INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17" x 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
DNA damage and cell lethality by photodynamically-produced oxygen radicals

Burch, Paula Ellen, Ph.D.
Rice University, 1989
RICE UNIVERSITY

DNA DAMAGE AND CELL LETHALITY BY PHOTODYNAMICALLY PRODUCED OXYGEN RADICALS

by

PAULA ELLEN BURCH

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

Ronald Sass, Ph.D., Chair
Professor of Biology

Joseph P. Martin, Jr., Ph.D.
Research Scientist, Upjohn Corporation

George N. Bennett, Ph.D.
Professor of Biochemistry

Stephen Subtelny, Ph.D.
Professor of Biology

Houston, Texas

April 21, 1989
ABSTRACT

DNA Damage and Cell Lethality by Photodynamically Produced Oxygen Radicals

Paula Ellen Burch

Synthetic dyes, including thiazines, acridines, xanthenes, and a phenazine, were used as models for studying the photodynamic effect. In the presence of physiological reductants, the illuminated dyes produced superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl free radical (OH·). Suitable reductants included NADH, glutathione, GMP, cysteine, tryptophan, and tyrosine. The production of OH· was dependent on chelated iron or on copper. Effective iron chelators included EDTA, DTPA, dipyridyl, and phenanthrolene, and biologically significant compounds such as ATP, ADP, succinate, citrate, and DNA. DNA intercalation of the dyes did not prevent oxidization of NADH or the production of O$_2^-$ or OH·. Hydroxyl radical scavengers competed effectively in the assays for the OH·. While in the Haber-Weiss reaction superoxide reduces iron, which is oxidized by H$_2$O$_2$ to produce OH·, excited state reduced dyes appeared capable of reducing the iron, so that SOD was only partially inhibitory of OH· production. Catalase prevented production of OH·.

Similar results were found for substrates and metals in an assay of single strand scission of DNA mediated by the dyes. Amounts of strand scission seen were dependent on concentrations of iron or dye. Cysteine, NADH, GTP, dGMP, tryptophan, and tyrosine were all able to provide electrons for the strand scission reaction. Furthermore, the single strand scission of DNA by the dyes was
prevented by scavengers of $O_2^-$, $H_2O_2$, or $OH\cdot$ which are poor scavengers for singlet oxygen ($^{1}O_2$), and substitution of deuterium oxide for water, which exacerbates any damage produced by $^{1}O_2$, did not increase damage.

The physiological reductant glutathione in *E. coli* was depleted by exposure to illuminated dye. Lethality of the dyes was reduced by enhanced levels of catalase or endonuclease IV provided by plasmid-coded genes, indicating that $H_2O_2$ is an important mediator of toxicity and that DNA is an important target, while $OH\cdot$ scavengers prevented kill, indicating that the $OH\cdot$ is also an important mediator of phototoxicity. Finally, DNA damage *in vivo* was reduced by an $OH\cdot$ scavenger. In conclusion, toxicity in the photodynamic effect is mediated by reduced oxygen species, particularly $OH\cdot$, and DNA damage probably underlies this toxicity.
ACKNOWLEDGEMENTS

I would like to express my appreciation to my advisor, Dr. Joe Martin, for his persevering in the lab at a time when many others would have given up; without his committment to research, I would not have been able to complete my doctoral work at this institution, rather than beginning anew at a different one after four years here at Rice.

I also wish to thank the other members of my committee: Dr. Ronald Sass, for being willing to assume the position of commitee director at the last moment; Dr. Stephen Subtelny, for becoming a member of my commitee also at the last moment; and Dr. George Bennett, whose encouragement and timely criticism were extremely helpful.

I am grateful to Dr. Kathy Matthews for opening her lab to me after my advisor's lab was no longer available to me, and to the members of her lab, especially Artemis, Ronit, and Kyle, for welcoming and encouraging me, and even helping with details of my work.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii

ACKNOWLEDGEMENTS ........................................................................................ iv

LIST OF FIGURES ................................................................................................... vii

LIST OF TABLES ....................................................................................................... xii

LIST OF ABBREVIATIONS ...................................................................................... xiii

CHAPTER

I. INTRODUCTION AND LITERATURE REVIEW ............................................. 1

   Oxygen Toxicity ..................................................................................................... 1
   Defenses Against Oxygen Toxicity ........................................................................ 14
   The Photodynamic Effect ..................................................................................... 27

II. REACTIONS OF DYES ....................................................................................... 57

   Introduction .......................................................................................................... 57
   Materials and Methods ......................................................................................... 59
   Results ................................................................................................................... 61

III. HYDROXYL RADICAL PRODUCTION IN VITRO ........................................ 82

   Introduction .......................................................................................................... 82
   Materials and Methods ......................................................................................... 83
   Results ................................................................................................................... 86

IV. DNA DAMAGE IN VITRO ............................................................................... 116

   Introduction .......................................................................................................... 116
   Materials and Methods ......................................................................................... 117
   Results ................................................................................................................... 118
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The partial reduction products of oxygen</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>The SOS regulon</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>The phototoxic spectrum</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>Structure of dyes</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>Dye reactions</td>
<td>43</td>
</tr>
<tr>
<td>6</td>
<td>Cyanide sensitive and resistant respiration</td>
<td>58</td>
</tr>
<tr>
<td>7</td>
<td>Change in spectrum of azure c with addition of DNA</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>Change in spectrum of methylene blue with addition of DNA</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>Change in spectrum of thionin with the addition of DNA</td>
<td>64</td>
</tr>
<tr>
<td>10</td>
<td>Change in spectrum of acridine orange with the addition of DNA</td>
<td>65</td>
</tr>
<tr>
<td>11</td>
<td>Change in spectrum of proflavin with the addition of DNA</td>
<td>66</td>
</tr>
<tr>
<td>12</td>
<td>Lack of change in spectrum of fluorescein with the addition of DNA</td>
<td>67</td>
</tr>
<tr>
<td>13</td>
<td>Oxidation of 0.5 mM NADH by 1 μM thionin</td>
<td>69</td>
</tr>
<tr>
<td>14</td>
<td>Oxidation of 0.5 mM NADH by 1 μM rose bengal</td>
<td>70</td>
</tr>
<tr>
<td>15</td>
<td>Oxidation of 0.5 mM NADH by 1 μM methylene blue</td>
<td>71</td>
</tr>
<tr>
<td>16</td>
<td>Oxidation of 0.5 mM NADH by 1 μM azure c</td>
<td>72</td>
</tr>
<tr>
<td>17</td>
<td>Reduction of 20 μM cytochrome c mediated by 0.5 μM methylene blue</td>
<td>74</td>
</tr>
<tr>
<td>18</td>
<td>Reduction of 20 μM cytochrome c mediated by 0.5 μM rose bengal</td>
<td>75</td>
</tr>
</tbody>
</table>
DNA as a chelator in the azure c mediated production of OH- as detected by the salicylate assay .................................................. 104
Production of OH- mediated by azure c in the presence or absence of EDTA or DNA ........................................................................ 105
Production of OH- mediated by DNA-intercalated azure c and methylene blue, as measured in the thiobarbituric acid assay ................................................................................................................................. 106
Production of OH- mediated by DNA-intercalated proflavin, acridine orange, fluorescein, and methylene blue, as measured in salicylate assay ................................................................................................................ 107
Production of OH- mediated by DNA-intercalated and non-intercalated methylene blue, neutral red, acridine orange, and fluorescein ........................................................................................................... 108
Competition between scavengers and salicylate in the salicylate assay ............................................................................................ 110
Azide as an OH- scavenger in the salicylate assay .............................................................................................................................. 111
Substrate limitation in OH- production .................................................................................................................................................. 113
Hydroxyl radical scavengers prevent enhancement of OH- production by hydrogen peroxide as assayed with salicylate .................................................................................................................................................. 114
DNA strand scission by illumination of dyes in presence and absence of light .......................................................................................... 119
Single-stranded scission of DNA by azure c ........................................................................................................................................ 121
Nicking of DNA by thionin in vitro ......................................................................................................................................................... 122
Nicking of DNA by toluidine blue o in vitro ........................................................................................................................................... 123
Nicking of DNA by methylene blue in vitro ........................................................................................................................................ 124
Nicking of DNA by acridine yellow in vitro ........................................................................................................................................ 126
Nicking of DNA by acridine orange in vitro ........................................................................................................................................ 127
Nicking of DNA by proflavin in vitro ...................................................................................................................................................... 128
Nicking of DNA by rose bengal in vitro .............................................................................................................................................. 129
Depletion of reduced glutathione in *E. coli* after exposure to dye, corrected for survival.......................... 166

Survival of azure c treatment by strains bearing plasmids encoding different repair enzymes............................. 168

Effect of azure c on survival on preinduced strains with and without the catalase plasmid.............................. 171

*In vivo* DNA damage by acridine orange........................................ 173

*In vivo* DNA damage by azure c is ameliorated by the OH- scavenger thiourea............................................... 174
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Bimolecular Rate Constants for Reactions Between Scavengers and the Hydroxyl Radical and Singlet Oxygen</td>
<td>12</td>
</tr>
<tr>
<td>II.</td>
<td>Ratio of moles of phosphate residues of DNA to moles of dyes at which dyes were fully intercalated</td>
<td>68</td>
</tr>
<tr>
<td>III.</td>
<td>Scavenger effects on measurement of hydroxyl radical production</td>
<td>99</td>
</tr>
<tr>
<td>IV.</td>
<td>Plasmids used in kill experiments</td>
<td>161</td>
</tr>
<tr>
<td>V.</td>
<td>Levels of superoxide dismutase, catalase, and peroxidase in cells used in kill experiment</td>
<td>169</td>
</tr>
<tr>
<td>VI.</td>
<td>Levels of superoxide dismutase, catalase, and peroxidase in cells used in preinduction/catalase plasmid kill experiment</td>
<td>172</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
<td></td>
</tr>
<tr>
<td>AY</td>
<td>acridine yellow</td>
<td></td>
</tr>
<tr>
<td>BPS</td>
<td>bathophenanthroline sulphonate</td>
<td></td>
</tr>
<tr>
<td>CCC</td>
<td>covalently closed circular</td>
<td></td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>copper- and zinc-containing superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5'-dimethylpyrroline-N-oxide</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
<td></td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
<td></td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>Endo</td>
<td>endonuclease</td>
<td></td>
</tr>
<tr>
<td>Exo</td>
<td>exonuclease</td>
<td></td>
</tr>
<tr>
<td>FeSOD</td>
<td>iron-containing superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>fluorescein</td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>hydroperoxidase</td>
<td></td>
</tr>
<tr>
<td>HpD</td>
<td>hematoporphyrin derivative</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td>minimal salts medium M9</td>
<td></td>
</tr>
<tr>
<td>MB</td>
<td>methylene blue</td>
<td></td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese-containing superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide-adenine dinucleotide</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide-adenine dinucleotide phosphate</td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>neutral red</td>
<td></td>
</tr>
</tbody>
</table>
Abbreviations, continued:

NTA  nitrilotriacetic acid
O$_2^-$  superoxide
OH-  hydroxyl free radical
OC  open circle
PR  proflavin
RB  rose bengal
SOD  superoxide dismutase
TB  toluidine blue o
TBA  thiobarbituric acid
TH  thionin
TSY  tripticase-soy yeast medium
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Oxygen toxicity

Oxygen has been known since the eighteenth century to be poisonous.¹ All organisms that use oxygen and are therefore exposed to it and to its various reduction products must protect themselves against oxygen toxicity. It is hardly necessary to point out that oxygen is toxic to anaerobic microorganisms, though its effect on these varies from immediate cell death to bacteriostasis. It is less obvious, however, that oxygen is poisonous to aerobic organisms, as well. High concentrations of oxygen, above the normal atmospheric level of 21%, slow plant growth², kill cultured animal cells³, and increase the rate of occurrence of blindness in premature infants⁴. Conversely, lowering ambient oxygen levels below 21% speeds the growth rate of both plants and cultured animal cells. Hyperbaric oxygen causes chromosome breakage in grain⁵ and mutations in bacteria⁶. Breathing pure oxygen is so toxic that there has been at least one human fatality resulting from medical treatment with hyperbaric oxygen⁷.
Figure 1. The partial reduction products of oxygen.⁸

Free radical theory of oxygen toxicity

The causes of the poisonous properties of oxygen were obscure before the publication of Gershman’s free radical theory of oxygen toxicity, which states that the toxicity of oxygen is due to partially reduced forms of oxygen⁹. The unusually high concentrations of oxygen found in tissues during exposure to pure oxygen encourages the formation of these reduced oxygen species. The toxic species are formed by one-electron reductions of oxygen by compounds and enzymatic reactions occurring in vivo⁸ (fig. 1). These reduction products include superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($OH^-$). These are commonly referred to as active oxygen species. Ordinary dioxygen is a biradical, since its two free electrons exist in two different orbitals and have parallel spins⁹. Dioxygen’s reactivity, however, is greatly lowered by the spin restriction: both of these electrons are of the same spin, so that if oxygen is to oxidize another molecule by accepting a pair of electrons from it, that pair must be of antiparallel spin, which cannot occur if both electrons occupy the same orbital. The spin restriction is removed when dioxygen is excited to the state known as singlet oxygen. In the cell, the spin restriction is removed
enzymatically by complexing the dioxygen with transition metals, which results in a delocalization of the electrons. However, the most common biological reductions of oxygen occur by the transfer of single electrons.\textsuperscript{10} All of these forms of oxygen are reactive as oxidants and reductants to a greater or lesser extent with a wide variety of compounds, both \textit{in vitro} and \textit{in vivo}.

\textbf{Superoxide}

\textbf{Sources of superoxide}

Superoxide is one of the species that has been implicated in the toxicity of oxygen. Generation of $\text{O}_2^-$ \textit{in vitro} has been observed in a number of different experimental systems. These systems include the enzyme xanthine oxidase acting upon the substrates xanthine or hypoxanthine\textsuperscript{11,12}, and the reduction of flavin mononucleotide and flavodoxin by ferredoxin-TPN\textsuperscript{+} oxidoreductase\textsuperscript{13}. Aqueous $\text{O}_2^-$ may also be generated \textit{in vitro} by dissolving tetramethyl-ammonium superoxide or potassium superoxide in water. Subcellular components such as cell nuclei\textsuperscript{14,15}, chloroplasts\textsuperscript{16,17}, mitochondria\textsuperscript{18}, human monocytes and neutrophils\textsuperscript{19}, macrophages\textsuperscript{20}, and microsomes\textsuperscript{21} have been demonstrated to produce $\text{O}_2^-$ \textit{in vitro}. The generation of $\text{O}_2^-$ by phagocytic cells has a microbicidal action essential to normal phagocytosis\textsuperscript{22}. The autoxidation of many biological compounds results in the production of $\text{O}_2^-$; among these compounds are oxyhemoglobin, which becomes methemoglobin\textsuperscript{23}, oxymyoglobin, which becomes metmyoglobin\textsuperscript{24}, clostridial and spinach ferredoxins\textsuperscript{25}, ascorbate, catecholamines, phenols, and reduced flavins\textsuperscript{26}. Other circumstances in which $\text{O}_2^-$ is generated include the photolysis of water\textsuperscript{27}, and the excitation of carboxyquinone, mitomycin c, and streptonigrin by visible light in the presence of oxygen\textsuperscript{28}. Superoxide is also produced by redox-cycling compounds \textit{in vivo}. As an
example, bipyridylum herbicides, such as paraquat, are reduced by an
NADPH-dependent cytoplasmic diaphorase in *E. coli*, and the monocation radi-
cals so produced reduce dioxygen to O$_2^-$.

**Reactions of superoxide**

Superoxide has been shown to be reactive to a number of compounds *in vitro*, as has its conjugate acid, the perhydroxyl radical, HO$_2$ *.* While less than 1% of O$_2^-$ will be present as HO$_2$ at the physiological pH of 7.4, the concentration increases dramatically as pH decreases. Since the pH is lower in the microenvironment adjacent to a membrane surface or in lysosomes, both the protonated and the unprotonated forms of O$_2^-$ should be considered. Among the reactions of O$_2^-$ observed *in vitro* are reactions with small molecules: ascorbate reacts with a rate constant of 1.5 $\times$ 10$^5$ M$^{-1}$s$^{-1}$ at pH 9.0$^{30}$. $\alpha$-tocopherol is oxidized by O$_2^-$ to 8-hydroxy-$\alpha$-tocopherone, which spontaneously forms $\alpha$-toco-
pherol quinone$^{31}$. NADH is oxidized by HO$_2$ and O$_2^-$, when bound to lactate
derhydrogenase, with rate constants at 23$^\circ$C of 1.2 $\times$ 10$^6$ M$^{-1}$s$^{-1}$ and 3.6 $\times$
10$^6$ M$^{-1}$s$^{-1}$, respectively$^{32}$. Superoxide also oxidizes and reduces many transition metal ions and their complexes$^{33}$. For example, it oxidizes Mn$^{2+}$ with a rate
constant of 6 $\times$ 10$^6$ M$^{-1}$s$^{-1}$ $^{34}$, and it reduces Fe$^{3+}$ complexed to EDTA with a
rate constant of 1.3 $\times$ 10$^6$ M$^{-1}$s$^{-1}$; it reduces Fe$^{3+}$ complexed to DTPA very
slowly, if at all$^{35}$. The reduction of cytochrome c by xanthine and xanthine ox-
dase is mediated by O$_2^-$ $^{36}$, and this reduction is used as the basis of assays for
both O$_2^-$ and superoxide dismutase (SOD). The rate constant for the reaction of
O$_2^-$ with ferricytochrome c is 2.6 $\times$ 10$^5$ M$^{-1}$s$^{-1}$, at pH 9.0$^{30}$. Other assays for
O$_2^-$ exploit the fact that it reacts with nitroblue tetrazolium, at a rate constant
of 5.9 $\times$ 10$^4$ M$^{-1}$s$^{-1}$. $^{30}$, $^{37}$ The perhydroxyl radical has been shown to react *in*
vitro with fatty acids; the rate constant for the reaction with linoleic acid is 1.2 x 10^3 M^{-1}s^{-1}; with linolenic acid, the rate constant is 1.7 x 10^3 M^{-1}s^{-1}; and with arachidonic acid, it is 3.0 x 10^3 M^{-1}s^{-1}. Sulfur compounds such as dithiothreitol, 2-mercaptoethanol, reduced glutathione, and ethyl mercaptan are oxidized in air; superoxide has been shown to play a part in these reactions and in the oxidation of phenols and catecholamines. Superoxide also reacts with certain proteins in vitro, inactivating them; among these are the enzyme catalase, and the selenium-containing glutathione peroxidase. Sinovial fluid, the breakdown of which is part of the pathology of arthritis, has been shown in vitro to be degraded by a O_2^{-}-generating system. In addition, O_2^{-} may react with the lipids of intact cell membrane. The O_2^{-}-generating reaction of xanthine oxidase oxidizing acetaldehyde damages resealed erythrocyte ghosts containing lipid hydroperoxides, while the enzyme superoxide dismutase protects against this damage. DNA strand scission is yet another form of damage caused by O_2^{-} generation in vitro.

In vivo effects of superoxide

Further, O_2^{-} has been demonstrated to cause some deleterious effects in vivo. Superoxide generated extracellularly by a xanthine oxidase/purine system has been shown to kill the bacterium *Staphylococcus epidermidis*. Potassium superoxide, dissolved in water to produce 2 nmoles/ml O_2^{-}, caused 50% cell mortality in cultured Chinese hamster ovary cells, and caused an increase of mutants in a dose-related fashion. O_2^{-}-generating systems have also been shown to cause mutations and cancerous tumors. C3H mouse fibroblast 10T^1/2 cells, exposed either to human neutrophils which had been stimulated to synthesize reactive oxygen intermediates, or to hypoxanthine and...
xanthine oxidase, then placed in tissue culture, were malignantly transformed in vitro.\textsuperscript{54} When injected into nude mice, the cells resulted in the production of malignant and benign tumors \textit{in vivo}.\textsuperscript{55}

\textbf{Arguments against the toxicity of superoxide}

Despite the aforementioned data concerning the reactivity of superoxide, certain investigators contend that $O_2^-$ is not toxic.\textsuperscript{56,57,58} Their conclusions are based largely on the limited lifespan of $O_2^-$ in aqueous media\textsuperscript{59}, and its unreactivity in aprotic solvents due to its relative insolubility in them\textsuperscript{33}. Furthermore, the reactivities of a number of specific compounds with $O_2^-$ in aqueous solution are low. The buffer Tris, the chelator EDTA, and certain metabolites such as $\alpha$-ketoglutarate, pyruvate, and succinate are all fairly unreactive, with second-order rate constants ranging from .001 to 0.3 M$^{-1}$s$^{-1}$.\textsuperscript{30} Superoxide is not very reactive toward amino acids, and neither is its protonated form, HO$_2$. For HO$_2$, the rate constants with amino acids range from 10 M$^{-1}$s$^{-1}$ for the aliphatic amino acids to about 60 M$^{-1}$s$^{-1}$ for the aromatic amino acids. For $O_2^-$, the range is from about 0.1 to 20 M$^{-1}$s$^{-1}$.\textsuperscript{60} Thus, it is evident that $O_2^-$ is not a universally reactive species.

\textbf{Hydrogen peroxide}

While it is possible that $O_2^-$ is not itself reactive enough to explain all of the biological damage resulting from systems that produce $O_2^-$, it is certainly evident that the damage does occur. Some reaction rates of $O_2^-$ given above are high enough to indicate that the species is potentially damaging in itself; additional damage may be caused by secondary species formed from $O_2^-$. One such potentially damaging species is H$_2$O$_2$. Hydrogen peroxide will be present
whenever $\text{O}_2^-$ is formed, because the dismutation of $\text{O}_2^-$ generates molecular oxygen plus $\text{H}_2\text{O}_2$, reaction (1):

$$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$  (1)

The spontaneous rate for this reaction at physiological pH is $5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, and the enzyme superoxide dismutase, which catalyzes this reaction, increases it by a factor of $10^4$. Thus, much of the damage that has been attributed to $\text{O}_2^-$ may actually have been caused by $\text{H}_2\text{O}_2$ that was formed from the $\text{O}_2^-$ that was initially generated.

**Biological sources of hydrogen peroxide**

Biological sources of $\text{H}_2\text{O}_2$ include lung mitochondria, whose release of $\text{H}_2\text{O}_2$ is increased ten-fold as the ambient oxygen concentration rises from 21% to 100% at one atmosphere pressure. Activation of phagocytic cells also results in $\text{H}_2\text{O}_2$ production through the dismutation of $\text{O}_2^-$ generated by a membrane-bound NADPH oxidase. Hydrogen peroxide is also formed during the one and two electron oxidations of numerous phenols, thiols, and catecholamines. Insulin stimulates intracellular $\text{H}_2\text{O}_2$ production in rat epididymal fat cells; $\text{H}_2\text{O}_2$ has been proposed to act as a second messenger for this hormone.

**Damage produced by hydrogen peroxide**

A number of forms of biological damage have been shown to be caused by $\text{H}_2\text{O}_2$. One of several sites at which damage has been clearly shown to be due to $\text{H}_2\text{O}_2$, and not to $\text{O}_2^-$ or $\text{OH}^-$, is the lens of the eye in organ culture. The autoxidation of oxyhemoglobin to metmyoglobin, mentioned above as a source of $\text{O}_2^-$, has been shown to be inhibited by catalase, the enzyme which breaks
down H$_2$O$_2$, suggesting that peroxide may initiate this oxidation. DNA is damaged by H$_2$O$_2$ in the presence of metals$^{66, 67, 68, 69}$; low concentrations of H$_2$O$_2$ induces resistance of procaryotic cells to degradation of DNA by a future challenge with H$_2$O$_2$. It also causes cell death in fibroblasts$^{22}$ and bacteria$^{70}$, mutagenicity in bacteria$^{70}$ and it has been shown to cause tumors in Drosophila embryos$^{71}$. Hydrogen peroxide also slowly but irreversibly inactivates the enzyme superoxide dismutase$^{72}$.  

**Metal catalyzed generation of the hydroxyl radical**

Hydrogen peroxide is not the only reactive oxygen species that may be formed from O$_2^-$. Haber and Weiss mentioned in a 1934 paper$^{73}$ the earlier proposal by Haber and Willstätter that O$_2^-$ could react with hydrogen peroxide to form OH$^-$:

$$O_2^- + H_2O_2 \rightarrow H_2O + OH^- + OH^-$$ (2)

This reaction has become known as the Haber-Weiss reaction. Hydroxyl radical, too, could be the source of part of the damage that has been attributed to O$_2^-$. However, the Haber-Weiss reaction as written is exceedingly slow, with a reaction rate of 3.0 M$^{-1}$s$^{-1}$ at pH 7.3. Chelated iron, on the other hand, which is frequently present in biological fluids, catalyzes this reaction, increasing the reaction rate to a significant degree$^{8, 75}$. Iron is chelated in vivo with phosphate esters such as ADP, ATP, GTP, and pyrophosphate$^{76}$, and other compounds such as citrate and oxalate$^{77}$. In vitro chelators such as EDTA and nitrilotriacetic acid (NTA) also allow iron to catalyze the reaction. In contrast, chelators that prevent iron catalysis of reaction (2) are diethylenetriaminepentaacetic acid (DTPA)$^{78}$ bathophenanthroline sulphonate (BPS)$^{79}$ phenanthroline,$^{79}$ and
bipyridine. The reaction for the iron catalyzed Haber-Weiss reaction is as shown in equations (3) and (4).

\[
\text{Fe}^{3+} + \text{O}_2^{-} \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad (3)
\]

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{-} + \text{OH}^{-} \quad (4)
\]

The rate constant for equation (3) when the iron is chelated with EDTA has been determined to be \( k_2 = 1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) at pH 7.0.\(^{35}\) The rate constant for equation (4), which is known as the Fenton reaction, for EDTA-chelated iron is given as \( k_3 = 10^4 \text{ M}^{-1}\text{s}^{-1} \) at pH 7.0.\(^{81}\) Thus, the catalytic properties of iron make the Haber-Weiss reaction quite possible \textit{in vivo} and the hydroxyl free radical (OH\(^-\)) is probably formed \textit{in vitro} and \textit{in vivo} mainly through the iron-catalyzed Haber-Weiss reaction (reactions 3 and 4).

