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"Mechanisms for Radiation Damage in DNA"

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I. Main Research Accomplishments

Overview: Over the last 8 years we have made significant advances impacting our understanding of radiation damage to DNA resulting in about 40 publications in the scientific literature, including three reviews which cover much of our work using electron spin resonance, product analyses and ab initio molecular orbital theory (See Bibliography).

The principal objective of our work is the elucidation of the fundamental mechanisms of radiation damage to DNA through the direct and indirect effects. We believe our work has been especially fruitful in this regard during the past four years. While some initial work on indirect attack of peroxy radicals and protection by thiols were investigated, in the last four years we have concentrated on the direct effect of radiation damage on DNA and DNA-peptide complexes. Our objective is to elucidate the ultimate radiation chemical damage to DNA arising from the direct effect. In this effort we have focused on the application of three techniques. ESR (electron spin resonance) spectroscopic measurement of initial radicals formed in DNA and its hydration layer at low temperatures. This technique gives an experimental measure of the initial events of radiation. Ab initio molecular orbital calculations are employed to give highly accurate theoretical predictions of early events such as electron and hole localization sites which serve to test and to clarify the experimental observations. HPLC and GC-mass spectroscopic assays (in joint work with Steven Swarts and Ken Wheeler) of DNA base products formation provide the ultimate chemical outcome of the initial radiation events. The bridge between the early ion radical species (seen by ESR) and the non radical products (observed in product analysis by GC mass spec) is made in ESR studies which follow the chemistry of the early species as they react with water and or other DNA bases. The use of these techniques we believe, has in the past three years resulted in a new and fundamental understanding of the radiation damage to DNA on a molecular scale. From this work, a working model for DNA damage from the initial ionization event to the eventual formation of molecular base damage products and strand breaks has been formulated. We believe that this model provides a focus for our and others future work. Below we describe our results over the past several years which have led to the formulation of this model.

Approximately 50% of the water in the nucleus is hydration water, i.e.,

water that is associated with biomolecules in the first few solvation layers. One of the major uncertainties about radiation damage to DNA in the cellular environment is whether this water behaves as bulk water or as an integral part of the DNA. Early workers suggested DNA hydration water actually transfer ionizations as electrons and hole directly into DNA. In our recent work we have thoroughly investigated the effect of DNA hydration water on yields of ion radicals and transfer of charge into the DNA biomolecule (publ. 26, 27, 36, 41). A recent breakthrough is the finding that the hydroxyl radical can be found in the hydration layer at low levels (36). It could not be detected previously at low temperatures due to the fact that it produces a different ESR spectrum in the glassy hydration layer than in bulk ice. This discovery will allow the mapping of the hole transfer-hydroxyl radical yields as a function of the number of water molecules/nucleotide. This work will clarify the dividing line between the direct and indirect effects of radiation.

Up to our recent work it had been difficult to distinguish the cytosine and thymine anions in DNA since both were doublet spectra of about the same coupling (22). However, we have found that in D_2O the resolution of the spectra improves so that they can be easily distinguished. In addition we found that the cation species, $G^{+\bullet}$ and $A^{+\bullet}$, could also be distinguished. In our work on dsDNA we showed through an extensive computer based analysis that the cytosine radical anion ($C^{-\bullet}$) and guanine radical cation ($G^{+\bullet}$) were the dominant ionic species at low temperatures.

We have also found that the radical ion distribution is a function of strandedness of the DNA. In single strand DNA we find that there is an increase in the contributions of thymine anion and adenine cation over that found in dsDNA. The strandedness of the DNA is also found to affect the subsequent chemistry of the ion radicals. For example less 5,6-dihydro-thymin-5-yl ($TH^{\bullet}$) radical is found in ssDNA than in dsDNA due to a reduced degree of ion transport.

Our recent work in this year (see progress report) confirms that after electron localization on cytosine, the dominant mechanism for electron reaction is transfer to thymine followed by protonation to form $TH^{\bullet}$ (31). No other anion radical protonation mechanism is found to be competitive due to differences in electron affinities which greatly favor the pyrimidines over the purines and the more rapid reaction of the thymine anion vs. cytosine anion radical. Recent product analyses do suggest at $37^{\circ}C$ there is some contribution from protonated

cytosine anion radical (42). This will be investigated in future efforts.

Over the past three years we have completed extensive ab initio molecular orbital studies of the ion radicals species involved in DNA radiation damage (24,25,29,30,33,37-39). In this effort the ionization energies (IE), electron affinities (EA), relative energies, fully optimized structural parameters and spin densities were determined. From calculations of the ionization potentials and electron affinities we predict the initial distribution of charge in irradiated DNA to be thymine anion radical and guanine cation radical as found in ssDNA (24). Calculations for the base pairs provide the explanation for the difference in the radical distribution in ss vs. dsDNA (25). In dsDNA, thymine anion cannot be protonated and stabilized whereas cytosine anion is predicted to be protonated from guanine. The initial ionization localizes the hole at guanine and the electron at thymine, but transfer of a proton from G to C causes the electron to localize on cytosine (28). We have studied the localization of charge on the DNA deoxyribose phosphate backbone which shows the ether oxygen as a site of localization (29). However, this IP is nearly 2 eV above that of the DNA bases which suggests hole transfer to the bases is quite likely. Each of the five possible sugar radicals in a model DNA backbone have been optimized and their relative stability and conformation investigated. The effect of the hydration layer on the electron affinities and ionization potentials has also been a focus of our efforts (30,37). The models used at this time for the bulk solvent are crude, but we have found that discrete solvation of the ionic species with a single hydration layer allows for good estimations of the effect of solvent.

