u>Enzyme</u>	<u>Aromatic Chromophore</u>	<u>Exposure</u>	η_{ox} ^(a)
Insulin	4 Tyr	2.3 (solv.pert.)	3.0
RNase A	6 Tyr	3 (titration)	3.5
Lysozyme	6 Trp	1.2 (x-ray)	1.5
Trypsin	4 Trp	2.3 (solv.pert.)	1.8
Papain	5 Trp	2.2 (solv.pert.)	~2

(a) flash photolysis data

Table 1. Photoionization yields of aromatic residues.

contribute to loss of enzymic activity.

The previous work was carried out with xenon flash lamps of $\sim 10^{-5}$ sec duration, making it difficult to resolve short-lived aromatic triplet states, which may be precursors of the radicals, and the details of the e_{aq}^- reactions. The measurement of accurate quantum yields was complicated by the broad band irradiation and the decay taking place during the excitation flash. The quadrupled Nd:glass laser system provides a 50 mJ pulse at 265 nm of 20 nsec duration. Initial e_{aq}^- yields of 50-100 μM were readily obtained, comparable to a pulse radiolysis dose of 25,000 rads in the production of e_{aq}^- from water. The quantum yields of e_{aq}^- generation from Trp, 1-MeTrp, and Tyr were determined by comparing the optical density changes at 630 nm with solutions of $Fe(CN)_6^{4-}$ and I^- of the same initial absorbance leading to the results in Table 2. The aromatic radical extinction coefficients were evaluated by extrapolating the e_{aq}^- and radical absorptions in air-saturated solutions back to the time of the laser flash and assuming that photoionization is the only photolysis process and e_{aq}^- reacts only with oxygen (e.g. Fig.3). The results in Table 3 are in satisfactory agreement with recent published values based on other techniques. The high values of the photoionization quantum yields of Tyr and Trp are found also in enzymes, particularly on a "per residue" basis corrected for the energy dissipated in the buried residues. However, the quantum yields of permanent Trp and Tyr destruction in aqueous solution are an order of magnitude smaller [e.g. ~ 0.05 for aqueous Trp at pH 2 to pH 11 (Pailthorpe et al., 1973)] indicating that back reactions are important.

It was expected that e_{aq}^- decay would follow the bimolecular reactions observed many times in pulse radiolysis with $k(e_{aq}^- + e_{aq}^-) = 0.5 \times 10^{10} M^{-1}sec^{-1}$. However, exponential decay was observed consistently in this work, which cannot be explained by an experimental artifact such as trace amounts of reactive impurities or inhomogeneous e_{aq}^- distribution. Figure 4 illustrates the good

<u>Solute</u>	<u>pH</u>	ϕ_e -(265 nm)	<u>Reference</u>
Fe(CN)_6^{4-}	7	0.52	Shirom and Stein (1971)
I^-	7	0.29	Jortner et al. (1964)
Trp	7	0.25 ± 0.02	This work
Trp	9-12	0.40 ± 0.02	This work
1-MeTrp	7	0.4_0	This work
Tyr	7-9	0.28 ± 0.02	This work

Table 2. Photochemical hydrated electron quantum yields.

Figure 3. Laser flash photolysis of 400 μM tryptophan at 265 nm.

Solute (a)	<u>Molar Absorbance</u> $\text{M}^{-1}\text{cm}^{-1}$			<u>Reference</u>
	330 nm	510 nm	410 nm	
Tryptophan	3100±300	1800±200	-	This work
	2800	1750	-	Redpath et al. (1975)
Tyrosine	-	-	2400±300 ^(b)	This work
	-	-	2750±200	Feitelson and Hayon (1973)

(a) pH ~7 unless specified

(b) pH 9

Table 3. Molar absorbance of aromatic radicals

Figure 4. First order decay of photochemical hydrated electron.

fit of the initial decay of e_{aq}^- in N_2 -saturated solutions to first-order kinetics. The average decay halftime for 12 different runs is $0.75 \pm 0.03 \mu\text{sec}$ with no apparent dependence on the solute (I^- , $\text{Fe}(\text{CN})_6^{4-}$, Tyr, Trp, and 1-MeTrp) or the irradiation conditions. The corresponding e_{aq}^- halftime in air-saturated solutions was $0.13 \pm 0.02 \mu\text{sec}$, in good agreement with the value expected from the sum of the anaerobic process plus the reaction $k(e_{aq}^- + \text{O}_2) = 2.0 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$. The first-order e_{aq}^- decay must be associated with the back reactions between the electron and radical co-products. If the radical disappears only by reacting with the electron, then the 510 nm absorption (for Trp) should parallel the 630 nm absorption displaced by the constant factor $(\epsilon_{e^-} + \epsilon_R)^{510 \text{ nm}} / \epsilon_{e^-}^{630 \text{ nm}}$. The data obtained with Trp and 1-MeTrp in N_2 -saturated solutions are in good agreement with this prediction (Fig.5).