\textbf{Evidence for the Haber-Weiss reaction}

Evidence for the occurrence of the iron-catalyzed Haber-Weiss reaction \textit{in vitro} includes the observation that more OH\(^-\) is detected from a xanthine/xanthine oxidase system when more iron or iron-EDTA is present, but that the iron-chelator DTPA, in suppressing the formation of the OH\(^-\) signal, maintains the presence of the O\(_2\)^\(-\) signal. This was shown by using electron spin resonance to detect products of the reaction of OH\(^-\) with compounds known as spin traps, such as 5,5'-dimethylpyrroline-N-oxide (DMPO).\(^{82}\) The hydroxylation of salicylate was used as an assay for OH\(^-\) formed by the xanthine/xanthine oxidase system, relying on the ability of OH\(^-\) to hydroxylate salicylate and form o-diphenols. Hyaluronic acid's depolymerization was found to be inhibited by inhibitors of the production or continued presence of OH\(^-\), namely the iron chelators BPS and DTPA, the enzymes catalase and SOD, and the OH\(^-\) scavengers mannitol and formate.\(^{79}\) The \textit{in vivo} formation of single strand breaks in
DNA by $\text{H}_2\text{O}_2$ was concluded to be mediated by the Haber-Weiss reaction when phenanthroline and bipyridine, strong chelators of iron, were found to protect the DNA from breakage by $\text{H}_2\text{O}_2$ in human fibroblasts.\textsuperscript{80} DNA binds iron (II) in a manner such that OH$^-$ formation from $\text{H}_2\text{O}_2$ is enhanced as compared to the amount formed without DNA.\textsuperscript{83} DNA strand scission in a system containing xanthine and xanthine oxidase increased as a function of iron concentration. Strand scission was stimulated by EDTA and inhibited by DTPA, the OH$^-$ scavengers mannitol and formate and the enzymes SOD and catalase.\textsuperscript{49} The iron carrying enzymes lactoferrin and transferrin have been found to catalyze the Haber-Weiss reaction when fully loaded with iron,\textsuperscript{84} but lactoferrin that is not fully loaded with iron was found to inhibit the reaction by inhibiting iron catalysis.\textsuperscript{85} Reduction of paraquat has been shown by gas chromatography to result in the production of OH$^-$\textsuperscript{86}.

Other chemical sources of hydroxyl free radical

Non-Haber-Weiss production of OH$^-$ is also possible. The antitumor drug talsomycin produces OH$^-$ without the production of intermediate $\text{O}_2^-$ or $\text{H}_2\text{O}_2$.\textsuperscript{87} The cardiotoxic antitumor drug adriamycin semiquinone reacts with $\text{H}_2\text{O}_2$, requiring neither metal catalyst nor $\text{O}_2^-$ for the production of OH$^-$.\textsuperscript{88} The Fenton reaction produces OH$^-$ whenever Fe$^{++}$ and $\text{H}_2\text{O}_2$ are present together.

Biological sources of hydroxyl free radical

Biological sources of OH$^-$ include liver microsomes,\textsuperscript{89} human neutrophils,\textsuperscript{90} and human monocytes during the phagocytosis of opsonized zymosan.\textsuperscript{90} Interestingly, monocytes from a patient with chronic granulomatosis disease, a hereditary disorder in which the patient suffers persistent and multi-
ple infections, failed during phagocytosis to generate the ethylene gas from methional used as an assay for OH- in this experiment.\textsuperscript{91}

**Reactivity of hydroxyl free radical**

The hydroxyl radical is extremely reactive with a wide variety of compounds. Second-order reaction rates are commonly on the order of $10^8$ to $10^9$ M$^{-1}$s$^{-1}$\textsuperscript{92}; the reaction rate of OH- with the enzyme catalase is $2.6 \times 10^{11}$ M$^{-1}$s$^{-1}$. As would be expected from its extreme reactivity, the OH- is capable of causing extensive and indiscriminate biological damage. It has been found to degrade DNA,\textsuperscript{67} form crosslinks between DNA molecules and between DNA and proteins,\textsuperscript{93} oxidize thymidine,\textsuperscript{94} oxidize and hydroxylate amino acids,\textsuperscript{92} kill bacterial cells,\textsuperscript{95} and mediate the induction of diabetes in mice by alloxan.\textsuperscript{96,97} Hydroxyl radical also mediates the degradation of the wood polymer lignin by the fungus *Phanerochaete chrysosporium*.\textsuperscript{98} Toxic oxygen species such as OH- may be involved in the normal senescence of leaf tissue.\textsuperscript{99}

**Hydroxyl radical scavengers**

Scavengers of OH- are compounds that react with OH- as rapidly as or even more rapidly than other compounds, and which thus, when added to a model system or a biological system in high enough concentrations, prevent other OH- mediated reactions from occurring. To be suitable for this purpose, compounds must not be especially reactive with substances other than the OH-; furthermore, the products of their reaction with OH- must not be particularly damaging in themselves. Substances commonly used as OH- scavengers include ethanol, mannitol, formate, benzoate, thiourea, and dimethylsulfoxide.
<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Hydroxyl Radical</th>
<th>Singlet Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Azide</td>
<td>7.5 x 10^9</td>
<td>5 x 10^8</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>7.0 x 10^9</td>
<td>~ 10^3</td>
</tr>
<tr>
<td>Sodium Benzoate</td>
<td>6.0 x 10^9</td>
<td>3 x 10^3</td>
</tr>
<tr>
<td>Thiourea</td>
<td>5.0 x 10^9</td>
<td>8 x 10^5</td>
</tr>
<tr>
<td>Sodium Formate</td>
<td>2.8 x 10^9</td>
<td>~10^3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.5 x 10^9</td>
<td>~10^3</td>
</tr>
<tr>
<td>Urea</td>
<td>~10^6</td>
<td>~10^3</td>
</tr>
</tbody>
</table>

(DMSO). The latter two scavengers are particularly useful for *in vivo* experiments, as they are capable of crossing cell membranes. Table I gives a more complete list of OH· scavengers, along with the rate constants of their reaction with OH·. Urea, a poor OH· scavenger, is frequently added as a control; the rate of its reaction with OH· is less than 7.0 X 10^5 M^-1s^-1. Some OH· scavengers, such as azide, also scavenge ^1^O_2_, but DMSO and ethanol only react with OH·.

**Oxygen toxicity summary**

In conclusion, the poisonous properties of oxygen to all organisms is due to the reactivity of oxygen species with many cellular components. Oxygen in
the molecular state is not very reactive. However, partially reduced forms of oxygen, known as active oxygen species, are considerably more reactive. The toxicity of oxygen is mediated by these species, which comprise $O_2^-$, $H_2O_2$, and $OH^-$. The active oxygen species are generated as byproducts of cellular metabolism, as well as by the activity of toxic agents. The reduction of molecular oxygen to form the more active species is effected by escaped electrons from the electron transport chain in respiring cells, by the action of enzymes such as the microbicidal oxidases of phagocytic cells, by the autoxidation of compounds such as hemoglobin, catecholamines, and reduced flavins, and by the redox cycling properties of certain herbicides and antibiotics.
Defenses against Oxygen toxicity

In vivo methods of studying the photodynamic effect can make great use of manipulations of the cell’s natural defenses. Discovering the necessary natural defenses also yields information on what forms of damage and damaging agents contribute to lethality.

Cells maintain a variety of defenses against oxygen toxicity. Among these are an array of enzymes that have evolved to deal with oxidative stress, including superoxide dismutase and catalase (described below). In animals, additional defensive enzymes include methionine sulfoxide reductase, which repairs methionine residues in proteins that have been damaged by OH-, glutathione peroxidase, and the glutathione reductase which regenerates the cofactor for the glutathione peroxidase. Still other defensive enzymes are endonucleases, exonucleases, and DNA polymerase, which repair single-stranded breaks and modified bases in DNA caused by oxidative and other stresses. In eucaryotes, uric acid, α-tocopherol, ascorbic acid, and β-carotene are among the compounds that function in vivo to scavenge active oxygen species and other organic radicals formed in the cell by reactions with the oxygen radicals; both procaryotes and eucaryotes contain high levels of glutathione, a scavenger of OH- and $^{1}O_{2}$.

Superoxide dismutases

Superoxide dismutase (SOD) catalyzes the reaction between two molecules of superoxide to form hydrogen peroxide and molecular oxygen (reaction 1, repeated below):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$  (1)
Superoxide dismutases are ubiquitous in aerobic organisms.\textsuperscript{102} Eucaryotes contain, typically, both a copper and zinc-containing superoxide dismutase (CuZnSOD) and another SOD that contains manganese (MnSOD); the two are unrelated, and the latter is compartmentalized in the mitochondria, while the former is in the cytoplasm.\textsuperscript{103} Bovine copper-zinc SOD has a molecular weight of 32,500\textsuperscript{104} and contains two atoms of copper and two atoms of zinc per molecule.\textsuperscript{105} It is composed of two identical subunits joined by at least one disulfide bond.\textsuperscript{105} The human CuZnSOD is similar to the bovine CuZnSOD.\textsuperscript{106} The mechanism of action of CuZnSOD involves alternate reduction and reoxidation of the Cu\textsuperscript{2+} at the active site during successive interactions with O\textsubscript{2}\textsuperscript{-}.\textsuperscript{102} The mitochondrial SOD, MnSOD, contains four subunits, each with a molecular weight of 20,000; it is located in the matrix of the chicken liver mitochondrion.\textsuperscript{102}

Prokaryotes such as \textit{E. coli} contain both an MnSOD and an iron-containing superoxide dismutase (FeSOD). The procaryotic MnSOD shows homology to the eucaryotic enzyme but contains two subunits rather than four and is consequently half as large, with a molecular weight of 40,000. The FeSOD is partially homologous to the MnSOD.\textsuperscript{107} It has two subunits, one Fe\textsuperscript{3+} ion per molecule of enzyme, and a molecular weight of 39,000. The mechanism of the Mn and FeSODs is probably similar to that of the eucaryotic CuZnSOD.\textsuperscript{102} Superoxide dismutases increase the spontaneous rate of O\textsubscript{2}\textsuperscript{-} removal according to equation (1) from about 5 x 10\textsuperscript{5} M\textsuperscript{-1}s\textsuperscript{-1} at physiological pH\textsuperscript{44} to about 1.8 x 10\textsuperscript{9} M\textsuperscript{-1}s\textsuperscript{-1} for the MnSOD at pH 7.8 and about 1.6 x 10\textsuperscript{9} M\textsuperscript{-1}s\textsuperscript{-1} for the CuZnSOD between pH 5.3 and 9.5.\textsuperscript{44}
Regulation of SOD levels

Of the two *E. coli* superoxide dismutases, FeSOD is constitutively expressed, while MnSOD is inducible. Studies on the activity of *E. coli* SODs indicate that levels of the FeSOD can be increased somewhat with iron supplementation, and decreased by iron chelators.\textsuperscript{108} Levels of the MnSOD are increased more dramatically by chelating agents, Mn(II), paraquat\textsuperscript{108} or oxygenation, and decreased by anaerobic conditions. It has been proposed that oxygenation and intracellular O$_2^-$ production induce MnSOD because O$_2^-$ oxidizes Mn(II) to Mn(III). Mn(II) has a higher affinity for the manganese-free apoMnSOD, and successfully competes with Fe(II) that would otherwise combine with the apoMnSOD to produce an inactive enzyme. This results in increasing MnSOD levels.\textsuperscript{108} Maximum levels of total SOD in *E. coli* are induced by growth in minimal media that has been supplemented by a combination of 100μM Fe(II), 100 μM Mn(II), 10 μM paraquat, and 100 μM 8-hydroxyquinoline; under these conditions, as much as ten percent of the total cellular protein is superoxide dismutase.

Compounds that are capable of entering cells and inducing superoxide dismutase include paraquat (methyl viologen), pyocyanine, phenazine methosulfate, streptonigrin, juglone, menadione, methylene blue, and azure c, all known redox-cycling compounds.\textsuperscript{109}

Low levels of the MnSOD are characteristically seen when cells have been grown in a glucose minimal medium. High levels of SOD are produced by growth in TSY, or by growth in minimal medium to which 100 μM iron(II), 100 μM manganese(II), 100 μM 8-hydroxyquinoline, and 10 μM paraquat have been added; levels are enhanced thirty-fold by the latter treatment.\textsuperscript{108} Similar induc-
tion levels are observed by the addition of paraquat and manganese alone at similar levels.

**Catalase**

The catalases belong to the family of enzymes which contains the hydroperoxidases and peroxidases. Catalase catalyzes the reaction:

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2, \]  

(5)

whereas peroxidase catalyzes the reaction:

\[ \text{AH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{A} + 2 \text{H}_2\text{O} \]  

(6)

where A is an electron donor such as diansidine or guiacol.\textsuperscript{110}

There are two main enzymes with catalase activity in *E. coli*; one, hydroperoxidase II (HPII), possesses only catalatic activity. The other enzyme, which migrates more slowly than HPII during electrophoresis, hydroperoxidase I (HPI), is both a catalase and a broad spectrum peroxidase. When electrophoresed on a polyacrylamide gel, HPI can be visualized as two isozymes, HPI-A and HPI-B.\textsuperscript{111} Peroxidase (HPI) has a molecular weight of 337,000, is composed of four subunits of equal size, and contains two molecules of protoheme IX per tetramer.\textsuperscript{110} Catalase (HPII) has a molecular weight of 240,000 and consists of four polypeptides, each of which is associated with a ferric protoporphyrin IX.\textsuperscript{112}

---

**Factors that affect expression of hydroperoxidases**

Catalase is induced by peroxide and ascorbate.\textsuperscript{113} It is repressed by glucose in a classical example of catabolite repression.\textsuperscript{114} Catalase is important in protecting the cell against ultraviolet lethality; in fact, it was some time after the discovery of *nur*, a gene named for its conferring resistance to near-ultraviolet light, that it was found to code for a hydroperoxidase.\textsuperscript{115} Exogenous catalase
provides protection against lethality due to acridine orange as well as to phenazine methosulfate. Catalase is inducible by treatment of log-phase cells for thirty minutes with 500 μM H₂O₂, or with 5 mM ascorbate, which gives rise to H₂O₂; catalase levels are enhanced as much as six-fold by this treatment and twenty- to thirty-fold by treatment with phenazine methosulfate and pyocyanine.

Low levels of catalase are observed in cells that have been grown in glucose minimal medium; levels four- to five-fold higher are seen when cells have been exposed for thirty minutes to 0.5 mM hydrogen peroxide or 5 mM ascorbic acid. The increase in catalase level is due to the induction of hydroperoxidase I, the broad spectrum peroxidase of E. coli. Enhancement of intracellular catalase is also seen when cells are pretreated with paraquat and manganese.

The oxyR regulon

Christman et al. have discovered a regulatory element, oxyR, which appears to regulate a group of enzymes that defend against oxidative stress, particularly HPI. Constitutive mutants in oxyR overproduce nine of the proteins that are induced by oxidative stress, including HPI-A and HPI-B, MnSOD, and glutathione reductase.

Cloning of superoxide dismutase and catalase genes

The genes for three of the E. coli enzymes of defense against oxidative stress, HPI (katG), MnSOD (sodA), and FeSOD (sodB) have been cloned onto multicopy plasmids. Loewen et al. screened the Clarke and Carbon E. coli colony bank by plating out the colonies and looking for high catalase levels, as
evidenced by an increase in the colony’s bubbling in response to $\text{H}_2\text{O}_2$. One strain, JA200/pLC36-19, proved to have a basal catalase level about twice the normal basal level.\textsuperscript{116} Loewen and Triggs have subcloned this gene to form plasmids BT22 and BT28, which lead to an increase in catalase and peroxidase levels of twenty to thirty-fold, compared to the parent strains.\textsuperscript{119} Nettleton et al.\textsuperscript{120} identified two clones in the Clarke and Carbon bank that contained an extra copy of the iron SOD gene, using a synthetic oligonucleotide probe constructed from information on the amino acid sequence of the protein. They found that the probe binds exclusively to a fragment contained in common by clones JA200/pLC18-11 and JA200/pLC13-47.\textsuperscript{120} Sakamoto and Touati also cloned the FeSOD gene, using immunoprecipitation of crude extracts to screen a cosmid bank they had constructed\textsuperscript{121}; they found that their cosmid contained a 6.6 kb $\text{Pst I}$ fragment which carried $\text{sodB}$. Touati similarly cloned and mapped the MnSOD gene from the same cosmid bank and subcloned it into plasmid DT1-5; crude extracts of a strain containing this plasmid contain four to five times as much superoxide dismutase as the parent strain when grown in rich media to late log phase.\textsuperscript{122}

**DNA repair**

The defensive enzymes superoxide dismutase, hydroperoxidase, and catalase act to remove toxic oxygen species before they can do intracellular damage. In the instance that these defenses fail, other systems are ready to repair damage, particularly DNA damage, that is done by the toxic oxygen species.
Kinds of DNA damage

The existence of stress agents such as ultraviolet light, chemical mutagens, intracellular oxygen or organic radicals that damage DNA requires cells to maintain enzymes to repair damaged DNA. DNA damage may include not only single- and double-stranded breaks, but also the production of damaged bases, such as thymine dimers or guanine residues that have been depurinated or hydroxylated, resulting in alkali-labile phosphodiester bonds.

Kinds of DNA damage that happens under the influence of light include altered or missing bases, single-strand breaks, double-strand breaks, and cross-linking. At least three kinds of damage are commonly defined: far ultraviolet kill, which seems mainly to result from the formation of thymine dimers; chemically-assisted ultraviolet kill (with psoralens for example), which results in DNA cross-linking; and the kind of kill seen as a result of exposure to near ultraviolet light or visible light in the presence of dyes, photodynamic kill, which includes modified bases, apurinic/apyrimidinic sites, and single stranded breaks. The differences in kinds of damage produced by these different forms of light were suggested by their being protected against by different repair systems.

Apurinic/apyrimidinic (AP) sites are generated spontaneously and by treatment with acid or as a result of chemical alkylation of deoxyguanosine, which weakens the N-glycosidic bond that attaches the base to the sugar-phosphate backbone of the DNA. They are also generated by DNA glycosylases, which recognize and cleave N-glycosidic bonds of damaged nucleotide residues in DNA.

When the DNA backbone is broken by ionizing radiation or hydrogen peroxide, the ends contain 3' terminal phosphoglycolaldehydes or thymine
glycols,\textsuperscript{126} or 3' terminal phosphates,\textsuperscript{125} which cannot act as substrates for DNA polymerase I or DNA ligase. These blocked 3' ends must be removed before repair of the DNA can take place, starting with the 3'-OH group of the primer DNA.\textsuperscript{127}

\textbf{DNA repair enzymes}

As \textit{E. coli} is the best studied of all organisms with respect to DNA repair,\textsuperscript{128} this discussion will concentrate on the repair systems in this organism. There is some redundancy of the DNA repair systems, which allow mutants in any one of the enzymes to function reasonably well under normal physiological conditions; this indicates their importance to the survival of the organism. Some strains of \textit{E. coli} are more sensitive to photodynamic kill than others; \textit{E. coli} B/s is ten times more sensitive than \textit{E. coli} K12 to photodynamic and paraquat toxicity.\textsuperscript{129} It is possible that the responsible difference between the two strains involves differential DNA repair ability.

DNA repair systems in \textit{E. coli} include photoreactivation repair, excision repair, recombination repair, and SOS repair. Photoreactivation involves the cleavage of thymine dimers into a pair of normal thymines by photolyase, an enzyme activated by visible light.\textsuperscript{130}

Excision repair is a process in which two cuts are made in the sugar-phosphate backbone of the DNA on either side of a distortion caused either by a thymine dimer or by a base with which no nucleoside can form a base-pair, producing a 3'-OH group on the 5' side. This allows DNA polymerase I to synthesize a new strand, displacing the defective strand; DNA ligase joins the new strand to the original strand. The original incisions are made in \textit{E. coli} by endonuclease I. \textsuperscript{130}
Recombination repair, also called daughter-strand gap repair, involves by-passing a block to DNA polymerase III, allowing replication to continue, leaving a gap opposite the block (e.g., a thymine dimer). The unpaired gaps are filled by excising the homologous piece of undamaged sister strand and inserting it into the gap. DNA polymerase I and DNA ligase then join the inserted piece to adjacent regions and fill in the gap left in the donor segment.\textsuperscript{130}

In SOS repair, binding of the recombination A (RecA) gene product to single-stranded DNA in the region of a thymine dimer or other distortion allows the insertion of adenines or mismatched bases by DNA polymerase III. The RecA protein also induces a series of other proteins, called din for damage inducible, which are kept repressed by the LexA protein in the absence of single-stranded DNA.\textsuperscript{130} A model of the SOS regulatory system is shown in figure 2. A fusion of one of the genes for a din protein and the coding region for the β-galactosidase gene has been made, allowing the induction of the SOS response to be easily studied by assaying for β-galactosidase levels after submitting the cells to an appropriate inducing stress agent, such as mitomycin c.\textsuperscript{131} It was thought at one point that the protein \textit{rec A} protected against both far and near ultraviolet and photodynamic killing in \textit{E. coli};\textsuperscript{132} later studies showed that \textit{rec A} did not protect at all against photodynamic killing, while \textit{nur} showed great protection.\textsuperscript{115}
Figure 2. Model of the SOS regulatory system. The open circles represent proteolytically inactive RecA molecules and closed circles represent proteolytically active RecA molecules. The semicircles represent LexA molecules. (From G. Walker, Microbiol. Rev. 48: 60-93.)
Endonucleases

The phosphodiester bonds at apurinic/apyrimidinic sites are cleaved by AP endonucleases as the first step in excision repair. These AP endonucleases have been found to be ubiquitous in both procaryotes and eucaryotes.\textsuperscript{133} At least four AP endonucleases have been identified in \textit{E. coli}.\textsuperscript{123} These include exonuclease III, endonuclease IV, and endonuclease V; little is known about endo V at this time.\textsuperscript{134} AP sites are not the only kinds of lesions that require repair by these enzymes. Single-stranded breaks must have a free terminal 3'-OH group in order for DNA polymerase I to initiate repair synthesis, so any ends which contain a phosphoglycolaldehyde end, a terminal phosphate, or otherwise lack a 3'-OH group, must be cut out using an exonuclease, before repair synthesis can occur.\textsuperscript{130} Those enzymes which have this ability include endonuclease III, endonuclease IV, and exonuclease III.\textsuperscript{134}

Exonuclease III

Exonuclease III is the major AP endonuclease in \textit{E. coli}, with about 85% of \textit{E. coli}'s measurable AP endonuclease activity.\textsuperscript{133} Its functions include cutting at AP sites, acting as a 3' to 5' exonuclease of double stranded DNA, cutting at the 5' side of a urea residue in DNA, removing 3' phosphates and phosphoglycolate esters, and hydrolyzing the RNA from a hybrid duplex in the 3' to 5' direction.\textsuperscript{134} Exonuclease III mutants are hypersensitive to \textit{H}_2\textit{O}_2 and to near ultraviolet light, both of which produce AP sites.\textsuperscript{115} Both exo III and endo IV can efficiently remove 3' terminal phosphoglycolaldehyde from DNA.\textsuperscript{127}
**Endonuclease III**

Endonuclease III cleaves 3' to AP sites and ring-damaged pyrimidines, especially hydroxylated thymine residues (thymine glycols), generating 3'-OH and 5'-P termini. It has no cofactor, so it is active in the presence of EDTA. Endo III does not remove terminal PO or phosphoglycoaldehyde. The gene for endonuclease III, *nth*, has been cloned onto plasmid pRPC53.

**Endonuclease IV**

In *E. coli*, endonuclease IV (endo IV) is a nuclease for AP sites; it catalyzes the cleavage of a phosphodiester bond 5' to an AP site, and has no other known activity. It is resistant to EDTA. Both exo III and endo IV can efficiently remove 3' terminal phosphoglycoaldehyde from DNA. Endo IV is induced in *E. coli* by paraquat, which is also known to induce SOD. Endo IV was identified as an EDTA-resistant endonuclease activity. Less than 10% of measurable AP endonuclease activity is due to endo IV in *E. coli* grown aerobically in rich medium, in the absence of an inducing agent such as paraquat. Strains mutant in endo IV have an increased sensitivity to alkylating agents and oxidants such as peroxides and bleomycin. The gene for endo IV has been cloned into plasmids pLC38-27 and pWB21.

**Uses of Defense and Repair Enzymes**

Defense and repair enzymes are useful in the study of damage produced by active oxygen in two ways. First, the existence of the enzymes shows what the cell finds damaging; if a substance is not harmful, the enzyme is unlikely to exist solely to remove it. The discovery of SOD was what led to the proposal that
superoxide is harmful.\textsuperscript{136} Secondly, levels of the enzymes can be manipulated, either by induction under appropriate stress conditions, or by subcloning of their genes; if increased concentrations of an enzyme are protective, then the enzyme's substrate must somehow mediate damage, and this substrate is likely to be produced during a stress episode.

The gene for the \textit{E. coli} HPI has been placed onto the plasmids pBT22 and pBT28 by Triggs-Raine and Loewen.\textsuperscript{119} The gene for the \textit{E. coli} MnSOD was placed onto the plasmid pDT1-5 by Danielle Touati.\textsuperscript{122} Bernard Weiss's group placed the endonuclease IV (\textit{nfo}) gene on a plasmid, pWB21.\textsuperscript{135} All three of these plasmids were used in this study.
The Photodynamic Effect

Oxygen-dependent toxicity that also requires the presence of light and a sensitizing compound is known as the photodynamic effect.\textsuperscript{137} It was first described by Raab in 1896.\textsuperscript{137} It affects all aerobic eucaryotic and procaryotic organisms and has been the subject of thousands of studies. The mechanism of the photodynamic effect remains mysterious\textsuperscript{138}. Visible light is not absorbed by most cellular components, and therefore does not usually have the toxic effects associated with X-rays or ultraviolet light. However, a sensitizer that absorbs visible light—a colored substance, or dye—may serve to transfer the energy of the light absorbed, usually through oxidation or reduction reactions, to a species which will then cause damage to cellular components. The wavelengths of light and their activities, as well as the spectra of representative photosensitizing dyes, are shown in figure 3. Photosensitizers are molecules which can absorb light to produce a chemical reaction which would not occur in their absence.\textsuperscript{139}

Biological examples of photosensitization

There are many instances of reactions for which this effect is responsible. For example, skin diseases of phototoxicity in humans,\textsuperscript{140} livestock, and experimental animals; light-activated pesticides;\textsuperscript{141} and medical treatments including photochemotherapy\textsuperscript{142} and phototherapy of jaundice,\textsuperscript{143} herpes simplex\textsuperscript{144} and psoriasis.\textsuperscript{145} Studies have shown that photosensitizers can cause cell death,\textsuperscript{146} DNA damage,\textsuperscript{147,148,149} protein damage,\textsuperscript{150,151} membrane damage,\textsuperscript{148} mutagenesis,\textsuperscript{146} and both tumor destruction\textsuperscript{152} and possibly carcinogenesis.\textsuperscript{153} It is thought that photodynamic action is responsible for the
Figure 3. The phototoxic spectrum.

