To the Graduate Council:

I am submitting herewith a dissertation written by Richard Joseph Brake entitled "Effects of Ionizing Radiation on DNA." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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EFFECTS OF IONIZING RADIATION ON DNA

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Richard Joseph Brake

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Emily paid my way and co-enjoyed many dogwoods.

ABSTRACT

The radiosensitivity of samples of bacterial DNA of several different base compositions was determined in aqueous solution. The number of single- and double-strand breaks induced by ^{60}Co γ-rays was independent of composition over a range of 38-72% guanine plus cytosine. An endonuclease from M. luteus that converts nonbreak damage in irradiated DNA (probably base alteration) into easily-detectable strand breaks was used to assess the number of such lesions in the same DNA samples. There were 10-30% more sites susceptible to this enzyme in DNA rich in adenine and thymine; the extent of the difference depended on the irradiation conditions. Strand breakage was independent of base composition for all conditions tested. The significance of these results in relation to the reported increase in radiosensitivity of GC-rich organisms is discussed.

The covalent circular DNA from bacteriophage PM2 was used in a sedimentation analysis system to quantitate three distinct classes of lesions: (1) strand breaks, (2) alkali-labile bonds, and (3) sites susceptible to the γ-endonuclease from M. luteus. This system was employed to study radiation damage to DNA (1) indirectly by the action of water radicals in dilute solution, (2) by direct deposition of photon energy in DNA, and (3) indirectly by secondary organic radicals. Each process probably contributes significantly to radiation action in vivo.

In dilute aqueous solution the OH-radical causes mostly strand breaks and alkali-labile bonds, but few if any enzyme sites. Conversely, the electron causes many enzyme sites, a few alkali-labile bonds and probably no strand breaks. The hydrogen atom accounts for a minor part of the total damage. The effect of oxygen in this condition is protection from radiation by scavenging of the aqueous electron.

In highly-protected conditions where the direct action of radiation becomes important the spectrum of damage is similar to the indirect effects, except that a new form of alkali-labile bonds (perhaps apurinic and apyrimidinic sites) that are susceptible to the γ-endonuclease are induced. Oxygen has no effect in this condition.

In the presence of thymidine all three classes of damage to DNA appear to be mediated by the thymidine-OH-radical adduct. Oxygen enhances each kind of damage 3- to 7-fold over the anoxic response. This oxygen effect apparently requires the aqueous electron, and is prevented by chloride. It seems likely that some critical sequence of reactions involving thymidine-peroxyl radicals is necessary for this potentiation of the oxygen effect. Irradiation in the presence of adenosine showed much less potentiation, and phenylalanine was completely unreactive in this respect. The probable importance of radiation damage mediated by organic radicals in vivo is discussed.

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CHAPTER I

THE EFFECT OF BASE COMPOSITION ON RADIOSENSITIVITY

Introduction

The mechanism by which ionizing radiation kills cells has been and remains today the central problem in radiation biology. It is generally, though not unanimously, accepted that DNA is the principal target of radiation action, but in the wide spectrum of DNA damage caused by radiation no lesion or class of lesions has been clearly demonstrated to be lethal to intact cells. In fact, extensive repair of virtually all types of lesions has been reported. The most convincing evidence currently points to base damage, without strand breakage, as the class of lesion that contributes most to lethality. Radiation chemists have radiation damage (e.g., Scholes et al., 1960), but until recently base damage was inaccessible to study at biological doses. This technical block was first overcome by methods of separating broken from unbroken DNA molecules, and assaying the biological activity of each form in a molecules, and assaying the biological activity of each form in a transfection system. Thus van der Schans et al. (1973) demonstrated that both single- and double-strand breaks contribute little to inactivation of PM2 and ØX174 replicative form DNA, while "base damage" (defined as nonbreak damage) accounts for about 90% of the inactivation. Their original work was in aerated solution, but they obtained very similar results when they irradiated anoxically at 77°K (van der Schans and Bleichrodt, 1974).

It is now well known that not all base damage is lethal, but that cells contain enzymes that recognize many of the damaged bases and initiate excision repair similar to that of UV-induced pyrimidine dimers. The first such enzyme was reported by Setlow and co-workers (Paterson and Setlow, 1972; Setlow and Carrier, 1973) and others have followed. (For review of this field see Grossman et al., 1975). Clearly it is important to determine which if any subset of these lesions are distinctly unrepairable, and thereby lethal.

A possible clue in this matter is found in the data of Kaplan and Zavarine (1962). They X-irradiated eight species of bacteria and found that the radiosensitivity of the organisms increased with the GC-content of the chromosomal DNA, suggesting that GC-lesions were relatively more important than AT-lesions in radiation-induced lethality. They subsequently extended this work to radiation inactivation of transforming DNA from B. subtilis, and found similarly that GC-rich markers were more sensitive not only to X-rays, but also to nitrogen mustard. With ultraviolet radiation an inverse correlation was found; i.e., the sensitivity increased with AT-content [Kaplan et al. (1964)]. Haynes and co-workers confirmed the results for both the alkylating agent and ultraviolet radiation (Haynes, 1964) in a series of bacteria similar to that used by Kaplan and Zavarine. The X-ray results were confirmed by Pihl and Sanner (1966), who irradiated bacteria anoxically and at 77°K (Kaplan and Zavarine irradiated in aerated suspension). Several of these authors speculated that the results with nitrogen mustard and UV light were explainable by the exceptional reactivity of guanine

residues in alkylation, and thymine residues in the formation of cyclobutyl dimers respectively, but all acknowledged that the X-ray result remains unexplained.

Exceptions to the above results, especially with X-rays have often been cited (notably M. radiodurans, GC-rich but very radioresistant), and have led many authors to suggest that the choice of bacterial species by Kaplan and Zavarine was fortuitous. The most comprehensive example of this is the work of Underbrink and Sparrow (1968) who examined 50 organisms (mostly viruses and bacteria), and concluded that base composition was generally not a factor in determining radiosensitivity. Examination of their data (Underbrink and Sparrow, 1968, Figure 1) reveals that, except for chick and human cells (the only higher eucaryotes tested) the radiosensitivity of all species was either close to or less than that predicted from their GC-content. These data clearly demonstrate that in many cases base composition is a minor factor in radiosensitivity, but also support the hypothesis that when all other factors contributing to radioresistance are equal (e.g., intracellular sulfhydryls and repair capacity) an intrinsic sensitivity of GC-rich DNA becomes important. The baseline for radioresistance in their data appeared to be just that found by Kaplan and Zavarine.

In the large body of radiation chemistry of the DNA bases (mostly quantitative ESR and chromosome destruction studies) there is no convincing evidence for an exceptional radiosensitivity of any particular base. There currently seems to be general agreement that at least the yield of initial radicals by both direct and indirect

action of radiation is not very different among the four bases (Ward, 1975; Meyers, 1973). Pihl and Sanner (1966) isolated DNA from several of the same organisms studied by Kaplan and Zavarine, irradiated them in vacuum at 77°K, and examined the formation of DNA radicals by ESR spectroscopy. AT-rich DNA showed an increased fine structure (attributable to the "thymine octet"), but there was no significant increase in the total yield of radicals. In comparative studies with monomer subunits they claimed some evidence for an increased yield, and decreased stability (on warming to room temperature) of radicals in guanine and cytosine bases and nucleosides, but this result is confusing since it was not found in either nucleotides or DNA. Other ESR studies are equally difficult to interpret (for review see Meyers, 1973), and must currently be discounted.

Quantitative comparisons of eventual products after irradiation of DNA or constituents is even more difficult. Studies of base destruction usually have relied on measurements of optical density, and necessarily (but perhaps incorrectly) assume that none of the products of radiation action absorb in the region monitored. Such studies led Weiss and co-workers (reviewed in Weiss, 1964) to conclude that the pyrimidines, and particularly thymine, were more sensitive than the purines. Rafi et al. (1968) irradiated DNA samples of various base composition, and found evidence for a slight increase in chromophore destruction in polymers of composition above 50% AT-content. It is not currently known whether these results are due to a real difference in base damage between AT and GC polymers, or are at least in part artifacts due to

UV-absorbing GC-products. In either case there is no apparent evidence for an increased sensitivity of GC-rich DNA, and therefore no explanation for the results of Kaplan and Zavarine. It seems likely that if the reported GC-dependence of radiosensitivity is correct that it must arise from some aspect of base damage, either quantitative or qualitative, not detected by the above methods.

I report here results of studies on the radiosensitivity of DNA purified from bacteria of various GC-contents for three classes of lesions that might be important for lethality: single-strand breaks, double-strand breaks, and sites susceptible to the γ-endonuclease from M. luteus. The last class of lesion has not been chemically characterized, but almost certainly represent damage to the DNA bases. The system provides the unique opportunity to reexamine the intrinsic sensitivity of DNA at doses within the range of biological relevance (the previously cited studies necessarily used extremely high doses), and to simultaneously determine several lesions whose biological consequences are otherwise accessible. It is shown that while strand breakage is independent of base composition, there is some increase in the yield of enzyme sites in AT-rich DNA.

Materials and Methods

Preparation of DNA. Purified bacterial DNA was prepared by the method of Marmur (1961), after growth and labeling of bacteria as outlined below. Escherichia coli B3T$^-$ (50% GC, all base composition data from Handbook of Biochemistry and Molecular Biology, 1975) was

grown at 37°C in M-9 salts (1 g NH_4Cl, 6 g $Na{\cdot}HPO_4$, 3 g KH_2PO_4, 5 g NaCl per 900 ml) plus 0.5% glucose, 0.25% caseamino, 0.01% $MgCl_2$, and 4 μg/ml thymidine for labeling with either [^{3}H-methyl]- or [2-^{14}C]-thymidine. For labeling with inorganic [^{32}P]-phosphate cells were grown in low phosphate medium (Grossman, 1967). Haemophilus influenzae (38% GC, acquired from J. K. Setlow) was grown at 37°C in the medium of Herriott et al. (1970) containing 10 μCi/ml [^{3}H-methyl]-thymidine (final specific activity was ~10^5 cpm/μg DNA). Micrococcus luteus (73% GC, ATCC 4698 obtained from Miles Laboratories as a freeze-dried preparation) was grown at 32°C in a medium prepared by adding 1 ml of 5% yeast extract, 1 ml 40% glucose, 0.05 ml 10% $MgSO_4$ and 0.05 ml of a biotin solution (400 mg $FeSO_4{\cdot}7H_2O$; 200 mg $MnCl_2{\cdot}4H_2O$; and 1 mg biotin per 100 ml H_2O) to 50 ml of buffered saline (8 g NaCl, 2 g K_2HPO_4, 1 g NH_4Cl, KH_2PO_4 added to pH 7.6, in 900 ml H_2O). All strains were grown aerobically in a shaker incubator, and labeled for several generations before harvest in the late exponential or early stationary phase of growth. DNA preparations of average single-strand molecular weights of 10-30 × 10^6 daltons were routinely obtained, and comparison of single- and double-strand molecular weights (determined as described below) indicated that there were few if any nicks in freshly prepared, unirradiated samples. Multiple-label buoyant density gradients (neutral CsCl) confirmed the identity and GC-content of each preparation, and were in agreement with the published values. Absorption spectrometry indicated that there was no large contamination of protein in the final DNA preparations.

Phage T4 DNA (34% G+ hydroxymethylcytosine) was prepared by the hot phenol extraction method of Massie and Zimm (1965) after growth in E. coli by the method of Wulff (1963).

All DNA samples were dialyzed extensively into 0.1 M Na-phosphate, 0.01 M Tris buffer (pH 7.5), and were stored at 4°C for irradiation experiments.

Irradiation procedure. Prior to irradiation DNA samples to be used in multiple-label experiments were mixed, adjusted to a total DNA concentration of 50 μg/ml, and dialyzed against 0.1 M NaCl, 0.01 M Na-phosphate buffer (pH 7). After dialysis the appropriate solution (e.g., yeast extract, KI) were added. This procedure removes the radioprotective Tris-buffer, and ensures an identical and well-defined radiochemical environment for each kind of DNA. Samples were irradiated at a dose rate of about 3 krad/min in a ^{60}Co Gammacell 200 (Atomic Energy of Canada, Ottawa) with bubbling of the appropriate gas (air or nitrogen) 15 min before and during irradiation. An exception to this was samples containing yeast extract, which, to avoid foaming, were either evacuated and sealed to obtain anoxia, or had air led over their surface during irradiation to maintain oxygenation. All samples were kept in an ice bath before, during and after irradiation until the assay procedure dictated otherwise.

UV-irradiations were performed as described by Carrier and Setlow (1966). The light source was a high-pressure mercury arc, passed through a quartz prism monochromator tuned to 280 nm. The incident intensity was about 240 J/m^2 per min. The DNA concentration was about

100 μg/ml, and dosimetry corrections for sample absorption were made as has been described (Morowitz, 1950).

Treatment with endonuclease. Irradiated samples were diluted at least 5-fold with 0.1 M Tris-Cl buffer (pH 8), containing 0.01 M 2-mercaptoethanol and 5 μg/ml tRNA (to inhibit nonspecific nucleases) and split into two equal fractions (usually 100-200 λ per fraction). One part received either UV- or γ-endonuclease from M. luteus, the other received no enzyme. Both enzymes were gifts from W. L. Carrier and have been characterized (Carrier and Setlow, 1974). Samples were incubated 20 min at 37°C, sufficient time for endonuclease action to go to completion. Reactions were stopped by the addition of NaOH (for alkaline assay) or NaCl (for neutral assay) to 0.5 M, EDTA to 0.5 M, and placement of samples in an ice bath. Unirradiated control DNA was slightly susceptible to both enzymes, due to damage incurred from decay of incorporated radioisotopes. (Storage of DNA in the presence of Tris-buffer, ethanol, or potassium iodide, all OH-radical scavengers, protected against this form of damage, and prolonged the useful life of the DNA samples.) The incubation procedure had no effect on samples containing no enzyme.

Assay for single- and double-strand breaks. Linear [5-20% (w/v)] sucrose gradients in polyallomer tubes (Beckman, SW-56 rotor) were used for sedimentation analysis of molecular weights. Alkaline gradients contained 0.5 M NaCl; 0.2 M NaOH; 0.01 M EDTA; neutral gradients contained 0.7 M NaCl, 0.01 M EDTA, 0.05 M Tris-Cl buffer (pH 8).

Gradients were prepared (6 at a time) through the use of a peristaltic pump (Technicon Autoanalyzer), and were adjusted to equal volumes (~3.8 ml) before DNA samples (usually in 0.1-0.2 ml) were layered on top. All centrigugations were carried out in the Beckman Model L, or L3-50 ultracentrifuge at 20°C, and speeds of 40-50,000 rpm as needed to sediment the DNA about half-way into the gradient. Samples were collected (again usually 6 at a time by pumping from the bottom through a Technicon Autoanalyzer) onto 1 × 57 cm Whatman #17 paper strips (Carrier and Setlow, 1971). The strips were washed in 5% (w/v) trichloroacetic acid, followed by two washings in 95% ethanol, dried (in a 60-80°C oven), cut into individual premarked fractions, and placed in disposable shell vials (Kimble, Toledo). Radioactivity was determined by liquid scintillation counting [cocktail was 12 g Permablend I (Packard) per 4 liters toluene] in a Packard Tri-carb scintillation spectrometer. The counter was equipped with a Teletype with paper-tape punch (Packard Instruments) which facilitated input of the data into a computer (PDP11) programmed to correct for background, channel spill-overs (in double- and triple-label experiments), and to calculate average molecular weights. The entire system was developed at Oak Ridge by R. B. Setlow and W. L. Carrier, and has been calibrated with phage DNA markers ranging in molecular weights from 1.6×10^6 (ØX174) to 1.1×10^8 daltons (T4). Strand breaks were calculated from the reciprocals of the average molecular weights, and changes in these values were attributed either to irradiation or enzyme action.

Although no detailed study was performed there did not appear to be significant postirradiation breakdown of DNA over periods of several days. Alkaline treatment (approximately 15 min at room temperature in 0.5 M NaOH before sedimentation) appeared sufficient to hydrolyze any alkali-labile bonds, as holding for periods up to 1 h caused no further decrease in molecular weights.

Results

UV-irradiated DNA. Ultraviolet light induces cyclobutyl pyrimidine dimers in DNA, which are susceptible to endonucleases that introduce a strand break at or near the dimer initiating the familiar process of excision repair. The isolation of the dimer-specific endonuclease from several sources has allowed widespread use of this enzyme in quantitation of dimers indirectly by monitoring enzyme-induced strand breaks. Since it has been shown by direct chromatographic analysis that the yield of dimers is a function of the base composition of the target DNA (Setlow and Carrier, 1966), the yield of UV-endonuclease-susceptible sites should vary accordingly. Figure I-1 shows that the expected base composition dependence in fact can be observed. Each data point represents the slope of a linear dose-response curve for UV-endonuclease-susceptible sites in the range of 0-600 J/m^2. For comparison the dimer yields previously determined by Setlow and Carrier (1966) using chromatographic analyses and the same three sources of DNA are also plotted. The chromatography experiments were performed on samples that had received much higher doses of UV (400 J/m^2) and, since dimer

Figure I-1. Yield of dimers (▲) and UV-endonuclease-sensitive sites (●) as a function of GC-content. The dimer values are taken from the chromatography data of Setlow and Carrier (1966).

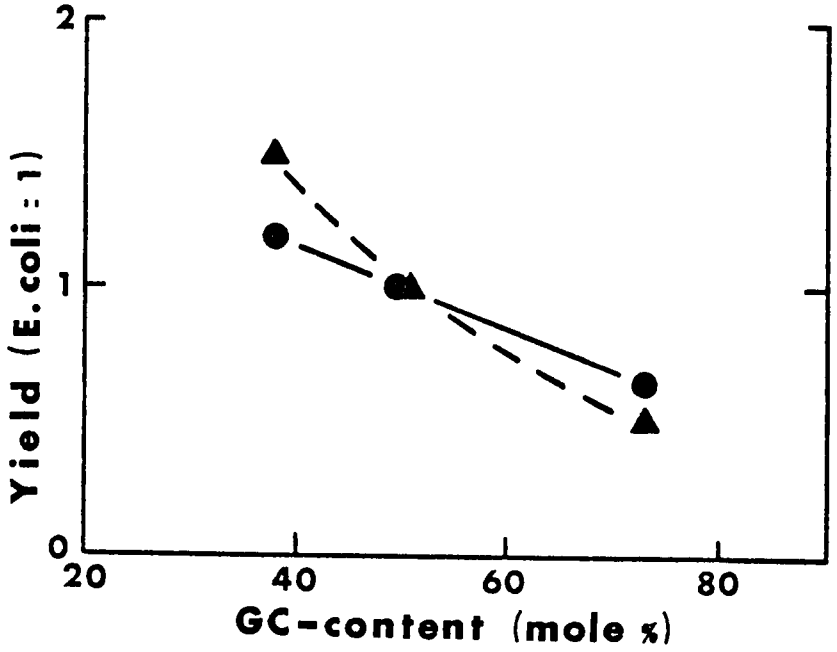


Figure I-1

induction curves are not linear in this region, the shape of the curves in Figure I-1 should not be expected to exactly coincide. Nevertheless both techniques demonstrate the increased sensitivity of AT-rich DNA toward ultraviolet light, which has been proposed as the molecular basis of the increased UV-sensitivity of AT-rich organisms.

Ionizing radiation. Experiments analogous to those described above were performed with ionizing radiation. In this case single-strand breaks, double-strand breaks and γ-endonuclease-susceptible sites were monitored. Several irradiation conditions were tested, and results are shown only for aerated 5% (w/v) yeast extract (Figure I-2), assumed to be a better model of the conditions in vivo than inorganic buffers. All conditions were qualitatively similar.

Both single- and double-strand breakage are independent of base composition, as might be expected since sugar and phosphate damage probably account for the majority of these lesions. Others have concluded that single-strand breakage was independent of base composition. Summers and Szybalski (1967), who measured the loss of renaturability of crosslinked DNA, and Poverennyi et al. (1972), who used the kinetic formaldehyde method found no difference in these strand break-sensitive parameters over a range of GC-content. The results presented here provide a much more direct measurement of breakage, and confirm this conclusion.