A possible mechanism consistent with these findings is based on the assumption that the photolysis act generates a loosely bound complex between the electron and radical in which the absorptions are not greatly different from the free species. The observed first-order decay can be attributed to recombination in the complex:

This mechanism appears to explain the major experimental observations, although it is surprising that the lifetime of $\sim 0.8 \mu\text{sec}$ is so similar for the various solutes. It should be emphasized that this postulated complex is quite different from the "solvent cage" of much shorter lifetime, as required by the ability of O_2 (and also H_2O_2) to scavenge e_{aq}^- during the initial decay stage. The excited state precursors of this intermediate have not ^{been} determined and are the subject of current work. However, the laser photolysis of Trp and 1-MeTrp are monophotonic, arguing against the involvement of the triplet state.

Figure 5. Linear relationship between electron and radical concentrations during initial stages of decay after laser flash photolysis at 265 nm.

The extension of these measurements to the aromatic residues in enzymes constitutes part of the proposed new work.

Sensitizing Properties of N-formylkynurenine

The ultraviolet irradiation of tryptophan (Trp) or tryptophyl residues in proteins leads to N-formylkynurenine (FK) as an oxidation product (Deschreider and Renard, 1955; Pirie, 1971; Asquith and Rivett, 1971; Pirie and Dilley, 1974). [FK also is a product of tryptophan radiolysis and metabolism]. Walrant and Santus (1974a) found that FK is itself a photodynamic sensitizer of amino acids for wavelengths above 320 nm where Trp is not excited. For example, the quantum yield for histidine oxidation sensitized by FK was enhanced by a factor of 5 on changing the solvent from H₂O to D₂O while the addition of 0.01 M NaN₃ led to complete protection, proving that $^1\Delta_g \text{O}_2^*$ is the major oxidizing agent (Nilsson et al., 1972). In the case of Trp, however, the same diagnostic tests indicate a competing pathway, postulated as oxidation of Trp by O₂⁻ from the dependence of the quantum yield on the Trp and O₂ concentrations. Similar reactions were proposed in connection with the photoinactivation of bovine carbonic anhydrase (BCA) by ultraviolet ($\lambda > 280$ nm) and sensitized by external FK ($\lambda > 320$ nm), where the addition of azide protected against loss of enzymic activity and histidine destruction under oxygenated conditions (Walrant and Santus, 1974b). However, Trp destruction in BCA was not altered in the presence of azide indicative of the alternate oxidation pathway. The present work was undertaken to investigate the "non-singlet oxygen" process by which FK sensitizes the photooxidation of Trp.

Spectral studies on FK (Walrant and Santus, 1974a) show the longest wavelength absorption band is located at 320 nm (pH 7.6, $\mu = 0.15$ M phosphate buffer). At 77 K the phosphorescence lifetime is 0.5 s (1:1 water-ethylene glycol) with undetectable fluorescence indicating a high intersystem crossing efficiency. Xenon lamp flash photolysis of air-free FK in the pH 7.6 buffer

Figure 6A. Flash photolysis transient spectrum from 250 μM FK ($\lambda > 320$ nm); N_2 -sat, pH 7.6 phosphate buffer.

Figure 6B. (a) Flash photolysis transient spectrum of 250 μM FK in N_2 -sat ethanol. (20 cm path) (b) Pulse radiolysis transient spectrum of 250 μM FK (30 MeV e^-); N_2O -sat pH 7.6 phosphate buffer with 0.1M sodium formate. (20 cm path, 1.3 krad/pulse)

led to the 435 nm band in Fig.6A attributed to the triplet state (^3FK). This transient was not observed in the presence of air and is quenched by KI according to Stern-Volmer kinetics with quenching constant $3 \times 10^3 \text{ M}^{-1}$. The triplet decay was 1st order with 2 μsec lifetime as determined with the quadrupled Nd:glass laser system. A different transient product was observed by flash photolysis of FK in N_2 -sat ethanol, with the absorption maximum at 465 nm (Fig.6B). The decay was 2nd order with $k/\epsilon = 8.5 \times 10^5 \text{ cm-s}^{-1}$ at 465 nm. This species is attributed to the FK semiquinone radical ($\text{FKH}\cdot$) formed by:

