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Viral inactivation using DNA intercalators and dyes for sterilization of blood products

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The Ohio State University, 1992
VIRAL INACTIVATION USING DNA INTERCALATORS AND DYSES FOR
STERILIZATION OF BLOOD PRODUCTS

Dissertation

Presented in Partial Fulfillment of Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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The Ohio State University
1992

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Copyright
Saroj Rai, 1992
To my Mommy
ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor, Dr. Matthew Platz for his guidance and encouragement during my research. I have enjoyed being his surrogate daughter and what a wonderful DAD he has been...

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CHAPTER I

INTRODUCTION

There is an urgent need to develop protocols for the sterilization of viruses present in the human blood supply. Transmission of viral diseases through blood products remains an unsolved problem in transfusion medicine. Although intensive donor pre-testing has improved the safety of the national blood supply, there is still some risk of virus transmission by single-donor blood components. This risk has increased even more with the very recent discovery of new viral strains of the AIDS virus for which there is no screening available. Such transmission can occur during early phases of infection as a result of the absence of detectable viral markers or because tests may not have adequate sensitivity to detect latent infection or low level of virus. Current estimates suggest that the risk of transfusion-transmitted human immunodeficiency virus (HIV) infection ranges from 1 in 40,000 to 1 in 150,000,\(^1,2\) and that the rate for hepatitis C virus (HCV) infection may be as high as 1 in 1000 depending on geographic location.\(^3,4\) It does not seem likely that these risks can be eliminated or even greatly reduced by improvements in testing or donor selection.

In the last several years there has been increased interest in the development of technology to inactivate bacteria and viruses present in blood products: red blood cells, platelets and plasma proteins. Selective inactivation of infectious agents in cellular transfusion products, with adequate preservation of red cell and platelet functions, represents a considerable challenge. The aim of this study is to identify conditions which
will allow light (visible and UV) and X-ray promoted inactivation of model viruses that could be present in the human blood products, without compromising the function and the structural integrity of the blood products.

The approach taken in this study is to inactivate model viruses by generating reactive free radicals in the vicinity of the viral target. It is well established that free radicals damage nucleic acids, RNA and DNA. A free radical such as hydroxyl can abstract a hydrogen atom from a C-H bond of the ribose or deoxyribose moiety of RNA or DNA. This initiates a chain of reactions leading to cleavage of a nucleic acid (Scheme 1), where B is a base such as adenine, thymine, uracil, cytidine or guanine.

There are many ways of generating organic radicals with UV light. Generation of free radicals by UV light in the presence of platelets is an acceptable approach, since UV light does not damage the platelets. However, this approach can not be used with red blood cells because of the optical properties of hemoglobin. Hemoglobin is so intensely absorbing that it will absorb all of the incident UV light, hence preventing activation of the sensitizer. Therefore, other radiation sources such as far visible light and/or X-ray radiation are the preferred sources of radiation.

A large number of studies suggest that X-ray irradiation of aqueous media produces hydroxyl radicals and it is the principle species responsible for damage to the nucleic acids. Irradiation of blood containing virus again produces hydroxyl radicals. Unfortunately, the hydroxyl radical is generated indiscriminately in blood thereby damaging the blood products as well as the viruses present in the blood products. Damage to the blood products can be eliminated by generating radicals only in the vicinity of viral nucleic acids. This study proposes to achieve selectivity by developing sensitizer molecules which bind to nucleic acids by intercalation. We have synthesized several new
\[ \text{HO} \cdot + \text{Base} \rightarrow \text{H}_2\text{O} + \text{Base} \]

\[ \text{CH}_3\text{CO}+\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CO} + \text{H}_2\text{O} \]

\[ \text{CH}_3\text{CO} + \text{Base} \rightarrow \text{CH}_3\text{CO} + \text{Base} \]

\[ \text{CH}_3\text{CO} + \text{Base} \rightarrow \text{CH}_3\text{CO} + \text{Base} \]

\[ \text{HO} \cdot = \text{hydroxyl radical} \]

\[ \text{OP} = \text{O} - \text{P} : \text{O} \]

\[ \text{Scheme 1} \]
water soluble, halogenated derivatives of psoralen and anthracene. The design of the sensitizer must incorporate several features. It must selectively bind tightly to nucleic acids of model viruses, relative to the blood products. It must contain a functional group which will not interfere with the binding to nucleic acids. Upon irradiation, the functional group must generate a highly reactive intermediate, such as a radical which can quickly react at the binding site in the nucleic acid. The sensitizer must selectively absorb the radiation to maintain the integrity and viability of the blood products. Lastly, it must be water soluble.

All viruses contain nucleic acids but red cells do not contain any RNA or DNA. Platelets contain only a small amount of mitochondrial DNA. Thus, it is hoped that our new sensitizers will selectively bind to the viral nucleic acids by design and induce damage to the virus without collateral damage to the structure and function of the blood products (Figure 1).

The sensitizers of this work contain a DNA intercalator, such as a psoralen or anthracene unit, a polyammonium ion tether for increased water solubility and bromine or iodine as the radiation sensitizer. The ammonium salts of these compounds, bind strongly to the nucleic acids, presumably by a mixture of intercalative and electrostatic interactions. Binding constants of these compounds to DNA were determined by the ethidium bromide fluorescence quenching assay.9 The cleavage products of the plasmid DNA pBR322 produced by photolysis of these compounds were analyzed by gel electrophoresis. The ability of these sensitizers to inactivate model viruses, λ and ϕ6, were analyzed by the plaque forming assay. The encouraging results obtained from these experiments have led to the evaluation of these sensitizers in clinical platelet concentrates by Cryopharm Corporation.
Figure 1: Cartoon like representation of the strategy for viral inactivation of blood products.
In addition to studying DNA intercalators, we have also used dyes which are halogenated derivatives of fluorescein (rose bengal, eosin) (Figure 2). These dyes are negatively charged and water soluble, but are lipophilic in nature. It is possible that fluorescein (FL), rose bengal (RB) and eosin Y (EY) may selectively bind to viral membranes because merocyanine-540 is also a negatively charged water soluble lipophilic dye and it is known to selectively bind to the membranes of the HIV virus relative to healthy human red cells. This is possible because red cells are covered with many negatively charged sialic acid residues. Thus a negatively charged sensitizer might not bind to red blood cells.
This series of dyes are excellent sensitizers for X-ray as well as visible light (528 nm) irradiation. We will demonstrate that these dyes do indeed bind model viruses and inactivate these viruses upon activation with X-ray or visible light (528 nm).

To utilize far visible light (658 nm) to inactivate viruses, we have employed another sensitizer, methylene blue (Figure 3). Methylene blue has an absorption maximum near 650 nm and photolysis of viruses with methylene blue at 658 nm caused dramatic reduction in viral activity. Viral inactivation using methylene blue was performed in solution and in frozen media. Much to our surprise, methylene blue completely inactivates viruses up to a depth of 10 mm of frozen samples.

![Figure 3: Chemical structure of methylene blue (MB)](image)

It will be necessary to explore the general areas mentioned above to gain a better perspective. We will first look at the relevant background of each sensitizer used in this study. This includes their binding to DNA and their uses as photosensitizers. Photosensitization will include photocleavage of DNA, as well as the effectiveness of these sensitizers as antiviral agents, if any. A discussion of general mechanistic aspects of each of these sensitizers, with an emphasis on the reactive intermediates involved will be mentioned. The effects of these reactions at the molecular and cellular level will be discussed.
Many biological effects have been attributed to the interaction of small molecules with DNA. There are three modes by which small organic molecules interact with nucleic acids; first, covalent binding, chemical carcinogens can become covalently attached to the bases of the nucleic acids. Second, groove binding, molecules can localize in the grooves of DNA by a mixture of hydrophobic, electrostatic and hydrogen bonding interactions. Lastly, intercalation, molecules with a planar aromatic chromophore can slide in between the base pairs of the double stranded polynucleotide.

Lerman in 1961 first proposed the intercalative binding of planar aromatic molecules to explain the observed behavior of DNA in the presence of acridines, in particular proflavin (Figure 4). According to this model of intercalation, the acridine molecule is sandwiched between two adjacent base pairs in the double helix (Figure 5). The planar intercalator is parallel to the base pairs and perpendicular to the helical axis. The binding is noncovalent and reversible.

Lerman's theory was initially formulated with reference to a phenanthridine derivative, ethidium bromide (etBr) (Figure 4). Since this time, intercalation has proven to be the primary mode of binding for small organic molecules with a planar structure. The interaction of aromatic molecules with DNA is of great importance in the area of medicine. The characteristic physical effects of intercalation on DNA are unwinding and lengthening of the DNA helix, an electronic interaction of the intercalator within the helix and increased rigidity and stabilization of the helix. DNA intercalating molecules, are commonly used as antibacterial, antiviral and antitumor agents. The effects of these drugs upon binding to cellular DNA are inhibition of DNA dependent enzymes and frameshift mutagenesis. To detect intercalation of a molecule to DNA, changes in the helical structure of the DNA is generally observed including the unwinding of the superhelical structure of the DNA. Changes in the electronic interactions between the intercalator and the DNA is also observed. A shift in the UV absorbance or fluorescence intensity maxima of the
intercalator is also generally observed upon intercalation. Indirect methods which demonstrate intercalation can be established by competition studies. A very potent intercalator, ethidium bromide which fluoresces more intensely in the presence of DNA than in its absence, is generally used in the indirect establishment of intercalation. Quenching of etBr fluorescence in the presence of DNA can be observed by the addition of a second molecule which binds by intercalation. The effect results from competition of the molecule of interest for binding to DNA with etBr, with the effective displacement of etBr from the binding site.

However, it must be noted, that each of the criterion mentioned above is not sufficient by itself to conclusively establish intercalation. An accumulation of the above evidence is necessary. The first X-ray evidence which firmly demostrated intercalation between a small molecule and DNA base pairs was presented in 1977 by Sobell. Sobell demostrated that ethidium bromide formed a crystalline complex with dinucleotides 5-iodo-CpG and 5-iodo-UpG (Figure 6). It is this property, intercalation, of the molecules of this work, psoralens and anthracenes, which we wish to exploit in the current study. It is our hope that the halogenated derivatives of these molecules will penetrate viral capsids or envelopes and viral nucleic acid, and, upon illumination with UV light generate free

![Ethidium Bromide and Proflavin Hydrochloride](image-url)
Figure 5: A side view and a top view, of the crystal structure of the acridine:CpG.

Figure 6: A portion of the etBr:iodoUpA crystal structure viewed parallel to the planes of the adenine:uracil base pairs.
radicals and inactivate the virus. The bacteriophage or viruses used in this study are lambda (λ) and phi (φ)6 viruses.

Bacteriophage φ6 is a double stranded RNA virus of *Pseudomonas syringae*. It has a mass of 99 mega daltons. The virus nucleocapsid is surrounded by a lipid-protein envelope. The envelope consists of phospholipids and virus specific proteins. The genome consists of three pieces of double stranded RNA located inside a polyhedral nucleocapsid (Figure 7). Rather detailed knowledge of the virus structure is available in a review article by Mindich.16 These structural elements of φ6 mimic those of the AIDS virus making it an excellent model system.17

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**Figure 7**: Diagram of the structure of φ6. The three pieces of genomic dsRNA are found in a toroidal coil inside and dodecahedral structure composed of P1, P2, P4 and P7 proteins. This is enclosed by a shell of P8 protein. Outside of this is the membrane composed of phospholipids and proteins P9, P10, P13, P6 and P3.
Bacteriophage λ has a mass of 3.2 mega daltons. The host of λ phage is E. coli strain C600. The genome of λ consists of double stranded DNA and its is surrounded by a protein coat. These structural features make λ a good model of non-A and non-B strains of the hepatitis virus.17

In addition to the intercalators, we have used dyes; methylene blue, fluorescein, rose bengal and eosin. These dyes are lipophilic in nature (Figure 2). It is anticipated that these dyes will bind to the viral membrane and upon illumination, cause damage to the virus. To understand the rationale for choosing to work with these sensitizers, it is now necessary to look at the literature background of each of the sensitizers individually.
Psoralens:

Psoralen is a furocoumarin derivative (Figure 7) which contains a furan ring fused to a coumarin molecule. The coumarins that are fused at the 2,3 bond of the furan and the 6,7 bond of pyrone are considered furocoumarins and are commonly called psoralens. Furocoumarins have been used for centuries in the form of crude and purified plant extracts to treat vitiligo.\textsuperscript{18-20} Photosensitization or photodermatitis can be caused by ingestion or contact with the plants that are used for this therapy or with a number of other plants including parsnip, parsley and celery.\textsuperscript{21,22} Progress in understanding the chemical reactions responsible for the photosensitization caused by these plants came slowly. Kalibruner, in 1834, was the first to report the isolation of the photosensitizer, 5-methoxypsoralen (5-MOP) (Figure 8) from bergamot oil.\textsuperscript{23} It was not until almost one hundred years later in 1931 that Phyladelphy demonstrated that sunlight was a necessary component in the action of these compounds.\textsuperscript{24}

Since then, psoralens have been used to treat a variety of skin disorders such as psoriasis\textsuperscript{25,26}, postular psoriasis\textsuperscript{27}, cutaneas lymphomas\textsuperscript{28} and others.\textsuperscript{29} Recently, the photochemistry of psoralen has received much attention due to their use in photochemotherapy. Furthermore, the psoralen derivatives have been very useful in the elucidation of nucleic acid structure and function.\textsuperscript{30} Since psoralens are such potent reagents for the modification of the nucleic acids, a major effort has been devoted to understanding psoralen-nucleic acid photochemistry and photobiology.

One of the desirable properties of psoralens is that they are planar tricyclic molecules that can intercalate between DNA base pairs. The structure of psoralen as well as some of its more popular derivatives along with the numbering of the rings are shown in figure 8. It has been demonstrated using three independent experiments that psoralen molecules do indeed intercalate. First, the \textit{cis-syn} stereochemistry of the isolated photoadducts of the planar psoralen was observed.\textsuperscript{31}
Figure 8: Structures of the psoralen derivatives mentioned in this study.
psoralens were intercalated. Second, direct fluorescence anisotropy measurements are also indicative of intercalation.\textsuperscript{32} Finally, nuclear magnetic resonance (NMR) studies using model compounds indicate that psoralens stack within the helix.\textsuperscript{33}

Several methods have been used to determine psoralen-DNA binding constants. The three psoralen derivatives, 8-MOP, TMP and AMT (Figure 8) that have been used clinically have significantly different DNA binding constants. The three added methyl groups in TMP have been observed to enhance DNA binding with respect to psoralen by more than an order of magnitude. Rodighiero \textit{et. al} showed that the sequential addition of methyl groups to a psoralen derivative resulted in a stepwise increase in the DNA binding constant.\textsuperscript{34} However the additional methyl groups tend to make the TMP less water soluble. This was alleviated by the addition of a short hydrocarbon chain containing a charged moiety (\textsuperscript{+}NH\textsubscript{3}) which significantly enhanced the water solubility. Such is the case in the psoralen derivative, AMT. AMT is four orders of magnitude more water soluble than TMP.\textsuperscript{35} The amino group of AMT is positively charged under physiological conditions, thereby enhancing its binding to negatively charged DNA. Evidence accumulated to this point suggests that the psoralens damage DNA by the formation of photoadducts, both monoadducts and cross-links (Figure 9). The magnitude of the binding constant of the psoralen with DNA determines the amount of the drug that interacts with DNA and ultimately the upper limit of photoadduct formation.

The effect of psoralens and UV light on nucleic acids have been extensively studied. The photochemically reactive sites in psoralens are located at each of the carbon-carbon double bonds in the furan ring and the pyrone ring. Generation of an excited singlet state of psoralen can occur in two ways. An electron in the carbonyl bond of the pyrone ring can be promoted to an antibonding \pi^* orbital, giving rise to the \textsuperscript{1}(n, \pi^*) state (Figure 10).\textsuperscript{36} Alternatively, an electron in the extended aromatic tricyclic ring system could be promoted to an antibonding orbital \textsuperscript{1}(\pi, \pi^*).\textsuperscript{36} The photoexcitation of an
intercalated psoralen leads to the photoexcitation of either the furan or the pyrone double bond. An intercalated psoralen which is adjacent to a pyrimidine base (thymidine) can form cyclobutyl type monoadducts (Figure 9) by a photocycloaddition reaction. The furan

![Diagram showing structures of psoralen photoadducts](image)

Figure 9: Structures of psoralen photoadducts.

monoadducts can absorb a second UV photon leading to a second four-center photocycloaddition at the pyrone end of the molecule and thus the formation of a diadduct
or cross-link (figure 9). However, pyrone monoadducts do not absorb in the UV region and hence can not form a cross-link with further UV irradiation.

Interstrand cross links can be formed in double stranded DNA with psoralens and this properties of psoralens has been exploited to investigate the structure of nucleic acids.\textsuperscript{30,38} Psoralens such as 3-carbethoxypsoralen (3-CPs) can not form a cross-link with DNA.\textsuperscript{37} One of the reactive sites in 3-CPs is blocked hence it can only form monoadducts with DNA.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{jablonski_diagram}
\caption{Jablonski energy diagram. This energy diagram indicates the relationship between ground and excited states. (Reference 37)}
\end{figure}
Based on molecular orbital calculations, the 3,4 bond of psoralen is the most reactive site. The greatest electron density is localized at this position. Thus, the 3,4 bond is the most reactive towards free pyrimidines in solution. In the case of double stranded DNA, the 4',5' adduct forms most rapidly and not the 3,4 adduct. This difference is attributed to the geometry of psoralen with respect to the pyrimidine when it is intercalated in DNA. But it is also possible that when psoralens are intercalated in DNA, reaction of singlet oxygen, which is predicted to react preferentially at the 4',5' bond may occur due to the close association of the psoralen and pyrimidine. Thus, when AMT is bound to DNA, the triplet state can not be detected due to depletion of the singlet state as a result of quenching by DNA.

The lethal effects of psoralens and UV light in microorganisms as well as viruses have been attributed to the modification of DNA. Although it is easy to see why DNA-binding is most often cited as the crucial reaction responsible for the bioeffects of psoralens, the possibility exists that some of the observed bioeffects may arise from other types of lesions. It has been shown that psoralens form adducts with proteins, amino acids and olefins. There is efficient photochemical formation of covalent adducts between unsaturated fatty acids and psoralen. The formation of adducts between a fatty acid and psoralen proceeds with a quantum yield comparable to that of psoralens and DNA. This phenomenon suggests that psoralens bind to fatty acids in cells probably at least as much as they bind to DNA. So, which of the two reactions, addition to nucleic acids or to lipids, is responsible for viral inactivation and tumor necrosis? It is suggested that the more hydrophobic psoralens, such as TMP, may associate more readily with the relatively hydrophobic environment of lipid membranes, while the planar, positively charged psoralen, AMT, might bind strongly to DNA.

Various bioeffects have been associated with the formation of DNA monoadducts and DNA cross-linking by the cycloaddition of psoralens to pyrimidines and the production
of singlet oxygen and triplet excited states. The mechanistic roles of monoadducts and cross-links of psoralen and UV light have not yet been clearly demonstrated. The quantum yield of cycloaddition varies from psoralen to psoralen depending on the substituents (Table 1). The type of psoralen photoadduct and its yields and distribution depend on the number and nature of the substituents on the tricyclic ring system. The addition of methyl groups results in dramatic enhancement in the DNA binding constant. It has also been shown that the addition of just a single methyl group can affect the relative reactivities of the 3, 4-pyrone double bond and the 4, 5'-furan double bond. As mentioned earlier, the larger binding constant of TMP to DNA is attributed to its three methyl groups. Of all the psoralens known to date, AMT has the greatest DNA binding constant. Although AMT interacts more strongly with DNA than TMP, it formed fewer cross-links suggesting that not every intercalation site is a potential cross-linking site. It has been shown that the photoreaction of psoralen with nucleic acid can occur far beyond the loss of inactivation. The formation of photoadducts and crosslinks in the DNA of intact HSV-1 is fifty times more than that required for inactivation.

The photobiological effects which results from photoactivation of a biomolecule depend in large part on the lifetime and the nature of the photoexcited state and the structural orientation of the activated species. The singlet state of an excited psoralen is very short lived (nanoseconds), thus the ground state may be repopulated before the photoexcited species encounters a suitable substrate for a reaction. When an intercalated psoralen is photoexcited to the short lived excited singlet state, it can immediately undergo photocycloaddition to the 5, 6-bond of pyrimidine. It is possible that the triplet state may also lead to the formation of photoadduct.
Some bioeffects of psoralens are oxygen dependent indicating the importance of the photooxidation reactions. It has been shown that the lethal effect of 3-carbethoxypsoralen (3-CPs) (Figure. 8) in yeast is reduced in the absence of oxygen$^{57}$ suggesting that 3-CPs is a potent source of singlet oxygen in aerobic solution and it is relatively slow to form nucleic acid adducts. However, not all psoralens show the same oxygen dependence. The greater bacteriocidal activity of 8-MOP in the absence of oxygen suggest that bacterial killing is due primarily to reactions that do not involve oxygen (Table 2).$^{58}$

The role of photooxidation however is not easily explained by the dependence of oxygen alone. Removal of oxygen demostrates that the formation of singlet oxygen is not required for increase in the rate of psoralen plus light induced killing of E. coli.$^{58}$ However, it is not a conclusive evidence that only an oxygen dependent mechanism is involved in the presence of oxygen. It is also possible for the chemical mechanism to change with reaction conditions. The quenching of a triplet state of an aromatic by oxygen often produces singlet oxygen in high yield$^{60}$ and it decreases the chances that the psoralen triplet state can take part in any other reaction.
Table 2: Effects of oxygen on the photoactivity of psoralens:

<table>
<thead>
<tr>
<th>Psoralen</th>
<th>Host</th>
<th>O2 effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-CPs</td>
<td>Yeast57</td>
<td>+</td>
</tr>
<tr>
<td>8-MOP</td>
<td>E. coli58</td>
<td>-</td>
</tr>
<tr>
<td>8-MOP</td>
<td>λ phage59</td>
<td>-</td>
</tr>
</tbody>
</table>
Anthracene (Figure 11) is a planar tricyclic aromatic molecule and thereby makes it an ideal nucleic acid intercalator. Historically, anthracenes have not been used as photocleavage agents or antiviral agents probably because of their mutagenicity and low solubility in water. Furthermore, bromoaromatic compounds are hydrophobic and an environmental problem. Photoinduced dehalogenation reactions of bromoanthracene have been studied. The data suggests that the photolysis of bromoanthracene gives rise to anion radicals of solute molecules in the presence of amines by the formation of exciplexes between the lowest excited singlet state of anthracene and the amines. Furthermore, this radical anion fragments to form aromatic radicals and halogen atoms (scheme 3).

\[
\begin{align*}
9\text{-bromoanthracene} + \text{Donor (amine)} & \xrightarrow{h\nu} \text{(radical anion)} \\
& \rightarrow \text{(aromatic radical)} + \text{Br}^- + \text{Donor}^{++}
\end{align*}
\]

Scheme 3
Dyes:

Xanthene dyes are traditionally used as tracing agents in water pollution and aerial pesticide spraying studies; as coloring agents in drugs, cosmetics, textiles and inks; and as laser dyes. Cationic xanthene dyes accumulate in the mitochondria of living cells and are generally non-toxic even at high concentrations. Some of these dyes are selectively cytotoxic to cancerous cells. Studies have shown that cationic dyes photosensitize the inactivation of viruses and bacteria (Table 3). Living organisms have been used to demonstrate the photodynamic action of these dyes. It is believed that DNA is the target of photosensitization for many dyes, such as acridine orange and proflavin, by means of intercalation. Furthermore, these dyes may accumulate in the viral membrane as well as bind externally, in the major and minor grooves of DNA in addition to intercalation.

Table 3: Photosensitization of microorganism by cationic dyes.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Microorganism Photosensitized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>Actinophage, E. coli, VSV</td>
</tr>
<tr>
<td>Acridine</td>
<td>E. coli</td>
</tr>
<tr>
<td>Rose Bengal</td>
<td>HSV-1</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Actinophage</td>
</tr>
</tbody>
</table>

The mechanism of photosensitized degradation of DNA by these dyes is the focus of much research today. Early studies have found that positively charged heteroaromatic dyes degraded DNA in solution in the presence of light and oxygen. It is concluded that guanine is the DNA base most susceptible degradation upon photosensitization by the dye.
probably because it is most easily oxidized. It is speculated that photocleavage of DNA occurs by one of three mechanisms: electron transfer, hydrogen abstraction or reaction with \( ^1\text{O}_2^* \). Electron transfer seems possible because the lowest singlet and triplet states of the dyes have been shown to act as good electron acceptors and guanine is the most easily oxidized DNA base. It is feasible that the triplet state or radical derived from the dyes can undergo a hydrogen abstraction reaction. Singlet oxygen-initiated cleavage requires the efficient formation of the triplet excited state of the dyes while bound to DNA.

**Methylene Blue:**

Methylene blue (MB) (Figure. 4) was first shown by Simon and Van Vunakis in 1962 to photosensitize the decomposition of DNA.\(^{71}\) MB has also been reported to inactivate E. coli upon exposure to visible light.\(^{66}\) Furthermore, it has been reported that MB can photosensitize the bacteriophage, actinophage.\(^{65}\) It has been shown that MB can generate base specific modification of nucleic acids at the guanine residues.\(^{72}\) Guanosine monophosphate (GMP) was more efficiently degraded than any other nucleotide in solution. It has been also shown that MB preferentially intercalates next to a G-C base pair rather than to an A-T base pair.\(^{73}\)

Many mechanisms have been proposed for methylene blue photosensitization of DNA. It is still not clear which of these proposed mechanisms best explains the bioeffects of methylene blue observed upon illumination with visible light. MB is a very effective sensitizer of singlet oxygen (\(^1\text{O}_2\)) with a reported quantum yield of singlet oxygen formation of greater than 0.55\(^{74}\) suggesting that the destruction of nucleic acids can occur by a singlet oxygen mediated mechanism. The formation of singlet oxygen occurs by energy transfer between the triplet state of MB and oxygen. It has been shown that a direct MB-substrate reaction is also responsible for the photochemical damage.\(^{74}\)
Kelly (1987) has observed that binding of MB to DNA or poly [d (G-C)] strongly quenches the formation of the triplet state. As mentioned earlier, since the cleavage occurs specifically at guanine residues, the data suggests that the photosensitization may involve direct electron transfer from guanine to the singlet state of methylene blue in the first step of the cleavage reaction.