Sites susceptible to the γ-endonuclease did show a dependence on base composition (Figure I-2). With the irradiation conditions of this experiment there was a 25% increase in enzyme sites as the AT-content

Figure I-2. Yield of γ-ray damage to DNA as a function of GC-content. Single-strand breaks (0), double-strand breaks (■) and sites susceptible to the γ-endonuclease (●) were assayed by sedimentation after DNA was exposed to ^{60}Co γ-radiation in the presence of 10% yeast extract, under air. Values are normalized to the yield of single-strand breakage in E. coli (50% GC).

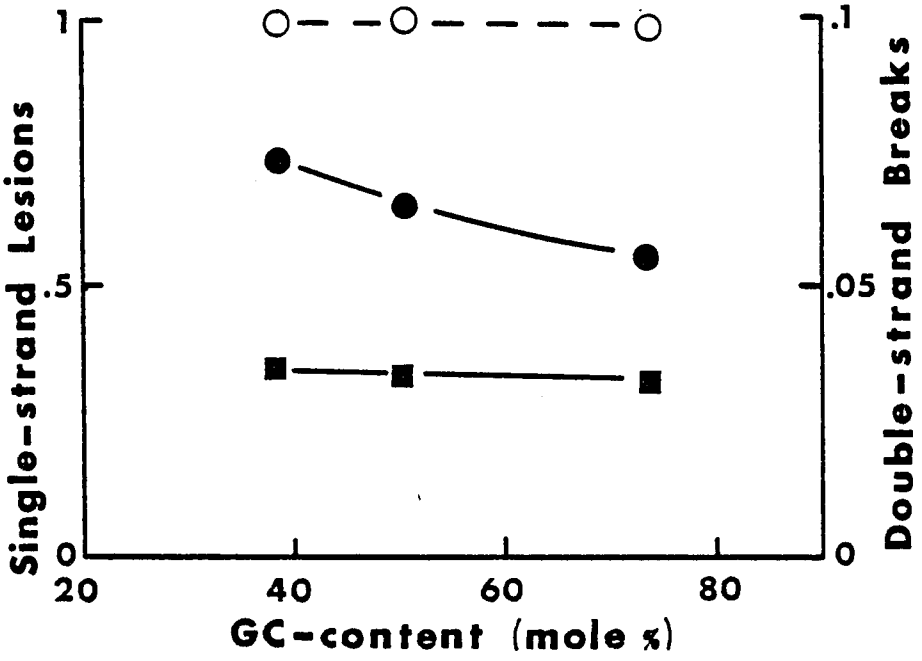

1
.5
0
.1
.05
0
Single-strand Lesions
Double-strand Breaks
20
40
60
80
GC-content (mole %)

Figure I-2

increased from 27% (M. luteus) to 62% (H. influenzae). In other conditions this increase in enzyme sites in AT-rich DNA was also evident, but the magnitude of the increase varied from 10% to nearly 30%. The reason for this irreproducibility is unknown.

It was discovered later (reported in subsequent chapters of this thesis) that conditions which favor the attack of the aqueous electron on DNA (e.g., potassium iodide) enhance the relative yield of enzyme sites, and thereby the resolution of the base composition dependence, while those that quench the electron (e.g., oxygen) decrease the resolution. The rate of strand breakage varied greatly among the several conditions tested, but was always independent of base composition.

An exception to the above results was that DNA from phage T4 (66% AT) was noticeably less sensitive than all other kinds of DNA tested to radiation induction of both strand breaks and γ-endonuclease sites. It was assumed that this result was attributable to the unusual nucleosides (either hydroxymethylcytosine or glucosylated residues) found in this DNA, rather than the overall AT-content. This assumption is supported by the fact that glucosylated T4 is uniquely resistant to exonuclease III of E. coli (Richardson, 1966). The possibility that the M. luteus γ-endonuclease is less active on T4 simply because it is heterologous DNA is eliminated since of all bacterial DNA tested, this enzyme is least active on its own homologous DNA (Figure I-2).

Discussion

In the search for evidence of a "lethal lesion" caused by ionizing radiation the results of this chapter are of little help. If GC-rich organisms are in fact generally radiation sensitive it cannot be attributed to a gross change in the sensitivity of their DNA. Kaplan and Zavarine (1962) observed about a 6-fold change in radiosensitivity over a range of GC-content studies in this work; no such variation in any lesion class could be demonstrated. Moreover, the only composition dependence found indicates an increased sensitivity of AT-rich DNA.

It is possible that the putative "lethal lesion" is simply too infrequent to be detected by the methods used here. For example, if lethality is a net effect of a large amount of damage followed by a large amount of repair, the small portion of damage that is wholly unrepairable would be quantitatively insignificant.

Another possibility is that lethality does not arise from distinctly lethal (unrepairable) lesions, but from a dynamic competition between the repair processes and some aspect of cellular physiology (e.g., DNA synthesis), such that death results when the repair machinery lags too far behind. If this is the case a reasonable hypothesis is that AT-rich DNA (shown here to contain more enzyme sites) is more easily (rapidly?) repaired than is GC-rich DNA. It would be interesting to know if the importance of base composition for radiosensitivity could be reduced by treatments that increase the time available for pre-replicative DNA repair (e.g., postirradiation liquid holding).

There are other suggestions of a generally decreased repairability of GC-rich DNA, particularly after damage by chemicals. An endonuclease II preparation from E. coli (probably a mixture of several enzymes involved in the repair of both X-irradiated and alkylated DNA) has been shown to degrade poly d(AT), but not poly dG:dC, even after extensive alkylation with methyl methanesulfonate (Hadi et al., 1973). This same enzyme preparation removes 3-methyladenine but not 7-methylguanine residues from DNA treated with the carcinogen N-methyl-N-nitrosourea (Kirtikar and Goldthwait, 1974), and removes the adenine adducts of 7-bromomethyl-12-methylbenz[a]anthracene three to four times faster than the guanine adducts (Kirtikar et al., 1975). Minor sites of alkylation (e.g., the O^6- and 3-position of guanine, and the 1- and 7-positions of adenine) are much more difficult to study, but enzymatic removal of some or all of these lesions seem likely. The status of the hypothesis that AT-lesions are removed more readily awaits the details of the repairability of these and other lesions.

Unfortunately much less is known about repair of specific radiation-induced base alterations. The most studied lesion is the 5,6-hydroperoxide of thymine, for which extensive repair has been reported (Swinehart and Cerutti, 1975), but comparable information for cytosine and the purines is not yet available. It seems likely that a hierarchy of repairability analogous to the situation with chemical damage remains to be elucidated.

The base compositions of the DNA of living organisms shows a remarkably broad distribution. While E. coli, the standard unit of life

to many biochemists and geneticists, resides at the median (50% AT), values for bacteria alone range from 25% to 77%. Barring the possibility that secondary factors (e.g., cellular amino acid or RNA content) are involved, it seems likely that this distribution results from selection for DNA resistant to the deleterious effects of environmental agents. Singer and Ames (1970) proposed that natural exposure to ultraviolet light provided the evolutionary pressure toward high GC-content, and suggested that ionizing radiation or naturally occurring alkylating agents might explain the low GC-content species. If this hypothesis is correct it appears that mammals (including man, with 60% AT) have been strongly influenced by one or both of the latter agents. The current literature and the data of this chapter indicate that the critical factor in such a scheme is not likely to be strand breakage, but rather base damage, and moreover the repairability rather than yield of such damage.

CHAPTER II

THE ROLE OF WATER RADICALS

Introduction

When a living cell is exposed to ionizing radiation the incident energy is deposited in a rather unselective way, leading to an approximately uniform distribution (by weight) of free radicals among the cellular components. Thus DNA, the apparent critical target, absorbs less than 1% of the energy (for bacteria), while most (80%) is deposited in cell water. The radiolysis of water involves a sequence of very fast reactions that may be summarized by the following equation (Blok and Loman, 1973):

$$H_2O \xrightarrow{100\ eV} 2.7\ OH + 2.65\ E + 0.55\ H + 0.70\ H_2O_2 + 0.45\ H_2 .$$

The important species for this thesis (referred to as "water radicals") are the highly reactive OH-radical (OH), aqueous electron (E), and hydrogen atom (H). Each can diffuse and react with a variety of organic and inorganic solutes, including DNA, giving rise to secondary adducts and eventually, after radical recombination, stable chemical products. The entire process probably takes about 10^{-6} seconds in aqueous solutions.

A great deal of evidence has accumulated that suggests indirect effects, particularly those mediated by the OH-radical, account for most of the lethal effects of ionizing radiation in living systems. Most

of these studies involve radiation protection with radical scavengers that selectively quench the individual water radicals, but have no effect on the direct action of radiation. In the bacterium E. coli alcohols and other OH-radical-scavengers have been shown to prevent at least 50% of the cell killing, and the molar efficiency of the scavengers in this protection correlated directly with their rate constants for reaction with the OH-radical (Johansen and Howard-Flanders, 1965; Sanner and Pihl, 1969). No such correlation was found for either H or E. Roots and Okada (1972) performed similar experiments with a series of alcohols in mouse leukemia cells and concluded that the OH-radical was responsible for 70% of the strand breaks in this system while H and E contributed little. Cell killing in these cells was also prevented by OH-scavengers, but the correlation with rate constant was not as convincing as the bacterial work (Roots, 1970, referenced in Ward, 1975). This may be due to secondary reactions of certain alcohol-OH radical adducts important for protection (Ewing, 1976), or to difficulty in determining intracellular alcohol concentrations, and does not weaken the argument for the general importance of the OH-radical.

Studies involving the irradiation of biologically active DNA in dilute solutions arrive at similar conclusions. In these conditions the damage is known from strictly physical consideration to be almost exclusively due to water radicals. Blok and Verhey (1968) measured the loss of transfection capacity of single-stranded ØX174 DNA, and found that the OH-radical accounts for 95% of the biological effects, especially at high concentrations of DNA (greater than 1 mg/ml). At low

DNA concentrations the reducing radicals (E and H) contributed significantly (50%) to inactivation, but due to the presence of phosphate buffer (0.01 M) during irradiation they were uncertain which of the species was responsible. It is probable that in these conditions there is at least a partial conversion of E into H ($H_2PO_4^- + E \rightarrow HPO_4^= + H$, $k \simeq 10^7\ M^{-1}\ sec^{-1}$, Jortner et al., 1962), which in turn activates the DNA. At the higher DNA concentration most E react directly which the DNA, and as concluded in vivo are apparently noninactivating. The exact role of the hydrogen atom remains undetermined and presents some difficulty in this thesis which will be discussed later.

Quite converse to the above findings, it has been shown that the electron affinity of a series of compounds correlates well with their ability to sensitize biological systems to radiation (Simic and Powers, 1974). This finding is difficult to interpret (see Chapter IV), but is certainly consistent with the hypothesis that E-induced damage (quenched by electron-affinic compounds) contributes little to lethality.

To date, no satisfactory chemical or molecular explanation of the apparent lethality of the OH-radical or the unimportance of E-damage has been offered. At the chemical level, much data has been compiled about the second order rate constants for the initial attack of water radicals on nucleic acid subunits (nucleotides and free bases), and is summarized in Table II-1 (for detailed reviews see Meyers, 1974; Scholes, 1968; Scholes et al., 1960). It is important to note that the great majority of primary attack occurs at the aromatic base, only 10% or less at the deoxyribose moiety. The reactions generally occur at or near

TABLE II-1

THE RADIATION CHEMISTRY OF NUCLEOSIDES

Radical	Site of Reaction in Nucleosides	Reaction Rate ($\underline{M}^{-1}$ sec^{-1})	Products
OH	80-90% aromatic base 20-10% deoxyribose	Approx. 5×10^9	Addition to double bonds (5,6-pyrimidines; 4,5-purines) Abstract H-atoms from sugar
H	100-80% aromatic base 0-20% deoxyribose	Approx. 5×10^8	Similar to OH
E	100% aromatic base	$1\text{-}2 \times 10^{10}$ (diffusion-limited)	Anion radicals lead to unknown products

diffusion-limited rates (somewhat slower for H-atoms), and are approximately equal for the four DNA bases. Native DNA reacts similarly, but about an order of magnitude more slowly. Ward and Kuo (1970) observed a shoulder in the dose response for base destruction and proposed that the stacked, internalized bases in double-stranded DNA are shielded from attack until some disruption of the helix, probably single-strand breakage, has taken place. Others have calculated that native DNA reacts at the collision-limited rate with the OH-radical (Michaels and Hunt, 1973), and only slightly slower for the aqueous electron (Shragge et al., 1971). They attributed the decrease in absolute reaction rate almost entirely to the decrease in mobility of the macromolecule, discounting steric effects of the structure. Clearly more work is needed before the details are understood, but there is currently little dispute that the bases are indeed the major site of initial reaction with all three water radicals, even in native DNA.

The subsequent reactions of the DNA-radical adducts are varied and for the most part unknown. The same limitations mentioned above still apply—most work deals with monomers, and at very high doses (Table II-1, column 3). Pyrimidine reactions are much better understood than those of the purines and deoxyribose. OH- and H- adducts of the bases saturate to give hydroxy-hydro- and dihydro-derivatives, respectively (often at the 5,6-position of pyrimidine, and probably the 4,5-position in purines). Both OH and H can abstract hydrogen atoms from deoxyribose, and reactions of the resultant carbon radical are likely to be important

in the formation of strand breaks and alkali-labile bonds (Dizdaroglu et al., 1977).

The electron adducts of nucleosides are almost exclusively delocalized π-radicals of the base moiety and react very rapidly ($2\text{-}3 \times 10^9$ $\underline{M}^{-1}$ sec^{-1}) by electron transfer and recombination reactions leading to oxidation or addition products. In the absence of electron affinic compounds (e.g., anoxic irradiation) the fate of the adduct remains unclear. Restitution of all or part of the aromatic system seems to be possible (Ward, 1975), but chromophore destruction certainly occurs with some efficiency (Hayes et al., 1974).

Although studies with native DNA are few, there is currently no apparent reason to doubt that the information in Table II-1 is a general guide to the products formed by reaction of water radicals with the macromolecule. There is no obvious explanation from available radiation chemistry for the unique importance of the OH-radical in radiation lethality.

Since most of the models of radiation lethality promote a certain class of "molecular damage" (see Chapter I) it becomes important to know the contribution of water radicals to these classes. Most of the information that has been reported deals with strand breaks. As mentioned above Roots and Okada (1972) attribute 70% of strand breaks in mouse cells to the OH-radical, and this has been confirmed in bacteria (Donta and Freifelder, 1970), and in vitro (Achey and Duryea, 1974), but single-strand breaks do not seem to contribute to lethality. Glycerol, an OH-scavenger, has been reported to protect against 60-70% of the

double-strand breaks in E. coli (Bonura and Smith, 1976) and λ phage (Donta and Freifelder, 1970), but, at least with phage, double-strand breaks are too infrequent to account for lethality (Freifelder, 1965; van der Schans et al., 1973). It is implicit in some studies of strand breaks (those that use alkaline sucrose gradients) that alkali-labile bonds are likely due to OH-radicals but no explicit study has been reported. The only information about base damage comes from Donta and Freifelder (1970), who attribute about 35% of base damage (lethality unaccounted for by double-strand breaks) to the OH-radical. Clearly more information is needed about the roles of water radicals in producing each type of damage. To this end, the work reported in this chapter examines the contribution of OH, H, and E toward each of the three classes of single-strand damage—strand breaks, alkali-labile bonds, and damaged bases. I report here only work with dilute, anoxic solutions of DNA; the effects of oxygen (Chapter III) and protective solutes (Chapter IV) will be considered later. During the course of this study very similar work appeared (Strniste and Wallace, 1975; Armel, Strniste and Wallace, 1977), which will be discussed where appropriate.

Materials and Methods

DNA preparation. Phage PM2 was grown according to the method of Espejo and Canelo (1968), and isolated by low-speed configuration after aggregation in polyethylene glycol (Carbowax 6000) by the method of Yamamoto et al. (1970). Phage were lysed in 1% sodium lauryl sulfate and deproteinized by extraction with either chloroform-isoamyl

alcohol (24:1) or buffered phenol. Both techniques gave, after extensive dialysis, DNA which was 80-95% unbroken (form I) and pure by UV-spectral analysis. Stock DNA was routinely stored in Tris buffer, but dialyzed against 0.01 M sodium phosphate (pH 7) before use in these experiments. The guidance of W. L. Carrier, and frequent gifts of phage DNA were greatly appreciated.

Irradiation of DNA. Irradiations were performed with either a ^{60}Co source (approximately 3 krad/min), or a 250 kVp X-ray machine (dose rates from 10 rads/min to 4 krad/min). Prior to irradiation additional solutes were added to DNA samples as required by the experiment, and the samples were bubbled at least 20 minutes with the appropriate water saturated gas (N_2, O_2, or N_2O). Bubbling was continued during and at least 1 minute after irradiation to insure constant irradiation conditions. Other details of irradiations are noted in the figure legends.

Assay for damage. Following irradiation DNA was either (1) incubated in the irradiation buffer, (2) treated with the γ-endonuclease from M. luteus (as in Chapter I of this thesis), or (3) exposed to alkali, before determination of strand breakage by conversion of supercoils (form I) to relaxed (form II) molecules on sucrose gradients. This protocol allowed determination of (1) strand breaks, (2) γ-endonuclease-sensitive sites, and (3) alkali-labile bonds, respectively.

The appropriate control experiments showed that the above classes of lesions were valid distinctions in that the numbers of lesions were

additive under most conditions tested. For example treatment of the DNA with enzyme followed by alkali gave the number of strand breaks expected from the sum of each treatment alone. In addition the product of enzyme action was found to be a strand break (expressed on a neutral sucrose gradient), verifying that the enzyme is indeed an endonuclease rather than N-glycosylase as reported for some forms of chemical damage. Conversely, the alkali-labile lesions measured here were not susceptible to the enzyme preparation. This fact together with their extreme sensitivity to alkali (they are expressed immediately in 0.1 M NaOH) leaves doubt that these lesions are apurinic or apyrimidinic sites (see Lindahl and Andersson, 1972). An exception to these generalizations is discussed in Chapter IV of this thesis.

Finally, all three classes of lesions appeared to be stable at neutral pH, in that no interconversion or hydrolysis to strand breaks was observed under several conditions of postirradiation incubation (e.g., 120 minutes at 60°C). The unstable forms of radiation damage reported by others (e.g., Ward and Kuo, 1976) must either not be formed or go undetected in the system used here.

Radical scavengers. A list of second-order rate constants for reactions with OH, H, and E of the important compounds in this and subsequent chapters is found in Table II-2. To assess the role of the individual water radicals in DNA damage selective radical-scavengers are needed. The principal scavengers used in this study are I^- (for OH) and NO_3^- (for E). It should be noted that both compounds react significantly with the hydrogen atom (Table II-2), as do almost all

TABLE II-2

RATE CONSTANTS FOR IMPORTANT RADIOCHEMICAL REACTIONS

Reactant	Rate Constant (M^{-1} sec^{-1})		
	$OH^{\cdot}$	$H^{\cdot}$	E
Double-stranded DNA	4×10^{8}[a]	$\sim 10^{8}$[i]	1.3×10^{8}[d]
Single-stranded DNA	1×10^{9}[a]	--	5×10^{8}[d]
I^-	1.6×10^{10}	4×10^{7}[f]	$<2.4 \times 10^{5}$
t-butanol	5×10^{8}	1×10^{5}	nil[j]
NO_3^-	$<5 \times 10^{5}$[b]	$\sim 10^{7}$[b]	1.1×10^{10}
N_2O	nil[j]	1×10^{5}	9×10^{9}
Cl^-	$<10^{3}$[g]	$<10^{5}$	$<10^{4}$
ClO_4^-	nil[b]	nil[b]	1.2×10^{7}
$H_2PO_4^-$	2×10^{6}[e]	5×10^{5}	$\sim 10^{7}$[c]
O_2	nil	2×10^{10}	1.9×10^{10}
Thymidine	5×10^{9}	2.5×10^{8}	1.7×10^{10}
Phenylalanine	6×10^{9}	8×10^{8}[h]	1.5×10^{8}
Deoxyribose	2×10^{9}	2.3×10^{7}	1×10^{7}
Adenosine	4.2×10^{9}	1.4×10^{8}	9.2×10^{9}

All values at pH 7.