where Q is ethanol or another oxidizable substrate. The identification is supported by pulse radiolysis experiments carried out with the system located at Michael Reese Medical Center in which a similar band was formed by irradiating FK with 30 MeV electrons in the presence of 0.1 M *t*-butanol to scavenge OH radicals or in N_2O -sat, pH 7.6 phosphate buffer containing 0.1 M HCOONa (Fig.6B). The corresponding radiolytic reactions are:

with $k_2 = 3 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ($\pm 20\%$) based on e_{aq}^- decay and $k_3 > 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ based on $\text{FKH}\cdot$ growth. The molar absorbance of $\text{FKH}\cdot$ was estimated as $1.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, assuming that all e_{aq}^- reacted with FK, and $k/\epsilon = 6.6 \times 10^5 \text{ cm-s}^{-1}$ at 465 nm leading to the average value $k_4 = 6.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ($\pm 25\%$) for:

The photosensitizing ability of FK was investigated by carrying out flash photolysis experiments with a filter of 26 g/l naphthalene in isooctane which cuts off at 320 nm. Figure 7 gives results obtained by irradiating 250 μM FK in the presence of 500 μM Trp. The transient obtained under N_2 -sat (7A) shows the 465 nm $\text{FK}\cdot$ band and shoulder near 510 nm. Only the 510 nm Trp band was

Figure 7. Transient spectra from flash photolysis($\lambda > 320$ nm) of 200 μ M FK(pH 7.6) in the presence of 500 μ M tryptophan.(A) N_2 -sat (B) air-sat, 5 μ sec delay.

Figure 8. Transient spectra from flash photolysis ($\lambda > 320$ nm) of 200 μ M FK (pH 7.6) in the presence of 500 μ M 1-methyltryptophan. (A) N_2 -sat, 5 μ sec delay (B) air-sat, 5 μ sec delay (C) N_2 -sat, 75 μ sec delay.

observed with air-sat solutions (7B), while no transient bands were detected without FK. The same Trp product is formed when triplet state eosin reacts with Trp (Grossweiner and Zwicker, 1963) which parallels the present case. The mechanism is supported by flash photolytic results where FK was irradiated in the presence of 1-Methyltryptophan (Fig.8) and the 510 nm band is replaced by the corresponding radical cation absorption near 590 nm (Santus and Grossweiner, 1972). These reactions exemplify (1) where Q is Trp or 1-MeTrp. In similar experiments the FKH $\cdot$ band was observed by irradiating 250 μ M FK in the presence of 500 μ M adenosine, 250 μ M guanosine, or 500 μ M thymine dimer, indicative of sensitized oxidations. However, irradiation of 250 μ M FK in the presence of 500 μ M histidine or 500 μ M thymine at pH 7.6 gave only the 435 nm 3 FK absorption.

The above results confirm that FK photosensitizes the oxidation of Trp via the triplet state under anaerobic conditions. The quenching of the 465 nm FKH $\cdot$ absorption by air (Figs. 7B and 8B) suggests that (1) is followed by:

Reaction (5) provides a possible mechanism for the "non-singlet oxygen" oxidation of Trp in BCA, where "direct" photooxidation of Trp ($\lambda > 280$ nm) leads to FK which is excited to 3 FK ($\lambda > 320$ nm). The oxidation of histidine is attributed to singlet oxygen populated by energy transfer from 3 FK. This process contributes to Trp oxidation in competition with O_2^- formation via (5) which, in turn, oxidizes additional Trp. The last step is supported by the recent work of Walrant et al. (1975) showing that O_2^- generated by pulse radiolysis or electrochemically oxidizes Trp (or possibly Trp $\cdot$) to FK. The overall reaction sequence is summarized in Fig.9. According to this scheme FK is produced by "direct" photooxidation of Trp and from the attack of O_2^- on Trp, generated in turn by the reaction of primary product e_{aq}^- with oxygen and also the oxidation of FK $\cdot$. The reducing agent Q in BCA is not known and may be another tryptophyl

residue leading to additional Trp oxidation. Similar pathways of "internal photosensitization" may take place in other enzymes, but are likely to be less important than BCA which displays an unusual dependence of the photo-inactivation quantum yield on oxygen.