**Fluorescein, Rose Bengal and Eosin Y:**

Water soluble dyes of the xanthene group include fluorescein (FL), rose bengal (RB) and eosin Y (EY) (Figure 2). These dyes form a homologous series containing a non-halogenated control (FL) and brominated (EY) and iodinated derivatives (RB). RB has often been used in aqueous solution as a photochemical source of $^1\text{O}_2^*$. The photosensitization of DNA by RB probably does not involve singlet oxygen since in the absence of oxygen, or in the presence of singlet oxygen quenchers photolysis did not affect the yield of strand breaks in DNA. Furthermore, other studies have shown that there is an increase in the yield of single strand breaks as the oxygen concentration was decreased. It is possible that rose bengal triplet, or a radical derived from it, reacts directly with DNA in the absence of oxygen (Scheme 4). However, a different mechanism must be responsible in the presence of oxygen to explain the quenching effect of oxygen.

\[
\begin{align*}
\text{RB} & \xrightarrow{hv} \text{RB}^*^1 & \text{RB}^*^1 & \xrightarrow{hv} \text{RB}^*^3 \\
\text{RB}^* + \text{D}^+ & \xrightarrow{} \text{RB}^- + \text{D}^* \\
\end{align*}
\]

*Scheme 4*
RB has also been used in the diagnosis of external ocular diseases. In vitro studies have demonstrated that RB inhibits bacterial and viral polymerases and is cytotoxic to some bacteria and protazoa. The antiviral activity of RB has been demonstrated against herpes simplex type 1 (HSV-1) in the absence of light. The non-halogenated control, fluorescein had minimal antiviral effect. The exact mechanism of the non-photochemical antiviral properties of rose bengal has not yet been established.

Eosin Y has received even less attention than RB in the area of viral inactivation. Eosin is found to be an excellent sensitizer for viral inactivation in this work along with rose bengal. Since these dyes are lipophilic molecules, they are expected to bind to viral membranes and to damage the virus upon photolysis or exposure to X-rays. Eosin Y has been used only once previously for the purpose of viral inactivation to inactivate a bacteriophage, actinophage.
Our interest has been focused on halogenated intercalators and halogenated fluorescein dyes which produce reactive species upon photolysis/radiolysis and which promote strand breaks in DNA that reduce the activity of model phages. It occurred to us that light initiated generation of a free radical center in a DNA intercalating molecule complexed to DNA would be an efficient strategy of creating strand breaks and inducing viral inactivation. Aromatic type radicals can be easily generated from iodinated and brominated precursors upon photoactivation either by direct homolysis or by an electron transfer sensitized fragmentation mechanism (Scheme 2).

**Direct homolysis**

\[
\text{Ph-Br} \xrightarrow{\text{hv}} \text{Br} + \text{Ph}^* \quad \text{RH} \rightarrow \text{Ph} - \text{H} + \text{R}^*
\]

**Electron transfer**

\[
\text{Ph-Br} + \text{Donor} \xrightarrow{\text{hv}} \text{Ph}^* \text{Br}^- + \text{Donor}^* \\
\quad \text{Ph}^* + \text{Br}^- + \text{Donor}^*
\]

Scheme 5
It is well established that aryl bromides and aryl iodides undergo light induced homolytic cleavage to generate an aryl radical and a halogen atom. Photolysis of bromobenzene (Ph-Br) forms the phenyl radical (Ph·) which is one of them most reactive of all organic radicals. Benzene has a C-H bond dissociation energy of 111.0 Kcal/mol which is comparable to that of water (H-OH BDE=119.3 Kcal/mol). Thus the phenyl radical should approach the reactivity of hydroxyl radical towards the C-H bonds of typical hydrocarbons (RH) and sugars. In the presence of electron donors aryl halides can undergo light induced electron transfer reactions. The resulting aryl halide anion will dissociate to form an aryl radical and a halide ion.

The best precedent of the halogen effect involving photolysis has been provided by early studies of the photochemistry of bromouracil (Figure 12). It has been shown that the radiosensitization by bromouracil is dependent on its incorporation into cellular DNA in place of thymine. Substitution of bromouracil for thymine in DNA of a variety of organisms has been shown to increase the yield of radiation induced DNA strand breaks. The incorporation of 5-bromouracil in the DNA of bacteria photosensitized cell necrosis upon UV illumination. The types of damage which are frequently evoked as potentially lethal events in irradiated cells are breaks in the polynucleotide strands of the DNA double helix, either as a break in only one strand (single break) or as nearby adjacent breaks in both strands of the double helix. The formation of strand breaks is a readily detected manifestation of cellular DNA damage.
It was anticipated that bromouracil would substitute for thymine in the DNA of *E. coli*. Since the Van der Waals radii of bromine (1.95 Å) is comparable to the thymine methyl group (2.0 Å), bromouracil can be readily incorporated into DNA. Circular dichroism and X-ray diffraction studies have shown that the introduction of the halogen atom does not appreciably alter the overall DNA conformation. Photolysis of bromouracil at 254 or 313 nm leads to carbon-bromine bond homolysis.

It was established that the reactive species generated was the aromatic radical and the major photochemical product from the photolysis of 5-bromouracil is uracil. The uracylyl radical, formed from the photoinduced dehalogenation, can readily extract a hydrogen atom from the ribose ring to give uracil as a product. Oxygen in solution did not reduce the yield of uracil. Also, the addition of thiol compounds during irradiation did not effect the yield of uracil.

The effect of bromouracil substitution for thymine and the subsequent photosensitization have been the subject of many studies. The incorporation of 5-bromouracil into bacterial cells and viruses caused an increased sensitivity to UV light and X-rays. Irradiation with UV light led to increased mutagenicity and lethality to the cells relative to unsubstituted DNA.90
The aim of this study was to develop a new protocol for the sterilization of blood products. The approach taken in this study was to design a sensitizer which would either bind to the viral nucleic acids and/or would accumulate in the viral membrane. Halogenated water soluble psoralens and anthracenes were synthesized and shown to bind tightly to DNA by the ethidium bromide fluorescence quenching assay. The photocleavage products of the supercoiled DNA, pBR322, at 350 nm using these sensitizers were analyzed by gel electrophoresis. The ability of these sensitizers to inactivate λ and φ6 phages upon photolysis at 350 nm was demonstrated using the plaque forming assay. The photocleavage and the viral inactivation experiments were run in the presence and the absence of oxygen and dithiothreitol in an attempt to gain insight into the active photobiological intermediate.

The halogenated fluorescein dyes, rose bengal and eosin Y, are activated by visible light (528 nm) and X-rays and were shown to inactivate λ and φ6. Viral inactivation by FL, RB and EY were performed in the presence and the absence of oxygen. The cationic xanthene dye, methylene blue, was used to inactivate λ phage at 658 nm. The experiments were performed in frozen samples and in the presence and the absence of oxygen and histidine to gain insight into the mechanism of inactivation by methylene blue.
CHAPTER II

RESULTS AND DISCUSSION

As stated earlier, the aim of this study is to identify conditions which will allow light (visible and UV) and X-ray promoted inactivation of model viruses present in human blood products, without compromising the function and the structural integrity of the blood products; red blood cells, platelets and plasma proteins.

The impetus and precedent for this study derived from work done over 3000 years. The Egyptians, Chinese and the Indians have been using photosensitization for over 3000 years in attempts to cure such disorders as vitiligo, psoriasis, skin cancer and psychosis. Early studies in 1900 by Tappeiner discovered that low concentrations of dyes, such as acridine, were able to photoinduce the rapid killing of paramecium, in the presence of light and oxygen. The presence of light and oxygen were necessary requirements in order to obtain photosensitization by acridine. The word "photodynamic" was introduced by Tappeiner to distinguish this new phenomenon. Now, this word has been extended to "photodynamic therapy" by clinicians.

The photodynamic mechanism was soon reported to occur in most kinds of biological systems, including plants, animals, cells, viruses and specifically to molecules such as enzymes, toxins and proteins. Oginsky et al. (1959) showed that
8-methoxypsoralen photosensitized the killing of bacteria\(^9\) and later Mathews (1963) obtained similar results in the absence of oxygen.\(^{94}\)

There exist a variety of molecules which are photosensitizers in biological systems. Some of these molecules are naturally occurring such as chlorophylls, porphyrin derivatives, metallophthalocyanines, acridine dyes, anthraquinone dyes, xanthene dyes, tetracylenes, psoralens and chloropromazines to mention just a few. Currently, the search for ideal sensitizers which will better respond to present and future medical requirements, is the subject of much research.

One class of naturally occurring photosensitizers, the psoralens, have been used in many forms since the beginning of mankind. However, the structure and function of psoralens have only been revealed in the last 40 years. Psoralen derivatives are now used as photochemotherapeutic agents as well as in their traditional use for the treatment of a variety of dermatological conditions.

Several psoralen derivatives have been since synthesized (Figure 8) and are used successfully in clinical cases. The psoralen derivatives have been shown by many researchers, with independent experiments,\(^{31-33}\) to bind non-covalently to DNA by intercalation. Psoralens require activation by UV light for their therapeutic effect. Upon activation with light the double bonds in the periphery of the psoralen molecule undergo consecutive 2+2 cycloaddition reactions with the thymine and uracil moieties of nucleic acids. This leads to effective cross linking of nucleic acid strands when the site of psoralen intercalation contains two uracils or two thymines on different strands of the double helix. All experiments suggest that psoralens damage DNA primarily by the formation of photoadducts (Figure 9) with DNA.

The magnitude of the binding constant of the psoralens with DNA determines the amount of drug that can interact with DNA and ultimately the formation of photoadducts. In comparing the psoralen derivatives known to date, AMT (Figure 8) has the best affinity
for DNA. AMT contains three methyl groups on the nucleus of psoralen molecule enhancing the hydrophobicity. Furthermore, it also contains an ionized moiety (\(-\text{NH}_3^+\)) which significantly improves its water solubility. In addition to improving the water solubility, the ammonium group also enhances the binding of AMT to DNA. Under physiological conditions, AMT is positively charged, hence it has a strong affinity toward the electronegative DNA.

It is possible to damage DNA with free radicals. The abstraction of a hydrogen atom from a sugar C-H bond initiates a series of reactions which leads to DNA strand breaks.\(^{95}\) It occurred to us that light initiated generation of a free radical center on a DNA intercalating molecule such as psoralen complexed to DNA would be an efficient strategy of viral inactivation. It has been well established that aromatic type radicals can be easily generated from brominated and iodinated precursors upon photoactivation either by direct bond homolysis\(^{83}\) or by an electron transfer sensitized fragmentation mechanism\(^{84}\) (Scheme 2). The resulting phenyl type radical is one of the most reactive of all organic radicals\(^{96}\) Thus, the phenyl radical can abstract hydrogen atoms from the sugar moiety of the DNA or RNA in much the same way as the hydroxyl radical (Scheme 1).

In addition to psoralens, we also wish to study anthracenes. Anthracene, like psoralen, is a planar tricyclic aromatic molecule making it an ideal nucleic acid intercalator. Historically, anthracenes have not been used as photocleavage agents or antiviral agents probably because of their mutagenicity and low solubility. It is our hope that the anthracene derivatives will serve as model sensitizers.

This has led us to synthesize water soluble psoralens, anthracenes and their halogenated analogues. These sensitizers contains a nucleic acid intercalator, such as a psoralen or anthracene unit; a polyammonium tether for increased water solubility and halogens, chlorine, bromine or iodine as the radiation sensitizer. The binding of these sensitizers to DNA, \(\lambda\) phage and \(\phi 6\) virus were determined by the ethidium bromide
fluorescence quenching assay. The cleavage products of the plasmid DNA pBR322 produced by photolysis at 350 nm of these sensitizers were analyzed by gel electrophoresis. The ability of these compounds to inactivate model phages, λ and φ6, upon illumination with 350 nm light were analyzed by the plaque forming assay. The results obtained from this study has led to the evaluation of these sensitizers in clinical platelets concentrates by Cryopharm Corporation.

Inactivation of viruses by UV light in the present of platelets is an acceptable approach, since UV light alone does not damage the platelets. However, this approach can not be used with red blood cells because of the optical properties of hemoglobin. Hemoglobin is so intensely absorbing in the UV region that it will absorb all of the incident UV light, hence preventing activation of the sensitizer. Therefore, other sensitizers were selected which can be activated by other radiation sources such as visible light and/or by X-rays.

We have also used fluorescein, rose bengal, eosin Y and methylene blue dyes (Figure 2+3). These dyes can be activated by visible lights and/or by X-rays. It is possible that these dyes may selectively bind to viral membranes and damage the virus upon illumination with visible light or X-rays. FL, RB and EY form a homologous series containing a non-halogenated control (FL) and brominated (EY) and iodinated (RB) derivatives. FL, RB and EY were activated by visible light (528 nm) and X-ray to inactivate λ phage and φ6 virus. Viral inactivation of λ phage using methylene blue was performed with 658 nm light.
A) Synthesis:

PSR-H was synthesized using literature procedures (Scheme 5).\textsuperscript{97,98}

\begin{align*}
\text{8-Methoxypsoralen (8-MOP)} & \xrightarrow{\text{BBr}_3} \text{OH} \\
& \xrightarrow{\text{BrCH}_2\text{CH}_2\text{CH}_2\text{Br}}
\end{align*}

Scheme 6

8-methoxypsoralen was deprotected using two equivalents of boron tribromide.\textsuperscript{97} The resulting hydroxyl group was alkylated using 1,3-dibromopropane.\textsuperscript{98} The nucleophilic substitution reaction of the resulting alkyl bromide with diethylamine was performed.\textsuperscript{98}

The resulting amine was converted to the corresponding hydrochloride salt using concentrated HCl.

PSR-Br was synthesized by brominating at the 5-position of 8-MOP with bromine at room temperature (Scheme 6). Then, deprotection of the methoxy group to hydroxyl, alkylation, nucleophilic substitution and the formation of the amine salt was performed in
an identical manner to PSR-H. PSR-I was synthesized by first iodinating at the 5-position of 8-MOP using the procedure of Hendel et al. 8-MOP was treated with silver trifluoroacetate and then with iodine at RT to give 5-iodo-8-methoxypsoralen. Again, the deprotection, alkylation, substitution with diethylamine and the formation of the amine salt were performed as described for PSR-H.

Scheme 7

A-H was synthesized using the procedure of Van Armin and Czarnik (Scheme 7). 9-Methanolanthracene was converted to the corresponding methyl bromide using concentrated HBr at room temperature. The nucleophilic substitution reaction with diethylenetriamine was performed in toluene. The resulting polyamine was converted to the corresponding salt using conc. HCl.
Scheme 8

A-Br was synthesized by brominating 9-methylanthracene using a literature procedure (Scheme 9). 9-Methylanthracene was brominated with cupric bromide. The resulting 10-bromo-9-methylanthracene was converted to 10-bromomethylanthracene using N-bromosuccinimide (NBS). The nucleophilic substitution reaction with diethylenetriamine and the formation of the corresponding salt were performed in exactly the same manner as A-H. A-Cl was synthesized in the same fashion as A-Br but with one difference. 9-methylanthracene was converted to 10-chloro-9-methylanthracene using cupric chloride instead of cupric bromide.
Scheme 9

Scheme 10
A-Br₂ was synthesized (Scheme 10) by treating 2-methylantracene with bromine to give tribromoantracene (9,10-dibromo-2-bromomethylantracene). The nucleophilic substitution reaction with diethylamine and the formation of the corresponding salt were done in a manner identical to A-H.

A-I was synthesized by first treating antracene with mercuric oxide and then with trichloroacetic acid and the resulting product was treated with iodine to give iodoantracene (Scheme 11). Treatment of iodoantracene with chloromethyl methyl ether at RT to give 10-iodo-9-chloromethyl antracene. The nucleophilic substitution reaction and the formation of the corresponding salt were performed as described above.

Scheme 11
B) Binding of Sensitizers to DNA, λ and φ6 virus:

After synthesizing the psoralen and anthracene derivatives, it was necessary to show that these sensitizers bind to nucleic acids. The relative binding constants of the synthetic psoralens and anthracenes to calf thymus double stranded DNA, λ phage and φ6 virus were determined by fluorescence titration using the technique of Hansen. The assay takes advantage of the increased yield of fluorescence of ethidium bromide in the presence of DNA. The sensitizer competes with ethidium bromide for intercalative binding and thereby reduces the fluorescence of ethidium bromide.

The nature of the interaction of ethidium bromide with nucleic acids has been examined by a variety of techniques over the last 25 years. It is generally recognized that the strong mode of binding of ethidium bromide to double-stranded nucleic acids results in the intercalation of the planar phenanthridine ring between adjacent base pairs on the double helix. Direct evidence for the intercalation of ethidium into nucleic acids has been obtained from studies of the solution complexes of ethidium with deoxydinucleotide and ribodinucleoside monophosphates, as well as subsequent X-ray crystallographic studies of ethidium complex with 5-iodo U-A and 5-iodoG-C in the solid state. Furthermore, it has been shown that ethidium bromide preferentially binds to pyrimidine-purine sites rather than purine-pyrimidine sites.

Ethidium bromide in aqueous solution exhibits very little fluorescence. However, the fluorescence of ethidium bromide increases dramatically upon binding to nucleic acids. The increase in the fluorescence intensity of ethidium bromide when intercalated into double-helical nucleic acids is accompanied by a significant increase in the fluorescence lifetime. The fluorescence lifetime of ethidium bromide increases from 1.8 ns for free ethidium to approximately 23 or 19 ns for the complex of ethidium bromide with double stranded DNA or RNA, respectively. It is generally accepted that these lifetimes...
correspond to intercalated ethidium bromide. The mechanism for the enhancement of ethidium fluorescence upon intercalation involves a reduction in the rate of excited-state proton transfer from ethidium amino groups to solvent molecules.\textsuperscript{109} Although ethidium bromide does not have a requirement for any particular base in binding to DNA\textsuperscript{110}, it does show a definite binding preference in binding to pyrimidine-purine sequences as mentioned earlier.

Previous studies have shown that psoralen and its derivatives form intercalated complexes with DNA.\textsuperscript{31-33} Intercalation of our synthetic psoralens and anthracenes were established by an indirect method utilizing competition studies. In the competition studies, our sensitizers compete for binding to DNA with ethidium bromide. The competitive binding of ethidium bromide and other intercalators with DNA has become a convenient method of determining binding constants of the intercalators to DNA. Hansen et. al has determined the binding of acridine-psoralen amines using their competitive binding with ethidium bromide.\textsuperscript{9}

Therefore, we have determined the binding constants of our synthetic psoralens and anthracenes relative to ethidium bromide. The binding constants of our sensitizers are summarized in Table 4. As mentioned earlier, etBr in aqueous solution has very little fluorescence. However, the fluorescence of etBr increases significantly upon binding to DNA. This dramatic enhancement in fluorescence of etBr is quenched by the addition of our sensitizers at known concentrations (Figure 14). A decrease in etBr fluorescence in the presence of DNA was observed upon increasing the concentration of our sensitizers.
Figure 13: Changes in fluorescence spectrum of etBr upon addition of A-H.
Samples were prepared for the determination of binding constants as discussed in the Experimental section. Control samples containing only etBr and another sample containing etBr and DNA were always maintained. After incubation at RT overnight, the etBr fluorescence (λex 540nm, λem 610 nm) was measured. The fluorescence intensity at 610 nm of each sample was measured. The fluorescence intensity of free ethidium bromide was subtracted from each sample. The fluorescence intensity of the sample containing only etBr and DNA was taken to be 100% fluorescence. The percent fluorescence of all the samples were calculated relative to this 100% fluorescence. The concentrations of the sensitizers in μg/mL were then plotted against the percent fluorescence (%Fluor) to obtain the 50% "quenching" concentration (Figure 14), corresponding to the replacement of 50% of the etBr in the DNA by the added sensitizer. 50% "quenching" is defined as a 50% reduction of the fluorescence of bound etBr, relative to uncomplexed etBr, which is only slightly fluorescent. Since the concentration of the etBr, is known, the relative equilibrium constants of the sensitizers were calculated using the following equation.

\[ K_{rel} = K_{etBr} [etBr] [sensitizer at 50% fluorescence]^{-1} \]

where \( K_{etBr} = 1.5 \times 10^5 \text{ M}^{-1} \) (Ref. 9)

Examining the binding constants presented in Table 4, among the psoralen derivatives, PSR-Br(PIP) binds the tightest to the DNA (the larger the number, the tighter is the binding to DNA). Furthermore, the addition of a halogen atom dramatically improves the binding. PSR-Br binds slightly better than PSR-I. But both PSR-Br and PSR-I bind at least a thousand times better to the DNA than does PSR-H, which binds very weakly to the DNA (Table 4). The psoralens containing the ether side chains, PSR-H(Ether) and PSR-Br(Ether), were not soluble enough to determine their binding constants. These compounds precipitated upon overnight incubation at RT.

Of the anthracene derivatives, the dibromo derivative A-Br₂, binds most tightly to calf thymus DNA. The addition of a single halogen to the anthracene improves the binding
Figure 14: A plot of % Fluorescence vs. concentration (ug/mL) of A-H.
of the sensitizer to the DNA only slightly relative to the non-halogenated derivative, A-H. However, when there are two bromine substituents on the anthracene, the sensitizer binds DNA about ten times more tightly than does the monobromo substituent. The anthracene derivatives bind much better than the psoralen derivatives. It is not clear whether this is related to the fact that the anthracene bears three positive charges in the side chain whereas the psoralens are only mono and doubly charged. This is a concern because the doubly charged psoralen binds DNA about ten times more tightly than the singly charged compound. In addition to the positive charges, the addition of the halogens enhances the binding of the psoralen to the DNA dramatically. In the anthracene series, the addition of only one halogen just slightly enhances the binding to the DNA. However, the addition of two halogens, A-Br2, increases the binding at least ten times. It can be concluded that of all the synthetic sensitizers in this study, A-Br2 binds best to DNA. We suspect that these sensitizers bind to the DNA by a combination of intercalation of the aromatic moiety and of electrostatic interactions between the ammonium ion side chains and the phosphate backbone of the DNA.

Binding of the sensitizers to λ phage and to the φ6 virus were determined using the same assay utilized for DNA. The appropriate virus of known titre was added to the samples instead of the DNA. Values of 50% fluorescence "quenching" due to the presence of the sensitizer for λ phage and 10% fluorescence "quenching" for φ6 were determined and are reported in Table 4. It was not possible to obtain a binding constant for λ phage and the φ6 virus because the corresponding $K_{etBr}$ value is unknown. Thus, the results are reported in units of concentration (µg/mL) of the sensitizers. It is important to note that this assay measures total binding of etBr and sensitizer to hydrophobic regions of the virus and not just to viral DNA.

The 50% quenching concentration of the sensitizers with λ phage follows a trend similar to the DNA binding constants (Table 4). The concentration of PSR-Br required for
50% displacement is slightly less than PSR-I. However, both PSR-Br and PSR-I require about 22 times less than the amount of PSR-H to displace etBr from λ and φ6. The non-halogenated psoralens containing two positive charges, PSR-H(PIP) and PSR-Br(PIP), do not bind to λ phage at all. The ether side chains of PSR-H(Ether) and PSR-Br(Ether) are not sufficiently soluble to determine quenching concentrations. Of the anthracene derivatives, A-Br2 requires the least material to give 50% displacement of etBr from λ and φ6. Furthermore, there is not a dramatic difference between the non-halogenated control, A-H, and the mono-halogenated anthracene derivatives in terms of their binding to either virus (Table 4).

It is suspected that the sensitizers compete with ethidium bromide for binding to the viral DNA and to the hydrophobic regions of the viral protein capsid (λ) or lipid envelope (φ6) and thereby reduce the fluorescence of ethidium bromide. The results suggest that aromatic halogenation leads to an increase in the binding of the sensitizer to the λ phage. Halogenation substantially increases the binding of the psoralens to lambda phage relative to their non-halogenated controls. Factors of 19-22 fold in binding constant are realized in these systems. The psoralens with two positive charges are probably not able to penetrate through the protein capsid of lambda phage, hence there is no binding to viral nucleic acid. However, the anthracenes which contain three positive charges bind effectively to lambda phage. The ammonium ion side chains of the anthracenes are linear whereas the psoralens, with two positive charges, contain a cyclic amine. It is possible that this structural difference prevents the psoralens from binding to lambda phage. Furthermore, it is likely that the anthracenes are more hydrophobic than psoralen, hence they bind more effectively to the virus.

In a similar fashion the 10% quenching concentration of the synthetic psoralens and anthracenes were determined with φ6 (Table 4). Since the sensitizers bind so poorly to the φ6 virus, only a 10% quenching concentration was determined. The same trend are seen
<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>$K_B (M^{-1})^a$</th>
<th>$\lambda^{50%}(\mu g/ml)^b$</th>
<th>$\phi 6^{10%}(\mu g/ml)^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT</td>
<td>$1.86 \times 10^3$</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>PSR-H</td>
<td>$&lt;&lt;$</td>
<td>450</td>
<td>d</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>$6.02 \times 10^3$</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>PSR-I</td>
<td>$5.42 \times 10^3$</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>PSR-H(PIP)</td>
<td>$7.61 \times 10^2$</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>PSR-Br(PIP)</td>
<td>$4.89 \times 10^4$</td>
<td>d</td>
<td>37</td>
</tr>
<tr>
<td>PSR-H(Ether)</td>
<td>e</td>
<td>e</td>
<td>e</td>
</tr>
<tr>
<td>PSR-Br(Ether)</td>
<td>e</td>
<td>e</td>
<td>e</td>
</tr>
<tr>
<td>A-H</td>
<td>$1.74 \times 10^5$</td>
<td>18</td>
<td>0.21</td>
</tr>
<tr>
<td>A-Cl</td>
<td>$1.67 \times 10^5$</td>
<td>10</td>
<td>0.19</td>
</tr>
<tr>
<td>A-Br</td>
<td>$4.06 \times 10^5$</td>
<td>11</td>
<td>0.20</td>
</tr>
<tr>
<td>A-I</td>
<td>$5.03 \times 10^5$</td>
<td>11.5</td>
<td>0.20</td>
</tr>
<tr>
<td>A-Br2</td>
<td>$4.59 \times 10^6$</td>
<td>0.25</td>
<td>0.12</td>
</tr>
</tbody>
</table>

a) Binding to calf thymus DNA  
b) The amount of sensitizer needed to displace etBr from the $\lambda$ phage virus resulting in a 50% decrease in fluorescent intensity. The concentration of $\lambda$ was $10^9$ pfu.  
c) The amount of sensitizer needed to displace etBr from the virus resulting in a 10% decrease in fluorescent intensity. The concentration of $\phi 6$ was $10^{10}$ pfu.  
d) These sensitizers do not bind to the calf thymus DNA or to the viruses at all.  
e) These sensitizers precipitated out of solution upon incubation.
with φ6 as with λ phage. The anthracenes bind much better than psoralens to φ6. The two non-halogenated psoralens, PSR-H and PSR-H(PIP), do not bind to φ6 at all. The bromine substitution of the psoralens clearly increases the hydrophobicity of the sensitizers and they are better able to localize in hydrophobic portions of the φ6 virus.