In joint effort with Steven Swarts and Ken Wheeler of Wake Forest University, γ -irradiated DNA hydrated to various levels has been analyzed for 14 types of lesions (22,42). These DNA lesions include the release of unaltered bases, and specific DNA base damage. Perhaps the most outstanding finding is that the base damage yields are mainly confined to two products, one from reduction, dihydrothymine, and one from oxidation, 8-hydroxyguanine, properly called 8-oxoguanine; whereas, the base release yields are nonspecific. This product distribution has led us to the overall scheme for the radiation damage to DNA, starting with the initial ionization events assayed by ESR spectroscopy to the final products assayed in this work. In Scheme 1 (see current year (94-95) progress report or proposal) we give an overview for the oxidative radiation chemical damage to DNA. The numbers on the right give the percentage yields for dry and hydrated DNA (which are similar) and those on the left give the percentage of

expected ionizations based on the total valence electrons. Clearly, transfer of the hole from other DNA bases to guanine must occur at an early time and is shown in the figure; however, transfer from the sugar to the base is apparently not as efficient and this nicely explains the near uniform yield of unaltered bases. Scheme I therefore delineates the oxidative radiochemical processes from early processes (ionization) through radical intermediates some seen by ESR, to late processes which yield the damaged bases and base release. A similar scheme for the reductive component in DNA is hoped to result from our future investigations.

We have investigated the effect of binding of polycationic species such as spermine, polylysine and polylysine copolymers on the distribution of charge within the DNA strand and between the DNA strand and the polypeptide. For all polylysine-DNA copolymers we find that the DNA has a disproportionate amount of anion radical likely from electron transfer from the polypeptide. Typically our analyses suggest a 40% increase in anion radical for the DNA polypeptide complexes. Hole transfer from the polypeptide is apparently not as facile. These results have implications for DNA irradiated with histones. The histones likely provide a protective effect from the direct effect of radiation by transfer of negative charge to DNA. Since it has been well established that the electron does not produce strand breaks, it is clear that increasing the number of anionic radicals on the DNA will likely result in ion recombination and a loss of potential strand break sites. Further work is planned to extend these works to actual histone-DNA complexes before publication.

Some of our most fundamentally important work to DNA radioprotection was performed in our early work on this DOE grant. This work now funded by the NIH is in the area of the interaction of thiyl radicals and oxygen (5,8,9,11,14,17) as well as (DNA) peroxy radicals with thiols (13,18). This work has given a wealth of information not known previously which we believe has important implications for radiation damage *in vivo*. Up to our work in this area, the sulfinyl radical, $\text{RSO}\cdot$, was not known to be a product of thiol or carbon based peroxy reaction with RSH . The structure of the thiyl peroxy radical, $\text{RSOO}\cdot$, was uncertain. The conversion of $\text{RSOO}\cdot$ to the sulfonyl radical, $\text{RSO}_2\cdot$, and the subsequent reaction with oxygen to form the sulfonyl peroxy radical, $\text{RSO}_2\text{OO}\cdot$, had never been suggested in the literature. In our recent efforts we have investigated the reactions of thiols with primary ion radicals in γ -irradiated DNA and observed electron transfer from the thiol to the guanine cation radical. This is believed to be the major protective effect of thiols from the direct effect of

radiation. Our work with DNA-thiols in the presence of oxygen shows the formation of thiol peroxy and sulfoxyl radicals. The formation of these species, instead of the radioprotective disulfide anion, is considered to be the explanation for the chemical oxygen enhancement effect.

Our work with peroxy radicals has also established that the ESR measured O-17 hyperfine couplings give a good estimate of the relative reactivity of the peroxy group and shows that the thiol peroxy radical cannot be an effective hydrogen abstracting agent (16).

Below, we outline some of the main features of our published work performed over the past three years. For the current years work, the accompanying Progress Report (94-95) should be consulted. Recent original publications and preprints being sent as part of the renewal package should be consulted for details.

a. Relative Abundance and Reactivity of Primary Ion Radicals in γ -Irradiated DNA: Single vs. Double Strand DNA.