Radiation Sensitivity of Carbonic Anhydrase

The flash photolysis studies on photosensitization by FK described in the previous section were motivated by recent work of Walrant and Santus (1974) on bovine carbonic anhydrase (BCA), showing that irradiation at $\lambda > 280$ nm oxidizes Trp to FK, which in turn photosensitizes inactivation induced by $\lambda > 320$ nm. The latter process requires oxygen and was completely quenched by 0.01M azide, indicating that singlet oxygen is the inactivating entity. Inactivation was accompanied by loss of 3.4 Trp residues (out of 7) and 8.9 His residues (out of 11) under O_2 -saturation. The Trp destruction was unchanged while the His loss was negligible with N_3^- present, indicating that His residues are the essential targets. BCA is a metallo-enzyme containing 1 zinc atom liganded to 3 His residues at the active site. However, the functional importance of the aromatic residues is not known and it was deemed interesting to apply the radical anion probe method to investigate the radiation sensitivity. This technique exploits the differential reactivity of radical anions such as Br_2^- and $(CNS)_2^-$ for specific amino acids and makes it possible to identify the residues essential for enzymic activity (Adams et al., 1972). The role of the zinc atom in BCA was investigated by studying the metal-free apoenzyme and cobalt-BCA which has similar activity as the zinc enzyme. This work was carried out in collaboration with Dr. J.L. Redpath and Dr. R. Santus of the Department of Medical Physics, Michael Reese Medical Center.

The BCA samples (Schwarz-Mann and Miles-Seravac) were irradiated with 25 MeV electrons at ~ 2 krad/min and assayed at pH 7 using paranitrophenyl acetate as the substrate. The apoenzyme and cobalt-BCA were prepared according

to the method of Lindskog and Malmstrom (1962). Intact Trp was assayed by the method of Spies and Chambers (1949) and also by fluorescence of the denatured enzyme excited at 295 nm. His and Tyr were determined with the method of Sokolovsky and Vallee (1966). The radiation sensitivities are reported as $10^3/D_{37}$ krad s^{-1} based on the exponential dose-response curves for activity and residue destruction.

The results obtained at pH 7 are summarized in Tables 4 and 5. The higher radiation sensitivity of the apo-enzyme in neutral, scavenger-free solutions (Table 5, Row 1) indicates that the metal atoms protect against both loss of activity and Trp loss. The systems providing oxidizing species show that the metal atoms do not greatly alter the sensitivity towards $(CNS)_2^-$, but the cobalt enzyme is much more sensitive to Br_2^- (Table 4, Rows 2-4). It has been shown that Br_2^- reacts with His >15 times faster than $(CNS)_2^-$ (Adams et al., 1972) suggesting a direct attack on the active site, where the liganding His residues may be more exposed in Co^{2+} -BCA because of small conformation changes. The higher sensitivity of the three enzyme forms to Br_2^- compared with $(CNS)_2^-$ is consistent also with the direct attack on essential His. The direct measurements of residue loss (Table 5) show that His destruction is 3 times more sensitive to Br_2^- than to $(CNS)_2^-$. Similarly, Br_2^- reacts with Trp and Tyr more rapidly than $(CNS)_2^-$ by factors of 3 and 4, respectively, consistent with the corresponding higher yields of residue destruction.

In alkaline solutions the strong selectivity of $(CNS)_2^-$ for Trp relative to Tyr (~50 times at pH 7) diminishes to ~1.5 because of the high reactivity of the tyrosinate anion. The comparison of the $(CNS)_2^-$ data in Table 6 with Row 3 of Table 4 shows that the radiation sensitivity is about the same at pH 7 and pH 11 for the three enzyme forms. However, Trp loss was negligible at pH 11 indicative of sensitive residue other than Trp or His, since $(CNS)_2^-$

Scavenger System (a)		Reactive Radicals			Radiation Sensitivity (b)					
					apo-BCA		Zn ²⁺ -BCA		Co ²⁺ -BCA	
					act.	Trp	act.	Trp	act.	Trp
1	none	e _{aq} ⁻	OH	H	22.2	18.0	9.3	4.6	6.3	2.2
2	N ₂ O		OH	H	16.0	9.4	16.0	7.7	10.0	2.2
3	N ₂ O + CNS ⁻	(CNS) ₂ ⁻		H	12.0	17.2	12.0	7.7	9.5	4.0
4	N ₂ O + Br ⁻	Br ₂ ⁻		H	23.9	13.5	25.0	13.7	59.0	10.2
5	N ₂ + t-butanol	e _{aq} ⁻		H	10.5	7.5 (pH 5)	3.7	-	2.8	-
6	N ₂ O + t-butanol			H	-	6.6 (pH 5)	1.2	-	2.1	-
7	N ₂ + HCO ₂ ⁻	e _{aq} ⁻		CO ₂ ⁻	4.7	6.6 (pH 5)	2.9	2.8	-	2.2
8	N ₂ O + HCO ₂ ⁻	CO ₂ ⁻		H	2.0	4.0 (pH 5)	<1		<1	-

(a) pH 7 and 20°C unless otherwise noted.