Kinds of Toxicity:
- Direct DNA Damage
- Sunburning
- Drug Phototoxicity

Solar Spectral Intensity:
- 3 mW/cm²
- 9 mW/cm²
- 52 mW/cm²

Absorption (arbitrary units)

Oxygen-dependent cell damage

Oxygen-independent molecular alteration
conditions that bring about the end of the toxic red tide, as photosensitizing compounds are released by the deaths of creatures killed by the red tide bloom.¹⁵⁴

**Examples of photosensitizers**

Among the chemicals known or suspected to induce skin phototoxicity are sweeteners, both cyclamates and saccharin,¹⁵⁵ cosmetics including perfumes¹⁵⁶ and lipsticks,¹⁵⁷ germicidal soaps,¹⁵⁷ industrial products including pitch, liquor picis detergents, and crude coal tar,¹⁴⁰ agricultural products such as celery,¹⁵⁸ and hypericin, which is found in a toxic agricultural pest eaten by livestock, St. John's wort.¹⁵⁹ Other inadvertent inducers of skin phototoxicity are drugs such as the phenothiazines, which are prescribed as antipsychotics;¹⁶⁰,¹⁶¹ tetracyclines;¹⁶²,¹⁶³,¹⁶⁴ sulfonamides, which were the first drugs recognized as having phototoxic side effects;¹⁶⁵,¹⁶⁶,¹⁶⁷ psoralens;¹⁶⁸,¹⁶⁹ chloroazepoxide, a tranquilizer that can cause liver damage due to photoproduts formed in the skin;¹⁷⁰ phenazines, drugs used for the treatment of psychotic disorders;¹⁶¹ nalidixic acid¹⁷¹, an antibiotic used in the treatment of urinary tract infections;¹⁴⁵ methotrexate, an anti-cancer drug;¹⁷² and benoxaprofen, an anti-inflammatory and analgesic drug.¹⁷³ Phototoxicity is also observed in diseases that cause the production or accumulation of photosensitizing metabolites, such as hyperbilirubinemia.¹⁷⁴ Despite all of these known phototoxic activities, the underlying mechanisms remain poorly understood.¹³⁸

**Phototherapy**

The photodynamic effect is not restricted to deleterious effects, but may be used therapeutically. Niels Finsen was awarded a Nobel prize in 1903 for
the treatment of lupus vulgaris, facial lesions seen in tubercular patients, with a
carbon arc lamp.\textsuperscript{175} Phototherapy has long been used in the treatment of psori-
asis, with the application of photosensitizers such as coal tar followed by ultra-
violet light exposure.\textsuperscript{145} Partially refined versions of coal tar are available on
prescription under a variety of proprietary names.\textsuperscript{145} Phototherapy with neutral
red\textsuperscript{144} and proflavin\textsuperscript{176,177} has been used in the treatment of herpes simplex,
although not without harmful side effects.\textsuperscript{153,178} Phototherapy has also been
used in the treatment of idiopathic vitiligo, seborrhea, eczema, and chronic
lichen simplex.\textsuperscript{145}

\textbf{Phototherapy of cancers}

A major recent impetus to the study of photodynamic effect has been the
potential use of dye photosensitizers in phototherapy of cancers.\textsuperscript{142} Leukemic
cells show greater sensitivity to phototoxicity than normal cells. Tumors in mice
treated with photochemotherapy have shown regression of greater than
90\%.\textsuperscript{179} Derivatives of hematoporphorin are at present undergoing extensive
clinical trials as sensitizers for photodynamic therapy of neoplasms\textsuperscript{180}. At the
present time one of the major problems with photochemotherapy is the current
poor understanding of the basic mechanisms involved.\textsuperscript{152}

\textbf{Photosensitizers in this study}

The classes of photosensitizers considered in this study include:
acridines; other synthetic dyes including thiazines, xanthenes, and phenazines;
porphyrins, and a naphthalamide (see figure 4, structures of dyes). The extinc-
tion coefficients, given here parenthetically where known, are from Houba-
Figure 4. Structure of dyes.
Herrin (1982).\textsuperscript{181} The acridines studied include acridine orange (wavelength maximum 492 nm, extinction coefficient 41,600 M\textsuperscript{-1}cm\textsuperscript{-1}), acridine yellow (wavelength maximum 442 nm, extinction coefficient 29,000 M\textsuperscript{-1}cm\textsuperscript{-1}), and proflavin (wavelength maximum, 444; extinction coefficient, 34,300 M\textsuperscript{-1}cm\textsuperscript{-1}). Xanthenes included were fluorescein (wavelength maximum 496 nm) and rose bengal (wavelength maximum 550 nm, extinction coefficient 99,800 M\textsuperscript{-1}cm\textsuperscript{-1}), which possesses one of the highest absorption coefficients known.\textsuperscript{182} The phenazine studied was neutral rod (wavelength maximum 540 nm). Thiazines included methylene blue (wavelength maximum 665 nm, extinction coefficient 78,000 M\textsuperscript{-1}cm\textsuperscript{-1}), azure c (wavelength maximum 616 nm), thionin (wavelength maximum 598 nm), and toluidine blue (wavelength maximum 626 nm).

Many, but not all, of these dyes are capable of penetrating into the pro-caryotic cell. Those dyes which cannot enter the cell will be able to damage only the cell membrane, which can itself cause cell death; however, in the absence of a reductant, dyes that cannot enter the cell are not lethal.\textsuperscript{183} The porin channels in the outer membrane of \textit{E. coli} freely allow the passage of small positively charged molecules, but keep out large, hydrophobic or negatively charged compounds\textsuperscript{184} in order to exclude the hydrophobic and anionic bile salts that are a threat to \textit{E. coli} in its natural habitat.\textsuperscript{185} In the drawing in figure 4, the thiazines and the phenazine have positive charges, while the other dyes are neutral. In experiments by Martin and Logsdon (1987), the dyes toluidine blue and acridine orange penetrated the cell, while fluorescein and lucifer yellow did not.\textsuperscript{183} Neutral red and methylene blue are accumulated in \textit{E. coli} at neutral pH, but rose bengal only at acidic pH.\textsuperscript{186} In general, the acridines, thiazines, and phenazine enter the cell, whereas the xanthenes and the napthalimide lucifer yellow do not.
Most of the dyes used in this study intercalate in between the bases of DNA. The acridines, thiazines, and phenazines all have planar, tricyclic structures, as seen in figure 3; however, the xanthenes are bulkier and cannot fit in between the bases of the DNA. In addition, the xanthines are negatively charged and are probably repelled by the sugar-phosphate backbone of the DNA. Xanthenes, unlike acridines, thiazines, and phenazines, do not intercalate into DNA. Since intercalating dyes will be largely intercalated into the DNA once they enter the cell, it is important to study dye reactions when the dyes are in the intercalated state, as well as alone.

Why these dyes are included in this study

These organic dyes have been chosen for this study because they are the most widely studied compounds in photochemistry and the physiology of photodynamic action. Although they vary widely in their structures they are all capable of oxidation reduction reactions, and, because of this fact, of giving rise to oxygen radicals.\textsuperscript{183} Most of them are known to generate singlet oxygen ($^1\text{O}_2$) as well.\textsuperscript{146} Extensive studies have been done illustrating the effects of these particular dyes in \textit{E. coli}, before the significance of $\text{O}_2^-$ was known. Many of these dyes are structurally related to known redox active compounds such as pyocyanine and phenazine methosulfate, which are known to give rise to $\text{O}_2^-$ by redox-cycling within \textit{E. coli}.\textsuperscript{187} Reasons why so many structural classes of dyes were considered in this study were in order to determine whether their individual toxicities could be explained by a common mechanism; to determine the relative importance of cell localization in determining their toxic effects; and to illustrate that all forms of light, from near ultraviolet through the red, are sufficiently energetic to promote phototoxic effects in the presence of an appropriate
photosensitizing agent and to do so through the generation of reduced oxygen species.¹⁸³

**Uses of photosensitizers**

The dyes used in this study have a variety of functions and origins. Some acridines occur in coal tar.¹⁷⁵ Acridine orange and acridine yellow are used as adjuncts to laser surgery of cells, and as stains for nucleic acids,¹⁸⁸,¹⁸⁹ while proflavin is used as a topical antiseptic.¹⁹⁰ Porphyrins occur as metabolic byproducts, and are used in photochemotherapeutic agents.¹⁸⁰ Fluorescein (D & C Yellow No. 7) is commonly used to test for corneal abrasion.¹⁹⁰ Rose bengal has become one of the most widely used of all photodynamic sensitizers.¹⁹¹ Rose bengal is used as a biological stain, as a dye for straw and wood chips, as an ingredient in inks, for coloring edible products, and in cosmetics.¹⁹² Related xanthenes make up a number of dyes approved by the FDA for use in drugs and foods.¹⁹² Neutral red is used as a pH indicator and as a biological stain in Golgi apparatus studies¹⁹⁰ and is related structurally to phenazine methosulfate and pyocyanine, potent, known redox cycling compounds.¹⁸⁷ Methylene blue is used as a stain in bacteriology, as an antimethemoglobinemic and cyanide antidote in humans, and as an antiseptic and disinfectant in veterinary use. Eye drops containing methylene blue are available over the counter in some countries.¹⁹³ Thionine is used in general nuclear staining, in the counting of bacteria in milk, and as an antioxidant for linseed oil.¹⁹⁰ Azure c and toluidine blue o are used as biological stains.
Organisms affected by the photodynamic effect

The toxicity of photosensitizers is nearly universal; among those organisms sensitive to inactivation by the photodynamic effect are viruses, bacteriophage, bacteria, protista, yeasts, insects, and cultured mammalian cells.

Damage to viruses and bacteriophage by the photodynamic effect

Viruses and bacteriophage are susceptible to inactivation by the photodynamic effect. Phage particles are inactivated by light plus methylene blue,\textsuperscript{194} toluidine blue,\textsuperscript{195} and proflavin.\textsuperscript{196} Other isolated viruses, including adenovirus, vaccinia virus, SV40 papovavirus, influenza A, parainfluenza-3, measles virus, reovirus 1, and herpesvirus are all inactivated by toluidine blue o. Neutral red inactivated measles virus, and herpesvirus, while proflavin inactivated reovirus 1, influenza A, measles virus, and herpesvirus.\textsuperscript{197}

Damage to bacteria by the photodynamic effect

Experiments involving the exposure of bacteria to photosensitizing dyes have shown them to be killed as a result. Rose bengal immobilized on polystyrene beads was able to kill \textit{E. coli} upon exposure to light.\textsuperscript{198} Toluidine blue killed \textit{E. coli} cells.\textsuperscript{199} Thiazines, xanthenes, phenazines, acridines, and naphthalimides that kill \textit{E. coli} include azure c and thionin;\textsuperscript{200} rose bengal\textsuperscript{200} and fluorescein;\textsuperscript{183} neutral red;\textsuperscript{200} proflavin,\textsuperscript{200} acridine yellow,\textsuperscript{183} and acridine orange;\textsuperscript{183} and lucifer yellow.\textsuperscript{183} Other bacteria suffer similarly; for example, methylene blue inactivated \textit{Acholeplasma laidlawii}\textsuperscript{201} and toluidine blue inactivated \textit{Micrococcus roseus}.\textsuperscript{202}
Damage to yeast cells by the photodynamic effect

Yeast have been used extensively to study photodynamic lethality. Acridine orange in combination with 410-600 nm light inactivates yeast. The inactivation curves are the same shape as for X-ray and FUV killing of yeast, suggesting that there is at least some overlap between the targets and sites of these three forms of radiation. Ascorbate, a biological reductant, caused an oxygen-dependent enhanced lethality in yeast cells of the thiazines toluidine blue, methylene blue, and thionin, but not the xanthenes rose bengal and eosin yellow, though it had no lethality in absence of dye.\textsuperscript{203} Proflavin led to inactivation of yeast.\textsuperscript{146} Rose bengal, thionin, methylene blue, toluidine blue, acridine orange, proflavin, and acriflavin all inactivated yeast cells.\textsuperscript{146}

Damage to single-celled eucaryotes by the photodynamic effect

Other eucaryotic cells have been studied for sensitivity to photodynamic effects, as well. Paramecia are killed by acridine in the light, but not in the dark; Raab's discovery of this effect led to the first published example of the photodynamic effect, in 1900.\textsuperscript{137} Hematoporphorin derivative photosensitizes DNA strand breakage in Chinese hamster ovary cells.\textsuperscript{147} The crustacean \textit{Artemia salina} was immobilized by the photodynamic effect of polycyclic aromatic hydrocarbons.\textsuperscript{204} The activity of nerve cells is altered by acridine orange plus light,\textsuperscript{205} as well as by fluorescein\textsuperscript{206} and lucifer yellow\textsuperscript{207} while erythrocytes are lysed when exposed to dyes such as rose bengal, methylene blue, and neutral red plus light of the appropriate wavelength.\textsuperscript{137}
Other systems susceptible to the photodynamic effect

Other systems in which damage due to photosensitization occurs include multicellular organisms, both plants and animals. For example, strong illumination with blue and ultraviolet light photolyses rhodopsin, leading to the ultrastructural degeneration of the photoreceptors in the Drosophila eye.208,209 Rose bengal caused breakdown of chlorophyll and carotenoids and a decrease in CO2 uptake in leaves.210 Chlorophyll is among the most effective of photosensitizers for the oxygenation of organic substances.211

Human photoreactions

Some of the dyes used in this studied have been observed for their effects on human skin. Neutral red has been reported to cause acute contact dermatitis in humans.177 Rose bengal, when injected into the skin before light exposure, causes erythema and edema.166 Fluorescein and eosin, applied to the skin and then rubbed off before sun exposure, resulted in whealing.137 Not much is known about the mechanisms of action of photodermatoses.212

Cellular targets of photodynamic damage

The targets of photodynamic damage in the cell have been examined, both in vivo and in vitro, using isolated components. Subcellular components including mitochondria and cell membranes are degraded by photosensitizers plus light. Mitochondria that are exposed to light plus hematoporphyrin or hematoporphyrin derivative (HpD) successively lose coupling, calcium ion transport and respiration.213 Isolated chloroplasts lose their ability to carry out the Hill reaction (photochemical water-splitting process) following photodynamic treatment.214 Acridine plus ultraviolet light damages cell membranes.148
The photodynamic treatment with rose bengal of ribosomes isolated from *E. coli* causes a rapid loss of their ability to incorporate amino acids into polypeptides. Photodynamic damage to stained membranes, which occurs with many dyes in the presence of oxygen and intense illumination, is not well understood and is thought to involve production of free radicals by $^{1}\text{O}_2$.  

**Damage to proteins by the photodynamic effect**

Among those compounds which have been found to be degraded *in vitro* are amino acids, such as tryptophan, which is destroyed by methylene blue in the presence of light and oxygen. Photosensitization has been shown to degrade proteins, denaturing proteins such as egg albumin and inactivating enzymes such as catalase and invertase.

**Damage to DNA by the photodynamic effect**

DNA has been determined to be a major target of the photodynamic damage. From an analysis of yeast survival curves, it was concluded that the principle damage is probably mediated through the DNA. Direct evidence of DNA damage *in vitro* has included loss of viscosity, single stranded breaks, apurination, dimerization, and loss of ability to function as a template for DNA polymerase. The viscosity of calf thymus DNA is reduced by rose bengal and methylene blue in the light. Acridine plus ultraviolet light damages DNA, causing it to sediment more slowly. Acridine orange plus light causes lowered viscosity, a decreased sedimentation coefficient, and a decrease in the thermal denaturation temperature of salmon sperm DNA. This is due to depolymerization, as shown by CsCl banding experiments; depolymerization is due mainly to single-stranded scission. The damage is maximized when the
acridine is bound to DNA; this implies the formation of a short-lived interme-
diate.\textsuperscript{218} Selective degradation of the guanine residues might be the primary
damage responsible for the formation of both alkali-labile bonds and strand
scissions.\textsuperscript{220} Acridine produces not only thymine dimers but also other
lesions.\textsuperscript{221} Acridine orange and methylene blue activate phage lambda in lyso-
genic \textit{Escherichia coli}, which indicates that DNA damage is occurring.\textsuperscript{222} DNA
synthesis is inhibited by acridine orange or methylene blue in the presence of
light. Dark reactivation repair cannot repair after damage by acridine orange
plus light.\textsuperscript{222} In the light, methylene blue, rose bengal, eosin Y, thionin, azure c,
toluidine blue o, acridine orange, and neutral red all decreased melting tem-
perature of DNA, suggesting that the DNA had been degraded.\textsuperscript{149}
Photosensitization has been shown to destroy deoxyguanosine and
deoxyadenosine.\textsuperscript{223}

Proflavin plus light caused single stranded breaks in the bacteriophage
\( \Phi X174 \), where any single-stranded break is a lethal lesion. The packaging of
DNA inside the phage head increases damage over isolated DNA.\textsuperscript{224} Strand
breakage has been demonstrated to be caused by acridine orange,\textsuperscript{218} methy-
lene blue,\textsuperscript{219} and ethidium bromide.\textsuperscript{225}

Proflavin nicks inhibited template activity of DNA polymerase in
\( \Phi X174 \),\textsuperscript{223} indicating that the broken ends of the DNA do not contain a free 3'-
OH group, which is required for the initiation of repair synthesis of the broken
strand. The loss of infectivity of \( \Phi X174 \) following photodynamic treatment with
proflavin can be explained by a block in DNA polymerase reaction; termination
occurs one base before a damaged guanine residue.\textsuperscript{226} The photodynamic
reaction of methylene blue with deoxyribonucleic acid led to the rapid loss of
ultraviolet absorbance accompanied by the uptake of one mole \( O_2 \)/mole deriva-
tive; the reaction occurred with the guanine compounds, while thymine compounds reacted very slowly. Photodynamic reaction of methylene blue with DNA was more rapid with denatured than with native DNA. Strand scission of DNA in vivo has been shown for acridines with bacteria and cultured mammalian cells. There is a peak in DNA damage for human cells but not B. subtilis when irradiated at 450 nm even without the addition of exogenous sensitizers, indicating that endogenous riboflavin probably acts as a sensitizer in the human cells.

Mutations caused by the photodynamic effect

Unsurprisingly, considering the evidence for photodynamic DNA damage, mutations have been detected in photosensitizer-treated bacteriophage, bacteria, yeast, and isolated DNA used in transformations.

Mutations in phage caused by the photodynamic effect

The addition and deletion frameshifts induced by proflavin in phage T4 were used by Crick et al. to determine the nature of the genetic coding unit. Proflavin caused a 2-fold increase in rate of mutation in phage T4; the mutations were of the base-substitution type. The visible light treatment of bacteriophages sensitized by acridines has been found to induce transitions and transversions.

Mutations in bacteria caused by the photodynamic effect

Acridine orange causes mutations in bacteria. Neutral red plus light results in mutations of the base-substitution type in Salmonella typhimurium; methylene blue has the same effect in the same system. S. typhimurium Ames strains TA 1535 (base substitution-sensitive) and TA1538
(frameshift mutation-sensitive) were used to show the presence of base-substitution type mutations caused by acridine orange; no frameshift mutations were seen in this study.

Mutation in *E. coli* to resistance to bacteriophage T5 was induced by visible light (>408 nm) and black light (300-400 nm), causing mutation rates to increase more than 18-fold. In *Escherichia coli* B strain S, mutagenesis was produced by acridine orange and proflavin, both of which are radiomimetic, i.e., they induce cross-resistance to ultraviolet radiation and to each other; this suggests a similarity in the mechanisms used to protect the cells against the two forms of damage. All radiomimetics are mutagens in *E. coli* B.\(^{236}\) Acridines induced mutation in *E. coli* tenfold greater than the normal rate in the absence of dyes.\(^{237}\)

**Mutation in yeast caused by the photodynamic effect**

Rose bengal caused inactivation and gene conversion of yeast;\(^{238}\) as did thionin; proflavin also led to gene conversion of yeast. Acridine orange, proflavin, and acriflavine mutated yeast cells.\(^{146}\) Strong mutagenic action was noted for acridine orange, acridine yellow, methylene blue, and toluidine blue in yeast, as well as in phage and isolated transforming DNA.

**Reactions of photosensitizing dyes**

The toxic and damaging effects produced by the photosensitizers described above require the presence of light and oxygen. The effect of the interaction of light with a photosensitizer is to add energy to the sensitizer molecule. The energy taken up by the sensitizer changes the energy level of
one of its electrons,\textsuperscript{239} resulting in the formation of an excited singlet state. The singlet state rapidly decays to a triplet state, which has a longer lifespan than the singlet state.\textsuperscript{240} The primary photochemical reactions of acridine, xanthene and thiazine dyes are the formation of the singlet and then a longer-lived triplet state. For example, the triplet excited state of fluorescein lasts 10\textsuperscript{-4} second, which is very long compared to the lifetime of the initial singlet excited state, around 10\textsuperscript{-9} second.\textsuperscript{241} Secondary reactions include a triplet-triplet electron-transfer process, leading to the semi-oxidized and semi-reduced forms of the dye,\textsuperscript{242} or the interaction of triplet state dye with oxidants, reductants, or energy transfer compounds (such as the interaction with ground state oxygen (O\textsubscript{2}) to form singlet oxygen(1O\textsubscript{2})). The ability of various dyes to sensitize the photodynamic degradation of nucleic acids is correlated with their ability to form electronically excited dye molecules in a metastable state, in which case the nucleic acids act as reductants.\textsuperscript{149} The excited triplet-state sensitizer is capable of reactions which the ground-state sensitizer would not take part in, or which would occur much more slowly with the ground-state. Photoreduction of excited dye molecules is frequently observed, and the reduced dye donates electrons to other substances, returning to the oxidized state, from which the process may be repeated.\textsuperscript{239}

**Type I and type II reactions of photosensitizers**

The triplet state photosensitizer may react directly with a target molecule, causing damage in an oxygen-independent oxidation or energy transfer reaction, or react with an oxidizable substrate, gaining an electron (equation 8). Subsequently, the semi-reduced dye reacts with dioxygen to yield O\textsubscript{2}\textsuperscript{-} (eq. 9),
\[ ^0 \text{dye} + h\nu \rightarrow ^3 \text{dye} \]  
\[ ^3 \text{dye} + AH_2 \rightarrow \text{dye}^- + AH + H^+ \]  
\[ \text{dye}^- + O_2 \rightarrow \text{dye} + O_2^- \]  
\[ ^3 \text{dye} + O_2 \rightarrow \text{dye}^+ + O_2^- \]  
\[ ^1 \text{O}_2 + \text{NADH} \rightarrow O_2^- + \text{NAD}^+ \]

Figure 5. Dye reactions. "AH_2" represents a reductant; "^3 \text{dye}" represents a triplet state dye.

which secondarily damages the target; in either case, the initial reaction with the sensitizers is known as a Type I reaction (fig. 5). Alternatively, the triplet state photosensitizer may be quenched directly by oxygen, yielding either ^1O_2 or the O_2^- radical by electron transfer, in what is known as a Type II reaction (equations 10-11). For most sensitizers triplets, even in the absence of a reaction with oxidizable substrate, a small but significant percentage of interactions between the sensitizers and oxygen result in electron transfer, which produces O_2^-\text{243}. Either of the two dioxygen radicals, the singlet oxygen or the O_2^- anion, may then go on to react with a target molecule. Superoxide, as previously indicated, may dismute to form H_2O_2 and may ultimately give rise to the OH^- species exert deleterious effects in biological systems.
Factors that influence whether type I or type II reactions occur

Whether the type I or the type II pathway will be followed depends on the relative rates of reaction between the sensitizer and substrate and the sensitizer and oxygen, since these reactants compete for triplet state dye molecules, which are the common substrates of the two reaction pathways (fig. 5.). Also important are the relative concentrations of oxygen and substrate. In aerobic cells, the concentration of physiological reductants are quite high. For example, the concentration of NAD(P)H is about 1.0 mM, and that of glutathione about 6.0 mM. However, the level of oxygen is limited to 0.24 mM by solubility constraints and is much lower than this in respiring cells. The rates of reaction between dye and physiological substrates have not been determined for many of the dyes being investigated in this study, but for those that have been, reactions proceed with rate constants similar to that of the triplet state dye with O₂. For example, while excited triplet thionine reacts with dioxygen at a rate constant of $4.5 \times 10^8$ M⁻¹s⁻¹, it reacts with tryptophan at a rate constant of $3.9 \times 10^9$ M⁻¹s⁻¹.²⁴⁵ The rate constant of the reaction between excited methylene blue and oxygen is $3 \times 10^9$ M⁻¹sec⁻¹.²⁴⁶ The semiquinone form of anthraquinone reacts with oxygen at rate constant of $10^8$M⁻¹sec⁻¹.²⁴⁷