C) **Photocleavage of plasmid DNA pBR322**:  

After determining the binding constants of our synthetic sensitizers, we examined the ability of these sensitizers to nick the supercoiled plasmid pBR322 DNA by gel electrophoresis. pBR322 is double stranded supercoiled DNA of approximately 4300 base pairs in length. The supercoiled form of the DNA is a twisted closed circular form. Damage to the DNA by a nick or cut relaxes the DNA superhelical structure. A single strand scission (nick) relaxes the supercoiled DNA to the circular DNA (Figure 15). A double strand break converts the DNA to the linear form. The different forms of the DNA can be resolved by agarose gel electrophoresis.

![Image](image.png)

**Figure 15:** Mechanism of formation of form II (open circular) and form III (linear) from nicks in form I (supercoiled) DNA.
Plate I: A photograph of photolysis at 350 nm of pBR322 DNA as a function of time.

Lanes:
Lane 1: DNA -hv
Lane 2: DNA +hv 1 min
Lane 3: DNA +hv 3 min
Lane 4: DNA +hv 5 min
Lane 5: DNA +hv 10 min
Lane 6: DNA +hv 15 min
Lane 7: DNA +hv 20 min
Lane 8: DNA +hv 30 min
The first set of photocleavage experiments were conducted with the synthetic psoralens. Solutions of pBR322 DNA with PSR-Br (3μg/mL), or PSR-H (3μg/mL) or without any sensitizer, were prepared and photolyzed at 30°C in a rayonet reactor equipped with 8 lamps with principal emission of 350 nm. Aliquots from the samples were removed after 3, 5, 10, 15, 20 and 30 minutes of photolysis and resolved by agarose gel electrophoresis at room temperature as shown in Plate II. The plate is divided into two tiers. In both tiers, lane 2 contains lambda DNA digested with the restriction endonuclease Hind III as a molecular weight marker. The band from top to bottom are fragments of DNA of: 23,000, 95,00, 2300 and 2000 base pairs, respectively.

The top lanes are as follows; lane 3=unphotolyzed DNA, lanes 5-11 contain PSR-Br:DNA corresponding to 1, 3, 5, 10, 15, 20 and 30 minutes of photolysis, respectively; lane 12=pBR322 DNA photolyzed for 30 minutes and lane 14=PSR-Br:DNA unphotolyzed. The bottom lanes are exactly as the top lanes with PSR-H instead of PSR-Br.

Lane 2 on the top and bottom contains DNA without any sensitizer and which has not been exposed to light. This lane contains two bands, one on the top which is extremely faint and represents the relaxed open circular form of the DNA which is a contaminant in the untreated pBR322. The lower, more intense band, is the supercoiled form of the DNA. Lanes 5-11 of the top series are DNA containing PSR-Br:DNA with increasing photolysis time. A slow progression in nicking from the supercoiled (SC) to the relaxed circular (C) form is observed with increasing photolysis time. After 15 minutes of photolysis (top lane 9), all of the supercoiled DNA has been converted to the relaxed circular form by PSR-Br as evident by the absence of the lower band corresponding to the supercoiled form of the DNA. PSR-Br:DNA before photolysis (top lane 14) does not do any damage in the dark to the DNA.
Lanes 5-11 on the bottom tier are the non-halogenated control and DNA mixtures, PSR-H:DNA, photolyzed for the same time intervals as per PSR-Br:DNA. After 15 minutes of photolysis of PSR-H:DNA (bottom lane 9), most of the DNA is in the supercoiled form. There is not much sensitized nicking of the DNA by PSR-H after 15 minutes of photolysis. Furthermore, 30 minutes of photolysis of DNA in the presence of PSR-H (bottom lane 12) does not significantly nick the supercoiled DNA.

Lane 14 both on the top and bottom tier contain DNA, without any sensitizer, photolyzed for 30 minutes as a control. Clearly light (\(\lambda=350\) nm) alone does not damage the DNA under these conditions as evident by the presence of the lower band corresponding to the supercoiled form of the DNA. As a control experiment, a reaction profile of the DNA photolyzed with 350 nm light was conducted. Plate I shows the results of this experiment. Lane 1 contains just the DNA before photolysis. Lanes 2-8 contain DNA, no sensitizer and after photolysis of 1, 3, 5, 10, 15, 20 and 30 minutes, respectively. It is clear from this experiment that light alone under these conditions does not damage the DNA.

In another set of experiments, the ability of PSR-Br and PSR-I to sensitize nicks of the pBR322 DNA upon photolysis with 350 nm light were compared. Four different solutions were prepared containing DNA:PSR-Br (3mg/ml), DNA:PSR-I (3 mg/mL), DNA:PSR-H (3\(\mu\)g/mL) and DNA without any sensitizer. The solutions were photolyzed as before for 1, 3, 5, 10 and 15 minutes. The photograph of the plate is shown in Plate III.

The top tier of lanes contain the PSR-Br samples with the appropriate controls and the bottom tier of lanes contain PSR-I samples with the same controls as the tier of lanes on the top. After 15 minutes of photolysis of plasmid DNA pBR322 in the presence of both PSR-Br and PSR-I (top and bottom lane 10, respectively), the supercoiled DNA is completely nicked and converted to the relaxed circular form. Therefore, PSR-Br and
Plate II: A photograph of the photocleavage of pBR322 DNA with PSR-H (3 μg/mL) and PSR-Br (3 μg/mL) at 350 nm.

<table>
<thead>
<tr>
<th>Top lanes:</th>
<th>Bottom lanes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: -</td>
<td>1: -</td>
</tr>
<tr>
<td>2: DNA marker</td>
<td>2: DNA marker</td>
</tr>
<tr>
<td>3: -</td>
<td>3: -</td>
</tr>
<tr>
<td>4: PSR-Br +hv, 1 min</td>
<td>4: PSR-H +hv, 1 min</td>
</tr>
<tr>
<td>5: PSR-Br +hv, 3 min</td>
<td>5: PSR-H +hv, 3 min</td>
</tr>
<tr>
<td>6: PSR-Br +hv, 5 min</td>
<td>6: PSR-H +hv, 5 min</td>
</tr>
<tr>
<td>7: PSR-Br +hv, 10 min</td>
<td>7: PSR-H +hv, 10 min</td>
</tr>
<tr>
<td>8: PSR-Br +hv, 15 min</td>
<td>8: PSR-H +hv, 15 min</td>
</tr>
<tr>
<td>9: PSR-Br +hv, 10 min</td>
<td>9: PSR-H +hv, 10 min</td>
</tr>
<tr>
<td>10: PSR-Br +hv, 20 min</td>
<td>10: PSR-H +hv, 20 min</td>
</tr>
<tr>
<td>11: PSR-Br +hv, 30 min</td>
<td>11: PSR-H +hv, 30 min</td>
</tr>
<tr>
<td>12: DNA +hv, 30 min</td>
<td>12: DNA +hv, 30 min</td>
</tr>
<tr>
<td>13: -</td>
<td>13: -</td>
</tr>
<tr>
<td>14: PSR-Br -hv</td>
<td>14: PSR-H -hv</td>
</tr>
</tbody>
</table>
Plate III: A photograph of the photocleavage of pBR322 DNA with PSR-Br (3 μg/mL) and PSR-I (3 μg/mL) at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: PSR-Br -hv
6: PSR-Br +hv, 1 min
7: PSR-Br +hv, 5 min
8: PSR-Br +hv, 7 min
9: PSR-Br +hv, 10 min
10: PSR-Br +hv, 15 min
11: -
12: PSR-I +hv, 15 min
13: DNA +hv, 15 min

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: PSR-I -hv
6: PSR-I +hv, 3 min
7: PSR-I +hv, 5 min
8: PSR-I +hv, 7 min
9: PSR-I +hv, 10 min
10: PSR-I +hv, 15 min
11: -
12: PSR-H +hv, 15 min
13: DNA +hv, 15 min
Plate IV: A photograph of the photocleavage of pBR322 DNA with PSR-Br(PIP) (3 μg/mL) and PSR-Br (3 μg/mL) at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: PSR-Br(PIP) -hv
5: PSR-Br(PIP) +hv, 2 min
6: PSR-Br(PIP) +hv, 4 min
7: PSR-Br(PIP) +hv, 6 min
8: PSR-Br(PIP) +hv, 8 min
9: PSR-Br(PIP) +hv, 9 min
10: PSR-Br(PIP) +hv, 12 min
11: PSR-Br(PIP) +hv, 15 min
12: PSR-Br(PIP) +hv, 18 min
13: DNA +hv, 18 min

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: PSR-Br -hv
5: PSR-Br +hv, 2 min
6: PSR-Br +hv, 4 min
7: PSR-Br +hv, 6 min
8: PSR-Br +hv, 8 min
9: PSR-Br +hv, 9 min
10: PSR-Br +hv, 12 min
11: PSR-Br +hv, 15 min
12: PSR-Br +hv, 18 min
13: DNA +hv, 18 min
Plate V: A photograph of the photocleavage of pBR322 DNA with PSR-Br(Ether) (3 μg/mL) and PSR-H(Ether) (3 μg/mL) at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: PSR-Br(Ether) -hv
6: PSR-Br(Ether) + hv, 3 min
7: PSR-Br(Ether) + hv, 7 min
8: PSR-Br(Ether) + hv, 10 min
9: PSR-Br(Ether) + hv, 15 min
10: PSR-Br(Ether) + hv, 20 min
11: PSR-Br(Ether) + hv, 30 min
12: -
13: DNA + hv, 30 min

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: PSR-H(Ether) - hv
6: PSR-H(Ether) + hv, 3 min
7: PSR-H(Ether) + hv, 7 min
8: PSR-H(Ether) + hv, 10 min
9: PSR-H(Ether) + hv, 15 min
10: PSR-H(Ether) + hv, 20 min
11: PSR-H(Ether) + hv, 30 min
12: -
13: DNA + hv, 30 min
Plate VI: A photograph of the effect of argon on the photocleavage of pBR322 DNA with PSR-Br (3 μg/mL) at 350 nm.

Top lanes:
1: -
2: -
3: DNA marker
4: -
5: -
6: PSR-Br -hv
7: PSR-Br +hv, 5 min
8: PSR-Br +hv, 10 min
9: PSR-Br +hv, 15 min
10: PSR-Br +hv, 20 min
11: PSR-Br +hv, 30 min
12: -

Bottom lanes:
1: -
2: -
3: DNA marker
4: -
5: -
6: DNA -hv
7: DNA +hv, 5 min
8: DNA +hv, 10 min
9: DNA +hv, 15 min
10: DNA +hv, 20 min
11: DNA +hv, 30 min
12: -
Plate VII: A photograph of the effect of DTT on the photocleavage of pBR322 DNA with PSR-H (3 μg/mL) and PSR-Br (3 μg/mL) with 25 min at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: DNA +hv
5: -
6: PSR-Br -hv
7: PSR-Br - DTT +hv
8: PSR-Br + DTT (.5M) +hv
9: PSR-Br + DTT (.1M) +hv
10: PSR-Br + DTT (.01M) +hv
11: PSR-Br + DTT (.001M) +hv
12: PSR-Br + DTT (.0005M) +hv
13: PSR-Br + DTT (.00001M) +hv

Bottom lanes:
1: -
2: DNA marker
3: DNA + DTT (0.05M) -hv
4: DNA + DTT (0.05M) +hv
5: -
6: PSR-H -hv
7: PSR-H - DTT +hv
8: PSR-H + DTT (.5M) +hv
9: PSR-H + DTT (.1M) +hv
10: PSR-H + DTT (.01M) +hv
11: PSR-H + DTT (.001M) +hv
12: PSR-H + DTT (.0005M) +hv
13: PSR-H + DTT (.00001M) +hv
PSR-I are nearly identical in their ability to sensitize the nicking of pBR322 DNA at equal concentrations by weight.

The sensitizing ability of the other synthetic psoralens, PSR-H(PIP), PSR-Br(PIP), PSR-H(Ether) and PSR-Br(Ether) were compared to that of PSR-Br at equal concentration by weight. Solutions of DNA:PSR-Br (3mg/ml) and DNA:PSR-Br(PIP) (3mg/ml) were prepared and photolyzed as before. The results of this experiment are presented on Plate IV.

The top lanes correspond to PSR-Br(PIP) and the bottom lanes correspond to PSR-Br. Lane 2, top and bottom, is the molecular weight marker and lane 3 is the DNA, without any sensitizer before photolysis. Lanes 5-12 of the top tier of lanes contain PSR-Br(PIP), after photolysis of 2, 4, 6, 9, 12, 15, and 18 minutes, respectively. After 15 minutes of photolysis in the presence of PSR-Br(PIP) (top tier of lanes 11), all of the supercoiled form has been converted to the relaxed circular form of the DNA. However, the bottom tier of lanes 5-12, which correspond to PSR-Br do not sensitize any nicking of pBR322 DNA under these conditions.

PSR-Br(PIP) sensitizer nicks the DNA much more efficiently than does PSR-Br. This enhancement in nicking is probably due to the tighter binding of PSR-Br(PIP) to the DNA. PSR-Br(PIP) binds at least ten times better to the DNA than does PSR-Br (Table 4). An important point that is necessary to mention here is that PSR-Br is shown on Plates II and III to nick the DNA to the relaxed form in 15 minutes. However, this is not seen on Plate IV. This is due to the fact that the UV bulbs get weaker after prolonged usage.

The polyether psoralen derivatives, PSR-H(Ether) and PSR-Br(Ether), were also tested for their ability to nick the plasmid DNA. Solutions of DNA:PSR-H(Ether) (3mg/ml), DNA:PSR-Br(Ether) (3μg/mL) and DNA, without any sensitizer, were prepared and photolyzed as above. The results are presented on Plate V. The top tier of lanes correspond to PSR-Br(Ether) and the bottom tier of lanes correspond to PSR-H(Ether).
The top tier of lanes 6-11 contain DNA:PSR-Br(Ether), after photolysis of 3, 7, 10, 15, 20 and 30 minutes, respectively. Even after 30 minutes of photolysis (top lane 11), PSR-Br(Ether) does not nick the DNA to a significant amount. Most of the DNA is still in the supercoiled form. The bottom tier of lanes 6-11 correspond to DNA:PSR-H(Ether), after photolysis of 3, 7, 10, 15, 20 and 30 minutes. There is no nicking with PSR-H as expected. The lack of photocleavage by PSR-Br(Ether) is due to its poor binding to the DNA.

Three additional control photocleavage experiments with PSR-Br were performed. First, the photocleavage of DNA with PSR-Br as sensitizer was performed in the absence of oxygen by bubbling the samples with argon before photolysis. The results of this experiment are presented on Plate VI. The top tier of lanes 7-11 contain PSR-Br:DNA in the presence of argon, after photolysis of 5, 10, 15, 20 and 30 minutes, respectively. The bottom tier of lanes 7-11 show the photolysis of the DNA, without any sensitizer, after photolysis of 5, 10, 15, 20 and 30 minutes, respectively.

The photolysis experiment in the absence of oxygen was performed to determine the effect of oxygen on the DNA nicking by the sensitizer. The goal was to determine if the scission chemistry is due to singlet oxygen or due to photolytically generated aryl radicals. It is evident from the results presented on Plate VI that the absence of oxygen did not inhibit photocleavage of the DNA by PSR-Br. Therefore, singlet oxygen is not the mechanism responsible for the photocleavage effect of PSR-Br. It is likely that photolytically generated aromatic radicals are damaging the supercoiled DNA.

The photocleavage experiment was conducted in the presence of a radical scavenger, dithiothreitol, DTT in an attempt to determine whether the reactive species is generated within the binding site of the DNA. Any radical species formed free in solution should be quickly quenched by DTT before damaging the DNA. If the reactive species is
generated while intercalated into the DNA, it should be protected from the scavenger and will still induce strand breaks.

Solutions of DNA:PSR-Br (3μg/mL), DNA:PSR-H (3μg/mL) and DNA, without any sensitizer present but in the presence of variable concentrations of DTT (0.5-1x10^{-4} M) were prepared. The samples were photolyzed for 25 minutes and the results are shown on Plate VII. The top lanes contain PSR-Br and the bottom lanes contain PSR-H. Top lane 2 is the molecular weight marker, lane 3, is the DNA before photolysis and lane 4 is the DNA after photolysis. Lane 6 contains PSR-Br without any DTT, before photolysis and lane 7 is plasmid DNA exposed to 25 minutes of photolysis. Lanes 8-13 of the top tier contain PSR-Br:DTT, 0.5, 0.1, 0.01, 0.001, 0.0005 M, respectively, after photolysis for 25 minutes. Similarly, the bottom lanes 8-13 contain PSR-H:DTT in the same concentrations as above after photolysis of 25 minutes.

It is clear from the results of Plate VII that even a million fold excess of DTT (0.1M, lane 9) does not inhibit nicking of the DNA by PSR-Br. Only at very high concentrations of DTT (0.5 M) is the DNA protected from nicking. Therefore, the aromatic radical generated photolytically is probably intercalated into the DNA. Very high concentrations of DTT (0.5M) may simply repair free radical damage to the DNA.

Lastly, photocleavage by PSR-Br was run in the presence of a competitive binder, spermidine. Spermidine binds to the backbone of the DNA by interacting with the negatively charged phosphate groups. Therefore, it is expected that spermidine would competitively inhibit binding of the sensitizers to the DNA and protect the DNA from photodegradation by the sesitizer.

Solutions of the DNA:PSR-Br with varying concentrations of spermidine (1.0-1x10^{-3} M) were prepared. The samples were photolyzed for 25 minutes at 350 nm at 30°C, and the results are presented in the bottom tier of lanes of Plate XIV. Bottom lane 2 contains the molecular weight marker. Lane 3 is the DNA before photolysis and lane 4 is
the DNA after photolysis. Lane 5 is DNA:spermidine (1M), before photolysis and lane 6 is this sample after photolysis of 25 minutes. Lane 7 contains DNA:PSR-Br, without any spermidine, before photolysis and lane 14 is after photolysis. Lanes 8-13 contain DNA:PSR-Br and varying concentrations of spermidine, 1.0, 0.5, 0.1, 0.05, 0.01 and 0.001 M, respectively. Spermidine (1.0M) completely protects the DNA from photocleavage by PSR-Br (lane 8) and 0.001 M of spermidine does not protect at all.

The psoralen photocleavage results are summarized in Table 5. Of all the synthetic psoralens, PSR-Br(PIP) photocleaves the plasmid DNA most efficiently. PSR-Br and PSR-I are about equally efficient in nicking the DNA and PSR-Br(Ether) does not damage the DNA to any significant amount under identical conditions. The trend observed for photocleavage among the psoralens could be due to their affinity for the DNA. PSR-Br(PIP) binds the DNA tightest and it also is the most efficient sensitizer for nicking. Furthermore, the absence of oxygen has no effect on the photocleavage of the DNA by PSR-Br. A million fold excess of DTT (0.1M) does not protect the DNA from photocleavage, but spermidine (10^{-3}M) does protect the DNA. These results suggest that aromatic radicals are generated within the DNA binding sites.

Photocleavage experiments using the synthetic anthracenes were also performed. Comparison of the different halogens, iodine, bromine, chlorine, dibromo and the non-halogenated anthracene, was demonstrated. An important difference between the psoralens and anthracenes is that the psoralens damage DNA by both free radical and cycloaddition mechanisms but the anthracene can only generate radicals. Therefore, the effect of argon, DTT and spermidine on the ability of A-Br to photodamage the DNA was also studied.

Solutions of DNA:A-I (50μg/ml), DNA:A-Br (50μg/mL), DNA:A-Cl (50μg/mL), DNA:A-H (50μg/mL) and DNA, without any sensitizer were prepared and photolyzed at 30°C in a rayonet reactor equipped with 8 lamps of output of 350 nm. The results are summarized on Plates VII-X. A comparison of photocleavage sensitized by A-I and A-Br
is presented in Plate VIII. The top tier of lanes correspond to A-I and the bottom lanes correspond to A-Br. The top tier of lanes 6-12 contain DNA:A-I, after photolysis of 2, 4, 6, 7, 8 and 9 minutes. After 8 minutes of photolysis (lane 11), A-I completely nicks the supercoiled DNA to the relaxed circular form. However, after 8 minutes of photolysis (bottom lane 11), A-Br only slightly nicks the DNA.

On Plate IX, the photocleavage of the DNA sensitized by A-Br and A-Cl is shown. The top tier of lanes correspond to A-Br and the bottom tier of lanes correspond to A-Cl. Top lanes 5-10 contain DNA:A-Br, after photolysis of 3, 6, 9, 15 and 18 minutes. A-Br completely nicks the supercoiled DNA to the relaxed circular form in 15 minutes (top lane 9). A-Cl does not nick the DNA to a significant amount after this time (bottom lane 9). Furthermore, even after 27 minutes of photolysis, A-Cl does not nick the DNA completely (Plate X, top lane 13). After 27 minutes of photolysis with A-Cl, approximately 60% of the DNA has been converted to the circular form. In addition, A-Cl and A-H are about equally effective in photodamaging the DNA (Plate XI).

A comparison of the ability of mono-halogenated (A-Br) vs. dihalogenated anthracene (A-Br\textsubscript{2}) to sensitize photodamage to plasmid DNA was performed. A solution of DNA:A\textsubscript{Br\textsubscript{2}} (25\mu g/mL), DNA:A-Br (25\mu g/mL) and DNA, without any sensitizer present was prepared and photolyzed as before. The results are displayed in Plate XI. The top tier of lanes correspond to A-Br\textsubscript{2} and the bottom tier of lanes correspond to A-Br sensitization. The top tier of lanes 6-13 are DNA:A-Br\textsubscript{2}, after photolysis of 2, 4, 5, 6, 7, 8, 9 and 10 minutes, respectively. A-Br\textsubscript{2} sensitization completely converts the DNA to the relaxed circular form only after 5 minutes of photolysis (top lane 8). However, A-Br sensitization at this time nicks the DNA to only a small extent under the same conditions (bottom lane 8).

The effect of argon was studied using A-Br. Solutions of DNA:A-Br (70 \mu g/mL) and DNA without any sensitizer were prepared and degassed by bubbling with argon. The two solutions were photolyzed as before and the results are presented on Plate XII. The
top tier of lanes correspond to A-Br and the bottom tier of lanes correspond to DNA. Lane 2 of the top tier and the bottom tier is as usual the molecular weight marker. Lane 4 (top tier) is the DNA:A-Br sample, before photolysis. The top tier of lanes 5-12 contain DNA:A-Br, after photolysis of 1, 2, 3, 4, 5, 6, 7 and 8 minutes. The photocleavage of the DNA sensitized by A-Br is not reduced in the absence of oxygen. A-Br nicks the DNA to the circular form just as efficiently in the absence of oxygen as in the presence of oxygen. In this experiment, the concentration of A-Br used is much higher than that used on Plates IX and X and the UV bulbs were new and brighter. Therefore, the time required to nick the DNA by A-Br is much shorter than on Plate XII than Plate IX.

Photocleavage experiments by A-Br were performed in the presence of varying concentrations of DTT and spermidine. DTT was again used to determine the nature of the reactive species responsible for the photodamage of the DNA. Solutions of DNA:A-Br, DNA:A-H and DNA, without any sensitizer, in the presence of varying concentrations of DTT (0.05-5x10^-5 M) were prepared. The three solutions were photolyzed for 18 minutes at 350 nm and the results are presented on Plate XIII. The top lanes correspond to A-Br and the bottom lanes correspond to A-H. Top lanes 7-13 contain DNA:A-Br with DTT, 0.05, 0.005, 0.001, 0.0005, 0.0001 and 5x10^-5 M, respectively. Likewise, the bottom lanes 7-13 contain DNA:A-H with DTT of the same concentrations as above.