(b) 10³/D₃₇ (krad⁻¹) for activity loss and tryptophan loss.

Table 4. Radiation Sensitivity and Trp Loss at pH 7.

<u>Scavenger System</u>	<u>Reactive Radicals</u>	<u>Residue Loss Sensitivity</u> (a)		
		Trp	Tyr	His
$N_2O + CNS^-$ (pH 7)	$(CNS)_2^-$ H	7.7	7.5	3.7
$N_2O + Br^-$ (pH 7)	Br_2^- H	13.7	16.4	11.4

(a) $10^3/D_{37}$ (krad⁻¹)

Table 5. Residue Loss in Zn^{2+} -BCA at pH 7.

is not reactive towards His. Although Tyr has not been implicated in BCA activity, it is the likely target in alkaline solutions. This conclusion is supported by pulse radiolysis spectra taken of Zn^{2+} -BCA in the presence of N_2O and CNS^- which show the 510 nm Trp band at pH 7 and a strong Tyr 410 nm band at pH 11.5. The apparent extinction coefficients indicate that about half of the available $(\text{CNS})_2^-$ reacts with Trp at pH 7 and that all $(\text{CNS})_2^-$ reacts with tyrosinate at pH 11.5.

The results obtained with the reducing radicals (Table 4, Rows 5-8) provide new information about the role of metal atoms in radiation sensitivity. The protective effect of Zn^{2+} and Co^{2+} is evidenced by the higher sensitivity of the apoenzyme towards e_{aq}^- and CO_2^- . It is known that e_{aq}^- reduces the divalent cations to unstable monovalent cations, which may subsequently hydrolyze restoring the activity. The net effect is to "channel" the reactions away from the sensitive targets. The higher inactivation at pH 5 in the apoenzyme indicates that protonation of the His residues ($\text{pK}_2 = 6.0$) sensitizes for e_{aq}^- and CO_2^- . (The control activity was unchanged at pH 5.) The lower Trp loss at pH 5 in apo-BCA (Row 5) comparable to the "protected" Zn^{2+} -BCA and Co^{2+} -BCA can be explained by postulating that the electron attacks the "hole" and reaches a sensitive Trp residue.

In summary, these results indicate that the presence of the metal atoms protects BCA against the reductive attack of e_{aq}^- (and probably CO_2^-) but not against the oxidative attack of $\text{OH}^\cdot$ and the radical anions. The importance of His residues for activity is confirmed, while new evidence for sensitive Tyr and Trp residues under certain irradiation conditions has been obtained. The major role of His is consistent with the photochemical results.

Scavenger System (a)	Reactive Radicals	Radiation Sensitivity (b)					
		apo-BCA		Zn ²⁺ -BCA		Co ²⁺ -BCA	
		act.	Trp	act.	Trp	act.	Trp
N ₂ O + CNS ⁻	(CNS) ₂ ⁻ H	10.6	0	10.6	0	14.0	(c)
N ₂ O + Br ⁻	Br ₂ ⁻ H	14.7	8.7	8.0	10.2	6.0	4.5

(a) pH 10.5-11.5

(b) 10³/D₃₇ (krads⁻¹)

(c) non-exponential

Table 6. Radiation Sensitivity and Trp Loss at pH 10.5 - 11.5 .

Photodynamic Damage to Natural and Model Membranes

The plasma membrane of living cells provides the permeability barrier which controls the flow of nutrients and ions into and out of the cell. It acts as the first defense against harmful agents in the external environment and is the site of vital biochemical and metabolic functions. Photodynamic action is known to cause irreversible damage to red blood cell membranes (Lamola et al., 1973) and to phospholipid "liposomes" as model membrane systems (Anderson and Krinsky, 1973; Anderson et al., 1974). The role of singlet oxygen has been found to be crucial in both cases. Spin label ESR techniques are being used in this work to probe photodynamic damage. Preliminary studies on stearic acid in the previous Progress Report (COO-2217-5) showed that the rotational correlation time of spin label "2N19" (below)

is influenced by irradiation with visible light in the presence of eosin indicative of changes in local order. The continuation of this work and extension to oleic acid and triolein are described below. New spin label studies have been initiated on yeast cell membranes subjected to photodynamic inactivation.