Knowledge of the chemical nature and the distribution of the radiation induced damage sites in DNA is important to the understanding of the effect of radiation on biological systems. Until recently most investigations suggested that the thymine anion and guanine cation were the principal species found in γ -irradiated DNA at low temperatures. The fact that the final reaction product found in DNA is mainly the 5,6-dihydrothymine-5-yl radical, TH•, was a major piece of evidence in support of the thymine anion as the likely anionic primary radical. However, Bernhard, in work with oligonucleotides in aqueous glasses, found that the cytosine anion was the dominant anion radical; he suggested cytosine anion may also be the dominant anion in DNA as well. In previous work, the cytosine anion and thymine anion were difficult to distinguish in both DNA model systems and DNA since the spectra of both are doublets of about equal coupling. In last years effort, we found that the cytosine and thymine anion spectra were considerably more resolved and distinguishable in D₂O (vs. H₂O) matrices and through a series of experiments on polynucleotides, pdAdT and pG·pC, DNA, as well as computer reconstructions we concluded that cytosine anion is the dominant anion in γ -irradiated DNA. Our recent ab initio calculations (see below) and Steenken's measurements of ion radical pK's suggests that the cytosine anion is stabilized by protonation from its complementary base guanine, so that it is better designated as a neutral species,

$C^-(H^+)^\bullet$. For simplicity we will use the symbol $C'^-\bullet$ for both the anion and the protonated species.

In this work an electron spin resonance investigation of the free radical distribution in γ -irradiated frozen samples of single stranded DNA (ssDNA) and double stranded DNA (dsDNA) is reported. Computer analysis of the ESR spectra of γ -irradiated ssDNA in D_2O at 100K suggests a more uniform distribution of the radical ions on the DNA bases than found for dsDNA, with the approximate ssDNA composition: thymine anion, $T^-\bullet$ (30-35%), cytosine anion, $C'^-\bullet$ (20-28%), guanine cation, $G^+\bullet$ (26-28%) and adenine cation, $A^+\bullet$ (8-17%) with small amounts of purine anions or pyrimidine cations. Upon annealing γ -irradiated dsDNA, we find that the electron transfers from $C'^-\bullet$ to T, and the $T^-\bullet$ formed protonates at C-6 to produce the 5,6-dihydrothymine-5-yl radical ($TH^\bullet$, $TD^\bullet$). The rate of deuteration of $T^-\bullet$ is shown to be 20-fold less than the rate of protonation. Electron transfer in ssDNA appears to be less facile than in dsDNA. Holes transfer from adenine to guanine in ssDNA over the temperature range 130-190 K, whereas in dsDNA, the transfer of holes from adenine to guanine is nearly complete at 100K. The results suggest that DNA strandedness, which affects DNA conformation, base stacking and cross strand interactions, is important in determining the initial ion radical sites as well as the subsequent ion radical transfer and reactions. A calculation of the ion radical abundances as a function of base transfer, starting from a random distribution in a model DNA strand, suggests base pairing greatly augments hole and electron transfer through the DNA strand and that on average, ion transfer over only ca. 2 bases is sufficient to produce the ion distribution found in ssDNA and dsDNA.

b. Protonation of Nucleobase Anions in γ -irradiated DNA and Model Systems. Which DNA Base is the Ultimate Sink for the Electron?

Reversible and irreversible protonations of the nucleobase anions $C^{\bullet-}$, $T^{\bullet-}$, $G^{\bullet-}$ and $A^{\bullet-}$ have been followed with great interest since they play an important role in the mechanisms of radiation damage to DNA. Cullis et al. have recently reported works on the subject of the stabilization and reaction of primary ion radicals in DNA in which they suggest that the electron migration from $C^{\bullet-}$ to thymine does not occur on annealing and that the $T(C6)H^\bullet$ arises only from the original $T^{\bullet-}$ present in irradiated DNA (40kGy) at 77 K. The major support for this contention is the fact that only about 18% of the original signal converts to $T(C6)H^\bullet$ in contradiction to the 30% conversion found in our work. In this work

we show that the conversion is dose dependent and varies from 30% at low doses to 13% at high doses as described below.

In this work we attempt to better understand details of the protonations for the nucleobase anions in DNA from investigations of frozen solutions of dGMP•dCMP, dAMP•dTMP, polyG•polyC, poly[dGdC]•poly[dGdC], poly[dAdT]•poly[dAdT] and DNA itself. Analysis of the ESR spectra at a dose of 22 kGy shows that fractional conversion of total radicals to carbon protonated species on annealing is in the order: dAMP•dTMP (43%) > pdAdT = DNA (ca. 23%) > dGMP•dCMP (15%) > polydGdC•polydGdC (6%) > polyG•polyC (3%). Two hydrogen addition radicals make contributions to the polyG•polyC, poly[dGdC]•poly[dGdC] and dGMP•dCMP spectra in H₂O on annealing. They are those formed by protonation at C6 of cytosine anion radical, C(C6)H•, and at C8 of the guanine anion radical, G(C8)H•. Computer analysis reveals that anion protonation reaction in dGMP•dCMP results in mainly C(C6)H•, whereas protonation reaction in polyG•polyC and poly[dGdC]•poly[dGdC] yields mainly G(C8)H•. In dAMP•dTMP and poly[dAdT]•poly[dAdT] as in DNA itself the only DNA base found to undergo an irreversible protonation at a carbon site is thymine resulting in T(C6)H•. The conversion of DNA anion to T(C6)H• is found to be dependent on dose. At low doses (5 kGy), ca. 30% conversion to T(C6)H• is found whereas at high doses (94 kGy), only 13% conversion is found. Results show that at 40 kGy the 18% value reported by Cullis et al. is found in this work as well. The dose dependence is ascribed in part to ion radical recombinations whose probabilities are increased at high doses. A consideration of the rates of protonations of the purine and pyrimidine anion radicals as well as the differences in electron affinities suggest carbon protonation reactions of DNA base anions in irradiated stacked dsDNA at 37°C would be predominantly at thymine and perhaps guanine, whereas in ssDNA all bases could contribute. We suggest electron migration may be expected to be a mechanism for DNA radioprotection.