As seen on Plate XIII, a thousand fold excess of DTT (top lane 10) does not protect the DNA from photocleavage by A-Br. Only at a very high concentration does DTT (0.05M) protect the DNA from photodamage (top lane 7). The results suggest that aromatic radicals which are generated photolytically are intercalated to the DNA.
Plate VIII: A photograph of the photocleavage of pBR322 DNA with A-I (50 μg/mL) and A-Br (50 μg/mL) at 350 nm.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: A-I -hv
6: A-I +hv, 2 min
7: A-I +hv, 4 min
8: A-I +hv, 5 min
9: A-I +hv, 6 min
10: A-I +hv, 7 min
11: A-I +hv, 8 min
12: A-I +hv, 9 min
13: A-H +hv, 9 min
14: DNA +hv, 9 min

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: A-Br -hv
6: A-Br +hv, 2 min
7: A-Br +hv, 4 min
8: A-Br +hv, 5 min
9: A-Br +hv, 6 min
10: A-Br +hv, 7 min
11: A-Br +hv, 8 min
12: A-Br +hv, 9 min
13: A-H +hv, 9 min
14: DNA +hv, 9 min
Plate IX: A photograph of the photocleavage of pBR322 DNA with A-Br (50 μg/mL) and A-Cl (50 μg/mL) at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: A-Br -hv
5: A-Br +hv, 3 min
6: A-Br +hv, 6 min
7: A-Br +hv, 9 min
8: A-Br +hv, 12 min
9: A-Br +hv, 15 min
10: A-Br +hv, 18 min
11: -
12: A-H +hv, 18 min
13: DNA +hv, 18 min
14: -

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: A-Cl -hv
5: A-Cl +hv, 3 min
6: A-Cl +hv, 6 min
7: A-Cl +hv, 9 min
8: A-Cl +hv, 12 min
9: A-Cl +hv, 15 min
10: A-Cl +hv, 18 min
11: -
12: A-H +hv, 18 min
13: DNA +hv, 18 min
14: -
Plate X: A photograph of the photocleavage of pBR322 DNA with A-Cl (50 µg/mL) and A-H (50 µg/mL) at 350 nm.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: A-Cl -hv
5: A-Cl +hv, 3 min
6: A-Cl +hv, 6 min
7: A-Cl +hv, 9 min
8: A-Cl +hv, 12 min
9: A-Cl +hv, 15 min
10: A-Cl +hv, 18 min
11: A-Cl +hv, 21 min
12: A-Cl +hv, 24 min
13: A-Cl +hv, 27 min
14: DNA +hv, 27 min

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: A-H -hv
5: A-H +hv, 3 min
6: A-H +hv, 6 min
7: A-H +hv, 9 min
8: A-H +hv, 12 min
9: A-H +hv, 15 min
10: A-H +hv, 18 min
11: A-H +hv, 21 min
12: A-H +hv, 24 min
13: A-H +hv, 27 min
14: DNA +hv, 27 min
Plate XI: A photograph of the photocleavage of pBR322 DNA with A-Br$_2$ (25 µg/mL) and A-Br (25 µg/mL) at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: A-Br$_2$ -hv
6: A-Br$_2$ +hv, 2 min
7: A-Br$_2$ +hv, 4 min
8: A-Br$_2$ +hv, 5 min
9: A-Br$_2$ +hv, 6 min
10: A-Br$_2$ +hv, 7 min
11: A-Br$_2$ +hv, 8 min
12: A-Br$_2$ +hv, 9 min
13: A-Br$_2$ +hv, 10 min
14: A-H +hv, 10 min
15: DNA +hv, 10 min

Bottom lanes:
1: -
2: DNA marker
3: DNA -hv
4: -
5: A-Br -hv
6: A-Br +hv, 2 min
7: A-Br +hv, 4 min
8: A-Br +hv, 5 min
9: A-Br +hv, 6 min
10: A-Br +hv, 7 min
11: A-Br +hv, 8 min
12: A-Br +hv, 9 min
13: A-Br +hv, 10 min
14: A-H +hv, 10 min
15: DNA +hv, 10 min
Plate XII: A photograph of the effect of argon on the photocleavage of pBR322 DNA with A-Br (70 μg/mL) at 350 nm.

Top lanes:
1: -
2: DNA marker
3: -
4: A-Br -hv
5: A-Br +hv, 1 min
6: A-Br +hv, 2 min
7: A-Br +hv, 3 min
8: A-Br +hv, 4 min
9: A-Br +hv, 5 min
10: A-Br +hv, 6 min
11: A-Br +hv, 7 min
12: A-Br +hv, 8 min

Bottom lanes:
1: -
2: DNA marker
3: -
4: DNA -hv
5: DNA +hv, 1 min
6: DNA +hv, 2 min
7: DNA +hv, 3 min
8: DNA +hv, 4 min
9: DNA +hv, 5 min
10: DNA +hv, 6 min
11: DNA +hv, 7 min
12: DNA +hv, 8 min
Plate XIII: A photograph of the effect of DTT on the photocleavage of pBR322 DNA with A-H (50 μg/mL) and A-Br (50 μg/mL) with 18 min at 350 nm.

<table>
<thead>
<tr>
<th>Top lanes:</th>
<th>Bottom lanes:</th>
</tr>
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<tbody>
<tr>
<td>1: -</td>
<td>1: -</td>
</tr>
<tr>
<td>2: DNA marker</td>
<td>2: DNA marker</td>
</tr>
<tr>
<td>3: DNA -hv</td>
<td>3: DNA + DTT (0.05M) -hv</td>
</tr>
<tr>
<td>4: DNA +hv</td>
<td>4: DNA + DTT (0.05M) +hv</td>
</tr>
<tr>
<td>5: -</td>
<td>5: -</td>
</tr>
<tr>
<td>7: A-Br + DTT (.05M) +hv</td>
<td>7: A-H + DTT (.05M) +hv</td>
</tr>
<tr>
<td>8: A-Br + DTT (.01M) +hv</td>
<td>8: A-H + DTT (.01M) +hv</td>
</tr>
<tr>
<td>9: A-Br + DTT (.005M) +hv</td>
<td>9: A-H + DTT (.005M) +hv</td>
</tr>
<tr>
<td>10: A-Br + DTT (.001M) +hv</td>
<td>10: A-H + DTT (.001M) +hv</td>
</tr>
<tr>
<td>11: A-Br + DTT (.0005M) +hv</td>
<td>11: A-H + DTT (.0005M) +hv</td>
</tr>
<tr>
<td>12: A-Br + DTT (.00001M) +hv</td>
<td>12: A-H + DTT (.00001M) +hv</td>
</tr>
<tr>
<td>14: A-Br - DTT +hv</td>
<td>14: A-H - DTT +hv</td>
</tr>
</tbody>
</table>

A-Br

A-H
Lastly, the photocleavage of the DNA was performed with A-Br in the presence of a competitive binder, spermidine. Solutions of DNA:A-Br and DNA, without any sensitizer, in the presence of varying concentrations of spermidine (1.0-1x10^{-3} M) were prepared. The solutions were photolyzed for 18 minutes at 350 nm at 3°C and the results are shown on the top tier of lanes of Plate VIII. Lane 2 is the molecular weight marker. Lane 3 is the DNA before photolysis and lane 4 is the DNA after photolysis of 18 minutes. Lane 5 is the DNA:spermidine(1M) sample, before photolysis and lane 6 is this solution after photolysis. Lane 7 contains DNA:A-Br, no spermidine, before photolysis and lane 8 is this sample after photolysis. Lanes 8-13 contain DNA:A-Br and variable amounts of spermidine, 1.0, 0.5, 0.1, 0.05, 0.01 and 0.001 M, respectively. Spermidine (1.0 M) protects the DNA from photocleavage by A-Br. However, 0.01 M spermidine does not protect the DNA from photocleavage by A-Br. It is important to note that higher concentrations of spermidine are required to protect A-Br than PSR-Br. This could be due to the fact A-Br binds DNA tighter than does PSR-Br.

The anthracene photocleavage results are summarized in Table 5. A-Br_2 is the best sensitizer of all of the synthetic anthracene derivatives for photocleavage of superhelical DNA. The trend observed with the other anthracenes is as follows; A-I > A-Br >> A-Cl = A-H. The trend observed among the anthracenes could be due to their affinity for the DNA. A-Br_2 binds the tightest to the DNA and it is the best sensitizer for the photocleavage of the DNA. However binding can not be the only factor since the other anthracenes, A-I, A-Br, and A-Cl, have very similar binding constants (Table 4). The difference in the photocleavage abilities of these anthracenes could be due to the bond dissociation energy of the carbon-halogen bond and the ability to generate free radicals. The actual bond dissociation energy of anthracenes are not known. But based on the bond dissociation energy of iodobenzene and brombenzene, A-I should undergo light induced homolytic carbon-iodine bond cleavage more readily than A-Br to generate the
Plate XIV: A photograph of the effect of spermidine on the photocleavage of pBR322 DNA with A-Br (50 μg/mL) and PSR-Br (50 μg/mL) with 18 min and 25 min photolysis, respectively at 350 nm.

Top lanes:
1: -
2: DNA marker
3: DNA +hv
4: DNA +hv
5: DNA + SP (1M) -hv
6: DNA + SP (1M) +hv
7: A-Br - SP -hv
8: A-Br + SP (1M) +hv
9: A-Br + SP (.5M) +hv
10: A-Br + SP (.1M) +hv
11: A-Br + SP (.05M) +hv
12: A-Br + SP (.01M) +hv
13: A-Br + SP (.001M) +hv
14: A-Br - SP +hv

Bottom lanes:
1: -
2: DNA marker
3: DNA + DTT (0.05M) -hv
4: DNA + DTT (0.05M) +hv
5: -
6: PSR-Br -hv
7: PSR-Br - SP +hv
8: PSR-Br + SP (1M) +hv
9: PSR-Br + SP (.5M) +hv
10: PSR-Br + SP (.1M) +hv
11: PSR-Br + SP (.05M) +hv
12: PSR-Br + SP (.01M) +hv
13: PSR-Br + SP (.0015M) +hv
14: PSR-Br - SP +hv
anthracene radical. Furthermore, A-Cl and A-H are not able to undergo this homolytic bond cleavage hence no photocleavage of the DNA is observed. The combination of large binding constants and relatively high photoreactivity of the anthracenes makes them particularly powerful sensitizers for photocleavage of plasmid DNA.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Photolysis time (min) required to nick pBR322 DNAa</th>
<th>Binding of sensitizer DNA, $K_b$ (M$^{-1}$)</th>
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<tbody>
<tr>
<td>PSR-H (3µg/mL)</td>
<td>&gt;&gt; 30</td>
<td>&lt;&lt;</td>
</tr>
<tr>
<td>PSR-Br (3 µg/mL)</td>
<td>15</td>
<td>6.02x10$^3$</td>
</tr>
<tr>
<td>PSR-I (3 µg/mL)</td>
<td>15</td>
<td>5.42x10$^3$</td>
</tr>
<tr>
<td>PSR-H(PIP) (3 µg/mL)</td>
<td>&gt;&gt; 30</td>
<td>7.61x10$^2$</td>
</tr>
<tr>
<td>PSR-Br(PIP) (3 µg/mL)</td>
<td>15$^b$</td>
<td>4.89x10$^4$</td>
</tr>
<tr>
<td>PSR-H(Ether) (3 µg/mL)</td>
<td>&gt;&gt; 30</td>
<td>c</td>
</tr>
<tr>
<td>PSR-Br(Ether) (3 µg/mL)</td>
<td>&gt;&gt; 30</td>
<td>c</td>
</tr>
<tr>
<td>A-H (50 µg/mL)</td>
<td>&gt;&gt; 27</td>
<td>1.74x10$^5$</td>
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<tr>
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<td>A-I (50 µg/mL)</td>
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<tr>
<td>A-Br2 (25 µg/mL)</td>
<td>5</td>
<td>4.59x10$^6$</td>
</tr>
</tbody>
</table>

a) The photolysis was performed at 350 nm at 3°C.
b) The UV bulbs were very old hence not as bright as during other photolysis.
c) These samples do not bind to calf thymus DNA.
D) **Viral Inactivation:**

The encouraging results obtained from the photocleavage of the DNA by our synthetic sensitizers led us to screen these sensitizers as antiviral agents. The ability of our synthetic sensitizers to inactivate λ phage and the φ6 virus using UV light was determined by the plaque forming assay. In addition to the synthetic sensitizers, the ability of the dyes, FL, RB and EY were also evaluated using visible light (528 nm) and X-ray. Visible light of 658 nm was used to induce viral inactivation by methylene blue.

**UV Light: (350 nm)**

The ability of PSR-H, PSR-Br and PSR-I to inactivate λ phage is reported in Table 2. Of the three psoralens, PSR-Br is clearly more effective at the inactivation of λ phage at equal sensitizer concentration and light dose. PSR-I is slightly better, about 2 logs₁₀, than PSR-H (Table 6). PSR-Br produces almost 7 logs₁₀ of inactivation of λ phage more than PSR-H.

Increasing the concentration of PSR-Br with constant light dose leads to a steady increase in the inactivation of λ phage (Table 7). Increasing exposure of λ phage to 350 nm light at a constant concentration of PSR-Br also leads to a smooth increase in the inactivation of λ phage. Increasing the concentration of PSR-Br at constant light exposure also leads to a smooth increase in the inactivation of λ phage (Table 7). The optimum conditions of PSR-Br to inactivate λ phage is 25 μg/mL with photolysis time of 25 seconds at 350 nm.
### Table 6: Logs of reduction of λ phage of PSR-Br, PSR-I and PSR-H

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Concentration (µg/ml)</th>
<th>Δlog_{10} of reduction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSR-H</td>
<td>25</td>
<td>0.16</td>
</tr>
<tr>
<td>PSR-I</td>
<td>25</td>
<td>2.10</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>25</td>
<td>6.93</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples were photolyzed at 350 nm for 25 seconds with light flux of 6.03 mW/cm².
Table 7: Logs of reduction of λ phage activity as a function of the concentration of PSR-Br at constant light exposure and of light intensity at constant PSR-Br concentration.

<table>
<thead>
<tr>
<th>Constant Light Intensity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Constant PSR-Br Concentration&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con (μg/ml)</td>
<td>Δlogs&lt;sub&gt;10&lt;/sub&gt;</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>3.23</td>
</tr>
<tr>
<td>10</td>
<td>3.93</td>
</tr>
<tr>
<td>15</td>
<td>3.65</td>
</tr>
<tr>
<td>20</td>
<td>4.22</td>
</tr>
<tr>
<td>25</td>
<td>7.29</td>
</tr>
<tr>
<td>30</td>
<td>7.14</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples were photolyzed for 25 seconds at 350 nm at RT with the light flux of 6.01 mW/cm<sup>2</sup>.

<sup>b</sup> The constant concentration of PSR-Br was 25 μg/ml and photolyzed at 350 nm with light flux of 6.03 mW/cm<sup>2</sup>.

The ability of other psoralens, PSR-H(PIP), PSR-Br(PIP), PSR-H(Ether) and PSR-Br(Ether), to inactivate λ phage was also determined and the results are reported in Table 8. AMT (Figure 2) was used as a reference compound. Among all the psoralens, including AMT, PSR-Br is the best sensitizer to inactivate λ phage under these conditions.
Table 8: Logs of reduction of λ phage activity as a function of psoralen

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Concentration (μg/ml)</th>
<th>Δlogs_{10} of reduction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT</td>
<td>25</td>
<td>0.98</td>
</tr>
<tr>
<td>PSR-H</td>
<td>25</td>
<td>0.36</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>25</td>
<td>3.52</td>
</tr>
<tr>
<td>PSR-H(PIP)</td>
<td>25</td>
<td>0.71</td>
</tr>
<tr>
<td>PSR-Br(PIP)</td>
<td>25</td>
<td>1.52</td>
</tr>
<tr>
<td>PSR-H(Ether)</td>
<td>25</td>
<td>0.47</td>
</tr>
<tr>
<td>PSR-Br(Ether)</td>
<td>25</td>
<td>0.49</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples were photolyzed at 350 nm for 30 seconds with light flux of 3.58 mW/cm<sup>2</sup>.

An important point to note in Table 7 is that the light flux is half of that in Table 5.
Therefore, logs of inactivation by PSR-Br are considerably different in the two tables. As mentioned before, the UV bulbs get weaker after prolonged usage.

Since PSR-Br was the best sensitizer of all the psoralens, viral inactivation of λ phage was performed in the presence of a radical scavenger, DTT (5x10<sup>-4</sup> M) and in the presence of argon to gain an insight into the mechanism of viral inactivation by PSR-Br.
The results of viral inactivation by PSR-Br in the presence of DTT and in the presence of argon are summarized in Table 9.

Table 9: The effect of DTT (5x10^{-4} M) and argon on the λ phage activity after treatment with PSR-H and PSR-Br at 350 nm.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Con. (µg/ml)</th>
<th>Δlog_{10} of reduction(^a)</th>
<th>Δlog_{10} of reduction(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSR-H</td>
<td>25</td>
<td>0.05</td>
<td>0.55</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>25</td>
<td>5.55</td>
<td>6.65</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>0</td>
<td>6.23(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Samples were photolyzed for 25 seconds with light flux of 5.99 mW/cm\(^2\).
\(^{b}\) See Table 7, light flux of 6.01 mW/cm\(^2\).

It is evident from the results presented in Table 9 that viral inactivation is only slightly affected by the addition of a radical scavenger, DTT. In addition, the absence of oxygen does not reduce the viral inactivation mediated by these sensitizers.

The ability of all the synthetic psoralens to inactivate the φ6 virus, with the bacterial host, was determined using the plaque forming assay. AMT was again used as a reference sensitizer. The results of φ6 inactivation by the psoralens are presented in Table 10. The results show that of all the synthetic psoralens, PSR-Br is the best sensitizer for φ6 virus. However, the reference sensitizer, AMT is slightly better (0.5-1.0 logs) than PSR-Br.
Table 10: Logs of reduction of φ6 virus after treatment with psoralens at 350 nm.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Conc. (µg/ml)</th>
<th>Δlogs10 of reduction(^a) 3 min, hv</th>
<th>Δlogs10 of reduction(^b) 5min, hv</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT</td>
<td>100</td>
<td>0.99</td>
<td>1.63</td>
</tr>
<tr>
<td>PSR-H</td>
<td>100</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>100</td>
<td>0.00</td>
<td>0.19</td>
</tr>
<tr>
<td>AMT</td>
<td>175</td>
<td>2.37</td>
<td></td>
</tr>
<tr>
<td>PSR-H</td>
<td>175</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>PSR-Br</td>
<td>175</td>
<td>2.19</td>
<td></td>
</tr>
<tr>
<td>AMT</td>
<td>200</td>
<td></td>
<td>2.62</td>
</tr>
<tr>
<td>PSR-H</td>
<td>200</td>
<td></td>
<td>1.57</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>200</td>
<td></td>
<td>2.25</td>
</tr>
<tr>
<td>PSR-H(PIP)</td>
<td>200</td>
<td></td>
<td>1.04</td>
</tr>
<tr>
<td>PSR-Br(PIP)</td>
<td>200</td>
<td></td>
<td>1.40</td>
</tr>
<tr>
<td>PSR-H(Ether)</td>
<td>200</td>
<td></td>
<td>1.38</td>
</tr>
<tr>
<td>PSR-Br(Ether)</td>
<td>200</td>
<td></td>
<td>1.73</td>
</tr>
<tr>
<td>PSR-H</td>
<td>250</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>PSR-Br</td>
<td>250</td>
<td>7.74</td>
<td></td>
</tr>
<tr>
<td>PSR-H(PIP)</td>
<td>250</td>
<td>4.77</td>
<td></td>
</tr>
<tr>
<td>PSR-Br(PIP)</td>
<td>250</td>
<td>4.48</td>
<td></td>
</tr>
<tr>
<td>PSR-H(Ether)</td>
<td>250</td>
<td>2.95</td>
<td></td>
</tr>
<tr>
<td>PSR-Br(Ether)</td>
<td>250</td>
<td>4.01</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Samples were photolyzed for 3.0 minutes at 3°C with light flux of 1.09 mW/cm\(^2\).

\(^b\) Samples were photolyzed for 5.0 minutes at 3°C with light flux of 1.09 mW/cm\(^2\).
PSR-Br is the best of our synthetic psoralen sensitizers for the inactivation of λ phage and the φ6 virus. It gives at least 6 logs of inactivation relative to its non-halogenated control, PSR-H. Furthermore, PSR-Br is a more powerful sensitizer than AMT against λ phage. AMT is the most potent psoralen-UV sensitizer known for the sterilization of blood products. The increased effectiveness of PSR-Br relative to PSR-H is not due to its extinction coefficient, since the extinction coefficients of PSR-H and PSR-Br are very similar (Table 15). However, the increased effectiveness could in part be due the enhanced binding of PSR-Br to lambda phage relative to PSR-H (Table 4). It was our hope that the iodinated psoralen, PSR-I, would be more effective in reducing the viral activity than the brominated psoralen, PSR-Br. But from the results presented in Table 6, it is clear that the brominated psoralen, PSR-Br is a much more effective sensitizer than PSR-I. Therefore, other brominated psoralens, PSR-Br(PIP) and PSR-Br(Ether), containing different side chains were synthesized and evaluated as antiviral agents.

PSR-H(PIP) and PSR-Br(PIP) contain a cyclic amine with two positive charges in their side chains. It is evident from the binding of λ phage and the results of viral inactivation that sensitizers with two positive charges on the side chains are much less effective than sensitizers containing only one positive charge, PSR-Br and PSR-H. PSR-H(PIP) and PSR-Br(PIP) neither bind to λ phage nor do they inactivate the virus under the conditions where PSR-Br produces 6 logs of reduction. This is despite the fact that PSR-H(PIP) and PSR-Br(PIP) bind calf thymus DNA tightly than PSR-H or PSR-Br, respectively (Table 4). It is possible that the presence of the two positive charges on PSR-H(PIP) and PSR-Br(PIP) makes it difficult for the sensitizer to penetrate through the protein capsid to reach the viral nucleic acid of λ phage, hence no viral inactivation. Similarly, PSR-H(Ether) and PSR-Br(Ether) have a linear polyether side chain as opposed to the amines. Thus, these psoralens are very hydrophobic in nature. It is possible that
they are probably not able to penetrate through the charged protein coat of λ phage to reach the viral nucleic acid, hence no viral inactivation.

PSR-Br is again the best of our synthetic sensitizers for the inactivation of the φ6 virus. However, AMT, the reference sensitizer, is slightly better (0.5-1.0 logs) than PSR-Br against the φ6 virus. Increasing the concentration and the light dose leads to increased viral inactivation of φ6 mediated by the psoralen derivatives (Table 10). It was expected that the psoralens containing the hydrophobic side chain, PSR-H(Ether) and PSR-Br(Ether), would be the most effective sensitizers against the enveloped φ6 virus. The hydrophobic polyether side chains of these psoralens were anticipated to aid penetration through the hydrophobic environment of the lipid membrane of the φ6 virus and reach the viral nucleic acid to induce damage. Unfortunately, these psoralens are only slightly better than those containing the two positive charges, PSR-H(PIP) and PSR-Br(PIP) but clearly they are less powerful than PSR-Br. Clearly, PSR-Br is the best sensitizer of all of our synthetic psoralens.

The increased effectiveness of PSR-Br in terms of viral inactivation may be a result of its high affinity towards the viruses. Perhaps it is able to penetrate through the protein capsid of λ phage and the lipid membrane of the φ6 virus to induce viral damage. Psoralen theme selves will inactivate λ phage by consecutive cycloaddtion reaction between isolated double bonds on the periphery of the psoralen molecule and two uracil or two thymine residues. It is not clear whether it is the excited singlet state (S₁) or the triplet state (T₁) or both excited states of psoralen which are responsible for the cross linking of DNA in vivo. If T₁ is the reactive site in vivo one could argue that the bromine substitution has merely increased the efficiency of the 2+2 cycloaddition mechanism by heavy atom catalysis of intersystem crossing from S₁ and T₁. To test this possibility water soluble, halogenated anthracenes and their non-halogenated analogs were synthesized. The
anthracenes can undergo light induced cleavage of carbon-halogen bonds but cannot undergo consecutive cycloaddition reaction as per psoralen.

The results of viral inactivation of λ phage mediated by the synthetic anthracene derivatives are summarized in Table 11. A-Br$_2$ is the most powerful sensitizer in the series. A-Br is slightly better than A-I which is slightly better than A-Cl. Therefore, the trend for viral inactivation of λ phage is A-Br$_2$ $>>$ A-Br $>$ A-I $>$ A-Cl.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Concentration (µg/ml)</th>
<th>$\Delta$log$_{10}$ of reduction$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-H</td>
<td>25</td>
<td>0.18</td>
</tr>
<tr>
<td>A-Cl</td>
<td>25</td>
<td>0.29</td>
</tr>
<tr>
<td>A-Br</td>
<td>25</td>
<td>1.23</td>
</tr>
<tr>
<td>A-I</td>
<td>25</td>
<td>0.49</td>
</tr>
<tr>
<td>A-Br$_2$</td>
<td>25</td>
<td>6.20</td>
</tr>
<tr>
<td>A-H</td>
<td>50</td>
<td>0.33</td>
</tr>
<tr>
<td>A-Cl</td>
<td>50</td>
<td>6.15</td>
</tr>
<tr>
<td>A-Br</td>
<td>50</td>
<td>6.23</td>
</tr>
<tr>
<td>A-I</td>
<td>50</td>
<td>6.09</td>
</tr>
<tr>
<td>A-Br$_2$</td>
<td>50</td>
<td>6.96</td>
</tr>
</tbody>
</table>

$^a$) Samples were photolyzed for 25 seconds with light flux of 6.09 mW/cm$^2$. 
A-Br\textsubscript{2} is clearly the most powerful sensitizer in this series. Therefore, the minimum concentration required for A-Br\textsubscript{2} to completely inactivate \(\lambda\) phage at constant light dose was determined and the results are presented in Table 12. The minimum concentration required for A-Br\textsubscript{2} to completely inactivate \(\lambda\) phage is 25\(\mu\)g/mL for 25 seconds of photolysis (Table 12). However, A-Br, A-I and A-Cl, all require twice this amount (50\(\mu\)g/mL) of sensitizer at the same photolysis time to completely inactivate \(\lambda\) phage (Table 11).

**Table 12:** Logs of reduction of \(\lambda\) phage activity as a function of the concentration of A-Br\textsubscript{2} at constant light exposure (25 sec, 350 nm).

<table>
<thead>
<tr>
<th>Concentration ((\mu)g/ml)</th>
<th>(\Delta\log_{10}) of reduction\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.13</td>
</tr>
<tr>
<td>15</td>
<td>3.33</td>
</tr>
<tr>
<td>25</td>
<td>6.20</td>
</tr>
<tr>
<td>40</td>
<td>6.40</td>
</tr>
<tr>
<td>50</td>
<td>6.96</td>
</tr>
</tbody>
</table>

\textsuperscript{a) Samples were photolyzed for 25 seconds with light flux of 6.09 mW/cm\textsuperscript{2}.}

Furthermore, the effect of a powerful radical scavenger, DTT (5\(\times\)10\textsuperscript{-4} M), and oxygen on viral inactivation of \(\lambda\) phage was studied using A-Br. To study the effect of oxygen, the samples were degassed by bubbling with argon prior to photolysis. The results of DTT and argon are summarized in Table 13. A thousand fold excess of DTT
Table 13: The effect of DTT (5x10^{-4} M) and argon on the λ phage activity of after treatment with A-H and A-Br at 350 nm.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Conc. (μg/ml)</th>
<th>Δlog_{10} of reduction^a</th>
<th>Δlog_{10} of reduction^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-H</td>
<td>50</td>
<td>0.14</td>
<td>0.55</td>
</tr>
<tr>
<td>A-Br</td>
<td>50</td>
<td>5.69</td>
<td>5.64</td>
</tr>
</tbody>
</table>

^a) Samples were photolyzed for 25 seconds with light flux of 5.99 mW/cm².

b) Table 12, light flux of 6.09 mW/cm².