Spin Label Methods Spin label spectroscopy relies on the characteristic directional dependence of the nitroxide ESR spectrum (Keith et al., 1973; McConnell and McFarland, 1970). The three-line nature of the signal arises from

the hyperfine interaction of the unpaired electron in the nitroxide group with the spin-1 nitrogen nucleus, while the separation distance between lines in the spectrum in a homogeneous environment depends on the orientation of the nitroxide molecular orbitals with respect to the D.C. magnetic field of the spectrometer (Carrington and McLachlan, 1967). Changing the orientation of the nitroxide group with respect to the magnetic field alters the hyperfine splitting in a way which depends on the direction of the change (Libertini and Griffith, 1970; Snipes et al., 1974). If this change occurs rapidly, so that the nitroxide assumes more than one orientation during the signal absorption-emission lifetime of the probe ($\sim 10^{-9}$ sec), motional averaging is observed. The spectrum consists of a partial average of the orientation-dependent spectra, in which the averaging procedure is analogous to a quantum mechanical average (Williams et al., 1971). Measurements of the heights and widths of the peaks can then be employed to compute the rotational correlation time of the probe according to the expression

$$\tau_c = 6.5 \times 10^{-10} W_0 \left[\left(h_0/h_{-1} \right)^2 h_{-1} \right]$$

(Kivelson, 1960; Snipes and Keith, 1970). The quantities in this expression are defined in Fig.10. For rotational correlation times less than $\sim 10^{-10}$ sec, the averaging of orientation-dependent spectra is complete and the recorded spectrum consists of three lines of identical heights and widths. In terms of the above equation, h_{-1} approaches h_0 and thus τ_c becomes very small.

These motional variations of spin label spectra are illustrated in Fig.11 which displays spectra of a small roughly spherically-shaped label tempol dissolved in glycerol. There is no preferred axis of mechanical rotation for this probe, so motional averaging effects are not complicated by non-isotropic tumbling. Glycerol does not form an ordered solid as its temperature is reduced

Figure 10. Measurement of tumbling times from first derivative ESR spectra.

Figure 11. ESR spectra of Tempol in glycerol.

but rather becomes a more viscous fluid. The ESR spectra reflect this increased viscosity as a rotational hindrance to the probe which impedes motional averaging, so that the relative line heights and widths change. At the lowest temperature the tumbling time has decreased to well below 10^{-9} sec; this spectrum corresponds to an absence of motional averaging and is indicative of a collection of randomly oriented, slowly moving spin labels. Similar analyses can be applied to larger probes, but here the motional effects reflect the mobility only of the portion of the molecule near the nitroxide-containing ring (Smith, 1971).

Spin labels can experience the local magnetic field arising from neighboring spin label molecules in addition to the external and hyperfine fields (Keith and Snipes, 1974). When the spin label concentration becomes large enough for these effects to be observed, the relative heights and widths of the three peaks are altered characteristically. By measuring the extent of these effects, the translational diffusive mobility of the probes can be determined in solutions (Keith et al., 1975) and in membrane systems (Cohn et al., 1974; Scandella et al., 1972).

Membrane phospholipids can have a variety of saturated and unsaturated fatty acid residues in their hydrocarbon regions. In order to isolate the disruptive effect on the order of the hydrocarbon region of the phospholipid bilayer, we have undertaken the investigation of eosin-sensitized photodynamic damage in pure lipids. The technique adopted consists of irradiating the lipid in the presence of eosin at temperatures high enough to keep the mixture melted. Following irradiation the sample is spin labeled and spectra are recorded both as the sample is slowly cooled by increments from the liquid state and as the sample is slowly heated by increments after being thermally quenched from the melt. Measurements of spectral parameters at the different temperatures can be combined with qualitative observations of spectral features

Figure 12. Spin label rotational mobility in photodynamically damaged stearic acid-slow cooling.

Figure 13. Spin label rotational mobility in photodynamically 'damaged' stearic acid-quenched.

to detect any alterations in spin label mobility.

Photodynamic Damage in Fatty Acids. We have improved the preliminary results on photodynamic damage to stearic acid reported in 1974 (Cohn and Grossweiner, 1975). The present measurements were made with sufficiently small concentrations of 2N19 to preclude any interactions between nitroxides until well below the melting point. In order to reduce the possibility of significant interactions between eosin and 2N19 molecules, the concentration of eosin was kept approximately fifty times less than that of 2N19. Samples were irradiated with visible light as described previously, with a specially constructed temperature regulator keeping the sample at approximately 75°C. Since the ratio (h_0/h_{-1}) reflects changes in motion quite accurately, this quantity was plotted on Arrhenius plots to follow these effects. The results in Figs. 12 and 13 for 2N19 in stearic acid with slow cooling and quenching show lower label mobility at lower temperatures for quenching than for slow cooling, indicating that quenching has partly trapped the probe molecules dispersed through the sample, rather than allowing them to migrate into local zones of higher 2N19 concentration as could occur in slow cooling. The similarity of the spectra at all temperatures with the dye present indicates that eosin has not caused significant disruption of the stearic acid matrix. When the sample was irradiated with visible light, substantially different results were obtained. Much greater motional hindrance was observed at low temperatures, and different features were obtained for irradiation in the presence of oxygen than nitrogen. These data indicate that dye-sensitized irradiation effects can be detected even in a saturated hydrocarbon matrix. Rotational freedom is substantially reduced and the partial pooling of 2N19 at lower temperatures is affected. The difference between nitrogen- and oxygen-saturated samples suggests that the reduced mobility and altered pooling depend on the presence of oxygen.

