The Biochemical Reactions Of Dry State Dna

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ABSTRACT

The biochemistry of dry state DNA is of interest to the fields of forensics, ancient DNA, and DNA storage. The exact chemical nature of the degradation of the DNA molecule in the dry state has not been studied prior. If determined what chemical changes the DNA molecule undergoes, to what degree and in what time frame then protocols can be implemented to bypass the impact of this damage or to repair it when necessary. It is suspected that similar reactions occur to the dry state DNA molecule as does to the hydrated molecule. It cannot be assumed, however that these types of chemical processes occur to the same extent and at the same rates. In general the generic process of hydrolysis encompasses two important reactions, that of deamination and of base loss from the 2′-deoxyribose backbone. Base loss is believed to ultimately lead to chain scission. It is also suspect that reactive oxygen species (ROS) have an important role in the chemistry associated with DNA. Species such as hydroxyl radicals (OH•) and singlet oxygen (1O2) can lead to strand scissions and chemically modified bases.
Throughout this project various techniques were used to determine damage to DNA and its molecular constituents under conditions leading to hydrolytic and oxidative damage. Novel techniques used in this study include ion-pairing chromatography and denaturing HPLC (DHPLC) to measure glycosidic bond cleavage and strand breaks. The extent to which the macromolecule haemoglobin (Hb) can lead to oxidative damage of DNA in dried blood stains by acting as a Fenton chemistry catalyst was evaluated. Additionally the enzymatic activity of the extracellular nuclease from Alteromonas espejiana, BAL 31 was studied as it pertains to the degradation of single-stranded short homopolymeric oligonucleotides.

This study serves as the basis for future, more in depth experimentation into the more specific nature of dry state DNA biochemistry. It was found that to a large extent the same degradation reactions (base hydrolysis, base modifications, and strand breaks) do occur in the dry state as in the hydrated state when heat and UV radiation are used as energy sources. Reaction rates indicate that base hydrolysis and deamination occur much more slowly, yet have the same energies of activation in both states. Single
strand breaks of dry state duplex DNA occur with a half life of 24 ± 2 days and appears to occur in a mechanistic manner which could be of interest when attempting to repair such damage. In addition, base loss alone does not correlate with the extent of single strand breaks detected. Thermodynamic data can lead to the conclusion that DNA degradation in both dry and hydrated states is not a spontaneous process. It is also concluded that though the Hb molecule undergoes oxidative changes over time, these changes do not impact its ability to become a more aggressive Fenton reagent. However, the presence of Hb in the vicinity of DNA does create the opportunity for OH• induced damage to the deoxyribose sugar, and most likely the DNA bases themselves. This study also reveals that the general purpose BAL 31 nuclease commonly used in molecular genetics exhibits a hitherto non-characterized degree of substrate specificity with respect to single-stranded DNA oligomers. Specifically, BAL 31 nuclease activity was found to be affected by the presence of guanine in ssDNA oligomers.
This work is dedicated to my family. First of all to my daughter Baily who has literally sat with me through almost eleven years of courses and spent numerous hours entertaining herself while I worked towards this goal. To my husband Josè who attended my research presentations, spent numerous weekends and weeknights without me, and of course didn’t complain about driving with me across town just to spend a few minutes checking on an experiment, or about my graduate student stipend. And of course to my son Zane whose birth made my defending semester much more stressful, but also more enjoyable than it ever would have been without him.
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xxii
Abbreviation

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CHAPTER 1: GENERAL SCOPE AND PURPOSE OF WORK

Introduction

The use of DNA analysis in forensic casework has made a revolutionary impact on the criminal justice system and has allowed for the exoneration of the innocent and prosecution of the guilty. Forensic biological samples can differ from those obtained in vivo in the essence that they are found in a dry state and many times in small quantities (i.e. nanogram). These conditions can lead to evidentiary samples having a low molecular weight (LMW), low copy number (LCN), and in many cases degraded structures. For those samples that have undergone degradation the result can be the unsuccessful amplification during the PCR process, or possibly the mistyping of a sample. One example of the consequences of degraded samples is that Taq Polymerase used in the PCR technique has an error associated with it per nucleotide per cycle. Artifacts, or mistakes in the sequence alignment can occur by misinsertions of 2’-deoxynucleotide triphosphates (dNTPs) and frameshift errors in the primers. The frequency of sequencing artifacts has been found to be proportional to
the decay of the PCR template, or original DNA sample. Ambiguities found in the PCR process due to degraded samples can be overcome by sequencing both strands, but in some cases the problem cannot be resolved\textsuperscript{1}.

The current knowledge of the biochemical nature, and the extent of DNA damage in dried biological stains is rudimentary. Precise remedies for such damage will be dependent on its chemical nature. By looking at the effects of hydrolysis and oxidation on dry state DNA it will be possible to determine what biochemical changes occur and to what extent over time. It has been shown that in dry state DNA the main source of damage induced by UVC radiation is strand breaks as opposed to pyrimidine dimers that are seen in living tissues\textsuperscript{2}. Thus, it is not plausible to assume that damage seen \textit{in vivo}, or \textit{in vitro} conditions that simulate \textit{in vivo} conditions is the same for dry state biological samples.

There is hardly an atom on the pyrimidine and purine rings of DNA which has not been shown to react with some physical or chemical damaging agent. It is possible to predict the type of lesions which are likely to be formed from basic
chemical principles such as electrophilic properties of damaging agents that will react with the electron-rich centers of the heterocyclic bases. The primary structure of DNA can exhibit a variety of different lesions indicative of damage, including oxidation products, single- and double-strand breaks, UV-induced photoproducts, DNA or protein crosslinks, and chemical-agent-induced covalent adducts. In addition, over time hydrolysis products caused by spontaneous deamination, depurination, and depyrimidination can form. DNA in forensic type samples lack the repair mechanisms available in vivo due to cellular metabolism, thus dried biological fluid stains should experience a different rate of DNA lesion formation compared to the situation in situ. Varying environmental insults such as UV irradiation, heat, humidity, and genotoxins may cause or facilitate damage to the DNA molecule due to the lack of homeostatic processes available to tissue outside of the body.

Numerous studies have assessed the ability to obtain a genetic profile after exposure of dry state biological material to various environmental factors. DNA-typing systems look at forensic samples exposed to environmental
insults as a part of the validation process for use by the forensic science community. The common conclusion is that environmentally impacted DNA in biological samples results in a progressive loss of signal and allelic drop out with extended or intense exposure. No studies are known that determine the precise molecular nature of damage in forensic biological stains and related thermochemical and kinetic data.

The thermal and kinetic aspects of solid state reactions can be complex. Solid state chemistry involves the study of the synthesis, structure, and properties of solid materials. Solid state chemistry generally includes the synthesis of novel materials and their subsequent characterization. Inherent of such studies, a solid-state reaction is one in which the system is in the absence of a solvent. In conventional chemistry the reasons behind wanting a reaction to occur as a dry media can include economic considerations (no cost associated with solvents), ease in product purification, higher reaction rates due to high concentration of reactants, and environment considerations (i.e. no solvent to dispose of). Here, motivations were different than the conventional ones given
for studying dry media reactions. Unlike most solid-state chemists, the knowledge sought was simply that of knowing what is the difference between a reaction occurring in an aqueous medium versus a dry medium. It just so happens that the particular reactions of interest revolve around one of the most complex molecules found in nature, the large molecule that carries the genetic instructions for all known life forms.

To aid in preparing for the scope of this work, a simple chemistry reaction was used to model what a comparative study of a dry vs. hydrated reaction will entail. The simple study was performed on Al by the ferrous ion as shown in Scheme 1.

\[
3\text{Fe}^{2+} + 2\text{Al(s)} \rightarrow 2\text{Al}^{3+} + 3\text{Fe(s)}
\]

This reaction under standard state conditions has a potential of 2.07 V. Solutions of Fe\(^{2+}\) were made by dissolving ammonium Fe(II) sulfate hexahydrate in water with a resistivity of greater than 18 Ω/cm\(^2\). Aqueous reaction conditions were setup up by placing an excess
amount of Al foil pieces in 10 ml of a 0.0211 M Fe$^{2+}$ solution in a 15 ml screw top test tube. Aliquots (5 µl) were removed in triplicate daily for 10 days and frozen. Dry reaction conditions were setup up by lining 1.5 ml microcentrifuge tubes with Al foil and placing 10 µl of a 0.0207 M Fe$^{2+}$ solution inside the foil. Samples were then dehydrated in a vacuum centrifuge and placed in a laboratory drawer in ambient conditions (22.8ºC, 60% relative humidity). Samples were taken in triplicate every other day for 30 days and re-hydrated with 1 ml H$_2$O. Of this assay, 50 µl was removed and frozen until time of analysis. The detection of Fe$^{2+}$ was done using a ferroine assay (150 mg ferrozine, 150 mg neocuproine, 2 ml concentrated HCl diluted to 50 mL). Ferrozine complexes with Fe$^{2+}$ forming a magenta colored chromophore with an absorption maximum at 562 nm. The results are shown in Figure 1.

It can be seen that there was a significant difference in the behavior between the two different types of media used to carry out the reaction. In aqueous medium a first order reaction rate was easily determined by plotting the $-\ln([Fe^2]/[Fe^{2+}]_0$ vs. time to be $2.04 \pm 0.35 \times 10^{-7}$ s$^{-1}$. 
However; in the dry medium there was no ongoing reaction detected at all. It is noted that the dehydration process must have caused some oxidation of the aluminum to occur due to the decrease in [Fe$^{2+}$] at the initial time point which was measured after the dehydration process. The vacuum centrifuge does employ a heater set at 40$^\circ$C to try to keep the sample from freezing under the low pressure conditions created by the process. The total time required to dehydrate all of the samples took approximately 30 minutes. But after that initial step, there is no further reduction noted.

Figure 1: Results of the reaction shown in Scheme 1. In the hydrated state (■) Fe$^{2+}$ easily oxidizes Al with a measurable rate of 2.0 ± 0.4 x 10$^{-7}$ s$^{-1}$. In the dry state (●) however, there is not much of a reaction seen to occur at all.
Thus it can be seen that the removal of a solvent system does at the very least alter the rate at which reactions will occur. Though the presented example showed a decrease in rate, this might not always be the case. There must also be an impact on thermodynamic parameters as well.

Figure 2: Expected target sites for DNA damage to occur. In this short segment representing the full double helix, only the four nucleobases are shown (from top: adenine, cytosine, thymine, and guanine). Oxidative damage is shown by segmented green and curved yellow arrows. Hydrolytic damage is shown by orange lightening bolts (base hydrolysis), red arrows (deamination), and blue arrows (strand breaks).
The mentioned aspects of dry state reactions will be compared to hydrated reactions in this report in detail where the main reactant is DNA and its molecular components. Specific types of damage will be assessed as outlined in Figure 2.

It is hypothesized that hydrolytic reactions will occur to dry state DNA and its molecular components when ambient relative humidity conditions are available. These reactions are assumed to occur much less readily due to the absence of an aqueous environment and thus must require more stringent conditions for their induction. Therefore, hydrolytic reactions will need to be induced using elevated thermal conditions and results will have to be extrapolated to model ambient atmospheric conditions. It is thought that similar thermally induced reactions will occur to both hydrated and dry state molecules and be measurable to an extent to lead to the calculation of kinetic and thermodynamic parameters.

It is also hypothesized that photo-induced oxidative reactions may vary between the dry and hydrated states of DNA and its molecular components. To what extent this
hypothesis is true will be determined via UV radiation induced reactions. It can be assumed however, that aqueous environments will absorb photons differently than dry state molecules which are more likely to undergo scattering or photon deflection. This difference in quantum efficiency between the two states may generate different degradation products.

General Experimental Procedures

In this document experiments that are related to the general topic are presented uniquely. Thus, an experimental methods section is included that is specific to each chapter. However; some general calibration and instrument control procedures were performed to ensure the greatest amount of knowledge of the instruments used and their limitations. This data is presented here.

High Performance Liquid Chromatography and Associated Equipment

One of the main instruments used throughout these studies was an HPLC system consisting of a SpectraSystem P2000 pump and autosampler and a UV6000LP diode array detector
(ThermoElectron USA), equipped with a 5 cm light-path flow cell. Data were acquired and analyzed by a PC using the XCalibur® software package provided by the HPLC manufacturer. Quantities were determined using chromatogram peak areas based on known quantities of known standards. Standards of all deoxynucleotides, deoxynucleosides, nucleobases, and any other reagent necessary were made and calibration curves were generated (Figure 3). The linear dynamic range (LDR) of the UV detector in general has a maximum concentration of 35 ppm. The minimum concentration in the LDR exceeded the minimum concentration detectable in standards. The instrument has a very high signal to noise (S/N) ratio and in most instances analytes were detectable as low as on the order of $10^{-4}$ ppm (Figure 4). The retention times when applicable, stayed consistent enough to easily allow for analyte identification with variation being less then 0.1% of the retention time.

The autosampler pulls up the same amount of solution each time with a standard deviation of less than 2%. Samples of cytosine were injected in triplicate at concentrations of 5, 20, and 30 ppm. The average areas of the chromatogram
peaks were $6.355 \pm 0.06878 \times 10^5$, $1.757 \pm 0.028 \times 10^6$, and $2.291 \pm 0.018 \times 10^6$ respectively. The instrument response was monitored over a period of approximately 1.5 years. A 10 ppm cytosine standard whose spectrum was taken regularly for calibration was examined and plotted in Figure 5. The average chromatogram peak area over this time period was $1.13 \pm 0.08 \times 10^6$. This indicates that the instrument is very stable in its performance over long periods of time.

Figure 3: Example calibration curves for the four nucleobases. All curves are linear with a linear correlation greater than 0.99. Shown here the resulting curves are: $I = 95105[Cytosine]$ (R = 0.993); $I = 113072[Guanine]$ (R = 0.992); $I = 102520[Thymine]$ (R = 0.999); $I = 170671[Adenine]$ (R = 0.999).
The flow accuracy of the pump was determined at a flow of 1 ml/min using a NIST calibrated timer and filling three 5 ± 0.02 ml volumetric flasks. The timer was stopped when the flasks were full. The time required was 5.006 ± 0.01 min. Using standard error analysis in the form shown in Equation 1, the flow rate has an error of 0.004 ml/min, which is insignificant.

$$\delta f(r_1, r_2, ..., r_n) = \sqrt{\sum_{i}^{n} \left( \frac{\delta f}{\delta r_i} \right)^2}$$

Equation 1

Figure 4: (Left) Five samples run consecutively of dehydrated dCMP incubated at 72.8°C. The cytosine can be detected at very low levels (10⁻⁴) with an almost infinite S/N ratio. (Right) The retention times between runs varies by an acceptable amount, where the variation increases with increasing retention time. The same samples whose retention time of cytosine varies by only 0.02 minutes has a variation of retention time for dCMP of 0.11 minutes.
The wavelength calibration was confirmed using a 10 ppm caffeine standard in the UV range below 300 nm. The determined spectrum maxima were found at 203 nm and 271 nm where the true maxima are found at 205 nm and 273 nm respectively. Thus there is a ± 2 nm error on the wavelength below 300 nm.

![Figure 5: (Left) Instrument response for a 10 ppm cytosine sample measured over a 1.5 year time period. The average response is 1.13 ± 0.08 X 10^{-6}. (Right) Gradient verification of pump. The test involved water and a UV active solution consisting of 1 μl acetone in 100 ml methanol. The gradient program is overlaid on top of the normalized UV signal produced by the trace solution. The response of the UV detector is approximately 0.5 min behind the gradient program.]

The gradient accuracy of the pump was determined using two valves of the pump and a flow rate of 1 ml/min. There was no column in line for this experiment and the solvent flowed straight from the pump to the UV detector. In one
reservoir was pure Millipore water and in the other reservoir was a trace solvent consisting of 100 ml methanol with 1 μl acetone as a trace solute that absorbs in the UV region with an absorption maximum at 263 nm. A step gradient from the pure water to trace solvent was formed as follows: 2 min at 100% water; 1 min at up to 100% trace solvent; 2 min at 100% trace solvent; 1 min at up to 20% water; 2 min at 20% water; 1 min at up to 40% water; 2 min at 40% water; 1 min at up to 60% water; 2 min at 60% water; 1 min at up to 80% water; 2 min at 80% water, 1 min at up to 100% water; 2 min at 100% water. The resulting spectrum is shown in Figure 5. It can be seen that the pump responds well to the gradient program; however, each step down should be a decrease in 1/5 the initial absorption. The maximum absorption is 1.19 X 10^5 μAU, therefore every step down should be a decrease of 2.4 X 10^4 μAU. The first and second steps down to 80% and 60% trace solution respectively have an absorption error of 3.7% each where the signal is too low. The next step down to 40% trace solution has an absorption error of 1.1% where the signal is too low. The last step down to 20% trace solution has an absorption error of 5.0% where the signal is too high. Though the expected absorption has some error associated
with it, gradient programs used in this work should be portable to other systems.

**Experimental Error Determination and Analysis**

Experimental data was collected in triplicate unless otherwise stated. In all cases the standard deviation among the measurements far exceeds the error on each individual measurement as determined via the error of the slope of the calibration curve. Obtained data points shown in subsequent graphs and figures are thus an average of the three experimental values and error bars correspond to the standard deviation of the data set comprising each data point solely.

In many instances data obtained in the manner described is further used to determine such values as reaction rates and activation energies via the determination of the slope of said data points. In these situations the error on the slope is determined based upon the best fit to the averaged data without further consideration of the error on each individual data point. In instances where the slope is well defined (high linear correlation) and each data point
has a small standard deviation associated with it this method is adequate. In some instances where reaction rates are small and data have large standard deviations, this method less accurately reports the true error associated with the measurements.

When a calculated value depends on other values obtained, the error on the calculated value is determined via standard error regression Equation 1.
CHAPTER 2: BIOCHEMICAL REACTIONS IN THE DRY STATE: HYDROLYSIS OF DNA AND ITS MOLECULAR COMPONENTS

Introduction

In this report a fundamental chemistry approach was taken to determine dry state DNA component degradation and examine general reactions of hydrolysis on the free nucleobases, nucleosides, nucleotides, dN_{10}, and a forty base pair (bp) duplex. The potential impact of this work is to aid in the possible recovery of genetic information from damaged dry state biological samples commonly encountered by the forensic and ancient DNA communities. If determined what chemical changes the DNA molecule undergoes, to what degree and in what time frame then protocols can be implemented to bypass the impact of this damage or to repair it when necessary. Biobanking applications could also benefit from the data reported here.

It is suspected that similar reactions occur to the dry state DNA molecule as does to the hydrated molecule. It cannot be assumed however that these types of activities
occur to the same extent and at the same rates. Previous studies on the DNA molecule have concluded that in addition to strand separation upon heat exposure there is also the possibility of depurination and chain scission.\textsuperscript{6,7} In general the generic process of hydrolysis encompasses two important reactions, that of deamination and of base loss from the 2’-deoxyribose backbone. Base loss is believed to lead to chain scissions. Many studies have been done in the past on the hydrolysis of DNA in biological conditions.\textsuperscript{5,8-14} Deamination can occur through hydrolysis to three of the four primary bases normally present in DNA (cytosine, adenine, and guanine) (\textbf{Figure 6}). The loss of the exocyclic amino group results in cytosine transforming to uracil, adenine transforming into its oxidized reciprocal hypoxanthine (6-oxy purine), or guanine transforming into xanthine (2-oxy-6-oxy purine). The deamination of DNA purines is a minor reaction compared to that of hydrolytic conversion of cytosine to uracil under biological conditions. The main form of base loss in hydrated DNA occurs through depurination, but depyrimidination can occur as well.\textsuperscript{14,15}
Figure 6: Possible hydrolytic deamination reactions

Figure 7: Possible pathways for hydrolytic deamination and base loss to occur.
The mechanism by which base loss and deamination occurs can involve deamination $\Rightarrow$ de-phosphorylation $\Rightarrow$ base loss, base loss $\Rightarrow$ deamination, de-phosphorylation $\Rightarrow$ deamination $\Rightarrow$ base loss, or de-phosphorylation $\Rightarrow$ base loss $\Rightarrow$ deamination (Figure 7). Here it was examined to what extent base loss, hydrolytic deamination, single strand chain scissions, and de-phosphorylation occurs in the dry state. The determined rates (if applicable) are compared to rates obtained with hydrated samples prepared in the exact same manner only with the addition of pH neutral, sterilized water.

**Methods and Materials**

**Reagents**

All nucleobases, nucleosides, and nucleotides were purchased from Sigma Aldrich (St. Louis, MO, USA). Single stranded oligomers (dT$_{10}$, dC$_{10}$, dA$_{10}$, dG$_{10}$, poly (A$_7$, G$_{13}$, C$_7$, T$_{13}$), co-poly (A$_{13}$, G$_7$, C$_{13}$, T$_7$), poly (A$_6$, G$_8$, C$_5$, T$_{11}$), co-poly (A$_{11}$, G$_5$, C$_8$, T$_6$), poly (A$_4$, G$_5$, C$_4$, T$_7$), co-poly (A$_7$, G$_4$, C$_5$, T$_4$), poly (A$_2$, G$_5$, C$_3$, T$_5$), co-poly (A$_5$, G$_3$, C$_5$, T$_2$), poly (A$_2$, G$_2$, C, T$_2$), co-poly (A$_2$, G, C$_2$, T$_2$)) were cartridge purified (Invitrogen, Carlsbad, CA, USA). The nuclease BAL
31 was purified from the culture medium of *Alteromonas espejiana* BAL 31 containing a mixture of “fast” and “slow” species. One unit of BAL 31 catalyzes the removal of 200 base pairs from each end of linearized pBR322 DNA in a 20 µl reaction mixture in 10 minutes at 30°C at a DNA concentration of 50 µg/ml. The HpyCH4III nuclease was purified from an *E. coli* strain that carries the cloned HpyCH4III gene from *Helicobacter pylori* CH4. One unit is defined as the amount of HpyCH4III required to digest 1 µg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 µl. Both enzymes were purchased from New England BioLabs (Ipswich, MA, USA).

From batches of powdered reagents, 10-20 mg of nucleobases, nucleosides, and nucleotides was dissolved in 10 ml sterilized Millipore water to make stock solutions. The nucleobases are not water soluble at neutral pH. For these, the pH was adjusted to ~12 to solubilize. For the most part, batch reagent nucleobases were used as calibration standards, and as such the aliquot volume compared to the final dilution volume was so small that the final pH variation was negligible. Oligonucleotides were diluted to 1 ml using sterilized Millipore water directly in the vials they came in to make stock solutions. All
experiments were carried out in pure sterilized Millipore water unless otherwise stated.

**Duplex DNA**

A random oligomer, poly (A₇, G₁₃, C₇, T₁₃) was designed to be approximately 50/50 A•T/G•T content. The oligomer sequence is complementary in two regions with a 4 base dimerization possible in both regions. The melting temperature of poly (A₇, G₁₃, C₇, T₁₃) and co-poly (A₁₃, G₇, C₁₃, T₇) was determined to be ~70.8°C. The lack of secondary structures at the melting temperature was verified using free energy calculation with DNA parameters provided by SantaLucia. Annealing occurred via a step down procedure (95°C for 5 minutes; 80° - 60°C for 2 minutes/°C) on a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). To ensure that annealing had occurred, 20 µl of a 28 ng/µl annealed test solution was incubated overnight at 37°C with 1 U HpyCH4III with 2.5 µl buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9 at 25°C). HpyCH4III has a recognition site of 5’..AGNGT..3’ and 3’..TGNCA..5’, where it cleaves in between the nucleobases in bold (there
is one cleavage site available in poly (A₇, G₁₃, C₇, T₁₃) and its co-polymer).

Figure 8: PAGE gel showing double stranded digested poly(A₇, G₁₃, C₇, T₁₃) products. Lane 1: 680 ng 50 bp standard; lane 2: 150 ng duplex; lane 3: 150 ng digested duplex; lane 4: 150 ng poly(A₇, G₁₃, C₇, T₁₃) oligonucleotide; lane 5: 150 ng co-poly(A₁₃, G₇, C₁₃, T₇) oligonucleotide. Gel is 12% polyacrylamide, 120 V supplied for 1 hour. SyberGold stain used to visualize.

Both single stranded oligomers were subjected to the same digestion for control purposes. The 40 bp duplex was successfully digested with the exonuclease and the expected fragments were produced (16/15 bp and 24/25 bp) as verified by PAGE (12% polyacrylamide, 120 V for 1 hour). Neither
single stranded oligomer produced any fragments. It is also worth noting that the single stranded oligomers have different migration rates, probably due to secondary structures, than does the duplex further confirming the successful creation of duplex DNA (Figure 8). The extent of annealing was determined to be 90% using known quantities of one of the single stranded oligomers and comparing band intensity after ethidium bromide staining of the polyacrylamide gel to the un-annealed portion of the duplexed DNA.

Sample Preparation

Samples of nucleobases, nucleosides, and nucleotides were prepared by allocating stock solutions to 1.5 ml microcentrifuge tubes in an amount to supply 90 nmol of the analyte. Ten base homopolymeric oligomer samples were prepared by aliquoting the necessary volume to produce 9.0 nmol quantities into 1.5 ml microcentrifuge tubes. Aliquots of duplexed DNA were allocated to 1.5 ml microcentrifuge tubes in 0.20 nmol amounts. Dry state samples were then created by dehydration using a vacuum centrifuge. Hydrated samples were brought to a 100 µl
volume using steril Millipore water. PCR quality mineral oil was added to the samples to prevent evaporation during incubation.