Substrate concentrations in vivo

If the rate constants of reaction between the triplet dye and singlet oxygen are not very different from that of the reaction of triplet dye with an oxidizable substrate, the factor that will determine which reaction predominates will be the concentration of substrate versus that of oxygen. Oxygen is soluble in water to a concentration of 0.24 mM at 37°C and atmospheric pressure; in a respiring cell, the level will be much lower, as the oxygen is continually being
used up by the electron transport chain. Substrates under investigation in this study include NAD(P)H, whose in vivo level is around 0.4 to 1 mM, and reduced glutathione, whose in vivo level is around 6 mM.\textsuperscript{248} Clearly, the relative concentration of substrates and oxygen in vivo would tend to encourage type I reactions. Cadet et al. wrote in a recent review (1986) that “the exact role and the relative contribution of these two competitive mechanisms [type I versus type II] in the photodynamic effects remains to be determined at the cellular level.”\textsuperscript{223}

Production of singlet oxygen

For many years attention has centered on the type II production of singlet oxygen, which has frequently been suggested to be the major intermediate for photodynamic actions both in vitro and in vivo. Each of the photosensitizing dyes involved in this study is almost certainly capable of the production of singlet oxygen under certain circumstances, especially in organic solvents where the solubility of O$_2$ is high and in which many of the photochemical studies have been carried out. According to Ito,\textsuperscript{249} while the theory that singlet oxygen is a major intermediate in the photodynamic effect has served as a valuable stimulant to investigations in the field of photobiology, no photodynamic action so far investigated in vivo is solely explained by the singlet O$_2$ mechanism. Toluidine blue,\textsuperscript{250,251,252} methylene blue,\textsuperscript{253} thionin,\textsuperscript{146} acridine yellow,\textsuperscript{254} proflavin,\textsuperscript{254} neutral red, and rose bengal\textsuperscript{255,256} have all been claimed to produce singlet oxygen, while the damage they produce has been claimed to be mediated entirely by this singlet oxygen.
Questionable arguments for singlet oxygen’s mediation of photodynamic damage

In the general enthusiasm to determine that singlet oxygen is the major intermediate for photodynamic action, some rather questionable evidence has been pressed into service. Frequently, the shakiness of the argument is produced by the apparent belief that there are only two possibilities to be selected between: the type II reaction in which the only oxygen species produced is singlet oxygen, and a type I reaction in which any damage that occurs is caused only by a direct reaction between the triplet state dye and the target of damage. This scheme ignores altogether the possibility of other oxygen intermediates, such as $O_2^-$, $H_2O_2$, or $OH^-$ being involved. For example, one of the most widely used methods to demonstrate that singlet oxygen is the sole mediator of damage consists of demonstrating that the reaction is inhibited by the presence of azide. Unfortunately, the efficacy of azide as an indicator of singlet oxygen mediated damage is compromised by the even greater rate of reaction to be found between azide and $OH^-$. While azide reacts with singlet oxygen at a rate constant of $k=2.2 \times 10^{8}$ M$^{-1}$sec$^{-1}$, $^{246}$ azide reacts with $OH^-$ fifty times faster, at a rate constant of $1.08 \times 10^{10}$ M$^{-1}$sec$^{-1}$. $^{92}$ Moreover, none of the techniques used to assay for singlet oxygen involvement in photodynamic effects, including detection of luminescence, spin-trapping, and increasing reaction rate in D$_2$O, is completely specific for singlet oxygen.$^{257}$

Reactions of azide

The mere inhibition of reaction by azide has frequently been produced as 'proof' that singlet oxygen alone is the mediator of phototoxicity. When azide protected against killing by hematoporphorin, neutral red, methylene blue, rose
bengal, and fluorescein, it was taken as evidence of singlet oxygen involvement.\textsuperscript{154} Because azide reacts with singlet oxygen faster than it reacts directly to quench many triplet-state dyes, such as methylene blue, inhibition by azide is explicitly used as proof that the reaction proceeds by the type II pathway.\textsuperscript{246} Without confirmation from other methods, azide inhibition was used as evidence that singlet oxygen participates as a major intermediate in the photodynamic induction of genetic changes in yeast by acridine orange.\textsuperscript{258} Even in a recent (1986) paper, Kraljic used inhibition by sodium azide as sole proof that photodestruction occurs “exclusively or predominantly via the singlet oxygen mechanism”, “since the triplet state of HP does not react measurably with azide in aerated solution.”\textsuperscript{259}

**The deuterium oxide effect**

The exacerbation of damaging effects of the dyes by the substitution of deuterium oxide for water is often used as evidence that singlet oxygen mediates the damage in question. Singlet oxygen in aqueous solution will be quenched (and removed) by water molecules at a fast rate, \(10^6 \text{ M}^{-1}\text{sec}^{-1}\) at a water concentration of about 55 M. This quenching reaction is in direct competition with other reactions involving singlet oxygen. However, deuterium oxide (\(\text{D}_2\text{O}\)) reacts with singlet oxygen much more slowly than water does. When deuterium oxide replaces water, the rate of singlet removal by the quenching reaction is very low relative to the rate of singlet reaction with other available targets. The solvent lifetime of singlet oxygen is 10 to 17 times longer in \(\text{D}_2\text{O}\) than in \(\text{H}_2\text{O}\), thus allowing it more time to exert any deleterious effects on targets.\textsuperscript{44, 260, 261}
Lack of evidence for singlet oxygen’s mediation of photodynamic damage

Frequently, other data has failed to confirm azide’s demonstration of singlet oxygen involvement in photodynamic action. As exacerbation of damage by D$_2$O was looked for and not conclusively seen, in 1977 Ito and Kobayashi were unable to conclude that singlet oxygen was solely responsible for the toxic effects of xanthenes, thiazines, and acridines in yeast cells, although azide protected.$^{146}$ When the deuterium effect was not as large as predicted from in vitro experiments with singlet oxygen, it was hypothesized that natural quenchers in the cell masked the deuterium effect, and that therefore it could still be concluded that singlet oxygen must be the major intermediate for photodynamic actions in acridine orange-sensitized yeast cells.$^{260}$ Deuterium oxide did not enhance the gene conversion rate caused by acridine orange in yeast cells.$^{260}$ It has been stated that it is unlikely that the attack of singlet oxygen on DNA will give rise to either single stranded breaks or alkali-labile bonds.$^{223}$ However, both type I and type II reactions act in the photodynamic destruction of guanine.$^{262}$

When Nilsson et al. (1978) observed that “until now there has been no conclusive evidence for the participation of singlet oxygen in any biological photooxidation in solution,” they claimed that now they had unambiguous evidence for the participation of singlet oxygen in photodynamic oxidation of amino acids, but their unambiguous evidence turned out to rely on the observation of the destruction of histidine, since there is a low rate constant for reaction of histidine with triplet dye—without considering rate constants for alternative reactions of histidine with O$_2^*$, peroxide, or OH$^-$. Singlet oxygen has been assayed as the decrease in tryptophan absorbance at 280 nm, without confirming assays$^{264}$, when clearly there are more species than singlet oxygen
capable of degrading tryptophan, and some of them, such as OH-, are very likely to be produced by singlet oxygen-generating systems. In fact, tryptophan degradation has been used as a direct assay for the production of OH-. Deuterium oxide enhancement is very small in toxicity in yeast with acridine orange, proflavin, and acriflavin, though somewhat larger for rose bengal.\textsuperscript{265} The role of singlet oxygen was investigated but could not be established in the methylene blue-photosensitized strand cleavage of DNA, because there was little increase in reaction in the presence of deuterium oxide.\textsuperscript{266}

**Production of oxygen radicals and peroxide by the photodynamic effect**

Evidence does exist for the production of $O_2^-$, H$_2$O$_2$, and OH- by the photodynamic effect. For example, hemolysis in vitro by light-activated benoxaprofen results in production of free radicals, singlet oxygen and $O_2^-$ anion.\textsuperscript{267} Other evidence concerning the production of $O_2^-$, H$_2$O$_2$, or OH is given below.

**Production of superoxide by the photodynamic effect**

Superoxide is difficult to observe directly in photosensitized irradiations since most sensitizers absorb light strongly in the 250-300 nm region where $O_2^-$ absorbs;\textsuperscript{262} nevertheless, indirect assays have demonstrated production of $O_2^-$ by excited, semi-reduced dye molecules. The superoxide dismutase-inhibitable reduction of cytochrome c was used to demonstrate that $O_2^-$ is produced by acridine yellow, acridine orange, fluorescein, and lucifer yellow, when illuminated.\textsuperscript{183} A reduced oxygen radical formed from the fluorescein-photosensitized autoxidation of tyrosine was mentioned by Kasche in 1967.\textsuperscript{268} Kasche and Lindqvist suggested that the reaction between the triplet state of fluorescein and oxygen most likely produces $O_2^-$.\textsuperscript{269} This observation was made before the
discovery that oxygen radicals are commonly produced in biological systems. Photochemical generation of $O_2^-$ by rose bengal in the presence of sulfites was suggested in 1978$^{270}$. Both singlet oxygen and $O_2^-$ are produced by polymer-bound rose bengal through direct oxidation of rose bengal by dioxygen.$^{271}$ Superoxide is produced by the methylene blue-sensitized photooxidation of epinephrine; it was hypothesized to be produced from singlet oxygen.$^{272}$ but others maintain that $O_2^-$ is most likely produced by a different path than singlet oxygen.$^{273}$ However, in the presence of appropriate reductants, singlet oxygen actually proves to be a source of $O_2^-$. NADH reacts with singlet oxygen to produce $O_2^-$ and a semi-reduced NAD radical (fig. 5).$^{274}$ Though rose bengal and its derivatives have been used extensively as a singlet oxygen source (type II), their strong absorption at ~550 nm suggests a number of other possible photochemical processes, none of which have been fully exploited or explored.$^{182}$ The photooxidation of rose bengal in non-polar solvents is predominantly type-I.$^{182}$ Using benzoquinone to detect $O_2^-$, Lee and Rodgers (1987) found that about 20% of activated oxygen molecules resulting from reaction with rose bengal in the absence of an oxidizable substrate was $O_2^-$. The light-activated phenothiazine drug chlorpromazine gave a DMPO-OH adduct indicative of OH· whose intensity was decreased 50% by SOD.$^{275}$ Pсорalens have been observed to produce $O_2^-$ directly.$^{276,277,168}$ Both light-activated linear psoralens and isopsroralens produced both singlet oxygen and $O_2^-$ in varying proportions. When riboflavin, psoralen, benzoyl peroxide, and hematoporphyrin derivative were irradiated with ultraviolet light, they each produced superoxide.$^{278}$ When water was replaced with deuterium oxide, the enhancement of guanosine oxidation by the dyes acridine orange, rose bengal, thionin, and methylene blue was less than expected, suggesting a mixture of
singlet oxygen and O$_2^-$ involvement.$^{279}$ The production of single stranded breaks in $\Phi$X174 DNA is thought to occur by a two photon process—absorption at 440 nm and between 320 & 360 nm, followed by an electron transfer from the excited dye molecule to the substrate, DNA.$^{220}$

**Production of hydrogen peroxide by the photodynamic effect**

Specific evidence for H$_2$O$_2$ production in photodynamic effects has also been found. The toxicity of ultraviolet-irradiated tryptophan has been shown to be largely due to H$_2$O$_2$.\textsuperscript{280} The formation of H$_2$O$_2$ by methylene blue in the presence of ascorbate has been observed.\textsuperscript{281} Hydrogen peroxide is produced by the reaction between hematoporphyrin derivative and ascorbate in the presence of light.\textsuperscript{282} This is of physiological significance since some of the tissues targeted for photochemotherapy contain ascorbate at levels of 2-3 mM—such as the mammalian lens. Hydrogen peroxide is produced by xanthene and thiazine dyes in the presence of oxidizable substrates.\textsuperscript{283} It has been suggested that the same mechanism is responsible for near ultraviolet inactivation and photodynamic inactivation of $E$. coli;\textsuperscript{115} the H$_2$O$_2$ scavenger catalase, when incorporated into plating medium protects against near ultraviolet lethality\textsuperscript{284} and ammeliorates photodynamic killing by acridines and thiazines.\textsuperscript{183} The proflavin-sensitized breakdown of DNA may occur by a 'radical pathway', which requires a two-photon absorption and leads to the formation of peroxide free radicals in the DNA moiety of the DNA-proflavin complexes, leading to strand breakages.\textsuperscript{285}
Production of hydroxyl free radical by the photodynamic effect

The production of OH\textsuperscript{\cdot} has also been observed in dye photooxidation. Butylated hydroxytoluene's observed ability to protect against photodermato-toxicity had been explained on the basis of a hypothesized ability to change the structure of the skin, but when this was disproved its antioxidant effects were suggested to be responsible.\textsuperscript{286} This suggests that free radicals are responsible for the phototoxic effect in vivo. Vitamin E is changed to its radical form in the presence of hematoporphyrin derivative and light, indicating that it has been attacked by a free radical.\textsuperscript{287} Both singlet oxygen and OH\textsuperscript{\cdot} have been shown to be produced by HpD \textit{in vitro}.\textsuperscript{147} The OH\textsuperscript{\cdot} scavengers DMSO, sodium benzoate, and thiourea protected \textit{E. coli} B against the toxic effects of illuminated acridine orange.\textsuperscript{183} Both singlet oxygen and hydrogen abstraction mechanisms are involved in the photosensitization of substituted phenylalanines and tyrosines, but the hydrogen abstraction mechanism, a typical OH\textsuperscript{\cdot} reaction, predominates at pH 8.\textsuperscript{288}

The formation of OH\textsuperscript{\cdot} by methylene blue in the presence of ascorbate has been observed.\textsuperscript{289} When exposed to the spin trap compound DMPO, photoexcited psoralens were found to produce a DMPO-OH EPR signal that is abolished when the reaction is carried out in the presence of OH\textsuperscript{\cdot} scavengers.\textsuperscript{168} Hydroxyl free radicals are produced by hematoporphyrin derivative, ascorbate, and light.\textsuperscript{290} In the dye-sensitized photooxidation in methanol of polyunsaturated fatty acids, using the dyes methylene blue, erythrosin, hematoporphyrin, and riboflavin, the isomer product distribution was interpreted in terms of a dual singlet oxygen and radical mechanism.\textsuperscript{142} In the photoactivated reaction of tartrazine, which is mutagenic only in the presence of light, there is no singlet oxygen participation, according to the cholesterol reaction assay, in which
singlet oxygen is detected by its reaction with cholesterol, which results in reaction products specific to this reaction. However, superoxide dismutase & catalase inhibited OH· production, as measured by EPR using the spin-trap DMPO.

Other photodynamic reactions that produce the hydroxyl radical

Assorted other reactions appear to be dependent on photodynamically-produced OH·, whether or not the photosensitizer has been identified. When hematoporphyrin derivative was illuminated in the presence of methionine or tryptophan and Fe-EDTA, OH· were detected using both the salicylate and thio-barbituric acid assays. Tryptophan yielded a photoprotect that could substitute for H₂O₂ in the iron-catalyzed Haber-Weiss reaction, while porphyrin radical could replace O₂⁻. Light-induced chromatid damage in cells being cultured for other studies was found to be prevented by the H₂O₂ scavenger catalase or the OH· scavenger mannitol; after filters were installed to protect cells and medium from light of wavelengths less than 500 nm, the unwanted damage to the cultured cells was eliminated. DNA stand-breaking activity of lipid peroxides is dependant on metal ions and partly inhibited by catalase. The formation of fluorescent products from DNA is increased by metal ions and ascorbate.

Dye photosensitization reactions in vitro may produce singlet oxygen or O₂⁻, or both species, depending on the prevailing reaction conditions. In order to determine the identity of oxidizing species in vivo, it is important to 1) use in vitro model systems that mimic in vivo conditions as closely as possible, with
regard to presence and concentration of targets, substrates, chelators, and trace 
metals; and 2) utilize in vivo methods of study as much as possible.

I would like to emphasize why we need to know, in as 
much detail as possible, about the identity of the oxidiz-
ing species and how they are produced. Without such 
information, our attempts to understand the biological 
role of these species and then to modify them are likely to 
fail. This is particularly important for strongly oxidizing 
species that act like the hydroxyl radical.

—H.M. Swartz

Choice of organism

The reasons why *Escherichia coli* is the organism of choice for 
these studies have to do with the ease of manipulating it and its extensive use 
in previous work on oxygen toxicity. The bacterial cell is a single compartment 
(or at most a dual compartment) organism whose intracellular targets are all 
approximately equally accessible by oxygen radicals. *E. coli* is the best studied 
of all systems with respect to the regulation of SOD and catalase, as well as the 
role of SOD and catalase in preventing oxygen toxicity, and has been a very 
useful organism for previous studies because the cellular levels of the enzymes 
superoxide dismutase and catalase are easily manipulated by the choice of 
media or additions to the media. *E. coli* is also the best-studied system as far as 
the role of redox-cycling compounds in generating oxygen radicals is 
concerned, as well as systems of defensive enzymes. The genes for one of the 
catalases, both of the superoxide dismutases, and nucleases involved in DNA 
repair have already been cloned onto multicopy plasmids. *E. coli* is an organ-
ism that is easily grown and counted, one that can be easily manipulated, both 
physiologically and genetically. Finally, *E. coli* has a long history of study in 
connection with the phototoxicity of dyes used in this work. As all aer-
obic organisms are subject to similar problems as a result of oxygen toxicity, and their defense mechanisms have much in common, *E. coli* should provide a useful model for all aerobic cells.

**Summary**

Studies on the photodynamic effect have concentrated on singlet oxygen for historical reasons. The observation that oxygen was necessary for the toxicity of the photodynamic effect led naturally to the conclusion that an activated form of oxygen was the mediator. The study of singlet oxygen chemistry antedates the discovery of oxygen radicals and their effects on biological systems. McCord and Fridovich’s discovery of superoxide dismutase in 1969 led to the recognition of the toxic effects of the superoxide radical. These toxic effects were subsequently found to be at least in part due to the production of hydrogen peroxide and hydroxyl radicals via the iron-catalyzed Haber-Weiss reaction. These two bodies of knowledge, dye-mediated photosensitization and oxygen radical toxicity, have grown up in parallel without much cross-fertilization. This study was undertaken in order to show that many biological effects previously attributed to singlet oxygen can be explained by free radical mechanisms. The work described in this thesis follows up on observations already made by our lab in this respect and also extends previous observations on photosensitization.

Lethality due to oxygen radicals is an interesting phenomenon worthy of study; so is the production of these oxygen radicals by the photodynamic effect. This investigation is intended to shed light on the identity of cellular targets attacked by toxic oxygen species, on which of the oxygen species are involved
in toxicity, and on factors involved in the photodynamic effect's production of oxygen species.

A major cellular target attacked by toxic oxygen species is likely to be DNA. Single-stranded nicking of DNA will be examined in this study in order to determine which factors are involved in this damage. Among the factors to be examined are the effects of scavengers of superoxide, hydrogen peroxide, and of hydroxyl free radical on the DNA damage; effectiveness of a wide range of dyes in classes including the xanthenes, acridines, thiazines, and a phenazine; effect of different amounts of iron and copper and of a wide range of biologically significant potential iron chelators; and the effects of a series of biological reductants on the production of DNA damage by the photodynamic effect.

The production of toxic oxygen species will be examined in this study by means of a series of assays. This study will use assays for oxidation of NADH and reduction of cytochrome c. It will examine the spectral shifts caused by intercalation into DNA of individual dyes to study intercalation, and the dyes' ability to reduce cytochrome c and oxidize NADH even when intercalated will be investigated. Production of hydroxyl radicals as assayed by two different assays will be examined in the presence of various scavengers of $\text{O}_2^-$, $\text{H}_2\text{O}_2$, and $\text{OH}^-$, as well as in the presence of different metal concentrations, different dyes, different chelators, and different reductants. Nicking of DNA will be examined similarly. Cellular levels of one biologically important reductant, glutathione, will be measured after exposure of the cells to dyes. Amelioration of kill levels will be determined for the presence of plasmids coding for catalase, SOD, and endonuclease IV, as well as preinduction of protective enzymes by PQ and Mn and the presence of thiourea. Finally, in vivo breakage of plasmid DNA will be looked at.
CHAPTER 2. REACTIONS OF DYES

Introduction

In the type I reaction, a dye will become excited, oxidize a substrate, such as NADH, and then either the semioxidized substrate or the semi-reduced dye will reduce dioxygen, resulting in the production of superoxide. These reactions were examined in vitro by following the reduction of cytochrome c by illuminated dyes, and by following the oxidation of NADH by illuminated dyes.

These reactions in which dyes are reduced and oxygen is consumed have been followed in vivo by Martin and Logsdon. Dyes were found to be reduced by E. coli, and then, after the living cells were separated form the dye solution by filtration, the dyes were found to reduce cytochrome c. Furthermore, drawing on the fact that normal respiration of oxygen is inhibited by cyanide, while the consumption of oxygen by dye reactions is not and is therefore known as cyanide-resistant respiration, it was shown that oxygen consumption by cells is increased by the addition of dyes in the presence of light. This cyanide resistant respiration in the presence of illumination and dyes may equal or even exceed the level of oxygen consumption seen in normal respiration by the cell (fig. 6).
Figure 6. Cyanide-sensitive and cyanide-resistant respiration.
In this study, the reactions between specific dyes and NADH were followed in vitro. The oxidation of NADH in the presence of dyes was observed, showing whether NADH is capable of serving as an oxidizable substrate for each of the dyes, and the SOD-inhibitable reduction of ferricytochrome c was followed similarly in order to determine whether each of these reactions was capable of $O_2^-$ production. These reactions were followed with four dyes in solution and with dyes that had been intercalated into a target molecule so that they were potentially inaccessible to reductants and/or oxygen. The shifts in the spectra of dyes as they became intercalated into DNA$^{303}$ were followed in order to determine how much DNA was required to ensure that most of the dye molecules were in the intercalated state, so that the dye reactions could be studied under the condition of being bound to DNA, as the intercalating dyes normally would be if present within a bacterial cell.

**Materials & Methods**

**Materials** Methylene blue, rose bengal, fluorescein, acridine orange, proflavin, azure c, reduced NADH, cytochrome c type III, EDTA, high molecular weight double stranded calf thymus DNA, and superoxide dismutase were purchased from Sigma. Other reagents, including thionin, buffers, thiobarbituric acid, salicylic acid, and ether, were from Fisher.

**Absorption spectra of dyes** The absorption spectra of the dyes azure c, methylene blue, thionin, proflavin, and acridine orange were observed on an IBM scanning spectrophotometer. DNA intercalation leads to a red shift and decrease in absorbance of the spectrum of methylene blue.$^{304}$ Spectra were recorded in the absence of DNA and then in the presence of increasing amounts of purified calf thymus DNA at room temperature. When changes in the
spectra stopped, except for the lowering of amplitude due to the dilution effect of adding the additional DNA, the dye was taken to be fully intercalated. Ratios of DNA to dye were expressed in terms of moles of phosphates in the DNA to moles of the dye. Dye concentrations were: methylene blue, 5 μM; thionin, 40 μM; azure c, 7.5 μM; proflavin, 10 μM; acridine orange, 5 μM; fluorescein, 10 μM. Buffer was 100 mM potassium phosphate, 0.1 mM EDTA, pH 7.6.

The calf thymus DNA was purified by dissolving 50 mg in 10 mls proteinase K buffer 305 (10 mM Tris, pH 7.8, 5 mM EDTA, 0.5% SDS) and incubating with 50 μg/ml proteinase K (Sigma) for one hour at 50°C. The DNA was then extracted twice with 5 mls phenol, once with 10 mls chloroform (1:1 phenol:chloroform), twice with 10 mls chloroform (chloroform:isoamyl alcohol, 24:1 v/v), and twice with ether. Each extraction involved adding the solvent to the aqueous DNA layer, shaking, centrifuging 3 minutes at 1600 G, and transferring aqueous phase to a new centrifuge tube; ether was removed by pipetting it from the top of the aqueous phase followed by blowing nitrogen gas over the aqueous layer to remove remaining traces of ether. This DNA was devoid of associated proteins and should be similar in its dye-binding characteristics to bacterial DNA. The absorption of the DNA did not contribute to the spectra obtained for the dyes.

**Cytochrome c reduction** The reduction of cytochrome c by O₂⁻ was followed spectrophotometrically at 550 nm. Cuvettes were filled with a solution of dye, oxidizable substrate, and 20 μM cytochrome c, and illuminated by being placed in direct contact with a 24-inch GE Warm White fluorescent light bulb for the indicated time intervals. The dyes studied were methylene blue, rose bengal, fluorescein, acridine orange, proflavin, thionin, and azure c; all were at a concentration of 0.5 μM. The oxidizable substrate present was 0.125 mM NADH.
The concentration of reduced cytochrome c at the beginning of each experiment was 20 \( \mu M \), and all reactions took place in 0.05 M potassium phosphate buffer, 0.1 mM EDTA, pH 7.8. DNA concentrations were chosen to be at least twice the values at which most of the available dye molecules would be intercalated, in order to ascertain that the results obtained in the absence of DNA would still hold true for dyes that were intercalated into DNA. 40 units (as defined by McCord and Fridovich)\(^{104} \) of SOD were added to test for its ability to inhibit the reduction of cytochrome c.

**NADH oxidation** The oxidation of NADH was studied similarly; the cuvettes contained dye, 0.05 M potassium phosphate buffer, 0.1 mM EDTA, pH 7.4 and 0.5 mM NADH. Thionin, rose bengal, methylene blue, and azure c were all studied at 1 \( \mu M \). Bovine superoxide dismutase was added as an inhibitor of cytochrome c reduction, to determine the percentage of the reduction reaction that was dependent on \( O_2^- \).

**Results**

The spectra for azure c, methylene blue, thionin, acridine orange, proflavin, and fluorescein are shown in figures 7-12. The DNA phosphate:dye ratios at which the dyes were fully intercalated are shown in Table II. Fluorescein, which was not expected to intercalate—the xanthene dyes are not intercalators—did not exhibit a change in its spectrum as DNA was added. For the other dyes, however, intercalation caused a decrease and a red-shift in absorption spectra, with a clear isosbestic point in most cases indicating a shift between the two states.