Table 14: Logs of reduction of φ6 virus activity after treatment with anthracenes at 350 nm.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Conc. (μg/ml)</th>
<th>Δlog_{10} of reduction^a</th>
<th>Δlog_{10} of reduction^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.5 min, hv</td>
<td>3.0 min, hv</td>
</tr>
<tr>
<td>AMT</td>
<td>50</td>
<td>0.12</td>
<td>0.99</td>
</tr>
<tr>
<td>A-H</td>
<td>50</td>
<td>0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>A-Cl</td>
<td>50</td>
<td>0.13</td>
<td>7.11</td>
</tr>
<tr>
<td>A-Br</td>
<td>50</td>
<td>0.60</td>
<td>9.63</td>
</tr>
<tr>
<td>A-I</td>
<td>50</td>
<td>0.17</td>
<td>9.63</td>
</tr>
<tr>
<td>A-Br2</td>
<td>50</td>
<td>6.63</td>
<td>9.63</td>
</tr>
</tbody>
</table>

^a) Samples were photolyzed for 1.5 minutes at 3°C with light flux of 0.72 mW/cm².

b) Samples were photolyzed for 3.0 minutes at 3°C with light flux of 0.72 mW/cm².
does not protect the virus from photoinactivation. In addition, the absence of oxygen has no effect on the viral inactivation by A-Br.

The ability of the anthracene derivatives to inactivate the φ6 virus was evaluated and the results are reported in Table 10. AMT was used as a reference compound. A-Br₂ is much more potent than AMT. The trend for the φ6 virus is the same observed with λ phage as with the anthracene derivatives; A-Br₂ >> A-Br > A-I > A-Cl. As A-Br₂ is the best sensitizer for the φ6 virus, the optimum concentration and photolysis time were determined for the inactivation of the φ6 virus (Table 15). The minimum concentration required for A-Br₂ to inactivate φ6 completely is 50 µg/mL photolyzed for 1.5 minutes at 350 nm (Table 15). Of all the synthetic sensitizers used in this study including the psoralens, A-Br₂ is the most effective sensitizer against the φ6 virus.

Table 15: Logs of reduction of φ6 virus activity as a function of the concentration of A-Br₂ at constant light exposure and of light intensity at constant A-Br₂ concentration at 350 nm.

<table>
<thead>
<tr>
<th>Constant Light Intensityᵃ</th>
<th>Constant PSR-Br Concentrationᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con (µg/ml)</td>
<td>Δlog₁₀</td>
</tr>
<tr>
<td>25</td>
<td>2.99</td>
</tr>
<tr>
<td>50</td>
<td>6.24</td>
</tr>
<tr>
<td>75</td>
<td>9.63</td>
</tr>
<tr>
<td>100</td>
<td>9.63</td>
</tr>
<tr>
<td>200</td>
<td>9.24</td>
</tr>
</tbody>
</table>

ᵃ) Samples were photolyzed for 1.5 minutes at 3°C with the light flux of 0.72 mW/cm².
ᵇ) The constant concentration of A-Br₂ was 50 µg/mL, photolyzed at 3°C with the light flux of 0.72 mW/cm².
In interpreting the anthracene data, it is important to note that neither light alone nor the sensitizers in the dark damage the \( \lambda \) phage and the \( \phi 6 \) virus. The improved ability of the halogenated anthracenes to sensitize viral inactivation can not simply be due to binding (Table 4) or to molar absorbtivity (Table 16). The binding of the mono-halogenated anthracenes, A-Cl, A-Br and A-I, is quite similar to that of non-halogenated control, A-H. The molar absorbtivity of these derivatives are also similar.

The anthracenes cannot cross link viral nucleic acids as per the psoralens, thus the bromine effect with anthracene is not related to changes in the efficiency of the excited state cycloaddition reaction. It is possible that the bromine substituent creates a new reaction pathway leading to the formation of free radicals. It is known that the photolysis of anthracene itself in water leads to ionization (Scheme 5).

![Scheme 12](image)

Photolysis of anthracene derivatives in the presence of an electron donor can lead to electron trasfer as demostrated by Nagamura with 9-bromoanthracene\textsuperscript{111} (Scheme 6). The radical ion pair (A-Br\textsuperscript{−}/Donor \textsuperscript{+·}) can undergo back electron transfer to regenerate A-Br and Donor in their ground states. This is the likely pathway with A-H\textsuperscript{−}/Donor \textsuperscript{+·}. However, the halogenated anthracene radical anions (A-Br \textsuperscript{−}) can fragment to form a radical localized on the anthracene moiety (A·). If the sensitizer is bound to viral DNA, guanosine
is the likely donor and the radical produced is born intercalated into the viral nucleic acid. The guanosine radical cation can also undergo a series of reactions leading to DNA cleavage and to viral inactivation. If the sensitizer is localized in the protein capsid or the lipid membrane of the virus, the donor is likely to be an electron rich amino acid (cysteine, tryptophan) or the polar head groups of the lipid membrane, respectively. It is possible that viral inactivation results from lesions to both the viral nucleic acid and the protein capsid or the lipid membrane.

![Diagram](attachment:image.png)

**Scheme 13**

Furthermore, neither oxygen nor a free radical scavenger, DTT, inhibit photosensitized viral inactivation (Table 15). This demonstrates that singlet oxygen formation is not responsible for viral inactivation by these sensitizers and that the free radicals generated in bulk solution are not responsible for the effect. In addition, the dramatic improvement in viral inactivation by A-Br2 is also related to the increased affinity of A-Br2 towards the virus (Table 4).
Photophysics of the sensitizers:

The photophysical properties of the psoralens and anthracenes are presented in Table 16. Bromine substitution of psoralen and anthracene has little effect on the extinction coefficient of the sensitizers, thus an increase in absorptivity is not responsible for increases in its antiviral activity.

The bromine substituent has little influence on the fluorescence yield or triplet yield of the psoralens. The yield of triplet PSR-H and PSR-Br were measured by laser flash photolysis (308 nm) in degassed aqueous solution. The transient spectrum of triplet PSR-H is similar to the transient spectrum of PSR-Br. The relative triplet yields were determined assuming that the bromine substituent has little effect on the extinction coefficient of the triplet-triplet absorption. Thus it is clear that bromine has essentially no effect on the photophysics of the psoralen moiety. Furthermore, the fluorescence of PSR-H in water is readily quenched by diethylamine, calf thymus DNA or λ phage or φ6 demonstrating the ease with which the excited state of psoralen accepts an electron from a suitable donor.

A bromine substituent greatly quenches the fluorescence of anthracene but it has little effect on the yield of the excited triplet state of A-Br based on laser flash photolysis experiments. It is unlikely that the fluorescence quenching of A-Br is due to fragmentation of the carbon-bromine bond due to the low energy of the excited singlet state of anthracene. Bromine may effect the yield of ionization of the excited singlet state of A-Br. It is known that photolysis of anthracene itself in water leads to ionization.

The fluorescence of A-H in water is quenched by diethylamine, calf thymus DNA φ6 and λ phage. This demonstrated the ease of reduction of the excited singlet states of these sensitizers.
<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Absorption&lt;sup&gt;a&lt;/sup&gt; $\varepsilon$ (M&lt;sup&gt;-1&lt;/sup&gt;, cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Fluorescence&lt;sup&gt;b&lt;/sup&gt; $\lambda_{\text{max-em}}$ (Int&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Triplet yield&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSR-H</td>
<td>2288</td>
<td>505 (1)</td>
<td>1</td>
</tr>
<tr>
<td>PSR-Br</td>
<td>2188</td>
<td>512 (0.73)</td>
<td>1.14</td>
</tr>
<tr>
<td>PSR-I</td>
<td>3031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-H</td>
<td>3839</td>
<td>415 (1)</td>
<td>1</td>
</tr>
<tr>
<td>A-Br</td>
<td>3764</td>
<td>427 (0.08)</td>
<td>0.91</td>
</tr>
<tr>
<td>A-Cl</td>
<td>2788</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-I</td>
<td>2747</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-Br&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2139</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Extinction coefficients ($\varepsilon$) were calculated at 350 nm.
b) Fluorescence measurements were recorded with $A = 0.1$ at the excitation wavelength of 350 nm.
c) Triplet-triplet absorptions were measured by laser flash photolysis XeCl, 308 nm, 55mJ, 10 ns with $A_{308} = 1.0$. Triplet yields were calculated assuming that the bromine substituent has no effect on the extinction coefficient of the triplet-triplet absorptions of the psoralens and anthracenes.
Visible Light (528 nm):

The extreme optical density of hemoglobin (200-600 nm) prohibit the use of UV-VIS activated sterilization of virus. This led us to consider using far visible light and X-ray radiation for viral inactivation.

Halogenated derivatives of fluorescein dye (rose bengal, eosin Y) were used to inactivate \( \lambda \) phage and the \( \phi 6 \) virus upon illumination with 528 nm light. Fluorescein is the non-halogenated control of rose bengal and eosin Y both of which are iodinated and brominated, respectively. The results of viral inactivation of \( \lambda \) phage and \( \phi 6 \) virus by FL, RB and EY are summarized in Table 17.

From Table 17, it is clear that the non-halogenated control, FL, does not inactivate either \( \lambda \) or the \( \phi 6 \) virus to any significant extent (entries 1-3). Neither light alone nor the dyes in the dark lead to viral inactivation. RB and EY are not as effective against \( \lambda \) phage as they are against \( \phi 6 \) virus. However, RB is much better than EY in inactivating \( \lambda \) phage. At equal concentration by weight (150 \( \mu \)g/mL), RB is 2 logs better than EY in inactivating \( \lambda \) phage (entries 9,17). The absence of oxygen significantly inhibits viral inactivation of \( \lambda \) phage by RB (entry 9). RB (150\( \mu \)g/mL) produces 5.46 logs of inactivation of \( \lambda \) phage in the presence of oxygen but in the absence of oxygen at the same concentration, it only produces 2.73 logs of reduction in the viral activity. Therefore, the formation of singlet oxygen is probably involved in at least one pathway leading to viral inactivation of \( \lambda \) phage by RB. EY sensitization of \( \lambda \) phage was not affected to a significant amount by the absence of oxygen (entry 17).
Table 17: Logs of reduction of λ and φ6 virus activity of FL, RB and EY with visible light (528 nm).

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Conc.(μg/ml)</th>
<th>Δlogs_{10} of reduc. of λ</th>
<th>Δlogs_{10} of reduc. of φ6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ O₂</td>
<td>- O₂</td>
</tr>
<tr>
<td>1) FL</td>
<td>100</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>2)</td>
<td>150</td>
<td>0.71</td>
<td>1.09</td>
</tr>
<tr>
<td>3)</td>
<td>400</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>4) RB</td>
<td>15</td>
<td></td>
<td>4.12</td>
</tr>
<tr>
<td>5)</td>
<td>20</td>
<td></td>
<td>3.96</td>
</tr>
<tr>
<td>6)</td>
<td>25</td>
<td></td>
<td>4.98</td>
</tr>
<tr>
<td>7)</td>
<td>50</td>
<td></td>
<td>6.57</td>
</tr>
<tr>
<td>8)</td>
<td>100</td>
<td></td>
<td>7.66</td>
</tr>
<tr>
<td>9)</td>
<td>150</td>
<td>5.46</td>
<td>2.73</td>
</tr>
<tr>
<td>10)</td>
<td>400</td>
<td>5.91</td>
<td>3.60</td>
</tr>
<tr>
<td>11) EY</td>
<td>15</td>
<td></td>
<td>3.39</td>
</tr>
<tr>
<td>12)</td>
<td>20</td>
<td></td>
<td>3.54</td>
</tr>
<tr>
<td>13)</td>
<td>25</td>
<td></td>
<td>6.22</td>
</tr>
<tr>
<td>14)</td>
<td>50</td>
<td></td>
<td>6.05</td>
</tr>
<tr>
<td>15)</td>
<td>75</td>
<td></td>
<td>2.99</td>
</tr>
<tr>
<td>16)</td>
<td>100</td>
<td></td>
<td>5.66</td>
</tr>
<tr>
<td>17)</td>
<td>150</td>
<td>3.46</td>
<td>2.79</td>
</tr>
<tr>
<td>18)</td>
<td>400</td>
<td>3.00</td>
<td></td>
</tr>
</tbody>
</table>

a) Samples were photolyzed for 3.0 minutes at RT with light flux of 0.21 mW/cm².
b) Samples were photolyzed for 3.0 minutes at 3°C with light flux of 0.08 mW/cm².
Viral inactivation of φ6 by RB and EY is concentration dependent (Table 17). Increasing the concentration of RB and EY leads to increased viral inactivation of φ6. FL does not sensitize viral inactivation of φ6 (entry 1). EY is more effective in sensitizing the φ6 virus than RB, the opposite of the results obtained with λ phage. EY requires only 25 µg/ml to completely inactivate the φ6 virus (entry 13) while RB requires 50 µg/ml (entry 7) after three minutes of photolysis at 528 nm. Furthermore, inactivation of φ6 with EY and RB is not oxygen dependent (entries 7, 13). Therefore, the viral inactivation of φ6 by EY and RB must not involve the formation of singlet oxygen.

RB and EY are negatively charged and lipophilic in nature. They probably accumulate in the lipid membrane of the φ6 virus and damage the membrane upon activation with light. These dyes are probably not able to easily accumulate in the protein capsid of the λ phage. EY requires only 25 µg/ml to completely inactivate φ6 (Table 17, entry 13) but even 400 µg/ml of EY does not inactivate λ phage completely (entry 18). Similarly, RB requires 50 µg/ml to inactivate φ6 (entry 7) and 150 µg/ml to inactivate λ phage (entry 9) completely.

RB is more effective against λ phage than EY and the reverse is true against the φ6 virus. EY is more effective against φ6 than RB. Furthermore, viral inactivation of λ phage with RB is oxygen dependent suggesting that the mechanism of sensitization involves the formation of singlet oxygen. This is not the case with φ6 inactivation, sensitization with RB and EY of φ6 is not oxygen dependent. Therefore, another mechanism, not involving singlet oxygen formation, must be in play.
X-ray Irradiation:

In the experiment with UV and visible light discussed to this point the radiant energy is by design absorbed only by the sensitizer and not by the virus. This is not the case ith X-ray radiation. Water is the primary target which absorbs X-ray radiation in these experiments.

\[ \text{H}_2\text{O} + \text{X-ray} \rightarrow \text{H}_2\text{O}^{+*} + \text{e}^{**} \]

The primary event is ionization of water and the formation of an energetic electron. The high energy electron can ionize other molecules of water.

\[ \text{e}^{**} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^{+*} + 2\text{e}^{-} \]

This chain process can continue many thousands of times until the ejected electrons are thermolyzed.

\[ \text{e}^{-*} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^{+*} + 2\text{e}^{-} \text{ (aq)} \]

Thus scattering of a single X-ray by a molecule of water creates a cluster of radical cations and solvated electrons.

The sensitizer now functions as a free radical generator by virtue of its favorable electron affinity. The sensitizer absorbs a solvated electron to form an aryl radical and iodide ion (Scheme 14). This mechanism is only available for sensitizers bound to the outside of a virus, which can then react with a solvated electron. A sensitizer bound to viral nucleic acid probably cannot be activated with a solvated electron in solution. It is unlikely that the electron will penetrate the viral protein capsid or lipid envelope. As the electron affinity of iodine is more favorable than bromine, the iodinated sensitizer is expected to be superior.
Table 18: Logs of reduction of λ and φ6 virus activity of FL, RB and EY with X-rays.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Conc.(μg/ml)</th>
<th>Δlogs₁₀ of reduct. of φ₆</th>
<th>Δlogs₁₀ of reduct. of λ⁺O₂</th>
<th>Δlogs₁₀ of reduct. of λ⁻O₂</th>
<th>Δlogs₁₀ of reduct. of λ⁺O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>50</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td>50</td>
<td>6.40</td>
<td>5.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EY</td>
<td>50</td>
<td>5.63</td>
<td>5.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>25</td>
<td>0.08</td>
<td></td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td>25</td>
<td>3.58</td>
<td></td>
<td>8.45</td>
<td></td>
</tr>
<tr>
<td>EY</td>
<td>25</td>
<td>2.08</td>
<td></td>
<td>0.98</td>
<td></td>
</tr>
</tbody>
</table>

a) Samples were irradiated for 10 minutes, approximately 50,000 rads of radiation at RT.
RB and EY have been previously shown to inactivate virus, HSV-1\textsuperscript{68} and actinophage\textsuperscript{65}, respectively (Table 3 Introduction section) with visible light. In the current study, the non-halogenated control, FL, does not inactivate the viruses. Also, X-rays alone do not damage the virus to any significant amount. RB is the best sensitizer in this series against both viruses using X-rays. The minimum concentration needed to completely inactivate \(\lambda\) phage with RB is 25 \(\mu\text{g/mL}\) with approximately 10,000 rads. EY at this concentration does not inactivate \(\lambda\) phage. RB is slightly better than EY in sensitizing the \(\phi6\) virus and the minimum concentration required is 50 \(\mu\text{g/mL}\). The viral inactivation of \(\phi6\) with RB and EY using X-rays is not oxygen dependent.

Viral inactivation of \(\lambda\) phage using X-rays with PSR-Br and A-Br sensitizers in solution were performed unsuccessfully. Concentration as high as 100 \(\mu\text{g/mL}\) of each sensitizer was used with approximately 50,000 rads of radiation in the absence and presence of oxygen. Under these conditions, there was no loss in the viral activity of \(\lambda\) phage. In addition to studies of viral inactivation in solution, viral inactivation of \(\lambda\) phage with A-Br\textsubscript{2} sensitizer was performed in the frozen state with X-ray activation. The concentration of A-Br\textsubscript{2} used was 25 \(\mu\text{g/mL}\) with approximately 50,000 rads of radiation in the frozen state. Again, even in the frozen state, there was no significant loss of viral activity of \(\lambda\) phage with A-Br\textsubscript{2}.
Visible Light (658 nm):

To utilize far visible light (658 nm) to inactivate viruses, we have employed another sensitizer, methylene blue (Figure 3). Methylene blue (MB) has an absorption maximum near 650 nm. Viral inactivation of \( \lambda \) phage with MB has led to dramatic reduction in viral activity. However, inactivation of \( \phi 6 \) with MB as sensitizer was unsuccessful. MB has no activity upon illumination with 658 nm light against the \( \phi 6 \) virus.

Methylene blue has been shown previously to inactivate viruses, actinophage\(^{65}\) and VSV\(^{67}\), and bacteria, E.coli\(^{66}\) (Table 3 Introduction section). In the current study, the photolysis of \( \lambda \) phage with MB at 658 nm led to a dramatic reduction in the viral activity. Viral inactivation of \( \lambda \) phage with MB was performed in solution and in frozen medium. The results of viral inactivation of \( \lambda \) phage in solution are summarized in Table 19. The optimum concentration of MB required to completely inactivate \( \lambda \) phage is 50 \( \mu \)g/mL. Concentrations higher than this are actually less effective (Table 19). The concentration dependence of the inactivation mediated by MB demonstrates that the sensitizer, MB, binds to the virus. Viral inactivation of \( \lambda \) phage with MB in the absence of oxygen is inhibited. Therefore, the mechanism of sensitization of MB is oxygen dependent and likely proceeds with the formation of singlet oxygen as the reactive species.
### Table 19: Logs of reduction of λ virus activity of MB with 658 nm light.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Conc. (μg/ml)</th>
<th>Δlogs$_{10}$ of reduction of λ$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ O$_2$</td>
</tr>
<tr>
<td>MB</td>
<td>50</td>
<td>8.39</td>
</tr>
<tr>
<td>MB</td>
<td>100</td>
<td>6.68</td>
</tr>
<tr>
<td>MB</td>
<td>150</td>
<td>5.19</td>
</tr>
<tr>
<td>MB</td>
<td>200</td>
<td>4.19</td>
</tr>
<tr>
<td>MB</td>
<td>250</td>
<td>3.39</td>
</tr>
</tbody>
</table>

a) Samples were photolyzed for 3.0 minutes at RT with light flux of 0.12 mW/cm$^2$.

### Table 20: Logs of reduction of λ virus activity in the presence of histidine with 658 nm light for 3 minutes with MB (50 μg/mL).

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Histidine (M)</th>
<th>Δlogs$_{10}$ of reduction$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>10$^{-2}$</td>
<td>0.51</td>
</tr>
<tr>
<td>MB</td>
<td>10$^{-3}$</td>
<td>0.82</td>
</tr>
<tr>
<td>MB</td>
<td>10$^{-4}$</td>
<td>4.46</td>
</tr>
<tr>
<td>MB</td>
<td>10$^{-5}$</td>
<td>5.56</td>
</tr>
<tr>
<td>MB</td>
<td>0</td>
<td>8.48</td>
</tr>
</tbody>
</table>

a) Samples were photolyzed for 3.0 minutes at RT with light flux of 0.14 mW/cm$^2$. 
Viral inactivation of MB in the presence of histidine was performed. The results are reported in Table 20. Histidine is a powerful quencher of singlet oxygen. Viral inactivation via singlet oxygen will be inhibited in the presence of histidine since histidine scavenges singlet oxygen. It is evident from the results presented in Table 20 that very low levels of histidine ($10^{-4}$ M) do not inhibit viral inactivation. But concentrations higher than $10^{-4}$ M of histidine protects λ phage from viral inactivation sensitized with MB. Therefore, viral inactivation of λ phage with MB likely proceeds via a singlet oxygen pathway.

Viral inactivation of λ phage with MB in the frozen state was also performed and the results are presented in Table 21. The samples were prepared in a pyrex petri dish and frozen using a dry ice-acetone bath. The samples were maintained frozen during photolysis by placing them under a dry ice-acetone bath. The depth of the frozen sample was varied by increasing the total volume of the sample.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Δlog$_{10}$ of reduction of λ$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (nm)</td>
<td>Volume (mL)</td>
</tr>
<tr>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>8.0</td>
<td>9.0</td>
</tr>
<tr>
<td>10.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

a) Samples were photolyzed for 10.0 minutes with light flux of 0.12 mW/cm$^2$. 
This experiment was performed to determine if the visible light will penetrate the frozen sample and cause viral inactivation. The depth of the frozen samples was varied to determine the extent of light penetration. It is clear from the results in Table 21 that the visible light can penetrate frozen samples of up to a depth of 1.0 cm leading to viral inactivation in the presence of MB. Furthermore, it is also evident that MB binds to the virus since viral inactivation of λ phage proceeds in the frozen state. In the frozen state, neither MB nor singlet oxygen is free to diffuse in the solution. MB must be either bound to the virus or in the immediate vicinity of the virus in order for viral inactivation to occur in the frozen state.

The concentration and the photolysis time needed to inactivate λ phage in the frozen state of MB is much higher than in solution. The minimum concentration of MB required is 1mg/mL in the frozen state with a minimum photolysis time of 10 minutes, whereas in solution, 50 μg/mL of MB with 3 minutes of photolysis time is needed to completely inactivate λ phage. The results presented in Table 21 clearly suggest that the 658 nm light is able to penetrate through a frozen sample with a thickness of 1.0 cm. An important difference between the frozen state and solution is that oxygen is not a factor in viral inactivation of MB in the frozen state. The lack of an oxygen in the frozen medium does not inhibit viral inactivation.

The effect of a singlet oxygen quencher, histidine, was studied in the frozen state on viral inactivation mediated by MB. The results are summarized in Table 22. The effect of histidine in the frozen state is not as profound as it is in the solution. Even at high concentrations, histidine does not hinder viral inactivation of λ phage in the frozen state with MB. This result, together with the fact that the absence of oxygen does not effect viral inactivation of λ phage in the frozen state of MB (Table 21), confirm that viral inactivation in the frozen state does not proceed through the singlet oxygen pathway. An alternate mechanism must be involved.
Table 22: Logs of reduction of \(\lambda\) virus activity in the presence of histidine with 658 nm light of MB (1 mg/mL) in the frozen state.

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Histidine (M)</th>
<th>(\Delta\log_{10}) of reduction(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>0</td>
<td>7.25</td>
</tr>
<tr>
<td>MB</td>
<td>(10^{-2})</td>
<td>5.23</td>
</tr>
<tr>
<td>MB</td>
<td>(10^{-4})</td>
<td>5.93</td>
</tr>
<tr>
<td>MB</td>
<td>(10^{-6})</td>
<td>6.65</td>
</tr>
</tbody>
</table>

\(^a\) The depth of the frozen samples was 3.5 nm and photolysis time was 10 minutes with light flux of 0.12 mW/cm\(^2\).

MB is a powerful sensitizer against \(\lambda\) phage, however, it does not significantly reduce the viral activity of the \(\phi6\) virus. The optimum concentration of MB in solution containing \(\lambda\) phage is 50 \(\mu\)g/mL with 3.0 minutes of photolysis while it is 1 mg/mL with 10 minutes of photolysis in the frozen state of \(\lambda\) phage. Since oxygen is a necessary component in the viral inactivation of MB in solution, singlet oxygen must be the reactive species responsible for viral inactivation. This is further confirmed by the addition of a singlet oxygen quencher, histidine. Histidine retards the sensitization of MB of \(\lambda\) phage in solution. However, neither the absence of oxygen nor the presence of histidine hinders the inactivation of \(\lambda\) phage with MB in the frozen state (Table 21 and 22). Thus, the formation of singlet oxygen is not the primary pathway leading to viral inactivation of \(\lambda\) phage in the frozen state. Also, the sensitizer, MB, must be either bound to the \(\lambda\) virus or is present in the vicinity of the \(\lambda\) virus in order to effect viral inactivation in the frozen state.
F) **Summary:**

Water soluble halogenated psoralens and anthracenes were synthesized along with their non-halogenated controls. These sensitizers contain a DNA intercalator, such as a psoralen or anthracene unit, a polyammonium tether for increased water solubility and bromine or iodine as the radiation sensitizer. The ammonium salts of these compounds, bind strongly to the nucleic acids, presumably by a mixture of intercalative and electrostatic interactions.

The binding constants of the synthetic psoralens and anthracenes to calf thymus double stranded DNA, λ phage and the φ6 virus were determined using the ethidium bromide fluorescence quenching assay. The binding constants to the DNA are in the range of 10^{-10} M^{-1}. In general, the anthracenes bind better than the psoralens. Substitution of a halogen, bromine or iodine, on the psoralen (PSR-Br and PSR-I), dramatically improves the binding of the DNA relative to their non-halogenated control (PSR-H). Substitution of bromine, iodine or chlorine on the anthracene (A-Br, A-I or A-Cl) does not lead to a significant enhancement in the binding to the DNA. However, substitution of two bromines on the anthracene moiety (A-Br2) leads to at least a ten fold increase in the binding of the sensitizer to the DNA relative to its non-halogenated control (A-H).