Except as noted values are taken from National Standard Reference Series NSRDS-NBS Volumes 43, 43 supp., 46, 51, and 59.

[a] Michaels and Hunt, 1973.

[b] Anbar and Neta, 1967.

[c] Jortner et al., 1962.

[d] Shragge, Michaels, and Hunt, 1971.

[e] Grabner et al., 1973.

[f] Draganic and Draganic, 1972.

[g] Ward and Kuo, 1970.

[h] Neta and Schuler, 1971.

[i] Scholes and Simic, 1968.

[j] Commonly reported to be unreactive.

radical scavengers. Unfortunately no selective scavenger for the hydrogen atom is currently known. The scavenging reactions are found in the following equations:

$$I^- + OH^\bullet \rightarrow I^\bullet + OH^-$$

$$NO_3^- + E + H^+ \rightarrow NO_2 + OH^-$$

The reaction products have been shown to decay by reactions that do not involve DNA, and thus do not confuse the simple protection against water radical damage. It was also advantageous to choose KI and KNO_3 since they are similarly fully ionized salts in aqueous solution. Neither was found to effect the postirradiation chemistry or biochemistry (γ-endonuclease assay) required for assay of damage. Other scavengers used are t-butanol (for OH) and nitrous oxide (for E). All chemicals used were reagent grade. Nitrogen and nitrous oxide gases were obtained from Linde (Union Carbide) and contained less than .02% and .003% oxygen, respectively, according to the supplier. No further deoxygenation was attempted.

Kinetic model of radical scavenging. When several solutes (S_i) are competing for the same radical (X) the fraction of X that reacts with "solute i" is given by:

$$f_{X+S_i} = \frac{k_{X+S_i}[S_i]}{\sum_{\text{all solutes}} k_{X+S_i}[S_i]}$$

Thus when DNA (10^{-3} M) and iodide (10^{-4} M) compete for the OH-radical the distribution is as follows (rates are from Table II-2):

$$f_{DNA+OH^{\bullet}} = \frac{k_{DNA+OH^{\bullet}}[DNA]}{k_{DNA+OH^{\bullet}}[DNA] + k_{I^{\bullet}+OH}[I^{-}]}$$

$$= \frac{(4\times10^{8})(10^{-3})}{(4\times10^{8})(10^{-3}) + (7\times10^{9})(10^{-4})}$$

$$= 0.36 .$$

That is 36% of the OH-radicals react with DNA, 64% are scavenged by iodide. Calculations such as these were performed where necessary and are discussed where appropriate.

Results

Role of the OH-radical. Figure II-1 demonstrates the effect of 0.1 M potassium iodide on the three classes of damage to DNA by anoxic X-irradiation. While the relative amounts of the three classes of damage are very different at the two DNA concentrations shown (note that the ordinates have been adjusted) the pattern of iodide protection is remarkably similar. Iodide prevents 70-90% of the strand breaks and 60-90% of the alkali-labile bonds, but actually increases the yield of enzyme sites about 20%. The effect of iodide, and therefore probably the role of the OH-radical appears greater at the higher DNA concentration. Control experiments showed that iodide had to be present at the time of irradiation to have these effects, consistent with a

Figure II-1. Effect of potassium iodide on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). X-irradiations were performed under anoxic conditions (N_2 bubbling) in 0.01 M Na·phosphate buffer (pH 7), plus either 0.1 M KCl (solid symbols), or 0.1 M KI (open symbols). DNA concentration was (a) 12 μg/ml and (b) 500 μg/ml.

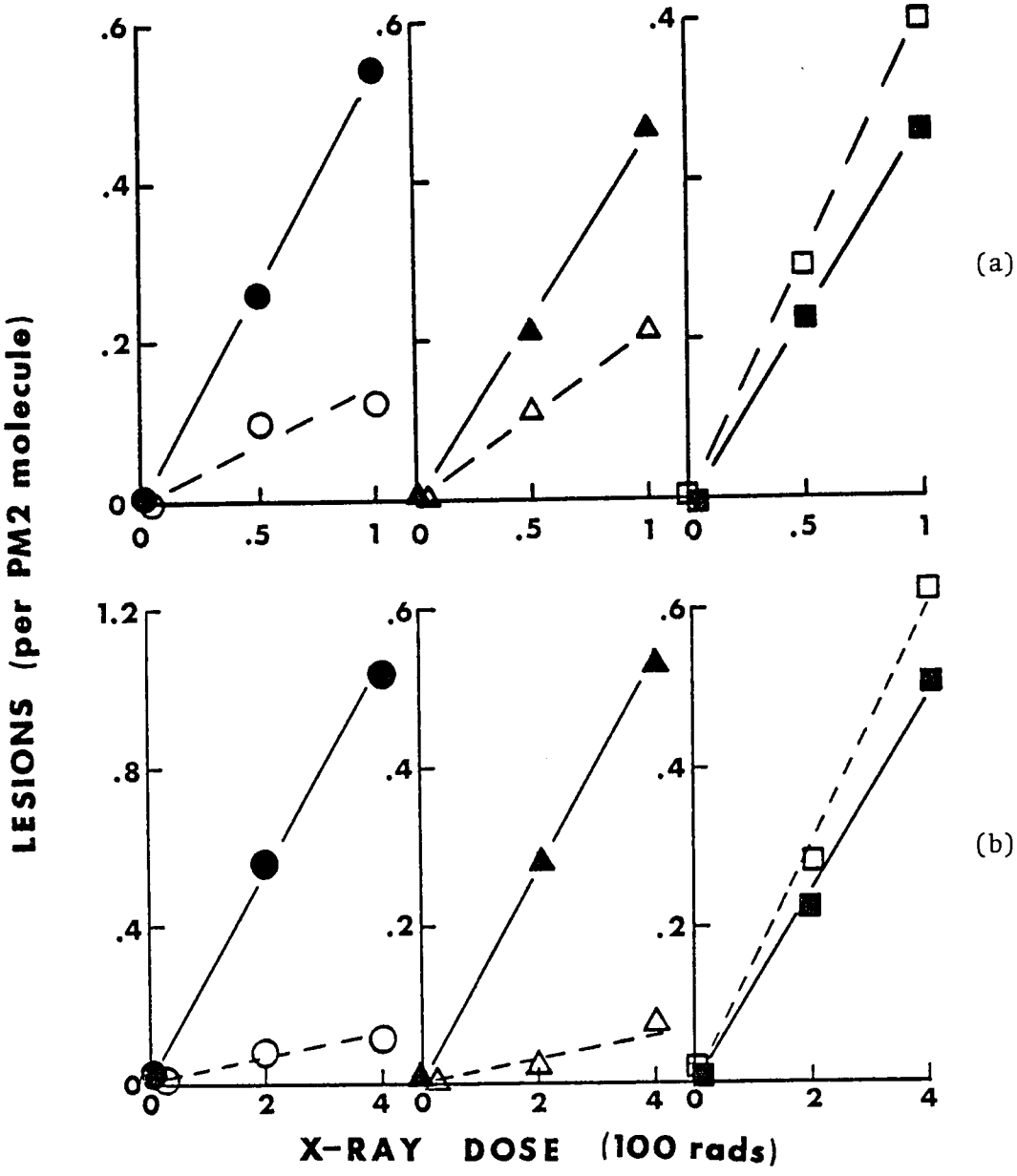


Figure II-1

simple competitive scavenger model. Iodide added immediately after irradiation did not influence either the enzyme or alkali treatments necessary for assay of the damage. Potassium chloride (unreactive with water radicals) had no effect when substituted either during or after irradiation, thus eliminating effects of ionic strength. Iodide has frequently been used as a selective scavenger for the OH-radical, but also reacts well with the less-frequent hydrogen atom ($k = 4 \times 10^7\ M^{-1}\ sec^{-1}$, Table II-2, page 29). Since t-butanol, which is nearly unreactive with the hydrogen atom gave very similar effects (Table II-3), it is probable that the protection seen in Figure II-1 is mainly due to quenching of the OH radical.

Further evidence that competitive scavenging of OH-radicals is involved in iodide protection can be seen by examining the dependence on iodide concentration (Figure II-2). At two different concentrations of DNA the effect of iodide over a wide range in scavenger concentration was determined. The concentration of iodide to give 50% OH-scavenging should be given by:

$$[I]_{50} = \frac{k_{DNA+OH^{\cdot}}}{k_{I^-+OH^{\cdot}}} [DNA] = \frac{1}{16} [DNA]$$

(Rate constants from Table II-2.) In Figure II-2 protection against strand breaks and alkali-labile bonds is half-maximal at an iodide concentration about an order of magnitude below the concentration of DNA, in rough agreement with this calculation. Similarly, scavenging of H-atoms should not reach 50% until the iodide concentration is about

TABLE II-3

EFFECTS OF RADICAL SCAVENGERS ON DNA DAMAGE

Lesion	Additional Solutes[a]					
	None	KCl	KI	KNO_3	t-Butanol	N_2O
Strand Breaks	1.0[b]	1.1	0.09	0.96	0.14	1.3
Alkali-labile bonds	0.90	1.1	0.45	0.63	0.42	1.2
Enzyme sites	2.1	2.2	3.0	0.72	2.4	1.1

[a]All solutions 0.01 M $Na \cdot PO_4$ pH 7; irradiated anoxically.
All solutes 0.1 M, except N_2O approx. 0.04 M.
DNA concentration 40 µg/ml.

[b]Values taken from dose response curves and normalized to strand breakage in phosphate buffer.

Figure II-2. Dependence of radiation protection on potassium iodide concentration. Symbols represent strand breaks (●), alkali-labile bonds (▲), and enzyme sites (■). Irradiations were anoxic (N_2 bubbling). (a) X-irradiation of DNA (35 μg/ml) in 0.01 M $Na{\cdot}PO_4$ pH 7. (b) ^{60}Co γ-irradiation of DNA (400 μg/ml) in 0.01 M $Na{\cdot}PO_4$ pH 7, 10^{-5} M EDTA.

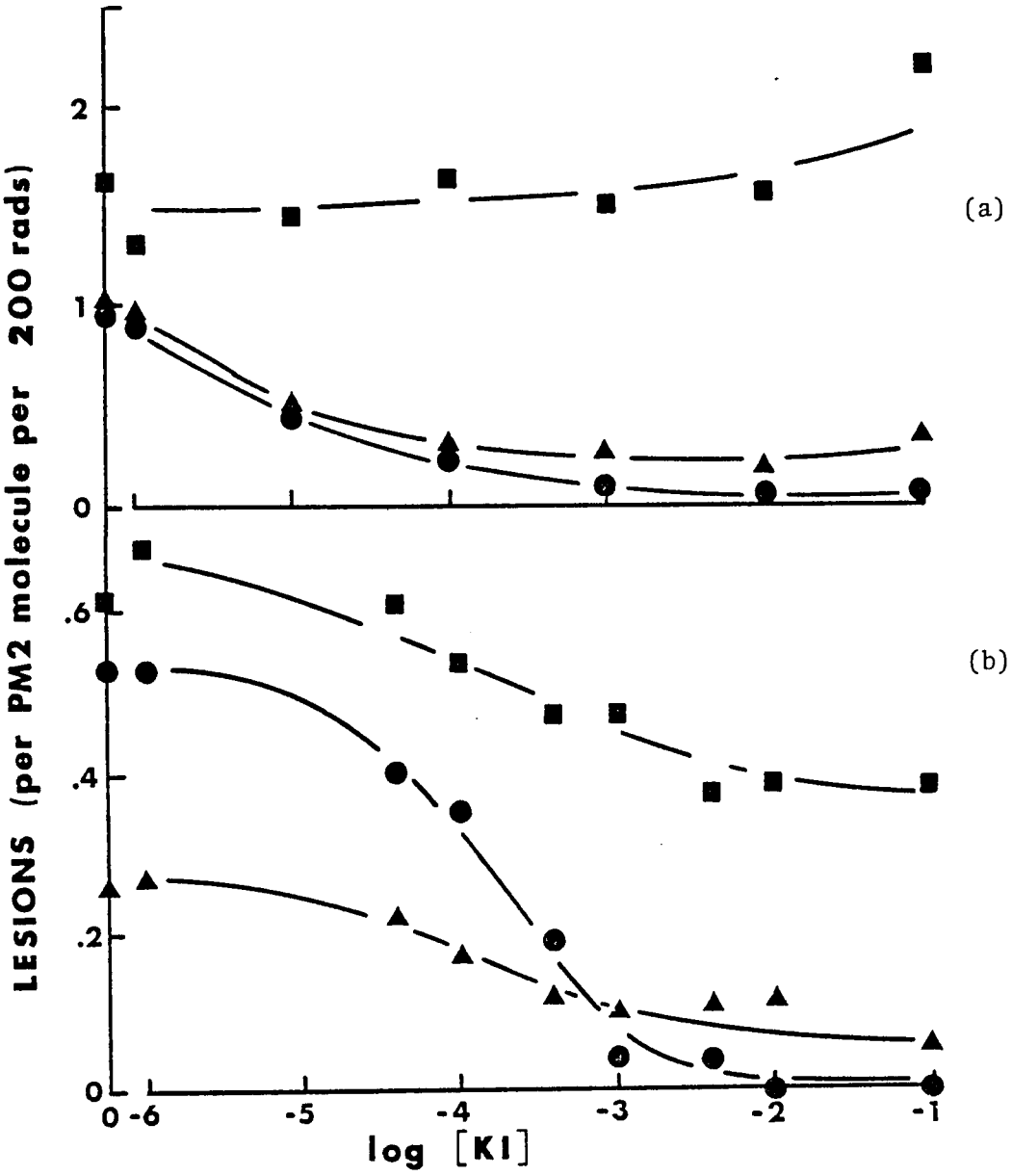

LESIONS (per PM2 molecule per 200 rads)
2
1
0
.6
.4
.2
0
(a)
(b)
0 -6
-5
-4
-3
-2
-1
log [KI]

Figure II-2

three times that of the DNA. There is no evidence of further protection in this region for any of the three classes of damage, further indicating that iodide protection does not involve this radical.

The increase in enzyme sites found with iodide in Figure II-1 (page 32) is also evident in Figure II-2a, and was a general result under the conditions of these experiments (250 keV X-irradiation in phosphate buffer). The experiment in Figure II-2b involved ^{60}Co γ-radiation in the presence of 10^{-5} M EDTA, and in these conditions enzyme sites appeared to decrease about 30% in iodide. This result remains unexplained, but is not necessarily in disagreement with experiments at the other conditions.

Another means of examining the role of the OH-radical is to irradiate DNA in the presence of nitrous oxide (N_2O) which converts electrons into OH-radicals by the following reaction (Blok and Loman, 1973):

$$N_2O + E + H_2O \rightarrow OH^{\cdot} + N_2 + OH^{-} \ .$$

Thus in N_2O the OH-radical should be the only damaging species, except for any minor contribution from the hydrogen atom. Figure II-3 shows that in nitrous oxide all three classes of damage are still produced, but in altered amounts. Compared to nitrogen there are relatively more strand breaks and alkali-labile bonds, but fewer enzyme sites in nitrous oxide, indicating that the OH-radical is more efficient than the electron in the former two, but less efficient in the latter reaction. The fact that the increases are not 2-fold as expected from complete

Figure II-3. Effect of potassium iodide on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). X-irradiations were performed under anoxic conditions in the presence of nitrous oxide (bubbling at room temperature), in 0.01 M $Na \cdot PO_4$ (pH 7) plus either 0.1 M KCl (solid symbols), or 0.1 M KI (open symbols). DNA concentration (a) 12 µg/ml, (b) 500 µg/ml.

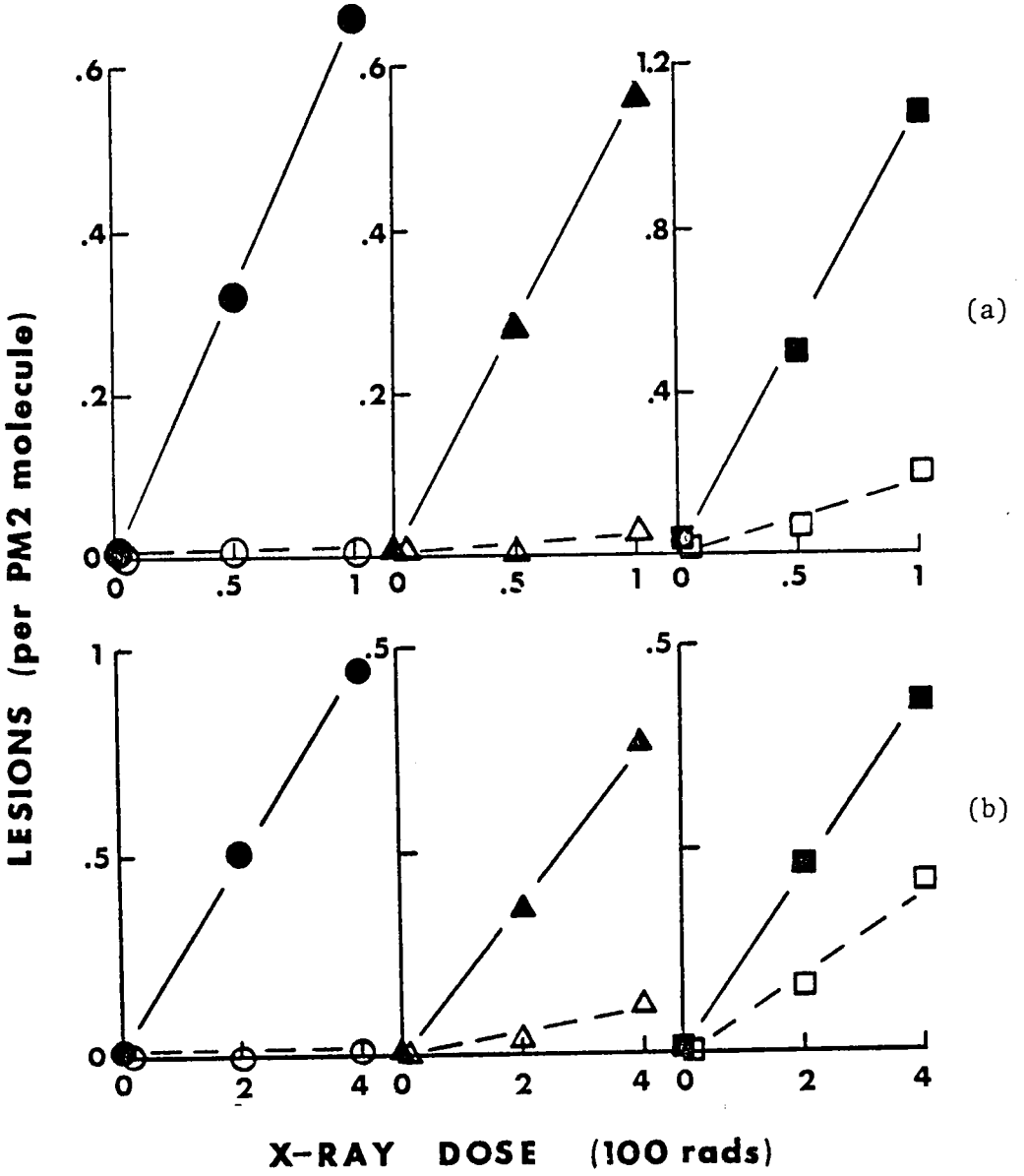

LESIONS (per PM2 molecule)
.6
.4
.2
0
.6
.4
.2
1.2
.8
.4
0 .5 1 0 .5 1 0 .5 1
(a)
1
.5
0
.5
.5
0 2 4 0 2 4 0 2 4
(b)
X-RAY DOSE (100 rads)

Figure II-3

conversion of electrons into OH-radicals (the primary yield of the two are equal) is not unlike results of others, and remains unexplained.

