The Direct Study of the Reaction of the Tamoxifen Carbocation with DNA

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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For

Mom and Dad
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Master of Science. 2001

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Abstract

Of the women administered tamoxifen (TAM) to treat breast cancer, a small percentage developed endometrial adenocarcinomas. The mechanism of carcinogenesis involves the formation of a carbocation intermediate (TAM\(^-\)) that binds to cellular DNA causing frameshift mutations. To examine the binding that leads to the formation of adducts, a detailed study of TAM\(^-\) in the presence of DNA was conducted.

TAM\(^-\) was shown, by kinetic measurements, to form non-covalent association complexes with DNA. This electrostatic binding was confirmed by varying pH and ionic strength. Decreasing pH and added salt increased the binding constant and observed rates. The TAM\(^-\)-DNA complex reacts with water and DNA to form alcohols and adducts respectively. The selectivity ratios (rate of reaction with DNA vs. water) were: by HPLC, 7.31 \(\times\) 10\(^3\) M\(^{-1}\) and 3.75 \(\times\) 10\(^3\) M\(^{-1}\) at 22°C and 37°C respectively; by laser flash photolysis, 2.76 \(\times\) 10\(^4\) M\(^{-1}\) and 1.86 \(\times\) 10\(^4\) M\(^{-1}\) at 22°C and 37°C respectively. DNA is a better nucleophile than polyguanylic acid or deoxyguanosine alone.
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Chapter One:

Tamoxifen
1.1 **Background**

The incidence of female breast cancer has increased substantially recently in developed countries, yet mortality rates have not.\(^1\) This is partly a result of early diagnoses by breast cancer screening with mammography, as well as a consequence of the introduction of tamoxifen (TAM, \(\text{trans}^\ast\)-1-(4-(2-(dimethylamino)ethoxy)phenyl)-1,2-diphenyl-1-butene. 1) as an effective treatment that improves the survival of women with early disease and of patients with advanced breast cancer.\(^1\) This drug has been in clinical use for over 25 years and is the most widely used adjuvant therapy drug for the treatment of breast cancer in the world.\(^2\) The efficacy of TAM in reducing the incidence of breast cancer in women has led to its recent use as a chemopreventative agent, i.e. use by healthy women with a family history of the disease who are at an increased risk of developing it.\(^3\)

TAM can act in two ways, as an estrogen-antagonist or as an estrogen-agonist, and this is a function of the estrogen receptor complex present in a particular cell or tissue. The antitumor effects of TAM are due to its antiestrogenic activity by competitively inhibiting

\[\text{cis} - \text{trans} \text{ nomenclature is used to indicate the location of the phenyls relative to each other. If neither } \text{cis}-\text{nor } \text{trans}- \text{ is denoted, then the } \text{trans}- \text{isomer is implied.}\]
the binding of estrogen to its receptor sites. Consequently, TAM inhibits the expression of estrogen-regulated genes, such as growth factors that are secreted by the tumor and may stimulate growth. The net result is blockage of the G1 phase of the cell cycle and the slowing of cell production.4

The alkylaminoethoxy side chain of TAM is an essential structural feature needed for biological activity (along with conjugation). The nitrogen and the oxygen must be separated by two carbon atoms and the basicity of the nitrogen is also critical, as the pKₐ of the protonated amine must be about 7.9.5 Removal of the side chain results in loss of activity and estrogenic properties.
1.2 **Cancer Risk via DNA Adducts**

It was previously believed that TAM was "the cure" for breast cancer because of all the advantages associated with it. Not only is it highly effective, but it is extremely easy to administer (orally ingested in a tablet form daily), has limited side effects and, with continued treatment, reduces the rate of occurrence of new, contralateral breast cancer. In an unfortunate turnaround, studies in the last decade have shown a carcinogenic effect of TAM that comes with long-term treatment of breast cancer. Administration of TAM to women is associated with an increased risk of endometrial cancer in both breast cancer patients and in healthy women. This conclusion causes great concern in using it prophylactically against human breast cancer.

Early experiments with rats have shown that TAM is a potent liver carcinogen, forming DNA adducts in several rat organs. The highest levels thus found being in the liver. TAM induced DNA adducts have also been seen in human endometrial samples using \(^{32}\)P-postlabeling techniques coupled with high-performance liquid chromatography (HPLC).

### 1.2.1 Rodent Studies

The formation of DNA adducts in the livers of rats administered with TAM strongly suggests that TAM is carcinogenic by a genotoxic mechanism. These DNA adducts were identical to those isolated and characterized by *in vitro* studies of the reaction of DNA with \(\alpha\)-acetoxytamoxifen\(^{10}\) (TAMOAc. 2). Beyond identification of these adducts, the studies performed on rats assisted in determining the mode of activity of TAM.
Several TAM related compounds were synthesized by Brown et al. to assess their ability to cause DNA damage in the liver of rats using a $^{32}$P-postlabeling assay, ultimately leading to an understanding of the genotoxic potential and possible mechanisms of carcinogenicity of TAM.