c. The Influence of Hydration on the Absolute Yields of Primary Ionic Free Radicals in Irradiated DNA at 77K.

1. Total Radical Yields and the Role of Water.

In this work, an ESR investigation of the effect of hydration water on the

absolute yields (G) of ion radicals in γ -irradiated DNA at 77 K is reported. The total DNA radical yield is found to increase by over four fold on addition of the primary hydration layer (20 water molecules/nucleotide) to DNA. At hydrations greater than ca. 20 waters/nucleotide the excess water freezes into a separate and apparently radiologically independent bulk ice phase. Ice formation steals ca. 5 waters from the hydration layer which causes a proportionate (by weight) drop in the total DNA radical ion yield. Hydroxyl radicals ($OD\bullet$) are not observed even at great signal amplification in the primary hydration layer but are found only in the ice phase, which suggests efficient transfer of holes and electrons from the hydration layer to the DNA; as a consequence, DNA and its associated hydration water form the target mass for radiation damage. These results show that water of hydration is critical to radical formation and stabilization in DNA; however, the ice surrounding DNA does not contribute to direct DNA damage and is found to have the same properties as bulk ice. At 18 waters per nucleotide ($\Gamma=18$) about 1/4 of all ion radicals produced by the radiation are trapped at 77 K. The k value for destruction is found to vary only slightly with hydration. Based on the measured G and k values for hydrated DNA a trapped radical ion cluster size is estimated to be about 6 nm. For hydrated DNA the cluster size suggests electron migration distances of about 17 bases (for migration along the DNA strand).

2. Yields of Individual Radicals with Dose.

In this work an ESR investigation of the yields of the individual free radicals formed in γ -irradiated frozen DNA as a function of hydration and dose at 77K is performed. Analysis of the ESR spectra taken at low hydration shows that the ion radical composition remains nearly constant with dose, and that few secondary radicals are formed even at high doses (above ca. 50 kGy). For fully hydrated samples the radical composition changes dramatically with dose. Thymine anion radical ($T^-\bullet$) is found in abundance at low doses but nearly disappears at higher doses, with a corresponding increase in the N-3 deuterated cytosine anion radical ($C_D^-\bullet$). Guanine cation radical ($G^+\bullet$) decreases at high doses, with a concomitant increase in secondary radical species ($S\bullet$). Analysis of the dose response data for G values (the yields in $\mu\text{mol/J}$), k values (the destruction constants) and k' values (a new constant that characterizes the change in G with dose) were performed for each of the DNA base free radicals present at 77 K. The G value for each of the base radicals increases with the hydration level. The k values for $C_D^-\bullet$ and $G^+\bullet$ increase slightly with hydration;

however, that of $T^{\bullet}$ increases substantially. Destruction constants for neutral radicals such as $TH^{\bullet}$ and $C_D^{\bullet}$ are found to be substantially smaller than those for ion radicals and provide an indication of the radical charge state. A negative k' value for $T^{\bullet}$ and a positive k' value for $C_D^{\bullet}$ are explained in terms of radiation effects that result in the formation of a deuterated cytosine base, i.e. $C(N3)D^+$, which greatly increases cytosine's electron affinity. The ratio of anion radical to cation radical concentrations is found to be ca. 1.6 and is invariant with hydration. A speculation on the imbalance based on hole-hole combinations in spurs is presented.

d. Ab Initio Molecular Orbital Calculations of DNA Base Pairs and Their Radical Ions .

Considerable interest has arisen of late in the primary radicals formed in DNA as a result of several reports that the initial ion radical distribution may be dependent on proton transfer reactions between base pairs. Steenken first suggested that proton transfer reactions may be important in the initial stabilization of DNA ion radicals. Proton transfer from G to $C^{\bullet}$ has been suggested to stabilize the electron on cytosine and account for the dominance of the cytosine anion in DNA.