Addition of iodide in the presence of nitrous oxide (Figure II-3) verifies that the OH-radical is responsible for almost all of the damage in these conditions. Iodide quenches greater than 90% of all three classes of damage in nitrous oxide, except at the higher DNA concentration (Figure II-3b) where some alkali-labile bonds and enzyme sites remain. This might indicate that some electrons escape N_2O and react with the DNA under these conditions, although the rate constants and expected concentration of N_2O (0.05 M at saturation) argue against this. The possibility that toxic products known to be produced in nitrous oxide play a role cannot be excluded (Brustad and Wold, 1976). Regardless, it is clear that the OH-radical reacts with DNA to cause mostly strand breaks and alkali-labile bonds, but apparently also a few enzyme sites.

Role of the aqueous electron. Analogous to the above experiments, potassium nitrate was used as a selective scavenger to determine the role of the aqueous electron in producing DNA damage. The dose response curves are shown in Figure II-4. At the lower DNA concentration (Figure II-4a) it appears that nitrate has very little influence on strand breakage, but prevents about 35% of the alkali-labile bonds, and about 65% of the enzyme sites. At the higher DNA concentration the effect of nitrate is similar but less evident. Here there is clearly no protection against strand breakage, and only 15% and 35% protection against alkali-labile bonds and enzyme sites, respectively. The role

Figure II-4. Effect of potassium nitrate on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). X-irradiations were performed under anoxic conditions (N_2 bubbling) in 0.01 M $Na{\cdot}PO_4$ (pH 7), plus either 0.1 M KCl (solid symbols) or 0.1 M KNO_3 (open symbols). DNA concentration was (a) 40 μg/ml, (b) 500 μg/ml.

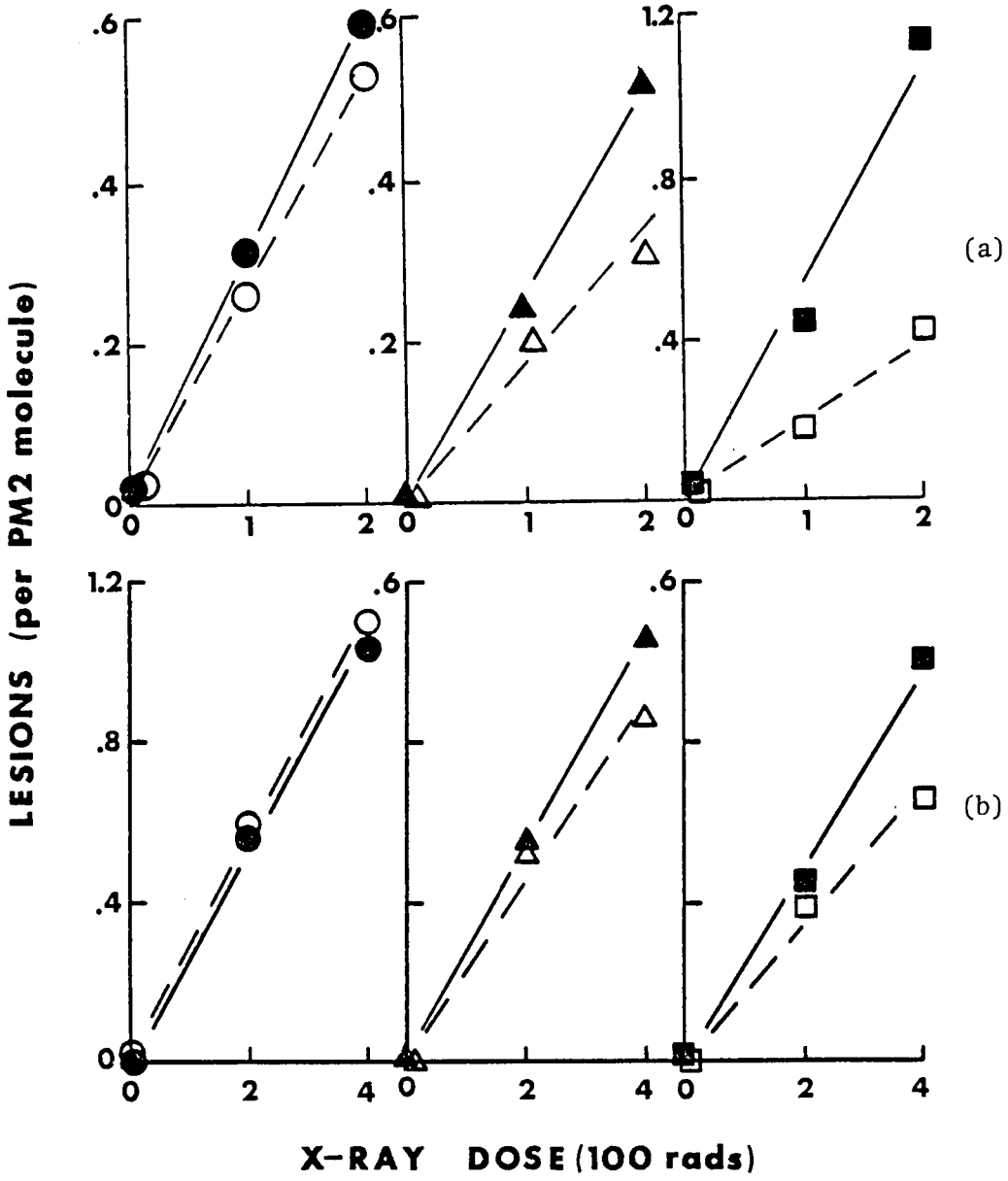


Figure II-4

of the electron apparently decreases with increasing DNA concentration. As with iodide, nitrate had to be present during irradiation, and none of the protective effects described were found in potassium chloride.

The dependence of this protection on nitrate concentration was examined only at the lower DNA concentration where the effects were greater and more easily studied. Figure II-5 shows that, unlike with iodide where definite regions of inflection were observed, protection by nitrate occurs over a very broad range in concentration. This is possibly due, at least in part, to the dependence of the rate of reaction of E with both NO_3^- and DNA (also negatively charged) on the ionic strength of the medium, which obviously varies with KNO_3 concentration. There is currently insufficient knowledge of this dependence to offer any quantitative explanations.

To examine the effects of the aqueous electron under conditions where it is the only damaging species irradiations were done in the presence of sufficient iodide to remove all the OH and H. The results, again for a low concentration (12 μg/ml) of DNA, are shown in Figure II-6. Although enzyme sites are by far the predominant form of damage (accounting for more than 90% of the total lesions), a few strand breaks and alkali-labile bonds are also formed. Addition of nitrous oxide to scavenge electrons abolishes all three kinds of damage almost completely, verifying that not only the enzyme sites, but the residual strand breaks and alkali-labile bonds were electron-induced. Similar results were obtained with nitrate but are not shown.

Figure II-5. Dependence of radiation protection on potassium nitrate concentration. Symbols represent strand breaks (●), alkali-labile bonds (▲), and enzyme sites (■). DNA (40 μg/ml) was X-irradiated under anoxic conditions (N_2 bubbling), in 0.01 M $Na{\cdot}PO_4$ (pH 7). The open symbols represent the 0.1 M KCl ionic strength control.

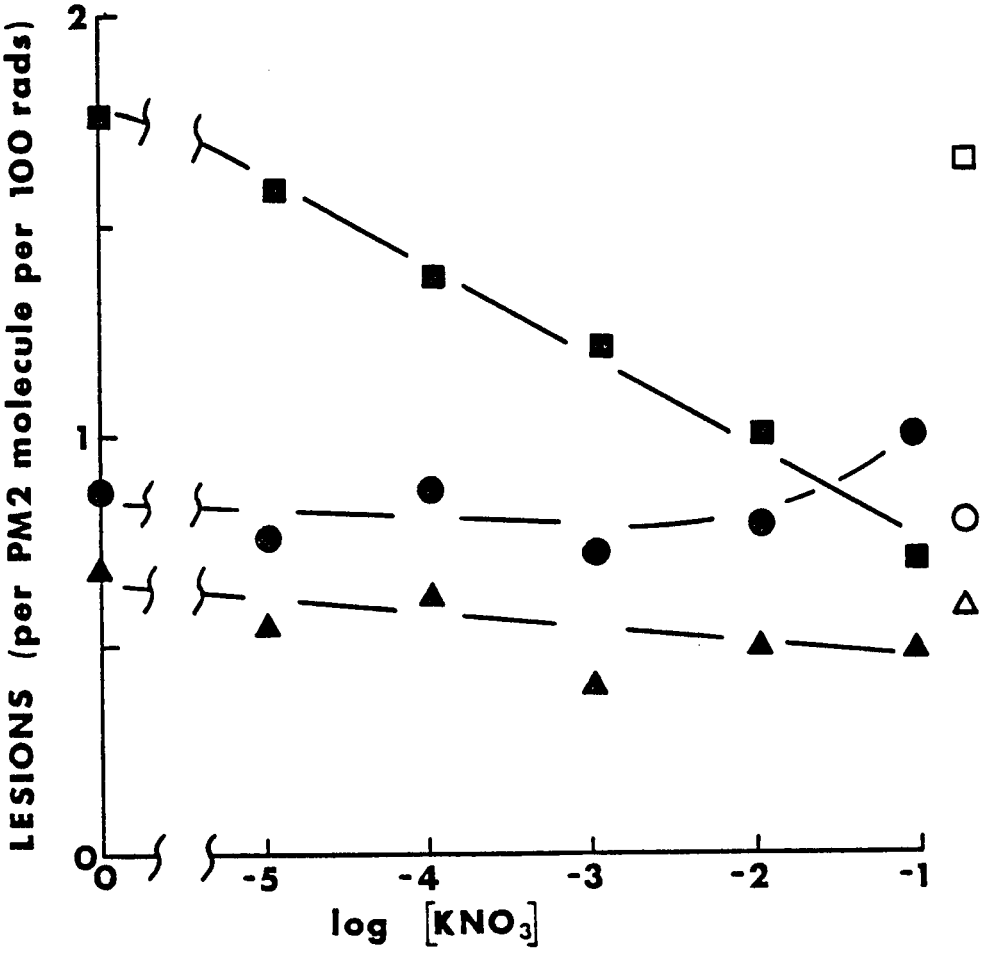


Figure II-5

Figure II-6. Effect of nitrous oxide on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). DNA (12 μg/ml) was X-irradiated under anoxic conditions in 0.01 M $Na \cdot PO_4$ (pH 7), 0.1 M KI. Solutions were bubbled with either nitrogen (solid symbols) or nitrous oxide (open symbols) 30 min before and during irradiation.

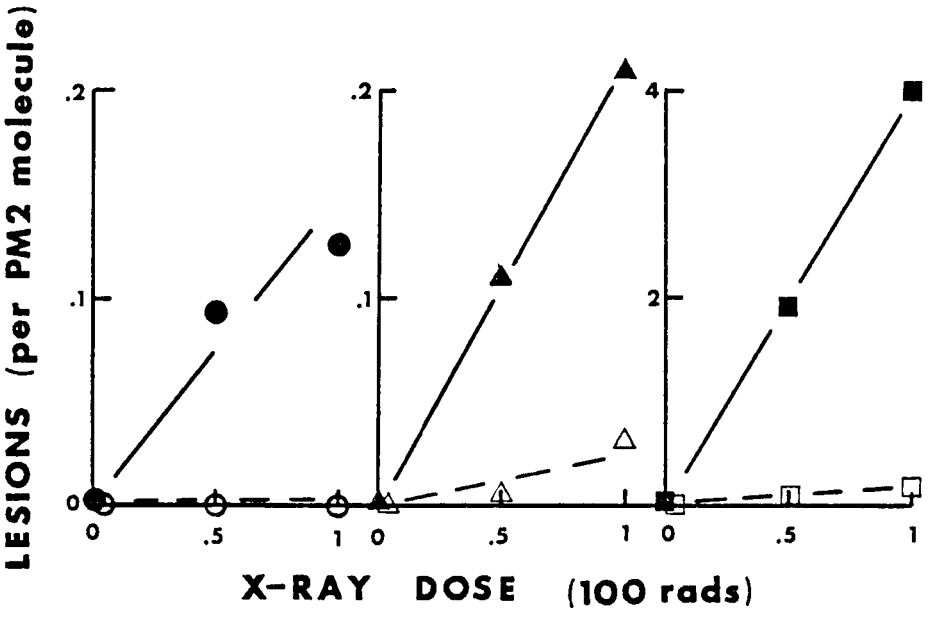

LESIONS (per PM2 molecule)
.2
.1
0
.2
.1
4
2
0
.5
1
0
.5
1
0
.5
1
X-RAY DOSE (100 rads)

Figure II-6

Role of the hydrogen atom—a difficulty with phosphate. Considering the generally complementary nature of the protection afforded by scavengers of the OH-radical and aqueous electron, it is tempting to discount completely the role of the hydrogen atom. As has been mentioned, both nitrate and iodide react with H, but the concentration-dependence curves for neither of these scavengers give any indication of protection in the region predicted by published rate constants. It should be noted, however, that the final product of neither of the scavenge reactions is known, and at least in the case of iodide may involve a net conversion of H-atoms into aqueous electrons (Draganic and Draganic, 1972). If this were true no effect of iodide would be expected if the damage due to attack on DNA by H and E were similar. This is not unlikely. H and E are often classified as the "reducing radicals" because of their similarities and frequent interconversion in solution. Therefore these data alone cannot be considered as proof that hydrogen atoms are unimportant.

The presence of phosphate buffer in this study compounds the problem. It must be pointed out that according to the published rate constants (Table II-2, page 29) the conversion of electrons into hydrogen atoms by $H_2PO_4^-$ (Jortner et al., 1962) should be a very significant reaction, especially at the low DNA concentrations used here. Phosphate buffer was chosen because it was thought to be radiochemically less reactive than most other commonly used buffers (Tris-buffer, for example, is highly radioprotective; Achey et al., 1974). During the course of the work a report appeared which showed

that phosphate sensitized the biologically active single-stranded DNA of ØX174 to γ-rays under anoxic conditions, presumably by conversion of noninactivating E into H atoms (Lafleur et al., 1975). This result has subsequently been confirmed, but in contrast the double-stranded replicative form of ØX174 DNA was found to be protected 2-fold by phosphate, as compared to unbuffered sodium chloride (Achey and Hallam, 1978). This currently seems very confusing, and must be understood before the separate roles of E and H can be known with certainty.

In order to test the effect of phosphate in this system preliminary experiments were performed in which the concentration of $H_2PO_4^-$ (the proposed reactive species) in the irradiated sample was varied by either changing pH or total phosphate concentration. The results are shown in Table II-4. In both experiments there was an increase in all three classes of damage with increasing $H_2PO_4^-$ concentration. The increase was greatest for alkali-labile bonds (100%) and enzyme sites (50%). If one accepts the proposed scheme for conversion of E into H the total yield of the latter radical would be expected to increase from the primary radiolytic value to about six times this value over the range of phosphate concentrations studied. The observed increases are much smaller, but may indicate that the hydrogen atom contributes significantly to the production of alkali-labile bonds and enzyme sites, especially in phosphate buffer. Like the electron, hydrogen atoms probably account for few if any strand breaks. In the absence of phosphate, or if the proposed conversion scheme does not occur, the role of the H-atom would, for strictly quantitative reasons, be very minor.

TABLE II-4

THE ROLE OF PHOSPHATE IN H-ATOM RADIOCHEMISTRY

Class of Lesion	Irradiation Conditions					
	a			b		
	10^{-5} M $Na \cdot PO_4$ (f = 0.003)	10^{-3} M $Na \cdot PO_4$ (f = 0.25)	10^{-1} M $Na \cdot PO_4$ (f = 0.97)	pH 8.2 (f = 0.91)	pH 7.2 (f = 0.98)	pH 6.2 (f = 0.99)
Strand breaks	1.0*	0.97	1.18	1.0*	1.27	1.39
Alkali-labile bonds	1.0*	1.28	2.15	1.0*	1.44	1.83
Enzyme sites	1.0*	0.96	1.64	1.0*	1.39	1.38

All irradiations were single dose of 200 rads, anoxic (N_2 bubbling. "f factors" are fraction of E calculated to convert to H from rate constants of Table

*Values within lesion class normalized to this value.

[a]All samples pH 7.2; DNA conc. 40 µg/ml.

[b]All samples 10^{-1} M $Na \cdot PO_4$; DNA conc. 40 µg/ml.

Discussion

The intent of this work was to classify the damage caused by the water radicals, and in so doing gain some insight to the importance of the OH-radical (and unimportance of $H^{\cdot}$ and E) for radiation lethality. The results at first seem paradoxical.

It is clear from conditions where only one radical species attacks the DNA that OH-radicals make mainly strand breaks, and electrons (and probably their reducing partner $H^{\cdot}$) cause mostly enzyme sites. The alkali-labile bonds seem to split between the radicals more evenly, perhaps consistent with the idea that there is more than one kind of these lesions. The scheme in Figure II-7 serves as a general summary of these results. This scheme is in general agreement with one proposed by Armel, Strniste and Wallace (1977), who used a similar enzyme extract from E. coli and classified radiation damage just as I have done here. Since they irradiated only under air, their data will be considered in more detail in Chapter III of this thesis.

The paradox is that the most accepted current theory on "lethal lesions," especially in phage DNA, favors base damage (monitored here as enzyme sites) rather than strand breaks, while maintaining the importance of the OH-radical. As I mentioned earlier, OH-radicals have been shown by others to be responsible for strand breakage, so this result was as expected. What is surprising is the relatively low yield of endonuclease-susceptible damage with the OH-radical, which, like all three water radicals, reacts primarily at the base moiety. A possible explanation for this is that the OH-radical does react primarily with

Figure II-7. Model proposed for the contribution of water radicals to DNA damage in dilute, anoxic solutions.

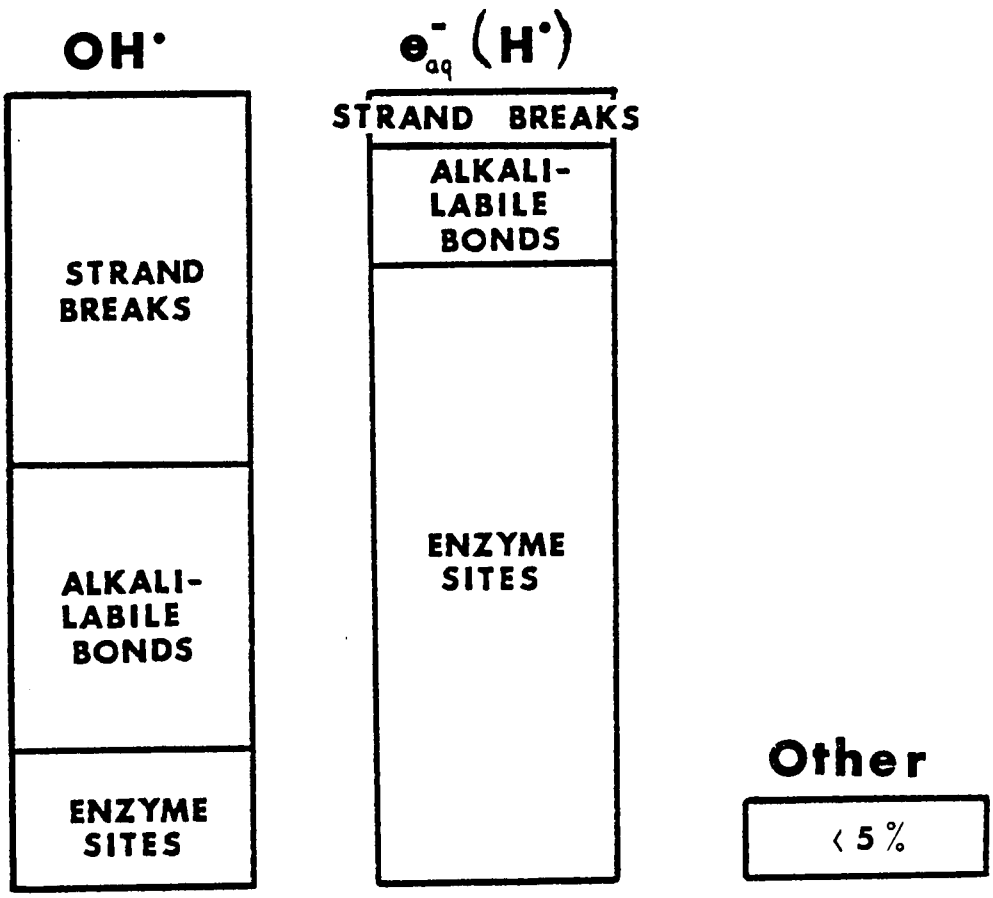

OH·
STRAND BREAKS
ALKALI-LABILE BONDS
ENZYME SITES
e^-_{aq} (H·)
STRAND BREAKS
ALKALI-LABILE BONDS
ENZYME SITES
Other
< 5 %

Figure II-7

the base, but that the resultant damage is simply not as easily recognized by the enzyme, and is therefore not repairable (i.e., lethal). Conversely, electron-induced damage appears highly repairable, and nonlethal. The possibility mentioned previously (Ward and Kuo, 1970), that the OH-radical cannot react with the base until some strand breakage has occurred is unlikely, since there is no shoulder in the dose response for enzyme sites under any of the conditions tested.