On administration of equimolar doses of tamoxifen 1,2-epoxide (3) or bromotamoxifen (4) to rats, no $^{32}$P-postlabeled DNA adducts could be detected. The absence of these adducts in the liver of rats indicate that these compounds are unlikely to be involved in the activation pathway. Furthermore, the inactivity of bromotamoxifen supports the view that $\alpha$-hydroxylation is a necessary first step in the activation of TAM to form $\alpha$-hydroxytamoxifen (TAMOH, 5). TAMOAc shows an approximate 200-fold increase in adduct formation relative to TAMOH, supporting the need for conjugation of
TAMOH to generate the electrophilic species.\textsuperscript{10} These electrophiles (e.g. carbocations and nitrenium ions) are known to be the metabolites many known carcinogens.

\textbf{1.2.2 Patient Studies}

The results from the rat studies sparked interest in searching for TAM-DNA adducts in human samples, as there was concern that the administration of TAM may be linked to an increased risk of liver cancer. In 1985, an association was made between TAM therapy and the development of endometrial cancer.\textsuperscript{13} In the earlier stages of this research, no DNA adducts were found in the liver, leucocytes,\textsuperscript{14} nor in endometrial tissues\textsuperscript{15} of subjects administered 20 mg of TAM daily (standard recommended dose). This was thought to be a result of a poor detection assay used by these researchers, so new, more sensitive techniques were developed.

In 1996, the International Agency for Research on Cancer (IARC) concluded that TAM was in fact carcinogenic in humans, and added it to the IARC list of known human carcinogens.\textsuperscript{16} Hemminki \textit{et al.}, using \textsuperscript{32}P-postlabelling coupled with HPLC, were one of the first to report the identification of TAM-induced adducts in human leucocytes\textsuperscript{17} and in endometrial tissue.\textsuperscript{9} The use of HPLC rather than thin layer chromatography (TLC) helped reduce the background radioactivity, thereby boosting the effective level of detection to approximately 1 adduct / 10\textsuperscript{9} nucleotides. These adducts were not characterized, but matched by retention times on HPLC using rat liver DNA as the positive standard. Bartsch \textit{et al.}, using similar detection techniques and standards, found no evidence of TAM-DNA adducts on lymphocyte DNA from female patients treated with therapeutic levels of TAM.\textsuperscript{18}
Shibutani et al. developed an ultrasensitive procedure for quantifying TAM adducts in endometrial DNA; they used a $^{32}$P-postlabeling TLC/HPLC technique combined with a butanol extraction procedure. The TAM-DNA adducts were isolated, purified and identified as cis- and trans- isomers of $\alpha$-(N$^2$-deoxyguanosinyl)tamoxifen (TAMdG, trans-6, cis-7. N.B. dR = deoxyribose sugar). The epimers of these two forms were also separated and quantified for a total of four (4) TAMdG adducts.

![Chemical structures](image)

There are several other metabolites observed in women administered TAM. TAMOH, TAM N-oxide (8), N-desmethyl TAM (9), and 4-hydroxytamoxifen (4-TAMOH, 10) were the major metabolites found. Several of these human metabolites have been modified by N-demethylation, 4-hydroxylation, or N-oxidation. It has been thought that $\alpha$-hydroxylation can lead to the formation of DNA adducts and the possible carcinogenicity of TAM.
1.2.3 Summary

The discussed studies display how TAM exhibits an unusual pattern of carcinogenicity. It causes cancer in the livers of rats, whereas women treated with TAM develop cancer in leucocytes and the endometrial tissue, but not in the liver. Human endometrial cancer has been attributed to the partial agonist effects of TAM and/or to the genotoxic effects of the drug.21
1.3 Metabolism

Several hypotheses have been proposed for the precise mechanism of formation of DNA adducts leading to the carcinogenic effect of TAM. They have been put forward in an attempt to explain the metabolites found in rats and women that have been administered TAM.

1.3.1 Tamoxifen

There have been three main metabolic pathways that have been proposed for the activation of TAM to a reactive electrophile. The general scheme of these three pathways is displayed in Figure 1.1.

![Metabolic activation pathways proposed for TAM](image)

**Figure 1.1.** Metabolic activation pathways proposed for TAM where the only major DNA adduct is shown.22
1.3.1.1 Quinone Methide

Studies of deoxyguanosine (dG) with tamoxifen quinone methide (TAMQM, 11) in vitro resulted in a very significant amount of adducts formed. Aromatic hydroxylation of TAM, producing 4-TAMOH, leads to the highly electrophilic TAMQM by a P450-catalyzed direct two-electron oxidation mechanism shown in Figure 1.2.

![Figure 1.2](image)

**Figure 1.2.** The formation of TAMQM, where R = O(CH$_2$)$_2$N(CH$_3$)$_2$.

These dG adducts in vitro were not confirmed in rodent or human subjects. No DNA or dG adducts were observed in the liver of rats treated with TAM or TAMOH, nor in the studied endometrial tissue of patients.