In this work, *ab initio* molecular orbital calculations of various protonation states of DNA base and DNA base radical ions were performed to aid our understanding of primary DNA radiation processes such as proton transfer between base pairs. Each of the structures was optimized at the STO-3G and 3-21G levels of theory, and single point calculations were performed at the 6-31G**/3-21G and 6-31+G(d)/3-21G levels. Each of the protonation states important to proton transfer reactions in base pair radical ions found in irradiated DNA was considered. Calculations for three protonation states for the cytosine reduced radical were performed and the most stable is found to be the N-3 ring protonated species. Calculations for the vertical ionization energies of the individual DNA bases yielded the following order $T > C > A > G$ with the same order found for the adiabatic electron affinities of the bases. The best fit to experiment was found with Koopmans theorem ionization potentials. The energy for proton transfer in the GC and AT base pair radical cation and radical anions were estimated from the energies of the individual species. All proton transfers were found to be energetically unfavorable except for the GC anion radical which is favored by 13 kcal.

The results of these calculations combined with previous work lead to a revised model for the initial localization of charge in irradiated DNA. Our results predict that thymine has the highest electron affinity and guanine the lowest ionization potential of the four DNA bases. As a consequence, the electron in irradiated DNA is more likely to be found on thymine and the hole on guanine. Results obtained with base pairs and four base stacked systems again suggest that the electron will localize on thymine. However a simple proton shift from guanine to cytosine also shifts the electron from thymine to cytosine. No such shift from A to T is predicted to be energetically favorable. For the hole, proton shifts are not predicted to alter the hole site due to the fact that guanine hole in the G-C base pair is calculated to be lower in energy than the adenine hole in the A-T base pair either before or after proton shift within the A-T base pair.

These results therefore suggest that electron attachment will initially favor thymine and subsequent proton transfer reactions between G-C base pairs will shift the electron to cytosine. Previous results in our laboratory have shown that in irradiated double stranded DNA at 100 K, the anion is mainly on cytosine and the hole on guanine. However, very recent results have shown that in single stranded DNA where proton transfer between base pairs is not possible, the thymine anion is found to increase its contribution as expected from the results of this work.

1. Effect of Hydration Water on EAs and IEs.

In this work we have performed calculations on DNA base pairs and their ion radicals at the 3-21G level which include waters of hydrations at probable sites of hydration in DNA. We have found that the waters of hydration have a small effect on both the ionization potentials and electron affinities. Both the EAs and IEs are slightly increased by the waters of hydration but their relative order is unchanged from the order without waters of hydration, i.e., C>T>A>G. The evidence also shows that the waters of hydration stabilize the anion radicals more than cation radicals.

2. Cation Radical Sites on the DNA Sugar Phosphate Backbone.

Up to this time ESR spectroscopy of irradiated DNA at temperatures from 4K to 77K has not shown any radicals other than those on DNA bases. DNA is composed of 55% by weight of sugar phosphate backbone and at least an equal weight to the DNA in hydration water. Thus although they make up less than

25% of the target mass, DNA bases apparently have all the free radicals formed in irradiated DNA. An explanation for this is that ionization in the hydration layer and sugar phosphate backbone transfer spin and charge to the DNA bases. Another explanation is that ionization on the deoxyribose phosphate backbone results in very broad radical species which are not easily observed by ESR. We find no solid evidence for the latter in our ESR spectra. Since the most damaging effect on radiation is the DNA double strand break it is critical to understand the possible mechanisms of production of radiation lesions on the DNA deoxyribose phosphate backbone. The calculations show that there are two sites where the cation or hole can localize before subsequent reaction or transfer to the DNA base. These are: 1. localization to the ring ether oxygen in the deoxyribose group or 2. localization to one of the singly bonded oxygens on the phosphate group. The site of localization varies with the backbone structure and conformation and result from the fact the the phosphate and deoxyribose portions of the backbone have nearly the same ionization energy. Most calculations favor the deoxyribose portion of the backbone.

The IE of the sugar phosphate backbone is found to be about 2 ev higher than that of the bases. This explains the transfer of the charge to the bases and the lack of observation of a large fraction of sugar phosphate radicals in DNA. It has become clear in recent pulse radiolysis work that excited DNA base cations may result in DNA strand breaks. It is clear that a DNA base excited state could undergo a charge transfer to the sugar which could then react to form the strand break. Such mechanisms are now under investigation.

e. Radiation-Induced DNA Damage as a Function of Hydration.

1. Release of Unaltered Bases.

In this joint effort with Steven Swarts and Ken Wheeler of Wake Forest University, γ -irradiated DNA hydrated to various levels has been analyzed for several types of lesions. These DNA lesions include the release of unaltered bases, specific DNA base damage, and nucleoside damage. These studies are reviewed briefly below.

The release of unaltered bases from irradiated DNA, hydrated between 2 and 33 moles of water per mole of nucleotide (Gamma) was investigated by HPLC. The objective of this study was to elucidate the yield of the four DNA bases as a function of dose, extent of hydration, and the presence or absence of oxygen. The increase

in the yield of radiation-induced free bases was not constant with Gamma over the range of hydration examined. For DNA with Gamma between 2 and 15 the yield of free bases was nearly constant. However for DNA with Gamma > 15 the yield increased rapidly. We suggest that the release of bases originating from irradiation of the hydration water is obtained predominantly by charge transfer from the direct ionization of the first 12-15 water molecules and predominantly by the attack of hydroxyl radicals generated in the outer more loosely bound water molecules.