Prepared samples were kept in a hybridization incubator (Robbins Scientific, Sunnyvale, CA, USA - model 310) at the specified temperatures noted in the text (duplexes were kept at 65.0°C). In general, temperatures ranged from 100°C - 37°C. Samples incubated at 37.4°C were kept in a NAPCO CO₂ 6000 incubation chamber without the use of the CO₂ environment functionality. Samples were incubated over various time periods depending on the detected rate of reactions. For instance, dG₁₀ undergoes depurination very rapidly, so the average incubation period for dry samples was four days. The dCMP molecule undergoes depyrimidination very slowly, so the average incubation period for dry samples was two weeks. The incubator was set digitally to temperatures of 95.0°C, 85.0°C, 75.0°C, 68.0°C, 60.0°C, and 52.0°C. By using two NIST calibrated thermometers it was determined that the actual temperature in the oven is slightly higher than the programmed temperature. A calibration curve was generated (Figure 9), and the actual temperature can be determined by Equation 2.
Figure 9: Real temperature in the hybridization incubator corresponds to the set temperature by: 

\[ \text{Real } T = -6.37(0.76) + 1.17(0.01)(\text{Set } T) \]

Real temperature (circles) in the column heater corresponds to the read temperature by: 

\[ \text{Read } T = 4.97(0.82) + 0.99(0.01)(\text{Actual } T) \]

Values in parenthesis are the errors on the slope and intercept.

\[ \text{Real } T = (-6.37 \pm 0.76) + (1.17 \pm 0.01)\text{Set } T \]

Equation 2

As samples were removed from the incubator they were stored in a -20°C freezer until the whole sample set was ready for analysis. Dry state samples were re-hydrolyzed with 1 ml sterilized water (100 µl for duplexed DNA) and placed in a 56°C water bath for one hour to ensure they were in a homogeneous solution. The samples were then transferred to autosampler vials. Hydrated samples were carefully removed from the microcentrifuge tubes so as to avoid pulling up
the mineral oil. The samples were then transferred to autosampler vials and 0.9 ml of sterilized Millipore water was added to bring the final volume to 1 ml (except for duplexed DNA samples where the volume was kept at 100 µl). The dA₁₀ and dC₁₀ hydrated samples were dehydrated in a vacuum centrifuge once the mineral oil was removed. The samples were then digested by the BAL 31 enzyme in a 20 µl volume overnight with 0.5 U at 37°C in buffer (20 mM Tris-HCl (pH 8 at 25°C), 600 mM NaCl, 12 mM CaCl₂, and 12 mM MgCl₂). The dry state dA₁₀ and dC₁₀ samples were digested by the BAL 31 enzyme in the same manner without the dehydration step required for the hydrated samples. The samples were then transferred to autosampler vials along with 0.98 ml sterilized Millipore water to bring the final volume to 1 ml.

**High Performance Liquid Chromatography (HPLC)**

Samples were analyzed using ion-pairing HPLC. The HPLC apparatus consisted of a SpectraSystem P2000 pump and a UV6000LP diode array detector (ThermoElectron USA) equipped with a 5 cm light-path flow cell and data was collected at wavelengths between 200 and 300 nm. Data were acquired and
analyzed by a PC using the XCalibur® software package provided by the HPLC manufacturer. Separation of the nucleotides was carried out using a Pinnacle II 250 X 4.6 mm, 5 μm particle size C₁₈ column with a 10 X 2.1 mm guard column. The ion-pairing technique was employed using buffers designed by Tavazzi et al.¹⁷ Buffer A (10 mM KH₂PO₄, 0.125% methanol, 12 mM tetrabutyl ammonium hydroxide, pH 7.00), and buffer B (100 mM KH₂PO₄, 30% methanol, 2.8 mM tetrabutyl ammonium hydroxide, pH 5.50) were used in various (v:v) isocratic combinations dependent on what was being separated. A flow rate of 1.0 ml/min was maintained throughout the analysis at ambient temperature (~22°C). For the analysis of hydrolysis of nucleotides, nucleosides, and dN₁₀ various isocratic combinations of buffers A and B were used. Various retention times vs. the ratio of A to B are shown in Figure 10. For dCMP, dC, and dC₁₀ samples an 80:20 A:B combination was used. This allowed for a slower separation and could determine not only depyrimidination products, but deamination products as well. The deamination products looked for were uracil, deoxyuridine (dU), and 2’-deoxyuridine 5’-monophosphate (dUMP). For dAMP, dA, and dA₁₀ samples a 60:40 A:B combination was used. This system allowed for the detection of depurination and
deamination products. The deamination products looked for were hypoxanthine, 2′-deoxyinosine (dI), and 2′-deoxyinosine 5′-monophosphate (dIMP). For dGMP an isocratic 70:30 A:B buffer combination was used. This system allows for the detection of depurination products and will also allow for the detection of xanthine; which is the deamination product of guanine. There was never any xanthine detected in any samples, so it was not looked for after sets of preliminary experiments. For the analysis of dTMP, dT, dT₁₀, and dG₁₀ an isocratic 50:50 A:B buffer system was used. Chromatograms and UV spectra of standard molecular species is shown in Figure 11. Molecular species identification was determined by matching retention times and absorption spectra to prepared standards. The peak areas of hydrolysis products obtained from HPLC-UV absorption measurements were quantified using the program XCalibur® applying an Avalon algorithm of peak detection.

All four naturally occurring nucleobases, 5′-methylcytosine, hypoxanthine, and uracil were incubated in the dry state at temperatures ranging from 54.4°C to 104.8°C. With the exception of cytosine and 5′-methylcytosine, which showed signs of deamination at the
two highest temperatures, the nucleobases did not show any signs of decomposition. The measurement of how much nucleobase is present is the basis for many of the rates determined. Thus, if the nucleobase was deteriorating, the true rate would be higher than observed. This stability allows quantification of the nucleobase concentration as a means to rate determination.

Figure 10: Various retention times of DNA components based on the ratios of buffers A and B. The more buffer A used, the longer the retention time becomes.
Figure 11: Chromatograms and UV spectra of the nucleotides, nucleosides, and nucleobases. The ratios given on the chromatograms detail the type of buffer system used to perform the chromatography.

The recovery percentage was calculated by dividing the number of moles of species recovered by the maximum number of moles possible and multiplying by 100%. For example, 6 nmol of a poly 10-oligomer will theoretically yield a possible 60 nmol of nucleotides. Statistical analysis of data was carried out using ANOVA analysis where the between sample and within sample variances were compared using a one-sided F-test.\textsuperscript{18}

Denaturing HPLC (DHPLC)

Denaturing HPLC is a technique that separates DNA fragments based on size and stability of the duplex.\textsuperscript{19-25} Complete
denaturing was utilized here to detect strand breaks of duplexed poly (A$_7$, G$_{13}$, C$_7$, T$_{13}$). A silica based Helix DNA column (50 X 3.0 mm) by Varian was held at a constant 80 ± 1°C with a Flatron Eppendorf CH-30 column heater with TC-50 controller. Calibration of the heater is shown in Figure 9.

![Denaturing HPLC separation of various sizes of oligonucleotides](image)

Figure 12: Denaturing HPLC separation of various sizes of oligonucleotides (sizes indicated above each peak). Column temperature is 80°C.

An ion-pairing buffer (100 mM triethylammonium acetate, 0.1 mM EDTA) was used to separate DNA fragments based on size. The triethylammonium acetate was made by mixing 1:1
stoichiometric amounts of triethylammine and glacial acetic acid. A gradient program was used with the buffer and acetonitrile in the following manner; 7 minutes at 98-92% buffer; 4 minutes at 92% buffer; 7 minutes at down to 89% buffer. An example of a resulting standard chromatogram is shown in Figure 12. The largest error in elution times occurs with the larger fragments (i.e. 30-40 bases). Peak heights were determined by the distance from the peak maximum to the peak base, measured perpendicular to the ordinate after baseline correction.

**Results**

Dry (and hydrated control) samples comprising appropriate mononucleosides, mononucleotides, and decamers were subjected to heat-induced hydrolysis at various temperatures (~54°C, 63°C, 73°C and 81°C) over periods ranging from 4 days - 2 weeks depending upon the nature of nucleobase. The hydrolysis products were separated and identified by ion-pairing HPLC and were the result of base loss (depurination and depyrimidination), deamination and strand breaks.
Depurination of dRMP and dR_{10}

Adenine was released from all of the dAMP and dA_{10} samples at rates sufficient to be measured (Table 1). The rate of depurination from dAMP in the hydrated state was approximately two orders of magnitude that of the dry state for all temperatures examined. The rates of depurination of dry state dA_{10} are very similar to those for dry state dAMP. The rates of the hydrated dA_{10} were approximately one order of magnitude slower than those for hydrated dAMP. Thus for the dA_{10} samples, the rates of base loss from dry state samples were one order of magnitude slower than for the hydrated samples.

Table 1: Depurination rates (s\(^{-1}\)) of dAMP and dA_{10} (X10\(^7\)).

<table>
<thead>
<tr>
<th></th>
<th>54.4°C</th>
<th>63.4°C</th>
<th>72.8°C</th>
<th>81.2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry State dA_{10}</td>
<td>0.42 ± 0.04</td>
<td>1.6 ± 0.1</td>
<td>2.6 ± 0.7</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>Hydrated dA_{10}</td>
<td>9.8 ± 1.5</td>
<td>12 ± 1</td>
<td>43 ± 8</td>
<td>90 ± 20</td>
</tr>
<tr>
<td>Dry State dAMP</td>
<td>0.32 ± 0.07</td>
<td>0.70 ± 0.09</td>
<td>1.1 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Hydrated dAMP</td>
<td>32 ± 2</td>
<td>180 ± 8</td>
<td>320 ± 50</td>
<td>510 ± 3</td>
</tr>
</tbody>
</table>

The dGMP molecule has shown a much slower rate of depurination than that of dAMP (Table 2). It can be seen that dry state dGMP does not exhibit base loss until the...
highest temperature tested (81.2°C). Hydrated dGMP lost guanine at rates three orders of magnitude slower than for the other purine nucleotide. The release of guanine from dG10 occurred at similar rates to the other purine homopolymeric oligomer in the dry state. The hydrated samples underwent depurination an order of magnitude faster than dA10. Depurination of hydrated dG10 occurred at a rate three orders of magnitude faster than that of hydrated dGMP. These rates show that there is a significant increase in the reactivity of the homopolymeric G oligonucleotide over the dGMP nucleotide. The difference in reactivity between the oligonucleotide and nucleotide was much greater than for the corresponding adenine molecules. The rate differences between dry and hydrated dG10 samples were one to two orders of magnitude, where the hydrated samples underwent depurination faster. In contrast the dGMP underwent depurination 1000 times faster in the hydrated state.

Table 2: Depurination rates (s⁻¹) of dGMP (×10¹⁰) and dG₁₀(×10⁷)

<table>
<thead>
<tr>
<th></th>
<th>54.4°C</th>
<th>63.4°C</th>
<th>72.8°C</th>
<th>81.2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry State dG₁₀</td>
<td>2.6 ± 0.4</td>
<td>2.9 ± 0.6</td>
<td>3.7 ± 0.4</td>
<td>9.4 ± 1.3</td>
</tr>
<tr>
<td>Hydrated dG₁₀</td>
<td>15 ± 1</td>
<td>100 ± 5</td>
<td>110 ± 8</td>
<td>500 ± 40</td>
</tr>
<tr>
<td>Dry State dGMP</td>
<td>N.M.</td>
<td>N.M.</td>
<td>N.M.</td>
<td>0.34 ± 0.7</td>
</tr>
<tr>
<td>Hydrated dGMP</td>
<td>67 ± 5</td>
<td>160 ± 6</td>
<td>520 ± 30</td>
<td>970 ± 50</td>
</tr>
</tbody>
</table>
Figure 13: Base release from the homopolymeric ten base oligomers at 37.4°C. (A) Adenine; \( k = 1.1 \pm 0.1 \times 10^{-8} \text{ s}^{-1} \); \( R = 0.979 \) (B) Guanine; \( k = 2.0 \pm 0.2 \times 10^{-8} \text{ s}^{-1} \); \( R = 0.969 \) (C) Cytosine; \( k = 1.0 \pm 0.05 \times 10^{-9} \text{ s}^{-1} \); \( R = 0.978 \) (D) Thymine; \( k = 1.5 \pm 0.2 \times 10^{-11} \text{ s}^{-1} \); \( R = 0.883 \) (C) Cytosine; \( k = 1.0 \pm 0.05 \times 10^{-9} \text{ s}^{-1} \); \( R = 0.978 \).

In an attempt to measure depurination at more relevant physiological temperatures, dry state depurination was measured at 37.4°C for dG, dGMP, dG10, dA, dAMP, and dA10 for up to a 43 week time period. The nucleosides and nucleotides did not present measurable base loss at this
temperature despite the long period over which samples were monitored. In contrast, depurination of the single stranded decamers was measurable at rates of 1.1 ± 0.1 X 10^{-8} s^{-1} and 2.0 ± 0.15 X 10^{-8} s^{-1} for dA_{10} (Figure 13A) and dG_{10} (Figure 13B) respectively.

Depyrimidination of dYMP and dY_{10}

In general dTMP or dT_{10} were intractable to hydrolytic depyrimidination (Table 3). It can be seen that depyrimidination was only measurable at 81.2ºC for dry state samples. Rates of base loss from hydrated samples occurs on the order of 10^{-9} s^{-1}, which is very slow when compared to adenine and guanine containing molecules. In the hydrated samples, the rates are a little higher for dT_{10} compared to the nucleotide, but on the same order of magnitude. The difference in dry state vs. hydrated state at 81.2ºC is more pronounced with dT_{10} than for the nucleotide. The rate of depyrimidination from hydrated dT_{10} was faster by two orders of magnitude than in the dry state. For hydrated dTMP the rate of base loss was one order of magnitude larger than in the dry state.
The measured rates for the release of cytosine from dCMP and dC₁₀ are given in Table 4. Dry state samples lost cytosine an order of magnitude faster than dry state thymine containing samples lost thymine. The hydrated dCMP underwent depyrimidination two orders of magnitude faster than dTMP. Dry state dCMP underwent base loss two to three orders of magnitude slower than hydrated dCMP. The rate of base loss from hydrated dC₁₀ occurred approximately one order of magnitude slower than from hydrated dCMP. This is similar to what was seen when comparing hydrated and dry state dA₁₀. The difference in rates between hydrated and dry state dC₁₀ was only on the order of one magnitude.

Table 3: Depyrimidination rates (s⁻¹) of dTMP and dT₁₀(X10⁹)

<table>
<thead>
<tr>
<th></th>
<th>54.4°C</th>
<th>63.4°C</th>
<th>72.8°C</th>
<th>81.2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry State dT₁₀</td>
<td>N.D.</td>
<td>N.M.</td>
<td>N.D.</td>
<td>0.65 ± 0.15</td>
</tr>
<tr>
<td>Hydrated dT₁₀</td>
<td>N.M.</td>
<td>8.3 ± 1.0</td>
<td>17 ± 1</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Dry State dTMP</td>
<td>N.D.</td>
<td>N.M.</td>
<td>N.M.</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Hydrated dTMP</td>
<td>1.0 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>7.1 ± 0.3</td>
<td>20 ± 1</td>
</tr>
</tbody>
</table>

Table 4: Depyrimidination rates (s⁻¹) of dCMP and dC₁₀ (X10⁸).

<table>
<thead>
<tr>
<th></th>
<th>54.4°C</th>
<th>63.4°C</th>
<th>72.8°C</th>
<th>81.2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry State dC₁₀</td>
<td>0.35 ± 0.02</td>
<td>0.97 ± 0.09</td>
<td>2.8 ± 0.03</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>Hydrated dC₁₀</td>
<td>3.0 ± 0.1</td>
<td>16 ± 2</td>
<td>25 ± 1</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Dry State dCMP</td>
<td>N.D.</td>
<td>N.M.</td>
<td>0.40 ± 0.04</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Hydrated dCMP</td>
<td>11 ± 2</td>
<td>84 ± 2</td>
<td>130 ± 20</td>
<td>250 ± 40</td>
</tr>
</tbody>
</table>
Like the depurination studies, depyrimidination in the dry state was measured at the more physiologically relevant temperature of 37.4°C for up to a 43 week time period. The nucleosides and nucleotides did not exhibit measurable base loss at this temperature despite the long period over which samples were monitored. However, depyrimidination was measured to occur to dC10 (Figure 13C) at a rate of $1.0 \pm 0.05 \times 10^{-11}$ s$^{-1}$ and to dT10 at a rate of $1.5 \pm 0.2 \times 10^{-9}$ s$^{-1}$ (Figure 13D).

Deamination

Only hydrated samples of dCMP and dC10 produced deamination products with measurable rates. There was no indication of deamination occurring to guanine or adenine containing molecules. At high temperatures of 104.8°C and 93.2°C deamination of dry MeC was detected with low reaction rates of $\sim 10^{-9}$ s$^{-1}$ and $\sim 10^{-10}$ s$^{-1}$ respectively (Figure 14). Dry MeC samples incubated at 37.4°C over a forty-three week time period demonstrated no evidence for the presence of the thymine deamination product.

The deamination of the dry state base cytosine occurred at temperatures of 104.8°C and 93.2°C, but due to the
formation of only trace quantities of uracil it was not possible to determine an accurate reaction rate. It can be seen in Figure 15 that at 104.8°C the original mole percentage of uracil is 0.027% and increases to approximately 0.11% in a two week time period for a net change of 0.083%. At 93.2°C the initial mole percentage of uracil is 0.041% and increases up to 0.068% over the same time period for a net change of 0.027%. At 81.2°C the original mole percentage of uracil is 0.0083% and the final mole percentage is 0.0080% indicating that there is no new uracil formation during the two week time period.

Figure 14: Deamination of MeC. The rate at 104.8°C is $k = 1.7 \pm 0.2 \times 10^{-9}$ s$^{-1}$ (R = 0.959), at 93.2°C thymine increases at a rate of $k = 2.1 \pm 0.7 \times 10^{-10}$ s$^{-1}$ (R = 0.748), and at 81.2°C there is variation in thymine detected from the original trace amount.
Dry state dCMP and dC₁₀ molecules did not produce deamination products at any temperature tested (54.4°C – 81.2°C). In the hydrated state, deamination of dCMP and dC₁₀ was measurable at rates listed in Table 5.

Table 5: Deamination rates (s⁻¹) of dCMP and dC₁₀ (X10⁸).

<table>
<thead>
<tr>
<th></th>
<th>54.4°C</th>
<th>63.4°C</th>
<th>72.8°C</th>
<th>81.2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry State</td>
<td>N.M.</td>
<td>N.M.</td>
<td>N.M.</td>
<td>N.M.</td>
</tr>
<tr>
<td>dC₁₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>1.6 ± 0.1</td>
<td>8.2 ± 0.7</td>
<td>9.9 ± 0.6</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>dC₁₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry State</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>dCMP</td>
<td>3.1 ± 0.1</td>
<td>18 ± 1</td>
<td>28 ± 4</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Hydrated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 15: The deamination of cytosine is seen to occur ever so slightly at temperatures of 104.8°C and 93.2°C, but not at 81.2°C.
De-Phosphorylation and Nucleoside Hydrolysis

To answer the question of whether the nucleobase is released from the intact nucleotide or whether there is an intermediate step of de-phosphorylation to produce the nucleoside and then the nucleobase, the amount of nucleoside present in the reaction mixtures was looked at. In all nucleotide samples the corresponding nucleoside was present at time $t = 0$. In all cases that dealt with the nucleotides, the concentration of nucleoside changed throughout the incubation time period. Sometimes it increased and other times it decreased. Based on the evidence, it can be hypothesized that there is a correlation between how easy the nucleotide underwent base loss and how much nucleoside was produced. The chromatogram area was looked at directly as opposed to converting the chromatographic peak area into molar quantities. This was adequate for these purposes because a trend was being looked for only, and the peak area is proportional to the quantity present.

It can be seen in Figure 16 that the amount of dT increased over time for both hydrated and dry state dTMP across the three highest temperatures. This is interesting because in
the dry state, base hydrolysis was not measurable with the exception of at 81.2°C. As is the case with dTMP, dGMP does not hydrolyze that readily to release its nucleobase. Here too there was a trend noted for the formation of dG. It increased in a linear fashion for both dry and hydrated states; though more readily for the hydrated state. The trend was not noted in the dry state at 54.4°C. These results are shown in Figure 17. Both the dT and dG species were examined to see how readily they undergo base hydrolysis, and both species did exhibit this phenomenon in the temperature range from 104.8°C down to 63.4°C for dT and 54.4°C for dG (Table 6). Measurements made at 37.7°C did not show any hydrolysis products.

Hydrolysis occurred to dAMP the most rapidly of all the nucleotides. The presence of dA was affected by this, particularly in the hydrated samples. For the dry state samples, it appeared that either the dA amount stayed approximately the same or it decreased slightly with increasing temperature. This is believed to be due to the nucleoside itself undergoing hydrolysis to release adenine. This was most pronounced at the highest temperature of 81.2°C. The hydrated samples lost almost all of the dA
over time. This can only be explained by dA being hydrolyzed itself completely. Across all temperatures the dAMP decomposed completely to adenine. It is not until the adenine amount reached 100% of the original dAMP content did the dA disappear as well. This is explainable by the dAMP hydrolyzing first (Figure 18). Adenine is lost from dry state dA at measurable rates from 72.8°C – 104.8°C.

Figure 16: Formation of dT from dTMP at various temperatures.
Figure 17: Formation of dG from dGMP at various temperatures.

The dCMP molecule did not undergo de-phosphorylation in the dry state (Figure 19). In the hydrated state there was a change in the initial amount of dC present in the samples at 63.4°C, 72.8°C, and 81.2°C; however, the amount present did not appear to follow any trend. For example, at 63.4°C the amount increased, but then at 72.8°C the amount decreased. Even though the amount of dC changed in the hydrated state, the amount of dC produced is not substantial in any case compared to the amount of dCMP.
Figure 18: Formation of dA from dAMP at various temperatures.

When examining the homopolymeric oligonucleotides (dN10) it became clear that hydrolysis did occur without the prerequisite of de-phosphorylation. Where the formation of dT was seen to occur in dry state dTMP samples, it was not seen to occur in the dry state dT10 molecule to an extent that was measurable (Figure 20). Hydrated dT10 samples were not probed for dT. In the dry state, dG was looked for at 93.1°C and at 81.2°C from the dG10 decamer. There were no peaks in either temperature’s chromatogram that would correspond with the presence of dG. The hydrated samples were not probed for dG.
Both the dC₁₀ and dA₁₀ oligomers had their respective nucleoside present. The trend for dA₁₀ was similar to what was seen with the dAMP nucleotide (Figure 21), only not so pronounced. The dry state samples kept a constant amount of dA at the two lower temperatures, but at the two higher temperatures there was a trend suggesting the dA was hydrolyzing into adenine.
Figure 20: The amount of dT mononucleoside detected in dry state dT_{10} samples at 54.4°C, 63.4°C, and 72.8°C.

Figure 21: The presence of dA in dA_{10} dry state and hydrated samples at various temperatures.
The dry state dC_{10} samples had dC present at all temperatures and it decreased over time presumably due to hydrolysis as well since there were no other products detected with this method. The hydrated samples had a steeper decline in dC present than did the dry state samples (Figure 22).

![Figure 22: presence of dC in dC_{10} dry state and hydrated samples at various temperatures](image)
Table 6: Rates and activation energies for the de-phosphorylation of dTMP and dGMP and the rates of hydrolysis of the deoxynucleosides

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Dry state dTMP $\rightarrow$ dT</th>
<th>Dry state dGMP $\rightarrow$ dG</th>
<th>Dry state dT $\rightarrow$ Thymine</th>
<th>Dry state dG $\rightarrow$ Guanine</th>
<th>Dry state dC $\rightarrow$ Cytosine</th>
<th>Dry state dA $\rightarrow$ Adenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T$ (ºC)</td>
<td>104.8 93.2 81.2 72.8 63.4 54.4</td>
<td>104.8 93.2 81.2 72.8 63.4 54.4</td>
<td>104.8 93.2 81.2 72.8 63.4 54.4</td>
<td>104.8 93.2 81.2 72.8 63.4 54.4</td>
<td>104.8 93.2 81.2 72.8 63.4 54.4</td>
<td>104.8 93.2 81.2 72.8 63.4 54.4</td>
</tr>
<tr>
<td>Rate $X_{10^9}$ (s$^{-1}$)</td>
<td>620 ± 230 150 ± 22 57 ± 11 4.2 ± 0.3 4.1 ± 0.4 2.8 ± 0.5</td>
<td>850 ± 20 500 ± 60 330 ± 33 31 ± 7 9.7 ± 1.1 N.M</td>
<td>100 ± 15 56 ± 12 34 ± 9 25 ± 4 7.6 ± 1.2 N.D.</td>
<td>2.7 ± 0.9 1.9 ± 0.5 1.5 ± 0.1 N.M. N.M. 0.80 ± 0.16</td>
<td>43 ± 10 64 ± 24 17 ± 4 N.M. N.D. N.D.</td>
<td>32 ± 2 27 ± 4 11 ± 1 6.1 ± 1.0 N.M. N.M.</td>
</tr>
<tr>
<td>$E_a$ (kJ/mol)</td>
<td>120 ± 18</td>
<td>120 ± 24</td>
<td>61 ± 8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Hydrolysis of Duplex DNA

Hydrated State

As revealed by DHPLC, heat induced hydrolysis of hydrated 40 bp duplex DNA at 65°C caused strand breaks that resulted in degradation of the molecule over time (Figure 23 and Figure 24). Initially (at t = 0) the 40 bp duplex was intact except for the presence of trace quantities of short 5–20 base fragments. After 3.5 hours the duplex was still primarily intact but some fragmentation to 17, 18 and 20 base fragments was evident. After 6.5 hours of heat treatment the initial peak associated with the intact duplex was diminished approximately 10,000 µAU (Figure 23).

Figure 23: (Right) Dimunition of the 40 bp duplex over time. (Left) Increase in the amount of free bases over time. The inlay shows the actual rate increase.
After 22 hours the 40 bp duplex was heavily fragmented with a concomitant increase in 16-21 base fragments. At the 30.5 hour time point the 40 bp duplex was no longer present, with the largest observable fragment being approximately 34 bases in length. A distinct fragment approximately 15 bases in length was visible as well as an increase in the 16-21 base fragment class seen earlier. After 45.2 hours the largest fragment was only approximately 20 bases in length and this started to decrease and at 54.5 hours there was an increase in fragments 15, 7, 6 and 5 bases long. After 68.8 hours there was an increased presence of a 12 base fragment and in 5-7 base fragments. After 93.8 hours peaks corresponding to approximately 10, 7, and 6 base fragments were dominant in the chromatogram. These peaks become more pronounced after 117.5 hours along with a peak corresponding with a 4 base fragment. For the remainder of the time up to 478 hours the peaks kept diminishing to a steady state in which peaks corresponding with fragments in the 7-10 base range were visible. Ultimately by 500 hours there were no discernable peaks except for a large peak at 0.8 minutes, which corresponded in retention time with a single mononucleotide. This peak was faintly visible at
time \( t = 0 \) hours and increased steadily during the denaturing process (Figure 23).