Figure 14. Spin label rotational correlation times in unsaturated lipids-slow cooling.

Figure 15. Spin label rotational correlation times in unsaturated lipids-quenching.

Measurements have been performed on the motion of hydrocarbon spin labels in oleic acid (MP = 16°C) the mono-unsaturated analog of stearic acid which has a cis-double bond at the ninth carbon.

Slow cooling and quenching series have been recorded for the labels 7N14 and 2N19 which should locate their nitroxide groups in the hydrocarbon zone and near the polar region of the solid lipid. From the Arrhenius plots of correlation time in Fig.14 it appears that slow cooling once again allows some degree of rotational mobility to persist below the melting point whereas quenching does not. The break in the graph for 2N19 suggests that the ordering process is different in the two different regions of the matrix. Spectra of 7N14 in triolein show

a reduction in motional freedom as the temperature decreases to the melting point (-5°C). At this temperature an immobilized spectrum appears and becomes more pronounced as the temperature is reduced further. Identical spectra were obtained at each temperature following quenching (Fig.15). Apparently the increased order of the triolein matrix traps 7Nl4 molecules at lower temperatures and does not allow them to diffuse translationally. Photodynamic irradiation of these unsaturated lipids is now being pursued so that the effects at double bond sites can be examined by ESR. These data will allow us to interpret the yeast results (to be described) more clearly in terms of damage to the lipid structure of membranes as opposed to other effects.

Photodynamic Damage in Yeast Cell Membranes The yeast cells were a diploid wild-type strain of Saccharomyces cerevisiae grown in a standard medium (Difco) with dextrose (YNBD medium). Growth curves were measured spectrophotometrically and the doubling time for exponential growth ("log phase") determined from the growth curves. The non-toxicity of eosin was demonstrated by measuring the doubling time in liquid medium containing 1 μM to 25 μM eosin which was not changed. Further tests were carried out by harvesting cells in mid-log phase by centrifugation, washing in distilled water, and suspending the cells in 5 μM eosin for different staining times. The cells were washed in distilled water and transferred to the YNBD medium and grown again. The doubling time at 30°C was constant at 120 ± 3 min for 0 to 4.5 hours staining time. Furthermore, irradiation of stained and washed cells with visible light ($\lambda > 400$ nm) at dosage sufficient to cause photodynamic inactivation (with the dye present) did not lead to cell killing or a change in the subsequent doubling time in the medium. The optical spectrum of the stained yeast cells was the same for all staining times, consisting of the 518 nm eosin band superimposed on a flat background caused by light scattering from the yeast. These measurements indicate that the eosin was located outside the cell or on the plasma membrane.

This point is particularly important in view of previous work of Ito (Ito et al., 1967; Ito and Kobayashi, 1974; Ito, 1974), in which the photodynamic inactivation of yeast sensitized by acridine orange (AO) was attributed to penetration of the cell and binding to DNA. Typical dose-response data obtained by irradiating the cells in the presence of 10 μM eosin are given in Fig.16. The approximately exponential response is strikingly different from the earlier work with AO in which the semilogarithmic plot shows a pronounced shoulder followed by linear response. (The occurrence of a shoulder is consistent with multi-target or multi-hit kinetics and sub-lethal, repairable damage, but there is no direct evidence leading to detailed models for the case in question).

Figure 16. Photodynamic inactivation of diploid yeast cells photosensitized by Eosin dye.

Ito and Kobayashi (1974) found that the addition of $0.023M$ N_3^- protected against photodynamic inactivation in air and irradiation in D_2O led to an enhanced killing rate, which they attributed to the participation of singlet oxygen. Furthermore, there was no inactivation when the cells were irradiated in N_2 -sat H_2O or D_2O . The examination of their data indicates that the sensitizing effect of D_2O was primarily on the shoulder suggesting that more than one oxygen-dependent (Type II process) inactivation mechanism is involved. The inactivation of N_2 -sat solutions found with eosin in the present work indicates that the Type I process does contribute, possibly the direct attack of triplet eosin on the cell. The diagnostic tests for singlet oxygen are being employed in current work.