Binding to λ phage and the φ6 virus follow the same trend as to the DNA. The actual binding constants to λ and φ6 were not determined. The concentrations where 50% and 10% fluorescence quenching of ethidium bromide occurred were determined for λ phage and the φ6 virus, respectively. Only the 10% fluorescence quenching concentration of the φ6 virus was determined since the sensitizers did not bind to φ6 very strongly. In general, the anthracenes required much less than the amount of psoralens to give 50% or 10% quenching of λ phage and the φ6 virus, respectively. The substitution of halogens on...
the psoralen nucleus (PSR-Br and PSR-I) dramatically improves binding of the sensitizer to the viruses but this is not true with the anthracenes. Substitution of one halogen on the anthracene (A-Br, A-I or A-Cl) does not improve the quenching concentration but the addition of two halogens (A-Br2) significantly improves the quenching concentration to the viruses. The psoralens containing two positive charges, PSR-H(PIP) and PSR-Br(PIP), do not bind to λ phage at all.

The ability of these synthetic sensitizers to nick the supercoiled DNA, plasmid pBR322, with 350 nm of light was determined and analyzed by gel electrophoresis. Among the psoralens, the psoralen containing the two positive charges PSR-Br(PIP), is the best sensitizer in nicking the DNA. PSR-Br and PSR-I are about equally efficient in nicking the plasmid DNA at equal concentration by weight relative to their non-halogenated control, PSR-H. Among the anthracene derivatives, the anthracene molecule containing two halogens, A-Br2, is the best in nicking the plasmid DNA. A-Cl and A-H are not very effective and A-I is better than A-Br in nicking the DNA. Therefore, the trend among the anthracenes in their ability to sensitize nicks of the plasmid supercoiled DNA is as follows; A-Br2 >> A-I > A-Br >> A-Cl = A-H.

Furthermore, the absence of argon and the presence of a radical scavenger, DTT, have no effect on the DNA nicking by the psoralens and anthracenes. A million fold excess of DTT did not inhibit DNA nicking by the sensitizers. Only at very high concentrations, (> 0.1M) does DTT inhibit DNA nicking. In addition, the presence of a DNA competitive binder, spermidine whose binding to calf thymus DNA is $10^3$ M$^{-1}$, protects the DNA from the sensitizers upon activation with UV light.

After determining their ability to nick the plasmid DNA, the ability of these sensitizers to inactivate model viruses that could be present in blood products was evaluated by the plaque forming assay at 350 nm. Two model viruses, λ phage and the φ6 virus,
were used in this study. A reference compound, AMT, was used to compare our synthetic
sensitizers to this literature reference compound.

PSR-Br is the best sensitizer against λ phage and the φ6 virus among the synthetic
psoralens of this work. PSR-Br is profoundly better than AMT against λ phage but AMT
is slightly better than PSR-Br against the φ6 virus. Among the anthracenes, A-Br2 is clearly
the best sensitizer with λ phage and the φ6 virus. A-Br2 is superior to AMT against both λ
phage and the φ6 virus. A-Br is slightly better than A-I which is slightly better than A-Cl
against both viruses. Of all the synthetic sensitizers, A-Br2 is the best sensitizer against λ
phage and the φ6 virus. Furthermore, the absence of oxygen and the presence of a radical
scavenger, DTT, do not inhibit viral inactivation of λ phage by these sensitizers.

In addition to studying DNA intercalators, we have also used dyes which are
halogenated derivatives of fluorescein (rose bengal, eosin Y). We have shown that these
dyes successfully inactivate λ phage and φ6 upon photolysis with 528 nm light or
irradiation with X-ray. Fluorescein (FL) is the non-halogenated control of iodinated rose
bengal (RB) and brominated eosin Y (EY). FL does not damage the viruses upon
photolysis or radialysis under the conditions used in this study.

RB is superior to EY against λ phage upon photolysis or irradiation with X-rays.
However, EY is much better than RB against φ6 virus upon photolysis with 528 nm light.
But upon irradiation with X-rays, RB is again more effective than EY against the φ6 virus.
Sensitization of λ phage with RB is oxygen dependent. In the absence of oxygen, RB
does not inactivate λ phage. However, the mechanism of sensitization of EY is not oxygen
dependent with λ phage and the φ6 virus.

To utilize far visible light (658 nm) to inactivate viruses, we have employed another
sensitizer, methylene blue (MB). MB does not inactivate the φ6 virus but it dramatically
reduces the viral activity of λ phage upon photolysis with 658 nm light. Viral inactivation
using MB against λ phage was performed in solution and in frozen medium. The optimum
concentration of MB required to inactivate λ phage is 50 μg/mL with 3 minutes of photolysis in solution and 1 mg/mL with 10 minutes of photolysis in frozen medium. The inactivation of MB is oxygent dependent in solution but not in the frozen state. The absence of oxygen does not inhibit viral inactivation in the frozen state. Furthermore, MB completely inactivates viruses up to a depth of 10 mm of frozen samples.

G) Conclusion:

The aim of this study was to identify conditions for the sterilization of viruses present in the human blood supply. The approach taken in this study is to inactivate model viruses by generating reactive free radicals in the vicinity of the viral target. The design of sensitizers needed to accomplish this aim incorporated several features. The sensitizers must selectively bind to the nucleic acids of the model viruses, relative to the blood products. They must contain functional groups which will not interfere with its binding to nucleic acids. Upon irradiation, the functional group must generate a highly reactive intermediate, such as a radical which can quickly react at the binding site in the nucleic acid. The sensitizers and not the blood products must selectively absorb the radiation to maintain the integrity and viability of the blood products. Lastly, they must be water soluble.

We synthesized water soluble halogenated psoralens and anthracenes. The nucleus of psoralen and anthracene serve as the DNA intercalator, the halogens, iodine, bromine and chlorine, serve as the radiation sensitizer and the ammonium salts provide the water solubility. These sensitizers were shown to bind to calf thymus DNA very strongly. Increasing the number of positive charges of the ammonium ion side chain on the nucleus of psoralen and anthracene, improved the binding of the sensitizers to the DNA. The anthracene derivatives, which contain three positive charges bind calf thymus DNA more strongly than the psoralens. Those psoralens which contain two positive charges bind
better than those containing one positive charge to the DNA. In addition, the incorporation of halogens also improves binding of these sensitizers to the DNA. The incorporation of two halogens is much better than one halogen in terms of binding.

The substitution of halogens makes the sensitizers more hydrophobic in nature hence, they are better able to slip between the hydrophobic interior of the helix. The presence of the positive charges on the sensitizers create the electrostatic binding of the sensitizers to the negatively charged DNA in addition to water solubility. Therefore, of all the synthetic sensitizers, A-Br2 which contains two bromines and three positive charges in its side chain, has the strongest binding to the DNA.

Nicking of the supercoiled DNA, plasmid pBR322, follows the trend of the DNA binding. Of the halogenated synthetic psoralens, the trend for nicking the plasmid DNA is as follows; PSR-Br(PIP) >> PSR-Br = PSR-I >> PSR-Br(Ether), which is similar to the binding trend. The trend for the anthracenes are: A-Br2 >> A-I > A-Br >> A-Cl. Furthermore, the nicking of the DNA by psoralen and anthracene derivatives is not oxygen dependent. Also since the presence of a million fold excess of a radical scavenger, DTT, does not inhibit the nicking of the DNA by these sensitizers, the aromatic radicals generated upon photolysis are intercalated within the helix. If the aromatic radicals were generated in bulk solution, then the presence of DTT would have completely inhibited the nicking of the DNA by the sensitizers. The presence of a competitive binder, spermidine, inhibited nicking of the DNA. Spermidine binds to the exterior grooves of the DNA and thus, prevents our sensitizers from intercalating to the DNA.

In addition to nicking of the DNA, halogen substitution on the nucleus of anthracene and psoralen greatly increases the effectiveness of viral inactivation with UV activation. Among the psoralens, PSR-Br is the best sensitizer in inactivating λ phage and the φ6 virus. Among the anthracenes, A-Br2 is the best sensitizer in inactivating λ phage.
and φ6 virus. Furthermore, of all the sensitizers, A-Br2 is the best sensitizer against the φ6 virus. Of all the halogen substituents, bromine is the most effective.

Viral inactivation of the sensitizer is not affected by the presence of DTT or argon. Therefore, the results are consistent with photochemically induced carbon-halogen bond cleavage to form a free radical center on the DNA intercalating molecule, possibly by an electron transfer type of mechanism or by direct homolysis. The sensitizer radical damages viral nucleic acid and/or the viral protein capsid or membrane. The encouraging results obtained from these experiments have led to the evaluation of these sensitizers in clinical platelet concentrates by the Cryoparm Corporation.

Since UV light damages red cells, we have used other sensitizers which are activated by visible light and/or X-rays. Viral inactivation of the halogenated fluorescein dyes, rose bengal and eosin Y with 528 nm light and X-rays has also produced encouraging results. These dyes are much more effective against the membrane enveloped virus, φ6, than the non-enveloped virus, λ phage. These dyes are lipophilic in nature and may bind selectively to the viral membrane and induce damage upon photolysis or radiolysis.

Bromine is once again better than iodine in viral inactivation with light. Eosin Y is the brominated derivatives of fluorescein and rose bengal is the iodinated derivative of fluorescein. Eosin Y is more effective against the φ6 virus than RB upon photolysis with light. Furthermore, viral inactivation of EY is not oxygen dependent. Therefore, the formation of singlet oxygen is not the primary pathway leading to viral inactivation. A new mechanism must be mediating the viral inactivation, possibly by the formation of aromatic type radicals resulting from the fragmentation of a carbon-bromine bond of EY.

Viral inactivation of φ6 virus with RB and EY upon irradiation with X-rays gives the trend opposite to that of photolysis. RB is the better sensitizer against φ6 upon
radiolysis. The mechanism of viral inactivation mediated by X-rays is quite different than that of photolysis.

Far visible light (658 nm) was utilized to effect viral inactivation with methylene blue. Methylene blue did not cause any damage to the φ6 virus but dramatically reduced the viral activity of λ phage. Viral inactivation of λ phage with MB is oxygen dependent and therefore must involve the formation of singlet oxygen. Viral inactivation of λ phage with MB was performed in frozen state. Much to our surprise, MB completely inactivates λ phage up to a depth of 1.0 cm in frozen samples. Furthermore, inactivation in the frozen state is not oxygen dependent. Thus, the formation of singlet oxygen is not the primary pathway of viral inactivation in the frozen state. MB must be bound to the virus or be in the vicinity of the virus in the frozen state.

Mechanistic studies to determine the precise origin of the halogen effect on DNA nicking and on viral inactivation are in progress. Since bromine is the best halogen for substitution of the aromatic nucleus, the effect of two bromines on the psoralen nucleus on viral inactivation would be worth studying. Variation in the side chains of the psoralen is currently in progress. A-Br₂ has three amino groups present. The effects of varying the amine group are currently in progress.
CHAPTER III

EXPERIMENTAL

Materials: Plasmid DNA pBR322 DNA was purchased from Boehringer Mannheim. Double strand calf thymus DNA was purchased from Sigma (St. Louis, MO). All bacterial and viral strains were obtained from The American Tissue Type Culture (ATTC) (Rockville, MD). Buffer solutions were either obtained from The Ohio State University Reagent Lab or prepared in our laboratory. AMT was purchased from HRI Inc. Dextrose saline was purchased from The Ohio State University Hospital. All other chemicals required for the synthesis and assay were of reagent grade or higher and were obtained from commercial sources.

General Procedure: Melting points were obtained with an electrothermal melting point apparatus and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker AM-250 spectrophotometer (1H at 270 MHz and 13C at 62.7 MHz). Infrared spectra were recorded on a Perkin Elmer model 1710 fourier transform infrared spectrophotometer. Ultraviolet spectra were recorded on a Perkin Elmer Lambda 3B UV-VIS spectrophotometer. Mass spectra and exact masses were obtained on a Kratos MS-30 mass spectrometer at the Campus Chemical Instrumentation Center. Lyophilizations were accomplished using a Viritis Freeze mobile-6 lyophilizer.
Binding constants of all the sensitizers to DNA were determined by the ethidium bromide fluorescence quenching assay\(^9\) using a Perkin Elmer LS-5 Fluorescence Spectrophotometer. The slit width was set to 10 mm on the fluorimeter. A total of 5 \(\mu\)g of calf thymus DNA (Sigma) and 5 \(\mu\)g of ethidium bromide (etBr) (Sigma) was mixed with a variable amount of the sensitizer in 4 mL of 10 mM Tris-HCl and 0.2 M NaCl, pH 7.4, in a test series. After equilibration at room temperature overnight the ethidium bromide fluorescence (\(\lambda_{ex}\) 540 nm, \(\lambda_{em}\) 610 nm) was measured. The fluorescence intensity was plotted against the concentration of the sensitizer. The concentration of synthetic intercalators resulting in 50% fluorescence "quenching", corresponding to replacement of 50% of the ethidium bromide in the DNA by the added sensitizer was determined. The term 50% "quenching" is defined as a 50% reduction of the fluorescence of bound etBr, relative to uncomplexed etBr, which is only slightly fluorescent. Since the concentration of ethidium bromide is known, the equilibrium constant of the sensitizer relative to ethidium bromide was calculated:

\[
K_{rel} = K_{etBr} \frac{[etBr]}{[\text{sensitizer at 50\% fluorescence}]}\]

where \(K_{etBr} = 1.5 \times 10^5 \text{M}^{-1}\).

Binding of sensitizers to \(\lambda\) phage and the \(\phi 6\) virus were determined using the same assay as described above for calf thymus DNA. The appropriate virus of known titre (\(10^7\) to \(10^{11}\) pfu) (133 \(\mu\)L of \(\lambda\) and 50 \(\mu\)L of \(\phi 6\)) was added to the test series instead of the DNA. The value of 50% fluorescence "quenching" due to the presence of sensitizer for \(\lambda\) virus and 10% fluorescence "quenching" for \(\phi 6\) were determined. It was not possible to obtain a binding constant for \(\lambda\) and \(\phi 6\) because the corresponding \(K_{etBr}\) value is unknown. Thus these results are reported in units of concentration of the sensitizer.

The photolysis solution containing DNA (pBR322) used in nicking experiments were prepared by mixing 1 part DNA solution (1.25 \(\mu\)g/mL) with 9 parts of sensitizer solution of variable concentrations and/or buffer solution as necessary. All psoralen derivatives solutions were prepared in 1% ammonium acetate buffer, pH 5.5. The
anthracene derivatives were prepared in TE buffer, pH 8.4. The concentration of the pBR322 used was 0.25 μg/μL. The dithiothreitol (DTT) (Aldrich) was prepared as a 1.0 mM solution in the appropriate buffer. The photolysis solutions were prepared in 300 μL pyrex HPLC samples injection vials (35 x 3 mm) and placed in a homemade pyrex sample holder. The photolysis experiments were conducted using a Rayonet reactor equipped with 8 lamps of 350 nm in a cold room at 3°C. Aliquots of 10 μL were removed as necessary and resolved by agarose gel electrophoresis.

The agarose gels were prepared in a 30 mL volume and cast in a Bio-rad mini sub gel apparatus. The size of the sub gel was 9 x 6 x 0.75 cm. Agarose was made up as 1.0% (w/v) solution in Plate preparation buffer (Appendix A). The electrode buffer used was running buffer (Appendix A). Samples were prepared for electrophoresis by the addition of 5 μL of the tracking dye (Appendix A) and 15 μL of TE buffer (Appendix A) to each irradiated, 10 μL, aliquots. Typically, 5 μL of sample volume was loaded into a well. DNA samples were conventionally run first at 65 Volts for 5 minutes and then at 45 Volts for typically one hour. Lambda DNA was digested with restriction endonuclease Hind III (Boehringer Mannheim) was used as a molecular weight marker. After the electrophoresis was complete, the Plates were stained in a bath containing 250 ml of water and 25 μl of 10 mg/ml ethidium bromide for 1 hr. The gels were then generally destained for another hour and visualized using a Fotodyne model 3-4400 Transilluminator and photographed with a Polaroid MP-4 land camera using P667 film.

**Bacteria, viruses and viral assays:**

The protein coated Lambda virus, the enveloped bacteriophage phi 6 virus and their hosts E. coli strain C600 and pseudomonas syringae, respectively, were obtained from the American Type Culture Collection (ATCC, Rockville, MD).
The dehydrated E. coli. strain bacteria was reconstituted by placing 1.0 ml of LB media (Appendix A) to the ATCC pellet of E. coli and vortexed until dissolved. The resulting suspension was transferred into a centrifuge tube containing 25 mL of LB media. The tube was loosely capped and placed in a shaking bath incubator (Blue M, magniwhirl constant temp bath) overnight at 37°C at 120 rpm. The overnight culture was streaked on a LB plate (Appendix A) and incubated (Precision Inst. model 4) at 37°C overnight to obtain isolated colonies. Single colonies were picked up and placed into 25 mL of LB and incubated at 37°C in the shaking bath incubator overnight. The resulting bacteria was used as such and was good for 10-14 days. After this, a fresh stock from previously grown stock had to be grown since the bacteria loses its potency after this time.

The pseudomonas syringae pellet obtained from ATCC was broken into many smaller pellets using an autoclaved sterile spatula and placed in small vials. One of these small pellets was placed in 0.3 mL of NBY media (Appendix A) and vortexed until dissolved. It was then streaked onto a NBY plate (Appendix A) and incubated at room temperature for 48-64 hrs to obtain isolated colonies. Single colonies of P. syringae were removed from the plate with sterile loops (Fisher Sci.) and placed in flasks containing 50 mL of NBY media (Appendix A), one colony per flask. The flasks were incubated at RT for 48 hrs on a water bath shaker (Baxter). Stocks of plating bacteria were prepared and tested against the phi 6 virus using the plaque forming assay (see plaque assay below) to choose the correct strain of the bacteria for the virus. It is necessary to plate out a new stock of the bacteria with the corresponding virus as this bacteria has a tendency to mutate. Therefore, colonies of mutated bacterial strains have been isolated when growing the bacteria. If the wrong bacterial colonies were obtained from the plates, plaques will not form after plating the bacteria. The flasks of bacteria which show plaques after plating are taken as the desired bacterial strain. The desired bacterial suspension was transferred to a
cenrifuge tube and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 0.1 M of MgSO\textsubscript{4} solution to obtain an OD of 2.0-2.2 at 600 nm. This stock was useable for 10 days, after this time, a new stock had to be grown from single colonies. The originally streaked plate can only be used for two weeks to isolate single bacterial colonies. Furthermore, only two generations of bacteria can be grown from a single stock. Therefore, it is necessary to restreak a plate of P. syringae from the original ATCC pellet every two weeks and repeat this procedure everytime. This bacteria has the tendency to mutate after two weeks hence it is no longer the host of \( \phi 6 \) virus. Because of this problem, only two generations of bacteria can be grown from a single stock.

**Viruses**

The dehydrated viruses, lambda and phi 6 were reconstituted by adding 0.5 mL of LB and NBY media (Appendix A), respectively. Pellets were vortexed until dissolved and a plaque assay was prepared using the appropriate media. Phage was extracted from the plates with confluent lysis by adding 2-3 mL of the appropriate media onto each plate. The plates were stored in a refrigerator overnight at 4°C. The resulting suspension was transferred with a sterile pipette to a centrifuge tube and spun at 5000 rpm for 10 min at room temperature. The supernatant was decanted and the plaque assay (see plaque assay below) was prepared to determine the viral titre. Typically, the stock suspensions of the viruses had titres of \( 10^7 \) to \( 10^{10} \) plaque forming units (pfu) per mL.

**Light sources and photochemistry**

Samples were photolyzed at the appropriate wavelength in a home made photolysis chamber equipped with 6 bulbs of the appropriate wavelength at RT for \( \lambda \) virus and with 4 bulbs of the appropriate wavelength at 30° C for \( \phi 6 \) virus at the appropriate time intervals. The bulbs were arranged in a horizontal array. Screening studies were performed in
covered (pyrex) petri dishes that were approximately 2.5 inches from the lightbulbs. A sheet of plate glass was placed between the lamps and the sample. The amount of light delivered to the samples was determined using an optical power meter (International Light Inc., model 1L1400A) fitted with a 230 to 850 nm radiometric sensitive element. The light flux was measured after every experiment.

**Viral assay (plaque forming assay)**

Inactivation of viruses by the sensitizers were determined using the plaque forming assay. Stock solutions (1mg/ml) of each sensitizer were prepared. For λ phage, solutions of sensitizers were prepared in dextrose-saline (5% dextrose and 0.9% NaCl) and in PBS buffer, pH=7.4 for φ6 virus. Due to solubility problems, some samples had to be prepared in DMSO. Screening studies were performed by adding 100 µL of a titred stock suspension of the virus and the desired concentration of the stock solution of the sensitizer. The total volume was adjusted to 3.0 mL with the appropriate buffer. Control experiments in the dark and with light without any sensitizer were always maintained.

Serial tenfold dilutions were made from samples of control and experimental petri dishes by taking 100 µL of the sample and placing it in a test tube marked zero dilution. Another 100 µL of the sample was taken and mixed with 0.9 mL of storage media (SM) (Appendix A) for the λ phage and 100 µL of this mixed solution was placed in an another test tube and marked as dilution one. Another 100 µL of this mixed solution was taken and mixed with another 0.9 mL of SM to give a total of seven dilutions (0-6). To 100 µL of the diluted samples, 100 µL of the appropriate bacteria was added to each dilution and these tubes were incubated at 37°C (λ) and at RT (φ6) for twenty minutes. Plaques were visualized by adding 3.0 mL of LB top agar (λ) and NBY top agar (φ6) (Appendix A) to each tube and the solutions were poured onto previously prepared agar plates and allowed to set at room temperature. Plates were then placed in an incubator at 37°C (λ) and at RT
(φ6) overnight. Plaques were counted manually after incubation. For the φ6 virus, the serial dilutions were performed in identical fashion as λ phage except the NBY medium (Appendix A) was used instead of SM.

**Calibration of the X-ray machine**

Dosimetry calibration of the X-ray source (Norelco, MG 150 tungsten tube) was accomplished using Fricke's dosimetry. Fricke's dosimetry solution contains ferrous ammonium sulfate hexahydrate (10^{-3} \text{ M}), sodium chloride (10^{-3} \text{ M}) and sulfuric acid (0.4 M) in 4.0 mL. The absorbance (OD) of this solution is taken at 304 nm before irradiation and then after exposure at known time intervals. The dose in rads is calculated using the following equation:

\[
\text{Dose} = D_A = 9.65 \times 10^6 \left( \frac{\text{OD}_i - \text{OD}_n}{\Delta \epsilon \ell p G} \right) \text{ (Gy)} \times 100
\]

where \text{OD}_i is the absorbance after irradiation, \text{OD}_n is the absorbance before irradiation and \( \Delta \epsilon \ell p \) is the difference in molar extinction coefficient between Fe^{+3} and Fe^{+2} at the monitoring wavelength (2201 M^{-1} cm^{-1}). G is the number of molecules changed per 100 eV energy absorbed (15.6). Dose in rads is plotted against the corresponding time intervals to give a linear plot.

A sample calculation:

\[
\text{OD}_i = 0.083 \text{ after one minute of irradiation; } \text{OD}_n = 0.061
\]

\[
\text{Dose} = D_A = 9.65 \times 10^6 \left( \frac{0.083 - 0.061}{(2201 \text{ M}^{-1} \text{ cm}^{-1} \times 1 \times 1 \times 15.6) \times 1} \right) \times 100
\]

\[
\text{Dose} = D_A = 618.3 \text{ rads}
\]
Viral inactivation using X-rays

Samples for viral inactivation using X-ray were prepared in a manner identical to that used for UV-VIS photolysis as described earlier. The pyrex petri dish is covered with a piece of saran wrap instead of a pyrex cover.

Deoxygenated samples

For the experiments that were conducted in the absence of oxygen (in the presence of saturating argon), the samples were degassed by bubbling the sample in a vial with argon for two minutes. The sample were then placed in a pyrex petri dish and degassed by bubbling with argon for an additional minute before photolysis/irradiation.
Synthesis of the sensitizers:

PSR-H (PIP), PSR-Br (PIP), PSR-H (Ether) and PSR-Br (Ether) (figure 11, p. 127) were kindly synthesized by Dr. S. Park in our laboratory. The NMR spectra of all the sensitizers used in this study are included in Appendix B.

8-(w-diethylaminopropyloxy)psoralen hydrochloride (PSR-H):

8-Methoxypsoralen (2.5g, 12.5mmol) was dissolved in dry methylene chloride (50ml) and added to a three-neck round bottom flask fitted with a reflux condenser under nitrogen. Boron tribromide in hexane (2.5ml, 25mmol) was then added in one portion to the flask via syringe. The resulting reaction mixture was stirred at RT for 7 hours after which some yellow solid precipitated. Water (2ml) was added cautiously, causing an exotherm, which subsided after a few minutes. Additional water (125ml) was added and the heterogeneous mixture was stirred at RT overnight. The crude product was collected by vacuum filtration and air dried. The crude product was recrystallized from acetonitrile (50ml) to give the product, 8-hydroxypsoralen, (1.45g, 7.03mmol, 56% yield); m.p. 247-249°C, literature m.p. 247-249°C. 1H NMR (DMSO-d6) δ: 6.25 (d, 1H), 7.05 (s, 1H), 8.15 (ds, 2H), 10.6 (s, alcohol-H).

8-Hydroxypsoralen (1.0g, 4.9mmol) was dissolved in anhydrous acetone (100ml). 1,3-Dibromopropane (2.0ml) and anhydrous K2CO3 (5.0g) were added. The resulting
mixture was refluxed for 48 hours. After cooling, the acetone solution was filtered and the solid residue was washed twice with acetone (50ml). The combined acetone solutions were concentrated to yield an oil which was dissolved in benzene (150ml). This was chromatographed through a silica Plate column containing water (5%). The product eluted with benzene and was concentrated to give a white solid. This material was recrystallized from benzene:hexane (1:5) to give the product, 8-(w-propyloxy)psoralen, (1.2g, 3.7mmol, 76% yield); m.p. 103-105°C, literature m.p. 102°C98. 1H NMR (CDCl₃) δ: 2.30-2.45 (m, 2H), 3.75 (t, 2H), 4.65 (t, 2H), 6.35 (d, 1H), 7.8 (s, 1H), 7.4 (s, 1H), 7.75 (d, 2H).