Several quantitative anomalies still exist in the experiments that have been described. The increase in enzyme sites upon addition of iodide indicates that the OH-radical contributes nothing to this class, but in nitrous oxide this is evidently not the case. The possibility that iodide converts hydrogen atoms to electrons (Draganic and Draganic, 1972), which are very efficient enzyme site producers still remains, especially in phosphate. There is also evidence of a slight increase in strand breakage with nitrate addition. Both of these anomalous increases occur at high scavenger concentration where some effect might be expected on the so-called "primary yields" in the diffusion model of radiation chemistry (Kupperman, 1967).

A major unexplained aspect of the present work is the apparent increase in the relative importance of the OH-radical with increasing DNA concentration. A similar effect in biological inactivation was seen by Blok and Verhey (1968), the reducing radicals were important only at the low DNA concentration. Another equally puzzling result with solutions of phage DNA is that the observed D_{37} for biological effects (dose required to give an average of one lethal hit per

molecule) is much higher than would be expected from complete scavenging of water radicals by the DNA. Thus "G values" (radiochemical yield = events per 100 eV absorbed) in the range of 0.06-0.13 have been reported for inactivation in dilute (3-5 μg/ml), unprotected conditions (Blok and Verhey, 1968; Lafleur et al., 1975), compared to a yield of about 6 for primary water radicals. They suggest that most of the damage is simply nonlethal, even in the single-stranded DNA of ØX174 used in their system. Blok and Loman (1973) reviewed the inactivation studies, and noted that the yield of inactivation increases with DNA concentration (G = 1.6 at 4 mg/ml). They were unable to offer a satisfactory explanation of the phenomenon. The data presented here suggest that the low yield and the role of the OH-radical, both dependent on the DNA concentration, may be closely related.

It is evident from Figure II-1 (page 32) that the yield of molecular damage is dependent on DNA concentration. At 12 μg/ml (about 1.2×10^{12} molecules of PM2 per ml) the D_{37} for total lesions is 25 rads (1 rad = 6.25×10^{13} eV/g), corresponding to a radiochemical yield of 0.08. This value increases to 0.4 at 400 μg/ml. Simultaneously the OH-induced (iodide-protectable) fraction increases from 15% to 75% of the total damage, and the repairable fraction (enzyme sites) decreases drastically. In the scheme of Figure II-7 (page 53) this can be visualized as a 15-fold increase in the size of the OH-radical "box," from one fifth to three times the size of the reducing radical box over the range of DNA concentrations in Figure II-1. The most straightforward explanation for this would be a contaminating impurity (or phosphate, see Black and

Hayon, 1970; Nakashima and Hayon, 1970) that competes with the DNA for the OH-radical but not the aqueous electron. Currently there is no independent proof that this is the case. It appears that the experiments of the Dutch groups with biologically active DNA, and the molecular studies such as this thesis are very comparable not only in positive aspects, but in shortcomings as well.

It is interesting to note that by manipulating the DNA concentration, and adding selective scavengers the ratio of strand breaks:alkali-labile bonds:enzyme sites ranges from 5:2:1 (in nitrous oxide at high DNA concentration) to 1:2:30 (in KI at low DNA concentration). Since intracellular DNA is surrounded by a myriad of radical scavengers it is difficult to guess what the ratio is in vivo. The data of Paterson and Setlow (1972) show that in E. coli minicells there are about an equal number of "strand breaks" (alkaline assay, therefore strand breaks plus alkali-labile bonds) and enzyme sites assayed with the same enzyme used in this thesis. Several laboratories have measured the relative number of strand breaks and alkali-labile bonds in vivo, and values range close to 1:1. Taken together these data suggest that the intracellular radiation environment lies intermediate to the two extremes above, perhaps at a damage ratio of 1:1:2. In Figure II-7 (page 53) this translates to roughly equal roles for OH and the reducing radicals in total DNA damage. This prediction is quantitatively compatible with the proposal that the OH-radical dominates lethality because of the repairability (rather than small amount) of electron-induced damage.

There are two complications in this simple scheme of the indirect action of radiation. First, the effect of oxygen, the most potent and well-known radiation sensitizer, are not yet explained. Secondly, the reactions of DNA with transient radical adducts of intracellular scavengers (e.g., nucleotide and amino acid radicals) probably cannot be ignored. These questions are the subject of the remainder of this thesis.

CHAPTER III

THE ROLE OF OXYGEN

Introduction

It is generally accepted in radiation biology that oxygen sensitizes living cells to the effects of ionizing radiation. Thus the term oxygen enhancement ratio (OER = response in oxygen/response in nitrogen) has found widespread use, and for biological endpoints usually has a value of about three. The mechanism of oxygen sensitization is the subject of much debate, because the problem is not only confusing on its own merits, but is compounded by the general ignorance of which kinds of damage are important for radiation lethality. Most discussions of the oxygen effect begin with strand breaks, the most studied and probably least important lesion, and become increasingly incomplete in progression to the more subtle forms of damage. Oxygen sensitization of every class of lesion has been reported, but there is little agreement among investigators as to the ingredients necessary for sensitization, let alone the sequence of events.

Strand breakage in vivo, measured soon after a dose of radiation is clearly greater if oxygen was present during the irradiation. It is not clear whether oxygen actually increases the initial yield of breaks, or diminishes the repairability and thereby increases only the apparent yield after some repair has taken place. The evidence for the latter possibility has been reviewed (Town, Smith and Kaplan, 1973), and recently supported (Srivastava, 1974, 1976). The heart of the argument

is whether there exists an "ultra-fast" system to repair a large fraction of breaks produced in anoxia. Johansen and his collaborators have published convincing data using rapid postirradiation lysis that conclude ultra-fast repair, if it exists, must be complete by 200 msec after irradiation (Johansen and Boye, 1975). Fox et al. (1976) performed similar experiments and concluded 10 msec was the maximal time for ultra-fast repair. This seems so preposterous that one must currently believe that there is no such repair system, but instead a real sensitization by oxygen of the initial number of strand breaks. Ironically, work of the latter group (Johansen, 1971) is probably the strongest evidence that oxygen sensitization of strand breakage has little to do with the enhancement of lethality. This was based on the finding that the molar efficiencies of oxygen in the two processes were very different, even though the final levels of sensitization were about the same.

Several investigators have assayed alkali-labile bonds in the presence and absence of oxygen. Estimates of the oxygen effect on this lesion range from a strict requirement for oxygen (i.e., no alkali-labile bonds in anoxia, Frey and Hagen, 1974) to an OER only slightly greater than unity (Paterson, Roozen, Setlow, 1973). The data of Ward (reviewed in Ward, 1975) suggest that not only the quantity, but the kind of alkali-labile bonds depends on the presence of oxygen. This was not considered in the above studies.

Base damage, assayed by chromophore destruction, has generally been reported to show some enhancement by oxygen (Weiss, 1964), but when

specific cases have been examined the effect does not appear to be simple. Hoard et al. (1974) found that while oxygen sensitized the free base thymine to damage by X-rays (loss of UV absorption), the nucleoside, nucleotide and polydT all showed protection by oxygen. In the purines, oxygen has been shown to prevent the radiation-induced opening of the imidazole ring (van Hemmen, 1975; van Hemmen and Bleichrodt, 1971), and although the products have not been fully characterized some protection of the chromophore could be demonstrated. Clearly oxygen modifies the course of radiochemical damage to the bases, but the general enhancement ratio contains little information.

The radiation chemistry of model systems (e.g., nucleotides) forms the basis of much recent speculation about the important reactions of oxygen in sensitization. The transient radicals of most DNA constituents have been found to react with oxygen either by addition and formation of peroxyradicals, or oxidation reactions (electron transfer from the organic radical to oxygen). The work of Adams and collaborators is prominent in this field and has been reviewed (Adams, 1972). They proposed that oxygen and other electron-affinic compounds radiosensitize by electron transfer mechanisms, fixing damage that otherwise might be restituted by recombination reactions. Although the original proposal involved radical ion pairs produced directly in DNA (Adams and Cooke, 1969) subsequent studies indicate that radicals produced by the indirect action of water radicals undergo similar reactions. Greenstock and Dunlop (1973) have shown that the oxidation reactions with several radiosensitizers are limited to electron adducts of DNA, while the

OH-adducts react almost exclusively by addition. Only the former reaction appears important for enhancement of strand breakage (Greenstock et al., 1973), but the latter certainly leads to major forms of base damage (Hariharan and Cerutti, 1971). In studies with simple single-stranded polynucleotides it has been shown that oxygen does react by simple addition to OH-adducts of pyrimidines, and by some more complex mechanism with purines (Michaels and Hunt, 1977a) but no (or very slow) reaction could be demonstrated with double-stranded polymers or DNA (Michaels and Hunt, 1977b). These results agree with earlier work of Willson (1970), who showed that among DNA constituents the OH-adduct radicals of guanylic acid, bromuracil, and macromolecular DNA appeared uniquely unreactive toward oxygen. Subsequent work (see Ward, 1975) indicates that the permanent products formed in oxygen absorb UV in the same region as the DNA-OH transients, thus the spectroscopic techniques used to monitor the decay of the latter may be misleading. Nevertheless, there appear to be some interesting effects of oxygen on water radical damage to DNA, which probably must be studied in the native macromolecule.

These results are just a sampling of a large body of studies involving recent techniques of pulse radiolysis in vitro. Much less has been done in vivo, but the results of Simic and Powers (1974) clearly indicate that the sensitization of an aqueous suspension of bacterial spores by oxygen and other compounds involves the affinity of the sensitizer for electrons, and therefore almost certainly some subset of the oxidation reactions described above.

The evidence cited earlier in this thesis that indirect effects of radiation play a major role in lethality apparently applies under oxygenated conditions as well (e.g., Johansen and Howard-Flanders, 1965). In fact, there have even been claims that some fraction of oxygen sensitization requires the OH-radical (Ewing and Powers, 1976). It is obviously important to know more about the effect of oxygen on the decay of water-radical adducts of DNA, and on the resultant molecular damage. I report here that DNA in dilute solution is not sensitized by oxygen, but rather is protected from the reducing radicals. The results seem disconcerting, but an explanation compatible with this thesis and the current literature is offered.

Materials and Methods

The preparation of PM2 DNA, irradiation procedures, and assay for strand breaks, alkali-labile bonds and γ-endonuclease sites were as described in Chapter II. All DNA samples were in 0.01 M phosphate buffer, pH 7, and were bubbled with the appropriate water saturaged gas (N_2, O_2, N_2O) 30' before and during irradiation. Pure oxygen was chosen over air to avoid ambiguities that might arise due to the heterogeneity of the latter (e.g., CO_2 and impurities).

In experiments where only one gas was bubbled the expected solution concentrations (at room temperature) should be approximately 1.3 mM for O_2, 0.66 mM for N_2, and 40 mM for N_2O (International Critical Tables, 1928). In several experiments oxygen and nitrous oxide were bubbled simultaneously, and at approximately equal rates by volume (no flow

meters were used), thus giving approximately half the above stated concentration of each chemical.

There have been several reports of postirradiation breakdown of DNA, especially if oxygen was present during irradiation (Weiss, 1964; Ward, 1975). In my experiments this does not seem to occur. There was no significant change in any of the three classes of damage measured here within 24 hours after fully oxygenated irradiation. No attempt was made to examine long-time courses in all experiments, rather assays were completed in a uniform and as short as possible time after irradiation.

Results

Oxygen has long been known to scavenge the reducing radicals in water, giving rise to the superoxide radical anion O_2^- by the following diffusion-limited ($k \simeq 2 \times 10^{10}$ $\underline{M}^{-1}$ sec^{-1}) reactions:

$$O_2 + e^-_{aq} \rightarrow O_2^-$$

$$O_2 + H \rightarrow HO_2 \rightleftarrows H^+ + O_2^- \quad (pK_a = 3.8) \ .$$

The superoxide anion is involved in oxygen toxicity (for review see Fridovich, 1976), and claims have been made for a requirement for both O_2^- and H_2O_2 in radiation sensitization in vivo (Misra and Fridovich, 1976) and in vitro (van Hemmen and Meuling, 1975). Superoxide alone is relatively unreactive with DNA ($k < 5 \times 10^6$ $\underline{M}^{-1}$ sec^{-1}; van Hemmen and Meuling, 1975) and does not appear to be responsible for any of the lethan effects on solutions of biologically active DNA (Blok et al., 1967).

At low DNA concentration where it has been shown (Chapter IV of this thesis) that the reducing radicals contribute significantly to DNA damage we might expect oxygen to protect the DNA. Figure III-1 shows that this is the case. Oxygen prevents the formation of a significant fraction of strand breaks and alkali-labile bonds, and of most of the enzyme sites. This result is consistent with the expected effect of a simple reducing radical scavenger in the model of Figure II-7 (page 53). Observations such as these prompted Blok and Loman (1973) to propose an "ambiguous role of oxygen": protection from reducing radical damage and sensitization (by peroxidation) of OH-induced damage. They observed that, as the model predicts, at high DNA concentration where the OH-radical dominates, oxygen does sensitize the biologically active DNA of phage ØX174. It was interesting to determine whether a basis for this sensitization is evident in the molecular damage assay used here. Thus the same experiment was performed at a DNA concentration of 500 μg/ml.

No oxygen sensitization of any of the three classes of damage was observed (Figure III-2a). At this high DNA concentration the OH-radical is the principal damaging species, but oxygen still protects. As would be expected for a reducing radical scavenger the oxygen protection was not as great as that found at the lower DNA concentration, particularly for strand breaks and alkali-labile bonds. In potassium iodide (Figure III-2b), where the damage is almost exclusively electron-induced, oxygen protection is especially evident. Enzyme sites, the predominant lesion in iodide, are almost completely eliminated by oxygen. At higher doses where the yield of strand breaks and alkali-labile bonds in

Figure III-1. Effect of oxygen on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). DNA (12 μg/ml) was X-irradiated in 0.01 M Na·phosphate buffer (pH 7) under anoxic (N_2 bubbling, solid symbols) or fully oxygenated (O_2 bubbling, open symbols) conditions.

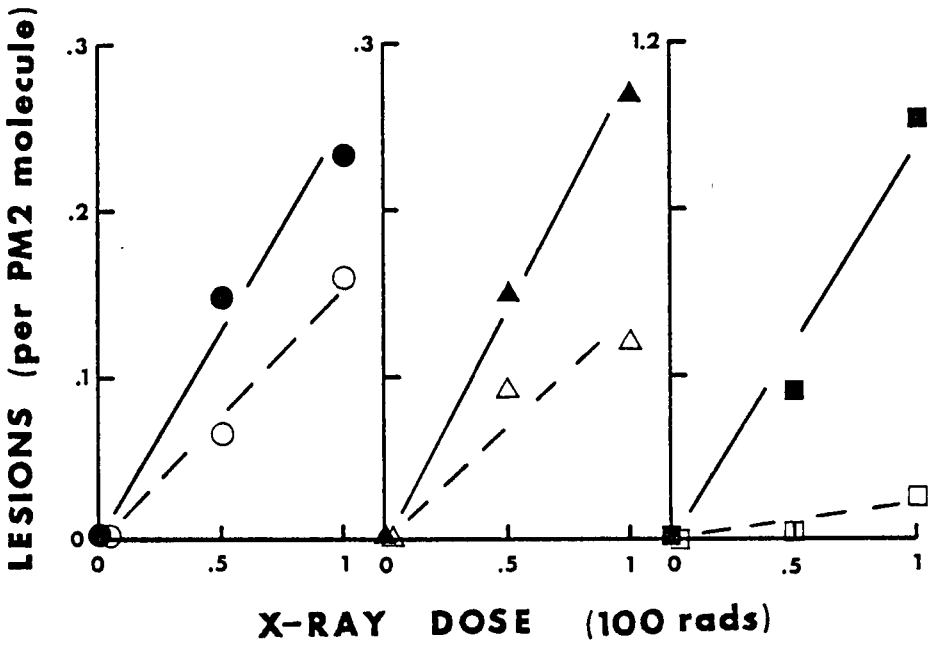

LESIONS (per PM2 molecule)
.3
.2
.1
0
.3
1.2
0
.5
1
0
.5
1
0
.5
1
X-RAY DOSE (100 rads)

Figure III-1

Figure III-2. Effect of oxygen in the presence of radical scavengers on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). DNA (500 μg/ml) was X-irradiated in 0.01 M Na·phosphate buffer (pH 7) under anoxic (N_2 bubbling, solid symbols) or fully oxygenated (O_2 bubbling, open symbols) conditions. Additional solutes were (a) 0.1 M KCl, (b) 0.1 M KI, (c) 0.1 M KNO_3.

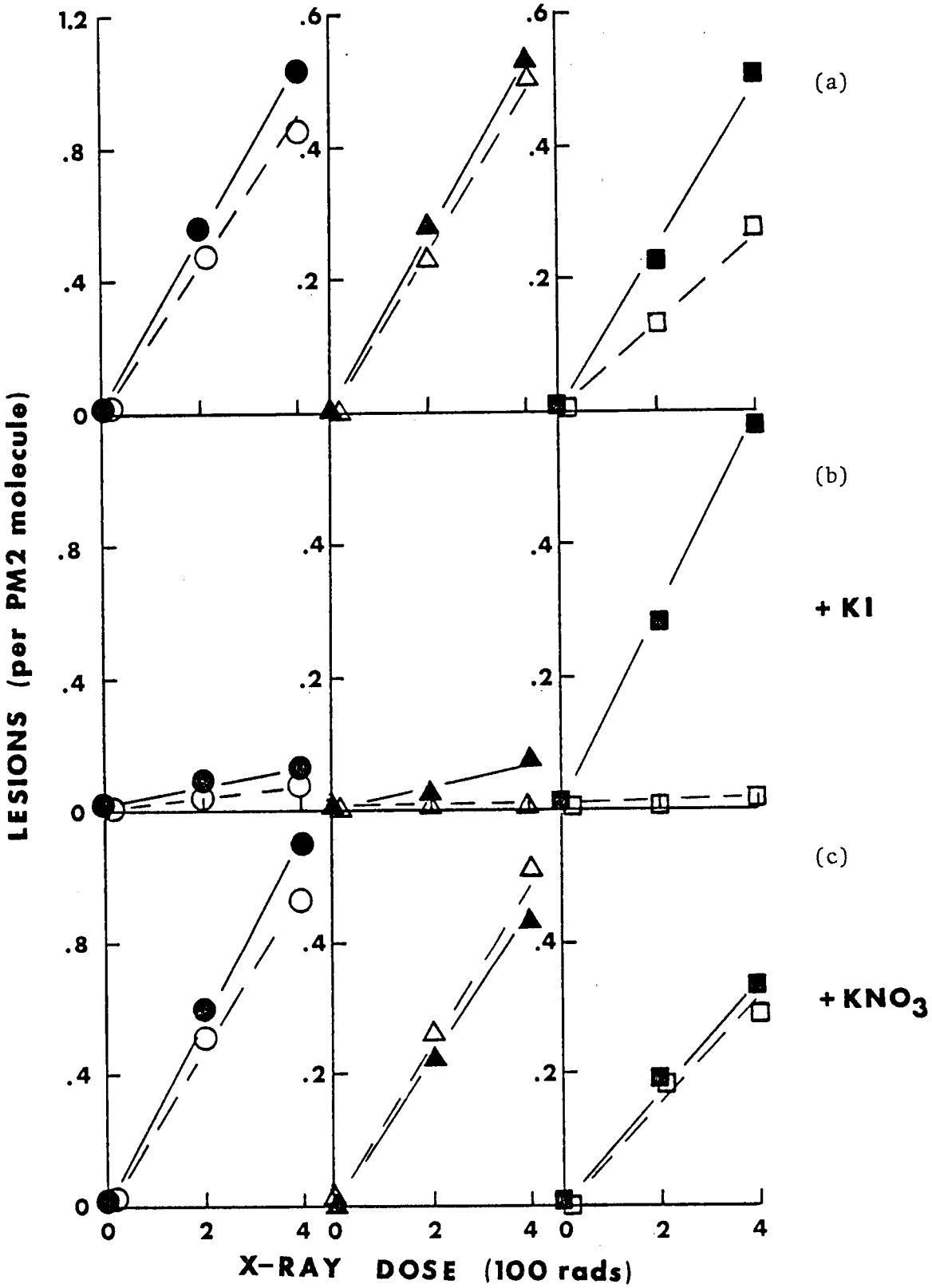


Figure III-2

potassium iodide can be measured better it is clear that oxygen prevents greater than 95% of these lesions and that the residual damage is very close to the expected direct effects on DNA (see Chapter IV). Apparently neither O_2^- nor I_2^- (the products of the E- and OH-scavenging reactions, respectively) are damaging to DNA in these conditions.