As the dyes exhibit a change upon intercalating into DNA, it is important to study their reactions in both the absence and presence of DNA. Experiments
Figure 7. Shift in the absorption spectrum of azure c with addition of calf thymus DNA. Top line is spectrum of 7.5 μM azure c in 50 mM phosphate buffer, pH 7.6. The second line from the top is the spectrum of the same solution with the addition of 71 μM DNA (DNA concentration expressed in phosphate residues); third, same with 142 μM DNA; fourth, same with 213 μM DNA; fifth, same with 284 μM DNA; sixth (occupying same position on graph as line number five), same with 355 μM DNA.
Figure 8. Shift in the absorption spectrum of methylene blue with addition of calf thymus DNA. Top line is spectrum of 5.0 µM methylene blue in 50 mM phosphate buffer, pH 7.6. The second line from the top is the spectrum of the same solution with the addition of 14 µM DNA; third, with 29 µM DNA; fourth, with 43 µM DNA; fifth, with 57 µM DNA; sixth, with 71 µM DNA; seventh, with 85 µM DNA; eighth, with 98 µM DNA; ninth, with 112 µM DNA; tenth, with 139 µM DNA.
Figure 9. Shift in the absorption spectrum of thionin with addition of calf thymus DNA. Top line is spectrum of 40 μM thionin in 50 mM phosphate buffer, pH 7.6. The second line from the top is the spectrum of the same solution with the addition of 36 μM DNA; third, same with 71 μM DNA; fourth, same with 107 μM DNA; fifth, same with 142 μM DNA; sixth, same with 178 μM DNA; seventh, same with 214 μM DNA; eighth, same with 249 μM DNA.
Figure 10. Shift in the absorption spectrum of acridine orange with addition of calf thymus DNA. Top line is spectrum of 5.0 μM acridine orange in 50 mM phosphate buffer, pH 7.6. The second line from the top is the spectrum of the same solution with the addition of 12 μM DNA; third, same with 23 μM DNA; fourth, same with 35 μM DNA; fifth, same with 46 μM DNA; sixth, same with 57 μM DNA; seventh, same with 68 μM DNA.
Figure 11. Shift in the absorption spectrum of proflavin with addition of calf thymus DNA. Top line is spectrum of 10.0 μM proflavin in 50 mM phosphate buffer, pH 7.6. The second line from the top is the spectrum of the same solution with the addition of 2.9 μM DNA; third, same with 14 μM DNA; fourth, same with 26 μM DNA; fifth, same with 37 μM DNA; sixth, same with 49 μM DNA; seventh, same with 71 μM DNA; eighth, same with 93 μM DNA; ninth, same with 115 μM DNA.
Figure 12. No shift in the absorption spectrum of fluorescein with addition of calf thymus DNA. Top line is spectrum of 10.0 μM fluorescein in 50 mM phosphate buffer, pH 7.6. The second line from the top is the spectrum of the same solution with the addition of 29 μM DNA; third, same with 139 μM DNA. The latter amount of DNA was added in a volume of 50 μl; this represents a dilution of the dye of 5%.
TABLE II. RATIO OF MOLES OF PHOSPHATE RESIDUES OF DNA TO MOLES OF DYSES AT WHICH DYSES WERE FULLY INTERCALATED.

<table>
<thead>
<tr>
<th>Dye</th>
<th>DNA/dye ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azure c</td>
<td>38</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>25</td>
</tr>
<tr>
<td>Thionin</td>
<td>5</td>
</tr>
<tr>
<td>Proflavin</td>
<td>7</td>
</tr>
<tr>
<td>Acridine Orange</td>
<td>11</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>0</td>
</tr>
</tbody>
</table>

on reactions with NADH and cytochrome c were performed in both the absence and presence of concentrations of DNA greater than required for complete intercalation in order to make sure that the dyes are active as oxidants and reductants even when intercalated into the DNA.

NADH oxidation was followed as the decrease in absorbance at 340 nm. Even when dye was complexed with a saturating quantity of calf thymus DNA, it was capable, upon illumination, of oxidizing NADH (fig. 13 to 16.) The ability of thionin (fig. 13) to oxidize NADH was greatest in the absence of DNA, inhibited slightly by a small amount of DNA, and inhibited more by a saturating concentration of DNA; twice as much DNA as was required for saturation caused the solution to be fairly viscous, slowing the oxidation of NADH markedly, but not to zero. Rose bengal (fig. 14), which does not intercalate into DNA, showed very similar slopes for NADH oxidation curves for all four treatments; an amount of DNA equivalent to that needed to saturate methylene blue was used, since there is no such quantity for rose bengal. Methylene blue (fig. 15) and azure c (fig. 16), like thionin, showed a decreasing but not insignificant rate of NADH
Figure 13. Oxidation of 0.5 mM NADH by 1 μM thionin. DNA:dye ratio (moles DNA phosphates/moles dye): line 1, zero; line 2, 35; line 3, 175; line 4, 350.
Figure 14. Oxidation of 0.5 mM NADH by 1 μM rose bengal. DNA:dye ratio (moles DNA phosphates/moles dye): line 1, zero; line 2, 35; line 3, 700; line 4, 1400.
Figure 15. Oxidation of 0.5 mM NADH by 1 μM methylene blue. DNA:dye ratio (moles DNA phosphates/moles dye): line 1, zero; line 2, 35; line 3, 700; line 4, 1400.
Figure 16. Oxidation of 0.5 mM NADH by 1 μM azure c. DNA:dye ratio (moles DNA phosphates/moles dye): line 1, zero; line 2, 35; line 3, 285; line 4, 570.
oxidation as the DNA concentration increased. For methylene blue, the presence of the smallest ration amount of DNA appeared to increase reactivity to some extent.

Cytochrome c reduction was observed in the presence and absence of DNA and SOD (fig. 17-23). Every treatment included 20 μM cytochrome c in buffer and 0.5 mM NADH to reduce the dyes. An important question concerned whether SOD would inhibit cytochrome c reduction when the dyes were intercalated into DNA, thus showing that the dyes were accessible to reductants and to O₂ when intercalated and capable of producing O₂⁻ when intercalated. This O₂⁻ could diffuse away from the site of production to reduce cytochrome c. A concentration of DNA was used that was at least twice that sufficient to intercalate all of the dye. The reduction of cytochrome c was observed for methylene blue (fig. 17), rose bengal (fig. 18), fluorescein (fig. 19), acridine orange (fig. 20), proflavin (fig. 21), thionin (fig. 22), and azure c (fig. 23). For all dyes cytochrome c was reduced and in every case the rate of the cytochrome c reduction curve was diminished in the presence of SOD, showing that O₂⁻ was being produced by dye-mediated NADH oxidation. The non-intercalating xanthene dyes, rose bengal and fluorescein, showed a minor decrease in the rate of reduction of cytochrome c when DNA was present, presumably due to an increase in solution viscosity, although this effect could also be due to some interaction between the DNA and the cytochrome c or NADH. The reduction of cytochrome c in the presence of the acridine dyes, acridine orange and proflavin, both showed a somewhat greater decrease in reaction due to the presence of DNA, but less than 50%. The cytochrome c reduction mediated by thiazine dyes was greatly reduced by the addition of DNA to the reaction mixture, indicating that
Figure 17. Reduction of 20 µM cytochrome c mediated by 0.5 µM methylene blue in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 2800:1.
Figure 18. Reduction of 20 μM cytochrome c mediated by 0.5 μM rose bengal in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 200:1.
Figure 19. Reduction of 20 µM cytochrome c mediated by 0.5 µM fluorescein in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 200:1.
Figure 20. Reduction of 20 µM cytochrome c mediated by 0.5 µM acridine orange in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 200:1.
Figure 21. Reduction of 20 μM cytochrome c mediated by 0.5 μM profavin in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 200:1.
Figure 22. Reduction of 20 μM cytochrome c mediated by 0.5 μM thionin in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 5:1.
Figure 23. Reduction of 20 μM cytochrome c mediated by 0.5 μM thionin in the presence of 0.125 mM NADH. Line 1, no additions; line 2, + 40 units SOD; line 3, + DNA; line 4, + 40 units SOD and DNA. DNA phosphate to dye molar ratio was 75:1.
intercalation of the dye into the DNA markedly reduces its accessibility to
cytochrome c, whether due to the interception of \( O_2^- \) by the DNA or due to
different chemistry of thiazines when existing in the hydrophobic environment
inside the DNA. Nonetheless, even when intercalated in DNA, the thiazines are
capable of superoxide dismutase-inhibitable reduction of cytochrome c. The
observation that some of the reduction of cytochrome c is not SOD-inhibitable is
explainable by mediation of cytochrome c reduction directly by reduced dye or
by semi-oxidized NADH.\textsuperscript{306}

Discussion

The spectra of a representative of each of the dye classes including the
thiazines, acridines, and phenazines were altered by the addition of purified
DNA, as the dyes intercalated between the bases of the DNA. Illuminated dyes
reduce NADH and, in the presence of NADH, lead to the production of super-
oxide, which oxidizes cytochrome c. Earlier studies\textsuperscript{306} in our lab showed
photocmodation of NADH and production of superoxide by illuminated dyes, but
this has been extended in the present study to intercalated dyes. This is
important because the bacterial cell contains nucleic acids which will intercalate
the thiazine, acridine, and phenazine dyes \textit{in vivo}. In studying DNA damage by
photodynamically produced active oxygen, it is necessary first to establish
whether reactions are altered by the presence of the target molecule.
CHAPTER 3. HYDROXYL RADICAL PRODUCTION IN VITRO

Introduction

The protective effects in *E. coli* of the hydroxyl radical scavengers thiourea and DMSO *in vivo* strongly suggest that the agent directly responsible for cell damage and death as a result of the photodynamic effect is the hydroxyl radical (OH·).\(^{200}\) In addition, protection by extracellular SOD and catalase in the cell incubation medium suggest that both O\(_2^−\) and hydrogen peroxide are involved in photodynamic kill.\(^{200}\) In order to investigate which reactants are necessary for the production of OH· by dye mediated photooxidation *in vivo*, direct *in vitro* assays were utilized under a variety of experimental conditions. The production of OH· as a result of reactions by the illuminated dyes requires the presence of an oxidizable substrate, such as NAD(P)H; other oxidizable substrates of potential *in vivo* relevance were also studied for their ability to substitute for NADH in this reaction. In addition, the ability of various iron chelators to catalyze the Haber-Weiss production of OH· was assessed, as was the ability of Cu++ to catalyze the reaction. A number of different dyes were investigated using these assays to demonstrate that all were capable of OH·
production. Two different OH- assays were used, as described below. These assays are the thiobarbituric acid assay and the salicylate hydroxylation assay. The thiobarbituric acid assay is more sensitive, both to interference and to detection of small amounts of OH- production, than the salicylate assay, while the salicylate assay is more quantitative, yielding better estimates of the actual amount of OH- produced.

Materials & Methods

OH- production was assayed using two methods: the salicylate assay and the thiobarbituric acid assay, both according to the methods of Halliwell and Gutteridge\textsuperscript{307}. All assays were carried out at room temperature; heating of solutions by light sources never increased temperatures over 25°C.

Salicylate assay. The salicylate assay involves exposing salicylic acid to a source of OH-. In a total reaction volume of 2.00 ml of phosphate buffer (150 mM potassium phosphate buffer, pH 7.6), 4 mM sodium salicylate (400 µl of 20 mM in the same phosphate buffer), dye, oxidizable substrate at a concentration of between 0.1 and 2.0 mM, scavenger if indicated, 100 µM EDTA (40 µl of a 5 mM solution), and 100 µM FeCl₃ (40 µl of a 5 mM solution) were combined in a glass tube and exposed to light. Iron levels used in the in vitro assays are realistic in view of the ability of E. coli to sequester iron using enterochelin and related compounds.\textsuperscript{308} The phosphate buffer was treated with Chelex 100 before use to remove as much contaminating iron as possible, by stirring with Chelex (8 grams per liter of buffer) for eight hours and then filtering out the Chelex granules with Whatman #1 filter paper. The light source was two 20 W. fluorescent bulbs, one on either side of the row of tubes and in direct contact with the tubes; one tube was a GE Warm White bulb, while the other was a
Figure 24. Set-up of hydroxyl radical assay experiments. The Warm White bulb on the left and the Easy-Gro bulb on the right are both in direct contact with the outsides of the tubes.

Sears Easy-Gro plant grow light bulb (fig. 24). After this incubation, the colorimetric assay was performed to quantify the presence of 2,3-dihydroxybenzoic acid. Salicylic acid is specifically hydroxylated by OH• and not by peroxides such as H₂O₂ or by O₂•. The primary hydroxylation product is the ortho diphenyl, 2,3-dihydroxybenzoic acid, which is detected in the assay, although some hydroxylation in the meta and para positions does occur. Hydrochloric acid (80 μl of an 11.6 M solution), 0.5 g NaCl, and then 4 ml chilled ether were added to the original reaction mixture, and vortexed for 30 seconds. Three mls of the upper, ether layer were then removed to another tube and evaporated until no trace of liquid remained. The residue was dissolved in 0.25 ml cold distilled water, and trichloroacetic acid (0.125 ml 10% (w/v) in 0.5 M HCl), sodium tungstate (0.25 ml 10% (w/v) in H₂O), and sodium nitrite (0.25 ml 0.5% (w/v) in H₂O) were added, in that order. The solutions were left standing for five minutes,
and then KOH (0.5 ml of 0.5 M) was added; exactly sixty seconds after the addition of KOH, the absorbance at 510 nm was determined. 2,3-dihydroxybenzoate was added to mock reaction mixtures, and the same extraction procedure followed in constructing the standard curve. With practice, I obtained higher yields on this assay, mainly through increasing the amount of vortexing at the step when the residue after ether evaporation was dissolved in water; unfortunately, this means that numbers cannot be compared between assays performed at different times, but only within a given experiment, which usually comprised 18 to 36 assays. Each figure shows the result only of assays performed at one time. The salicylate assay never yielded a value of zero; typically, dye alone and oxidizable substrate alone each yielded a small but real amount of absorbance. In most experiments, therefore, a background value, generally around 10 to 20% of the total, was subtracted. This value was obtained by adding the yield of dye alone to the yield of substrate alone and subtracting the value obtained in the absence of either dye or substrate. Since all of these values were altered by the presence of different chelators or the use of different experimental times, however, and since practical considerations limited the total number of assays that could be done in each experiment, the background value was not known in certain experiments; these experiments are indicated in their respective figure legends.

**Thiobarbituric acid assay** The thiobarbituric acid assay is performed using deoxyribose as the detector molecule, with a colorimetric assay that detects the formation of malondialdehyde, a fragmentation product of deoxyribose. Fragmentation occurs by a decomposition reaction initiated by abstraction of a hydrogen atom from carbon 4 of the deoxyribose ring by OH-. The reaction is initiated only by very strong oxidants such as OH-, not by O₂⁻ or H₂O₂ alone.
1.5 mM deoxyribose (200 µl of a 5 mM solution in phosphate buffer), 220 µM FeCl₃ (50 µl of a 1 mM solution), dye, oxidizable substrate (0.5 mM reduced NADH unless otherwise indicated), 220 µM chelator (EDTA, 20 µl of a 5 mM solution, unless otherwise indicated), and scavenger (if indicated) were combined in a total volume of 680 µl phosphate buffer (150 mM potassium phosphate buffer, pH 7.6) and exposed to light for thirty minutes as described for the salicylate assay. Chelator concentrations were always kept ≥ Fe³⁺ concentrations to ensure that all Fe³⁺ stayed soluble in the buffer solution. After light exposure, 0.3% thiobarbituric acid (500 µl of a 1% (w/v) solution in 0.05 M NaOH) and 0.83% trichloroacetic acid (500 µl of a 2.8% (w/v) solution) were added, the tubes were placed in boiling water for 10 minutes and then allowed to cool. Under these conditions malondialdehyde combines with thiobarbituric acid to form a pink chromophore. The absorbances at 532 nm were determined on a Gilson spectrophotometer. Malondialdehyde was used to prepare a standard curve in order to convert absorbance to number of moles of product.

Both assays were tested for specificity in determining OH⁻ by competition with a variety of known OH⁻ scavengers.

Results

Dyes. A number of dyes were surveyed for their ability to mediate OH⁻ production. In the presence of 1 mM NADH, 100 µM Fe(III), and 100 µM EDTA, thionin, proflavin, neutral red, fluorescein, toluidine blue o, rose bengal, methylene blue, and acridine orange all stimulated the production of large amounts of OH⁻ relative to the sensitivity of the salicylate assay; lucifer yellow and quinacrine produced very low levels (fig. 25).
Figure 25. Hydroxyl radical production mediated by ten dyes using salicylate assay as described in Materials and Methods. Light exposure was for thirty minutes. Concentrations were: all dyes, 5 μM; NADH, 1 mM; EDTA, 100 μM; FeCl₃, 100 μM.
Time. The OH• production mediated by azure c and proflavin was studied as a function of time (fig. 26). Most of the reaction sensitized by either dye was completed by thirty minutes; thus, subsequent experiments were performed using thirty minute incubations. As the dye reactions are cyclic, resulting in the regeneration of the dye in its original form, it is probably the exhaustion of reductants or of dissolved oxygen that limit OH• production.

Concentrations of dyes. Azure c, proflavin, and neutral red generated increasing amounts of OH• as dye concentration increased (fig. 27). The thiazine azure c mediated more OH• production at a given concentration than did proflavin, which in turn was more effective than the phenazine neutral red; the substituted acridine quinacrine did not produce OH• in the presence of 1 mM NADH. This pattern of reactivity parallels the relative reactivities of these dyes toward NADH under the illumination conditions employed, as determined in another study.183

Oxidizable substrates. Several potentially physiological substrates were tested with each of the dyes azure c, proflavin, and rose bengal (fig. 28). These substrates were tested at concentrations similar to those at which they occur in vivo, and under in vivo pH conditions. NADH and NAD(P)H are oxidized with equal effectiveness by dyes, and all experiments requiring NAD(P)H were carried out with NADH for the sake of economy. Reduced glutathione and NADH were both effective substrates for all three dyes. Under the same conditions, cysteine was an effective substrate for azure c and proflavin, but not for rose bengal; tryptophan and tyrosine were effective, albeit weaker, substrates for azure c, but failed to produce enough OH• when reacted with proflavin or rose bengal to be detected by the relatively insensitive salicylate assay.
Figure 26. Hydroxyl radical production mediated by azure c and proflavin as a function of time in salicylate assay. Concentrations were: azure c, 1.25 μM; proflavin, 5 μM; NADH, 1 mM; EDTA, 200 μM; FeCl₃, 100 μM. Background values were not subtracted.
Figure 27. Production of OH· increased as the concentration of dye increased in the salicylate assay. Other concentrations: NADH, 1 mM; EDTA, 100 μM; FeCl₃, 100 μM. Time of light incubation was 30 minutes as described in Materials and Methods. Azure c, □; proflavin, ■; neutral red, ×; quinacrine, ◆.
Figure 28. Effectiveness of various substrates in the salicylate assay. Concentrations were: azure c, 1.25 μM; proflavin, 10 μM; rose bengal, 10 μM; cysteine, tryptophan, glutathione, tyrosine, and NADH, 1 mM; EDTA, 100 μM; FeCl₃, 100 μM.
While glutathione was not found to be as active as NADH when the salicylate assay was used to study it, in reactions with the four dyes methylene blue, neutral red, acridine orange, and fluorescein, it did serve as a substrate for all four of these dyes (fig. 29). Moreover, glutathione levels within the cytoplasm of E. coli exceed those of NAD(P)H by 5 to 10-fold.

Glutathione was nearly as active as NADH in a thiobarbituric acid assay (fig. 30); cysteine, methionine, tryptophan, tyrosine, and GMP were also capable of acting as oxidizable substrates for azure c. Ascorbate appears to have no effectiveness due to the subtraction of no-dye backgrounds; actually, ascorbate's effectiveness in the absence of dye was very marked, resulting in an assay value equivalent to the production of 25 nmoles OH-. Evidently ascorbate was so powerful as a reductant of Fe-EDTA in the absence of dye that there was no more capacity for reaction left for the dye to enhance, with amount of detector molecule as the likely limiting factor. Cysteine was also quite active in the absence of dye, producing 7.0 nmoles of OH-; however, it was even more active in the presence of the dye, as the figure shows. In the absence of dye, glutathione produced very little positive assay, equivalent to the production of 0.2 n mole OH-; other low values were: methionine, 0.06; tryptophan, 0.06; tyrosine, 0.05; histidine, 0; GMP, 0.29; and ATP, 0.20. NADH produced more, as usual, about 2.7 nmoles.

**Metals and chelators** The catalysis of the dye-mediated production of OH- was studied to determine metal ions and chelators of in vivo relevance. Two metal ions, iron(III) and copper (II), were examined. Iron(III) is most effective in catalyzing the Haber-Weiss reaction when it is chelated by EDTA; however, copper is more effective when unchelated than when chelated with EDTA (data not shown). As trace amounts of metals were not removed from the dyes or
Figure 29. Glutathione and NADH as a substrate with four dyes in the thiobarbiturate assay. Concentrations were: methylene blue, 1.25 μM; neutral red, 5 μM; acridine orange, 2.5 μM; fluorescein, 5 μM; NADH, 0.5 mM; glutathione, 2 mM; EDTA, 200 μM; FeCl₃, 100 μM.
Figure 30. Effectiveness of various substrates in mediating \( \text{OH}^- \)-production in the presence of azure c as measured by the thiobarbiturate assay. Concentrations were: NADH, 0.5 mM; ascorbate, 2 mM; glutathione, 2 mM; cysteine, 0.5 mM; methionine, 0.5 mM; tryptophan, 0.5 mM; tyrosine, 0.5 mM; histidine, 0.5 mM; GMP, 2 mM; ATP, 2 mM; azure c, 2.1 \( \mu \text{M} \); EDTA, 200 \( \mu \text{M} \); FeCl\(_3\), 100 \( \mu \text{M} \).
other reagents, the treatments probably contained micromolar quantities of iron, although the buffer had been treated with Chelex-100 to remove iron. The effectiveness of EDTA-chelated iron appears to reach a maximum at around 10 μM; at the highest concentration tested, 50 μM, unchelated copper had not yet reached a maximum, and was about as effective as 2.5 μM iron (fig. 31).

The presence of added iron (50 μM) had no significant effect on the levels of OH· detection in the absence of chelator, or in the presence of 100 μM deferoxamine, an iron chelator known to prevent the Haber-Weiss reaction; however, in the presence of DTPA levels of OH· detection were increased over 3-fold by addition of iron, while in the presence of EDTA, 50 μM iron increased OH· to an even higher level, over 3½-fold to 125 nmoles per assay (fig. 32). Deferoxamine diminished the detection of OH· only slightly in comparison to the no-chelator reactions.

The chelator DTPA is known to be incapable of chelating iron so to allow it to catalyse the formation of OH· when the reductant is O₂⁻, as in the Haber-Weiss reaction, and yet, for most of the dyes studied, it was found to allow the production of nearly as much OH· as EDTA did (fig. 33); lucifer yellow was an exception, showing no OH· production in the presence of DTPA. This result is not surprising in light of the fact that Winterbourne has found that paraquat performs in this way, too: evidently the paraquat monocation radical (and by extension the semireduced dye radical) is a more potent reductant than is the O₂⁻ radical, and therefore substitutes for the latter in the reduction of the chelated iron. Superoxide is not capable of reducing the DTPA-chelated iron, which would permit the reduced iron to react with hydrogen peroxide to produce OH·. However, the excited, reduced dye can reduce the DTPA-chelated iron.
Figure 31. Increasing concentrations of iron and copper catalyzed increasing amounts of \( \text{OH}^- \) formation in salicylate assay. Concentrations were: azure c, 1.25 \( \mu \text{M} \); NADH, 0.5 mM. Copper reactions did not have added chelator, while iron reactions contained 200 \( \mu \text{M} \) EDTA.
Figure 32. Chelators with and without iron in the salicylate assay. Concentrations were: azure c, 1.25 μM; NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM; DTPA, 200 μM; deferoxamine, 200 μM.
Figure 33. Comparison of DTPA with EDTA in the mediation of OH⁻ production by several dyes in the salicylate assay. Concentrations were: NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM; DTPA, 200 μM; thionin, 1.25 μM; methylene blue, 1.25 μM; fluorescein, 5 μM; proflavin, 10 μM; acridine orange, 10 μM; neutral red, 50 μM; lucifer yellow, 100 μM.
<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Methylene blue</th>
<th>Neutral Red</th>
<th>Proflavin</th>
<th>Fluorescein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TBA 1.25</td>
<td>SAL 1.25</td>
<td>TBA 5</td>
<td>SAL 50</td>
</tr>
<tr>
<td>None</td>
<td>7.2 59</td>
<td>8.6 79</td>
<td>7.7 57</td>
<td>10.4 52</td>
</tr>
<tr>
<td>Urea</td>
<td>6.7 (NA)</td>
<td>6.9 (NA)</td>
<td>4.2 74</td>
<td>9.4 (NA)</td>
</tr>
<tr>
<td>Thiourea</td>
<td>0 0</td>
<td>0 (NA)</td>
<td>0.2 0</td>
<td>0 (NA)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0 0</td>
<td>0 (NA)</td>
<td>0.2 0</td>
<td>0 (NA)</td>
</tr>
<tr>
<td>Benzoate</td>
<td>1.6 22</td>
<td>1.5 0</td>
<td>4.2 0</td>
<td>1.8 0</td>
</tr>
<tr>
<td>DMSO</td>
<td>0 0</td>
<td>0 0</td>
<td>0.3 0</td>
<td>0 11</td>
</tr>
<tr>
<td>SOD</td>
<td>5.9 22</td>
<td>6.9 105</td>
<td>6.6 (NA)</td>
<td>7.7 23</td>
</tr>
<tr>
<td>Catalase</td>
<td>0 0</td>
<td>0 26</td>
<td>3.5 (NA)</td>
<td>0 16</td>
</tr>
</tbody>
</table>

aValues are in nmoles of OH-. Dye concentrations were as indicated. Other concentrations were: NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM. NA = data not available.

The Fenton reaction, which is the reduction of H₂O₂ to OH-, is readily catalyzed by iron chelated by either EDTA or DTPA. Superoxide, then, serves solely as a source for hydrogen peroxide in the DTPA-catalyzed reaction. This result also explains the repeated inability of superoxide dismutase to inhibit the production of OH-. In many reactions, SOD appears to increase OH- production; this may be due to the fact that SOD actually accelerates the production of hydrogen peroxide, a necessary substrate for the Fenton reaction.
When a number of potential biological chelators were tested for the ability to chelate iron in such a way that it would be capable of catalyzing the Haber-Weiss reaction, several were found to have this activity.