![Figure 24: Chromatograms of strand break fragments from hydrated 40 bp DNA.](image)

The samples were analyzed for individual components including nucleobases, nucleosides, and nucleotides. No nucleosides or nucleotides were detected, with the exception of a faint peak consistent with dAMP in several
samples (data not shown). Thus the large peak at 0.8 minutes could be caused by single nucleobases. The release of adenine and guanine from the hydrated duplex occurred at statistically the same rate \( k = 1.15 \pm 0.1 \times 10^{-6} \) and \( k = 1.3 \pm 0.1 \times 10^{-6} \text{ s}^{-1} \) respectively and was complete within 200 hours (Figure 25A). The release of cytosine was detected over this time period and occurred at a rate one order of magnitude lower than the purines \( k = 1.3 \pm 0.1 \times 10^{-6} \text{ s}^{-1} \). The release of thymine was not detected in any samples at any time point. When the individual contributions from adenine, guanine, and cytosine to the total absorption were added it appeared that all three of the liberated nucleobases were the main contributors to the 0.8 min peak up to approximately 200 hours (data not shown). After the 200 hour time period all of the possible adenine and guanine had been released, but the release of cytosine continued. However, the rate of increase in the 0.8 min peak's absorption could not be totally accounted for by cytosine release, and although no thymine or thymine derivatives were detected, it is not possible to preclude the possibility that a thymine containing species was also being released.
Figure 25: (A) Release of three of the natural nucleobases from hydrated 40 bp dsDNA samples. The rates of hydrolysis of the purine bases are statistically the same with $k = 1.2 \pm 0.1 \times 10^{-6}$ and $k = 1.3 \pm 0.1 \times 10^{-6}$ s$^{-1}$ for adenine and guanine respectively and $k = 1.1 \pm 0.05 \times 10^{-7}$ s$^{-1}$ for cytosine. Thymine release was not detected. (B) Release of adenine from dry state 40 bp dsDNA. The rate of release was $k = 5.8 \pm 0.9 \times 10^{-9}$ s$^{-1}$ (R = 0.783); (C) Decrease in the amount of dry state 40 base fragment as measured by decreased absorption. The rate indicated by the (---) line is $k = 3.4 \pm 0.3 \times 10^{-7}$ s$^{-1}$ (R = 0.898).
Dry State

The hydrolysis of duplex DNA in the dry state mediated by incubation at 65°C exhibited a number of significant difference compared to that of its hydrated analogue, including a lower rate of hydrolysis. Initially the 40 bp duplex was essentially intact except for minor fragmentation observed at $\leq 23$ bases (Figure 26) with the main product peaks occurring at 18-20 bases including a prominent 16 base fragment. After 30.5 hours the amount of 14-23 base fragments increased by approximately 200 $\mu$AU, and by 54.5 hours they had increased in concentration as evidenced by an increase in absorption of approximately 900 $\mu$A. The amount of >16 base fragments appeared to stay constant from then on but the main peak corresponding to ~16 bases continued to increase up to the 558 hour time point. Beyond 558 hours few additional changes were observed, except for marginal increases in the concentration of the 16 and 6 base fragments. In contrast to the hydrated state, the amount of base loss detected for the dry state DNA was minimal. The dehydration process itself produced depurination of ~2.5 mole % of the available adenine and guanine. Subsequently, the release of adenine occurred at a relatively slow rate ($k = 5.8 \pm \ldots$)
0.9 \times 10^{-9} \text{ s}^{-1}) (\text{Figure 25B}) with no pyrimidines or further release of guanine detected.

Figure 26: Chromatograms of strand break fragments from dry state 40 bp DNA.

The kinetics of the degradation of the 40 bp duplex was examined in more detail. The reduction in the natural logarithm of the 40 base peak normalized height ratio (\ln H/H_0) over time should follow a linear relationship since the process is expected to be first order and should permit
determination of the rate of formation of single strand breaks. Interestingly, loss of the 40 base peak did not occur at the same rate throughout the entire time period (Figure 25C). The rate was much faster up to the ~200 hour time period and then decreased significantly for the remainder of the experiment (up to 800 hours). It was hypothesized that the initial rate corresponded to rapid depurination that occurred during the dehydrating process itself. Thus, dehydration produced an initial loss of about 5% of the purine bases and the resulting apurinic (AP) sites were susceptible to single strand breaks. Further strand breaks might then be dependent upon the production of more abasic sites that would occur at a significantly lower rate, similar to that measured by the slow rate of adenine release. The measured rate for heat induced strand breaks in the dry state DNA at 65°C was $k = 3.4 \pm 0.3 \times 10^{-7} \text{ s}^{-1}$ ($R = 0.898$). Significantly, this is faster by two orders of magnitude than the release of adenine and is consistent with the hypothesis that the creation of strand breaks is not solely dependent on the creation of abasic sites in the dry state.
Discussion

From the data presented it can be concluded that base loss does occur to DNA components at a measurable rate in the dry state. The incorporation of nucleotides into single-stranded oligomers or duplexes does not prevent such base loss. Depurination has been reported in the past under various conditions to occur more readily than depyrimidination and to occur at similar rates to guanine and adenine analogues.\textsuperscript{14,15,26} Here it was seen that guanine and adenine were not always released at comparable rates from their respective nucleotides in the dry or hydrated states. The release of guanine from dry state dGMP occurred so slowly that it was not measurable in most instances, whereas dry state dAMP released adenine at all temperatures with measurable rates. In hydrated samples at the same temperatures the release of adenine was similar in dA\textsubscript{10} and duplex DNA (poly(dA)\cdot poly(dT)), whereas guanine release from dG\textsubscript{10} was an order of magnitude faster than from the duplex (poly(dG)\cdot poly(dC)). The duplex DNA rate of adenine release differed by three orders of magnitude between the hydrated and dried states. As with dGMP, the rate for guanine release was not measurable with the dry state duplex DNA. For dG\textsubscript{10} and dA\textsubscript{10} oligomers, the rates of
base loss were in agreement for the dry state with slightly more guanine being released.

Table 7: Activation energies associated with base loss and deamination of various molecular components of DNA

<table>
<thead>
<tr>
<th>Entity</th>
<th>State</th>
<th>( E_a ) (kJ/mol)</th>
<th>( A ) (s(^{-1}))</th>
<th>Linear Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>dA(_{10})</td>
<td>Dry</td>
<td>76 ± 8</td>
<td>6.6 \times 10^4</td>
<td>-0.984</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>85 ± 16</td>
<td>2.7 \times 10^7</td>
<td>-0.968</td>
</tr>
<tr>
<td>dAMP</td>
<td>Dry</td>
<td>70 ± 7</td>
<td>5.3 \times 10^3</td>
<td>-0.990</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>96 ± 21</td>
<td>1.0 \times 10^4</td>
<td>-0.957</td>
</tr>
<tr>
<td>dG(_{10})</td>
<td>Dry</td>
<td>73 ± 14</td>
<td>5.4 \times 10^4</td>
<td>-0.949</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>110 ± 26</td>
<td>2.2 \times 10^{12}</td>
<td>-0.952</td>
</tr>
<tr>
<td>dGMP</td>
<td>Dry</td>
<td>N.M.</td>
<td>N.M.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>99 ± 6</td>
<td>4.4 \times 10^7</td>
<td>-0.996</td>
</tr>
<tr>
<td>dT(_{10})</td>
<td>Dry</td>
<td>78</td>
<td>2.5 \times 10^2</td>
<td>-1*</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>71 ± 1</td>
<td>7.7 \times 10^2</td>
<td>-1</td>
</tr>
<tr>
<td>dTMP</td>
<td>Dry</td>
<td>N.M.</td>
<td>N.M.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>110 ± 7</td>
<td>1.8 \times 10^8</td>
<td>-0.996</td>
</tr>
<tr>
<td>dC(_{10})</td>
<td>Dry</td>
<td>84 ± 6</td>
<td>9.8 \times 10^4</td>
<td>-0.993</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>110 ± 17</td>
<td>3.7 \times 10^7</td>
<td>-0.973</td>
</tr>
<tr>
<td>dCMP</td>
<td>Dry</td>
<td>168</td>
<td>-</td>
<td>-1*</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>100 ± 25</td>
<td>8.5 \times 10^7</td>
<td>-0.948</td>
</tr>
<tr>
<td>dC(_{10})</td>
<td>Hydrated - Deamination</td>
<td>89 ± 20</td>
<td>4.3 \times 10^6</td>
<td>-0.954</td>
</tr>
<tr>
<td></td>
<td>Hydrated - Deamination</td>
<td>79 ± 24</td>
<td>1.8 \times 10^5</td>
<td>-0.895</td>
</tr>
</tbody>
</table>

*Only 2 data points available

\( E_a \) = Activation Energy
\( A \) = pre-exponential factor

For hydrated state duplex DNA the rates of release of both purines were in good agreement and were in accord with previous reports. However, the measured depurination rates of dAMP and dGMP were not in agreement with prior
reports. Differences were noted in the rate of release of the pyrimidines with cytosine hydrolysis occurring much faster than with thymine. Indeed for duplex DNA the release of thymine was not measurable in either state at the temperature used. The release of cytosine from duplex DNA occurred at a similar rate in the hydrated state to that of dC10, but was not measurable in the dry state.

The activation energies (Table 7) associated with depurination from dAMP, dGMP, dA10 and dG10 were all statistically indistinguishable by ANOVA analysis ($F_{6,6} = 1.07 \ (p < 0.05, F_{cr} = 4.28)$). Though the rates of base loss for the hydrated state were greater than those for the dry state, the activation energies do not differ significantly. Activation energies determined by Greer and Zamenhof26 for depurination from DNA using two different buffers with differing ionic strength and measurements carried out in the vacuous dry state (Table 8) were statistically indistinguishable by ANOVA to those reported here ($F_{5,5} = 1.94 \ (p < 0.05, F_{cr} = 5.05)$). When these values are compared to the values obtained in the study here all values are statistically the same ($F_{12,12} = 1.11 \ (p < 0.05, F_{cr} = 2.69)$). Additionally Nyberg14 reported the activation
energies for depurination of native DNA to be $129.7 \pm 8.4$ and $121.3 \pm 12.6$ kJ/mol in a Mg$^{2+}$ physiological ionic strength buffer and a 0.1M NaCl, 0.01M NaHPO$_4$, 0.01M Na Citrate buffer respectively. These values are also statistically indistinguishable from the activation energies reported here ($F_{14,14} = 1.14$ (p < 0.05, $F_{cr} = 2.49$)).

Table 8: Activation energies reported by Greer and Zamenhoff$^{26}$ for DNA degraded by heat

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Conditions</th>
<th>$E_a$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine Liberation</td>
<td>0.005M Phosphate buffer I = 8.7 X $10^{-3}$</td>
<td>*112.6 ± 10.7</td>
</tr>
<tr>
<td></td>
<td>0.14M NaCl - 0.015M Na Citrate I = 0.26</td>
<td>129.5 ± 45.4</td>
</tr>
<tr>
<td></td>
<td>Dry State Vacuo</td>
<td>90.3 ± 10.1</td>
</tr>
<tr>
<td>Guanine Liberation</td>
<td>0.005M Phosphate buffer I = 8.7 X $10^{-3}$</td>
<td>121.1 ± 13.1</td>
</tr>
<tr>
<td></td>
<td>0.14M NaCl - 0.015M Na Citrate I = 0.26</td>
<td>109.7 ± 34.3</td>
</tr>
<tr>
<td></td>
<td>Dry State Vacuo</td>
<td>83.7 ± 3.3</td>
</tr>
</tbody>
</table>

†These values were obtained by fitting the author’s data to Arrhenius plots of lnk vs. 1/T.

*This value was reported by the author to be 28 kcal/mol (117.2 kJ/mol) which is not in agreement with my calculation. There was no error reported by the author’s.

The activation energies associated with depyrimidination from dCMP, dTMP, dC$_{10}$, and dT$_{10}$ were also statistically indistinguishable ($F_{4,4} = 2.02$ (p < 0.05, $F_{cr} = 6.39$)).
summary, all activation energies determined in this study for base loss were statistically indistinguishable ($F_{11,11} = 1.26$ ($p < 0.05$, $F_{cr} = 3.82$)). Though the rates of base loss differed depending on the type of base, these results show that the energy necessary for such a process to happen is the same. This is true regardless of whether the molecule containing the base is in the hydrated or dry state.

The thermodynamic formulation of transition state theory can be used to determine thermodynamic parameters. The standard molar Gibbs energy of reaction is given by Equation 3.

$$
\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger
$$

Equation 3

The reaction rate can be expressed by the Eyring equation (Equation 4), similar to the Arrhenius equation, only in this instance the pre-exponential factor is defined as the Boltzman ($k_b$) constant times the absolute temperature divided by Plank’s constant ($h$). Substituting Equation 3 into Equation 4, the rate constant can be defined as in Equation 5. By dividing the rate by the absolute
temperature and taking logarithms of both sides of Equation 5, Equation 6 is generated.

\[ k = \frac{k_B T}{h} e^{-\frac{\Delta G^\ddagger}{RT}} \]

Equation 4

\[ k = \frac{k_B T}{h} e^{-\frac{\Delta H^\ddagger}{RT}} e^{\frac{\Delta S^\ddagger}{R}} \]

Equation 5

\[ \ln \frac{k}{T} = \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT} \]

Equation 6

By plotting \( \ln(k/T) \) vs. \( 1/T \); the resulting linear curve can be used to approximate \( \Delta H^\ddagger \) as a temperature independent value. The entropy is then determined at a relevant temperature (25°C) by determining the rate of reaction at 25°C and entering that value of \( k \) along with 298K as the temperature back into the Eyring equation with the determined enthalpy value. To determine the rate of reaction at 25°C, the Arrhenius plot is extrapolated to that point using the determined activation energy and pre-
exponential factor. Enthalpy and entropy values determined in this manner are given in Table 9.

Table 9: Determinations of enthalpy and entropy values for hydrolytic reactions. The entropy and free energy are evaluated at 25°C.

<table>
<thead>
<tr>
<th>Entity</th>
<th>State</th>
<th>$\Delta H^\ddagger$ (kJ/mol)</th>
<th>$\Delta S^\ddagger$ (J/K•mol)</th>
<th>$\Delta G^\ddagger$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP</td>
<td>Dry</td>
<td>68 ± 6.5</td>
<td>-180 ± 32</td>
<td>120 ± 12</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>94 ± 21</td>
<td>-60 ± 100</td>
<td>110 ± 36</td>
</tr>
<tr>
<td>dA₁₀</td>
<td>Dry</td>
<td>72 ± 17</td>
<td>-170 ± 63</td>
<td>120 ± 25</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>82 ± 16</td>
<td>-110 ± 76</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>dGMP</td>
<td>Dry</td>
<td>N.M.</td>
<td>N.M.</td>
<td>N.M.</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>96 ± 6</td>
<td>-110 ± 28</td>
<td>130 ± 10</td>
</tr>
<tr>
<td>dG₁₀</td>
<td>Dry</td>
<td>40 ± 16</td>
<td>-270 ± 71</td>
<td>120 ± 27</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>110 ± 30</td>
<td>-15 ± 123</td>
<td>120 ± 45</td>
</tr>
<tr>
<td>dTMP</td>
<td>Dry</td>
<td>N.M.</td>
<td>N.M.</td>
<td>N.M.</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>110 ± 10</td>
<td>-94 ± 33</td>
<td>130 ± 12</td>
</tr>
<tr>
<td>dT₁₀</td>
<td>Dry</td>
<td>N.M.</td>
<td>N.M.</td>
<td>N.M.</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>69 ± 1.5</td>
<td>-200 ± 6</td>
<td>130 ± 2</td>
</tr>
<tr>
<td>dCMP</td>
<td>Dry</td>
<td>170</td>
<td>110*</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>100 ± 30</td>
<td>-58 ± 120</td>
<td>120 ± 43</td>
</tr>
<tr>
<td></td>
<td>Hydrated$^\dagger$</td>
<td>76 ± 29</td>
<td>-150 ± 130</td>
<td>120 ± 48</td>
</tr>
<tr>
<td>dC₁₀</td>
<td>Dry</td>
<td>95 ± 6</td>
<td>-110 ± 28</td>
<td>130 ± 10</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>100 ± 20</td>
<td>-68 ± 81</td>
<td>120 ± 29</td>
</tr>
<tr>
<td></td>
<td>Hydrated$^\dagger$</td>
<td>87 ± 20</td>
<td>-120 ± 95</td>
<td>120 ± 35</td>
</tr>
</tbody>
</table>

* only 2 data points to determine $\Delta H^\ddagger$

$^\dagger$ Deamination reaction data

The mechanism by which base loss occurs via hydrolysis has been a subject of debate. The entropies of the transition state can be used to distinguish between a unimolecular and a bimolecular reaction mechanism.$^{10,28,29}$ It has long been believed that hydrolysis is acid catalyzed, yet it still occurs at neutral pH levels. Shapiro and Kang$^{10}$ found that
at neutral pH (3-7) rates of hydrolysis of deoxyuridine, thymidine, and 5-bromodeoxyuridine remain constant. The rates at 95°C and pH < 3 are faster than those in the pH range 3-7, which in turn are faster than rates in the pH range 9-11. Based on their analysis of entropies of activation they determined that an $S_n1$ mechanism was responsible for the uncatalyzed hydrolysis of deoxyuridine derivatives including thymidine. This is in conflict with the data presented here. The entropies calculated are all negative in sign (though could be positive due to the error calculation) with the exception of that for dry state dCMP, for which only two rates were able to be determined. This indicates that there is a different reaction mechanism at play in the scenario here than that previously reported; however, only pyrimidine hydrolysis was examined in the cited literature. It can be noted that if the standard deviation of the curve used to generate the enthalpy values by Shapiro and Kang\textsuperscript{10} is taken into consideration, there is the possibility that the entropies of activation are negative in sign as well. Additionally, it is logical to assume a negative entropy value and a positive free energy value since the DNA molecule should not be compromised easily, thus sustaining life as we know it. This proposed
mechanism suggests rates are truly pseudo first order. Through calculation of the half lives at 25°C (Table 10) the significant stability afforded by the dry state is evident. Since the activation energies are statistically the same regardless of state, the determining factor in the rate difference is the pre-exponential factor (frequency factor). In the dry state, the availability (frequency) of water with sufficient energy to cause a reaction is expected to be significantly reduced.

Table 10: Half lives of dNMP and dN₁₀ at 25°C

<table>
<thead>
<tr>
<th>Entity</th>
<th>State</th>
<th>t₁/₂ (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP</td>
<td>Dry</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>0.15</td>
</tr>
<tr>
<td>dA₁₀</td>
<td>Dry</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>0.65</td>
</tr>
<tr>
<td>dGMP</td>
<td>Dry</td>
<td>N.M.</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>110</td>
</tr>
<tr>
<td>dG₁₀</td>
<td>Dry</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>0.64</td>
</tr>
<tr>
<td>dTMP</td>
<td>Dry</td>
<td>N.M.</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>1000</td>
</tr>
<tr>
<td>dT₁₀</td>
<td>Dry</td>
<td>41000</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>80</td>
</tr>
<tr>
<td>dCMP</td>
<td>Dry</td>
<td>18000</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Hydrated¹</td>
<td>8.6</td>
</tr>
<tr>
<td>dC₁₀</td>
<td>Dry</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Hydrated¹</td>
<td>20</td>
</tr>
</tbody>
</table>
It has been noted that when examining nucleotides, there was a yield of nucleosides. This yield could then contribute to the amount of nucleobase once the nucleosides themselves underwent hydrolysis. The concern then becomes, can a true picture of nucleotide hydrolysis be derived from this data without the interference of nucleoside hydrolysis? When examining dTMP, de-phosphorylation seemed to occur more readily than the release of the base, thymine. Graphs of $[dT]/[dTMP]_0$ vs. time produced curves with measurable rates and subsequently an activation energy of $120 \pm 18$ kJ/mol. It can be concluded from these results that the loss of the 5’ phosphate group is the primary chemistry that occurs to dTMP in the dry state. Since this is the primary reaction noted, it was examined to see if dT produces thymine at all, and again it was determined that hydrolysis does not occur that readily. The maximum amount of thymine produced occurred at 104.8°C, and that was up to ~10% over a fourteen day time period.

De-phosphorylation of the dGMP mononucleotide was the only reaction noted in the dry state. The ability to measure hydrolysis at temperatures up to 72.8°C have been unsuccessful. Graphs of $[dG]/[dGMP]_0$ vs. time produced
curves with measurable rates and subsequently an activation energy of 120 ± 24. Note that the activation energy for the de-phosphorylation of dTMP and dGMP are the same. This is reasonable because the phosphate group is attached to the 5’ position of the deoxyribose backbone and not the base itself. In the dry state the ability for molecules to cluster and interact is hindered by the lack of a fluidic medium. Therefore, it can be hypothesized that the loss of the phosphate group would be a localized event that does not depend on what is attached to the molecule at minimum four bonds away. It was not possible to measure the rate of de-phosphorylation of the dAMP and dCMP mononucleotides because they both underwent measurable hydrolysis. Not only was the nucleotide hydrolyzing, but the nucleoside must have been as well. This would account for the decrease seen in the presence of dC and dA in the time lapsed chromatograms.

Though there was little thymine seen in dry state dT samples, the rates across several temperatures can be deduced and an activation energy of 61 ± 8 kJ/mol can be determined. Similarly for dry state dG, the hydrolysis was measurable leading to the determination of an activation
energy of 25 ± 1 kJ/mol. The hydrolysis of both dG and dT occurred with such small rates, it is believed that there just wasn’t enough dG and dT produced from their respective nucleotides for there to be enough of the nucleobases in the assays for detection. This could explain why there were no measurable hydrolysis rates for the dry state dTMP and dGMP samples at most of the temperatures surveyed. The hydrolysis of dC to produce cytosine only occurred at 104.8°C, 93.2°C, and 81.4°C to a measurable extent. The hydrolysis of dA to produce adenine was measurable and an activation energy of 60 ± 10 kJ/mol was derived. Note that the activation energies for the nucleosides are very low compared to the activation energies required for hydrolysis from the nucleotides and ten base homopolymeric oligonucleotides.

Nucleosides were detected from dA₁₀ and dC₁₀, but not so much from dT₁₀ or dG₁₀. One reason this could be is that the dA₁₀ and dC₁₀ oligomers were digested with the BAL 31 enzyme prior to chromatographic analysis. It has in fact been noted that nucleosides are present in assays that have been digested with the BAL 31 enzyme (author’s data). However; in the hydrated dC₁₀ and dA₁₀ samples the amount of
nucleoside present decreased over time at temperatures in the range of 63.4°C – 72.8°C. If the digestion alone is creating the nucleoside, then there should be a constant amount seen in all samples. Since the loss of the nucleoside was much more pronounced in the dAMP and dCMP samples compared to the dA_{10} and dC_{10} samples it can be concluded that the nucleoside is produced much more readily from the nucleotides.

Detecting nucleoside products from mononucleotides, but not to the same extent for the dN_{10} oligomers was expected. Greer et al.\textsuperscript{26} found that the nucleotides yield nucleosides and inorganic phosphate as well as the bases, but no nucleosides, inorganic phosphate, organic phosphate or deoxyribose are liberated from DNA. This does not mean that it does not occur, it could just occur at such a slow rate that it was not detectable by the methods used. That can explain why deoxynucleosides have been detected in the dN_{10} samples studied here in some instances. Lindahl et al.\textsuperscript{14} found ethanol-soluble material in \textit{B. subtilis} \textsuperscript{14C} DNA after heating at 70°C for eight hours in a saline buffer that did not consist solely of free purine bases. Their method did not allow for the separation of
oligonucleotides, mononucleotides, free pyrimidines, or pyrimidine nucleosides. The method did allow for the detection of purine nucleosides, which none were detected. While studying heat-induced depyrimidination of DNA, Lindahl et al. found the presence of deoxycytidine in their assays after incubation in buffer for fourteen days at 80°C, but the presence of thymidine was not detected. It can be concluded from results here and published elsewhere that the release of nucleosides from DNA and DNA components larger than the mononucleotides is not the primary form of degradation under aqueous conditions. There is evidence presented here that supports the same conclusion for the dry state.

The results here show that hydrolytic deamination does not occur appreciably to individual DNA components studied in the dry state. Cytosine and its derivative were the only samples seen to undergo hydrolytic deamination, and even then either very high temperatures or hydration was required to permit rate measurements. Hydrated samples of dCMP and dC$_{10}$ exposed to heat at 54.4°C - 81.2°C produced deamination reaction rates on the order of $10^{-8}$ to $10^{-7}$ despite the corresponding activation energies being lower
than that required for depurination/depyrimidination. In a study of alkali-induced deamination of DNA, McCarthy et al.\textsuperscript{30} found that deamination of the purine components of DNA occurred at a rate only 7\% of cytosine. It was also noted that deoxycytidine underwent deamination faster than deoxycytidylic acid whose rate was faster than cytosine residues in DNA. This was speculated to be due to electronic repulsion of the hydroxyl ion. Similar results are exhibited here at neutral pH where dCMP underwent deamination slightly faster than dC\textsubscript{10}. In accord with previous studies we found no evidence of deamination occurring to the purine containing molecules.

Lindahl and Nyberg\textsuperscript{11} looked at deamination at a more neutral pH of 7.4, similar to the conditions present in this study. The rates for dC\textsubscript{10} and dCMP are slower at 95°C than what is reported here for dC\textsubscript{10} and dCMP at 81.2°C. The activation energies are much higher as well in accord with the slower rates. In the studies of Lindahl and Nyberg\textsuperscript{11} it was noted that increasing the phosphate buffer concentration did not appreciably increase deamination rates. It was thus hypothesized that deamination events occur by an addition-elimination mechanism with water rather than buffer anion
as the principal reaction. The data here supports this because there was only pure water available to the hydrated samples. The lack of deamination noted for dry state species support this hypothesis as well.