8-(w-bromopropyloxy)psoralen (0.70g, 2.1mmol) was disssolved in anhydrous ethanol (10ml). Diethylamine (2.1ml) was added and heated at 65°C for five hours. After cooling, the brown solution was poured into water (100ml) and extracted twice with chloroform (200ml). The organic layer was dried with MgSO₄ and concentrated to give an oil. The hydrochloride salt of the product was precipitated by adding anhydrous ethanol (2ml), conc. aqueous HCl (0.5ml) and ether (2ml). The resulting mixture was cooled in the freezer overnight and vacuum filtered to give a white solid which was recrystallized from anhydrous ethanol:hexane (1:5) to give the salt (.5g, 1.42mmol, 68% yield); M.P. 145-147°C, literature m.p.131°C98. 1H NMR (D₂O/DMSO-d₆) δ: 1.2 (t, 6H), 2.1 (m, 2H), 3.1 (q, 4H), 3.3 (m, 2H), 4.45 (t, 2H), 6.3 (d, 1H), 6.95 (s, 1H), 7.55 (s, 1H), 7.9-8.05 (ds, 2H). 13CNMR (DMSO) δ: 8.32, 23.85, 46.17, 46.49, 47.61, 70.83, 107.08, 114.11, 114.43, 116.36, 125.68, 130.73, 142.83, 145.16, 147.23, 147.80, 159.58. FAB mass spectrum, m/e 316 (M+1)⁺, calculated (C₁₈O₄NH₂₁) m/e 315.
5-Bromo-8-(w-diethylaminopropyloxy)psoralen hydrochloride (PSR-Br):

![Chemical structure]

To a solution of 8-methoxypsoralen (0.75g, 3.5mmol) in THF (75ml), was added bromine (3.5mmol, 1.5ml) in THF (5ml) dropwise. The resulting mixture was stirred at RT overnight. 10% Sodium thiosulfate (5ml) was added and the solution was basified using conc. aqueous NH₄OH, and then extracted twice with chloroform. The organic layer was recrystallized from chloroform to give the product, 5-bromo-8-methoxypsoralen, (0.75g, 2.55mmol, 73.5% yield); m.p. 178-180°C. NMR (CF₃CO₂D) δ: 4.4 (d, 3H), 6.6 (d, 1H), 6.9 (s, 1H), 7.75 (s, 1H), 8.4 (d, 1H). Exact mass spectrum m/e 295.9529, calculated (C₁₂O₄BrH₇) m/e 295.0956

5-Bromo-8-methoxypsoralen (0.70g, 2.4mmol) was dissolved in dry methylene chloride (50ml) and added to a three-neck round bottom flask fitted with a reflux condenser under nitrogen. Boron tribromide in hexane (5.1ml, 5mmol) was then added in one portion to the flask via syringe. The resulting reaction mixture was stirred at RT for 7 hours after which some yellow solid precipitated. Water (2ml) was added cautiously, causing an exotherm, which subsided after a few minutes. Additional water (125ml) was added and the heterogeneous mixture was stirred at RT overnight. The crude product was collected by vacuum filtration and air dried. The crude product was recrystallized from acetonitrile (45ml) to give the product, 5-bromo-8-hydroxypsoralen, (0.40g, 1.43mmol,
58% yield); m.p. 254-256°C. $^1$H NMR (DMSO-d$_6$) $\delta$: 6.5 (d, 1H), 7.0 (s, 1H), 8.1-8.2 (d, 2H), 10.8-11.1 (broad, alcohol-H).

5-Bromo-8-hydroxypsoralen (0.35g, 1.22mmol) was dissolved in anhydrous acetone (100ml). 1,3-Dibromopropane (1.5ml) and anhydrous K$_2$CO$_3$ (3.0g) were added. The resulting mixture was refluxed for 48 hours. After cooling, the acetone solution was filtered and the solid residue was washed twice with acetone (950ml). The combined acetone solutions were concentrated to yield an oil which was dissolved in benzene (150ml). This was chromatographed through a silica Plate column containing water (5%). The product eluted with benzene and was concentrated to give a white solid. This material was recrystallized from benzene:hexane (1:5) to give the product, 5-bromo-8-(w-bromopropyloxy)psoralen, (0.30g, 0.73mmol, 60% yield); m.p. 94-98°C. $^1$H NMR (DMSO-d$_6$) $\delta$: 2.20-2.30 (m, 2H), 3.75 (t, 2H), 4.5 (t, 2H), 6.5 (d, 1H), 7.05 (s, 1H), 8.15 (d, 1H), 8.25 (s, 1H).

5-Bromo-8-(w-bromopropyloxy)psoralen (0.30g, 0.73mmol) was dissolved in anhydrous ethanol (10ml). Diethylamine (2ml) was added and heated at 65°C for five hours. After cooling, the brown solution was poured into water (100ml) and extracted twice with chloroform (200ml). The organic layer was dried with MgSO$_4$ and concentrated to give an oil. The hydrochloride salt of the product was precipitated by adding anhydrous ethanol (2ml), conc. aqueous HCl (0.5ml) and ether (2ml). The resulting mixture was cooled in the freezer overnight and the precipitate was vacuum filtered to give a white solid which was recrystallized from anhydrous ethanol:hexane (1:5) to give the salt (.15g, .35mmol, 48% yield); M.P. 196-198°C. $^1$H NMR (D$_2$O/DMSO-d$_6$) $\delta$: 0-0.1 (t, 6H), 0.8-0.95 (m, 2H), 1.9-2.05 (q, 4H), 2.1-2.2 (m, 2H), 3.15 (t, 2H), 5.0 (d, 1H0, 5.5 (s, 1H), 6.5 (d, 1H), 6.6 (s, 1H). $^{13}$CNMR (DMSO) $\delta$: 8.36, 23.84 46.18, 47.60, 71.03, 105.63, 111.16, 115.40, 116.07, 127.34 130.29, 142.48, 143.67,
FAB mass spectrum, m/e 396 (M+1)$^+$, calculated (C$_{18}$O$_4$NBrH$_2$O) m/e 395.

5-Iodo-8-(w-diethylaminopropyl oxy)psoralen hydrochloride (PSR-I):

In a 500ml three-neck round bottom dried flask, silver trifluoroacetate (1.1g, 5mmol) was placed and a solution of 8-methoxypsoralen (1.1g, 5mmol) in anhydrous chloroform (50ml) was added slowly. The reaction mixture was stirred vigorously at RT under nitrogen. Iodine (1.25g, 5mmol) in dry chloroform (100ml) was added dropwise over 3hrs. The reaction was stirred for an additional one hour. The precipitated silver iodide was filtered off, washed well with chloroform and the filtrate was evaporated in vacuo to yield a pale yellow solid. This was recrystallized in MeOH/CHCl$_3$ to give the product, 5-iodo-8-methoxypsoralen, (1.0g, 3.0mmol, 62.0% yield); m.p. 189-191$^\circ$C, literature m.p. 191$^\circ$C.$^99$ 1H NMR (DMSO-D$_6$) d: 4.15 (s, 3H), 6.5 (d, 1H), 6.88 (s, 1H), 8.05 (d 1H), 8.25 (s, 1H).

5-Iodo-8-methoxypsoralen (0.95g, 2.9mmol) was dissolved in dry methylene chloride (50ml) and added to a three-neck round bottom flask fitted with a reflux condenser under nitrogen. Boron tribromide in hexane (6.4ml, 6.4mmol) was then added in one portion to the flask via syringe. The resulting reaction mixture was stirred at RT for 7 hours after which some yellow solid precipitated. Water (2ml) was added cautiously, causing an exotherm, which subsided after a few minutes. Additional water (125ml) was
added and the heterogeneous mixture was stirred at RT overnight. The crude product was collected by vacuum filtration and air dried. The crude product was recrystallized from acetonitrile to give the product, 5-iodo-8-hydroxypsoralen, (0.65g, 1.98mmol, 68.3% yield); m.p. 210-215°C. 1H NMR (DMSO-d6) δ: 6.45 (d, 1H), 6.82 (s, 1H), 8.00 (d, 1H), 8.19 (s, 1H) 10.95 (broad, alcohol-H).

5-Iodo-8-hydroxypsoralen (0.65g, 1.98mmol) was dissolved in anhydrous acetone (100ml). 1,3-Dibromopropane (0.81ml) and anhydrous K₂CO₃ (2.0g) were added. The resulting mixture was refluxed for 48 hours. After cooling, the acetone solution was filtered and the solid residue was washed twice with acetone. The combined acetone solutions were concentrated to yield an oil which was dissolved in benzene (150ml). This was chromatographed through a silica Plate column containing water (5%). The product eluted with benzene was concentrated to give a white solid. This material was recrystallized from benzene:hexane (1:5) to give the product, 5-iodo-8-(w-bromopropoxy)psoralen, (0.20g, 0.45mmol, 23% yield); m.p. 119-121°C. 1H NMR (DMSO-d6) δ: 2.20-2.35 (m, 2H), 3.75 (t, 2H), 4.5 (t, 2H), 6.48 (d, 1H), 6.95 (s, 1H), 8.07 (d, 1H), 8.25 (s, 1H).

5-Iodo-8-(w-bromopropoxy)psoralen (0.20g, 0.45mmol) was dissolved in anhydrous ethanol (10ml). Diethylamine (1ml) was added and heated at 65°C for five hours. After cooling, the brown solution was poured into water (100ml) and extracted twice with chloroform (200ml). The organic layer was dried with MgSO₄ and concentrated to give an oil. The hydrochloride salt of the product was precipitated by adding anhydrous ethanol (2ml), conc. aqueous HCl (0.5ml) and ether (2ml). The resulting mixture was cooled in the freezer overnight and vacuum filtered to give a white solid which was recrystallized from anhydrous ethanol:hexane (1:5) to give the salt (0.10g, 0.21mmol, 47% yield); m.p. 208-211°C. 1H NMR (D₂O/DMSO-d₆) δ: 0-0.1 (t, 6H), 0.8-0.95 (m, 2H), 1.9-2.05 (q, 4H), 2.1-2.2 (m, 2H), 3.15 (t, 2H), 5.0 (d, 1H), 5.5 (s,
1H), 6.5 (d, 1H), 6.6 (s, 1H). $^{13}$CNMR (DMSO) δ: 9.45, 25.02, 47.99, 49.33, 71.77, 82.77, 111.39, 116.54, 118.98, 131.56, 133.11, 143.63, 145.81, 148.50, 149.07. FAB mass spectrum, m/e 442 (M+1)$^+$, calculated (C$_{18}$O$_4$NiH$_2$O) m/e 441.

I-N-(9-Anthrylmethyl) Diethylenetriamine hydrochloride (A-H)$_{100}$:

![Chemical Structure](image)

A solution of 9-anthracenemethanol (1.0g, 4.8mmol) and con. HBr (50ml) stirred at room temperature for two days. The solution was vacuum filtered and air dried to give a lemon yellow solid (1.2g, 5.0mmol, 96%). $^1$H NMR (CDCl$_3$) δ: 5.55 (s, 2H), 7.5 (m, 2H), 7.65 (m, 2H), 8.0 (d, 2H), 8.3 (d, 2H), 8.45 (s, 2H).

A solution of 9-bromomethylanthracene (1.2g, 5.0mmol) and diethylenetriamine (5 ml, 40 mmol) in toluene (50 ml) was heated at reflux for 12 hrs. The solution was then cooled to room temperature and extracted twice with 6M NaOH, then twice with water. The organic layer was dried by filtration through Na$_2$CO$_3$. The solvent was evaporated at reduced pressure leaving an oily yellow residue which was taken up as a fine suspension in ethanol and treated with 10 ml of conc. HCl. The resulting mixture was stored in the freezer. After several hours the resulting precipitate was collected by vacuum filtration. The solid was treated with 6M NaOH and extracted with CHCl$_3$. The organic layer was filtered through Na$_2$CO$_3$ and the solvent removed by rotary evaporation. The orange residue was dissolved in a small amount of CHCl$_3$ and loaded onto a small column of neutral alumina (activity V). It was eluted first with CHCl$_3$ thereby removing non-basic
anthracene impurities at the solvent front, then with CHCl₃/4% MeOH. The yellow band which eluted was evaporated to yield a yellow oil and then dissolved in EtOH. Conc. HCl (2 ml) was added resulting in a light yellow precipitate which was vacuum filtered and air dried to give a yellow powder (0.9 g, 2.2 mmol, 44.9% yield) m.p. 269-270°C. ¹H NMR (D₂O/DMSO-d₆) δ: 3.15 (m, 2H), 3.3 (m, 2H), 3.4 (m, 2H), 3.6 (m, 2H), 5.15 (s, 2H) 7.5 (m, 2H), 7.65 (m, 2H), 8.0 (d, 2H), 8.2 (d, 2H), 8.55 (s, 1H). ¹³C NMR (DMSO-d₆) δ: 35.03, 42.67, 42.81, 43.68, 43.88, 122.77, 124.56, 125.53 127.07, 129.00, 129.93, 130.66, 130.85. FAB mass spectrum m/e 294 (M+1)⁺, calculated (C₁₉N₃H₂₂) m/e 293.

1-N-(9-Anthrylmethyl-10-Bromo) Diethylenetriamine hydrochloride (A-Br):

A solution of 9-methylanthracene (2 g, 10 mmol) and cupric bromide (4.4 g, 20 mmol) in CCl₄ (60 ml) was heated at reflux for 24 hrs in a round bottom flask connected to a condenser attached to a drying tube. The reaction mixture was cooled to room temperature and the cuprous bromide by-product was removed by vacuum filtration. The carbon tetrachloride solution was passed through a short column of neutral activity one alumina. The column was eluted with 200 ml of CCl₄. The eluant was evaporated to dryness and recrystallized from petroleum ether (b.p. 60-80°C) to give yellow needles
(1.1g, 4 mmol, 41% yield), m.p. 155-160°C. $^1$HNMR (CDCl$_3$) δ: 3.05 (s, 3H), 7.45-7.65 (m, 4H), 8.3 (d, 2H), 8.6 (d, 2H).

A solution of 10-bromo-9-methylanthracene (0.9g, 3.3 mmol) and N-bromosuccinimide (1,1 g, 6.6 mmol) in CCl$_4$ (50 ml) was heated at reflux for 12 hrs. Benzoyl peroxide was added in a catalytic portion as a radical initiator. The reaction mixture was cooled to room temperature and vacuum filtered to remove succinimide. The filtrate was dissolved in CCl$_4$ (15 ml) washed once with water, dried over anhydrous MgSO$_4$, filtered and evaporated to give a brown solid (1g, 3 mmol, 91% yield) which was used immediately. A solution of 10-bromo-9-bromomethylanthracene (1g, 3 mmol) and diethylenetriamine (1.6 ml, 15 mmol) in toluene (50 ml) was heated at reflux for 12 hrs. The solution was then cooled to room temperature and extracted twice with 6M NaOH, then twice with water. The organic layer was dried by filtration through Na$_2$CO$_3$. The solvent was evaporated at reduced pressure leaving an oily yellow residue which was taken up as a fine suspension in ethanol and treated with 10 ml of conc. HCl. The resulting mixture was stored in the freezer. After several hours the resulting precipitate was collected by vacuum filtration. The solid was treated with 6M NaOH and extracted with CHCl$_3$. The organic layer was filtered through Na$_2$CO$_3$ and the solvent removed by rotary evaporation. The orange residue was dissolved in a small amount of CHCl$_3$ and loaded onto a small column of neutral alumina (activity V). It was eluted first with CHCl$_3$ removing anthracene impurities at the solvent front, then with CHCl$_3$/4%MeOH. The yellow band which eluted was evaporated to yield a yellow oil and dissolved in EtOH. Conc. HCl (2 ml) was added resulting in a light yellow precipitate which was vacuum filtered and air dried to give a yellow powder (0.5g, 1mmol, 34.5% yield) m.p. 269-270°C. $^1$H NMR (D$_2$O/DMSO-d$_6$) δ: 3.2-3.5 (m, 8H), 4.9 (s, 2H), 7.38-7.58 (m, 4H), 7.9 (d, 2H), 8.15 (d, 2H). $^{13}$C NMR (DMSO-d$_6$) δ: 36.74, 44.85, 44.94, 45.14,
45.97, 122.35, 124.58, 127.91, 129.52, 130.61, 131.86. FAB mass spectrum m/e 374 (M+1)^+, calculated (C_{19}N_3BrH_{22}) m/e 373.

1-N-(9-Anthrylmethyl-10-Chloro) Diethylenetriamine hydrochloride (A-Cl):

![Chemical structure](image)

A solution of 9-methylanthracene (2.4g, 13 mmol) and cupric chloride (3.5g, 26 mmol) in CCl\(_4\) (60 ml) was heated at reflux for 18 hrs in a round bottom flask connected to a condenser attached to a drying tube. The reaction mixture was cooled to room temperature and the cuprous chloride by-product was removed by vacuum filtration. The carbon tetrachloride solution was passed through a short column of neutral activity one alumina. The column was eluted with 200 ml of CCl\(_4\). The eluant was evaporated to dryness and recrystallized from petroleum ether (b.p. 60-80°C) to give yellow needles of 10-chloro-9-methylanthracene (0.63g, 2.8 mmol, 21% yield), m.p. 164-166°C. \(^1\)HNMR (CDCl\(_3\)) d: 3.1 (s, 3H), 7.65 (m, 4H), 8.3 (d, 2H), 8.55 (d, 2H).

A solution of 10-chloro-9-methylanthracene (0.6g, 2.63 mmol) and N-bromosuccinimide (0.52g, 2.89 mmol) in CCl\(_4\) (50 ml) was heated at reflux for 12 hrs. Benzoyl peroxide was added in a catalytic portion as a radical initiator. The reaction mixture was cooled to room temperature and vacuum filtered to remove succinimide. The filtrate was dissolved in CCl\(_4\) (15 ml) washed once with water, dried over anhydrous MgSO\(_4\), filtered and evaporated to give a brown solid (0.79g, 2.58mmol, 98% yield).
which was used immediately. A solution of 10-chloro-9-bromomethylanthracene (0.79g, 2.58mmol) and diethylenetriamine (3.84 ml, 36 mmol) in toluene (50 ml) was heated at reflux for 12 hrs. The solution was then cooled to room temperature and extracted twice with 6M NaOH, then twice with water. The organic layer was dried by filtration through Na₂CO₃. The solvent was evaporated at reduced pressure leaving an oily yellow residue which was taken up as a fine suspension in ethanol and treated with 1.0 ml of conc. HCl resulting in a brown precipitate which was vacuum filtered and air dried to give a brown powder (0.63g, 1.44mmol, 56% yield). ¹H NMR (D₂O/DMSO-d₆) δ: 3.2-3.3 (m, 6H), 3.48 (m, 2H) 4.9 (s, 2H), 7.38-7.55 (m, 4H), 8.0-8.2 (dd, 4H). ¹³C NMR (DMSO-d₆) δ: 34.97, 42.69 43.67, 43.81, 123.38, 124.62, 125.44, 127.35, 127.58, 127.74 130.56, 131.01. FAB mass spectrum m/e 328 (M+1)⁺, calculated (C₁₉N₃ClH₂₂) m/e 327.

1-N-(2-anthrylmethyl-9,10-dibromo)diethylenetriamine hydrochloride (A-Br₂):

Bromine (0.6ml, 0.11mol) in CCl₄ (10ml) was added dropwise in a 250ml three-neck round bottom flask containing a suspension of 2-methylanthracene (1.0g, 5.2mmol) in CCl₄ (30ml) and stirred vigorously. After the addition of bromine was complete, the reddish solution was gently heated to reflux for one hour. The resulting solution was cooled to room temperature and concentrated to give an oily product. This oily substance was recrystallized from petroleum ether (60-80°C) to give the product, 9,10-dibromo-2-bromomethylanthracene (0.78g, 1.82mmol, 34% yield), m.p. 115-120°C.
bromomethylanthracene (0.78g, 1.82mmol, 34% yield), m.p. 115-120°C. $^1$HNMR (CDCl$_3$) $\delta$: 4.7 (s, 2H), 7.58 (m, 3H), 8.56 (m, 4H). Exact mass spectrum m/e 429.8040, calculated (C$_{15}$Br$_3$H$_9$) m/e 428.9652.

A solution of 9,10-dibromo-2-bromomethylanthracene (0.25g, 0.58mmol) and diethylenetriamine (0.63 ml, 5.8 mmol) in toluene (20 ml) was heated at reflux for 12 hrs. The solution was then cooled to room temperature and extracted twice with 6M NaOH, then twice with water. The organic layer was dried by filtration through Na$_2$CO$_3$. The solvent was evaporated at reduced pressure leaving an oily yellow residue which was taken up as a fine suspension in anhydrous ethanol and treated with 0.5 ml of conc. HCl resulting in a brown precipitate which was vacuum filtered and air dried to give a brown powder (0.28g, 0.54mmol, 92% yield). $^1$H NMR (D$_2$O/DMSO-d$_6$) $\delta$: 3.2-3.3 (m, 6H), 3.6(m, 2H) 4.5 (s, 2H), 7.55 (m, 3H), 8.15-8.4 (m, 4H). $^{13}$C NMR (DMSO-d$_6$) $\delta$: 35.04, 42.52, 43.75, 49.91, 57.43, 122.60, 127.73, 128.21, 129.57, 130.06, 130.47, 131.92. FAB mass spectrum m/e 452 (M+1)$^+$, calculated (C$_{19}$N$_3$Br$_3$H$_{23}$) m/e 453.

I-N-(9-anthrylmethyl-10-iodo) diethylenetriamine hydrochloride (A-I):

Yellow mercuric oxide (4.32g, 0.02mol) was added to a stirred solution of trichloroacetic acid (8.0g, 0.04mol) and anthracene (3.56g, 0.02mol) in methylene chloride (70ml) at room temperature. The mixture thickened appreciably and was shaken overnight and vacuum filtered. The yellow green solid was washed with methanol to give 9-
trichloroacetatomercurianthracene (6.3g, 0.012mol, 58.3% yield). The product was dissolved in acetone (60ml) and a solution of iodine (3.05g, 0.12mol) in acetone (10ml) was added rapidly. The mixture was brought to reflux briefly then filtered into aqueous KI (10g, 50ml water) and vacuum filtered to collect the product. The crude product was washed with KI and water and air dried to give iodoanthracene. The crude product was recrystallized from MeOH/CHCl₃ to give (1.3g, 4.09 mmol, 34.8% yield), m.p. 76-78°C, literature m.p. 78°C102. ¹H NMR (CDCl₃) δ: 7.4-76 (m, 4H), 7.95 (ds, 3H). Exact mass spectrum m/e 304.8954, calculated (C₁₄H₉) m/e 304.1315

Iodoanthracene (0.41g, 1.29mmol) was dissolved in acetic acid (10ml). Chloromethyl methylether (5ml) was added to this clear solution and stirred at room temperature for three days. Water (30ml) was added and the product was vacuum filtered, washed with some more water and air dried to give yellow solid (0.42g, 1.19mmol, 92.8% yield), m.p. 141-145°C, decom. 110°C. ¹H NMR (CDCl₃) δ: 5.55 (s, 2H), 7.54-7.7 (m, 4H), 8.3-8.4 (m, 2H), 8.55 (d, 2H). Exact mass spectrum m/e 351.9470, calculated (C₁₅HI₃) m/e 352.60435.

A solution of 10-iodo-9-chloromethylanthracene (0.35g, 1.0mmol) and diethylenetriamine (1.08 ml, 10mmol) in toluene (25 ml) was heated at reflux for 12 hrs. The solution was then cooled to room temperature and extracted twice with 6M NaOH, then twice with water. The organic layer was dried by filtration through Na₂CO₃. The solvent was evaporated to give an oily yellow residue which was taken up as a fine suspension in ethanol and treated with 0.5 ml of conc. HCl resulting in a yellow precipitate which was vacuum filtered and air dried to give a yellow solid (0.32g, 0.61mmol, 61% yield). ¹H NMR (D₂O/DMSO-d₆) δ: 3.2-3.3 (m, 6H), 3.48 (m, 2H) 4.9 (s, 2H), 7.38-7.55 (m, 4H), 8.0-8.2 (dd, 4H). ¹³C NMR (DMSO-d₆) δ: 34.972, 42.692, 43.668, 43.808, 123.376, 124.618, 125.441, 127.351, 127.582, 127.742 130.560, 131.008. FAB mass spectrum m/e 420 (M+1)⁺, calculated (C₁₉N₃H₂₂) m/e 419.
Figure 16: The chemical structures of additional psoralen derivatives used in this study.

![Chemical structures of additional psoralen derivatives](image)

X = H, PSR-H (PIP)
X = Br, PSR-Br (PIP)

X = H, PSR-H (Ether)
X = Br, PSR-Br (Ether)

**Determination of binding constants of the sensitizers:**

**a) Calf thymus DNA:** A total of 5 µg of calf thymus DNA and 5 µg of ethidium bromide (etBr) were mixed with variable amounts of the sensitizer in 4 mL of 10 mM Tris-HCl and 0.2 M NaCl, pH 7.4, in a test series (see an example below). After equilibration at room temperature overnight, the ethidium fluorescence (λ<sub>ex</sub> 540 nm, λ<sub>em</sub> 610 nm) was measured. The percent fluorescence (% Fluor) intensity was plotted against the concentration of the sensitizer and from this the concentration causing 50% fluorescence "quenching", corresponding to replacement of 50% of the ethidium bromide in the DNA by the added sensitizer was found. The relative binding constant was then determined as described earlier in the general procedure. A sample fluorescence quenching spectrum is presented in the Results and Discussion section (Figure 13) and the binding constants are listed in table 4 in the Results and Discussion section.
Table 23: A specific example of sample preparation for binding to DNA

Stock solutions of ethidim bromide (etBr) and DNA were 0.1 mg/mL in NaCl-Tris buffer. Stock solution of the sensitizer, A-H, was 1.0 mg in 50 mL of NaCl-Tris buffer.

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<th>DNA (µL)</th>
<th>A-H (µL)</th>
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<th>%Fluor</th>
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I_{610} is the fluorescence intensity of etBr measured at 610 nm of each sample. ΔI is the difference in intensity of free ethidium (1) and the intensity of each of solution. The percent fluorescence (% Fluor) is ΔI of solutions, (3)-(16), divided by the ΔI of solution (2). The concentration of the sensitizer added is calculated in μg/mL.