Thiobarbituric acid assays indicated that not only DTPA and EDTA were good chelators, but also ATP and ADP (fig. 34). Results for citrate, GTP, 2,3-dihydroxybenzoate (2,3-DHB), picolinic acid, 2,4-dipyridyl, oxalic acid, and pyrophosphate are less definitive, but suggest that these, too, may have some ability to chelate iron so that it has catalytic activity. Deferoxamine, isocitrate, phytate, and lactate appear to be without this ability. These compounds were chosen for their potential biological significance. 2,3-DHB, for example, is a structural component of the physiological chelator enterochelin found in *E. coli*; phytate is a natural chelator found in plant seeds which helps to prevent rancidity. Oxalic acid is found in many plant foods.\(^{311}\) Salicylate assays confirmed that, in addition to EDTA, ATP, ADP, and citrate were good chelators(fig. 35A). Succinate, picolinic acid, the antibiotic oxolinic acid, and the phototoxic antibiotic nalidixic acid, also appeared to be good chelators. In another salicylate experiment (fig. 35B), phenanthroline and dipyridyl were found to have some catalytic-chelator activity, but phytate, oxalate, histidine and the phototoxic chemotherapeutic agent methotrexate were without this activity.

A potential chelator of particularly noteworthy importance is DNA itself. The multitude of negatively-charged phosphate groups on the outside of the DNA suggests that it could have chelating abilities. Since DNA serves as both a major target of hydroxyl-radical produced damage, and actually holds thiazone, phenazine, and acridine dyes in place through intercalation,\(^{312}\) any chelating activity, however small, could be of enormous *in vivo* relevance. It was found in salicylate assays that purified calf thymus DNA promoted OH- production in the
Figure 34. Ability of assorted substances to act as chelators in OH- producing reactions sensitized by azure c in thiobarbiturate assays. Concentrations were: 1.25 μM azure c, 0.5 mm NADH, 100 μm FeCl₃; EDTA, 200 μM; DTPA, 200 μM; deferoxamine, 200 μM; citrate, 1 mM; isocitrate, 1 mM; ATP, 1 mM; ADP, 1 mM; AMP, 1 mM; GTP, 1 mM; histidine, 1 mM; phytate, 200 μM; 2,3 DHB, 500 μM; succinate, 1 mM; malate, 1 mM; ribose, 1 mM; picolinate, 200 μM; dipyridyl, 200 μM; phenanthroline, 200 μM; oxalate, 1 mM; lactate, 1 mM; pyrophosphate, 1 mM. Cross-hatched boxes indicate mean values; open boxes indicate one standard deviation of error. (Numbers in parentheses indicate number of experiments represented.)
Figure 35. Ability of assorted substances to act as chelators in azure c-sensitized OH production when measured in the salicylinate assay. A. Concentrations were: azure c, 1.25 μM; NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM; ATP, 400 μM; ADP, 400 μM; citrate, 400 μM; succinate, 400 μM; nalidixic acid, 200 μM; oxolinic acid, 200 μM; picolinic acid, 200 μM. B. Concentrations were: azure c, 1.25 μM, NADH, 1 mM; FeCl₃, 100 μM; histidine, 400 μM; phenanthroline, 200 μM; dipyridyl, 200 μM; phytate, 200 μM; oxalate, 200 μM; methotrexate, 200 μM.
absence of other chelators (fig. 36 and 37). While the addition of DNA to an EDTA-containing reaction seemed to reduce the amount of OH· seen, the substitution of DNA for EDTA gave significantly greater yield of OH· than did no chelator at all. When increasing concentrations of DNA were studied, it was found that beginning at concentrations of 12.5 μM of the phosphate groups of the DNA, OH· production began to increase; at a concentration of 31.25 μM (a DNA phosphate:dye ratio of 25:1), and above, the DNA and the 50 μM EDTA were similar in their effects.

All of the dyes were studied in the presence of concentrations of DNA at which most of the available dye molecules would be intercalated, in order to ascertain that the results obtained in the absence of DNA would still hold true for dyes that were intercalated into DNA. Increasing quantities of DNA slightly decreased the detection of OH· for both the thiobarbituric acid assay (fig. 38) and the salicylate assay (fig. 39). This was true even for the non-intercalating xanthene dye fluorescein (fig. 40), suggesting that the reduction in yield was not due to inaccessibility of intercalated dye. The small reduction in yield observed indicates that inaccessibility is not a major problem for intercalated dyes. Since DNA actually reacts with OH·, it probably competes with the indicating scavenger for the OH· which is generated by the dyes.

Scavengers A variety of OH· scavengers was used to confirm that the two OH· assays were measuring OH· instead of some other reactive species, such as singlet oxygen (see Table III, above). Thiourea, ethanol, benzoate, and DMSO all significantly reduced the reaction of OH· with the detector molecules. Urea did not, as was expected since it is a poor scavenger of the OH·. Catalase had a marked inhibitory effect on OH· production; superoxide dismutase was partially inhibitory for methylene blue and fluorescein but actually increased the
Figure 36. DNA as a chelator in the azure c mediated production of OH· as detected by the salicylate assay. Concentrations were: azure c, 1.25 μM; NADH, 0.5 mM; EDTA (when present), 50 μM. Light incubation was for ninety minutes. Purified calf thymus DNA was ethanol-precipitated and washed in ethanol to rid it of EDTA.
Figure 37. Production of OH• mediated by azure c in the presence or absence of EDTA or DNA. Concentrations were: azure c, 1.25 μM; NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM (when present); DNA, 90 μM (when present).
Figure 38. Production of OH· mediated by DNA-intercalated azure c and methylene blue, as measured in TBA assay. Concentrations were: methylene blue, 1.25 μM; azure c, 1.25 μM; NADH, 1 mM; FeCl₃, 125 μM; EDTA, 200 μM.
Figure 39. Production of OH· mediated by proflavin, acridine orange, fluorescein, and methylene blue, in the presence of DNA, as measured in salicylate assay. The dyes were observed in four separate experiments, one for each dye. Concentrations were: NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM; proflavin, 1.25 μM; acridine orange, 2.5 μM; fluorescein, 5 μM; methylene blue, 1.25 μM.
Figure 40. Production of OH\textsuperscript{•} mediated by DNA-intercalated and non-intercalated methylene blue, neutral red, acridine orange, and fluorescein. Concentrations were: NADH, 1 mM; FeCl\textsubscript{3}, 100 \textmu M; EDTA, 200 \textmu M; methylene blue, 1.25 \textmu M; neutral red, 5 \textmu M; acridine orange, 2.5 \textmu M; fluorescein, 5 \textmu M. DNA phosphate:dye molar ratio was 100:1 for methylene blue, 50:1 for neutral red, 100:1 for acridine orange, and 25:1 for fluorescein. Incubation time for methylene blue was 1/2 hour, as usual, but was extended to one hour for the other three dyes.
reactivity of neutral red. As discussed above, the semi-reduced neutral red dye radical probably reduces the chelated iron, substituting for the role of $O_2^-$ in the iron-catalyzed Haber-Weiss reaction. Methylene blue, proflavin, and fluorescein probably also act in this way, although with these dyes both of the pathways of $Fe^{3+}$-EDTA reduction (the $O_2^-$-dependent and the dye-•-dependent) probably make significant contributions, since SOD did show some degree of protection.

Four scavengers, DMSO, benzoate, formate, and ethanol, were assayed over a wide range of concentrations for their ability to compete with salicylate in reacting with $OH^-$ (fig. 41). The concentrations at which they competed equally with 4 mM salicylate, resulting in 50% inhibition of reaction, were approximately 4 mM for benzoate, 6 mM for DMSO, 11 mM for formate, and 40 mM for ethanol. This ordering is the same as would be predicted from their respective rate constants with $OH^-$, except for DMSO and benzoate, which are very close in either case. The rate constants for the reactions of these scavengers with $OH^-$, as given in Table I, above, are DMSO, $7.0 \times 10^9$; benzoate, $6.0 \times 10^9$; formate, $2.8 \times 10^9$; and ethanol, $1.5 \times 10^9$.

In view of its historically frequent use as a singlet oxygen scavenger, azide was tested for its ability to scavenge $OH^-$ in the salicylate assay (fig. 42). Sodium azide was found to be highly effective as a scavenger in this assay, which is not surprising since it has been shown that sodium azide reacts at nearly diffusion limited rates with $OH^-$ as determined in pulse radiolytic studies.

Addition of hydrogen peroxide. Hydrogen peroxide was added to the dye mediated photooxidation reactions to assess the role of substrate limitation in $OH^-$ production. The amount of $OH^-$ detected was increased dramatically by 100 $\mu\text{M}$ $H_2O_2$ for the thiazine dyes azure c and thionin, the acridines proflavin
Figure 41. Competition between scavengers and salicylate in the salicylate assay. Concentrations were: azure c, 1.25 μM; NADH, 0.5 mM; FeCl₃, 100 μM; EDTA, 200 μM.
Figure 42. Azide as an OH• scavenger in the salicylate assay. Concentrations were: azure c, 1.25 µM; fluorescein, 5 µM; proflavin, 10 µM; neutral red, 50 µM; NADH, 0.5 mM; FeCl₃, 100 µM; EDTA, 200 µM; DMSO, 350 mM; sodium azide, 25 mM.
and acridine orange, the xanthene fluorescein, the phenazine neutral red, and the napthalimide lucifer yellow (fig. 43). Catalase and the OH⁻ scavengers thiourea, benzoate, and DMSO were able to inhibit both the normal amount of reaction and the amount of excess reaction produced by the presence of hydrogen peroxide (fig. 44). This is consistent with the hypothesis that the dyes are producing O₂⁻, which dismutes to form hydrogen peroxide and ultimately OH⁻, with semi-reduced dyes substituting for O₂⁻ in the reduction of chelated iron(III). It is also consistent with the observation that SOD increases the amount of OH⁻ detected in the thiobarbituric acid assay reaction sensitized by neutral red.

**Discussion**

In this chapter, data have been presented which indicate that all of the dyes tested except for quinacrine and lucifer yellow, including the thiazines thionin, azure c, and methylene blue, the acridines proflavin and acridine orange, the xanthenes fluorescein and rose bengal, and the phenazine neutral red, produce significant quantities of hydroxyl radicals. The amount of OH⁻ produced was dependent on time and dye concentration. Oxidizable substrates were required for the production of large amounts of hydroxyl radicals, although some hydroxyl radical production was detected even in their absence; effective substrates for the dye azure c included NADH, glutathione, cysteine, tryptophan, tyrosine, methionine, and GMP. Metal ions were required as catalysts of OH⁻ production. Both copper and chelated iron proved to be effective catalysts. Chelators that could enable iron to act as a catalyst for OH⁻ production included EDTA and DTPA as well as the biologically significant compounds ATP, ADP, citrate, GTP, oxalic acid, 2,3-DHB, succinate pyrophosphate, and purified large
Figure 43. Substrate limitation in OH⁻ production—added hydrogen peroxide enhanced reaction. Concentrations were: azure c, 1.25 μM; thionin, 1.25 μM; methylene blue, 1.25 μM; proflavin, 10 μM; acridine orange, 10 μM; fluorescein, 5 μM; neutral red, 50 μM; lucifer yellow, 100 μM; NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM; H₂O₂, 20 mM. Light incubation was for only fifteen minutes.
Figure 44. Hydroxyl radical scavengers prevent enhancement of OH· production sensitized by azure c by hydrogen peroxide as assayed with salicylate. Concentrations were: azure c, 1.25 μM; NADH, 1 mM; FeCl₃, 100 μM; EDTA, 200 μM; H₂O₂, 20 mM; catalase, 1500 units/ml; thiourea, 25 mM; benzoate, 25 mM; DMSO, 350 mM; urea, 25 mM. Light incubation was for only fifteen minutes.
molecular weight calf thymus DNA. The $\text{H}_2\text{O}_2$ scavenger catalase prevented OH\textsuperscript{-} production, showing that $\text{H}_2\text{O}_2$ is required for the dye-mediated production of OH\textsuperscript{-}. However, the $\text{O}_2^-$ scavenger SOD did not prevent OH\textsuperscript{-} production, indicating that the semireduced dye can substitute for $\text{O}_2^-$ in reducing the chelated iron, as is also indicated by the fact that DTPA-chelated iron acts as a catalyst for the dye-mediated production of OH\textsuperscript{-}, although superoxide cannot reduce DTPA-chelated iron. Hydroxyl radical scavengers including thiourea, ethanol, benzoate, azide, and DMSO prevented the reaction of OH\textsuperscript{-} with the detector molecules used in the assays, confirming the specificity of the assays. Supplementation of the reactions with additional $\text{H}_2\text{O}_2$ increased the production of OH\textsuperscript{-}; with semi-reduced dye reducing the chelated iron, the Fenton reaction yield is increased.

In Chapter Two, we saw that the illuminated dyes were able to oxidize NADH and produce $\text{O}_2^-$ \textit{in vitro}; this chapter shows that they also produce OH\textsuperscript{-} \textit{in vitro}, and extends our knowledge to what the requirements for OH\textsuperscript{-} production are. All of the requirements outlined in this chapter, including those for oxidizable substrate, iron, and chelator, are met by the contents of normal cells, and are therefore of potential \textit{in vivo} significance.
CHAPTER 4. DNA DAMAGE IN VITRO

Introduction

Damage to DNA by the illuminated dyes was studied in detail using an \textit{in vitro} assay for DNA nicking. A similar assay has been used by Braun and Fridovich (1977) in a study of DNA damage produced by OH\textsuperscript{-} generated by the action of xanthine oxidase acting upon its substrate xanthine.\textsuperscript{49} The variables studied relative to the production of OH\textsuperscript{-} in \textit{in vitro} assays as described in chapter IV, above, were also studied for their effectiveness in mediating DNA damage in this system. Results were consistent with those seen in the OH\textsuperscript{-} assays: those reactants which were effective in mediating the production of OH\textsuperscript{-} were also effective in producing DNA damage, strongly corroborating the theory that photodynamic DNA damage is mediated by the OH\textsuperscript{-} and suggesting that DNA damage is one of the events that figures into the photodynamic effect \textit{in vivo}.

Nicking of DNA is a good assay for hydroxyl radical production because the hydroxyl radical reacts quite rapidly with DNA,\textsuperscript{67} causing single-stranded scission,\textsuperscript{49} but it is unlikely that the attack of singlet oxygen on DNA will give
rise to either single stranded breaks or alkali-labile bonds.\textsuperscript{223} The main value of DNA strand scission as an assay, however, is its high degree of applicability to the situation inside a cell undergoing photodynamic attack.

**Materials & Methods**

The target of the assay, 7.5 - 12.5 $\mu$g/ml supercoiled pBR322 DNA, purchased from Pharmacia or Boehringer Mannheim Biochemicals, was mixed in 20 $\mu$l 100 mM phosphate buffer, pH 7.6, with 50 $\mu$M ferric chloride, 0.4 mM NADH, and 2-50 $\mu$M dye, unless otherwise indicated, in a well of a microtiter assay plate. The wells were covered with a Wratten #2B pale yellow filter to exclude ultraviolet radiation under 410 nm\textsuperscript{313}, which otherwise caused the NADH to produce a noticeable degree of DNA damage in the absence of dye; NADH is known to produce oxygen radicals when exposed to ultraviolet light\textsuperscript{314}. (The glass of the test tubes prevented ultraviolet excitation of NADH in the OH-assays.) Illumination was provided by placing the microtiter assay plate under a 20 Watt GE Warm White fluorescent light bulb, with the fixture suspended so that the distance between the lamp surface and the surface of the reaction solution was 2.4 cm, resulting in illumination with 0.9 mW/cm\textsuperscript{2}, an intensity below what was used in the OH-assays and significantly below that of natural sunlight, which is around 52 mW/cm\textsuperscript{2}. Illumination took place for a period of ninety minutes, unless otherwise indicated. After illumination, 5 $\mu$l of loading buffer (50% glycerol, 1% bromophenol blue) were added to each of the DNA solutions, and each entire reaction solution was placed in one well of a 0.7% agarose minigel in which two rows of wells had been formed. The samples were then electrophoresed for approximately one hour at 40 volts and 100 milliamps. The gels were stained for at least one hour in 1 $\mu$g/ml ethidium, destained if necessary in
1 μM MgSO₄, and photographed using type 667 Polaroid film with a Polaroid MP4 Copy Camera while illuminating with a 302 nm transilluminator. The figures in this chapter were prepared using values obtained by digitizing the positive prints with a Thunderscanner® digitizer and a Macintosh® Computer, using Scanning Analysis® software. The program produces a print-out of the graph of the intensity and size of the peaks representing the various bands. The area under the peaks was integrated by cutting out and weighing each of the peaks on a Mettler balance.

In order to compare a reaction in water to the same reaction in deuterium oxide, every solution had to be made up from anhydrous reagents in deuterium oxide (Sigma 99.8% D₂O). It was necessary to use the formula

\[
\text{actual pH of D}_2\text{O solution} = \text{apparent pH} - 0.4
\]

in order to prepare buffers of comparable acidity. DNA was ethanol-precipitated and then redissolved in either aqueous phosphate buffer or phosphate buffer prepared in D₂O before its use in this experiment.

Results

The dyes azure c, acridine orange, proflavin, rose bengal, fluorescein, and neutral red all caused single stranded DNA nicking when illuminated in the presence of NADH and iron, with dark conditions resulting in very little strand scission in most cases; even quinacrine caused some nicking, at the exceptionally high NADH level of 2.0 mM used in this experiment (fig. 45). The dark reaction was much higher for azure c than for the other dyes; this was attributed to the high reactivity of the dye at low light levels and the difficulty in completely excluding the red wavelengths that activate azure c from the reaction system.
Figure 45. DNA strand scission by illumination of dyes in presence and absence of light. Concentrations were: DNA, 7.5 μg/ml; NADH, 2 mM; FeCl3, 50 μM; EDTA, 12.5 μM; azure c, 5 μM; acridine orange, 5 μM; rose bengal, 5 μM; quinacrine, 20 μM; fluorescein, 10 μM; neutral red, 20 μM; proflavin, 5 μM.
The single-stranded nicking of isolated pBR322 DNA was dependent on the presence of three reactants: dye, added iron, and a reductant such as NADH. As all reactions contained EDTA as provided by the DNA as supplied, it was not necessary to add a chelator to see nicking as long as other required reactants were provided. When the DNA was illuminated in the absence of these components (fig. 46), only a small degree of nicking was seen, present in the DNA mixture as supplied. When 400 μM NADH and 25 μM iron (III) were added, nicking was increased to a small degree, around 13%. In the absence of NADH but presence of iron and 5 μM azure c, nicking was around 10%. Without added iron, NADH plus dye yielded 37% nicking, due probably to contaminating trace metals in the DNA as supplied; with iron, nicking increased to 100%.

This DNA nicking was inhibitable by catalase, SOD, and the OH· scavengers dimethyl sulfoxide, ethanol, benzoate, and thiourea. Thus the DNA strand scission was prevented either by scavenging the essential reactants of the iron catalyzed Haber-Weiss reaction, O₂⁻ and H₂O₂, or by scavenging the ultimate product of the reaction, OH·. Catalase had a stronger inhibitory effect than did SOD; I believe that the lower level of inhibition by scavenging of O₂⁻ reflects the ability of the excited dye radical to substitute for O₂⁻ in the reduction of chelated ferric iron in the iron-catalyzed Haber-Weiss reaction. The addition of similar concentrations of bovine serum albumin did not inhibit the nicking, thus demonstrating that inhibition of nicking is not a general protein effect. Urea, which reacts with OH· more slowly than the other scavengers by a factor of 10⁴³¹⁶, and is thus a poor OH· scavenger, did not inhibit the DNA strand scission reaction at all.

Similar results were seen for other dyes, including the other thiazines, thionin (fig. 47), toluidine blue o (fig. 48), and methylene blue (fig. 49); the
Figure 46. Single-stranded scission of DNA by azure c. Concentrations were: DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl3, 50 μM; EDTA, 12.5 μM; azure c, 5 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 47. Nicking of DNA by thionin in vitro. Concentrations were: thionin, 10 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl₃, 50 μM; EDTA, 12.5 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 48. Nicking of DNA by toluidine blue o in vitro. Concentrations were: toluidine blue o, 10 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl3, 50 μM; EDTA, 12.5 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 49. Nicking of DNA by methylene blue in vitro. Concentrations were: methylene blue, 10 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl₃, 50 μM; EDTA, 12.5 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
acridines, acridine yellow (fig. 50), acridine orange (fig. 51), and proflavin (fig. 52); the xanthenes, rose bengal (fig. 53) and fluorescein (fig. 54); the naphthalimide lucifer yellow (fig. 55); and hematoporphorin derivative (fig. 56). All of these dyes followed the same pattern of less damage in the absence of reductant or in the presence of DMSO or thiourea, without significant decrease in damage conferred by urea. The substituted acridine quinacrine did no damage to the DNA (fig. 57). Control experiments in a previous paper\textsuperscript{200} show that \( \text{OH}^- \) scavengers do not interact directly with \( \text{O}_2^- \) or \( \text{H}_2\text{O}_2 \), and they do not slow the oxidation of NADH or glutathione, or the reduction of cytochrome c by illuminated dyes in the presence of the reductant.

Increasing concentrations of azure c led to increasing levels of DNA damage in the presence of NADH and iron (fig. 58). For NADH levels of 0.4 mM, the damage produced by the azure c leveled off after the concentration of azure c reached 8 \( \mu \text{M} \).

Different quantities of DMSO were added to a reaction including 5 \( \mu \text{M} \) azure c and 0.4 mM NADH (fig. 59, panel A). Increasing quantities prevented more and more of the DNA strand scission from occurring, as one would expect, until essentially all of the reaction was blocked at 1 M. When the log of the \% nicking is plotted against the log of the DMSO concentration (fig. 59, panel B), a nearly straight line is obtained, showing the competition of the DMSO with the DNA for the agent which causes the DNA nicking, \( \text{OH}^- \).

The level of iron present in the reaction also affected the degree of DNA strand scission seen (fig. 60). Increasing the concentration of the \( \text{FeCl}_3 \) dramatically increased the degree of DNA damage. This produced one difficulty
Figure 50. Nicking of DNA by acridine yellow in vitro. Concentrations were: acridine yellow, 10 µM; DNA, 7.5 µg/ml; NADH, 0.4 mM; FeCl3, 50 µM; EDTA, 1.25 µM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 51. Nicking of DNA by acridine orange in vitro. Concentrations were: acridine orange, 5 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl₃, 25 μM; EDTA, 12.5 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 52. Nicking of DNA by proflavin in vitro. Concentrations were: proflavin, 5 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl₃, 25 μM; EDTA, 12.5 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 53. Nicking of DNA by rose bengal in vitro. Concentrations were: DNA, 7.1 μg/ml; NADH, 0.4 mM; FeCl₃, 25 μM; EDTA, 11.8 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 54. Nicking of DNA by fluorescein in vitro. Concentrations were: fluorescein, 10 µM; DNA, 7.1 µg/ml; NADH, 0.4 mM; FeCl₃, 50 µM; EDTA, 11.9 µM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea 100 mM; urea, 100 mM.
Figure 55. Nicking of DNA by lucifer yellow in vitro. Concentrations were: DNA, 4.45 μg/ml; NADH, 0.4 mM; FeCl₃, 50 μM; EDTA, 5.9 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 56. Nicking of DNA by hematoporphyrin derivative in vitro. A 1:10 dilution of a saturated solution of hematoporphyrin derivative (HpD) was used. Other concentrations were: DNA, 7.5 µg/ml; NADH, 0.4 mM; FeCl₃, 50 µM; EDTA, 12.5 µM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M; ethanol, 1.68 M; benzoate, 100 mM; thiourea, 100 mM; urea, 100 mM.
Figure 57. Nicking of DNA by quinacrine in vitro. Concentrations were: quinacrine, 25 μM; DNA, 4.45 μg/ml; NADH, 0.4 mM; FeCl₃, 50 μM; EDTA, 5.9 μM; DMSO, 1.4 M; thiourea, 100 mM; urea, 100 mM.
Figure 58. Nicking of DNA by increasing concentrations of azure c in vitro. Other concentrations were: DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl3, 25 μM; EDTA, 12.5 μM.
Figure 59. Prevention of azure c mediated nicking of DNA by increasing concentrations of DMSO in vitro. Other concentrations were: azure c, 5 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl₃, 25 μM; EDTA, 12.5 μM.
Figure 60. Exacerbation of azure c mediated nicking of DNA by increasing concentrations of FeCl₃ in vitro. Other concentrations were: DNA, 7.5 μg/ml; NADH, 0.4 mM; EDTA, 12.5 μM.
in these experiments. The DNA as supplied contained different amounts of iron, and contained the chelator EDTA; however, except in the experiment comparing the solvent deuterium oxide to water, the DNA was used without dialysis or precipitation, in order to minimize preliminary strand scission of the DNA, so that iron contamination was not controlled for. The experiments shown in figures 66 and 68 were performed for shorter periods of illumination, to counter the increased amounts of damage seen in the reactions, evidently due to contamination with higher concentrations of iron in the Boehringer Mannheim DNA used in these experiments and in the experiment shown in figure 69, as opposed to the Pharmacia DNA used in the other experiments. Furthermore, the experiment shown in figure 65 showed more damage than would have been expected in the reactions performed in the absence of NADH, compared to similar experiments represented in other figures, because the Boehringer Mannheim DNA was used without adjusting the length of incubation accordingly.