In opposition, it was proposed that free cytidine is deaminated by a buffer anion dependent mechanism (Figure 27) where the base is protonated at N3 followed by the saturation across the 5,6-double bond by addition of buffer anion followed by hydrolytic deamination of the dihydrocytosine intermediate (II → III → IV → VI).9 The alternative route would be analogous to the hydrolysis of an amide (II → V → VI), but has not been reported to have supporting evidence in known reactions involving nucleophilic displacement of the amino group of cytosine. For example, the partial saturation of the cytosine ring is similar to the route established by Brown and Phillips31 for the reaction of hydroxylamine with cytosine derivatives. Intermediate III was found by Johns et al. to be briefly present during the deamination of dihydrocytosine.32 It has also been demonstrated reversible addition of a nucleophile across the 5,6-double bond of a uracil ring.33 In the
absence of buffer anions, deamination of cytidine has been reported on recrystallization from water.\textsuperscript{34}

![Diagram of mechanisms for deamination of the cytosine ring.](image)

Figure 27: Mechanisms for deamination of the cytosine ring. In the case that buffer anions are present the addition across the 5,6-double bond would be done by that species as opposed to water.

Cytosine deamination rate studies done by Lindahl and Nyberg\textsuperscript{11} on denatured E. coli DNA at 100°C and various pH values showed that above pH = 8 the rate increased greatly, but also at pH levels below pH = 8, the rates increases slightly as well. Thus the rate of deamination of cytosine residues in DNA is slowest at pH 8.0-8.5. The reason being
is that the DNA molecule is hypothesized to be the most stable in that pH range and thus has the ultimate amount of protection inherent on the duplex structure disallowing nucleophilic attack. The rates here are much higher because the species studied are not engaged in a duplex formation. A genetic assay developed by Frederico et al.\textsuperscript{35} produced deamination results very similar to those proposed by Lindahl with the same activation energy. It was possible to determine exact rate constants of cytosine deamination at 37°C for single and double stranded DNA as \( \sim 1 \times 10^{-10} \) and \( \sim 7 \times 10^{-13} \) using their assays respectively. Additional work by Frederico et al.\textsuperscript{36} showed that the rate of cytosine deamination in mismatched heteroduplexes resembles that of single stranded DNA due to the instability imposed on the duplexed molecule. It is concluded that the nature of the duplexed molecule provides protection from the deamination event.

The formation of apurinic sites in single and double stranded DNA can lead to chain breaks.\textsuperscript{37} In solution (predominantly B-form DNA) the abasic sites in DNA exist predominantly as a mixture of ring-closed \( \alpha \) and \( \beta \) hemiacetals with a minor percent possibly in the ring-
opened aldehyde and aldehyde hydrate form.\textsuperscript{38,39} The chain breaks are predominantly introduced at the 3’ side of the apurinic sugar moiety between the sugar and the phosphate residue through $\beta$-elimination.\textsuperscript{13} Molecular modeling and NMR studies have shown that hydrated duplexes with apurinic sites adopt a conformation with the neighboring base pairs being closer to one another than in B-form DNA.\textsuperscript{40} The abasic site is more perturbed when the base opposite the site is a pyrimidine resulting in a less thermodynamically stable situation.\textsuperscript{40,41}

Lindahl et al. reported that chain breakage at apurinic sites occurs at a rate of $k = 5.7 \times 10^{-5}$ s\(^{-1}\) at 70°C in 0.1 M KCl, 0.05 M Hepes-KOH, 0.01 M MgCl\(_2\), 0.0005 EDTA (Ph 7.4) buffer.\textsuperscript{13} Here it was reported that, at 65°C, chain breaks occur in the dry state at a minimal rate of $3.4 \pm 0.3 \times 10^{-7}$ s\(^{-1}\), which is much slower than that observed for hydrated samples. There was no evidence of an intact duplex after 33 hours in the hydrated state, where the dry state molecule was still in tact after 700 hours. The half life of a dry state sample based on this analysis is 24 ± 2 days. The rate of chain breakage in the dry state exceeded that of depurination. Thus there must be some other
mechanism by which the DNA molecule degrades into smaller components other than through hydrolysis of abasic sites. For example, oxidatively damaged sites undergo chain scission at a faster rate than apurinic sites.\textsuperscript{42} Thus, it is possible that oxidatively damaged sites or other lesions that were not detectable by the techniques employed here might be responsible for the noted discrepancy in the rates.

An interesting observation made during the course of detecting strand scissions in 40 bp duplex DNA with DHPLC was that the DNA molecule appeared to break down in the same manner for each sample. Each measurement was done in triplicate with three individual samples in their own test tube for both dry and hydrated states. With few exceptions, the chromatograms show the same fragmentation pattern with the same concentration at each time period. Thus the manner of degradation in the hydrated and dry states may not be a random event, but appears to proceed in a mechanistic fashion.

In summary, employing a variety of different purine/pyrimidine base containing monomeric and polymeric
substrates, the mechanistic chemistry of DNA hydrolysis appears to be the same for the hydrated and dry states. The thermodynamic parameters are also similar in both states in that the activation energies for base hydrolysis are indistinguishable. The principle difference between the two states is the rate at which hydrolytic degradation occurs. The dry state offers some protection to the DNA molecule’s integrity in the absence of metabolic repair mechanisms. The biggest threat to the structural integrity of the DNA molecule in dried specimens encountered in ancient and forensic studies appears to be depurination, especially that of the adenine base. Apurinic sites can cause inhibition of the PCR process by ‘jumping PCR’ and which, given sufficient time, will result in the formation of single strand breaks.
CHAPTER 3: BIOCHEMICAL REACTIONS IN THE DRY STATE: THERMALLY INDUCED DEGRADATION OF NAKED HUMAN DNA

Introduction

The break down of extracted intact DNA can be induced using thermal energy.\textsuperscript{6,7,11,26} This type of energy source is encountered over time in ambient natural conditions in warm climates which forensic stains could be subjected to and as DNA samples become buried deep within the earth as is the case with some ancient DNA type samples. Once the activation energy is achieved, the creation of strand breaks, base modifications, and base loss becomes evident in DNA samples not only under hydrated conditions, but under solid-state conditions as well.

Oxygen is a factor that affects DNA degradation. It is suspected that reactive oxygen species (ROS) play a role in the degradation of DNA not only in the case of creating oxidatively modified nucleobases, but through the induction of strand breaks as well.\textsuperscript{43} Though damage by the hydroxyl radical (OH\textsuperscript{•}) and superoxide radical (O\textsuperscript{2−•}) has been documented to potentially occur to all kinds of
biomolecules, it is suspected that singlet oxygen (\( ^1\text{O}_2 \)) plays a large role in DNA degradation.\(^{44-49} \)

Atmospheric molecular oxygen is preferentially in a triplet state (\(^3\text{O}_2\)). Singlet oxygen (\( ^1\text{O}_2 \)) is a metastable state of \(^3\text{O}_2\) with a higher energy. It is a forbidden transition to go from the triplet to the singlet state and cannot be induced by incident radiation based on probabilistic considerations. As such, the lifetime of \( ^1\text{O}_2 \) once formed is very long lived for a high energy molecular state (greater than an hour).\(^{50,51} \) \( ^1\text{O}_2 \) can react efficiently with DNA and it can be generated inside cells through photosensitization and enzymatic oxidation.

It was determined here to what extent heat causes strand breaks to occur to naked genomic DNA in the dry state using temperatures ranging from 65ºC – 100ºC. In addition to strand breaks, the amount of glycosidic bond cleavage causing base loss was measured at most temperatures. It was also determined to what extent \( \cdot\text{OH} \) and \( \text{O}^{2-} \) scavengers (mannitol and TEMPO respectively) and \( ^1\text{O}_2 \) quenchers (\( \text{NaN}_3 \), ascorbic acid, and histidine) could arrest the formation of
strand breaks. Aqueous DNA was also examined in order to have a comparison of dry state behavior.

**Methods and Materials**

In an effort to detect strand breaks of denatured DNA the median average length of the DNA was determined. Upon degradation due to strand breaks, the DNA molecule becomes increasingly shorter in length with increasing time. This degradation can be detected using gel electrophoresis with staining by a fluorescent dye where the resulting bands are degenerate in nature. It can be assumed that upon subjection to one-dimensional gel electrophoresis, fragments are separated solely as a function of their molecular lengths. When a fluorescent dye stained gel is imaged, the summation of DNA distributed through the z-axis at any point on the gel can be defined as a function $F(x,y)$ which is defined by Equation 7.$^{52}$

The limits of integration in Equation 7 are from the bottom to the top surface of the gel. The constant $K$ includes the effect of numerous experimental parameters including the fluorescence quantum yield of dye molecule per base/base
pair, the intensity of the exciting light, exposure time, efficiency of the optical system, and so forth. The variable \( \rho(x,y,z) \) is the number of base pairs per unit volume at the point specified by the coordinates \( x, y, \) and \( z \). The DNA is separated only as a function of its mobility in the direction of electrophoresis. A lane profile as determined by densitometric scanning will determine where and relatively how much DNA is present, and can reveal the median average length of fragments. The two-dimensional distribution \( F(x,y) \) can be reduced to a number of a one-dimensional profile by integrating between lane boundaries along the \( y \)-axis:

\[
F(x,y) = K \int \rho(x,y,z) dz
\]

Equation 7

\[
F(x) = \int_{y_0}^{y_1} F(x,y) dy
\]

Equation 8

where the lane is bounded by \( y_0 \) and \( y_1 \). A background subtraction can be done to offset contribution from non-DNA-specific fluorescence and detector offset.
Sample Preparation

Human placental DNA (Sigma Aldrich, USA) was used in these studies. The product is a highly polymerized DNA that may contain small amounts of mitochondrial DNA. The molecular weight is approximately 14,000 kDa. DNA from human placenta is 42 mole% G-C and 58 mole% A-T. A stock solution of 1.25 µg/µl DNA was prepared using sterilized deionized water (pH ~7.5). Samples were prepared by aliquoting 25 µl stock solution into individual 1.5 ml microcentrifuge tubes. Dry state samples were dehydrated in a vacuum centrifuge and hydrated samples were brought to a 50 µl total volume (625 ng/µl) and PCR grade mineral oil was floated on top to prevent evaporation. As samples were removed from the incubation chamber they were frozen at -20°C until time of analysis. After incubation at temperatures of 65°C, 72°C, and 80°C dry state samples were brought to a 50 µl total volume using sterilized water.

To incorporate additives, additional species were introduced to each sample (15 µg DNA/sample) prior to evaporation for dry state samples and prior to adjusting the final volume (100 µl) for hydrated samples. The following amounts of additives were used: 50 nmol TEMPO, 2
μmol NaN₃, 2 μmol ascorbic acid, 2 μmol histidine, 5 μmol mannitol. Samples were then incubated in a 100°C incubator to induce thermal degradation. Hydrated samples were left for a total time period of 4.5 hours where samples were pulled every ½ hour. Dry state samples were incubated over a total time period of 9 days where samples were pulled every 24 hours. Samples were stored in a -20°C freezer until time of analysis. Dry state samples were re-hydrolyzed to a total volume of 100 µl prior to analysis.

Electrophoresis and Glyoxal Treatment

Electrophoresis was used to determine fragment length (in base pair (bp) or in bases) of DNA samples and to determine PCR products. For the determination of the fragment size resulting from single strand breaks, samples were treated with a DMSO/glyoxal solution. The 1.0 M glyoxal (Sigma Aldrich, USA) solution contained 50% DMSO (v/v) in 10 mM sodium phosphate.

Glyoxal forms adducts with guanine residues in the duplex and inhibits the molecule from taking its native double helical shape (Scheme 1). It is thus possible for single-
stranded DNA (ssDNA) and double-stranded DNA (dsDNA) to be run on the same gel without denaturing conditions since the glyoxal is supplied directly to each individual sample. Also, due to the nature of the glyoxylated single strands, the treated DNA does not take on secondary structures and migrates at the same rate as its double-stranded counterpart. This is a true statement as discovered by experimentation to the extent that the slight variation in migration rates between treated single and non-treated double-stranded DNA generates a much smaller error in size determination than does treating a size standard and trying to decipher the different bands in the resulting "smear".

Scheme 1: The interaction of glyoxal with guanine

![Scheme 1: The interaction of glyoxal with guanine](image)

To demonstrate the nature of glyoxylated DNA, a test gel was stained with 1 µg/ml acridine orange (Figure 28). Acridine orange has a unique property where it will fluoresce green if intercalated to dsDNA, and will fluoresce red if electrostatically bound to ssDNA. This is
a good test for verification purposes, but the use of acridine orange as a stain was not found to be nearly as sensitive as ethidium bromide (EtBr) or SYBRGold®. In addition to the lack of sensitivity, staining of gels with acridine orange has to be done in the dark for long time frames (> 1 hour) and then destained overnight in the dark in an enameled pan, thus causing a much more time consuming protocol.

Figure 28: Gel demonstrates effect of the glyoxal denaturing solution on DNA. Lanes 1 and 2 are the same size fragment of DNA. The fragment in lane 1 was treated with glyoxal denaturing solution where the fragment in lane 2 was not. Same scenario with lanes 3 and 4, and lanes 5 and 6. It can be seen that the migration distance is affected slightly by the glyoxal denaturing solution, but as the fragment size becomes smaller, the difference in migration becomes smaller. The gel was stained with acridine orange which fluoresces red if the DNA is single stranded and fluoresces green if the DNA is double stranded.
Glyoxylation was carried out by the addition of 3 µl glyoxal denaturing solution to 5 µg of heat treated DNA. The reaction took place over one hour at 55°C. 2 µl of 6X loading dye was added to each sample prior to electrophoresis. For non-glyoxyl treated samples, approximately 300 ng of treated DNA was diluted in 5 µl sterilized H₂O along with 1 µl 6X loading dye. Two percent native gels were prepared by dissolving the appropriate quantity of molecular biology grade agarose (Fisher Scientific) in 1X TAE (40 mM Tris acetate, 2 mM EDTA, pH = 7.2). The pH was adjusted of the TAE buffer because as the pH approaches 8.0 glyoxal will dissociate from DNA. Electrophoresis was carried out at 150 V for 2.5 hours. A λ Hind III and 50 bp standard were included on each gel for size calibration. Gels were stained with EtBr (0.5 µg/ml) for ten minutes, and then SYBRGold® (Molecular Probes, Eugene, OR, USA) (0.1 µl/ml) was added and staining was continued for an additional 30 minutes. Gels were visualized on an Omega 12 gel documentation system (UltraLum, Claremont, CA, USA).

Densitometric scanning of gels was performed using the 1D Gel Analysis Software program (Scanalytics, Inc. Fairfax,
VA, USA). The software was instructed to insert one Gaussian peak to fit the densitometric scan for a single sample in a single lane. The maximum height of the Gaussian peak corresponds to the location in the gel where the average length resides. Calibration of a band’s size as a function of migration distance in the gel relative to the size standards was carried out using the 1D Gel Analysis Software. Both the $\lambda$ Hind III and 50 bp size standards were used and a sample calibration curve is shown in Figure 29. Calibration was performed for each gel individually.

Alkaline gel electrophoresis was done using 2% gels made by dissolving the appropriate quantity of agarose in alkaline gel buffer (50 mM NaCl, 1 mM EDTA). Upon hardening, the gel was presoaked for 30 minutes in alkaline running buffer (30 mM NaOH, 1 mM EDTA). To each DNA sample was added an equal volume of alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% glycerol, 0.5% bromocresol green) before loading the entire sample into the well. Electrophoresis proceeded at 60 V for 4.5 hours after which the gel was soaked in a neutralization buffer (1 M Tris-HCl Ph 7.6, 1.5 M NaCl) for 1 hour. The gel was stained using SYBRGold® and visualized.
Figure 29: The top two portions of the figure show the densitometric scan of the 50 bp ladder and the HindIII digested λ DNA along with the picture on the gel. The bottom portion of the figure shows the migration distance vs. the fragment size in a calibration curve.

**DNase I Treatment**

Placental DNA was randomly degraded using DNase I. DNase I is an endonuclease that non-specifically cleaves DNA to
release di-, tri- and oligonucleotide products with 5’-phosphorylated and 3’-hydroxylated ends. The reaction assays consisted of 5 µg DNA in 1X DNase I reaction buffer (10 mM Tris-HCl, 2.5 mM MgCl₂, 0.5 mM CaCl₂, pH 7.6 at 25°C) at 37°C. Varying the time of incubation and concentration of DNase I allowed for the desired amount of degradation to occur. Enzyme inactivation was done by incubating at 75°C for 10 minutes.

**Polymerase Chain Reaction – PCR**

PCR amplification was carried out using primers designed to produce a non-specific 188 and 236 bp product (236 amplimer: 5’-GCCTTTCTATAGCAGGCATGA; 118 amplimer: 5’-CAATTTTTCAAGCCACACC). A 25 µl reaction volume containing 1 ng template was submitted to 32 cycles of amplification using an Applied Biosystems GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA): 95°C for 10 min prior to cycling, 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, a 10 min hold at 72°C after cycling. The polymerase used was AmpliTaq Gold® (Applied Biosystems, Carlsbad, CA, USA). Amplified fragments were detected on a 2% native agarose gel after electrophoresis at 150V for 43
minutes in 1X TAE along with a 50 bp size standard after staining for 30 minutes with EtBr and de-staining in de-ionized water for 15 minutes. Densitometric scanning was performed to obtain the density of the fluorescent signal caused by the sample in the well. This density was then normalized to the density of a 1 ng standard control DNA amplified under the same conditions and run on the same gel.

**Glycosidic Bond Cleavage Detection Using HPLC**

The detection of free bases was carried using ion-pairing HPLC with UV detection. Aliquots were taken and diluted to a total volume of 50 µl producing a final concentration of 187.5 ng/µl which was then subjected to analysis. The HPLC apparatus consisted of a SpectraSystem P2000 pump and a UV6000LP diode array detector (ThermoElectron USA), equipped with a 5 cm light-path flow cell and data was collected at wavelengths between 200 and 300 nm. Data were acquired and analyzed by a PC using the XCalibur® software package provided by the HPLC manufacturer. Separation of the nucleobases was carried out using a Pinnacle II 250 X 4.6 mm, 5 µm particle size C18 column with a 10 X 2.1 mm
guard column. The ion-pairing technique was employed using buffers designed by Tavazzi et al.\textsuperscript{17} Buffer A (10 mM KH$_2$PO$_4$, 0.125% methanol, 12 mM tetrabutyl ammonium hydroxide, pH 7.0), and buffer B (100 mM KH$_2$PO$_4$, 30% methanol, 2.8 mM tetrabutyl ammonium hydroxide, pH 5.5) were used in a 75:25 A:B (v:v) isocratic combination. A flow rate of 1.0 ml/min was maintained constant throughout the analysis. The temperature was that of the ambient (~22ºC).

**Deamination Detection of Cytosine Residues**

Uracil-DNA Glycosylase (UDG) (New England BioLabs, Ipswich, MA, USA) catalyzes the release of free uracil from uracil-containing single and double-stranded DNA.\textsuperscript{56} Heat treated DNA (9.4 µg) was subjected to digestion by UDG overnight at 37ºC in an assay consisting of 5 µl 10X UDG buffer (200 mM Tris-HCL, 100 mM EDTA, 100 mM dithiothreitol, pH = 8.0 at 25ºC), and 5 U UDG. The final reaction volume was brought to 50 µl. One unit is defined by the manufacturer as the amount of enzyme that catalyzes the release of 60 pmol of uracil per minute from double-stranded uracil containing DNA. Quality control assays carried out by the manufacturer showed no degradation of 1 µg λ phage DNA held
overnight at 37°C with 50 U UDG. Also, incubation of 1 µg
\(^3\)H-DNA released < 0.1% radioactivity after 4 hours at 37°C
with 50 U UDG and incubation at 37°C of 1 µg øX174 RF I DNA
resulted in < 5% conversion to RF II proving little to no
endonuclease or exonuclease activity associated with the
enzyme. No deactivation of the enzyme was required, and
assays were analyzed without further preparation using ion-
pairing HPLC as described for base loss detection. A 20
base oligomer containing one uracil residue was designed
(5’-GTCTGAAUTTGGGTCATACT) to ensure digestion by UDG was
occurring as expected. Of four test assays, recoveries
ranged from 85-100%.

**Molecular Assignment and Calculations**

Molecular species identification was determined by matching
retention times and absorption spectra to prepared
standards. The peak areas of hydrolysis products obtained
from HPLC-UV absorption measurements were quantified using
the program XCalibur\(^\circ\) applying an Avalon algorithm of peak
detection. The mole % of material recovered was calculated
by determining the number of moles of each base present in
the sample determined by standard calibration curves and
then dividing by the number of moles possible for each nucleobase in the sample. The number of moles possible was determined using the average molecular weight of intact DNA (2 X 10^{12} \mu g/\mu mol) and the number of nucleobases per mole of DNA (6 X 10^{9} mole base/mole DNA). The number of moles of each nucleobase was then determined using the mole % of the G-C and A-T content of the DNA sample.

**Free Base Determination in Samples Containing ROS Interacting Additives**

Aliquots adequate to deliver 9 \mu g were combined with 10 \mu l 5 M NaCl and 50 \mu l of 100% ethanol. The DNA was allowed to precipitate overnight at -20°C. The free nucleobases do not precipitate and are recoverable at ~100% according to control studies performed using known amounts of the four nucleobases. After centrifugation on high for 15 min, 100 \mu l of the supernate was transferred to a clean microcentrifuge tube and dehydrated via vacuum centrifugation. Sterilized de-ionized water was added in the amount of 50 \mu l and samples were incubated at 55°C to re-hydrolyze. The degree to which free bases were released was determined by measuring the UV absorption at 260 nm of each sample. The molar absorptivity of free bases is
larger than for DNA fragments. The resulting UV absorption will increase as more free bases accumulate in the samples over time. The resulting area of the absorption peak found at 260 nm was determined for each sample. There were three individual samples for each time period of thermal incubation. The areas for samples incubated with DNA alone, mannitol, TEMPO, and NaN₃ were corrected in the following manner: due to differences in the absorption of the additives themselves, the absorption at the zero time point (A₀) was subtracted from each value; the range of absorption values for each additive was normalized to 1.

Results

Glycosidic Bond Cleavage

Over a twenty day time period, there was very little base loss detected for dry state DNA held at 65°C. What bases were detected were i) purines and ii) found in such small random quantities that rates could not be determined. The hydrated state DNA underwent base loss much faster than its dry state counterpart and produced measurable rates for cytosine, guanine, and adenine. Thymine was not detected
in any samples over the twenty days (Figure 30). The rates of base loss were recorded to be $1.4 \pm 0.1 \times 10^{-6} \text{ s}^{-1}$ for cytosine, $1.4 \pm 0.1 \times 10^{-5} \text{ s}^{-1}$ for guanine, and $1.0 \pm 0.1 \times 10^{-5} \text{ s}^{-1}$ for adenine.

Figure 30: (Left) The mole % of material released from hydrated DNA incubated at 65ºC. The rates are $1.4 \pm 0.1 \times 10^{-6} \text{ s}^{-1}$ ($R = 0.923$), $1.4 \pm 0.1 \times 10^{-5} \text{ s}^{-1}$ ($R = 0.921$), and $1.0 \pm 0.1 \times 10^{-5} \text{ s}^{-1}$ ($R = 0.917$) for cytosine, guanine, and adenine respectively. (Right) The mole % of material released from dry DNA incubated at 65ºC. Though adenine and guanine are detectable, the small amount (< 0.25%) does not allow for the determination of a rate.

Increasing the temperature to 72ºC did not result in significant base loss from dry state DNA. Any bases detected in the assays were in very minute amounts that were not formed in a manner that could lead to determination of a rate. Of the bases that were found to
be present in minute quantities in few of the samples, they were the purines.

Using the high temperature of 80ºC to incubate dry state samples, base loss for both adenine and guanine were determined to be $5.0 \pm 0.8 \times 10^{-7}$ s$^{-10}$ and $3.3 \pm 0.6 \times 10^{-7}$ s$^{-1}$ respectively (Figure 31). Note how slow both rates are for these two species when compared to the hydrated state samples. Both cytosine and thymine were detected in the assays, yet did not reveal a rate determining trend with their presence.

In contrast to dry state DNA, that in the hydrated state exhibited base loss at a measureable rate for adenine, guanine, and cytosine (Figure 31) as expected at 72ºC. As previously noted, thymine does not appear as a base loss product. The rates of release for both purine bases are very similar with guanine released at a rate of $1.45 \pm 0.2 \times 10^{-5}$ s$^{-1}$ and adenine released at a rate of $1.6 \pm 0.2 \times 10^{-5}$ s$^{-1}$. Cytosine is released less frequently than the two purine bases with a rate of $2.5 \pm 0.4 \times 10^{-6}$ s$^{-1}$. 
At 80°C, hydrated state DNA exhibited base loss at a rate where almost all purine nucleobases were in solution by eight days. The rates of release for guanine and adenine
are $1.1 \pm 0.1 \times 10^{-4}$ s$^{-1}$ and $1.2 \pm 0.1 \times 10^{-4}$ s$^{-1}$. Not only is base loss measurable for the purine nucleobases, but both pyrimidines are detected with measurable rates as well. The rate of release for cytosine is $2.2 \pm 0.2 \times 10^{-5}$ s$^{-1}$ and for thymine the rate is $4.1 \pm 0.8 \times 10^{-7}$ s$^{-1}$. The release of thymine has been hard to detect compared to the other three nucleobases, so it makes sense here that the rate is very slow even at such a high temperature.

**Strand Breaks**

It is not surprising that there is not a significant degree of strand breaks determined to occur to dry state DNA held at high temperatures since there is not much base loss seen. The formation of abasic sites is a major cause for strand breaks and there is little base loss detected. The median average fragment length was determined by calibrating to the standard lanes and the deterioration of the intact molecule is shown in Figure 32.

The dsDNA showed that it initially had an average length of ~10 kb. This is a little smaller than what is normally seen for placental DNA (~14 kb), but is believed to be a
possible side effect from the dehydration process. Some degradation is seen to occur to the 65°C dry DNA where it appears to be diminished to ~7 kb over the twenty day time period. The samples were still able to be amplified successfully at both 235 bp and 118 bp loci over the entire time period as would be predicted by the median average length and the lack of depurination detected.

Dry state DNA incubated at 72°C showed a little more degradation than it did at the lower temperature. Over the course of the experiment the lowest median average length was determined to be ~1 kb. These samples as well were still able to be amplified successfully at both loci (Figure 33).

The most significant amount of damage detected was expectedly seen in dry state DNA held at 80°C. The decrease in median average length reached ~ 0.5 kb by day 14 (357 hours) and only slightly decreased by ~0.1 kb over the rest of the time period. This degree of degradation did cause a loss in the ability to effectively amplify the 236 bp PCR products. Starting at around 9 days, the detection of products becomes almost too faint to see and
is minimally detectable up through 16 days before no signal is detected. Though in theory there is enough DNA still intact to be able to amplify this size of amplicon, it is believed that the presence of abasic sites in addition to the degraded template leads to PCR failure. The 118 bp amplicon is detectable throughout the entire incubation time period.