Preliminary spin label studies on irradiated yeast cells are now in progress using 2N19, which should orient in the membrane bilayer with the hydrocarbon tail extending into the interior and the oxazolidine group localized near the polar region. ESR spectra were recorded for control and photodynamically irradiated samples of the yeast grown and harvested as before. Spectra were taken at a series of temperatures for each sample and were examined for motional effects which would reflect the state of the membrane. Sample temperature is effectively a membrane parameter, since the degree of order, fluidity and rigidity of the phospholipid bilayer varies with temperature and reflects apparent phase transitions at temperatures which are characteristic of the preparation (Raison et al., 1971). The spectra for the control sample (Fig.17) exhibit a considerable amount of interaction between probe molecules at $0^\circ C$ which diminishes slowly as the temperature is raised and a free-tumbling signal appears. This suggests that many of the 2N19 molecules have migrated into local pools of high spin label concentration, since the overall concentration is too low to give rise to nitroxide-nitroxide interactions. The spectra for the irradiated culture (Fig.18) show some free tumbling at all temperatures,

Figure 17. Spin label ESR spectra of 2N19 in Unirradiated diploid yeast cells.

Figure 18. Spin label ESR spectra of 2N19
in photodynamically damaged
diploid yeast cells.

but now the suggestion of pooling is greater at the higher temperatures. These data indicate that photodynamic action can alter local membrane properties, perhaps by "loosening up" the bilayer structure. It is known that photodynamic action cleaves lipids at carbon-carbon double bonds in the alkyl chain (Rawls and van Santen, 1971), and this might result in a decrease in cooperative motion of the membrane phospholipids with an accompanying increase in fluidity. These experiments are presently being continued in order to determine the nature of the damage and to quantitate factors such as the dose dependence.

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): 1 month Summer 1974; 10% of time 1974-75 academic year.

Dr. Gerald E. Cohn (Assistant Professor): 33% of time 1974-75 academic year.

Dr. F. Dudley Bryant (Research Associate): 84% of time 9/1/74 - 6/30/75.

Dr. Arun G. Kaluskar (Research Associate): 100% of time 7/1/74 - 8/31/74.

Equipment Purchased

Holobeam doubler crystal for ruby laser rod (to be purchased).....\$1,000

Foreign Travel none

Reporting of Research

Meetings

"Photodynamic Action in Hydrocarbon Matrices." G.E.Cohn and L.I.Grossweiner, American Society for Photobiology, Vancouver, B.C., July 22-26, 1974.

"Radiolytic Cleavage of Thymine Dimer." L.I.Grossweiner, J.A.Vigil, and R.Santus, 5'th Int.Congress of Radiation Research, Seattle, Wash., July 14-20-, 1974.

"Motion of Impurity Molecules in Solids Unsaturated Lipids." G.E.Cohn and L.I.Grossweiner, Annual Meeting, The Biophysical Society, Philadelphia, Pa., February 18-21, 1975.

"Laser Flash Photolysis of Aqueous Tryptophan." F.Dudley Bryant, R.Santus, and L.I.Grossweiner, American Society for Photobiology, Louisville, Ky. June 22-26, 1975.

"The Role of the Hydrated Electron in the Photochemical Inactivation of Enzymes." L.I.Grossweiner, E.J.Hart Conf. on Aqueous Radiation Chemistry and Solvated Electrons, Argonne National Laboratory, July 7-9, 1975 (INVITED)

"Photochemical Inactivation of Enzymes." L.I.Grossweiner, VIII Int.Conf. on Photochemistry, Edmonton, Canada, August 7-12, 1975 (INVITED)

Journal Articles

"Thymine Dimer Splitting by Ionizing Radiation." L.I.Grossweiner, A.G.Kepka, R.Santus, and J.A.Vigil, Int.J.Radiat.Biol. (1974) 25 521-523.

"Sensitizing Properties of N-Formylkynurenine." P.Walrant, R.Santus, and L.I.Grossweiner, Photochem.Photobiol. (submitted).

"Laser Flash Photolysis of Aqueous Tryptophan." F.D.Bryant, R.Santus, and L.I.Grossweiner (in preparation).

"Photochemical Inactivation of Enzymes," L.I.Grossweiner, in CURRENT TOPICS IN RADIATION RESEARCH (Ed. M.Ebert and A.Howard), North-Holland Pub. Co., Amsterdam (in press).

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