Iron is not the only metal that can act as a catalyst for the Haber-Weiss reaction (fig. 61). Copper can, as well, although the effects of chelators are different. The lower band in each lane in the photograph contains supercoiled DNA, while the upper band contains nicked DNA; linearized DNA would occupy a band in a position between these two bands, and highly degraded DNA a smear. Lane one shows the DNA largely nicked after treatment with azure c, NADH, EDTA, and iron. Lane two shows a similar treatment, but with cupric chloride substituted for the iron, resulting in a smear of degraded DNA. Lane three shows that additional EDTA did not much affect the iron reaction, as the DNA looks much the same as in lane one. Lane four shows that additional
Figure 61. Nicking of DNA in the presence of different metals and chelators. All reactions contained 7.5 μg/ml DNA, 0.4 mM NADH, 5 μM azure c, and 12.5 μM EDTA in 100 mM phosphate buffer, pH 7.6. From left to right, the reaction whose results are shown in lane 1 contained 12.5 μM added FeCl₃ and 12.5 μM EDTA; lane 2, 12.5 μM cupric chloride and 12.5 μM EDTA; lane 3, 12.5 μM added FeCl₃ and 37.5 μM EDTA; lane 4, 12.5 μM cupric chloride and 37.5 μM EDTA; lane 5, 12.5 μM added Fe, 12.5 μM EDTA, and 25 μM deferoxamine; lane 6, 12.5 μM added Fe, 12.5 μM EDTA, and 25 μM DTPA; and lane 7, 12.5 μM added Fe, 12.5 μM EDTA, and 25 μM citrate.
EDTA partly inhibited the copper's ability to catalyze the production of OH-. (Lane five shows that the addition of the chelator deferoxamine, a chelator known for its ability to bind iron in such a way that it cannot catalyze the Haber-Weiss reaction, almost completely prevents DNA damage. Lanes five and six show that DTPA and citrate lack this ability, or are unable to competitively sequester the Fe$^{3+}$ from the EDTA that is present.)

The requirement for oxidizable substrates

When a series of substances of biological origin were tested for their ability to act as oxidizable substrates in the reaction with azure c, including asparagine, malate, isocitrate, cystine, succinate, citrate, lysine, cysteine, riboflavin, and NADH, only the latter four had any discernable effect (fig. 62). It must be remembered that the illumination in this experiment was much weaker than in the hydroxyl radical assays which detected more oxidizable substrates. Riboflavin did not require the presence of any added dye in order to completely nick the DNA, while cysteine had a marked effect in the absence of dye but an even greater effect with dye. Riboflavin is itself a chromophore and readily oxidizes under illumination. This property endows riboflavin with the ability to generate DNA strand scission.$^{317}$

The requirement for NADH in order to see substantial amounts of DNA strand scission varied from dye to dye (fig. 63). Acridine orange, methylene blue, and proflavin produced the most substantial amounts of strand scission in the absence of substrate; azure c, rose bengal, and neutral red produced far less damage without an oxidizable substrate. In most cases, however, the OH-scavenger DMSO prevented most of this damage, and in all cases it had some inhibitory effect, even in the absence of oxidizable substrate. While the amount
Figure 62. Abilities of several substances to act as oxidizable substrates in azure c mediated nicking of DNA in vitro. Concentrations of substrates were as indicated in figure; other concentrations were: azure c, 5 μM; DNA, 7.5 μg/ml; FeCl₃, 25 μM; EDTA, 12.5 μM.
Figure 63. Nicking of DNA by several dyes in the presence and absence of NADH and of the scavenger DMSO in vitro. Dyes were: AO, acridine orange; RB, rose bengal; NR, neutral red; MB, methylene blue; PR, proflavin; and AZ, azure c. Concentrations were: acridine orange, 10 μM; rose bengal, 10 μM; neutral red, 50 μM; methylene blue, 10 μM; proflavin, 10 μM; azure c, 10 μM; DNA, 7.5 μg/ml; NADH, 2 mM; FeCl₃, 50 μM; EDTA, 12.5 μM.
of DNA damage increased substantially in the presence of NADH, an even
greater proportion of the total damage was prevented by the OH- scavenger
DMSO. The presence of oxidizable substrate is important in deciding whether a
type I or type II reaction will predominate. Reactions occurring without sub-
strates but with dyes may have been caused by direct DNA oxidation by triplet
state dye or by the oxidation of dye to a more positive valence state with the
release of an electron to O_2 to form O_2^- in a type II oxidation (see Introduction).

DNA strand scission could be accomplished with a range of substrates in
addition to NADH (fig. 64). (As in figure 61, the lower band in each lane in the
photograph contains supercoiled DNA, while the upper band contains nicked
DNA.) Increasing concentrations of each of the oxidizable substrates resulted in
increasing amounts of DNA strand scission. Glutathione at 0.4 mM was as
effective in the reaction as the same concentration of NADH, in the presence of
25 μM added iron(III); 1 mM of either substrate resulted in complete
strand scission when exposed to 5 μM azure c. Tyrosine and tryptophan, which
were tested in the presence of 50 μM iron, were also capable of acting as sub-
strates, and gave increasing amounts of DNA strand scission as their concen-
trations were increased. DMSO at a concentration of 1.4 M completely inhibited
the single stranded DNA nicking, suggesting once again that OH- are the medi-
ators of damage.

Not all dyes reacted equally with the different substrates. In the presence
of sufficient dye to result in 100% strand scission with NADH, azure c showed a
marked degree of damage with tryptophan and tyrosine, while rose bengal,
Figure 64. Increasing concentrations of substrates have increasing effects. All reactions contained 5 μM azure c and were illuminated for 75 minutes. NADH and glutathione reactions contained 25 μM added iron (III); tryptophan and tyrosine, 50 μM added iron (III). Each reaction contained 125 ng of supercoiled pBR322 DNA dissolved in 20 μl phosphate buffer with 12.5 μM EDTA.
proflavin, and neutral red gave little, if any, more damage in the presence of tryptophan or tyrosine than in the absence of any oxidizable substrate (fig. 65). When used in place of NADH, GTP increased the amount of DNA damage over that seen without an oxidizable substrate particularly for the dye rose bengal, but also for methylene blue and azure c (fig. 66). For the dyes neutral red, proflavin, and fluorescein, however, GTP had no such effect; it acted only as a non-specific scavenger, instead (its reaction rate with OH· is 7.6 × 10⁹). When the damage resulting from the reaction of rose bengal with GTP was studied (fig. 67), the OH· scavenger DMSO was found to lend a protective effect, but the enzymes catalase and superoxide dismutase were not significantly protective, as they were in the presence of NADH, suggesting that this strand scission reaction proceeds via a different mechanism than that characteristic of the other substrates. Hydroxyl radical scavengers were far more protective against damage than were the enzymes SOD and catalase for the reactions between dGMP and rose bengal and between dGMP and azure c, as well (fig. 68).

**Deuterium oxide effect**

The exacerbation of damaging effects of the dyes by the substitution of deuterium oxide for water is often used as evidence that singlet oxygen mediates the damage in question. The solvent lifetime of singlet oxygen is 10 to 17 times longer in D₂O than in H₂O, thus allowing it more time to exert any deleterious effects on targets. However, when the DNA was exposed to 400 μM NADH and 5 μM rose bengal or azure c, or 10 μM proflavin or acridine orange, in the presence of illumination, this exacerbation was not seen. In fact, inhibition
Figure 65. Nicking of DNA by several dyes in the presence of NADH, tryptophan, tyrosine, or no reductant in vitro. Concentrations were: azure c, 5 μM; rose bengal, 5 μM; proflavin, 10 μM; neutral red, 20 μM; DNA, 7.1 μg/ml; NADH, 0.4 mM; tyrosine, 4 mM; tryptophan, 4 mM; FeCl₃, 50 μM; EDTA, 11.8 μM. Background nicking was high due to source of DNA.
Figure 66. Nicking of DNA by several dyes in the presence and absence of GTP in vitro. Dyes were: RB, rose bengal; NR, neutral red; PR, proflavin; FL, fluorescein; MB, methylene blue; and AZ, azure c. Concentrations were: rose bengal, 10 μM; neutral red, 25 μM; proflavin, 10 μM; fluorescein, 25 μM; methylene blue, 5 μM; azure c, 5 μM; DNA, 7.5 μg/ml; FeCl₃, 25 μM; EDTA, 25 μM; GTP, 1 mM. Illumination was for ninety minutes.
Figure 67. Substitution of GTP for NADH in the rose bengal mediated nicking of DNA in vitro. Concentrations were: rose bengal, 10 μM; DNA, 7.5 μg/ml; GTP, 1 mM; NADH, 0.4 mM; FeCl₃, 25 μM; EDTA, 25 μM; SOD, 350 units/ml; catalase, 3500 units/ml; BSA, 0.1 mg/ml; DMSO, 1.4 M. Illumination was for 15 minutes.
Figure 68. Ability of dGMP to act as oxidizable substrate for rose bengal and azure c mediated nicking of DNA in vitro. Concentrations were: rose bengal, 10 μM; azure c, 10 μM; DNA, 7.5 μg/ml; dGMP, 1 mM; NADH, 0.4 mM; FeCl3, 50 μM; EDTA, 25 μM; SOD, 350 units/ml; catalase, 3500 units/ml; DMSO, 1.4 M; formate, 100 mM; benzoate, 100 mM; urea, 100 mM. Illumination was for 20 minutes.
of damage was seen, instead (fig. 69). Inhibition of the reaction suggests that a protonation step, which is slower when the deuterium ion must be exchanged instead of the hydrogen ion. Protonation is an obligate part of two of the reactions that leads to the formation of OH· from superoxide.

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

\[ \text{Fe}^{2+}\text{EDTA} + \text{H}_2\text{O}_2 + \text{H}^+ \longrightarrow \text{OH} \cdot + \text{H}_2\text{O} \]

Moreover, 1.4 m DMSO protected against strand scission even in the presence of deuterium oxide. Since DMSO reacts very slowly with singlet O₂ but very rapidly with OH·, it is clear that deuterium is inhibiting free radical reactions leading to OH· production and DNA strand scission and not a reaction involving singlet oxygen as an essential intermediate. Interestingly, a similar experiment performed in the absence of oxidizable substrate gave the same result—inhibition of DNA damage by deuterium oxide.

**Discussion**

The data provided in this chapter reinforce the results of the previous chapter, as all of the requirements for DNA damage are the same as the requirements for OH· production. Most of the dyes tested mediated DNA strand scission, including the thiazines azure c, methylene blue, thionin, toluidine blue o, the xanthenes rose bengal and fluorescein, the acridines proflavin, acridine yellow, and acridine orange, as well as the phenazine neutral red, the naphthalimide lucifer yellow, and the metabolite derivative known as hematoporphyrin derivative. DNA strand scission appears to be an even more sensitive assay for hydroxyl radical production than either the salicylate assay or the thiobarbiturate assay. As was the case for hydroxyl radical production, DNA strand scission in the presence of dye was increased by the presence of an oxi-
Figure 69. Effect of solvent on single-stranded scission of DNA. Concentrations were: acridine orange, 5 μM; proflavin, 10 μM; rose bengal, 5 μM; azure c, 5 μM; DNA, 7.5 μg/ml; NADH, 0.4 mM; FeCl3, 50 μM. All buffers, dye solutions, iron solutions, DNA solutions, and NADH solutions were prepared separately from anhydrous reagents and dissolved in D2O or in H2O. Illumination was for thirty minutes.
dizable substrate. Appropriate compounds included NADH, glutathione, tryptophan, tyrosine, GTP, and dGMP. Scavengers including catalase, SOD, thiourea, formate, azide, benzoate, ethanol, and DMSO prevented damage to the DNA. The reason for SOD’s protective effect being so much greater than was seen in the hydroxyl radical assays is presumably the lower light level used in these experiments; the dyes, when excited by light, are reduced by the substrates and then react with oxygen to produce superoxide in either case; when the light is very bright, perhaps the supply of oxygen is a limiting factor, leaving semireduced dyes to react with the oxidized iron, while in dim light, oxygen reacts with most of the smaller amount of semi-reduced dye that is produced, leaving less to reduce the iron. In a very important experiment in this chapter, the deuterium oxide effect showed that, whether or not singlet oxygen is produced in quantity by the illuminated dyes, it is not responsible for the DNA damage they cause. Moreover, even the non-intercalating xanthenes nicked DNA, showing that intercalation is not essential for DNA damage by dyes. Hydroxyl radical mediates the DNA nicking effect of illuminated dyes.
CHAPTER 5. DNA DAMAGE IN VIVO

Introduction.

The *in vitro* work described in this thesis was extended by three different types of *in vivo* experiments. In the first, levels of glutathione in *E. coli* strain AB1157 were measured relative to survival following exposure to azure c. It was observed that the glutathione was depleted by acting as an oxidizable substrate for the dye *in vivo*. Secondly, strains of *E. coli* B containing plasmids coding for various protective enzymes were compared for their ability to survive exposure to azure c. Finally, single stranded scission of plasmid DNA *in vivo* was observed in rapid small-scale plasmid preps to be increased by exposure to azure c, but partially ameliorated by the presence of thiourea. These experiments provided insight into the molecular species that cause damage *in vivo* and to the molecular targets of attack.

**Glutathione levels in vivo.** The oxidizable substrates found in the *in vitro* studies described in earlier chapters to be capable of reducing the dye to the active semireduced form that reacts with oxygen were NADH, glutathione, cysteine, tryptophan, and tyrosine. All of these are of potential *in vivo* relevance,
as typical in vivo levels are similar to those used in the in vitro assays. One of
these substrates, glutathione, was the subject of in vivo assays to determine
whether this reducing potential translates to free radical generating activity in
the cell. Cells exposed to dyes were assayed for their glutathione content, to
determine whether the cell’s supply of reduced glutathione were depleted by
the action of the dye.

**Factors that affect survival of E. coli in vivo** Studies of the photodynamic
effect in vivo in E. coli suggest that the mediator of phototoxicity is OH-. Among
the results obtained in our laboratory that support this suggestion are the induct-
ion of the protective enzymes SOD and catalase by exposure to the dyes, and
of protection against the light and oxygen dependent lethality of dyes by pre-
induction of these enzymes prior to exposure. In addition, it was
determined that cell-permeant OH- scavengers protect against lethality in vivo.
Martin and Logsdon found micromolar levels of the dye toluidine blue in combi-
nation with illumination halted growth of E. coli cells in NB medium, but that
growth could begin again after a lag period, as though resistance were con-
ferred by the induction of protective enzymes. Greater concentrations of the dye
required correspondingly greater lag periods before growth could occur. Under the conditions of the growth experiments the dyes toluidine blue and
proflavin, when illuminated, induced superoxide dismutase and catalase, and
the enzyme levels induced increased as dye concentration was increased.

When the protective enzymes superoxide dismutase and catalase were
induced by pre-growth in rich, glucose-free media, or by pre-growth in the pres-
ence of small concentrations of paraquat in combination with manganese sup-
plementation in a glucose-containing minimal medium, dye lethality was dra-
matically reduced. Similarly, lethality was reduced in the presence of the cell-
permeant OH- scavengers thiourea, sodium benzoate, and DMSO. All of this data supports the theory that the lethality of illuminated dyes is due to OH-, which is produced by reactions requiring hydrogen peroxide and O2-, and presumably mediated by illuminated dyes in vivo. One treatment used to induce superoxide dismutase and catalase, pre-growth in the presence of small concentrations of paraquat, has been found to also induce endonuclease IV, which repairs DNA damage by removing apurinic or apyrimidinic sites and blocked 3’ termini. The fact that superoxide dismutase, catalase, and endonuclease IV are all coinduced by the same oxidative stress is highly suggestive of what the mediators of damage are and what a likely target of damage is, namely DNA, in the presence of oxidative stress. However, the possibility of multiple enzymes being induced by the same protective pre-treatment implies that protection by pre-induction does not definitively prove that superoxide dismutase and catalase are the only enzymatic agents that protect against lethality. In this study, the question of whether kill is ameliorated by supplementation with plasmids coding for the protective enzymes catalase and SOD was examined. In this way, unambiguous evidence was obtained that it is possible that not only the enzymes that protect against DNA damaging agents are involved in the protection of the cell against the lethal effects of illuminated dyes, but also that enzymes that protect against toxic oxygen species play a major role in protecting DNA and in defending the cell against oxygen toxicity. Therefore the toxic oxygen species, O2- and H2O2, and species derived from them, play a part in mediating photodynamic damage.

DNA damage in vivo The DNA damage that occurs in vivo as a result of photodynamic action has been studied in our laboratory by examining the
varying survival of strains deficient in several different DNA repair enzymes, in the presence of illuminated dyes. Differences in survival among these strains reflect different forms of DNA damage that require the action of distinct DNA repair systems for their correction. Strains deficient in certain DNA repair enzymes were much more sensitive to photodynamic killing than their otherwise isogenic parent strains (fig. 70.). An *E. coli* K12 strain, xthA, which is deficient in that organism’s major AP endonuclease, exonuclease III, showed great sensitivity to photodynamic kill, compared to otherwise identical K12 strains. The deficiency in exonuclease III rendered the strain as sensitive to kill as *E. coli* B, while addition of thiourea or preinduction of the protective enzymes SOD and catalase lent it complete protection against photodynamic kill. *E. coli* B is not deficient in SOD or catalase, but preinduction of these enzymes protect it against kill in just the same way. It seems an interesting possibility that the difference between K12 and B strains that renders the latter so much more sensitive to photodynamic kill may be a deficiency in DNA repair enzymes.

The SOS response, which is induced by DNA damage, was studied in an earlier investigation by Carla Bull of our laboratory with photodynamic action as a response trigger. Induction of the SOS response was seen in a din(damage-inducible)/β-galactosidase fusion strain when it was exposed to azure c plus light (fig. 71.). The fusion strain contains the structural gene for β-galactosidase, an enzyme which is convenient to assay, fused to a promotor which is activated by DNA damage. Exposure of the cells containing the gene fusion expressed β-galactosidase in response to a drug known to induce the SOS response, mitomycin c; it also expressed high levels of β-galactosidase in response to exposure to azure c and visible light. Light in the absence of dye and dye in the absence of light each induced expression more moderately.
Figure 70. Survival of *E. coli* xthA and *E. coli* B following incubation with 2 μM azure c. *E. coli* strains grown to the mid-log phase in M9 salts + 0.4% glucose were harvested and washed twice with M9 salts. The cells were diluted into three milliliter cuvettes containing M9 salts, pH 7.4, 0.4% glucose, 80 μg/ml chloramphenicol, 2 μM azure c and scavengers as indicated. Cuvettes were exposed to visible light at 2.1 mW/cm². Line 1, xthA grown in medium containing 100 μM Mn and 10 μM paraquat; Line 2, xthA, grown as in line 1 plus 100 mM thiourea; Line 3, AB 1157 (xthA⁺); Line 4, xthA plus 100 mM thiourea; Line 5, *E. coli* B; Line 6, xthA. The genotype of AB1157 is *leuB6 Δ(gpt-proA2) hisG4 argE3 lacY1 galK2 ara-14 mtl-1 xyl-5 thi-1 tsx-33 rpsL31 supE44 rac*; the genotype of xthA is the same except for the mutation *xthA*. 
Figure 71. Induction of the SOS response in *E. coli* GW 1040 by azure c and visible light. Cells were grown in M9 medium supplemented with 0.5% Casamino acids at 30°C while exposed to 1.95 mW/cm² visible light. Samples of bacteria were removed from exponential phase cultures at intervals and assayed for β-galactosidase activity.³¹⁹ Mit. C, cells exposed to mitomycin c plus light; Azure C Lt, cells exposed to azure c plus light; Lt, cells exposed to light but neither azure c nor mitomycin c; Azure C Drk, cells exposed to dye in the absence of illumination.
In this study, DNA damage caused by illuminated dyes was examined, using rapid isolation of plasmid DNA, to determine whether it involved single-stranded nicking. Protection from nicking was looked for in the presence of cell-permeant OH- scavengers, to determine whether damage was reduced by such treatment, giving information as to the mediator of the DNA damage.

**Materials & Methods**

**In vivo glutathione determination** Cellular levels of glutathione were determined by making extracts of the cells in boiling ethanol and then reacting these extracts with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). These extracts were made before and after treatment of the cells with azure c. Only the post-dye treatment assays were corrected for cell survival. The cells were grown in minimal media M9 with ampicillin to a density of 100 Klett units, then centrifuged 10 minutes at 6000 G to harvest and resuspended in 6 mls M9 salts, placed in small (60 mm) sterile disposable petri dishes (Falcon) with dye at the indicated concentrations, 20 μl 20% glucose per ml, and 20 μl of a 2 mg/ml aqueous solution of chloramphenicol per ml. Chloramphenicol was added to prevent the induction of defensive enzymes during dye exposure. The plates were placed at room temperature, with lids on, directly under either a Warm White fluorescent bulb, with minimal clearance between lid and lamp. A Thomas slow rotating platform was used to keep the cells continuously suspended. The light intensity at the solution surface was about 1 mW/cm².

In the hot ethanol extraction procedure, 1.5 mls of culture was centrifuged in an Eppendorf microcentrifuge for 3 minutes. The supernatant was removed
and the pellet was resuspended in 5 µl of a 150 mM potassium phosphate buffer, pH 7.6. 57 µl of 86% prewarmed ethanol were added and heated for 3 minutes at 70°C. The supernatant after centrifuging the extract as before was removed to another tube and the ethanol dried off in a Savant Speed Vac Concentrator. The same phosphate buffer was added to bring the total volume up to 1 ml, then DTNB (6.67 µl of a 3.92 mg/ml solution in 150 mM potassium phosphate buffer, pH 7.0) added. After about two minutes, absorbance at 412 nm was determined on a Gilson spectrophotometer. Concentration was determined using the molar extinction coefficient of 13,600 M⁻¹cm⁻¹.⁳²⁰ The levels were compared to cell survival by plating 50 µl samples of the cells on LB medium (1% NaCl, 0.5% yeast extract (Difco), 1% Bacto-tryptone (Difco), 1.5% agar (Difco)) prior to the extraction process. Cells were grown 24 hours at 37° and counted for survival.

Methods used in determining protective factors involved in survival

Preinduction of enzymes in cells In the kill experiments, some cells were subjected to preinduction treatments to increase their intracellular levels of SOD and catalase. Cells were grown overnight in minimal media, then inoculated into minimal media with a 2 to 4% inoculum. The cells were allowed to grow for one hour before the addition of 100 µM MnCl₂ and 10 µM paraquat (methyl viologen, Sigma). The preinduced cells were then grown for the same amount of time as the non-preinduced cells, until the non-preinduced cells had reached a turbidity of 100 to 150 Klett units and the preinduced cells had reached a turbidity of 20 to 50 Klett units.

Kill experiments Fifty ml of medium are inoculated with a 2% inoculum from a saturated overnight culture. Cells are grown to a Klett value of 100 to
125, equivalent to an O.D.\textsubscript{600} of 0.20 to 0.25. The cells are harvested by centrifuging at 6000 G for ten minutes, washed by resuspending in 40 mls phosphate buffer (50 mM, pH 7.6, with 0.1 mM EDTA), recentrifuging, and resuspending again in 40 mls phosphate buffer. Each of a number of sterile cuvettes is filled with 3 mls of sterile M9 minimal salts (6 g Na\textsubscript{2}HPO\textsubscript{4}, 3 g KH\textsubscript{2}PO\textsubscript{4}, 0.5 g NaCl, and 1 g NH\textsubscript{4}Cl, per liter), 60 µl sterile glucose (20%), 60 µl chloramphenicol (2 mg/ml in sterile water), plus the dye to be examined, the amount of which to use is predetermined by trial and error to provide a significant amount of kill. The chloramphenical is added to prevent induction of protective enzymes during the dye exposure; the glucose, to keep the cells metabolizing and maintaining cellular stores of oxidizable substrates. Cells were harvested in mid-log phase after inoculation from an overnight culture. About 75 µl of resuspended bacteria are used per cuvette: an amount in each cuvette calculated from the Klett absorbance to contain the same number of bacteria. The cuvettes are then exposed to visible light to cause photochemical activation. The light was provided by standing the cuvettes up in direct contact with the surface of a General Electric Warm White fluorescent bulb. The light intensity was 1.2 mW/cm\textsuperscript{2}.\textsuperscript{321} Samples are withdrawn, diluted, and plated out on LB agar (10 g Bacto Tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl, and 15 g Bacto Agar (Difco) per liter) to determine survival after fifteen, thirty-five, and sixty-five minutes. Plates were incubated for 24 to 48 hours at 37°C in the dark before counting surviving colonies. All dilutions were plated in duplicate or triplicate.
Strain Construction

The *E. coli* B strains were prepared by isolating the specific plasmids from strains that were obtained from the investigators who constructed them (Table IV) and inserting them into *E. coli* B 23226.

**Plasmid isolation** For small scale plasmid preparation, five mls of LB plus ampicillin (50 mg/L) were inoculated with the strain containing the plasmid of interest and grown overnight. The cells were harvested by spinning in an Eppendorf microcentrifuge for two minutes and resuspended in 0.35 mls Triton lysis buffer (0.5% Triton X-100, 8% sucrose, 50 mM EDTA, 10 mM Tris.Cl, pH 8.0). 25 μl of 10 mg/ml lysozyme in 10 mM Tris-HCl, pH 8.0, was then added, the microfuge tubes were vortexed for three seconds and then boiled for forty seconds. Each tube was spun for twenty minutes in the Eppendorf microcentrifuge, and the pellet removed with a sterile toothpick. Each tube was precipitated with 450 μl isopropanol and 25 μl 3 M sodium acetate, pH 5.2, at -20°C for several hours. After twenty minutes of centrifugation at 4°C, the pellet was dried in air with the tubes inverted, and resuspended in 50 μl TE.

TABLE IV. PLASMIDS USED IN KILL EXPERIMENTS

<table>
<thead>
<tr>
<th>plasmid</th>
<th>relevant genotype</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDT1-5</td>
<td>sodA⁺</td>
<td>122</td>
</tr>
<tr>
<td>pBT22</td>
<td>cat⁺</td>
<td>119</td>
</tr>
<tr>
<td>pBT28</td>
<td>cat⁺</td>
<td></td>
</tr>
<tr>
<td>pWB21</td>
<td>nfo⁺</td>
<td>135</td>
</tr>
</tbody>
</table>
**Transformation**  Cells were grown in LB from a 1% inoculum of a saturated overnight culture to a Klett of no more than 80, and 45 mls were centrifuged for ten minutes at 6000 G. They were then resuspended in ten mls of PC (50 mM CaCl$_2$, 1 mM PIPES, pH 6.8), held on ice 20 minutes, spun down again for ten minutes at 6000 G, and resuspended in ten mls PC. The DNA was diluted to a concentration of only 0.03 to 1.0 μg/ml. The competent cells (100 μl) were added to 50 μl of the DNA solution, shaken gently, held undisturbed on ice for 15 minutes, heat-shocked at 42°C for two minutes, held on ice for five minutes, and held at room temperature for ten minutes; 0.35 ml of prewarmed LB was then added to each tube, and the cells incubated for 45 minutes at 37°C before plating on LB + ampicillin. The plates were incubated at 37°C for 24 hours.