Figure 32: Median average length of dry state DNA after incubation at A) 65°C, B) 72°C, C) 80°C. Each time point consists of measurements made on three different samples.
Figure 33: Dry state DNA incubated at 72°C over ~20 days. The first lane on all gels is a 50 bp size standard. A) 236 bp amplicons: Lane 2: Standard DNA; Lane 3: Blank; Lane 4-6: DNA heated for 0 hours; Lane 7-9: DNA heated for 1 day; Lane 10-12: DNA heated for 2 days; Lane 13-15 DNA heated for 3 days. Lane 16-18: DNA heated for 4 days; Lane 19-21: DNA heated for 5 days; Lane 22-24: DNA heated for 6 days; Lane 25-27: DNA heated for 7 days; Lane 28-30: DNA heated for 8 days; Lane 31-33: DNA heated for 9 days; Lane 34-36: DNA heated for 10 days; Lane 37-39: DNA heated for 11 days. B) 236 bp amplicons: Lane 2: Standard DNA; Lane 3: Blank; Lane 4-6: DNA heated for 12 days; Lane 7-9: DNA heated for 13 days; Lane 10-12: DNA heated for 14 days; Lane 13-15: DNA heated for 15 days; Lane 16-18: DNA heated for 16 days; Lane 19-21: DNA heated for 17 days; Lane 22-24: DNA heated for 18 days; Lane 25-27: DNA heated for 19 days. C) 118 bp amplicons: Lane 2: Standard DNA; Lane 3: Blank; Lane 4-6: DNA heated for 0 hours; Lane 7-9: DNA heated for 1 day; Lane 10-12: DNA heated for 2 days; Lane 13-15 DNA heated for 3 days. Lane 16-18: DNA heated for 4 days; Lane 19-21: DNA heated for 5 days; Lane 22-24: DNA heated for 6 days; Lane 25-27: DNA heated for 7 days; Lane 28-30: DNA heated for 8 days; Lane 31-33: DNA heated for 9 days; Lane 34-36: DNA heated for 10 days; Lanes 37-39: DNA heated for 11 days. D) 118 bp amplicons: Lane 2: Standard DNA; Lane 3: Blank; Lane 4-6: DNA heated for 12 days; Lane 7-9: DNA heated for 13 days; Lane 10-12: DNA heated for 14 days; Lane 13-15: DNA heated for 15 days; Lane 16-18: DNA heated for 16 days; Lane 19-21: DNA heated for 17 days; Lane 22-24: DNA heated for 18 days; Lane 25-27: DNA heated for 19 days.
A very different scenario is seen with the break down of DNA in a hydrated state. As already discussed, there is significant base loss occurring to these samples at the temperatures used in these studies. The fragment size decreases greatly just over a twenty-four hour time period (Figure 34). By the time one day had passed the median average length was ~0.6 kb for both the ds- and ssDNA. This size further decreased to < 0.1 kb after 5 days. The 236 bp PCR products amplified very slightly. Again, it is believed that in addition to strand breaks, abasic sites lead to amplification loss. The 118 bp PCR products continued to amplify for another day.

Figure 34: Median average length of hydrated DNA after incubation at A) 65°C, B) 72°C, C) 80°C. Each time point consists of measurements made on three different samples.
Hydrated DNA incubated at 72°C degraded even faster than that at 65°C, and of course 80°C proved even more detrimental to the molecular integrity. At 72°C the median average length decreased to ~0.3 kb after 1 day and was down to ~0.2 kb by day 3. This caused the PCR to fail when attempting to amplify the 236 bp fragment at the 1 day time point. The 118 bp product was detected after 1 day, but was not detected anymore after that (Figure 35). Neither PCR product was able to be detected at the 1 day time point after incubation at 80°C. The median average length at this time point was ~0.1 kb.

Figure 35: Hydrated DNA incubated at 72°C over approximately 5 days. The first lane on all gels is a 50 bp size standard. A) 236 bp amplicons: Lane 2: Standard DNA; Lane 3: Blank; Lane 4-6: DNA heated for 0 hours; Lane 7-9: DNA heated for 1 day; Lane 10-12: DNA heated for 2 days; Lane 13-15 DNA heated for 3 days. Lane 16-18: DNA heated for 4 days; Lane 19-21: DNA heated for 5 days. B) 118 bp amplicons: Lane 2: Standard DNA; Lane 3: Blank; Lane 4-6: DNA heated for 0 hours; Lane 7-9: DNA heated for 1 day; Lane 10-12: DNA heated for 2 days; Lane 13-15 DNA heated for 3 days. Lane 16-18: DNA heated for 4 days; Lane 19-21: DNA heated for 5 days.
Fragment Sizes, Abasic Sites, and PCR Failure

It would be a reasonable assumption that a PCR product of a certain size should be able to amplify if the median average length of the template DNA was greater than or equal to the size of the amplicon. It was seen continuously here that fragment lengths well above the PCR amplicon size failed to amplify. Thus, there must be another underlying phenomenon in the thermal degradation process that inhibits PCR. It was suspected that the introduction of abasic sites in the template DNA that had not quite deteriorated into a strand break were ultimately inhibiting PCR. It has been reported prior that thermally induced error accumulation in the PCR process stems from depurination in addition to deamination and oxidative base damage.57

PCR of both 118 and 236 bp amplicons was attempted after holding hydrated DNA at 80°C for periods of 0-9 hours pulling samples every hour using not only the high fidelity AmpliTag Gold®, but the less faithfull Taq polymerase (ABgene, Rockford, IL, USA). The unmodified Taq, which is known to have a low fidelity can “skip over” abasic sites incorporating the usual adenine as is common in such
situations\textsuperscript{58} and the template will be able to amplify longer despite more degradation. The 236 bp product was not successful using either polymerase after 1 hour of incubation. The 118 bp product was able to amplify with decreasing efficiency up to 2 hours with the Amplitaq Gold\textsuperscript{\textregistered} polymerase, and did amplify for an additional hour longer using Taq polymerase.

Additionally, degraded samples were run on a denaturing agarose gel as described in the materials section. The alkaline conditions are conducive to cleaving abasic sites.\textsuperscript{13} This is the main reason why glyoxylation was used to determine median average length as opposed to denaturing gels. More damage would be induced in the alkaline environment. The fragment size of each piece of DNA after damage was noted on the alkaline gel and compared to the fragment size after glyoxal treatment and separation on a native agarose gel. These sizes were then compared to when the ability to amplify was lost. It can be seen in Figure 36 that when the abasic sites are cleaved the median average length is more in line with that which would not produce a PCR product of the indicated size.
Figure 36: Here it is shown DNA that was thermally degraded which leads to both strand breaks and base loss, specifically depurination. The median average length is measured after electrophoresis with native conditions (■), native conditions after glyoxal treatment (♦), and after alkaline denaturing conditions (▲). The dotted lines indicate where PCR products were no longer detected for the size of amplicons indicated. The dsDNA and ssDNA assumed by native gel conditions with and without glyoxal treatment respectively are of approximate size. However, once separated on an alkaline gel whose conditions cleave at abasic sites, the fragment sizes are seen to decrease greatly as indicated in the text to the right of the graph. The inlaid graph shows the same data with better resolution.

The question was considered when statistically will damage to a DNA template yield a sample that is not able to be amplified using the PCR technique when the damage is strictly strand breaks. To answer this it was obvious that thermally degraded samples could not be used due to the presence of abasic sites that caused read-through
inhibition of the polymerase. The degradation of DNA was carried out using DNase I which will randomly degrade DNA into smaller fragments until it finally reaches the point of di- and tri- nucleotides. By varying the amount of enzyme and the incubation time, fragments of various sizes were created and then subjected to PCR using the standard 236 and 118 bp amplicons used throughout this study.

A moment must be taken to speak of the difference between median average length and the maximum abundant length present. The median average length can be greater than the maximum abundant length because the breaking down of the DNA molecule by DNase I (and thermally induced strand breaks) does not always create a Gaussian distributed densitometric scan (Figure 37). This becomes more evident the smaller the molecule becomes and is believed to mimic the reality of DNA degradation based on the studies conducted here. With that being said, two fragment sizes were used to determine loss of amplification ability. The results are shown in Figure 38.

A normalized value of ‘1’ means that the degraded template DNA amplified as efficiently as the non-degraded control
DNA. A value greater than '1' indicates that it amplified more efficiently and a value less than 1 says that it amplified less efficiently. A value of zero indicates that no amplification product was detected on the gel. It can be seen that the 236 bp amplicon successfully amplified the majority of the time when the average fragment length was greater than 236 bases. However, the reduction in average length did not necessarily hinder the ability to obtain a PCR product until a length of roughly 150 bases.

Figure 37: Two different curves that have the same median average length, but different maximum abundant lengths.

Similar results were seen for the 118 bp amplicon. The ability to amplify continued after the average DNA fragment size was less than the product size. In fact there are still quite a few successful products from fragments that are less than 50 bases in size. Interestingly, there are
differences in the trends noted between the two differently sized PCR products. The larger product continues to amplify well below an average fragment size equal to the product’s size. There are no samples that did not amplify that are larger in average size compared to the product size. The reverse is true for the smaller PCR product. Once the average sample size is smaller than the 118 bases, the majority of samples fail to product a PCR product. In addition to this though, there are PCR failures before the average fragment size is less than 118 bases. It is not until the average size is greater than 135 bases that there are no PCR failures noted.

This was determined to be due to the intrinsic definition of median average length. The more degraded the DNA treated with DNase I becomes, the more the densitometric trace begins to look like the non-Gaussian representation in Figure 37. Thus it would be expected that the successful amplification of the smaller PCR products would decrease or cease prior to the median average length actually reaching the amplicon size. This is exactly what is seen to occur. When the maximum abundant length is
used, the ability to amplify continues well below the amplicon size for both PCR products.

Figure 38: The ability to amplify two different sized amplicons using the PCR technique vs. the average single stranded template size. The density is a measurement of the intensity of the band and is normalized by dividing by the intensity of a control sample amplified with all samples and run on each gel along with the test samples. The top figure corresponds with a 118 bp amplicon, and bottom to a 236 bp amplicon. The red dashed line indicates on the graph the size of the amplicon used.
Deamination of Cytosine Residues

A subset of DNA samples that were incubated at 80°C were treated with UDG as described under the materials section and ion-pairing HPLC was used to determine the molar amount of uracil released. DNA in the dry state did not show the presence of any uracil at the level of detection of the instrument (pmol). DNA that was incubated in aqueous conditions did exhibit uracil formation in a first order fashion (Figure 39).

Figure 39: Release of uracil from UDG treated DNA samples after incubation at 80°C. The percent amount of uracil detected was determined by dividing the moles of uracil by the moles of cytosine in each sample.
For hydrated DNA, the rate of cytosine deamination was calculated to be $8.5 \pm 0.2 \times 10^{-8}$ s$^{-1}$ when examined for up to one week of heat exposure. DNA incubated at temperatures lower than 80ºC were not tested since there was no reaction in the dry state.

**ROS Species Affect on Thermal Degradation**

This portion of the study was conducted on dry state and hydrated placental DNA incubated for 9 days and 4.5 hours respectively at 100ºC. Additives were used as described in the materials section above.

The hydrated DNA degraded very rapidly as expected over the incubation time period. As can be seen in Figure 40, treatment with TEMPO and mannitol did not produce results that were significantly different from the control DNA. The addition of NaN$_3$ did keep the DNA from degrading into smaller fragments as rapidly as the control samples. Both glyoxal denatured and non-glyoxal treated DNA samples showed almost identical degradation patterns in all cases examined. These results indicate that $^{1}$O$_2$ is possibly forming in the reaction environment and subsequently
playing a role in DNA degradation. Specifically when there is sufficient thermal energy supplied.

Figure 40: Hydrated DNA subjected to thermal degradation over 4.5 hours. The median average length was determined using densitometric scanning of glyoxal denatured fragments separated on a 2% agarose gel.

Due to the nature of the ‘dry state’ it was considered that the addition of additives to the dry state DNA would not influence the interaction of reactive species with the DNA. Surprisingly, similar results were obtained with the dry state samples as was obtained with the hydrated samples. Again NaN₃ was able to slow the degradation indicating \(^1\)O₂ as a possible source for damage (Figure 41). The other two additives showed the same amount of degradation via strand
breaks as did the control samples and it did not matter whether the samples had been treated with glyoxal or not.

![Dry State DNA graph]

**Figure 41:** Dry DNA subjected to thermal degradation over 9 days. The median average length was determined using densitometric scanning of glyoxal denatured fragments separated on a 2% agarose gel.

The TEMPO treated samples showed considerable damage at the initial time point corresponding to no incubation, but having been dehydrated. The TEMPO molecule itself could lead to DNA degradation under certain circumstances. This is important to realize that the additives themselves can lead to damage beyond what would be normally expected for DNA exposed to high temperatures. This was noted when attempting to use ascorbic acid as a singlet oxygen
quenching additive. The ascorbic acid degraded the DNA so fast that at the initial time frame there was very little left in tact and after ¼ hour of incubation hydrated samples could not be detected on the gel anymore.

In addition to determining that NaN₃ protects DNA from thermally induced strand breaks, it was also determined that it provides protection from base loss as well. This was determined by looking at the increase in absorption of DNA in both states at 260 nm (Figure 42). The hydrated state DNA clearly showed that the absorption increased the same with the addition of mannitol and TEMPO as it did without the addition of either additive. However, in the presence of NaN₃ there was no increase noted in the contribution to the absorption spectrum at all.

Dry state DNA samples without any additives had an absorption increase similar to that seen with the addition of mannitol and TEMPO. The NaN₃ treated samples showed a decrease in the release of contributing material, but it was not as pronounced as it was for the hydrated state samples. This is most reasonably due to the fact that the dry state samples do not experience base loss nearly as
rapidly as does hydrated DNA, thus there is less of an effect to have an impact on.

Figure 42: Increase in absorption at 260 nm of thermally degraded samples with various additives to quench or scavenge ROS (NA = no additive). (Left) Dry state DNA (Right) Hydrated DNA.

In addition to NaN₃, histidine was used as an additive to see if it could slow the rate of strand breaks in hydrated DNA incubated at 100°C since it is a known ¹O₂ quencher as well.⁴⁹ Some interesting phenomena was noted when attempting to look at histidine as a preservative. First, just by taking hydrated DNA in de-ionized sterilized water alone and adding 2 µmol histidine and then treating for 1 hour at 55°C with glyoxal denaturing solution degradation was noted to produce a smear of approximately 0.6 kb on an agarose gel. When the same was done without the glyoxal
denaturing step, the DNA was still in tact. However, after 30 minutes at 100°C in the presence of histidine without glyoxal treatment the DNA was degraded to a fragment of approximately 50 bases. It has been noted that at 100°C the oxygen effect on thermal induction of DNA degradation can be ignored. It is shown here that is not entirely the case. Though the DNA did degrade at this high of temperature in both dry and hydrated states, the preservation power of NaN₃ was still noted to occur indicating that ¹⁰₂ might play a role.

Zhang et al. reported that incubating naked DNA in sterilized water alone at 75°C for 90 minutes in the presence of both histidine and NaN₃ resulted in the DNA staying in tact. Without the presence of these additives or the addition of OH• and O²• scavengers degradation was measured. Why the addition of histidine at 75°C offers protection, but its addition at 100°C does not is not fully understood. It is also noted that histidine appears to have an adverse reaction when mixed with glyoxal and DNA that causes damage to the DNA molecule. Glyoxal itself can be an oxidizing agent on DNA as is exploited through is use here as a guanine binding reagent to separate and linearize
DNA. This dual behavior has been reported before for at least one $^{1}\text{O}_2$ quencher, cysteamine. Cysteamine is a potent antioxidant that will react with and protect against $^{1}\text{O}_2$ intracellularly, but enhances $^{1}\text{O}_2$ induced damage to isolated DNA in vitro.$^{48,59}$

**Discussion**

It can be determined that the temperature and duration of heating influence the thermal degradation of DNA. The studies done here were on naked human DNA. It is possible that the results given here could vary for cellular DNA. One argument could be that damage would occur to a lesser extent due to the added protection of the cellular environment. To the contrary, Zhang et. al.$^{43}$ provided evidence that cellular DNA is more damageable than isolated DNA due to the existence of lipids inside the cell which can undergo lipid peroxidation. The products of lipid peroxidation can lead to a propagation of free radical reactions$^{60}$ enhancing thermal degradation of the DNA molecule.
It has been reported in previous studies conducted by this author (see Chapter 2 of this thesis) that a short duplex DNA does not exhibit a high degree of base loss in the dry state when held at a temperature of 65°C (~10^{-9}/s). The studies on dry state human placental DNA conducted here hold further evidence that base loss occurs to an even lesser extent when the overall size of the DNA molecule is increased, as base loss was not even measurable at 65°C or 72°C. The temperature had to be held at 80°C in order to recognize depurination, and even then the rates were of magnitude 10^{-7}/s. Oddly enough, human DNA incubated at 65°C in the hydrated state released both purine bases an order of magnitude faster than did the short duplex (40 bp) DNA tested in prior studies (10^{-5}/s vs. 10^{-6}/s).

The median average length of dry state DNA is not impacted greatly when compared to its aqueous counterpart by heating. Hydrated human DNA degraded so quickly, that its length was decreased to only 7.5% of its original size overnight at 65°C. There was no significant size difference noticed between dsDNA and ssDNA for DNA in either state. Remarkably, the dry state DNA did not fragment worth noting at all after 20 days of being held at
65°C. It was not until 80°C did the DNA fragment to an extent that caused the loss of the ability to amplify a 236 bp PCR product, but the 118 bp product was still detected.

When the DNA was damaged to an extent to inhibit PCR, the median average length was high enough to where strand breaks alone could not be responsible. The 80°C dry state DNA stopped amplifying a 236 bp product when the length was ~0.6 kb. At some point between 1 and 2 days, hydrated DNA held at 65°C lost the ability to amplify a 236 bp PCR product. The median average length at 2 days was ~0.4 kb. The ability to amplify the 118 bp product was gone after 3 days when the length was ~0.2 kb. Hydrated DNA held at 72°C lost the ability to amplify the 236 bp product prior to 1 complete day when the fragment size was measured to be ~0.3 kb. The 118 bp product was seen to lose the ability at some point between 1 and 2 days. The median average length though measured at 1 day and 2 days are 0.32 kb and 0.31 kb respectively. Since there wasn’t much of a difference in the length during the time period, the loss of ability to amplify has another influential factor. After 1 day, 80°C hydrated DNA was no longer amplifying, though in theory there could still be enough intact
template to produce a 118 bp product (~0.1 kb). The fragment size when the DNA truly lost the ability to amplify was present prior to the 1 day time mark when analysis was done. It is believed based on this evidence in addition to the known rate of base loss, that abasic sites in addition to strand breaks play a big role in the successful amplification of degraded DNA samples. It has been reported elsewhere that after thermal degradation of naked DNA an 889 bp segment could not be amplified though 1 kb-sized fragments still existed. The authors explanation for this observation was that abasic sites could be responsible as well.

Deamination of human naked DNA was looked at to decide to what extent it played a role in the degradation of the macromolecule. In the dry state, even after 19 days of a constant 80°C temperature, dry state DNA did not exhibit even a hint of uracil present after treatment with UDG at the level of detection available. The hydrated DNA on the other hand had converted approximately 7% of its cytosine residues into uracil within a 1 week time period at the same temperature. Though deamination plays a role in hydrated DNA degradation, it does not for the dry state.
Previous studies using a genetic assay employing ung-bacterial strains lacking the enzyme uracil glycoylase to measure cytosine deamination determined that dsDNA converted cytosine to uracil at a rate of $7 \times 10^{-13} \text{ s}^{-1}$ at 37°C (pH = 7.4), which is a half-life of 30,000 years. The same authors reported ssDNA to convert cytosine to uracil at a rate of $1 \times 10^{-10} \text{ s}^{-1}$ (half-life of 200 years).

The extent that dry state DNA undergoes glycosydic bond cleavage and strand breaks is very minor when compared to hydrated DNA. It is rather amazing that a two week incubation at 80°C produced such little degradation of the dry state molecule. From the studies conducted here it can be concluded that naked genomic dry state DNA does not undergo deamination to any extent that would be believed to interfere with any current day typing technique. The cleavage of glycosidic bonds is detectable at temperatures that are much higher than would ever be expected to be encountered in the ambient, and again should not be of significant importance to dry state DNA degradation. The accumulation of strand breaks appears to be the major cause of concern for dry state DNA, but again is surprisingly minimal. The accumulation of strand breaks and base loss
collectively has been determined to lead to the inability to perform the common PCR on dry state forensic type samples. The consequence of course would be the loss of evidentiary data.

This study has provided evidence that $^{1}\text{O}_{2}$ may play a role in thermal DNA degradation. This evidence consisted of the use of NaN$_3$, a known quencher of $^{1}\text{O}_2$ in the preservation of not only hydrated DNA, but dry state DNA as well during incubation at 100°C. There was no direct measurement of $^{1}\text{O}_2$ in the experimental setup which consisted of a dark incubator holding a very high temperature. Since $^{1}\text{O}_2$ has an excitation energy associate with it (0.98 eV$^6$) its formation must be temperature dependent. Bleaching experiments using 1,3-diphenyl-isobenzofuran (DPBF), an efficient trapping agent of $^{1}\text{O}_2$, showed that in the dark $^{1}\text{O}_2$ can be generated in increasing amounts by increasing the temperature.$^{43}$ An additional consideration for the formation of $^{1}\text{O}_2$ could include the fact that samples were kept in polypropylene tubes that in some instances contain polynuclear aromatics (i.e. naphthalene and anthracene). Polynuclear aromatic compounds can induce the formation of
Further studies are needed to confirm the role of this species in thermal DNA degradation.
CHAPTER 4: BIOCHEMICAL REACTIONS IN THE DRY STATE: UV IRRADIATION INDUCED INSULTS TO DNA

Introduction

Oxidative DNA damage can be caused by ambient solar radiation. The extent of this damage can be dependent upon the energy associated with this radiation. Ultraviolet radiation from the sun is of the types UVA (320-400 nm), UVB (290-320 nm), and UVC (100-290 nm). The most damaging of these rays are absorbed by the atmosphere and do not have much affect on life at the planet’s surface. The type of radiation that does reach the planet’s surface is primarily UVA and is less damaging than the more energetic forms. The effects of UVA radiation can still cause damage to biomolecules such as DNA. Reports have established that UVA can induce photo-oxidation of guanine bases to form 8-hydroxy-guanine (8-oxoG) in both DNA and RNA.\textsuperscript{59,64,65}

Irradiation by UVC has been reported to substantially induce the formation of 8-oxoG in purified DNA when oxygen is present.\textsuperscript{66} 8-oxoG will mispair with A causing GC $\rightarrow$ TA transversion mutations. This could have an impact on single nucleotide polymorphism (SNP) analysis.
For forensic type samples found in the outside environment there is always a concern as to the extent of damage induced by the sun’s radiation. If biological samples become too damaged they lose their ability to be typed and subsequently used as evidence. It was examined here to what extent UVC and UVA radiation cause DNA damage in the dry state, such as would be encountered in most crime scene scenarios. Though UVC is more powerful than the ambient UVA, it can be used as a model to deduce possible damaging pathways caused in more realistic situations. Damage generated via UVA radiation upheld this hypothesis.

Nucleobases are targets for oxidation-reduction activities due to their intrinsic reactivities with electrons. The bases alone are favorable to undergo one-electron reduction reactions and this reactivity is conserved when incorporated into nucleosides. However, when the phosphate group is added, the reactivity decreases, most likely due to electronic repulsion by the phosphate group. A one-electron oxidation of nucleobases is a common reaction that is not discouraged by the incorporation into the larger DNA molecule. Results from ESR experiments have led to the hypothesis that centers of electron loss in the DNA
molecule end up on the purines (primarily guanine) and the ejected electron ends up primarily on thymine.\textsuperscript{71,72} It is believed that the electron gain and loss require overlap of the $\pi$-systems of the donor (guanine) and acceptor (thymine), a condition met by base stacking along the helical axis. This type of reaction is expected in DNA leading to the formation of oxidized guanine (8-oxoG). The radicalized form of this product further leads to the formation of 5-formamido-2,6,-diamino type products (FAPy).

Oxidative damage can be induced through a one-electron process involving reactive oxygen species such as the hydroxyl radical (OH•), hydrogen peroxide, and singlet oxygen (\textsuperscript{1}O\textsubscript{2}). Exposure of biological samples to agents such as solar radiation can induce the formation of superoxide and hydroxyl radicals. The main classes of damage include oxidized bases, abasic sites, DNA-DNA intrastrand links, DNA strand breaks, and DNA-protein cross-links.\textsuperscript{73-75} Here it has been examined to what extent DNA strand breaks are induced by UVC/UVA radiation and it is determined to what extent certain base damage occurs under the same conditions using formamidopyrimidine glycosylase (FPG).
FPG is a DNA glycosylase with associated lyase activity. It has the ability to recognize several oxidatively damaged lesions in DNA such as open ring forms of purines, 8-oxoG, 7-methylguanine, 5-hydroxycytosine, 5-hydroxyuracil, and 8-oxoadenine.\textsuperscript{76-78} The enzyme will remove damaged bases creating an apurinic site. The associated AP-lyase activity will then cleave both 3' and 5' to the site leaving a one base gap.

The level of FPG sensitive sites (ignoring AP sites) can be rather low. In addition to looking at naked human DNA in this study, short 30 base oligomers were used with a method developed by Margolin\textsuperscript{79} to examine oxidative base damage induced by UV radiation that offers a pathway to remove the background “noise” associated with DNA strand breaks which a large portion of the damage consists of. The oligonucleotides were designed with phosphorothioate-modified 3’ ends which are not susceptible to digestion by Exonuclease (Exo) III. Damaged oligomers were irradiated and then subjected to treatment with Exo III. Any oligomer that contained a strand break would be digested since the protective phosphorothioate 3’ terminals would be removed. Additionally, due to Exo III being the major AP
endonuclease of *Escherichia coli*, abasic sites will be digested as well.

Figure 43: Approach to look at oxidative base damage in 30 bp oligomers. ‘X’ represents an oxidatively damaged base and \( h\nu \) indicates UV radiation.