In Figure III-2c nitrate is used to remove the reducing radicals, leaving only the OH-radical to damage the DNA. In this condition there is no oxygen protection, but neither is there sensitization. Figure III-3 shows very comparable results in nitrous oxide. Again only the OH-radical reacts with DNA, but oxygen has no significant effect on the observed damage. In none of the conditions of this chapter was there an increase in any of the three classes of damage when oxygen was admitted during irradiation.

Discussion

These results clearly indicate that sensitization of cells by oxygen cannot be explained by these experiments. Either the critical precursors for the oxygen effect are not produced in these conditions, or the products of their reaction with oxygen are not detected by these methods. In previous work with the γ-endonuclease Setlow and Carrier (1973) observed that the number of endonuclease-sensitive sites was greater for DNA irradiated anoxically than with oxygen present, prompting them to support the hypothesis of Alper (1967) that N-type (anoxic) damage to DNA is more repairable than O-type (oxygen mediated). Although the correlation of enzyme sites with N-type damage remains attractive the results presented here suggest that the prevention of enzyme sites by

Figure III-3. Effect of oxygen in nitrous oxide on strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□). DNA (500 μg/ml) was X-irradiated in 0.01 M Na·phosphate buffer (pH 7) under N_2O in the absence (solid symbols) or presence (open symbols) of oxygen.

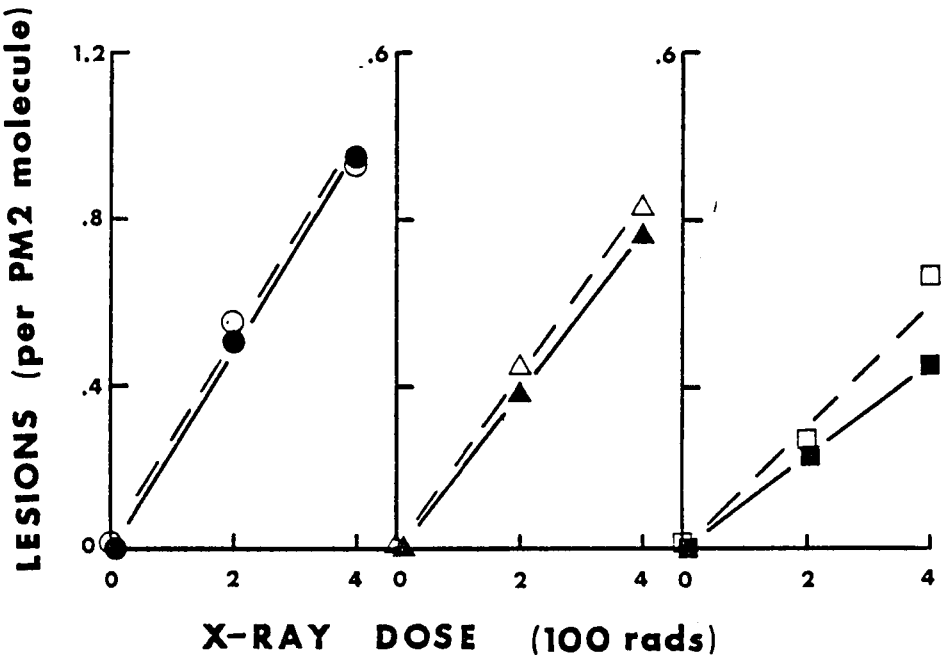

LESIONS (per PM2 molecule)
1.2
.8
.4
0
.6
.6
0
2
4
0
2
4
0
2
4
X-RAY DOSE (100 rads)

Figure III-3

oxygen represents simple protection by radical scavenging, rather than conversion of DNA damage from a repairable to nonrepairable condition. The similarity of protection by oxygen and the other electron scavengers used here (NO_3^-, N_2O) support this conclusion.

In many radiation chemistry studies oxygen is present during irradiation by default; researchers simply irradiate samples open to the air. This procedure should result in the quenching of reducing radical effects, and indeed is often claimed as a method for isolating the reactions of the OH-radical (assuming the unreactivity of O_2^-). I suggest that such irradiation conditions are quantitatively imprecise, probably resulting in some intermediate level of the oxygen-protection phenomenon demonstrated here. For example, Strniste and Wallace (1975; Armel, Strniste, and Wallace, 1977) used an extract of E. coli and similar damage classifications to those used here, and arrived at conclusions about the roles of the water radicals similar to those discussed in Chapter II of this thesis. They irradiated under air (without bubbling), and in the presence of Tris-buffer and EDTA. The similarity of our conclusions probably depends on both OH-scavenging by the latter solutes and reducing radical-scavenging by oxygen. They were only able to detect significant numbers of nonalkali-labile enzyme sites when iodide was present during irradiation. In these conditions iodide protection from the OH-radical outweighs oxygen protection, thus unmasking the residual damage caused by the reducing radicals. In the absence of iodide they observed a lesion that was both alkali-labile and susceptible to their enzyme extract. No such lesion was found in this

work, and it remains to be determined whether this is due to the differences in the two enzyme preparations (both of which may contain more than one enzyme), or the irradiation conditions.

The apparent absence of any oxygen effect on OH-induced damage to DNA is consistent with the previously-mentioned result that OH-adducts of double-stranded DNA are unreactive toward oxygen (Michaels and Hunt, 1977b). Some single-stranded regions might be expected at the high radiation doses needed to kill cells, therefore it would be interesting to know if oxygen does sensitize single-stranded DNA. These experiments have not been done. It seems an interesting possibility that the reactions of the OH-radical leading to chromophore destruction which have been reported to occur only after some disruption of the double-helical structure of DNA (Ward and Kuo, 1970, discussed in Chapter II) are closely related to a fraction of OH-induced damage that is potentially sensitizable by oxygen. Until these matters are settled the possibility cannot be ignored that the experiments reported here are below a threshold dose for some important radiobiological reactions.

There is some evidence that oxygen sensitization in vivo involves one or more oxygen-dependent species that are either not produced or are apparently inert in this system. For example Misra and Fridovich (1976) proposed a combination of O_2^-, H_2O_2, and OH in bacterial killing, and van Hemmen and Meuling (1975) showed that a combination of HO_2 and H_2O_2 can inactivate phage DNA. Powers and his collaborators have suggested a rather confusing scheme for oxygen sensitization of bacterial spores involving not only the OH-radical and H_2O_2, but also the aqueous electron

and its reactions with oxygen and other electron-affinic compounds (Powers, Richmond, and Simic, 1972; Powers, Cross, and Simic, 1972; Simic and Powers, 1974; Richmond and Powers, 1974). More recently they have resolved three components of sensitization based on oxygen concentration, only the first (low concentration component) of which depends on the OH-radical (Ewing and Powers, 1976). The gas-mixing experiments of van der Schans (see Blok and Loman, 1973) similarly showed evidence of oxygen sensitization of strand breakage and lethality only at low oxygen concentrations. Subsequently van Hemmen et al. (1973) reported that neither saturating amounts of oxygen (bubbling pure gas) nor chemical radiosensitizers increase radiation lethality in dilute solutions of phage DNA. If these results are substantiated experiments such as those reported here will have to be reevaluated, and the possibility that sensitization occurs only at low oxygen concentration examined.

Any of the above complex models could explain why sensitization by oxygen, known to occur in vivo was not detected in dilute solution. The current state of knowledge about the reactions of the several oxygen-dependent species that might be involved makes it difficult to test these hypotheses. Another possible explanation first suggested by Blok and Verhey (1968) is that some component(s) of the cellular environment must be present during irradiation for oxygen to sensitize. In order to test this hypothesis the radiation chemistry of DNA in highly protected conditions, perhaps comparable to those inside cells, and the roles of water radicals and oxygen in these conditions are addressed in Chapter IV.

CHAPTER IV

DAMAGE TO DNA UNDER HIGHLY RADIOPROTECTIVE CONDITIONS

Introduction

The preceding chapters have dealt exclusively with DNA in dilute solution, unprotected from attack by water radicals. The kinds of damage produced under these conditions were found to be not unlike those observed in vivo, and some explanation, though not altogether satisfactory, of the importance of the OH-radical in lethality was offered. However, the complete absence of an oxygen effect raises serious doubts about the validity of dilute DNA solutions as a model for radiation action in vivo. In this chapter I consider two components of intracellular radiation damage that are quantitatively insignificant in dilute solutions, and thus were ignored in the previous experiments. They are (1) direct effects due to primary deposition of photon energy in DNA, and (2) indirect effects mediated by organic solutes. Both effects can be enhanced if irradiations are carried out in the presence of an excess of well-chosen radioprotective compounds. For direct effects, saturating concentrations of the inorganic radical scavengers used in Chapters II and III suffice. In order to study the more complex organic radical reactions some knowledge of the radiation chemistry of the myriad of cellular solutes is required.

Direct effects have traditionally been studied by irradiating crystals or powders of DNA constituents, or dry DNA fibers. Radicals thus produced are detected by electron spin resonance (ESR) spectroscopy,

and evidence exists for species derived from homolytic bond cleavage ($DNA - H \rightarrow \dot{D}NA + \dot{H}$), and from ionic precursors ($DNA \rightarrow \dot{D}\overset{+}{N}A + e^-$). In the dry state the diffusible radical in each instance (H and E) will be trapped by neighboring target molecules and, depending on the temperature, some rearrangement due to charge or radical migration may occur. In solution the fate of directly produced DNA radicals may be quite different. The diffusible radical may escape the macromolecules and dissipate in the medium, and conversely the solvent or other solutes may influence the sequence of reactions of the DNA radical. These processes lead to greatly shortened radical lifetimes compared to dry or low-temperature conditions, and render the system virtually inaccessible to ESR spectroscopy. The cascade of reactions leading to DNA damage by direct effects in solution has thus been greatly ignored, even though the evidence suggests this process accounts for 30-50% of the lethal effects of radiation in vivo (see Introduction to Chapter II, this thesis). A significant question, addressed in this chapter, is whether the products of the direct action of radiation on DNA differ greatly from those of indirect action.

Indirect damage to DNA by organic radicals probably leads to DNA-DNA and DNA-protein crosslinks, as well as DNA adducts with small organic molecules. While the argument that indirect effects account for most radiation damage remains valid, the possibility that some or all of these effects are mediated by organic solutes cannot be ignored. Thus energy deposited in cell water produces diffusible radicals (OH, H, and E) as previously described, which in turn react with organic molecules, forming

transient organic radicals that might damage DNA. Radiation-induced binding of amino acids and nucleic acid bases with protein, RNA, and DNA has been demonstrated in vitro, and in cells and cell lysates (Yamamoto, 1967; 1975), and found to depend principally on OH-induced reactions (Yamamoto, 1973a; 1973b). While all the nucleic acid bases were reactive (pyrimidines more than purines), only the aromatic and sulfur-containing amino acids gave significant binding. Yamamoto proposed that resonance-stabilized aromatic radicals, produced by abstraction of a ring hydrogen atom were suitable intermediates in the binding reaction. Byfield, Lee, and Bennet (1970) demonstrated that radiation-induced binding of small molecules to DNA in solution was very nonspecific (they showed data for leucine and phenylalanine).

de Jong, Loman, and Blok (1972a) irradiated biologically active phage DNA in the presence of radioprotective phenylalanine, and found residual inactivation under conditions where a negligible fraction of water radicals react with the DNA. This inactivation was apparently due to reactions of phenylalanine radicals leading to nonbreak nucleotide damage. Subsequently they demonstrated that a majority of the phenylalanine mediated damage formed in anoxia was subject to uvr-dependent repair in E. coli (de Jong, Loman, Blok, 1972b). No such repair was in evidence after oxygenated irradiation, and the DNA was consequently more sensitive. Recently van der Schans, van Rijn and Bleichrodt (1976) quantitated the radiation-induced binding of phenylalanine and inactivation of PM2 DNA, and estimated that there were 25 phenylanine molecules bound per lethal hit in double-stranded DNA and about 5 for

single-stranded DNA, indicating a very efficient repair of these lesions. They further attributed the residual strand breakage, and thereby virtually all the damage in the presence of phenylalanine to the secondary amino acid radicals. Clearly "radioprotection" with phenylalanine represents a summation of actual protection from water radicals and residual phenylalanine-mediated damage.

Similar evidence exists for biologically significant damage by diffusible nucleotide radicals. DNA from the bacteriophage ØX174 was found to be protected by oxygen when irradiated in phosphate buffer, but in the presence of certain nucleotides, themselves highly radioprotective, oxygen sensitization was dramatic (Blok and Varkey, 1968). Of the four deoxynucleotides only the pyrimidines demonstrated this "potentiation" of the oxygen effect, the purines being equally radioprotective in the presence and absence of oxygen. They suggested that peroxy-radicals of the pyrimidines were the likely damaging species. A series of papers from the laboratory of van Hemmen supports this hypothesis, and provides encouraging evidence that similar reactions are important in oxygen sensitization in vivo. They showed (van Hemmen et al., 1973) that sensitization of bacteriophage T1 by oxygen and other radiosensitizers did not occur in the dry state (direct effects), or if the sensitizer was added within 1.5 msec after irradiation, both conditions designed to eliminate possible masking of the oxygen effect by trivial oxygen scavenging of reducing radicals. They subsequently found that the low molecular weight fractions of bacterial and human cells contained compounds capable of potentiating the oxygen effect, and demonstrated

that protection from water radicals (cell lysates are highly radioprotective) was not sufficient for this effect (van Hemmen, 1974a). Sulfhydryl compounds, certainly present in these extracts, are highly radioprotective (probably by reductive restitution of DNA radicals), and sensitization by oxygen does occur in their presence (e.g., Howard-Flanders, 1960). That sulfhydryl compounds were not responsible (or at most to a minor extent) for oxygen sensitization in cell lysates was convincingly demonstrated by the high resistance to radiation and N-ethylmaleimide of the potentiating substance, and the presence of an oxygen effect in lysates of a mutant of E. coli with extremely low levels of endogenous sulfhydryls (van Hemmen et al., 1974c). Although no further purification of the component(s) of lysates important for sensitization has been reported, it can be assumed that pyrimidine nucleotides account for at least part of the effect.

It is the purpose of this chapter to examine the kind and quantity of damage to DNA produced by radiation in the presence of several organic compounds suspect of radiochemical reactivity. In each case the role of the water radicals, and thereby of the organic radical-adducts is examined, and the effect of oxygen is determined. I report here results with phenylalanine, thymidine, and adenosine. In addition the kind of damage which might be attributed to direct effects in solution, and the contribution of such damage in the above highly protected conditions is estimated.

Materials and Methods

The preparation of PM2 DNA, irradiation procedures, and assay for strand breaks, alkali-labile bonds and γ-endonuclease sites were as described in Chapter II. All DNA samples were in 0.01 M phosphate buffer, pH 7, and were bubbled with either N_2 or O_2 30 min before and during irradiation.

All chemicals were reagent grade. dl-Phenylalanine (Merck), thymidine and deoxyadenosine (Sigma) were used as obtained from the supplier.

Results

Direct effects in solution. In order to observe the direct action of radiation on DNA in solution it is necessary that the indirect effects be almost completely removed. As described in previous chapters this can probably be accomplished by quenching the water radicals with a combination of scavengers such as iodide (H and OH) and nitrate (H and E). When radiation doses high enough to give significant amounts of direct effects are employed the stipulation that the products of scavenging reactions (e.g., iodine atoms) be unreactive with DNA becomes more stringent. A valid test of this unreactivity is whether protection of DNA to a level at or near that expected from direct effects can be achieved. The latter can easily be calculated if one knows the molecular weight of the target (6.4×10^6 daltons for PM2) and assumes a radiochemical yield for target radicals ($G \simeq 2$, or 50 eV per ionization for DNA; Ward, 1975). The following useful equation (see

Dertinger and Jung, 1970, p. 56) relates the D_{37} (dose required to give

$$D_{37} = \frac{1}{M}\left(\frac{9.7 \times 10^{11}}{G}\right) \text{ rads}$$

an average of one radical per target molecule) to these quantities, and upon substitution of the above values predicts a D_{37} of 76 krads for double-stranded PM2 DNA. In comparison, the D_{37} for molecular lesions in dilute unprotected solution (Chapter II) was 25 rads, or 3000-fold lower. There are many reports in the literature describing "fully protective conditions" that fall well short of these requirements.

In reality, even the highest concentrations of scavengers attainable can only reduce the indirect effects to a level comparable to the direct effects, and thus we must be satisfied to examine situations where the direct action is greatly enhanced but still only accounts for part of the damage. Such a condition is described in Figure IV-1. Here DNA (10 μg/ml) is protected by iodide (0.1 M) and either nitrate (0.1 M) or oxygen, both conditions where the direct effects are predicted from scavenger-DNA competition kinetics to contribute significantly to the total damage. For 1 krad of radiation, and a G-value of 2 for primary DNA-radicals, the DNA radicals resulting from direct action in Figure IV-1 (considering a liter of solution) are given by:

Figure IV-1. Radiation induction of strand breaks (●,○), alkali-labile bonds (▲,△), and enzyme sites (■,□) under highly protected conditions. DNA (10 μg/ml) was irradiated in 0.01 M Na-phosphate buffer (pH 7), 0.1 M KI plus either oxygen (continuous bubbling, solid symbols) or 0.1 M KNO_3 in anoxia (open symbols). Enzyme sites in 0.1 M KI plus oxygen, assayed on neutral sucrose gradients are also shown (X).

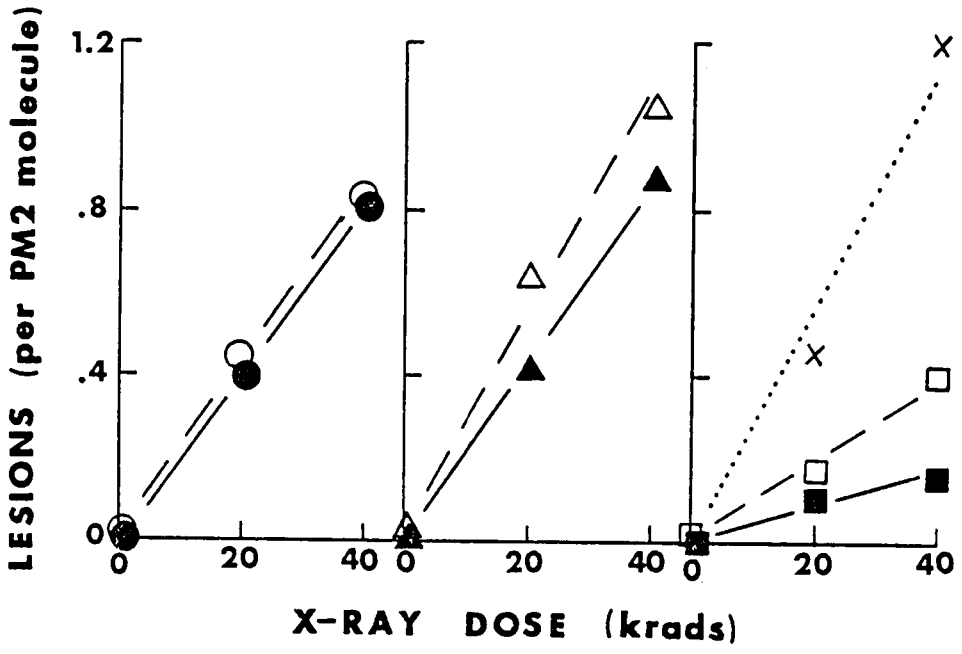

LESIONS (per PM2 molecule)
1.2
.8
.4
0
0
20
40
0
20
40
0
20
40
X-RAY DOSE (krads)

Figure IV-1

Direct DNA radicals (per liter) = (fraction of energy deposited in DNA) × ($G \times 10^{-6}$ moles)*

$= (10\ \mu g/g) \times (2 \times 10^{-6}\ \text{moles})$

$= 2 \times 10^{-11}$ moles.