2. Formation of Specific DNA Base Damage Products.

In this joint study we have examined the levels of radiation induced base damage produced in double stranded salmon sperm DNA hydrated between 2 and 33 moles of water per mole of nucleotide (Gamma). The base damage products are assayed using gas chromatography/mass selective detection techniques following formic acid hydrolysis of the DNA.

The DNA base damage products that are likely formed from the dry electron and positive hole, i.e. dihydrothymine and 8-hydroxyguanine, predominate at low DNA hydrations. For higher levels of DNA hydration (Gamma>13), the base damage products formed by reaction with hydroxyl radical (i.e., 5-hydroxymethyl uracil, thymine glycol, FAPyAdenine, and 8-hydroxyadenine are the major species observed in addition to 8-hydroxyguanine. In the presence of oxygen, greatly reduced levels of dihydrothymine are found at low hydration levels. This is likely due to scavenging of electrons by oxygen or the addition of oxygen to the thymine electron adduct preventing the formation of the dihydrocompound.

II. Future Plans in View of Past Results.

Our proposed effort (see proposal) is based on the past research accomplishments summarized above. Specifically, we plan to extend this work in several areas. First electron spin resonance investigations will be performed on: (1) The formation of the $\bullet\text{OH}$ in hydration layer to test the dividing line between hole transfer and hydroxyl radical formation, i.e., the division between the direct and indirect effects. (2) Investigations of possible deoxyribose radicals in DNA will be performed to elucidate effect of radiation on the deoxyribose phosphate backbone of DNA.

Our work with ab initio MO calculations of radiation damage to DNA will continue with investigations of radical and final molecular products of the initial

DNA ion radicals. MO calculations will be completed on the optimized geometries of the DNA base radical reaction products such as TH•, GOH•, AOH•, etc. The products dihydrothymine, 8-oxoguanine, fapyGuanine etc. will also be structurally optimized. Various properties such as spin densities, charge densities, dipole moments, ionization energies, electron affinities for all optimized structures will be computed. Further investigations on the energetics of the formation of the DNA strand break from the C3' and C4' sugar radicals will also be investigated. We applied for Cray time at the Lawrence Livermore supercomputer facility from the National Energy Research Supercomputing Center and have been awarded 450 hours. This time along with our own computing facilities will be ample to complete our proposed effort.

Finally, our cooperative investigations of the products formed by the direct effect of radiation on DNA will continue. We plan investigations of the effect of hydration and temperature on the reductive product distribution in DNA in order to compare product results with those of ESR investigations. The same samples used for ESR investigations will be analyzed for products. Thus the radical formation and reactions will be followed from the initial ionization event to the final product.

III. Students Trained, Degrees Granted

Undergraduate Students who participated in this program over the past eight years: 18.

Graduate Students who participated in this program over the past eight years: 5.

Two graduate students are now working on aspects of this project. One of these students A. O. Colson will obtain her degree next spring.

Degrees Granted:

Ph.D. (Spring 1995) - Anny-Odile Colson, Health and Environmental Chemistry. Thesis Title: "Ab Initio Molecular Orbital Calculations of Radiation Induced Radicals and Their Reactions in DNA."

Ph.D. Granted - Mengyao Yan, Health and Environmental Chemistry, Spring 1992. Thesis Title: "ESR Studies of Free Radical Mechanisms of Radiation Damage and Protection in DNA"

Ph.D. Granted - Steven Swarts, Ph.D. in Health and Environmental Chemistry, Dec. 1987. Thesis Title: "ESR Study of the Irradiation of Biologically Important Molecules"

IV. Bibliography of Publications Associated with this Grant. (1986-1995)

A. Publications 1986-1995

1. "An Electron Spin Resonance Investigation of Radical Intermediates in Cholesterol and Related Compounds: Relation to Solid-State Autoxidation," C. L. Sevilla, D. Becker, and M. D. Sevilla, J. Phys. Chem., 90, 2963-2974 (1986).
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B. Presentations 1986-1994.

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2. "An ESR Investigation of Radical Intermediates in Cholesterol and Related Compounds: Relation to Solid State Autoxidation," C. L. Sevilla, D. Becker, M. D. Sevilla, presented at 34th Annual Meeting of the Radiation Res. Soc., April 13-17, 1986, Las Vegas.
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4. "Electron Spin Resonance Investigation of Radical Intermediates in Cholesterol," C. L. Sevilla, D. Becker, and M. D. Sevilla, presented at the 9th International EPR Symposium of the 1986 Rocky Mountain Conference, August 3-7, 1986, Denver.
5. "EPR Spectroscopic Evidence for Spatially Sequential Amide Bond Formation in Plant Polygalactopyranuronides," P. L. Irwin and M. D. Sevilla, presented at the 9th International EPR Symposium of the 1986 Rocky Mountain Conference, August 3-7, 1986, Denver.