The samples after purification are then subjected to FPG digestion and separated electrophoretically to look for strand breaks caused by these treatments (Figure 43).

**Materials and Methods**

Naked DNA and short 30 bp synthetic duplexes in hydrated and dry states were exposed to UV light in a Stratalinker 1800 (Strategene, LaJolla, CA, USA). UVC and UVA radiation consisted primarily of sharp peaks at 254 and 365 nm respectively. The flux of the UVC and UVA lamps was 179 ±
9 and 147 ± 3 mJ/cm²/min respectively. After exposure samples were stored in the dark at -20°C until evaluation.

**UVC Exposure of Naked DNA**

Placental DNA was hydrolyzed to a concentration of 1.25 µg/µl and aliquoted into individual samples of 15 µg in polypropylene microcentrifuge tubes that offer some transparency to UVC rays. The lids were left open, and samples were positioned in such a manner that the radiation could access the sample without passing through the tube. The dry state samples were subsequently evaporated in a vacuum centrifuge prior to radiation exposure. The hydrated state samples were brought to a total volume of 100 µl before exposure. The time period that the hydrated samples were exposed was much shorter than that for the dry state samples. Any evaporation that occurred was determined to be negligible.

**Formamidopyrimidine Glycosylase (FPG) Treatment of DNA**

6 µg of each irradiated sample was incubated at 37°C overnight with 16 U FPG (New England Bioloabs, Ipswich, MA,
USA) in 1X reaction buffer (10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM EDTA, 0.1 mg/ml BSA) in a total volume of 50 µl. The enzyme was deactivated by incubation at 65°C for 20 minutes. After treatment samples were subjected to glyoxal denaturation as described below.

One unit is defined as the amount of enzyme required to cleave 1 pmol of a 34-mer oligonucleotide duplex containing a single 8-oxoguanine base paired with a cytosine in 1 hour at 37°C in a total reaction volume of 10 µl. Quality control was conducted by the manufacturer and it was determined that there was no nuclease degradation occurring to Hind III digested λ DNA after 16 hours incubation at 37°C. Exonuclease activity was also determined to be lacking where a mixture of single and double-stranded [³H] E. coli DNA was incubated for 4 hours at 37°C where < 1.0% of the total radioactivity was released.

**Denaturation and Agarose Gel Electrophoresis**

To determine median average length of UVC exposed DNA, samples were denatured using a glyoxal denaturing solution (1 M glyoxal, 50% DMSO, in phosphate buffer, react for 1 hr
at 55°C) and subjected to 150 V for 2.5 hrs on a 2% agarose gel (1X TAE pH 7.2 running buffer). A Hind III digestd λ DNA standard and a 50 bp ladder were loaded as sizing standards in their own lanes. Median average length was determined using densitometric scanning (1D Gel Analysis Software program; Scanalytics, Inc. Fairfax, VA, USA) of the gels after staining for 10 minutes with EtBr and 30 minutes with SybrGold® and visualized using an Omega 12 gel documentation system (UltraLum, Claremont, CA, USA). Calibration was done against the molecular weight standards.

**Glycosidic Bond Cleavage Detection Using HPLC**

Ion-pairing HPLC with UV detection was used to determine free natural nucleobases in solution after UVC exposure. Aliquots adequate to deliver 9 µg were combined with 10 µl 5 M NaCl and 50 µl of 100% ethanol. The DNA was allowed to precipitate overnight. The free nucleobases do not precipitate and are recoverable at ~100% according to control studies performed using known amounts of the four nucleobases. After centrifugation on high for 15 min, 100 µl of the supernate was transferred to a clean
microcentrifuge tube and dehydrated via vacuum centrifugation. Sterilized de-ionized water was added in the amount of 50 µl and samples were incubated at 55°C to re-hydrolyze.

The HPLC apparatus consisted of a SpectraSystem P2000 pump and a UV6000LP diode array detector (ThermoElectron USA), equipped with a 5 cm light-path flow cell and data was collected at wavelengths between 200 and 300 nm. Data were acquired and analyzed by a PC using the XCalibur® software package provided by the HPLC manufacturer. Separation of the nucleobases was carried out using a Pinnacle II 250 X 4.6 mm, 5 µm particle size C_{18} column with a 10 X 2.1 mm guard column. The ion-pairing technique was employed using buffers designed by Tavazzi et al.\textsuperscript{17} Buffer A (10 mM KH_{2}PO_{4}, 0.125% methanol, 12 mM tetrabutyl ammonium hydroxide, pH 7.0), and buffer B (100 mM KH_{2}PO_{4}, 30% methanol, 2.8 mM tetrabutyl ammonium hydroxide, pH 5.5) were used in a 60:40 A:B (v:v) isocratic combination. A flow rate of 1.0 ml/min was maintained constant throughout the analysis. The temperature was that of the ambient (~22°C).
Molecular species identification was determined by matching retention times and absorption spectra to prepared standards. The peak areas obtained from UV absorption measurements were quantified using the program XCalibur® applying an Avalon algorithm of peak detection. The mole % of material recovered was calculated by determining the number of moles of each base present in the sample determined by standard calibration curves and then dividing by the number of moles possible for each nucleobase in the sample. The number of moles possible was determined using the average molecular weight of intact DNA (2 X 10^{12} \mu g/\mu mol) and the number of nucleobases per mole of DNA (6 X 10^9 mole base/mole DNA). The number of moles of each nucleobase was then determined using the mole % of the G-C and A-T content of the DNA sample.

**Synthetic Oligonucleotide UV Exposure and Enzyme Treatments**

Oligonucleotides were designed as follows: 5’-Bio-CGT-ACT-CTT-TGG-TTG-ATG-GGT-TCT-T*C*T*A*T where the ‘*’ indicates a phosphorothioate linkage and the forward oligonucleotide was biotinylated at the 5’ end. Both forward and reverse oligonucleotides were ordered cartridge purified via
Invitrogen, USA. Annealing occurred via a step down procedure (95°C for 5 minutes; 80° - 60°C for 2 minutes/°C) on a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). 500 pmol quantities of double stranded oligonucleotides (ds-oligos) were either brought to a final volume of 20 µl or dehydrated using a vacuum centrifuge. Hydrated samples were exposed to up to 400/1200 J/cm² (UVC/UVA) radiation and dry state samples were exposed to up to 2/6 kJ/cm² (UVC/UVA) radiation as described above. Prior to analysis, dry state ds-oligos were re-hydrolyzed to 20 µl. Sodium azide was added to a second sample set assayed in the same manner only with the addition of 400 nmol of the additive.

A 25 pmol aliquot was taken from each sample for analysis prior to enzyme treatment. Samples were then digested with Exonuclease III (New England Biolabs, Ipswich, MA, USA) for 1 hour at 37°C in a 50 µl reaction volume (10 mM Bis-tris-propane-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.0 @ 25°C) containing 5 U enzyme. 2.5 µl of 0.5 M EDTA was added to stop the reaction and 5 µl of QuickClean™ enzyme removal resin (Clontech, CA, USA) was added. Samples were vortexed briefly and then centrifuged on high for 1 minute.
The supernate was then removed and the procedure was repeated to ensure removal of the enzyme prior to further treatment. 35 pmol aliquots were taken and subjected to ethanol precipitation overnight via the addition of 1.2 μl 0.5 M NaCl and 30 μl 100% ethanol. Aliquots were centrifuged on high for 10 min and the supernate was removed. A second precipitation step was done using 150 μl 95% cold ethanol. After precipitation, samples were evaporated and analyzed.

After cleanup of Exo III digested fragments, the assay was subjected to digestion with 8 U of FPG along with 0.1 mg/ml BSA in a 25 μl total volume for 2 hours. The FPG was deactivated by holding at 65ºC for 20 minutes. Samples were subjected to ethanol precipitation as described above prior to analysis.

**Polyacrylamide Gel Electrophoresis (PAGE)**

Samples were dehydrated via vacuum centrifugation and dissolved in 5 μl gel loading dye (47.5% formamide, 9 mM EDTA, and 0.0125% each of SDS, bromocresol and bromophenol blue). 20% polyacrylamide gels containing 8 M urea were
Southern Blotting of Oligonucleotides

Nylon membrane (Biodyne B, Pall Life Science, East Hills, NY, USA) transfer of the gel was carried out at 120V for 90 minutes and crosslinked using a Stratalinker 1800 (Stratagene, La Jolla, CA, USA). Immobilized nucleic acids were detected following procedures for the Chemiluminescent Nucleic Acid Detection Module offered by Pierce (Rockford, IL, USA (product no. 89880)). Fluorescence was captured using the CCD camera of a Omega 12 gel documentation system (UltraLum, Claremont, CA, USA).

Results

UVC Induced Base Loss and Strand Breaks in Naked DNA

The median average length was examined for both hydrated and dry state DNA after being exposed to increasing doses
of UVC radiation (Figure 44). The hydrated state DNA, as expected degraded rather rapidly through the induction of strand breaks. After approximately 500 J/cm² the molecule was degraded to an average size of 100 bases. The dry state DNA degraded as well, but only to a certain fragment length of approximately 450 bases which was achieved after about 600 J/cm². As the dose increased, the median average length did not decrease, the amount of DNA seen on the gel decreased indicating that degradation was occurring to produce fragments too small to stay on the gel during electrophoresis.

The treatment with FPG did produce fragments of different sizes. This is most evident with hydrated DNA where it appears the formation of oxidized bases occurred early on in the irradiation process. The difference noted for the dry state DNA is not so evident. There are some fragments that are much smaller after FPG treatment, but that is not seen over the entire exposure period. This method of trying to see damage through the large magnitude of strand breaks doesn’t offer the sensitivity desired to fully understand the extent to which base oxidation is occurring.
Nor is it possible to determine if the FPG cleaved the DNA in response to an oxidized base or an abasic site.

Figure 44: (Left) The median average length of both hydrated and dry state DNA before and after treatment with FPG. (Right) The release of adenine and guanine from both hydrated and dry state DNA after various doses of UVC radiation. DS = dry state, HS = hydrated state

When base loss was looked for in irradiated dry state DNA (Figure 44) it was seen that it occurred at almost the exact same rate as it did with hydrated DNA. The release of guanine and adenine were the only two bases detected which is not unexpected based on previous research (see Experiments 2 and 3). The release of adenine was more pronounced than that of guanine. There was again a leveling off effect that occurred around 700 J/cm² similar to what is seen with strand breakage. It could be that the
bases were being converted into oxidized bases such as 8-oxoG and 8-oxoA that are not detected using the HPLC method employed. It could also be that the degradation process reaches a steady-state after a certain point and this is supported by looking at both base loss and strand break phenomena. Another explanation could include the formation of DNA-DNA cross-links that make fragments appear larger than they really are.

**Induced Damage to Synthetic Oligonucleotides**

The condition of samples was monitored via PAGE (SybrGold® staining) and Southern blotting between each step of enzyme treatment. The fluorescently stained gels will show all fragment sizes present in each sample, and the Southern blots will only show those pieces that still have a biotinylated 5’-end present. Though oligonucleotides had been cartridge purified from the manufacturer, gels showed smearing of annealed pieces prior to any UV exposure.

**UVC Exposed**

Regardless of the initial fragments present in the samples, neither hydrated nor dry state ds-oligos showed any further
formation of strand breaks. After exposure to 50 J/cm\(^2\) the next dose measured was 200 J/cm\(^2\). At this dose both hydrated and dry state ds-oligos showed a slight decrease in the amount of smaller fragments present. This could indicate that those fragments were degrading further to smaller fragments that were too small to be detected on the gel, though the resolution was verified down to at least 10 bp, and a fragment as small as 5 bp should have been detectable. It could also be that those fragments were forming DNA-DNA cross-links and not migrating as far on the gel. Cross-linking of the DNA appears to be the major reaction occurring upon exposure to UVC irradiation. Surprisingly, for the hydrated ds-oligos the appearance of cross-links does not become evident until 50 J/cm\(^2\) was delivered. In contrast, the dry ds-oligos showed cross-linking after as little as 3 J/cm\(^2\) (Figure 45).

After treatment with Exo III there was not a noticeable increase or decrease in the amount of fragments seen that had smaller molecular weights than the original dry state ds-oligos. The hydrated state ds-oligos showed an increase in the amount of strand breaks present after treatment with Exo III, but only for samples that did not show significant
cross-links present. For those that did, the amount of strand breaks appeared to stay the same as before enzyme treatment (Figure 45). It could be that the enzyme is unable to fully digest strands that are involved in the cross-linked product, thus making it ineffective for those samples. This would explain why there was not a difference noted for dry state ds-oligos, they showed cross-links after only the first dose of radiation.

Figure 45: SybrGold® stained polyacrylamide gels of UVC damaged ds-oligos. The top set of pictures correspond to samples after UVC exposure alone. The bottom set of pictures correspond to samples after digestion with Exo III under conditions described in the text. Samples sets may not have all been run on the same gel.
Subsequent treatment with FPG should reveal oxidative base damage with regards to those species recognized by the enzyme. The fluorescently stained gels in neither dry nor hydrated states showed an increase in strand breaks after treatment. The hydrated ds-oligos did not show the presence of smaller fragments that would indicate the presence of an oxidized base.

The dry state samples showed different behavior. Dry state ds-oligos after undergoing the full procedure outlined in Figure 43 showed a decrease in the amount of DNA on the membrane and an increase in random signal on the lower portion of the membrane. It was expected that fragmentation due to the presence of oxidized bases would lead to the distinction of somewhat discrete bands once visualized after transfer to the membrane. This did not occur, but it could be that the fragments created by the FPG digestion were just too small to remain on the gel during electrophoresis, especially if there were multiple fragments generated from the remaining strands of template.

The use of the $^{1}\text{O}_2$ quencher, sodium azide revealed no difference due to its presence with the hydrated state ds-
oligos. There was no difference noted in comparing polyacrylamide gels and Southern blots of dry state ds-oligos either until the membranes were examined after Exo III and subsequent FPG digestion was carried out. The dry state samples that showed degradation under these conditions without the addition of sodium azide were still intact. This could be an indication that $^1\text{O}_2$ is involved in base oxidation, but not DNA-DNA cross-linking (Figure 46).

![Figure 46: Dry state UVC exposed ds-oligos after digestion with Exo III followed by treatment with FPG. The top gel shows samples of just DNA and the bottom gel shows samples that were made with 400 nmol of sodium azide. The first four sodium azide treated samples were run on a separate gel which ran a little crooked leading to the effect seen.](image)
**UVA Exposed**

Similar to ds-oligos exposed to UVC radiation, UVA exposed ds-oligos showed signs of DNA-DNA crosslinks as their major product (Figure 47 and Figure 48). Dry state ds-oligos were more prone to crosslinking than the hydrated state. In contrast, it did appear that there were more strand breaks present after treatment with FPG as visualized via PAGE. The increase in smaller fragments was not seen after membrane transfer and chemiluminescent detection. The fragments visualize via Southern blotting were only those with the 5′-biotin label end attached on the forward strand. The polyacrylamide gel showed all fragments. It could be that the fragments generated by cleavage at sites of oxidized bases did not give rise to detectable biotinylated fragments. FPG could also be active towards abasic sites in DNA-DNA crosslinks. If Exo III is not able to digest abasic sites that are contained in crosslinks and FPG is, then the slight increase in fragments seen could also be an artifact of that caveat.

The addition of sodium azide to ds-oligos exposed to UVA irradiation did not offer any protection from the insults inflicted. This is true for both hydrated and dry state
ds-oligos and is similar to what was seen with UVC irradiated samples.

Figure 47: Hydrated state ds-oligos after UVA irradiation.
Figure 48: Dry state ds-oligos after UVA irradiation.
Discussion

A thorough review of products arising from oxidative damage from guanine and thymine has been done by Cadet et. al.\textsuperscript{46} Though no thymine moieties were looked for in the current study, it is worth mentioning that the majority of oxidation products as previously determined are relatively stable hydroperoxides including the \textit{cis} and \textit{trans} diastereomers of 6-hydroperoxy-5-hydroxy-5,6-dihydrothymidine (1), 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (2), and 5-hydroxyperoxymethyl-2’-deoxyuridine (3) (\textbf{Figure 49}).

![Chemical Structures](image)

\textbf{Figure 49:} Major products of oxidation of thymine

When the hydroxyl radical is mediating base damage, the two most significant products from guanine are 2,2-diamino-4-[(2-deoxy-\(\beta\)-D-erythro-pentofuranosyl)amino]-5(2H)-oxazolone (4) and 2-amino-5-[(2-deoxy-\(\beta\)-D-erythro-pentafuranosyl)amino]-4H-imidazol-4-one (5). Surprisingly the presence of 8-oxoG (6) is minor when exposure to OH•

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without the presence of a reducing agent such as Fe$^{2+}$. When the guanine is incorporated into dsDNA the most predominant moiety is found to be 8-oxoG upon exposure to OH•. A cause for this could be due to transition metals bound to the dsDNA structure intracellularly. The oxidative damage measured here could be conservative in the sense that there are more variables involved in oxidative damage when DNA is in the presence of cellular components.

![Figure 50: Major products of oxidation of guanine (4 and 5) and 8-oxoG (6).](image)

Singlet oxygen can react efficiently with DNA and it can be generated inside cells through photosensitization and enzymatic oxidation. It is believed that the oxidative damage caused by UV radiation is not due to radiation absorption by the DNA, but rather by activation of photosensitizers generating $^{1}$O$_{2}$ species. When comparing UV induced base oxidative damage among UVC, UVB, and UVA it was found that though pyrimidine dimers are favorable under all circumstances, the induction of 8-oxoG relative to
pyrimidine dimers increases with the lower energy radiation (i.e. above 365 nm). Kvam et al. replaced water in a cell culture medium with D$_2$O and noted a significant increase in 8-oxoG formation upon exposure to UVA.$^{59}$ Similar results were obtained by Wei et al.$^{66}$ when inducing damage with UVC radiation. The lifetime of $^{1}$O$_2$ is prolonged in a D$_2$O environment indicating its involvement. This was via an intracellular test which could lead to the argument that intracellular photosensitizers lead to energy transfer of molecular oxygen which would not be available to naked DNA. It was also determined by Kvam et al. that interference with iron dependent Fenton reactions did not change the ability of UVA radiation to induce formation of 8-oxoG, implicating that the OH· does not play a role at the ambient wavelength.$^{59}$

It is believed that there are possibly different reaction mechanisms inducing oxidative DNA damage when the inducing radiation is of different wavelengths.$^{65}$ The use of UVC and UVB radiation to induce damage can only give a partial picture of what to expect when dealing with ambient UVA radiation because UVA is not absorbed by DNA where the lower frequency energies are. Therefore, with UVA
radiation the driving force for oxidation is more likely to be the formation of $^1O_2$ that then induces damage indirectly. There are also most likely differences in oxidative damage dependent upon the presence of the intact cell. Zhang et al. found that purified DNA was more susceptible to 8-oxoG formation than HeLa cells irradiated with UVA, UVB, and UVC energy sources. This indicates that there are protective mechanisms or species in place intracellulary, such as superoxide dismutase and catalase. Interestingly it was found by the same authors that the relative yield of 8-oxoG to cyclobutyl pyrimidine dimers increased nearly 1000-fold in both purified and cellular DNA upon exposure to UVA radiation compared to UVB and UVC. This indicates that the effect of UVA damage on forensic type samples could be more pronounced than originally expected in regards to base damage.

The use of hydroxyl radical scavenges such as DMSO and mannitol can quench the formation of 8-oxoG when DNA is subjected to ionizing radiation or the Fenton reaction, but does not when UVC radiation is causing the damage, indicating that the $OH^\cdot$ is not responsible for damage induced in this manner. The use of sodium azide which is
a specific $^1\text{O}_2$ quencher does substantially reduce levels of 8-oxoG formation.$^{66}$ Previous literature additionally reports that OH\textsuperscript{•} can induce CC $\rightarrow$ TT mutations via UV damage to DNA.$^{82}$ The frequency of this mutation is less that that for single base mutations by a factor of thirty.

Based on the information gathered during this study DNA-DNA cross-links and strand breaks are the most significant damage encountered by naked DNA induced by UVC radiation. Strand breaks were the most evident with genomic DNA, but with the 30 bp ds-oligos cross-links were the most evident. The methods used in this study were not adequate enough to make a judgment on the extent of oxidative base damage that occurred to genomic DNA. The more sensitive technique used in conjunction with the ds-oligos did indicate that oxidative base damage was occurring in the dry state. What was more interesting however, was that the formation of cross-links either i) halted the action of the Exo III enzyme and thus invalidated the rest of the detection method, or ii) actually offered protection from the subsequent damage of strand breaks and base loss. Irregardless of this uncertainty in the data, it is apparent that the insults induced by UVC radiation are very
similar to those induced by UVA radiation. Therefore, it can be concluded that UVC induced damage can be used as a model for what to expect with ambient lower energy UVA radiation. This of course may not hold to be true in the case that intracellular components are present.
CHAPTER 5: SEQUENCE SPECIFICITY OF BAL 31 NUCLEASE FOR SINGLE-STRANDED DNA REVEALED BY SYNTHETIC OLIGOMER SUBSTRATES CONTAINING HOMOPOLYMERIC GUANINE TRACTS

Introduction

The extracellular nuclease from Alteromonas espejiana, BAL 31 comprises several nuclease activities designated as “slow” (S) and “fast” (F) depending upon the relative rates with which they catalyze the terminally directed hydrolysis of duplex DNA. The displayed nuclease activity also includes hydrolysis of single-stranded DNA, cleavage of negatively supercoiled DNA to the linear duplex form, and cleavage of duplex DNA in response to the presence of apurinic sites. The use of BAL 31 is favored in molecular cloning techniques due to its stability upon extended storage and resistance to inactivation in the presence of high concentrations of salt or denaturing agents. Applications that benefit from the use of BAL 31 include those that require the progressive removal of nucleotides from both termini of dsDNA, complete digestion of ssDNA, restriction site mapping in DNA and
the detection of lesions or distorted structures in duplex DNA.\textsuperscript{\textluromaks89}

Although BAL 31 activity with duplex DNA is relatively well characterized,\textsuperscript{\textluromaks84,85} there appear to be no detailed reports of studies examining how the BAL 31 enzyme interacts with single stranded linear polymers of DNA, especially those containing homopolymeric tracts. Though it has been noted that the exonuclease activity is hindered by the presence of G\textbullet{}C sequence motifs \textsuperscript{\textluromaks84,85} in dsDNA, there has been no such hindrance noted for ssDNA. Here it is focused on how the BAL 31 enzyme degrades single stranded DNA, using homogeneous and heterogeneous 10-20 base oligomers. Specifically, it is determined the efficiency of hydrolysis of short 10-20 base oligomers and whether the enzyme exhibits any sequence specificity. It was found that homopolymeric guanine oligomers are not digested by BAL 31 and that the presence of short dG tracts in mixed sequence oligomers hinder BAL 31 enzyme activity.
Methods and Materials

Sample Preparation

Single stranded oligomers (dT<sub>10</sub>, dC<sub>10</sub>, A<sub>10</sub>, G<sub>10</sub>, 5'-GGATCATCGG (G<sub>2</sub>-CAP), 5'-ATCGGGGATC (G<sub>4</sub>-MID), 5'-ATATCATCAC (G-NO), 5'-GGGATCTCATCTCATCTGGG (G<sub>3</sub>-CAP-L), 5'-GGGGTCTCATCTCATCGGGG (G<sub>4</sub>-CAP-L), 5'-GGGATCTCA (G<sub>4</sub>-5’CAP), 5'-ATCTCAGGGG (G<sub>4</sub>-3'CAP) and 5'-ATCTCATCGGGGTCATCTCA (G<sub>4</sub>-MID-L)) were cartridge purified (Invitrogen, Carlsbad, CA, USA). The deoxycytidine 5’-monophosphate (dCMP), thymidine 5’-monophosphate (dTMP), deoxyadenosine 5’-monophosphate (dAMP), and deoxyguanosine 5’-monophosphate (dGMP) standards were obtained from Sigma Aldrich (St. Louis, MO, USA). The nuclease BAL 31 was purified from the culture medium of Alteromonas espejiana BAL 31 containing a mixture of “fast” and “slow” species (New England BioLabs, Ipswich, MA, USA). Quality control assays and double stranded endonuclease activity were checked for by the manufacturer.

Oligomer samples were prepared by aliquoting the necessary volume to produce 6.0 nmol quantities (unless otherwise noted) into 1.5 ml microcentrifuge tubes and dehydrating in a vacuum centrifuge. Reactions were carried out in a 20 μl
reaction volume containing 300 µM ssDNA oligomer, 20 mM Tris-HCl (pH 8.1 at 25°C), 600 mM NaCl, 12 mM CaCl₂, and 12 mM MgCl₂. Samples were incubated at 37°C for 24 hours unless otherwise indicated. Enzyme deactivation was accomplished by incubation at 95°C for 15 minutes.

**Sample Analysis**

Samples were analyzed using ion-pairing HPLC. The HPLC apparatus consisted of a SpectraSystem P2000 pump and a UV6000LP diode array detector (ThermoElectron, Waltham, MA, USA), equipped with a 5 cm light-path flow cell and data was collected between 200 and 300 nm. Data were acquired and analyzed by a PC using the XCalibur® software package provided by the HPLC manufacturer. Separation of the nucleotides was carried out using a Pinnacle II 250 X 4.6 mm, 5 µm particle size C₁₈ column with a 10 X 2.1 mm guard column (Restek Corporation, Bellefonte, PA, USA). The ion-pairing technique was employed using buffers described by Tavazzi et al.¹⁷ Buffer A (10 mM KH₂PO₄, 0.125% methanol, 12 mM tetrabutyl ammonium hydroxide, pH 7.0), and buffer B (100 mM KH₂PO₄, 30% methanol, 2.8 mM tetrabutyl ammonium hydroxide, pH 5.5) were used in a 50:50 (v:v) isocratic
combination unless otherwise stated. A flow rate of 1.0 ml/min was maintained constant throughout the analysis and the analysis was conducted at ambient temperature (~22°C). The use of HPLC to detect mononucleotides released by BAL 31 has been done previously with an alkaline salt gradient,\textsuperscript{90} however; the method took longer to elute all four dNMPs and the elution peaks were not well defined.

**Molecular Species Identification and Statistical Analysis**

Molecular species identification was determined by matching retention times and absorption spectra to prepared standards. The peak area of hydrolysis products obtained from HPLC-UV absorption measurements were quantified using the program XCalibur\textsuperscript{®} applying an Avalon algorithm of peak detection.