The residual reaction of DNA with OH-radicals (using rate constants from Table II-2, page 29, and taking a G-value of 2.7 for OH-radicals) yields the following:

OH-induced DNA radicals (per liter) = (fraction of OH reacting with DNA) × ($G_{OH} \times 10^{-6}$ moles)

$$= \frac{(3\times10^{-5})(4\times10^{8})}{(3\times10^{-5})(4\times10^{8}) + (10^{-1}\times1.6\times10^{10})} \times 2.7 \times 10^{-6}\ \text{moles}$$

$= 2 \times 10^{-11}$ moles.

That is, there should be about equal numbers of direct effects, and OH-induced effects in the DNA. Similar calculations predict that in nitrate there should be about 10^{-11} moles of E-induced and 3×10^{-10} moles of H-induced DNA radicals, while in oxygen the values are 5×10^{-10} moles and 7×10^{-11} moles respectively. Thus while OH should contribute a constant background of indirect effects, H-reactions should be a particular problem in nitrate, and both reducing radicals should dominate the direct effects in oxygen. The dose-response curves of

---

*There are 10^{-6} moles radicals/krad for G = 1.

Figure IV-1 indicate that the expected background of indirect effects is not as great a problem as anticipated. The D_{37} for total molecular lesions is about 20 krads in iodide plus oxygen, and only slightly less in iodide plus nitrate, consistent with a contribution of about 25% of the damage from direct effects. While such an enrichment (from 1/3000 to 25%) is not totally satisfactory it might be enough to observe qualitatively different kinds of damage, or effects of oxygen in direct action of radiation.

Since there are not great differences between nitrate and oxygen in this system, it seems possible that even for directly produced damage, oxygen does not influence the quantities of lesions in the classes measured here. The prevention by oxygen of some enzyme site production may be significant but cannot be confidently assessed based on these data.

Qualitatively the lesions produced here show some similarities, and one major difference from those produced in unprotected solution. All three kinds of lesions are evident as soon after irradiation as they could be measured, and appear stable to postirradiation incubation (24 hours at room temperature). However, the alkali-labile bonds are apparently of a different nature than those characterized in Chapter II, and subsequently quantitated under conditions of strictly indirect radiation action. This conclusion is based on their unique susceptibility to cleavage by the enzyme extract used here. Figure IV-1 demonstrates that after irradiation in the presence of oxygen and iodide, treatment of the DNA with the enzyme preparation breaks only a few sites when

assayed on an alkaline sucrose gradient, but a much larger number of enzyme sites when assayed on a neutral sucrose gradient. Furthermore, the number of enzyme sites in the neutral assay (about 1.1 per PM2 molecules at 40 krad) appears to be the sum of the alkali-labile bonds and alkali-stable enzyme sites (0.9 and 0.2, respectively, at the same dose), indicating that all of the alkali-labile bonds produced under these irradiation conditions are also enzyme-susceptible. Figure IV-2 further demonstrates this point. Treatment of DNA with the enzyme extract for 30 minutes induces some strand breakage even in unirradiated DNA due to damage incurred from decay of incorporated ^{3}H-thymidine. These enzyme sites are not alkali-labile, as evidenced by their persistence through assay on alkaline sucrose gradients. DNA X-irradiated in oxygen plus iodide shows no additional enzyme sites in alkaline assay, but a significant number of sites in neutral assay. Similar results were obtained after irradiation in anoxia with iodide plus either nitrate or nitrous oxide, indicating that oxygen is not required for production of these lesions.

Irradiation is known to cause release of intact and damaged bases from DNA, leading in some cases to apurinic or apyrimidine sites (collectively AP-sites) with an intact phosphodiester backbone (Ullrich and Hagen, 1971). Such AP-sites are expected to be alkali-labile and are susceptible to endonucleases present in all species yet tested. Preliminary results indicated that DNA heated at pH 5 to produce apurinic sites is a substrate for the enzyme extract used here. If the alkali-labile bonds unique to the above highly-protected conditions are indeed

Figure IV-2. Enzyme action on DNA (10 μg/ml) irradiated in oxygenated (continuous bubbling) 0.01 M Na-phosphate buffer (pH 7), plus 0.1 M KI. Assays were performed on alkaline (Δ,▲) or neutral (○,●) sucrose gradients. DNA received 20 krad (solid symbols) or sham irradiation (open symbols).

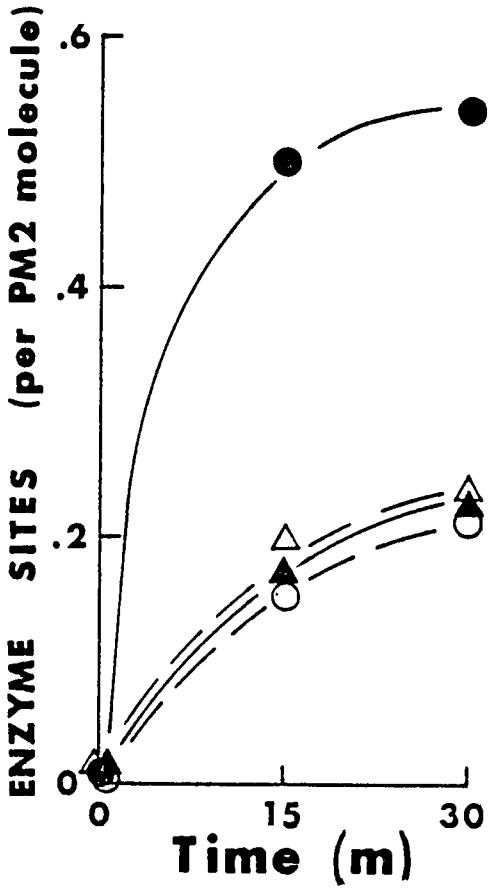


Figure IV-2

AP-sites, it must be concluded that indirect effects (e.g., OH-radicals) cause few intact AP-sites, but many alkali-labile bonds of another type not susceptible to the M. luteus extract. Wallace, Armel and Katcher (1978) recently reported evidence to support this possibility. They irradiated DNA in conditions very similar to those of Figure IV-1 (page 83) and found two kinds of alkali-labile bonds: (1) a class converted rapidly to strand breaks by alkali, and (2) a slow-converting class [$t_{1/2}$ = 4 h in alkali, similar to the kinetics of AP-site hydrolysis (Lindahl and Andersson, 1972)]. The second class was produced only in the presence of iodide (under air), and was cleaved by their enzyme preparation (from E. coli), in further agreement with the results presented here.

It is tempting to speculate that these enzyme-susceptible alkali-labile bonds are the result of direct effects (their D_{37} in Figure IV-1 is 50 krads), although secondary damage due to the products of scavenger reactions or indirect effects not significant in unprotected conditions (e.g., H-reactions) cannot be eliminated. Substitution of an OH scavenger other than iodide in the above experiments would help to resolve this question, but has not been done. In subsequent sections protection with organic scavengers does not attain the level described above, and the putative AP-sites are not observed.

Damage Mediated by Organic Radicals

Phenylalanine. Almost any compound added to a dilute solution of DNA will lead to some protection from damage by water radicals. If, as

in the previous section, the product of the scavenge reaction is unreactive with the DNA the observed protection will be precisely that calculated from competition kinetics. In cases where some DNA damage is induced by the secondary radical the observed protection will be less than the calculated value, the difference depending directly on the amount of secondary damage. The latter situation probably holds for most organic compounds, particularly the highly radiochemically active substances considered in this section. Table IV-1 demonstrates that this is certainly the case with phenylalanine. Under these irradiation conditions (DNA at 500 μg/ml) it was shown in Chapter II that the OH-radical is the principal damaging species. In the presence of 0.01 M phenylalanine it can be calculated that 99% of the OH-radicals react with the amino acid, predicting about a 100-fold protection from molecular damage. As can be seen in the table, the observed protection is less than half this value, indicating that the majority of the residual damage is caused by secondary phenylalanine radicals. The possibility that the aqueous electron, which reacts much more slowly with phenylalanine than does OH accounts for the excess damage in the presence of the amino acid is eliminated in Figure IV-3. In this experiment nitrate and iodide were added at concentrations sufficient to compete with phenylalanine for the water radicals. Nitrate (specific for the electron) has very little effect in this condition, while iodide (specific for the OH-radical) reduces strand breaks and alkali-labile bonds significantly. It is interesting that the effects of the inorganic scavengers in the presence of phenylalanine are not greatly

TABLE IV-1

RADIOPROTECTION BY PHENYLALANINE

	D_{37} (rads)[a]	
	0.01 Phosphate Buffer (pH 7) 0.1 M KCl	0.01 M Phosphate Buffer (pH 7) 0.1 M KCl 0.01 M Phenylalanine
Strand Breaks	380	12000 (32)[b]
Alkali-Labile Bonds	370	15000 (41)
Enzyme Sites	820	17000 (21)

All irradiations were anoxic; DNA concentration 500 µg/ml.

[a]Calculated from dose response curve.

[b]Dose-modification factor for phenylalanine protection.

Figure IV-3. Radiation induction in the presence of phenylalanine of strand breaks (circles), alkali-labile bonds (triangles), and enzyme sites (squares). DNA (500 g/ml) was irradiated in anoxic (N_2 bubbling) 0.01 M Na·phosphate buffer (pH 7) 0.01 M phenylalanine, plus either 0.1 M KCl (solid symbols), 0.1 M KI (half-filled symbols), or 0.1 M KNO_3 (open symbols).

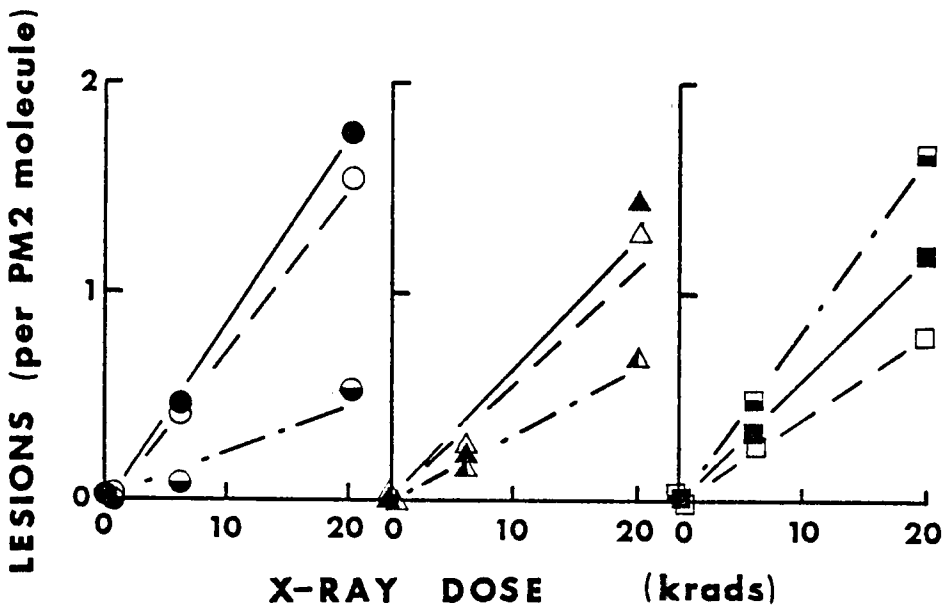

LESIONS (per PM2 molecule)
2
1
0
0
10
20
0
10
20
0
10
20
X-RAY DOSE (krads)

Figure IV-3

different than in its absence (Chapter II). Apparently the phe-OH adduct (or OH-induced phenyl radical) is the principal damaging species in anoxic irradiation.

de Jong, Loman and Blok (1972a) found that oxygen sensitized the DNA from phage ØX174 toward radiation inactivation if phenylalanine was present during irradiation, and proposed that a peroxy radical adduct (phe-OH-$O_2^{\cdot}$) was responsible for the inactivation. The effect of oxygen on molecular damage in the presence of phenylalanine is demonstrated in Figure IV-4. There is no evidence that oxygen increases the yield of any of the forms of molecular damage assayed here, nor under any condition of selective scavenging. Partial prevention of enzyme sites, again much as in the absence of phenylalanine, appears to be the only oxygen effect. Close examination of the phage DNA inactivation data, particularly the dependence on amino acid concentration (Figure 1 in de Jong, Loman, Blok, 1972a) reveals a complicated situation. Oxygen sensitization was observed only at intermediate phenylalanine concentrations, oxygen protection was found at extremely high or low concentrations. In order to test the possibility that the conditions of Figure IV-4 lay outside the sensitizing range, DNA at 10 µg/ml was irradiated in 0.01 M phenylalanine, to give a phe/DNA ratio of 300, well within the range calculated from their data. Under these conditions 99.98% of the OH-radicals react with the phenylalanine, and from the D_{37} it can be estimated that more than 90% of the damage is due to the secondary radicals. Still there was no significant oxygen effect.

Figure IV-4. Effect of oxygen on radiation induction in the presence of phenylalanine of strand breaks (circles), alkali-labile bonds (triangles), and enzyme sites (squares). DNA (500 μg/ml) was irradiated in anoxic (solid symbols) or oxygenated (O_2 bubbling, open symbols) 0.01 M Na·phosphate buffer (pH 7), 0.01 M phenylalanine plus either (a) 0.1 M KCl, (b) 0.1 M KI, or (c) 0.1 M KNO_3.

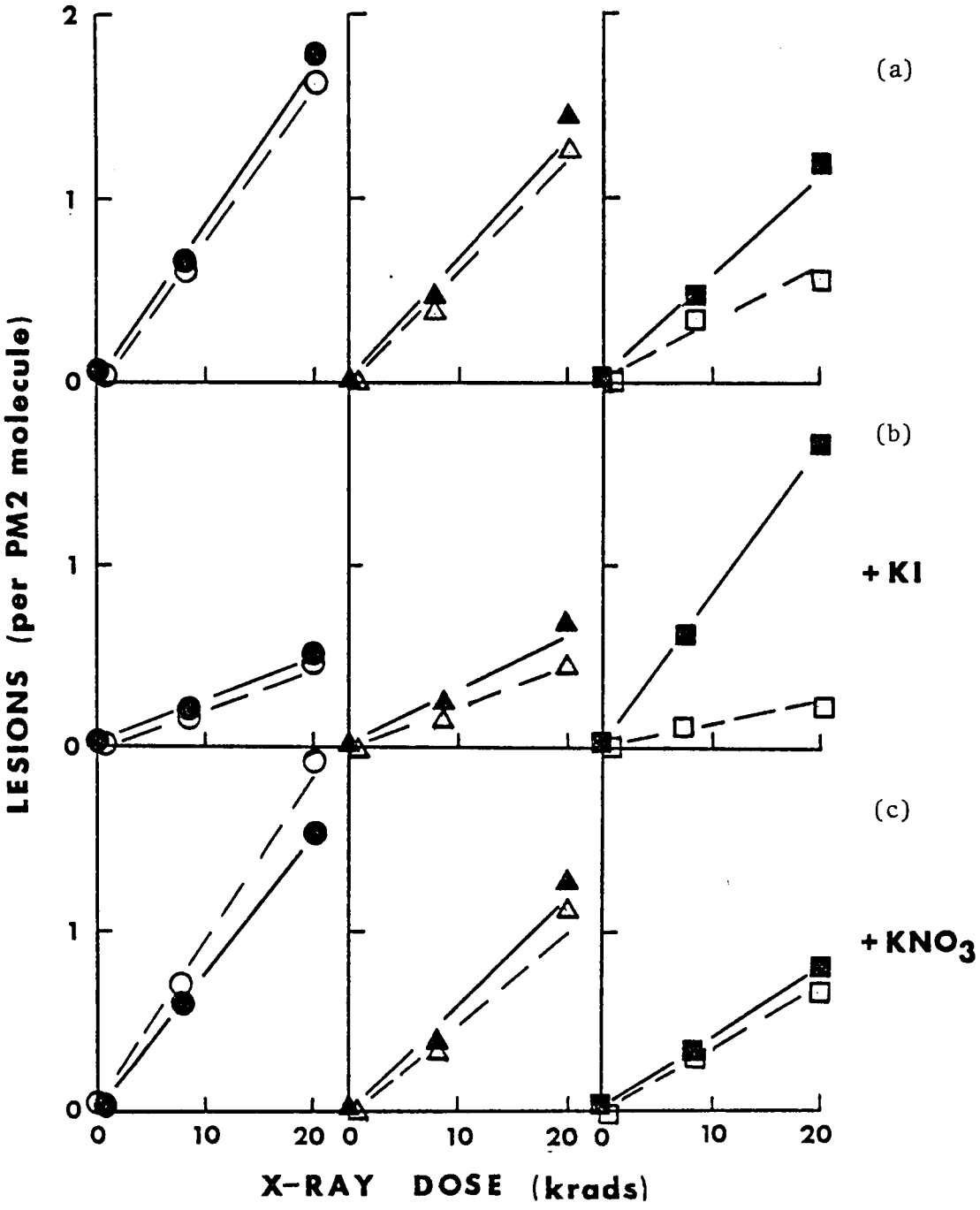

LESIONS (per PM2 molecule)
2
1
0
(a)
(b)
+ KI
(c)
+ KNO3
0
10
20
X-RAY DOSE (krads)

Figure IV-4

The failure to demonstrate an oxygen effect in phenylalanine remains unexplained. It should be kept in mind that the inactivation data are not necessarily comparable to the physical damage data presented here. In subsequent work de Jong, Loman, and Blok (1972b) reported that the repairability of phenylalanine-induced damage is an important factor in determining survival; the effect of oxygen resulted in part from decreased repair. The decrease in enzyme sites for irradiation in oxygen (Figure IV-4) might be an indication of this decreased repairability. The uvr-dependence of the repair observed in vivo, perhaps indicating excision of phenylalanine-DNA adducts, suggested the involvement of a uv-endonuclease rather than the γ-endonuclease used here. Preliminary results with the uv-endonuclease from M. luteus indicated that DNA irradiated in the presence of phenylalanine may contain a few lesions susceptible to this enzyme, but further experiments are needed. It remains possible that nonbreak nucleotide damage (according to de Jong, Loman, and Block, 1972a, the major lesion in phenylalanine) is produced here, but not recognized by either enzyme tested.

Thymidine and adenosine. When DNA was irradiated under anoxic conditions in the presence of thymidine a spectrum of lesions are produced as shown in Figure IV-5. Under the irradiation conditions of this experiment essentially all of the water radicals (99.98% of OH, 99.9% of H, and 99.998% of E) react with thymidine, predicting about a 5000-fold protection of the DNA. The observed protection is much less (80-200-fold, data not shown), indicating as with phenylalanine, that

Figure IV-5. Radiation induction in the presence of thymidine of strand breaks (circles), alkali-labile bonds (triangles), and enzyme sites (squares). DNA (10 μg/ml) was irradiated in anoxic (N_2 bubbling) 0.01 M Na·phosphate buffer (pH 7), 0.01 M thymidine, plus either (a) 0.1 M KCl, (b) 0.1 M KI, or (c) 0.1 M KNO_3.