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14. "The Radiation Chemical Consequences of Charge Transfer in DNA," Michael Sevilla, Symposium on Charge Transfer and Subsequent Reactions in DNA, 37th Annual Meeting of the Radiation Research Society, Seattle, Washington, March 18-23, 1989.
15. "Reaction of DNA Peroxyl Radicals with Cysteamine: Formation of Sulfoxyl Radicals," Michael D. Sevilla, M. Yan, D. Becker, and S. Gillich, 37th Annual Meeting of the Radiation Research Society, Seattle, Washington, March 18-23, 1989.

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17. "Kinetic Analysis of Free Radical Reactions in the Low-Temperature Autoxidation of Triglycerides," J. Zhu, and M. D. Sevilla, First International Conference on Eicosanoids and Bioactive Lipids in Cancer and Radiation Injury, Detroit, MI Oct. 11-14, 1989.
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37. "Ab Initio Molecular Orbital Calculations of the Structure and Energetics of DNA Deoxyribose Radicals" A. O. Colson and M. D. Sevilla, 42nd Annual meeting of the Radiation Research Society, Nashville, Tennessee, April 29-May 4, 1994.
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39. "Reactions of Thiols with Individual DNA base Radicals in DNA and DNA Model Systems: The Effect of Oxygen on Radioprotection" Michael D. Sevilla, David Becker and Wending Wang, Presented at the Workshop on Radiation Damage in DNA: Structure Function Relationships at Early Times, Salishan Lodge, Gleneden, Oregon, Oct 1-6, 1994.
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V. The Present State of Knowledge in This Area of Research.

This is an exciting time in the area of radical and molecular mechanisms of radiation damage to DNA. The initial ionization events leading to radiation damage are becoming increasingly well understood as are the radical reactions that follow and lead to the diamagnetic product formation. A new model for the initial events in the radiation damage to DNA has been formulated and a

fundamental understanding of the direct effect of radiation on DNA is progressing from the primary ion radical localization, through the stepwise formation of radical intermediates, to the products formed by the ion radical intermediates. The transition from molecular mechanisms to biological end points (mutagenesis and cell death) are now also beginning to be understood. An understanding of the effect of ionizing radiation on DNA from the initial ionization to biological consequences is the dream of the radiation physicist, chemist and biologist. Recent results by a number of groups have pointed the way toward its fulfillment.

Below we present a brief overview of this area. However, we have recently written three extensive reviews of radiation damage to DNA each from a different perspective. The first is from an overall perspective ("The Chemical Consequences of Radiation Damage to DNA" D. Becker and M. D. Sevilla, in *Advances in Radiation Biology*, Vol.17, (J. Lett, Ed.) Academic Press (1993)). The second concentrates on the recent contributions of electron spin resonance spectroscopy to this field ("Radiation Damage to DNA" , Michael D. Sevilla and David Becker, in *Royal Society of Chemistry Specialist Review, Electron Spin Resonance*, Vol. 14 (1994)). The third gives a review of the latest theoretical efforts which elucidate radiation damage to DNA ("Elucidation of Primary Radiation Damage in DNA through Application of Ab Initio Molecular Orbital Theory" A.O. Colson and M. D. Sevilla, submitted). These should be consulted for a more complete overview of this area.

Through our efforts and that of other workers (Bernhard et al., Steenken et al., and Hüttermann et al.) we now are confident that at low temperatures the most stable anion is on cytosine in dsDNA , whereas the most stable cation is on guanine in all DNA forms. We have also found that the radical ion distribution is a function of strandedness of the DNA. In single strand DNA we find that there is an increase in thymine anion radical. Modeling of the ion transport in DNA suggest that the base pairing provides a significant increase in electron and hole transport through DNA. Steenken has shown that it is the proton transfer within the GC base pair that stabilizes the anion on cytosine in dsDNA. Our ab initio calculations on electron affinities and ionization potentials of DNA bases and base pairs also confirm the overall model including the energetics of the proton transfer.

Our recent ESR efforts clearly show that at elevated temperatures the DNA anion radicals can migrate to other bases where irreversible reactions occur. For

example, these studies show that the thymine anion radical protonates at C6 to form $T(C6)H^\bullet$. From product analyses work from Cadet and ourselves it is clear that the guanine cation adds OH^- at C8 to form $G(C8)OH^\bullet$. Subsequent reactions of these and other intermediate species account for the diamagnetic base products formed in DNA. For example, reduction of $T(C6)H^\bullet$ results in 5,6-dihydrothymine and oxidation of $G(C8)OH^\bullet$ results in 8-hydroxyguanine; these are two major diamagnetic products found at low hydration levels (in our joint work with Swarts and Wheeler) for which the direct effect predominates. Our work and that of others (Bernhard et al., Hüttermann et al.) on radical and product formation as a function of hydration has aided our understanding of the contribution of the first few layers of water to radiation damage to DNA. Our most recent work shows that the first water layer (up to 12 waters/nucleotide) contributes mainly to the direct effect through electron and hole transfer whereas the subsequent layers contribute to the indirect effect. Our recent work which shows that the OH can be found in the glassy hydration layer needs to be further expanded to determine the yield of OH radical as a function of the number of waters/nucleotide.