The recovery percentage was calculated by dividing the number of moles of nucleotide recovered by the maximum number of nucleotide moles possible and multiplying by 100%. For example, 6 nmol of a 10-oligomer will theoretically yield a possible 60 nmol of nucleotides. Statistical analysis of data was carried out using ANOVA.
analysis where the between sample and within sample variances were compared using a one-sided F-test.\textsuperscript{18}

**Results**

**Hydrolysis of dN\textsubscript{10}**

BAL 31 hydrolysis of the four dN\textsubscript{10} homopolymers was carried out for varying periods (3, 9, 24, 48 and 72h) at 37\textdegree C and with two different enzyme concentrations (0.5 and 1.0 U). Samples were prepared in triplicate. Oligomer hydrolysis was monitored quantitatively by the separation and detection of dNMPs by HPLC (Figure 51). Of the four homopolymers the dT\textsubscript{10} oligomer was, in general, the most efficiently hydrolysed substrate. DT\textsubscript{10} incubated with 0.5 U BAL 31 resulted in ~ 68-100% recovery of dTMP monomers, whereas with 1 U enzyme the dTMP recover was ~61-100%. The dC\textsubscript{10} oligomer incubated with 0.5 U BAL 31 resulted in ~61-67% recovery of dCMP monomers, whereas with 1 U enzyme the dCMP recovery was ~63-68%. The dA\textsubscript{10} oligomer incubated with 0.5 U BAL 31 resulted in ~39-99% recovery of dAMP monomers, and with 1 U enzyme the dAMP recovery was ~60-100%. In contrast to the other homopolymers, the dG\textsubscript{10} oligomer proved
to be highly refractory to hydrolysis by BAL 31. Specifically, the dG$_{10}$ oligomer incubated with 0.5 U BAL 31 resulted in only ~0.1-1.4% recovery of dGMP monomers, with 1 U enzyme the dGMP recoveries were ~0.1-1.8%. The results were essentially the same for all four homopolymeric oligomers when the enzyme reaction took place at 30°C (Figure 52).

Figure 51: Hydrolysis of homodecameric oligomers. dC$_{10}$, dA$_{10}$, dT$_{10}$, and dG$_{10}$ were incubated at 37°C with 1 U, 0.5 U, and 0 U of BAL 31. The relative hydrolysis efficacy of each homopolymeric oligomer is indicated by the percentage recovery of their constituent 5’dNMPs over time (3-72 h).
Figure 52: Hydrolysis of homodecameric oligomers. dC$_{10}$, dA$_{10}$, dT$_{10}$, and dG$_{10}$ were incubated at 30°C with 1 U, 0.5 U, and 0 U of BAL 31. The relative hydrolysis efficacy of each homopolymeric oligomer is indicated by the percentage recovery of their constituent 5’dNMPs over time (3-72 h).

It was considered that the dramatic reduction in hydrolysis efficiency with dG$_{10}$ could be an artifact due to guanine homopolymer self aggregation to form higher order structures$^{91,92}$ that may not be efficient substrates for the enzyme. Lowering the salt concentration or increasing the temperature might be expected to decrease such aggregation. However, lowering the salt concentration would be counter
productive due to the requirement of Mg$^{2+}$ and Ca$^{2+}$ for enzymatic activity. Since the enzyme requires a large deactivation temperature (~85°C) and presumably is still active at elevated temperatures, the enzyme reaction temperature was increased from 37°C to 45°C, 50°C, and 55°C and the dG$_{10}$ oligomers were incubated over a twenty-four hour period with 0.5 U enzyme. DT$_{10}$ oligomers were incubated under the same conditions as a control. Samples were prepared in quintuplet. The dT$_{10}$ samples produced 82.6 ± 9.9%, 88.2 ± 5.9%, and 98.4 ± 8.4% dTMP at 45°C, 50°C, and 55°C respectively. The dG$_{10}$ samples produced 0 ± 0%, 1.31 ± 0.94%, and 0.78 ± 0.46% dGMP at 45°C, 50°C, and 55°C respectively (Figure 53).

Even if not all secondary structure was eliminated at these elevated temperatures, it would be alleviated which should lead to an increase in dGMP produced. These results indicated that the refractory nature of dG$_{10}$ to BAL 31 digestion was probably not due to the self aggregation of homoguanosine oligomers into higher order structures.
Figure 53: dG₁₀ homodecamers continue to be refractory to hydrolysis at elevated temperatures. dG₁₀ and dT₁₀ were hydrolyzed by BAL 31 at 45°C, 50°C, and 55°C for a twenty-four hour period. The relative hydrolysis efficacy of each homopolymeric oligomer is indicated by the percentage recovery of their constituent 5’dNMPs.

Position and Sequence Requirements for the Guanine Inhibition of ssDNA by BAL 31

To further delineate the sequence length and position requirements for the dGₙ mediated inhibition of BAL 31, a series of decamers were synthesized that (i) contained two guanines that ‘capped’ both the 5’ and 3’ ends (G₂-CAP), (ii) contained a homopolymeric stretch of four guanines in the middle of the decamer (G₄-MID), (iii) contained no guanine residues (G-NO), (iv) contained a homopolymeric
stretch of four guanines at the 5’ end of the decamer (G₄-5’CAP), and (v) contained a homopolymeric stretch of four guanines at the 3’ end of the decamer (G₄-3’CAP). A series of 20-mers were also used that (i) contained a homopolymeric stretch of four guanines in the middle of the 20-mer (G₄-MID-L), (ii) contained three guanines that ‘capped’ both the 5’ and 3’ ends (G₃-CAP-L), and (iii) contained four guanines that ‘capped’ both the 5’ and 3’ ends (G₄-CAP-L).

The oligomers G-NO, G₂-CAP, and G₄-MID were incubated at 37 °C with 0.5 U enzyme for a twenty-four hour period (in quintuplet) and, as before, their hydrolysis efficiency was measured by the recovery of the constituent nucleotides. An ANOVA analysis was conducted to determine whether the variation in recovery rates were significant. The G₂-CAP oligomer produced nucleotides at recovery rates similar to those seen with the non-G containing homopolymeric oligomers and with the G-NO oligomer (Figure 54). In contrast, G₄-MID produced non-G nucleotides at significantly lower recovery rates compared to G₂-CAP. G-tract hydrolysis, as measured by the formation of dGMP, occurred with both G₂-CAP and G₄-MID, but at lower amounts than the
other three nucleotides. Thus G di-nucleotides at the 5’ and 3’ ends of decameric oligonucleotides appear to be permissive for BAL 31 digestion of the decamer, whereas a G tetra-nucleotide tract in the middle of the decamer makes it more refractory to hydrolysis. This possibly indicates that the hydrolysis proceeds from one end of the ssDNA and/or that guanine tracts are difficult to fully hydrolyze.

Figure 54: Hydrolysis efficiency of oligonucleotide substrates is affected by the location of the guanine tract. The relative hydrolysis efficacy of each oligomer is indicated by the percentage recovery of their constituent 5’dNMPs. (A) 300 µM oligomer substrates containing either no guanine (G-NO) or guanine tracts at the ends (G2-CAP) or in the middle (G4-MID) of the polynucleotide chain were incubated at 37°C for twenty-four hours with 0.5 U BAL 31 enzyme. (B) The same experiment as in (A) except using 30 µM oligomers.
The above experiments used oligomers at a concentration of 300 µM and the hydrolysis proceeded at 37°C. In order to preclude the possibility that self aggregation of the G₄-MID oligomer₉¹₉² was responsible for the observed hindrance of enzymatic activity, the reactions were repeated in quintuplet using conditions that would reduce the possibility of self-aggregation. Specifically, hydrolysis proceeded using a ten fold less concentration of oligomer substrates (30 µM) and at an elevated temperature (55°C). The results obtained were similar to those found at the lower temperature and higher concentration, thus providing support for the hypothesis that self-aggregation is not the cause of the decreased digestion rate with tracts of G residues (Figure 54). In order to preclude the possibility that the aberrant enzyme activity noted with different substrates was due to peculiarities attached to one particular batch of enzyme (such as co-contaminants etc), some of the experiments were repeated using BAL 31 nuclease from a different manufacturer (USB Corporation, Cleveland, OH, USA). These additional experiments included the digestion of dT₁₀, dG₁₀, G₂-CAP, G₄-MID, and G-NO at temperatures of 37°C and 55°C using 0.5 U enzyme over a twenty-four hour incubation period. The results were
essentially the same as that obtained with the New England Biolabs enzyme used in the initial studies (Figure 55) indicating that the refractory nature of guanine tracts to BAL 31 digestion was an inherent property of the enzyme. Moreover, the USB Corporation purified the enzyme using SDS-PAGE and reported only seeing two bands corresponding to the ‘fast’ and ‘slow’ forms of the enzyme. In order for the activity seen to be due to a contaminant, the contaminating species would have had to have been of similar size as one of the species comprising the BAL 31 enzyme and co-elute as such.

Further studies were carried out using the oligomers G₄-5’CAP, G₄-3’CAP, G₄-MID-L, G₃-CAP-L and G₄-CAP-L to see if further insight could be obtained into the mechanism of action of BAL 31. For all five oligomers the mean recovery of each nucleotide after BAL 31 is shown in Table 11. An ANOVA analysis was conducted to ascertain the significance of the differences observed. According to the least significant difference, recovery of dCMP from G₃-CAP-L was the same as from the other four oligomers (G₄-5’CAP, G₄-3’CAP, G₄-MID-L, G₄-CAP-L). G₄-5’CAP was statistically the same as G₄-3’CAP and G₃-CAP-L, where G₄-3’-CAP was
additionally similar to G₄-MID-L. G₄-CAP-L, G₃-CAP-L, and G₄-MID-L had the same amount of dCMP recovery according to statistical analysis.

Figure 55: Results of BAL 31 digestion of dG₁₀, dT₁₀, G₂-CAP, G₄-MID, and G-NO with an enzyme supplied by USB Corp. Two temperatures were used (37°C and 55°C) with 0.5 U enzyme. The results are very similar to those obtained with the enzyme from New England BioLabs. Top: G = dGMP, T = dTMP and the temperature is indicated on the x-axis in parenthesis. Bottom Left: G₂-CAP, G₄-MID, and G-NO incubated at 37°C. Bottom Right: G₂-CAP, G₄-MID, and G-NO incubated at 55°C.
Recovery of dAMP from G₄-CAP-L, G₄-5’CAP, G₃-CAP-L, and G₄-MID-L did not differ significantly and recovery from G₄-3’CAP and G₄-MID-L did not differ significantly. In addition G₄-MID-L did not differ from G₄-3’CAP. G₃-CAP-L and G₄-MID-L do differ significantly. The recoveries of dTMP from G₄-CAP-L, G₄-MID-L, and G₄-5’CAP were statistically indistinguishable, and recovery from G₄-5’CAP was similar to G₃-CAP-L. Recovery from G₄-3’CAP differed from the other four oligonucleotides. Most importantly, recovery of dGMP differed significantly between all oligomers except G₄-CAP-L and G₃-CAP-L.

Table 11: Recovery of dNMPs after BAL 31 hydrolysis

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>% dAMP</th>
<th>% dCMP</th>
<th>% dTMP</th>
<th>% dGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₄-5’CAP (5’-GGGGATCTCA)</td>
<td>66.1 ± 6.4</td>
<td>100 ± 4.9</td>
<td>100 ± 8.0</td>
<td>29.3 ± 4.5</td>
</tr>
<tr>
<td>G₄-3’CAP (5’-ATCTCACGGG)</td>
<td>46.2 ± 1.0</td>
<td>97.5 ± 2.2</td>
<td>56.5 ± 1.5</td>
<td>100 ± 2.7</td>
</tr>
<tr>
<td>G₄-MID-L (5’-ATCTCATCGGGGTCATCTCA)</td>
<td>52.8 ± 4.6</td>
<td>85.4 ± 5.5</td>
<td>86.4 ± 5.5</td>
<td>74.1 ± 3.4</td>
</tr>
<tr>
<td>G₃-CAP-L (5’-GGGATCTCATCTCATCTGGG)</td>
<td>74.3 ± 6.7</td>
<td>96.0 ± 9.7</td>
<td>115 ± 10.0</td>
<td>53.0 ± 4.8</td>
</tr>
<tr>
<td>G₄-CAP-L (5’-GGGGTCTCATCTCATCGGG)</td>
<td>64.4 ± 4.6</td>
<td>75.5 ± 2.4</td>
<td>85.6 ± 3.7</td>
<td>41.3 ± 1.3</td>
</tr>
</tbody>
</table>

The decamer that has four guanines at the 5’ end (G₄-5’CAP) was refractory to G tract hydrolysis whereas, in contrast, almost complete recovery of dGMP occurred with the decamer with four guanines at the 3’ end (G₄-3’CAP). Surprisingly,
the hydrolysis of the other non-G nucleotides was more efficient with G₄-5’CAP than with G₄-3’CAP. This data supports the notion that, independent of any endonuclease activity it may possess, the BAL 31 acts as a ssDNA 3’ → 5’ exonuclease particularly when the substrate is of minimal length (10 mer) as previously reported with single stranded viral øX174 (wild type) DNA. With the longer 20-mer substrates, recovery of dGMP was greater when the G tract was in the middle (G₄-MID-L) as opposed to being capped at both ends (G₃-CAP-L and G₄-CAP-L). While ostensibly mimicking its shorter counterpart G₄-MID, the G₄-MID-L 20-mer, in contradistinction to its shorter counterpart G₄-MID, was not more refractory to hydrolysis than its capped end homologs (G₃-CAP-L, G₄-CAP-L). Thus an increase in the length of the polynucleotide chain from 10 to 20 appeared to overcome the G-tract mediated inhibition of hydrolysis when the G tract was in the middle of the polynucleotide chain.

Discussion

This study has revealed that the general purpose BAL 31 nuclease commonly used in molecular genetics exhibits a
hitherto non-characterized degree of substrate specificity with respect to single stranded DNA oligomers. Specifically, BAL 31 nuclease activity was found to be affected by the presence of guanine in ssDNA oligomers and the subsequent use of different G-tract containing substrates allows us to speculate on the likely mode of action of the enzyme. Minimal G tracts of four bases in the middle of short oligomers and at the 5’ end of decamers are refractory to hydrolysis. The enzyme does not appear to ‘skip over’ the difficult to digest tracts of guanine but appears to be hindered by them resulting in a loss of processivity momentum. Previous reports demonstrating that high G•C content in dsDNA hindered digestion hypothesized that this may be due to the greater difficulty in unwinding the thermodynamically more stable G•C rich DNA, a necessity for phosphodiester bond cleavage. However our data suggest an alternative explanation for the refractory nature of guanine rich regions of ssDNA to BAL 31 digestion and this may also pertain in part to the resistance of G•C rich regions of dsDNA.

The lack of digestion of the dG_{10} oligomer indicated an inhibition of the nuclease’s activity when guanine is
encountered. When guanine was present as a two base tract at the 5’ and 3’ ends of a decamer the result was the almost complete digestion of the adenine, thymine, and cytosine interstitial nucleotides, yet only a little over half of the guanine nucleotides were recovered. This result is consistent with a mechanism in which nucleotide hydrolysis begins at one end of the polynucleotide strand, as previously reported,\textsuperscript{90,94} including the first two guanines, then proceeds in a processive manner along the strand digesting the next six nucleotides, but then possessing insufficient momentum to “push through” the last two guanines. When guanine was present in a four base sequence in the middle of a decamer, approximately half or less of all bases were recovered as their respective 5’-mononucleotides. This too can be explained by digestion being hindered when encountering the guanine homopolymeric four base stretch. When four guanines were positioned at the 5’ end of a decameric oligomer the non-G nucleotides were hydrolyzed rather efficiently, yet less then 30% of the guanine mononucleotides were recovered. This is consistent with digestion beginning at the 3’ end and again being hindered when the four guanine tract was encountered at the other end of the polynucleotide chain. In contrast,
when the same decameric oligomer was fashioned where the four guanine tract was positioned at the 3’ end, all of the guanines were digested, yet the recovery of dAMP and dTMP was decreased. This is also consistent with the enzyme encountering a loss of processive momentum due to the initially resistant guanine tract. When the four guanine tract was located in the middle of a twenty base oligomer approximately 74% of the guanine nucleotides were hydrolyzed. When there were three or four base guanine tracts at each end of a twenty base oligomer approximately 53% and 41% of the guanines were recovered, respectively. Again this result is consistent with hydrolysis of the initial guanine tract but being ‘slowed down’ in the process with the resulting processive momentum loss such as to lessen the ability of the enzyme to digest the last tract of guanines encountered at the other end of the oligomer. The recovery of the other three 5’-mononucleotides from G₄-MID-L was lower than for G₃-CAP-L and G₄-CAP-L in general, which is consistent with the enzyme efficiently loading at one end and catalyzing hydrolysis of the non-G nucleotides at that end but subsequently being hindered in its processive track once it encounters the four base G tract in the middle of the oligomer. It is
worth mentioning that Lu and Gray\textsuperscript{94} provided evidence that removal of mononucleotides from very short oligomers (~3 bases) may not be solely processive.

It is unclear why the presence of guanine tracts in ssDNA oligomers hinders BAL 31 hydrolysis activity. It is noted, however, that guanine is the bulkiest of the four nucleotides, since it is purine based and possesses two exocyclic functional groups (a primary amine and carbonyl) compared with the single functional group on the other purine nucleotide, adenine. Thus one hypothesis is that the active site of the enzyme is spatially constrained in such a manner that the bulky guanine tracts are bound and catalyzed more inefficiently than the other nucleotides. The resulting loss of ‘processive momentum’ could explain the activities noted in this study.

Future studies could include the use of nucleotide analog substrates containing a variety of different exocyclic architectures to permit further testing of the postulated hypothesis of enzyme inhibition by bulky nucleotides. Such studies would also provide a more precise delineation of the steric impediments to efficient catalysis. Further
characterization of the noted ssDNA nuclease activity would include determining, after chromatographic fractionation, whether the activity was present in the F and/or S isoforms.
CHAPTER 6: CHANGES IN DRY STATE HEMOGLOBIN OVER TIME DO NOT INCREASE THE POTENTIAL FOR OXIDATIVE DNA DAMAGE IN DRIED BLOOD

Introduction

Hemoglobin (Hb) is the iron containing oxygen transport protein present in the red blood cells of vertebrates (Hemoglobin A for humans). Oxygenated Hb (oxyHb) is a low-spin ferrous compound that gives blood its characteristic red color. OxyHb is easily oxidized under the influence of external oxidants to methemoglobin (metHb) which is a high-spin ferric protein that can no longer bind elemental oxygen. Over time, the high-spin ferric compound can convert to various low-spin ferric forms called hemichromes (Scheme 2). Hemichromes are formed through changes of protein conformation so that atoms endogenous to the protein become bond to the iron as the sixth ligand. Because Hb is a major component of blood (a body fluid often subjected to forensic DNA analysis), it is important to understand molecular transformations of the Hb molecule that could lead to possible oxidative damage to the other components of a bloodstain, particularly DNA.
Scheme 2
In this notation the superscript denotes the number of d electrons in the iron atom and the subscript is the total spin of the iron atom.

\[ \text{Hb}(d^5) + \text{O}_2 \rightarrow \text{oxyHb}(d^6)\text{O}_2 \rightarrow \text{metHb}(d^5_{5/2}) - \text{O}_2 \rightarrow \text{hemichrome}(d^5_{1/2}) \]

For oxidative damage to occur, oxidizing agents must be available that can interact with biomolecules such as DNA. One of the most damaging of these agents is the hydroxyl radical (OH•), which can be produced during a biological Fenton type reaction catalyzed most likely by ‘free’ iron. Native Hb contains four heme groups, each of which contains an iron center (Figure 56). It is unlikely that the iron complexed with Hb itself produces OH• capable of interacting with other biomolecules; such radicals produced at the iron center would have to travel through the protein into free solution to react. Thus, the formation of OH• in this manner would most likely lead to oxidative damage of the parent Hb molecule.

There have been no previous studies conducted on how Hb in the dried state affects the oxidation of other cellular components, specifically DNA. It can be hypothesized, base on previous research on the destructive role of ionic iron
in vivo, that oxidative damage could be exacerbated by the presence of Hb and its potential release of ‘free’ iron. The handling of ‘free’ iron inside the living body is carefully regulated via metabolic pathways which help keep the formation of cytotoxic OH• under control.\textsuperscript{97,98} An overload of ionic iron is correlated with DNA oxidative damage.\textsuperscript{99} These metabolic pathways would not be functional in dried bloodstains. However, in the forensic context some damage to DNA in bloodstains is expected and is likely to be more pronounced in older samples.\textsuperscript{1,100} Upon recovery of a dried bloodstain from a crime scene, the potential role that Hb can play in subsequent damage to the sample can be inferred by this study.

This work sought to characterize the molecular species formed by Hb maintained in the dry state at ambient temperatures and humidity over a period of time. First, it was determined the presence and/or formation of Hb isoforms because these species will determine what type of iron (i.e. Fe\textsuperscript{2+}/Fe\textsuperscript{3+}/Fe\textsuperscript{4+}) is available to participate in further chemical reactions. It was also determined whether, and to what extent, free iron is released from Hb because this together with its oxidation status will affect the ability
of the system to undergo Fenton type reactions. Finally, the ability of Hb to inflict oxidative damage on a deoxyribose substrate, presumably through the formation of \( \text{OH}^\cdot \), was measured as a function of the age of the dried Hb.

Figure 56: (Left) Globular structure of Hb protein, (Right) Molecular structure of the heme center found in quadruplet in each Hb molecule.

**Methods and Materials**

**Sample Preparation and Analysis**

All dry state samples were created by vacuum centrifugation and then maintained at room temperature in the dark at ambient temperature and humidity conditions (22.0 ± 0.4°C, 54 ± 7 % relative humidity) for varying time periods up to
approximately three months. Samples were removed from the ambient environment at various time points in triplicate and frozen. Dry state samples were prepared for analysis by re-hydrating to a total volume of 50 µl (same volume as hydrated samples) unless otherwise stated.

All spectra were measured using a UV6000 diode array detector (ThermoElectron, Waltham, MA, USA), equipped with a 5 cm light-path flow cell. The detector was in line with a SpectraSystem P2000 pump and autosampler which supplied buffer (0.5 mM Tris HCl, 0.1 mM EDTA) at a flow rate of 1 ml/min through the system. Data were analyzed using the XCalibur® software package provided by the manufacturer.

Human A₀ stabilized Hb, human Hb mainly in the form of metHb, and 2-deoxy-D-ribose (deoxyribose) was purchased from Sigma Aldrich (St. Lois, MO, USA). Ammonium acetate, ammonium Fe(II) sulfate hexahydrate, ascorbic acid, ferrozine, neocuproin, thiobarbituric acid (TBA), trichloroacetic acid (TAA), and sodium dithionite were purchased from Fisher Scientific (Pittsburgh, PA, USA).
Oxidation of Human Hemoglobin

A stock solution of ferrous stabilized human A\textsubscript{0} Hb was made by diluting 5.0 g of product in 10 ml of 0.5 mM Tris HCl and 0.1 mM EDTA. The stabilized Hb product has less than 15% metHb and primarily in the oxyHb form as verified by spectral analysis. The means by which this product is stabilized is proprietary to the supplier, although ficoll and sucrose are present. Therefore, measuring the mass of the product does not allow for determination of the amount of Hb. The determination of Hb concentration in samples was determined using molar absorbtivities (Table 12) provided by Winterbourn.\textsuperscript{101} Individual samples were made with 50 µl aliquots of the stock solution. A 20 µg/µl stock solution of human Hb which was primarily in the form of metHb (both stated by the manufacturer and confirmed by spectral analysis) was used to make individual 100 µl samples containing 2 mg of Hb.

After incubation and re-hydration where necessary, oxyHb samples were analyzed without further preparation. Three distinct species were speculated to be prevalent; oxyHb, metHb, and hemichromes. FerrylHb was also considered to be a possible product. Using the millimolar extinction
coefficients of the Hb derivatives, equation sets 9 and 10 were used to determine the amount (µM) of each species present considering the 5 cm path length of the instrument.

Table 12: Millimolar absorption coefficients of Haemoglobin derivatives†

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxyHb\textsuperscript{102}</td>
<td>560</td>
</tr>
<tr>
<td>methHb\textsuperscript{103}</td>
<td>8.6</td>
</tr>
<tr>
<td>ferrylHb\textsuperscript{104}</td>
<td>4.30</td>
</tr>
<tr>
<td>Hemichrome</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
</tr>
</tbody>
</table>

† All values are expressed per heme group.

\[
\text{[oxyHb]} = 13.2A_{577} - 16A_{630} \\
\text{[methHb]} = -0.6A_{577} + 55.8A_{630}
\]

Equation 9

\[
\text{[oxyHb]} = 17.8A_{560} + 23.8A_{577} - 7.8A_{630} \\
\text{[methHb]} = -10A_{560} + 5.3A_{577} + 60.8A_{630} \\
\text{[hemichrome]} = 46.6A_{560} - 26.5A_{577} - 22.6A_{630}
\]

Equation 10

Dry metHb samples were re-hydrated with 1.1 ml of water. An additional 1.0 ml of water was added to the hydrated samples to bring their total volume to 1.1 ml as well. From each sample, 13.75 µl was taken and diluted to 100 µl.
to form 250 ppm solutions whose absorption spectra were measured from 200-800 nm.

To detect the presence of hemichromes against the background of oxyHb and metHb, samples were reduced and their spectra were measured to look for characteristic peaks at 529 and 558 nm indicative of hemochromes. Solid sodium dithionite was added directly to the Hb solutions and allowed to react at room temperature for 30 minutes. Samples were then injected into dialysis cassettes (Fisher Scientific, USA) and dialyzed against a 0.1 M phosphate buffer (pH 7.0) overnight.