The concentrations of the added sensitizer in μg/mL are plotted against the percent fluorescence to obtain the 50% "quenching" concentration (Appendix D).

50% quenching concentration of A-H = 1.1 μg/mL = 1.1x10^{-3} g/L = 2.73x10^{-6} M

\[ K_{rel} = K_{etBr} [etBr] [sensitizer at 50\% fluorescence]^{-1} \]

where \( K_{etBr} = 1.5\times10^5 \text{ M}^{-1} \)

\[ K_{rel} = 1.5\times10^5 \text{ M}^{-1}[3.17\times10^{-6}\text{M}] [2.73\times10^{-6} \text{ M}] \]

\[ K_{rel} = 1.74\times10^5 \text{ M} \]

b) λ and φ6 phage: Samples were prepared in a manner identical to that described above. However, 133 μL of λ and 50 μL of φ6 were added to the test solutions instead of calf thymus DNA. 50% (λ) and 10% (φ6) fluorescence "quenching" concentration were calculated and are presented in the Results and Discussion section.

DNA photocleavage experiments:

a) pBR322 DNA without sensitizer: A solution was prepared by diluting 10 μL pBR322 DNA (0.25μg/μL) to 100 μL with the appropriate buffer as described in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home made pyrex sample holder and photolyzed at 30°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were taken after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.
b) **pBR322 DNA with sensitizer:** A solution was prepared by taking 10 μL pBR322 DNA (0.25μg/μL) and the desired concentration of the sensitizer, diluted to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home made pyrex sample holder and photolyzed at 3°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.

**Preparation of deoxygenated samples:** To study the effect of oxygen, if any, the DNA photocleavage experiment was conducted in the presence of argon. The samples were prepared in the exact same manner as described above. The home-made pyrex sample holder was evacuated with argon by purging for three minutes. The sample in the HPLC vial was purged for two minutes, placed in the previously purged pyrex sample holder, wrapped with a piece of parafilm and photolyzed as per the oxygenated samples.

<table>
<thead>
<tr>
<th>Table 24: An example of sample preparation for DNA nicking with sensitizer, A-Br:</th>
</tr>
</thead>
</table>

A stock solution of A-Br was prepared in TE buffer (1mg/10mL).

<table>
<thead>
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<th>pBR322 DNA (μL)</th>
<th>Sensitizer (μL)</th>
<th>TE buffer (μL)</th>
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</thead>
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<tr>
<td>a) 10</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>b) 10</td>
<td>50 (A-Br)</td>
<td>40</td>
</tr>
</tbody>
</table>
The above samples were photolyzed at 30° C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were taken before photolysis of each solution and after photolysis at 3 min, 6 min, 9 min, 12 min, 15 min and 18 min. To each aliquot, 5 μL of the loading dye and 15 μL of TE buffer were added. 5 μL of this resulting solution was loaded on an agarose gel and resolved by electrophoresis as described in the general procedure.

DNA photocleavage with added quencher:

a) pBR322 DNA with no sensitizer and no quencher: A solution was prepared by diluting 10 μL of pBR322 DNA (0.25 μg/μL) to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 30° C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.

b) pBR322 DNA and sensitizer, no quencher: A solution was prepared by taking 10 μL pBR322 DNA (0.25 μg/μL) and the desired concentration of the sensitizer, diluted to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 30° C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.
c) **pBR322 DNA and dithiothreitol:** The effect as well as the threshold concentration of a radical quencher, dithiothreitol (DTT), was determined by preparing a series of samples containing 10 μL pBR322 DNA (0.25μg/μL), variable concentrations of DTT (1.0-5x10^-5M), and diluted to 100 μL with the appropriate buffer as mentioned in the general procedure. The solutions were prepared in 300 μL pyrex HPLC sample injection vials (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 30°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.

d) **pBR322 DNA, dithiothreitol and sensitizer:** A series of samples containing 10 μL pBR322 DNA (0.25μg/μL), varying concentrations of dithiothreitol (0.5-5x10^-5M) and the desired concentration of the sensitizer were diluted to 100 μL with the appropriate buffer as mentioned in the general procedure. The solutions were prepared in 300 μL pyrex HPLC sample injection vials (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 30°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after a known time interval and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.
A Stock solution of A-Br was prepared in TE (Appendix A) buffer (1mg/5mL).

Stock solutions of DTT (0.1, 0.01, 0.001M) were made in TE buffer.

<table>
<thead>
<tr>
<th>pBR322 DNA (μL)</th>
<th>A-Br (μL)</th>
<th>DTT (μL)</th>
<th>TE buffer (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 5</td>
<td>0</td>
<td>0</td>
<td>45.0</td>
</tr>
<tr>
<td>b) 5</td>
<td>12.5</td>
<td>0</td>
<td>32.5</td>
</tr>
<tr>
<td>c) 5</td>
<td>12.5</td>
<td>25 (.1M)</td>
<td>20.0</td>
</tr>
<tr>
<td>d) 5</td>
<td>12.5</td>
<td>25 (.1M)</td>
<td>7.5</td>
</tr>
<tr>
<td>e) 5</td>
<td>12.5</td>
<td>5 (.1M)</td>
<td>27.5</td>
</tr>
<tr>
<td>f) 5</td>
<td>12.5</td>
<td>2.5 (.1M)</td>
<td>30.0</td>
</tr>
<tr>
<td>g) 5</td>
<td>12.5</td>
<td>5 (.01M)</td>
<td>27.5</td>
</tr>
<tr>
<td>h) 5</td>
<td>12.5</td>
<td>2.5 (.01M)</td>
<td>30.0</td>
</tr>
<tr>
<td>i) 5</td>
<td>12.5</td>
<td>5 (.001M)</td>
<td>27.5</td>
</tr>
<tr>
<td>j) 5</td>
<td>12.5</td>
<td>2.5 (.001M)</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The above samples were photolyzed at 30°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were taken before photolysis of each solution and after photolysis of 18 min. To each aliquot, 5μL of the loading dye and 15μL of TE buffer were added. 5μL of this resulting solution was loaded on an agarose gel and resolved by electrophoresis as described in the general procedure.
DNA photocleavage in the presence of a competitive binder, spermidine:

a) pBR322 DNA with no sensitizer and no spermidine: A solution was prepared by diluting 10 μL pBR322 DNA (0.25μg/μL) to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 3°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.

b) pBR322 DNA and spermidine: A solution was prepared by taking 10 μL of pBR322 DNA (0.25μg/μL) and known concentrations of spermidine diluting to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 3°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were removed after known time intervals and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.

c) pBR322 DNA and sensitizer, no spermidine: A solution was prepared by taking 10 μL of pBR322 DNA (0.25μg/μL) and the desired concentration of the sensitizer and diluting to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 3°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were taken after a known time period.
and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.

d) pBR322 DNA, spermidine and sensitizer: A solution was prepared by taking 10 μL pBR322 DNA (0.25μg/μL), various concentrations of spermidine (1-1x10^{-3}M) and the desired concentration of the sensitizer and diluting to 100 μL with the appropriate buffer as mentioned in the general procedure. The solution was prepared in a 300 μL pyrex HPLC sample injection vial (35 x 3 mm), placed in a home-made pyrex sample holder and photolyzed at 30°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 μL) were taken after a known time period and resolved by agarose gel electrophoresis, as described earlier in the general procedure. Photographs of the Plates are presented in the Results and Discussion section.
Table 26: An example of sample preparation for DNA nicking with sensitizer, A-Br and a competitive binder, spermidine:

A stock solution of A-Br was prepared in TE buffer (1mg/5mL).

Stock solutions of spermidine (2.0, 0.2, 0.02M) were prepared in TE buffer.

<table>
<thead>
<tr>
<th>pBR322 DNA (µL)</th>
<th>A-Br (µL)</th>
<th>Spermdine (µL)</th>
<th>TE buffer (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 5</td>
<td>0</td>
<td>0</td>
<td>45.0</td>
</tr>
<tr>
<td>b) 5</td>
<td>0</td>
<td>25 (2M)</td>
<td>20.0</td>
</tr>
<tr>
<td>c) 5</td>
<td>12.5</td>
<td>0</td>
<td>32.5</td>
</tr>
<tr>
<td>d) 5</td>
<td>12.5</td>
<td>25 (2M)</td>
<td>7.5</td>
</tr>
<tr>
<td>e) 5</td>
<td>12.5</td>
<td>12.5 (2M)</td>
<td>20.0</td>
</tr>
<tr>
<td>f) 5</td>
<td>12.5</td>
<td>25 (.1M)</td>
<td>7.5</td>
</tr>
<tr>
<td>g) 5</td>
<td>12.5</td>
<td>12.5 (.2M)</td>
<td>20.0</td>
</tr>
<tr>
<td>h) 5</td>
<td>12.5</td>
<td>25 (.02M)</td>
<td>7.5</td>
</tr>
<tr>
<td>i) 5</td>
<td>12.5</td>
<td>2.5 (.02M)</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The above samples were photolyzed at 30°C in a Rayonet reactor equipped with eight 350 nm lamps. Aliquots (10 µL) were taken before photolysis of each solution and after photolysis of 18 min. To each aliquot, 5µL of the loading dye and 15µL of TE buffer was added. 5µL of this resulting solution was loaded on an agarose gel and resolved by electrophoresis as described in the general procedure.
Viral inactivation of λ and φ6 by the sensitizers using X-ray/photolysis:

Viral inactivation of Lambda phage by photolysis was performed using an array of 6 horizontal bulbs of the appropriate wavelength at room temperature. The Phi 6 virus was inactivated using a horizontal array of 4 of the appropriate bulbs in the cold room at 3°C.

a) Virus without sensitizer: A solution of 100 mL of the appropriate virus and 2.9 mL of the appropriate buffer as described in the general procedure was prepared. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed at the appropriate wavelength (350, 528 or 658nm) in a home-made photolysis chamber equipped with bulbs. For X-ray irradiation, the petri dish was wrapped with saran-wrap and the samples were placed as close to the X-ray tube as possible. After photolysis/irradiation, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

b) Virus and sensitizer: A solution of 100 μL of the appropriate virus and the desired concentration of the sensitizer were diluted to 3.0 mL in the appropriate buffer as described in the general procedure. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed at the appropriate wavelength (350, 528 or 658nm) in a home-made photolysis chamber equipped with the appropriate bulbs. For X-ray irradiation, the petri dish was wrapped with saran-wrap and the samples were placed as close to the X-ray tube as possible. After photolysis/irradiation, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.
Preparation of deoxygenated samples:

To study the effect of oxygen, if any, on viral inactivation by these sensitizers, the experiments were performed in the presence of argon. The samples were prepared as described above. The sensitizer solution was degassed with argon for two minutes in the sample vial, placed in a pyrex petri dish, the appropriate virus was added and degassed for an additional minute. The solution was covered or wrapped and photolyzed/irradiated as usual. The plaque assay was performed as above.

| Table 27: Preparation of solution for viral inactivation (Φ6) by sensitizer, PSR-Br: |

The viral titre was usually in the range of $10^8$-$10^{10}$ plaque forming units.

A stock solution of PSR-Br (1mg/mL) was prepared in DS.

<table>
<thead>
<tr>
<th>λ (µL)</th>
<th>PSR-Br (µL)</th>
<th>DS (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>2.900</td>
</tr>
<tr>
<td>100</td>
<td>75</td>
<td>2.825</td>
</tr>
</tbody>
</table>
Effect of a quencher (dithiothreitol) on viral inactivation:

a) Virus with no sensitizer and no quencher: A solution of 100 μL of the appropriate virus and 2.9 mL of the appropriate buffer as described in the general procedure was prepared. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed at the appropriate wavelength (350, 528 or 658nm) in a home-made photolysis chamber equipped with bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

b) Virus and sensitizer: A solution of 100 μL of the appropriate virus and the desired concentration of the sensitizer was diluted to 3.0 mL with the appropriate buffer as described in general procedure was prepared. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed at the appropriate wavelength (350, 528 or 658nm) in a home made photolysis chamber equipped with bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

c) Virus with dithiothreitol and no sensitizer: A solution was prepared by diluting 100 μL of the appropriate virus to 3.0 mL with 10 mM dithiothreitol (DTT) prepared in the appropriate buffer as described in the general procedure. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed at the appropriate wavelength (350, 528 or 658nm) in a home-made photolysis
chamber equipped with bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

d) Virus, dithiothreitol and sensitizer: A solution was made by diluting 100 μL of the appropriate virus to 3.0 mL with the desired concentration of the sensitizer and 10mM DTT prepared in the appropriate buffer as described in the general procedure. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed at the appropriate wavelength (350, 528 or 658nm) in a home-made photolysis chamber equipped with the appropriate bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

**Table 28: Preparation of solution for viral inactivation (λ) in the presence of DTT by sensitizer, PSR-Br:**

The viral titre was usually in the range of $10^7$-$10^9$ plaque forming units.

A stock solution of PSR-Br (1.0 mg/mL) was prepared in DS.

A stock solution of DTT (1.0 M) was prepared in DS.

<table>
<thead>
<tr>
<th>λ (μL)</th>
<th>PSR-Br (μL)</th>
<th>DTT (μL)</th>
<th>DS (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 100</td>
<td>0</td>
<td>0</td>
<td>2.900</td>
</tr>
<tr>
<td>b) 100</td>
<td>75</td>
<td>0</td>
<td>2.825</td>
</tr>
<tr>
<td>c) 100</td>
<td>0</td>
<td>30</td>
<td>2.870</td>
</tr>
<tr>
<td>d) 100</td>
<td>75</td>
<td>30</td>
<td>2.795</td>
</tr>
</tbody>
</table>
The effect of histidine on the inactivation of lambda phage by methylene blue (MB) at 658nm:

a) λ with no sensitizer and no histidine: A solution of 100 μL of lambda and 2.9 mL of DS was prepared. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

b) λ and methylene blue: A solution of 100 μL λ virus, 150 μL of methylene blue (1.0 mg/mL in DS) was diluted to 3.0 mL with DS. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for three minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

c) λ with histidine and no methylene blue: A solution was prepared by taking 100 μL λ virus, histidine (10^-2 M) and diluting to 3.0 mL with DS. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for three minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.
d) Virus, histidine and methylene blue: A series of solutions were prepared by taking 100 μL of virus, 150 μL of methylene blue (1mg/ml) and varying the concentration of histidine (10^-2-10^-5 M) in a total of 3.0 mL of sample. The sample was placed in a covered pyrex petri dish approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for three minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, ten fold serial dilutions were performed for the plaque assay as described in the general procedure.
Table 29: Solution preparation for viral (λ) inactivation in the presence of histidine by methylene blue (MB):

The viral titre was usually in the range of $10^7$-$10^9$ plaque forming units.

A stock solution of MB (1.0 mg/mL) was prepared in DS.

Stock solutions of histidine (.01, .001 M) were made in DS.

<table>
<thead>
<tr>
<th>λ (μL)</th>
<th>MB (μL)</th>
<th>Histidine (μL)</th>
<th>DS (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b)</td>
<td>100</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>c)</td>
<td>100</td>
<td>0</td>
<td>300 (.01M)</td>
</tr>
<tr>
<td>d)</td>
<td>100</td>
<td>150</td>
<td>300 (.01M)</td>
</tr>
<tr>
<td>e)</td>
<td>100</td>
<td>150</td>
<td>30 (.01M)</td>
</tr>
<tr>
<td>f)</td>
<td>100</td>
<td>150</td>
<td>300 (.001M)</td>
</tr>
<tr>
<td>g)</td>
<td>100</td>
<td>150</td>
<td>30 (.001M)</td>
</tr>
</tbody>
</table>

Inactivation of lambda phage in frozen samples by methylene blue:

a) λ without methylene blue: A solution of 100 μL of lambda and 2.9 mL of DS was prepared. The sample was placed in a pyrex petri dish and frozen evenly using a dry ice-acetone bath. The sample was maintained frozen by keeping the petri dish in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for ten minutes in
a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

b) λ and methylene blue: A solution of 100 μL λ virus and 2.9 mL of methylene blue (1.0 mg/mL in DS) was frozen evenly in a pyrex petri dish using a dry ice-acetone bath. The sample was maintained frozen by keeping it in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for three minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions was performed for plaque assay as described in the general procedure.

The effect of histidine in frozen samples on viral inactivation by methylene blue:

a) λ without methylene blue: A solution of 100 μL of lambda and 2.9 mL of DS was prepared. The sample was placed in a pyrex petri dish and frozen evenly using a dry ice-acetone bath. The sample was maintained frozen by keeping the petri dish in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for ten minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

b) λ with histidine and no methylene blue: A solution of 100 μL of lambda and 2.9 mL histidine (10⁻²M in DS) was prepared. The sample was placed in a pyrex petri
dish and frozen evenly using a dry ice-acetone bath. The sample was maintained frozen by keeping the petri dish in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for ten minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

c) \(\lambda\) and methylene blue: A solution of 100 \(\mu\)L \(\lambda\) virus, 1.0 mL of methylene blue (4.5 mg/mL in DS) and diluted to 3.0 mL with DS was frozen evenly in a pyrex petri dish using a dry ice-acetone bath. The sample was maintained frozen by keeping it in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for three minutes in a home made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

d) \(\lambda,\) histidine and methylene blue: A series of solutions with varying concentrations of histidine (10\(^{-2}\)-10\(^{-6}\)M), 100 \(\mu\)L \(\lambda\) virus and 1.0 mL of methylene blue (4.5 mg/mL in DS) in 3.0 mL total volume were prepared. The samples were frozen evenly in a pyrex petri dish using a dry ice-acetone bath. These were maintained frozen by placement in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The samples were photolyzed for three minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the samples were thawed and ten fold serial dilutions was performed for the plaque assay as described in the general procedure.
Table 30: Preparation of solution for viral inactivation ($\lambda$) in the presence of histidine by methylene blue (MB) in frozen sample

The viral titre was usually in the range of $10^7$-$10^9$ plaque forming units.

A stock solution of MB (4.5 mg/mL) was prepared in DS.

Stock solutions of histidine (1.0, $1 \times 10^{-3}$ M) were made in DS.

<table>
<thead>
<tr>
<th>$\lambda$ (µL)</th>
<th>MB (mL)</th>
<th>Histidine (µL)</th>
<th>DS (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 100</td>
<td>0</td>
<td>0</td>
<td>2.900</td>
</tr>
<tr>
<td>b) 100</td>
<td>0</td>
<td>150 (1M)</td>
<td>2.750</td>
</tr>
<tr>
<td>c) 100</td>
<td>1</td>
<td>0</td>
<td>1.900</td>
</tr>
<tr>
<td>d) 100</td>
<td>1</td>
<td>150 (1M)</td>
<td>1.750</td>
</tr>
<tr>
<td>e) 100</td>
<td>1</td>
<td>300 (.001M)</td>
<td>1.600</td>
</tr>
<tr>
<td>f) 100</td>
<td>1</td>
<td>3 (.001M)</td>
<td>1.8970</td>
</tr>
</tbody>
</table>

Inactivation of lambda phage by varying the depth of the frozen sample of methylene blue:

a) $\lambda$ without methylene blue: A solution of 100 µL of lambda and 2.9 mL of DS was prepared. The sample was placed in a pyrex petri dish and frozen evenly using a dry ice-acetone bath. The sample was maintained frozen by placing the petri dish in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The sample was photolyzed for 10 minutes in a
home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions were performed for the plaque assay as described in the general procedure.

b) λ and methylene blue: A series of solutions were prepared using λ and methylene blue (1mg/mL in DS). A solution of 3.0 mL volume was prepared by mixing 100 µL λ virus, and 2.9 mL of methylene blue (1.0 mg/mL in DS). A 6.0 mL sample was prepared by taking 200 µL λ and 5.8 mL of MB (1.0 mg/mL). In a similar fashion, a total of 9.0 mL and 12.0 mL samples were prepared. These samples were frozen evenly in a pyrex petri dish using a dry ice-acetone bath and the depth of each sample was measured in mm. The samples were maintained frozen by placement in a dry ice-acetone bath approximately 2.5 inches from the light source. A sheet of glass plate was placed between the lamps and the samples. The samples were photolyzed for 10 minutes in a home-made photolysis chamber equipped with six 658 nm bulbs. After photolysis, the sample was thawed and ten fold serial dilutions were performed for the plaque assay as described in the general procedure.
APPENDIX A

Preparation of the media and buffers used for the viral inactivation and gel electrophoresis
The following solutions are required for growing φ6 virus and Pseudomonas syringae bacteria:

**NBY medium**: (for culturing the bacteria and doing the serial dilutions)

- Nutrient Broth Dehydrated (Difco, Detroit, MI) 8.0g
- Yeast Extract (Difco, Detroit, MI) 2.0g
- K$_2$HPO$_4$ 2.0g
- KH$_2$PO$_4$ 0.5g
- MgSO$_4$.7H$_2$O 0.2465g
- Distilled Water 950ml

Sterilize by autoclaving for twenty minutes in the liquid cycle. Then add aseptically 50 mL of filter sterilized 10% glucose solution.

**NBY Plating medium**: (for preparation of plates)

- NBY medium
- Bacto-Agar (Difco, Detroit, MI) 15g/L

**NBY top agar**: (for plating bacteria)

- NBY medium
- Bacto-Agar (Difco, Detroit, MI) 4g/L
The following solutions are required for growing \(\lambda\) virus and *E. coli* bacteria:

**LB medium**: (for culturing the bacteria)

- Bacto-Yeast Extract (Difco, Detroit, MI) 5.0g
- Bacto-tryptone (Difco, Detroit, MI) 10g
- NaCl 10g
- Distilled Water 1000 ml

Sterilize by autoclaving for twenty minutes in the liquid cycle.

**LB Plating medium**: (for preparation of plates)

- LB medium
- Bacto-Agar ((Difco, Detroit, MI) 15g/L

**LB top agar**: (for plating bacteria)

- LB medium
- Bacto-Agar (Difco, Detroit, MI) 7g/L

**Storage Medium**: (for serial dilutions)

- NaCl 5.8g
- MgSO4:7H2O 2.0g
- 1M Tris (pH 7.5) 50mL
- 2% Gelatin 5.0mL

Adjust with distilled water to 1000mL and sterilize as above.
The following solutions were prepared to run the neutral agarose gel electrophoresis.

**Plate Preparation Buffer:** (1 Liter)

- NaCl  
  2.922g
- EDTA-Na₂  
  0.3362g
- Distilled Water  
  1000 mL

Sterilize through a 20 micron filter.

**Neutral Running (electrode) Buffer:**

- Trisma base (Sigma)  
  4.844g
- Sodium acetate  
  1.641g
- EDTA-Na₂  
  0.672g
- NaCl  
  1.052g
- Distilled Water  
  1000 mL

Adjust the pH to 8.05 with acetic acid and sterilize as above.

**TE Buffer:**

- 1M Tris base (pH 7.4)  
  10mL
- 0.5M EDTA (pH 8.0)  
  2mL

Bring to 1000mL with distilled water and adjust the pH to 8.4 and sterilize as above.

**Tracking (loading) Dye:**

- Xylene Cyanole FF (Sigma)  
  5%
- Bromophenol Blue (Sigma)  
  5%
- Orange G(sodium salt) (Sigma)  
  1%

Make the dye solution in 60% glycerol and distilled water solution.
APPENDIX B

$^1$HNMR and $^{13}$CNMR spectra of selected compounds
Figure 17: $^1\text{HNMR}$ of 8-Hydroxypsoralen (DMSO-d$_6$).
Figure 18: $^1$HNMR of 8-(ω-Bromopropyloxy)psoralen (CDCl$_3$).
Figure 19: $^1$HNMR of PSR-H (D$_2$O/DSMO-d$_6$).
Figure 20: $^{13}\text{C}NMR$ of PSR-H (DSMO-$d_6$).
Figure 21: $^1$HNMR of 5-Bromo-8-methoxypsoralen (CF$_3$CO$_2$D).
Figure 22: $^1$HNMR of 5-Bromo-8-hydroxypsoralen (DMSO-$d_6$).
Figure 23: $^1$HNMR of 5-Bromo-8-(w-bromopropoxy)psoralen (DMSO-d$_6$).
Figure 24: $^1$HNMR of PSR-Br ($D_2O/DSMO-d_6$).
Figure 25: $^{13}$CNMR of PSR-Br (DSMO-$d_6$).
Figure 26: $^1$HNMR of 5-Iodo-8-methoxypsoralen (DMSO-d$_6$).
Figure 27 ¹H NMR of 5-Iodo-8-hydroxypsoralen (DMSO-d₆).
Figure 28: $^1$HNMR of 5-Iodo-8-(w-bromopropyloxy)psoralen (DMSO-d$_6$).
Figure 29: $^1$HNMR of PSR-I (D$_2$O/DSMO-d$_6$).
Figure 30: $^{13}$C NMR of PSR-I (DSMO-d$_6$).
Figure 31: $^1$HNMR of 9-Bromomethylnanthracene (CDCl$_3$).
Figure 32: $^1$HNMR of A-H ($D_2O/DSMO-d_6$).
Figure 33: $^{13}$C NMR of A-H (DSMO-d$_6$).

$$\text{CH}_2\text{NH(CH}_2\text{)}_2\text{NH(CH}_2\text{)}_2\text{NH}_2\text{3HCl}$$
Figure 34: $^1$HNMR of 10-Bromo-9-methylanthracene (CDCl$_3$).
Figure 35: $^1$HNMR of A-Br (D$_2$O/DSMO-d$_6$).
Figure 36: $^{13}$CNMR of A-Br (DSMO-d$_6$).
Figure 37: $^1$HNMR of 9,10-Dibromo-2-methylbromoanthracene (CDCl$_3$).
Figure 38: $^1$HNMR of A-Br$_2$ (D$_2$O/DSMO-d$_6$).
Figure 39: $^{13}$CNMR of A-Br$_2$ (DSMO-$d_6$).
Figure 40: $^1$HNMR of 10-Chloro-9-methylnanthracene (CDCl$_3$).
Figure 41: $^1$HNMR of A-Cl (D$_2$O/DSMO-d$_6$).
Figure 42: $^{13}$CNMR of A-Cl (DSMO-d$_6$).
Figure 43: $^1$HNMR of 9-Iodoanthracene (CDCl$_3$).
Figure 44: $^1$HNMR of 10-Iodo-9-methylchloroanthracene (CDCl$_3$).
Figure 45: $^1$HNMR of A-I (D$_2$O/DSMO-d$_6$).
Figure 46: $^{13}$CNMR of A-I (DSMO-d$_6$).
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Filmed as received.
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