**Preparation of crude protein extracts**  Assays on crude protein extracts were used to aid in interpretation of results from kill experiments. The assays for the enzymes superoxide dismutase, catalase, and peroxidase were performed on crude cell-free extracts of *E. coli* cultures. The extracts were kept cold after harvesting, dialysis was carried out at 4°C, and dialyzed extracts were used immediately or frozen; rethawing and refreezing was kept to a minimum. Frozen cells, in our experience, retain their SOD, catalase, and peroxidase levels indefinitely. Plasmid-containing strains were grown in 50 mls inducing or non-inducing minimal media (M9), at 37°C, to late log phase (O.D$_{600}$ = 0.2 or 0.25), harvested by centrifuging ten minutes at 6000 G in 50 ml tubes, washed, sonicated at 35 watts, on ice, in 6 bursts of 45 seconds each with a Heat Systems-Ultrasonics, Inc., Sonifier Cell Disruptor, model W185, and dialysed against three 1 liter changes of a phosphate-EDTA buffer, then subjected to enzyme assays.
Catalase assay  The crude protein extract is added to 10 mM H₂O₂ in 3 ml of 0.05 M phosphate buffer, pH 7.5, 0.1 mM EDTA. Amounts to use are determined by trial and error; about 10 μl is a good amount to start with. The rate of decline in absorbance of the hydrogen peroxide is followed spectrophotometrically on a Gilford 2000 spectrophotometer at 240 nm. The molar extinction coefficient used is 43.6 M⁻¹cm⁻¹. One unit is the amount of enzyme required to break down 1 μmole of H₂O₂ per minute.³²²

O-dianisidine assay for peroxidase  The crude protein extract is added to 3 ml of 10 mM phosphate buffer, pH 6.5, 0.1 mM EDTA, containing 0.6 mg o-dianisidine, and 5 mM H₂O₂. Change in absorbance is followed at 460 nm, the absorption maximum of the colored product of o-dianisidine oxidation. The molar extinction coefficient of oxidized o-dianisidine is 11,300 M⁻¹cm⁻¹.³²²

Cytochrome c assay for superoxide dismutase  The crude protein extract was added to 3 ml of a cocktail consisting of 0.05 M phosphate buffer, pH 7.8, 0.1 mM EDTA, 50 μM xanthine, 10 μM oxidized cytochrome c, and xanthine oxidase. An amount of xanthine oxidase was used that gave a reduction rate of 0.025 A₅₅₀ units per minute. One unit of superoxide dismutase is defined as the amount of enzyme that results in a 50% inhibition of the reduction of cytochrome c. Different amounts of each extract were assayed until an amount was found that would inhibit this reduction rate by between 40 and 60%, and the amount of extract that would yield one unit was calculated from this degree of inhibition.¹⁰⁴

Methods used in determining strand scission in vivo

Plasmid-containing E. coli B 23226 was subjected to the presence of dyes while exposed to light. The plasmid used was either pBR322 or pJC7, a pBR322 derivative containing 2-3 kb of clostridial DNA. The cells were grown in
minimal media M9 with ampicillin to a density of 100 Klett units, then centrifuged
10 minutes at 6000 G to harvest and resuspended in 6 mls (fig. 75) or 3 mls
(fig.76) M9 salts, placed in small (60 mm) sterile disposable petri dishes
(Falcon) with dye at the indicated concentrations, 20 µl 20% glucose per ml, 20
µl of a 2 mg/ml aqueous solution of chloramphenicol per ml, and, if indicated,
1.4 M DMSO or 100 mM thiourea. The plates were placed, with lids on, directly
under either a Warm White fluorescent bulb, with minimal clearance between lid
and lamp. Dark treatments were placed under an inverted stainless steel pan. A
Thomas slow rotating platform was used to keep the cells continuously sus-
pended.

After light treatment, cells were placed in Beckman amber microfuge
tubes and harvested by spinning for one minute; the supernatant was removed
using a pasteur pipette, and 43 µl of Triton-sucrose lysis buffer (0.5% Triton X-
100, 8% sucrose, 50 mM EDTA, 10 mM Tris.Cl, pH 8.0) was added. The cells
were resuspended by vortexing, two tubes were combined, DMSO added to
stop DNA damage that might occur while the DNA was being isolated from the
cell debris, and 10 µl of 10 mg/ml lysozyme were added. The lysozyme was
mixed in by vortexing for 3 seconds, and the mixture then boiled for 40 seconds
in a boiling water bath. Up to thirty minutes of microcentrifugation served to
clarify the plasmid solution. 20 µl of this solution was mixed with 5 µl ‘blue juice’
(50% glycerol, 0.5% bromphenol blue) and loaded onto one well of a 0.8%
agarose minigel. Electrophoresis separated the bands of supercoiled, nicked,
or linearized plasmid DNA. The gel was photographed using Polaroid type 667
film, which was then digitized using a Thunderware® scanning device on a
Macintosh® computer. Scans of the digitized bands were printed out using
Scanning Analysis® software and integrated by cutting out the peaks and weighing them on a Mettler balance.

Results

In vivo substrate depletion

Reduced glutathione, as detected by assay with Ellman's reagent, was depleted in vivo after exposure of the E. coli B cells to different concentrations of azure c for 65 minutes (fig. 72). Increasing concentrations caused greater depletion of glutathione. 65 minutes exposure to 1 μM azure c reduced glutathione levels by 18%, while the same exposure to 5 and 25 μM azure c caused reductions of 68% and 83%, respectively. Ellman's reagent is not entirely specific, in that reduced cysteine also may react with it, but this in no way detracts from the conclusion that oxidizable substrates are depleted by reaction with azure c. Hydroxyl radical assays and DNA strand scission assays showed that oxidizable substrates such as glutathione react with dyes in vitro. In addition Martin and Logsdon demonstrated that the dye sensitized oxidation of glutathione led to oxygen consumption and H₂O₂ formation which was augmented by addition of SOD. This experiment shows that this reaction actually occurs in vivo. While assays on other oxidizable substrates of biological importance, such as NADH and tryptophan and other amino acids, were not performed, one would expect that they also are depleted after in vivo exposure to illuminated dyes.
Figure 72. Depletion of reduced glutathione in *E. coli* B after exposure to dye, corrected for survival. After growth and preparation as described above under Materials and Methods, cells were exposed to no azure c or to 1, 5, or 25 μM azure c for an illumination period of sixty-five minutes.
Kill experiment results

After sixty-five minutes of exposure to 1 µM azure c, there was too much kill to make significant distinctions between the plasmid-free *E. coli* B 23226 and the strains containing the control plasmid pJC7, the SOD plasmid pDT1-5, and the catalase plasmids pBT22 and pBT28 (fig. 73). The endo IV plasmid, however, conferred a significant degree of protection on the strain which contained it, while both preinduction of protective enzymes and the presence of the hydroxyl radical scavenger thiourea conferred enough protection that with their help *E. coli* B could survive considerably better than even the *E. coli* K12 strain AB1157. *E. coli* K12 strains such as AB1157 have a considerably greater resistance to dyes than *E. coli* B strains such as *E. coli* B23226, usually around two to three orders of magnitude greater.

After thirty-five minutes of exposure in the same experiment, kill levels are sufficiently lower than after sixty-five minutes that the relative levels of protection can be distinguished. Preinduction of protective enzymes, addition of thiourea, and the presence of the endo IV plasmid all gave essentially complete protection, equivalent to the control K12 strain AB1157. Following this group of survivors, both catalase plasmid strains conferred a significant degree of protection, showing a drop in survival of about twenty-fold compared to starting values. The plasmid-free and control plasmid strains came in with much lower survival rates, around 0.1% of starting values, as did the SOD plasmid strain.

The survival rates after fifteen minutes were too high to allow much distinction between classes of survivors.

These rates are exactly what was predicted from *in vitro* experiments. Catalase showed a noticeable degree of protection, while superoxide dismutase did not; this makes sense, since even if all of the $O_2^-$ is broken down into
Figure 73. Survival of azure c by strains containing plasmids with different repair enzymes coded for on them. Kill experiment was performed using 1 μM azure c in the presence of glucose and chloramphenicol as described in Materials and Methods.
hydrogen peroxide, there is still excited semi-reduced dye to take its place in
the reactions that lead to the formation of OH-, reducing ferric iron to ferrous iron
so that it can participate in the Fenton reaction, producing OH-. Preinduction
treatments, which increase catalase and superoxide dismutase approximately
ten-fold, and endonuclease IV probably two-fold (estimating from other results;
this value was not determined),\textsuperscript{133} give a significant degree of protection. Table
V lists the levels of the protective enzymes SOD, catalase, and peroxidase in
extracts made from the cells that were used in this experiment. 100 mM
thiourea, a cell-permeant hydroxyl radical scavenger, gave a great deal of
protection as well.

\textbf{TABLE V.}

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>CATALASE (units/mg protein)</th>
<th>PEROXIDASE (umoles/min-mg protein)</th>
<th>SOD (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 23226</td>
<td>0.729</td>
<td>0.116</td>
<td>20</td>
</tr>
<tr>
<td>B 23226/pBT28</td>
<td>20.9</td>
<td>2.74</td>
<td>15</td>
</tr>
<tr>
<td>K12 AB1157</td>
<td>0.126</td>
<td>0.064</td>
<td>5.4</td>
</tr>
<tr>
<td>B23226 (preind.)</td>
<td>7.55</td>
<td>0.701</td>
<td>141</td>
</tr>
<tr>
<td>B23226/pBT22</td>
<td>21.2</td>
<td>3.02</td>
<td>17</td>
</tr>
<tr>
<td>B23226/pJC7</td>
<td>0.53</td>
<td>0.132</td>
<td>15</td>
</tr>
<tr>
<td>B23226/pWB21</td>
<td>0.39</td>
<td>0.842</td>
<td>15</td>
</tr>
<tr>
<td>B23226/pDT1-5</td>
<td>1.41</td>
<td>0.21</td>
<td>57</td>
</tr>
</tbody>
</table>
A higher level of azure c, 3 μM, was used to distinguish the protective effects of the preinduction of protective enzymes in a control plasmid strain, *E. coli* B/pJC7, with the effects of a similar preinduction in a catalase plasmid strain, *E. coli* B/pBT22 (fig. 74). After 65 minutes of exposure, the higher concentration of dye resulted in complete killing of the strains that were not preinduced, and even in the complete killing of the preinduced control plasmid strain. Thiourea provided complete protection again at 65 minutes, and a dark control (3 μM azure c in the absence of light) was run this time, resulting in very little kill. The only other strain showing any protection at all was the preinduced catalase plasmid. At thirty-five minutes of exposure to the 3 μM azure c, survival levels were again too high to make meaningful distinctions between the treatments, except for the unpreinduced treatment of the control strain. Table VI shows the levels of the protective enzymes SOD, catalase, and peroxidase in extracts made from the cells that were used in this experiment; the levels are similar to but not the same as the levels shown for the previous experiment in Table V, showing the necessity of testing the enzyme levels in each experiment due to inevitable variation, although similar methods of cell growth and induction were followed as described under Materials and Methods.

It is noteworthy that, even against a background of preinduced repair enzymes, an increased HPI level can still have a protective effect against photodynamic killing. While only one dye, azure c, was used in these experiments, the effectiveness of this particular dye in producing oxygen radicals, penetrating
Figure 74. Effect of azure c on survival on preinduced strains with and without the catalase plasmid. Kill experiment was performed using 3 μM azure c in the presence of glucose and chloramphenicol as described in Materials and Methods.
TABLE VI.
LEVELS OF SUPEROXIDE DISMUTASE, CATALASE, AND PEROXIDASE IN
CELLS USED IN PREINDUCTION KILL EXPERIMENT

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>CATALASE (units/mg protein)</th>
<th>PEROXIDASE (µmoles/min-mg protein)</th>
<th>SOD (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 23226/pBT22</td>
<td>9.04</td>
<td>1.95</td>
<td>15</td>
</tr>
<tr>
<td>B23226/pBT22 (preinduction)</td>
<td>14.9</td>
<td>1.61</td>
<td>158</td>
</tr>
<tr>
<td>B23226/pJC7</td>
<td>0.083</td>
<td>0.099</td>
<td>15</td>
</tr>
<tr>
<td>B23226/pJC7 (preinduction)</td>
<td>2.59</td>
<td>0.27</td>
<td>159</td>
</tr>
</tbody>
</table>

E. coli, intercalating into DNA, damaging DNA, and killing cells has been repeatedly demonstrated; moreover, previous experiments have amply shown that the radical generation mechanism of all the dyes is essentially similar.

DNA strand scission results

E. coli strain xthA showed significantly increased plasmid nicking after exposure to 20 µM acridine orange (fig. 75) in the light, as compared to the same exposure in the light and as compared to no acridine orange exposure.

After treatment with 5 µM azure c, E. coli B/pJC7 showed noticeably greater nicking after illumination in the presence rather than the absence of dye. The hydroxyl radical scavenger thiourea, however, lent a significant degree of protection form the dye-induced damage (fig. 76). This result further underscores the significance of OH- in the single stranded scission of DNA in vivo.
Figure 75. In vivo DNA damage by acridine orange. Cells were exposed to dye as described in Materials and Methods for 65 minutes with or without light.
Figure 76. In vivo DNA damage by azure c is ameliorated by the OH scavenger thiourea. Cells were exposed to light as described in Materials and Methods for sixty-five minutes with or without 5 μM azure c, or with 5 μM azure c and 50 mM thiourea.
Discussion

Previous chapters showed that illuminated dyes reduce NADH in vitro, that they produce $O_2^-$, and that they produce OH- with $H_2O_2$ as an essential intermediate. These chapters also showed that the requirements for these reactions to occur in vitro could be met by compounds known to occur in vivo, at physiological levels. In this chapter, these results were extended to in vivo conditions. First, it was shown that an oxidizable substrate is indeed depleted by in vivo exposure to illumination plus dye. The reaction between dye and glutathione does occur, not only in the test tube as was shown in hydroxyl radical assays and DNA strand scission assays, but also in the conditions of the living cell. Secondly, it was shown that cell death is ameliorated by the presence within the cell of enhanced levels of catalase, endonuclease IV, or a cell-permeant hydroxyl radical scavenger. Both $H_2O_2$ and OH- are important mediators of photodynamic kill, and DNA damage is an important target of photodynamic attack within the cell. Finally, DNA damage caused by the photodynamic effect within the living cell was observed directly by electrophoresis of plasmids damaged in vivo, and a cell-permeant OH- scavenger helped to prevent this damage. Previous chapters elucidated the probable mechanism of dye-mediated phototoxicity by in vitro means; this chapter demonstrated that conclusions made in those chapters do extend to in vivo conditions.
CHAPTER VI.

CONCLUSIONS.

While it has long been known that the lethal effects of photosensitizing dyes such as the thiazine, xanthenes, and acridines involve oxygen, little has been known about the mechanism by which these dyes exert their lethality. This study presents evidence that toxicity due to the photodynamic effect is not primarily an effect of singlet oxygen, but that, instead, OH· is an important mediator of photodynamic lethality. The OH· is not produced directly by the dye upon illumination, but depends upon the presence of oxidizable substrates and appropriately chelated iron; little reaction occurs in the absence of oxygen, light, dye, iron, chelator, or oxidizable substrate. Evidently, the dye is first excited by the absorption of light of the appropriate wavelength; in this excited state, it is then capable of reduction by the substrate. After reduction, the dye reacts with triplet state dioxygen, producing O$_2$·$^-$ plus the ground state form of the dye. The O$_2$·$^-$ dismutes to form hydrogen peroxide, spontaneously or with the aid of SOD. Either O$_2$·$^-$ or the excited dye reduces the chelated iron(III) to iron(II). Hydrogen peroxide then reacts with the iron(II) to form OH·. This OH· then attacks any cellular components in the vicinity of the reaction, including, particularly, DNA, which forms an intercalated complex with many of the dyes.
Results which support this scenario include the following. Superoxide was found to be produced by all dyes tested, intercalated or not. NADH was reduced by the dyes, again whether intercalated or not. Hydroxyl radical was produced by the dyes, whether intercalated or not, and this production was inhibited by the presence of catalase. Moreover, dye sensitized production of OH. was augmented by addition of H₂O₂ to reaction mixtures. The strand scission of DNA by the dyes was prevented by catalase and, to some extent, superoxide dismutase, as well as by every hydroxyl radical scavenger tested. Lethality in E. coli was reduced by enhanced levels of catalase and of a DNA repair enzyme.

The dyes that have been found to act in this fashion include the thiazines, methylene blue, thionin, azure c, and toluidine blue o; a phenazine, neutral red; the acridines, proflavin, acridine orange and acridine yellow; and the xanthenes, rose bengal and fluorescein. While similar concentrations of these dyes have different degrees of effectiveness in these reactions, they all appear to act in much the same manner. Other dyes that have been investigated briefly and found to apparently act in a similar fashion include lucifer yellow and hematoporphyrin derivative. These dyes were all studied in order to determine whether their reaction mechanisms are similar. In general, they seem to be extremely similar. The greatest difference between the dye classes studied appears to be in their reactivity toward different oxidizable substrates, and their access to the intracellular compartment of E. coli. NADH and glutathione were both quite effective with all of the dyes studied except for quinacrine, while GTP and GMP were found to be particularly effective in the reaction with rose bengal, but less so in reactions with the thiazines, and even less so in reactions with neutral red. Tryptophan and tyrosine were much more effective with azure c than with rose
bengal, proflavin, or neutral red. Other reaction participants, however, appear to be identical in their reactions with all active dye classes, as would be expected since all of the reactions are identical after $\text{O}_2^-$ is produced and chelated iron reduced. One exception lies in the varying ability of different classes of excited dyes to react with iron(III), substituting for $\text{O}_2^-$, as would be expected since in this reaction the specific structure of the dye in question may affect the course of the reaction. Under the conditions of the DNA strand scission experiments, rose bengal, proflavin, acridine orange, lucifer yellow, neutral red, fluorescein, thionin, hematoporphyrin derivative, azure c, and toluidine blue all showed at least partial protection with SOD, while methylene blue did not; however, under the harsher conditions of the hydroxyl radical experiments—higher light intensity and iron and chelator concentrations—SOD showed less protection for methylene blue, neutral red, proflavin, and fluorescein, indicating that the semi-reduced dye is reducing the iron. More clearcut is the result showing that $\text{OH}^-$ is produced in the presence of DTPA-chelated iron by thionin, methylene blue, fluorescein, proflavin, acridine orange, and neutral red; the only exception was lucifer yellow. As superoxide is not capable of reducing DTPA-chelated iron as it does EDTA-chelated iron, it appears that all of these dyes, when excited and subsequently reduced, are capable of this.

Work by Martin et al.\textsuperscript{200,183} established that lethality by dyes is a radical phenomenon; therefore, it is clear that intracellular metals and chelators do exist to carry out the Haber-Weiss reaction \textit{in vivo}. However, until now there has been no suggestion as to what substances of biological relevance may act as chelators, or what substances besides NADH may act as oxidizable substrates for the dyes. \textit{In vitro} studies presented in this thesis provide a number of relevant suggestions as to which substrates and chelators may be important, how
much iron is necessary, and how important DNA may be as a target for the radicals. Substrates that can be oxidized by the photoexcited dyes include cysteine, tryptophan, tyrosine, glutathione, and GMP. Chelators that allow iron to catalyse the production of OH· include DTPA, EDTA, ATP, ADP, citrate, GTP, 2,3-DHB, picolinic acid, 2,4 dipyridyyl, oxalic acid, pyrophosphate, and DNA itself. Many of the above results were shown by multiple detection methods in order to avoid uncertainties in the interpretation of results.

It was also shown that some of our predictions about the *in vivo* behavior of dyes can be verified. *In vitro* results showed that reductants were used up by reactions with the dyes; *in vivo*, levels of reduced glutathione were depleted. *In vivo* studies presented in this thesis also add to the information we have on the mechanisms of dye-mediated lethality. While previous studies showed that preinduction of the defensive enzymes SOD and catalase protected against killing by photosensitizing dyes, this study adds specificity to that information by showing that increased intracellular levels of catalase alone or endonuclease IV alone also lend protection. Catalase plasmid protection demonstrates that peroxide is an important mediator in dye lethality, while endonuclease IV protection shows that DNA is an extremely important target in dye-mediated lethality since endonuclease IV has no other enzymatic function than the repair of damaged DNA. Protection against lethality by hydroxyl radical scavengers and reduction in strand scission *in vivo* in the presence of scavengers both show that OH· is an important mediator of lethality *in vivo*. All of the factors that influence OH· production & removal similarly affect the ability of dyes to damage plasmid DNA. Moreover, the *in vitro* results on SOD protection suggest a reason for the fact that SOD plasmids are not very effective at conferring protection *in vivo*. Thus, the *in vitro* and *in vivo* results are gratifyingly consistent.
Another finding concerns *E. coli* B's greater sensitivity to phototoxicity and free radical toxicity in comparison with *E. coli* K. The increased sensitivity may be due to a deficiency in repair enzymes. This idea had originally been suggested by the fact that a K12 strain mutant in exonuclease III was similar to *E. coli* B in sensitivity. The fact that *E. coli* B is more highly sensitive than *E. coli* K12 to kill by photodynamic action and to kill by the O2- generating redox active herbicide paraquat further suggests that a free radical generating mechanism underlies both effects and that targets of damage are similar. DNA damage by paraquat and paraquat's induction of the SOS response have been demonstrated by Brawn and Fridovich. Potential future experiments include placing the *nfo* plasmid in *E. coli* strain xthA to determine whether endonuclease IV confers resistance to dyes in an exonuclease III deficient strain, as both function as AP endonucleases and it is thought that it is this function that is important in repairing damage by oxygen radicals.

Oxygen free radical generation results from a variety of different sources, including the dye-mediated photodynamic effect as well as others. The natural defenses of the cell against these active oxygen species include the front-line defenses of the enzymes superoxide dismutase and the hydroperoxidases; in case of failure of these enzymes to prevent oxygen free radicals from attacking cellular components, photodynamically-damaged DNA can be repaired by nucleases which remove damaged single-stranded sections in order to allow replication of undamaged sequences. Different sources of damage—radiation, hydrogen peroxide, near-ultraviolet light, and dye-sensitized visible light—are defended against by the same systems, suggesting that they all involve similar mechanisms & similar targets. The protection of catalase against photodynamic DNA damage and killing found in this study parallels the fact that near ultravi-
let damage is protected against by catalase.\textsuperscript{115} The sensitivity of the xthA strain, mutant in exonuclease III, to photodynamic kill compares to its sensitivity to kill by hydrogen peroxide\textsuperscript{324} and by near uv.\textsuperscript{325} Thus, all light- and oxygen-dependent lethal effects in bacteria may have a common mechanistic basis involving free radical chemistry.

A number of agents are noted for their ability to generate oxygen free radicals, among them the antitumor antibiotic adriamycin, bleomycin, streptonigrin, the diabetogenic agent alloxan, and the herbicides paraquat and diquat. It appears from this study, comparing oxygen radical yields of these substances with those of the photosensitizing dyes in the classes including the xanthenes, acridines, thiazines, and phenazines, that these dyes may be even more potent producers of these highly toxic radicals. All of the requirements outlined in this thesis are met by the contents of normal cells, and are therefore of \textit{in vivo} significance; all, then, that should be required in order for these reactions to take place \textit{in vivo} is a supply of a dye that functions as the dyes considered in this investigation do. If the dyes used in cosmetics and foods are capable of entering cells, damage to DNA may occur, possibly leading to mutation or cancer if cellular defenses are inadequate. Whether this damage will occur in people for exposure to a given dye depends only on the accessibility of dyes to the interior of cells and the presence of the various components required within a single cellular compartment. The fact that toxicity has been seen for dyes including neutral red\textsuperscript{177} and rose bengal\textsuperscript{166} in humans indicates that these requirements are probably met, and suggests caution in the current use of related dyes in foods, drugs and cosmetics.

The major finding of this investigation, that the hydroxyl radical rather than singlet oxygen is a major mediator of photodynamic toxicity, suggests that
a large body of photodynamic-related research, performed in the years before the elucidation of biologically relevant reactions that produce OH•, may require some reinterpretation.
REFERENCES


267. Reszke, K., and C.F. Chignell. 1983. Spectroscopic studies of cutaneous photosensitizing agents—IV. The photolysis of benoxaprofen, an anti-


Curriculum Vitae
Paula E. Burch

Education:
Ph.D., Biology, 1989. Rice University, Houston, Texas.
B.S., Biochemistry, May 1982. University of Maryland, College
Park, Maryland.

Honors and Awards:
NIH predoctoral trainee, Cell and Molecular Biology Training
Grant, February 1, 1985, through May 1989.
Member, General Honors Program, University of Maryland, 1978-1982.

Professional Affiliations:
American Association for the Advancement of Science, 1984 to present.
Association for Women in Science, Gulf Coast-Houston Chapter, 1984 to
present.

Teaching Experience:
Teaching Assistant, Genetics, Rice University, January-May 1984 and
1985.
Teaching Assistant, Introductory Biology, Rice University, August-
December 1984.
Teaching Assistant, Experimental Biology Laboratory, Rice University,
August-December 1983.
Independent Tutor, General Honors Program, University of Maryland,

Additional Work Experience:
Lab assistant, Lipid Lab, Human Nutrition Laboratory, USDA Beltsville
Agricultural Research Center, 1981.

Publications:
1. Oxygen radicals are generated by dye-mediated intracellular pho-
Biology and Pathology,” I. Fridovich, J. McCord, and N. Cerutti (eds.), Alan Liss:
2. Production of superoxide by photosensitization. Martin, J.P., and P.E.
3. Oxygen Radical Generation in E. coli B by dye mediated photoxida-
4. Protection against dye mediated photodynamic effects is conferred by
DNA repair enzymes and oxygen radical scavengers. Burch, P.E., and J.P.