Release of Iron Cations from Hb

Free Fe(II) ions was determined using the ferrozine method of Carter. Ferrozine, a disodium salt of 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonyl)acid)-1,2,4-triazine, forms a stable magenta colored compound with the ferrous ion in a 3:1 ratio (Figure 57) and has an absorption peak at 562 nm. Neocuproine forms a complex with copper(I) and copper(II) that can be used in conjunction with ferrozine to keep copper ions from interfering with the ferrozine method.
and thus was incorporated into the reaction. Human Hb that was primarily in the form of metHb was incubated in dry and hydrated (20 µg/µl) states over a 42 day time period. Samples were then diluted to a concentration of 1.82 µg/µl and two aliquots each of 500 µl were taken from each sample. One of the aliquots was then reduced by the addition of 500 µl reducing agent (ascorbic acid) and allowed to sit at room temperature for five minutes. This will result in the reduction of any Fe(III) present to Fe(II). The other aliquot was not reduced and this sample represents the amount of Fe(II) present. The Hb was then removed from both aliquots by the addition of 500 µl protein precipitant and centrifuged at 3000 rpm for five minutes. A volume of 1 ml was then removed from the reduced samples and to this was added 400 µl buffer and 100 µl ferroin reagent. A volume of 500 µl was removed from the non-reduced samples and to this was added 200 µl buffer and 100 µl ferroin reagent. The magenta-colored chromophore formed within five minutes and the stability of the complex was sufficient enough to allow for spectrophotometric analysis at 562 nm up to at least one day after formation. The difference in the response curves
for the non-reduced (Fe(II)) and reduced (Fe(II) + Fe(III)) samples represents the amount of Fe(III) present.

Reagents
Reducing Agent: A 0.2% ascorbic acid in 0.2 N hydrochloric acid solution was made by dissolving 20 mg ascorbic acid in a 100 ml volumetric flask with 1.67 ml of concentrated hydrochloric acid and deionized water. The solution was stored at 4°C for not more than a three day period.

Protein Precipitant: An 11.3% trichloroacetic acid (TAA) solution was made by dissolving 11.3 g of TAA in a 100 ml volumetric flask with deionized water.

Buffer Solution: A 10% ammonium acetate buffer solution was made by diluting 10 mg of ammonium acetate with deionized water in a 100 ml volumetric flask.

Ferroin Color Reagent: The ferrion color reagent consists of both ferrozine and neocuproine in aqueous solution. 300 mg of ferrozine and 300 mg of neocuproine were dissolved in deionized water using a few drops of concentrated
hydrochloric acid to aid in dissolution and diluted to 100 ml in a volumetric flask.

Standard Iron Solutions: A standard iron solution was made by dissolving 702 mg Fe\((\text{NH}_4\text{)}_2\text{SO}_4\cdot6\text{H}_2\text{O}\) with 0.5 ml concentrated sulfuric acid into a 1 liter flask. Standards were then made consisting of 0, 0.195, 0.476, 0.909, 1.15, and 1.45 µg/ml Fe(II). These standards were used to construct a calibration curve for Fe(II) determination in samples.

Figure 57: (Left) Structure of generic ferroin grouping that has complexing capabilities, (Middle) The ferrozine molecule complexed with an Fe(II) ion in a 3:1 ratio, (Right) The structure of the neocuproine molecule.
Hydroxyl Radical Detection

A thiobarbituric acid (TBA) assay for hydroxyl radical (OH•) detection was used that is based upon the detection of hydroxyl radical attack on, and degradation of, the sugar deoxyribose (2-deoxy-ribose).\textsuperscript{45,47,110-129} When the resulting degradation product is heated under acidic conditions, malonaldehyde (MDA) is formed and that is detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen. This assay has a high degree of specificity for OH• detection since other oxidizing species such as peroxyl and alkoxy radicals do not release TBA reactive materials from deoxyribose.\textsuperscript{122}

Exposure of deoxyribose to OH• at 37°C creates a situation where the generated products degrade to form MDA upon heating in acidic conditions.\textsuperscript{119} The structure of the TBA-MDA adduct was determined by Nair and Turner\textsuperscript{118} to be that shown in Scheme 3 where the two most dominant resonance structures are presented as determined by UV-visible, FTIR, \textsuperscript{1}H NMR, and \textsuperscript{13}C NMR spectroscopies. Scheme 4 presents the probable situation of nucleophilic attack involving carbon-5 of TBA onto carbon-1 of MDA followed by dehydration.
The second TBA molecule is introduced in a similar fashion upon attack of the 1:1 intermediate.

Scheme 3

The formation of the TBA-MDA adduct starting from the oxidation of deoxyribose which degrades upon heating in acid.

Scheme 4

The nucleophilic attack on MDA by two TBA molecules. Scheme 2 - Schematic of likely attack of MDA by TBA.

Preliminary studies of the TBA reaction were conducted to optimize deoxyribose concentration and incubation parameters. Figure 58 shows the absorbance measurement at 532 nm using 60 µg Hb and varying the concentration of deoxyribose at 37°C. The assay concept was to reach a point where the deoxyribose is not continuing to absorb radiation regardless of the concentration. It can be seen
that the absorption increases linearly up until approximately 60 mM, where the absorption becomes somewhat constant until approximately 100 mM. Above 100 mM the absorption increases again linearly. This trend is not only noted for samples with 60 µg Hb in them, but for the blank samples containing no Hb. The absorbance of the blank samples containing only deoxyribose treated with TBA increases with deoxyribose concentration, especially at concentrations larger than 100 mM; therefore, the curve will always increase slightly as the concentration of deoxyribose increases without degradation by Hb. The same figure shows the absorbance curve holding the concentration of deoxyribose steady at 100 mM and increasing the amount of Hb. At 100 mM the increase is linearly responsive to Hb concentration. Based on these results it was decided to use a 100 mM concentration of deoxyribose in the assay.

The incubation time period for reaction with deoxyribose was looked at using an Fe(II) standard and oxyHb for up to 1 hour checking samples every 15 minutes (Figure 59). From this study it was determined that there was not much difference in incubating with deoxyribose over a period of 15 minutes or for 1 hour. It was decided to use the 15
minute time period so as not to reach a point where the maximum amount of decomposition occurs regardless of the hydroxyl radical producing capability of the sample.

Human Hb that was primarily in the form of metHb was incubated in dry and hydrated (20 µg/µl) states over a 42 day time period in ambient conditions. Samples were then diluted to a concentration of 1.82 µg/µl and 16.5 µl aliquots (30 µg) were taken from each sample. Aliquots were added to 300 µl of 100 mM deoxyribose and allowed to incubate at 37°C for fifteen minutes. To each sample was
added 400 µl of each 1% w/v TBA in 0.05 M NaOH and 2.8% w/v trichloroacetic acid (TAA). Samples were incubated at 95°C for 15 minutes.

Figure 59: Reaction of 20 mM deoxyribose with Fe(II) and ferrous Haemoglobin (Hb) incubated at 37°C over time periods of up to one hour. The sugar is degraded on exposure to hydroxyl radicals. The reaction mixture is heated under acidic conditions using TAA to form malondialdehyde (MDA) which reacts with TBA to form a pink chromogen. The absorption was measured at 532 nm after incubation of deoxribose (■), with 1.66 µg Fe(II) (●), and with 35 µg Hb (▲).

Due to a number of factors a buffer was not used for the reactions described above. Firstly, some buffers such as Tris and Hepes are scavengers of OH•. Secondly, though a phosphate buffer mimics an in vivo situation, iron ions can bind to the buffer, to the deoxyribose, or to other
components of the reaction mixture. Iron-phosphate complexes are weakly active in producing ‘free’ OH•. All reactions were carried out in quadruply filtered de-ionized water.

To detect the MDA-TBA chromagen, 200 µl of the reaction assay was added to 500 µl 10% ammonium acetate buffer. The addition of the buffer allowed the pink chromagen to remain stable over the time period required to conduct spectrophotometric measurements at 532 nm. The absorption was used to determine relative amounts of deoxyribose degradation by aged samples.

Results

Oxidation of Human Hemoglobin

The dry state Hb samples used in the initial experiments were in their oxidized form (oxyHb) according to their measured spectra. Initially, ferrous-stabilized Hb was used to measure the relative rates of oxyHb oxidation between hydrated and dry state Hb. Without the stabilization process, the Hb would be primarily in the
metHb oxidized ferric form upon receipt in the lab from the commercial vendor due to the inherent proclivity of the metalloprotein to undergo ferrous ion oxidation over time. OxyHb samples in the dried and hydrated states were maintained at ambient temperature (22.0 ± 0.4°C) and relative humidity (54 ± 7 %) in the dark for varying periods up to 3 months (2200 hours). Oxidation product formation was monitored by visible region absorption spectrophotometry. The concentrations of oxyHb, metHb, and presumed hemichrome were measured as a function of time.

The spectra of hydrated oxyHb over time (Figure 60A) indicated the likely presence of only two major species because two isosbestic points at 524 and 590 nm were identified. The spectra shown are an average of three separate samples incubated over the same time period and might account for the minor variation of spectra around the 524 nm isosbestic point. However, to further investigate the number of Hb species formed, it was compared the rates of formation of solely metHb or the formation of metHb and hemichromes as a second product with the rate of degradation of oxyHb. If only two species were present (viz. oxyHb and metHb), then oxyHb appeared to degrade at a
rate of $1.5 \pm 0.04 \times 10^{-7} \text{ s}^{-1}$ and metHb formed at a rate of $1.1 \pm 0.1 \times 10^{-7} \text{ s}^{-1}$. If there were a strict one to one relationship between reactant and product, the two rates should be the same. However, calculation of the t-statistic indicates that the rates are significantly different ($t = 33.3$) at the 95% confidence level. In Figure 61A the rate of oxyHb loss over time was determined under the assumption that hemichromes were also being formed. In this scenario oxyHb degraded at a rate of $1.7 \pm 0.1 \times 10^{-7} \text{ s}^{-1}$ in the hydrated state. MetHb formed at a rate of $1.1 \pm 0.1 \times 10^{-7} \text{ s}^{-1}$ and hemichrome formed at a rate of $6.3 \pm 0.4 \times 10^{-8} \text{ s}^{-1}$. The combined rate for both metHb and hemichrome formation was $1.7 \pm 0.1 \times 10^{-7} \text{ s}^{-1}$ which should be the same as oxyHb degradation if both products were being formed. Although the t-statistic indicated that the rates were different, the value ($t = 3.35$) was ten times smaller than the one calculated without assuming the presence of product species other than metHb. The sums of the estimated concentrations of the reactant (oxyHb) and proposed products (metHb and hemichromes) appeared to be reasonably constant over time (Figure 60B) although a slight decrease was discernible. It is possible that the
latter observation might indicate the presence of another minor as-yet-unidentified product.

Figure 60: All data comprise an average of three samples. (A) Spectra of hydrated Hb at various time periods where it is evident that the oxyHb is oxidizing to primarily metHb. (B) Concentration of oxyHb (■), metHb (●), and hemichromes (▲) from hydrated Hb incubated over a 2200 hour time period in ambient conditions. (C) Spectra of dry Hb at various time periods where it is evident that the oxyHb is oxidizing to not only metHb, but what is suspected to be hemichromes. (D) Concentrations of oxyHb (■), metHb (●), and hemichromes (▲) from dry Hb incubated over a 2200 hour time period in ambient conditions.
In contrast to the hydrated state, dry state oxyHb lacked the two isosbestic points in the time evolved spectra (Figure 60C). Thus, more than two species were present with the initial hypothesis being that, like hydrated Hb, hemichromes were being formed in addition to metHb but in larger quantities than with the hydrated samples. This hypothesis is supported by the approximately 2 fold increased rate of oxidation measured for dry state oxyHb \( (k = 3.6 \pm 0.2 \times 10^{-7} \text{ s}^{-1}) \) compared to the hydrated state (Figure 61B). Although a degradation rate can be determined for dry state oxyHb, the formation of metHb and hemichrome over time did not appear to be a first order reaction. The sum of the three species (i.e. the reactant, oxyHb and the products, metHb and hemichromes) did not remain constant over time (Figure 60D) and is consistent with the presence of a fourth species. The putative fourth species could be a denatured Hb derivative that is not detectable by the methods employed here. Considering the nature of the Hb metalloprotein, other potential species that might be formed include ferrylHb and choleglobin. FerryHb is an Fe(IV) complex formed from ferrous hemoglobin and \( \text{H}_2\text{O}_2 \) whereas choleglobin is denatured hemoglobin in which the porphyrin ring has been hydroxylated or broken.
open. The ferrylHb species was ruled out because: (a) there was no exposure to H₂O₂, nor were there any environmental conditions that would lead to such exposure; and, (b) the spectra do not show evidence of its existence, primarily by the lack of spectral broadening where the 577 nm shoulder drops of steeply around 585 nm. There was also no spectral evidence of the presence of choleglobin due to the lack of increased absorption at 700 nm characteristic of the species.¹³¹

Figure 61: All data are an average of three samples. (A) Rate determination for the formation of metHb (■) and hemichromes (●) from hydrated samples. The rates are \( k = 1.11 \pm 0.07 \times 10^{-7} \text{ s}^{-1} \) (R = 0.91735) and \( k = 6.29 \pm 0.4 \times 10^{-8} \text{ s}^{-1} \) (R = 0.92667) respectively. (B) Rate determination for the oxidation of oxyHb by plotting \(-\ln([\text{oxyHb}]/[\text{oxyHb}_0])\) vs. time for both dry (■) and hydrated (●) samples. The rates are \( k = 3.58 \pm 0.17 \times 10^{-7} \text{ s}^{-1} \) (R = 0.95618) and \( k = 1.69 \pm 0.06 \times 10^{-7} \text{ s}^{-1} \) (R = 0.97785) respectively.
Figure 62: Spectra of hydrated (A) and dry (B) metHb at various time periods (0–983 hours). All data are the average of three samples.

Figure 63: Spectra of dry metHb (A) and dry oxyHb (B) prior to (---) and after (——) reduction with sodium dithionite. All spectra were measured after ~1000 hours.

After oxyHb oxidation to metHb had taken place, there was no evidence in either hydrated or dry state for further significant structural transformations that would cause changes in the absorption spectrum (Figure 62). After
reduction with sodium dithionite, dry state metHb and dry state oxyHb (Figure 63); maintained at ambient temperature and humidity for 1000 hours were converted to Hb primarily in the oxyHb form. Hydrated state oxyHb and metHb samples similarly treated were also converted to oxyHb (data now shown). The characteristic spectrum of hemochrome was not detected in any sample after reduction. Thus, it was concluded that the formation of hemichromes was not responsible for the additional unknown species in degraded oxyHb.

Release of Iron Cations from Hb

Human Hb that was primarily in the form of metHb was incubated in the dry and hydrated states over a 1000 hour time period and the free iron released from the Hb measured at various time intervals. Fe(II) was measured directly by interaction with ferrozine whereas Fe(II) plus Fe(III) was determined after reduction with ascorbic acid. The amount of Fe(III) is thus indicated by the differences in the two response curves.
Free Fe(III) was detected in both the hydrated (Figure 64A) and dry (Figure 64B) state oxyHb samples. This was expected because it was known that the Hb had oxidized into primarily metHb at the time the experiments were performed. Though Fe(III) appeared to be the dominant form of free iron, some Fe(II) was present. It can be hypothesized that Fe(II) was released while the protein was still in its oxyHb form, but as the protein was oxidized to metHb the iron that continued to be released was in the +3 oxidation state.

What was surprising was over time the Hb samples did not continue to release free iron in either state, as is evidenced by the lack of an increase in free iron. The dry samples did exhibit an insignificant increase in the

Figure 64: (A) Free iron present in hydrated Hb, ■ = Fe$^{2+}$, ● = Fe$^{2+}$ and Fe$^{3+}$. (B) Free iron present in dry state Hb, ■ = Fe$^{2+}$, ● = Fe$^{2+}$ and Fe$^{3+}$.
release of free Fe(II), perhaps due to some configuration that the protein takes in the dry state that leads to more favorable release of the ion during the dehydration process. Overall, these results imply that dried bloodstains may provide reactive free Fe(II) that can engage in a Fenton type reaction but that the age of a bloodstain may not be a significant factor in its ability to do so.

**Hydroxyl Radical Detection**

Hb primarily in the form of metHb was incubated over time at ambient temperature (21.9 ± 0.1°C) and relative humidity (61.3 ± 1.0%). The samples were reacted with deosyribose and then TBA to detect oxidative damage to the deoxyribose. Oxidative damage due to OH• attack of deoxyribose was considered to have occurred if the absorption of the pink chromogen after incubation with metHb was greater than incubation in the absence of metHb (Figure 65). The peak area obtained by measuring the spectra at 532 nm was used to determine relative amounts of damage to each sample after blank subtraction. The hydrated state metHb caused the most hydroxyl radical damage at the initial time point.
before being left to sit in ambient conditions and the damage decreased over time. The dry state MetHb displayed much less oxidative ability and the reactivity did not change noticeably during the time period.

![Graph](image)

**Figure 65:** MetHb incubated over time at 21.9 ± 0.1 °C and 61.3 ± 1.0 relative humidity (■ dry state, ● hydrated state). The samples were reacted with deoxyribose and then thiobarbituric acid to detect oxidative damage to the deoxyribose. The absorption measured at 528 nm is given after blank subtraction. Measurements are the average of three different samples incubated at the same time point.

**Discussion**

The data presented in this study indicates that dry state Hb undergoes much more rapid oxidation than that in a hydrated state. In both states; however, the resulting
product is Hb in which the Fe center has been oxidized to Fe(III). At least one other species is believed to be present as the result of the oxidation of oxyHb in both the hydrated and dry states (particularly the dry state), but its identity has eluded the experimental schema employed. In the hydrated state at neutral pH, the oxidation of oxyHb to metHb and the reduction of metHb to oxyHb have approximately the same rate constant. However, over extended periods of time such as was experienced by the samples here, this equilibrium eventually ceases to hold and the metHb species becomes more prevalent. It is possible that dry state oxyHb forms metHb more rapidly than in its hydrated form due to the lack of dynamic equilibrium that the hydrated state offers.

Formation of OH• requires the presence of iron salts, specifically Fe(II). Other transition metals or iron-protein complexes including Hb are unable to catalyze the reaction. It is believed that the most likely route for oxidative damage to DNA caused by a Fenton type reaction involving Fe(II) is for the ferrous ion to bind to the deoxyribose molecule with a certain affinity and then induce site specific damage. This hypothesis is supported
by prior studies carried out by Gutteridge\textsuperscript{44,120} where it was noted in such systems the reaction of the carbohydrate with OH• was poorly inhibited by most OH• scavengers. In addition to experimental evidence, a theoretical analysis of the thermodynamics of a “Fenton type” reaction offers evidence for an inner-shell or bridged reaction mechanism.\textsuperscript{95}

It has been previously reported that OH• can be generated in a reaction that is independent of O\textsubscript{2}•− by the addition of Fe(II) salts alone (Reactions 1, 2 and 3 below).\textsuperscript{115,116} This was determined by Halliwell and Gutteridge by the inability of superoxide dismutase to prevent deoxyribose degradation. However catalase did prevent damage indicating that H\textsubscript{2}O\textsubscript{2} is involved in the reaction, despite it not being added to the reaction mixture.\textsuperscript{115} Ferric ion incapable of degrading the deoxyribose substrate without the addition of a superoxide-generating system (xanthine/xanthine oxidase). The O\textsubscript{2}•− most likely reduces Fe(III) to Fe(II) (Reaction 4) which then allows for Reaction 1 to occur.\textsuperscript{121,123} The net reaction of Reaction 3 and Reaction 4 is Reaction 5 (Haber Weiss reaction). The Haber Weiss reaction is the underlying phenomenon that is believed to be the principle source of OH• in biochemical systems.\textsuperscript{116} Thus, there is protection
offered to samples due to the inherent nature of iron to oxidize to the ferric state.

\[
\text{Fe}^{2+} + \text{O}_2 \rightleftharpoons \text{Fe}^{3+} + \text{O}_2^{-}
\]

Reaction 1

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

Reaction 2

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{-} + \text{OH}^{\bullet}
\]

Reaction 3

\[
\text{Fe}^{3+} + \text{O}_2^{\bullet} \rightarrow \text{Fe}^{2+} + \text{O}_2
\]

Reaction 4

\[
\text{O}_2^{\bullet} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^{\bullet} + \text{OH}^{-}
\]

(Net Reaction of Reaction 3 and Reaction 4)

Reaction 5

OxyHb and metHb have been shown previously to form hydroxyl radicals in the presence of hydrogen peroxide. Although studies were not carried out here with \( \text{H}_2\text{O}_2 \), it was noted by
Halliwell and Gutteridge\textsuperscript{112} that metHb does degrade deoxyribose, and this degradation was increased when ascorbic acid was added to the reaction mixture. The ascorbic acid would reduce any free Fe(III) and allow for a better catalyst for the formation of OH•.

Although it is believed that iron bound to Hb will not produce free OH• in solution, ‘free’ iron can do so. It is possible to degrade Hb using peroxides and release the metal center,\textsuperscript{124} allowing for Fenton type chemistry. It was shown here that in the absence of H₂O₂ or any other organic hydroperoxides, small amounts of free iron can be released from the Hb molecule. The small amounts of unbound iron are sufficient to degrade deoxyribose to an extent that exceeds that of deoxyribose heated in the absence of Hb. It was also observed that dry state metHb did not degrade deoxyribose as extensively as hydrated state metHb did. This was unexpected as the amount of ‘free’ Fe(II) was measured to be slightly greater in dry state metHb.

Overall, the age of metHb samples did not influence their ability to generate OH• and cause oxidative damage. Though it is apparent that Fenton type chemistry is likely to occur in a Hb-containing system such as a dried bloodstain,
the damaging capabilities of such a system do not appear to increase as the age of the system increases – at least with the relatively mild laboratory conditions studied here. Further studies would have to be performed to determine whether the same holds for samples maintained under conditions representative of more extreme climatic conditions. Additionally, due to the presence of all cellular components in bloodstains (including pools of non-Hb sources of ‘free’ iron) the dynamics of the oxidative damage process may differ from the one studied here in isolation.

Based on the findings here, the oxidative damage that would be incurred by a dried stain sample containing the Hb molecule would most likely occur prior to the receipt of such a sample by the analyst. Therefore, subsequent storage of such samples should not result in further damage induced by Hb-derived hydroxyl radicals.
CHAPTER 7: GENERAL CONCLUSIONS

This study has shown that there are similarities between reactions that occur to hydrated and dry state DNA and its molecular components over time when induced thermally and photochemically. In the dry state these reactions are able to be detected, kinetically measured, and thermodynamically analyzed. Similarly in both states, molecules undergo base hydrolysis and induced strand breaks after subjection to thermal degradation. It is also believed that though it was not able to be fully confirmed here, that deamination occurs in both states as well. The main difference between the aqueous and dry states is that hydrolytic reactions occur much more slowly in the dry state than they do in the hydrated state leading to the conclusion that when temperature is a lone factor, forensic type biological samples have an advantage.

UV induced DNA damage resulted in differences noted between dry and hydrated DNA. Particularly, dry state DNA appeared to form DNA-DNA cross-links more readily than its hydrated analogue. It was also noted that base loss occurs in the dry state after UVC irradiation in the same time frame as
it does in the hydrated state. This can lead to the conclusion that forensic type samples available to solar radiation could be more prone to abasic sites than those types of samples subjected to ambient temperatures alone.

Now that the types of reactions that occur most readily to forensic type samples have been revealed, the next step would be to determine in more detail the steps that need to be taken to bypass and/or repair such damage. One of the most noted insults to occur is the breakage of the N-glycosidic bond between the nucleobase and the deoxyribose sugar. Without taking into consideration the subsequent strand breaks resulting from such damage, it would be necessary to replace missing bases to ensure the ability of the DNA polymerase to readthrough such sites during PCR. A natural intracellular pathway for repair of DNA damage consists of a DNA glycosylase recognizing a damaged nucleobase and excising it from the molecule leaving an abasic site. An AP endonuclease then recognizes the abasic site and cleaves the strand leaving a 3’-OH upstream of the damaged site. A DNA polymerase will then recognize this damage and using the opposite strand as a template, replace the damaged base along with several downstream. The final
step in the repair pathway is for a DNA ligase to anneal the newly formed DNA. As long as there is not significant subsequent damage to the DNA template in question, this repair pathway can be duplicated in vitro leading to the recovery of the damaged template.

The most probably approach to repair of single strand breaks in forensic type samples will be dependent on the nature of the fragmented ends of the DNA template after their introduction via environmental degradation. The mode of formation of such sites could also play a role in the molecular structure at the site of strand breaks. Those that occur due to the presence of abasic sites could very well be different than breaks that occur without the initial occurrence of base loss. This research provided evidence that abasic sites alone do not account for the frequency of strand breaks noted in neither dry nor hydrated DNA molecules. Enzymatic repair would be largely dependent on which side (3’ or 5’) the phosphate group remained with during the degradation process. Of course it is possible that the phosphate group is lost all together leaving either an -OH group or some further degraded
structure that would not be recognizeable by naturally occurring repair enzymes.

It has been presented here that deamination does not play a large role in the degradation noted in forensic type biological samples. The use of STR analysis in basic forensic biological analysis is not reliant on the sequence of the template DNA, but rather the number of tandem repeats in specific regions of the genome. Thus the presence of a uracil residue will not affect the outcome of the PCR analysis unless it so occurs in the primer binding region of the template. In which case, the product should still amplify, but possibly with less efficiency due to the lower annealing temperature that would be associated with the affected primer. The conclusion for forensic type samples is that other damage would affect the integrity of the sample long before deamination of cytosine would become a problem.

The nature of crosslinking in DNA that has been exposed to ambient radiation will be key in its repair. Naturally occurring photolyases are active towards specific types of damage. It can be hypothesized that cyclobutyl pyrimidine
dimers (CPDs) or 6-4 photoproducts are responsible for
interstrand linking. Even if this is true, there are many
product isomers that can exist. It will require further
examination of damaged templates to determine the exact
molecular structure of crosslinks that lead to the
observations made. Only then can the correct repair
pathway be determined.
APPENDIX: RATES AND ACTIVATION ENERGIES FOR HYDROLYSIS MEASUREMENTS FROM PREVIOUSLY PUBLISHED LITERATURE
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Deoxyguanylic acid
Deoxyadenosine
Deoxyguanosine
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Buffers of physiological ionic strength: 7.4
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†Thymidine was probed at 155°C (for fifteen minutes), 100°C (up to four hours) and 37°C (over forty-eight hours), but no base loss was found to occur. Deoxycytidine did not show any base loss at 155°C (in fifteen minutes) or at 37°C over a forty-eight hour time period.
‡These values were obtained by fitting the author’s data to Arrhenius plots of lnk vs. 1/T.
*This value was reported by the author to be 28 kcal/mol (117.2 kJ/mol) which is not in agreement with my calculation. There was no error reported by the author’s.
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