Ab initio molecular orbital studies of the ion radicals species involved in DNA radiation damage have been quite helpful in confirming and elucidating mechanisms of ion radical localization and reaction in DNA. For example, these calculations correctly predict that the initial localization of charge in irradiated DNA is on thymine anion and guanine cation as found in ssDNA. Calculations for the base pairs (mimicked by results for individual DNA base radicals and their complements in their appropriate protonation states) predict that guanine will protonate the cytosine anion but adenine will not protonate the thymine anion. This provides the explanation for the difference in the radical distribution in ss vs. dsDNA. In dsDNA, thymine can not be protonated and stabilized whereas cytosine can. The ssDNA results are a result of thymine's slightly greater EA whereas in dsDNA the preferential protonation of the cytosine anion yields enough stabilization to overcome the greater EA of thymine and result in localization of the charge on cytosine. Many such insights have arisen from this work and it is planned that such calculations will be an integral part of our upcoming proposed effort. Molecular modeling work by others (Osman, Wallace) have aided our understanding of the effects of DNA lesions on DNA structure and recognition of the lesions by enzymes.

Joint work with Steven Swarts and Ken Wheeler of Wake Forest on yields of

products on irradiation of DNA has yielded important new insights to radiation damage to DNA. From the product yields, the overall picture of the radiation damage processes is brought to its ultimate chemical result. Up to this work little information which isolates the direct effect of radiation on DNA was available even though it comprises ca. 40% of the damage. Our investigation of the yield of damage and unaltered bases found for DNA at room temperature are supported by the results of the ESR experiments on irradiated DNA at 77K. The radicals found by ESR are the logical precursors to the products found in the GC-mass spectroscopic analyses. These product analyses need to be continued to address several other fundamental questions. For example the current result show an imbalance favoring oxidative over reductive products. The missing reductive products need to be found. We propose to do this in the project with Ken Wheeler and Steven Swarts described in the proposal.

For the indirect effect much more information about DNA in solution is available. A number of DNA product analysis studies have been reported by Dizdaroglu et al. and recently Box et al. has reported a series of elegant NMR papers which have nicely shown the products of the indirect effect on dinucleotides. The free radical mechanisms for strand breakage have been elucidated by Schulte-Frohlinde's group at Mulheim in laser pulse photolysis work. These experiments have shown that the guanine cation radical results in strand breaks with about one third the efficiency of a hydroxyl radical attack. However, little is now known of the chemistry of strand break formation from the guanine cation radical. Cadet has pointed out that the protonation state of the guanine cation radical actually affects the products formed. Further work is needed in understanding this mechanism as well as the initial ionization events that occur on very short time scales. Here, the possible formation of sugar radicals by the direct ionization still needs to be elucidated. This is planned in our research effort. Our product analyses studies suggest that hole transfer from the sugar group to the base may not be competitive with deprotonation of the sugar cation to form a carbon centered sugar radical. Here ironically it is the product analyses done at long times that gives our only present information as to the very early processes involving hole transfer.

In summary the hydroxyl radical is known to be the major cause of strand breaks by the indirect effect and the DNA (base and sugar) cation radicals are the

major cause from the direct effect. The electron has been shown to be ineffective in the production of strand breaks. Some mechanisms for strand break formation from base and sugar radicals have been elucidated but further work is needed here. The major DNA diamagnetic base products found from radiation damage are consistent with initial free radicals observed in ESR experiments. It has been demonstrated that the indirect effect produces many of the same radicals and diamagnetic products as the direct effect (28).

Often the type of work described above is criticized for not having a direct biomedical application. Yet it is clear that biology as a science is becoming more powerful by its understanding of biological mechanisms at the molecular level. To understand and control radiation damage will require its understanding at this level as well. The ultimate biological effect is a result of complex and intricate cellular mechanisms; however, the immediate effect of radiation can be explained solely on the physics of the system -ionizations and excitations. The next few microseconds involve the initial chemistry of the system in which biochemical mechanisms have little part. The result of this chemistry can be predicted and the damage which through cellular biochemistry ultimately leads to its biological endpoint elucidated. It is only in this manner that a full understanding of the processes of radiation damage will be obtained.

VI. Present Division of Federal Support for Overall Research Program

A grant from the NIH for a project entitled "Radiation Induced Sulfhydryl Autoxidation and the OER" was received for the period Jan. 1, 1991 to Dec. 31, 1995. The direct costs are \$65,782 for 1995. These funds are currently supporting: a post doctoral fellow (Yurii Razskazovskii) fully, a senior level Ph.D. in Electron Spin Resonance (Prof. David Becker) for 19 hr/week, and the principal investigator (M. D. Sevilla) 1.5 months during the spring and summer. In addition a grant of \$24000 was received for the period Sept. 1, 1994 to July 31, 1995, from the U. S. Army Natick Research and Development Laboratory to fund a post doctoral level person for 8 months on a project entitled "Commonality of Radical Intermediates in Irradiated Muscle Foods".