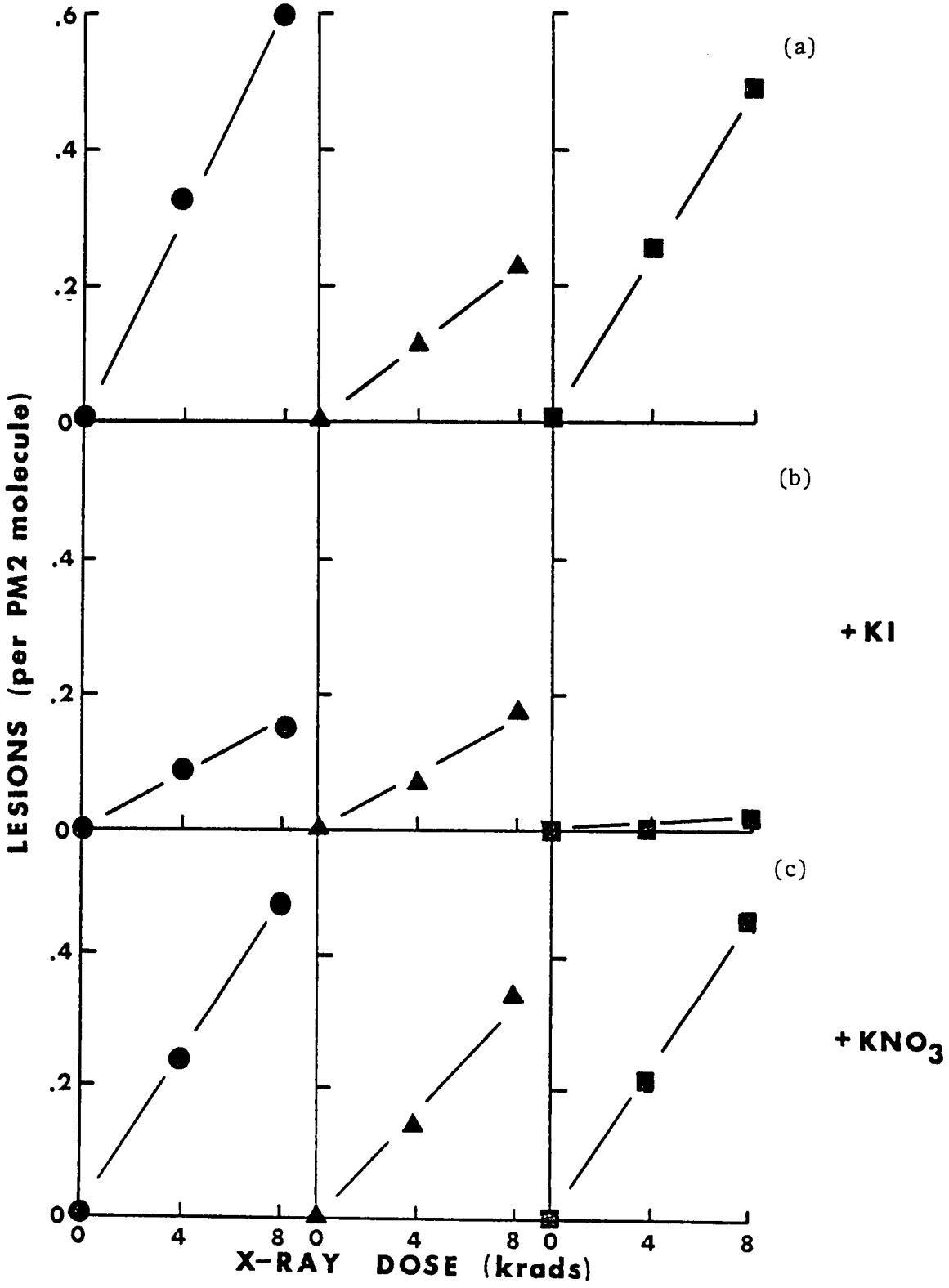

(a)
(b)
(c)
+KI
+KNO3
LESIONS (per PM2 molecule)
.6
.4
.2
0
0
4
8
X-RAY DOSE (krads)

Figure IV-5

most of the damage is mediated by the secondary thymidine radical. Addition of selective scavengers at sufficient concentrations to quench the water radicals before they react with the free nucleoside demonstrates which of the thymidine adducts is responsible for the damage (Figure IV-5). Nitrate, which should prevent the formation of the radical anion ($dT^{\cdot -}$) has very little effect, while iodide eliminates a significant fraction of the damage. Most of the strand breaks, and a few alkali-labile bonds, and essentially all of the enzyme sites are thus prevented. This last result is unique among the conditions tested in the present work, in that it implicates the OH-radical exclusively (apparently via the dT-OH adduct) in the production of base damage in DNA. A significant fraction of the residual, iodide-insensitive damage may be due to direct effects, raising the possibility that the aqueous electron (via the $dT^{\cdot -}$ anion radical) are not damaging to DNA in anoxia.

When oxygen is admitted during irradiation under the above conditions a dramatic effect is observed as shown in Figure IV-6. Oxygen enhances all three forms of damage when DNA is irradiated in phosphate buffer with thymidine. The level of sensitization (OER = 2.8 for strand breaks, 7.5 for alkali-labile bonds, and 6.0 for enzyme sites) is similar to that found for inactivation of ØX174 DNA under similar conditions (OER = 8, Blok and Verhey, 1968), and sufficient to account for sensitization commonly observed in vivo (OER $\simeq$ 3 for strand breaks and lethality). It is somewhat surprising that the addition of either KCl, KI, or KNO_3 (Table IV-2) completely eliminates this oxygen effect. While iodide conceivably removes the sensitizable precursor (OH-induced), neither of

Figure IV-6. Effect of oxygen on radiation induction in the presence of thymidine of strand breaks (circles), alkali-labile bonds (triangles), and enzyme sites (squares). DNA (10 µg/ml) was irradiated in anoxic (solid symbols) or oxygenated (O_2 bubbling, open symbols), 0.01 M Na·phosphate buffer (pH 7), 0.01 M thymidine.

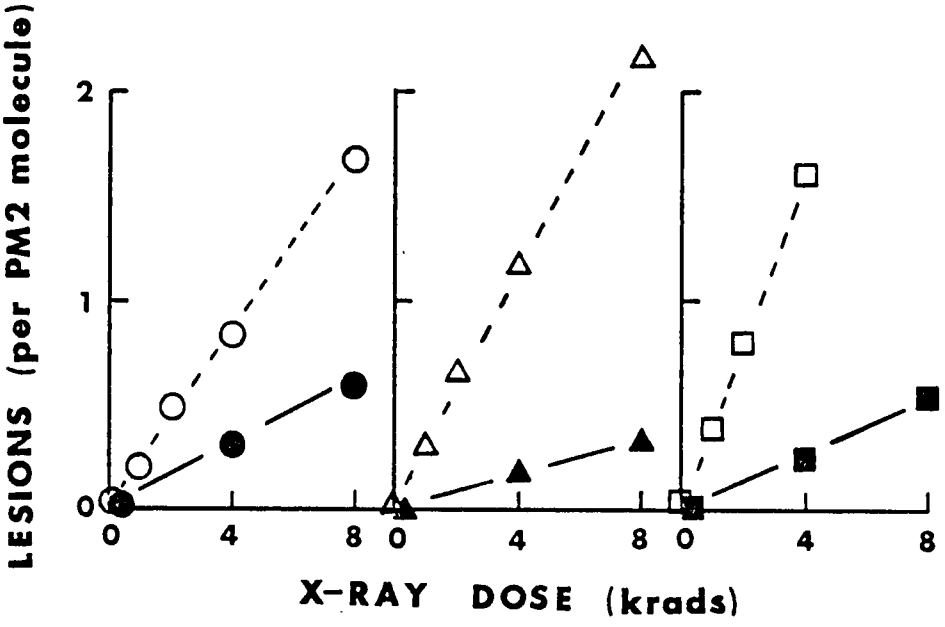


Figure IV-6

TABLE IV-2

EFFECT OF RADICAL SCAVENGERS ON THE OXYGEN EFFECT IN THYMIDINE

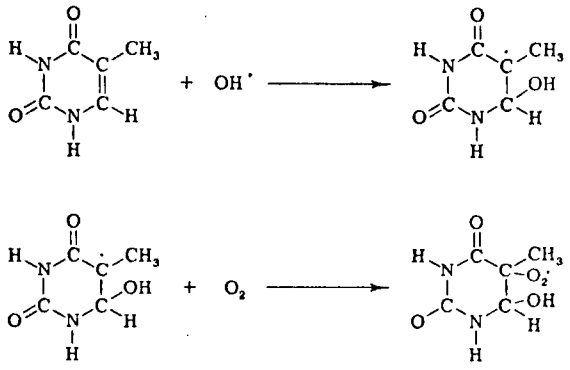
Additional Solutes[a]	Strand Breaks	Alkali-Labile Bonds	Enzyme Sites
None	2.8[b]	7.5	6.0
KI	1.0	1.2	1.0
KNO_3	1.4	0.9	0.7
KCl	1.1	1.3	1.2

[a]All irradiations at a DNA concentration of 10 μg/ml in 0.01 M Na·phosphate (pH 7), 0.01 M thymidine. All additional solutes at 0.1 M.

[b]OER calculated from dose response curves as in Figure IV-6.

the other solutes had significant effect in anoxia. Further experiments indicated that while NaCl behaved exactly as KCl, $NaClO_4$ had almost no effect (all salts at equal 0.1 M concentration), pointing to chloride as the common reactive species, and eliminating effects of ionic strength. Nitrous oxide behaved as nitrate, suggesting that scavenging of the aqueous electron was the mechanism for protection in both instances.

Considerable radiation chemistry is known about thymine in aqueous solution, and specifically about the thymine-, and thymidine-OH adducts (for review see Ward, 1975). While there is some indication of significant radiochemical differences between the free base and the nucleoside (see Discussion), the initial attack of the OH-radical, and the reaction with oxygen are apparently similar and proceed by the following mechanism:



If a peroxy-thymidine radical (e.g., the final product of the above sequence) is the damaging agent in the conditions of Figure IV-6, it may be that chloride or any of the compounds that prevent the oxygen effect influence reactions subsequent to those written above. Sodium chloride

is usually considered radiochemically inert, but has been reported to influence the decay kinetics of DNA peroxides (Schweibert and Daniels, 1971), and to protect double-stranded DNA from radiation-induced chromophore destruction (Ward and Kuo, 1970). It has also been suggested that iodide will reduce the peroxy-thymidine radical (see Simic, 1973). If this occurs it would add a second level of radioprotection by iodide in oxygenated conditions, but otherwise would not confuse its primary action here as an OH scavenger.

In the conditions of the above experiments the ratio [dT]/[DNA] was 330. Under less protected conditions at the same DNA concentration (10^{-4} M dT, [dT]/[DNA] = 3.3) oxygen sensitization was not observed. This might indicate that competition between several species for primary radicals is crucial for the oxygen effect. In the former condition (10^{-2} M dT) all the water radicals react with thymidine, then the adduct radicals react with oxygen (about 1 mM) or DNA. In the latter condition (10^{-4} M dT) most of the electrons react directly with oxygen producing O_2^-, which has been shown to reduce peroxy-thymidine radicals ($ROO^{\cdot} + O_2^{\cdot -} \xrightarrow{H^+} ROOH$, Scholes and Weiss, 1959). Unfortunately knowledge of possible reaction sequences, and rate constants to assess such competition is currently minimal.

If a significant buildup of thymidine peroxides occurs during irradiation some postirradiation damage to DNA by their decay might be expected. In some of the above experiments, particularly when KCl prevented the oxygen effect, there was some indication that this was the case. More work in these areas is needed.

Much less is known of the potential reactions of DNA damage mediated by purine radicals, but some similarities might be expected. Purine nucleotides demonstrated radioprotection equal to that of pyrimidines in ØX174 inactivation (Blok and Verhey, 1968), as would be expected from their similar reactivity with water radicals. However purines were unable to potentiate the oxygen effect in this system (OER = 1.2 for deoxyadenylate, < 1 for deoxyguanylate). When 0.01 M adenosine was tested in the molecular damage assay used here the radioprotection was verified, and a reduced oxygen effect (compared to thymidine) was observed. The OER for the three classes of damage ranged from 1.7 for strand breaks to 1.3 for enzyme sites, and again was prevented by chloride.

The results reported here with both nucleosides are similar to those reported for phage inactivation, indicating that these systems are probably assaying the same biologically important damage.

Discussion

It is clear from these results that under highly protected conditions DNA damage is not only quantitatively but in some cases qualitatively different from that caused by water radicals. This is evident in the data with both inorganic and organic protectors.

That direct effects are important in vivo was concluded from the inability to protect against a fraction of lethality and strand breakage with radical scavengers. The model system described here reveals a similarly unprotectable fraction of damage, principally strand breaks and a unique kind of alkali-labile bonds. Richmond and Zimbrick

(1975) reported the radiation-induced release of significant quantities of undamaged DNA fragments in vivo, including thymidine and the free base thymine (theoretical co-products with strand breaks and apyrimidinic sites, respectively). They considered that the radiochemical yields were somewhat high for strictly direct action, but by comparing N_2 and N_2O elimiated the OH-radical as the causative agent. Further work is indicated to determine the relationship between these kinds of damage and that measured here, and if either are in fact produced directly.

For strictly quantitative reasons direct effects can be concluded to play a minor role in the experiments with organic scavengers. For several reasons these conditions appear to be more analogous to those in vivo. With both phenylalanine and thymidine a large fraction of the damage was preventable by scavengers, and thus indirect as in vivo. Only thymidine met the two other criteria for in vivo-like conditions considered here: (1) dominance of OH-mediated reactions (at least in anoxia), and (2) sensitization by oxygen. Phenylalanine, even at an equivalent level of protection, failed to alter the unprotected condition with respect to either criterion. Since, as in vivo, the damage sequence in the presence of these organic compounds begins by reactions with water radicals, the rate constants of the two solutes considered here should be examined (see Table II-2, page 29). The compounds react comparably with both OH and H, but thymidine reacts about 100-fold faster than phenylalanine with the aqueous electron. This difference, together with the observations that quenching the electron with nitrate prevents the oxygen effect strongly implicate the electron (or $dT^{\cdot-}$) in sensitization.

A possible mediator, as suggested by Loman and Ebert (1970), is the ($dT\text{-}O_2^{\cdot-}$) oxidation intermediate.

It is impossible from the present data to propose a detailed mechanism for DNA damage in oxygenated, thymidine-containing solution, but peroxy radicals or peroxides are almost certainly involved. It is interesting that in the phage inactivation system both thymidine and thymidylate potentiated oxygen sensitization, but thymine did not (Blok, 1967), while in a chromophore destruction assay solutions of both thymidine and thymidylate were protected by oxygen, but thymine was sensitized (Hoard, Hayes, and Goad, 1974). Ward (1972) has reported that a major volatile product of oxygenated irradiation of thymine compounds is unique to those substituted in the 1-nitrogen position. Obviously the radiation chemistry of thymine derivatives, and the modes of decay of their peroxy radicals needs to be determined. Teoule and Cadet (1975) have isolated and characterized 20 radiation-induced peroxides of thymine and thymidine, indicating the problem is not a simple one.

A proposed model of radiation damage in thymidine-protected, anoxic conditions is shown in Figure IV-7. The general unimportance of the aqueous electron, particularly in the production of enzyme sites is the major difference between this model and the one proposed for unprotected conditions (Chapter II). From the data presented here it appears that phenylalanine-mediated damage does not fit this scheme; other purine and pyrimidine nucleosides might. The effect of oxygen, at least in the presence of thymidine, might be visualized as either an enhancement of

Figure IV-7. Proposed model for the contribution of thymidine radicals to DNA damage in anoxic, thymidine-protected solution.

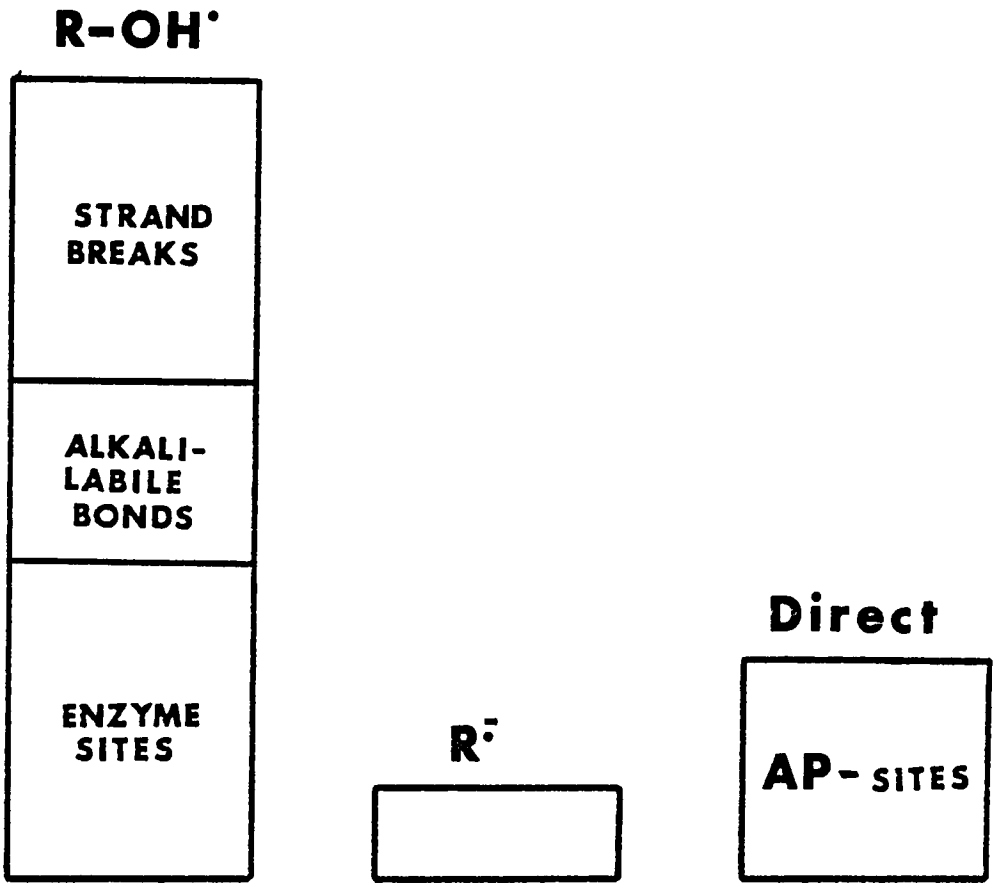

R-OH·
STRAND BREAKS
ALKALI-LABILE BONDS
ENZYME SITES
R·
Direct
AP-SITES

Figure IV-7

the R-OH-mediated damage, or to accommodate the current data, a potentiation of electron-initiated ($R^{\cdot}$) damage.

It is difficult to guess to what extent the radiation conditions inside a cell are approximated by these experiments. Local concentrations of organic solutes around the DNA are unknown, and the competition of alternate reaction sequences, found difficult in this work, seems prohibitive. Sulfhydryl compounds, present in cells but ignored in this work, might influence the fate of the organic radical adduct, or of subsequent transients to DNA damage. While the highly protected conditions studied in this chapter undoubtedly present a better model of cellular radiation chemistry, than unprotected solutions, they are at once the simplest protective conditions conceivable, and nearly unmanageable.

Finally, in attempting to explain radiobiological phenomena through assays of physical and chemical alterations as described here, it should be remembered that a significant fraction of DNA damage may go undetected by these methods. From the current literature and this thesis it is clear that differences exist in the substrate specificity of the E. coli extract of Wallace and co-workers (it attacks both fast- and slow-converting alkali-labile bonds, Strniste and Wallace, 1975; Wallace et al., 1978), and the M. luteus preparation of Setlow and Carrier (1973). Schon-Bopp, Schafer and Hagen (1977) recently reported five separate peaks of γ-endonuclease activity from M. luteus. If these results are substantiated, and these enzymes can be purified and characterized the techniques of this thesis could be used to obtain a more complete and detailed account of the broad spectrum of radiation damage to DNA.

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BIBLIOGRAPHY

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