1.3.1.2 Epoxides

A second pathway involves the oxidation TAM to form epoxide derivatives. In vivo studies with rats showed that neither the reactive intermediate tamoxifen 3,4-epoxide (12) nor tamoxifen 1,2-epoxide (13) caused any detectable DNA damage (relative to the control experiments).
1.3.1.3 Tamoxifen Esters

It is known that TAM requires metabolic activation before binding to DNA. Several metabolites have been observed (mentioned earlier in Section 1.2.2) which are mainly a result of the activity of cytochrome P450 enzymes in humans and other mammalian species.\textsuperscript{25} Oxidation of the ethyl side chain of TAM results in the formation of TAMOH (5), one of the major metabolites detected.

The TAMOH requires conjugation to provide a good leaving group, which, after bond cleavage, would result in a carbocation that is stabilised by conjugation. This conjugation is catalysed by sulfotransferases to form a sulfate ester. This process is displayed in Figure 1.3, along with some of the other metabolite activations. TAM N-oxide and N-desmethyl TAM can also undergo oxidation by P450 leading to DNA adducts, although it is thought that N-oxidation of TAM is reversible. Although TAM N-oxide and TAMOH N-oxide give rise to DNA adducts, some of these adducts appear to lack the oxygen at the N position.\textsuperscript{26}
Figure 1.3. Formation of cis- and trans- isomers of TAM-DNA adducts.²¹
After the sulfate group leaves to produce the carbocation intermediate, there can be rapid interconversion between the cis- and the trans- isomers. This is the reason why the cis-isomer is observed when the trans- isomer is reacted both in vivo and in vitro\textsuperscript{27, 28}. Hence, after the preferential reaction with the exocyclic amino group of the dG residue of DNA, 2 cis- and 2 trans- epimers of TAMdG are formed.

![Diagram of the interconversion of cis- and trans- isomers of the TAM carbocation.](image)

**Figure 1.4.** The interconversion of cis- and trans- isomers of the TAM carbocation.
It has been deduced by several different *in vitro* experiments that this proceeds through an $S_N1$ mechanism, where the rate determining step is the cleavage of the ester moiety to form the carbocation intermediate.\(^{27}\) This has lead scientists to believe the $\alpha$-hydroxylation pathway to be the mode of action of TAM in its genotoxic activity. Four major findings allowed for this conclusion to be made.

Firstly, Phillips *et al.* performed experiments comparing the rates of oxidation of [D$_5$-ethyl]-TAM (14) to TAM. They hypothesized that [D$_5$-ethyl]-TAM should be metabolically activated to DNA adducts at a slower rate *in vivo* and *in vitro*. This is due to the slightly higher bond energy of the C – D bond compared to the C – H bond of TAM, and consequently [D$_5$-ethyl]-TAM may be less genotoxic than TAM. It was found that female rats given 0.06 and 0.12 mmol/kg of [D$_5$-ethyl]-TAM resulted in a 2.5 and 1.7 fold reduction respectively in the levels of hepatic DNA adducts compared to TAM. *In vitro* studies showed a 2 to 3 fold decrease compared to the non-deuterated compound.\(^{29}\) These data strongly suggest that TAM is metabolized by $\alpha$-hydroxylation of its ethyl group.

Secondly, Phillips and his coworkers also investigated the effect of TAMOH vs. TAM alone. They administered female rats with each individually, and found that rat hepatocytes with TAMOH resulted in 15 – 63 fold higher levels of DNA adducts as
compared to those given TAM alone. These results have not considered the fact that some TAMOH may have not been detected because of rapid detoxification by glutathione S-transferases.\textsuperscript{30}

The third reason is the confirmation of the formation of the sulfate ester and its necessity in forming the carbocation intermediate. Randerath \textit{et al.} added sulfotransferase inhibitors along with the TAM, and found that few adducts were formed \textit{in vivo}. They concluded that the sulfotransferase inhibitors prevented sulfonation by steroid sulfotransferases, thereby inhibiting the formation of the proximate carcinogen, leading to the reactive carbocation.\textsuperscript{31}

Lastly, a TAM derivative toremifene (TOR. 15) that has a $\beta$-Cl atom substituted for a hydrogen atom in the ethyl group of TAM was found to produce no adducts in rat liver DNA.\textsuperscript{32} It has been suggested by the authors that the $\beta$-Cl atom inhibits the P450-catalyzed $\alpha$-hydroxylation. However, it has been proposed that it may prevent carbocation formation after hydroxylation. It has also been shown by computational analysis that the carbocation intermediate of toremifene is less stable than that of TAM, suggesting that toremifene is not activated as often to the intermediate as TAM is.\textsuperscript{33} Figure 1.5 shows the stability of the reactive carbocation intermediates of some TAM derivatives.
Figure 1.5. The relationship of the stability of the reactive carbocation intermediates of TAM derivatives.33

1.3.2 Other Electrophiles

Benzopyrene (BP), a common environmental pollutant, is metabolized in mammalian cells to a highly reactive and mutagenic diol epoxide (BPDE, 4 stereoisomers) which is prone to attack by nucleophiles like DNA. Their binding to DNA causes mutations by the transformation of protooncogenes to oncogenes. Only one of the stereoisomers (of the four formed) is biologically important, the (+)-anti isomer ((+)-anti-BPDE, 16). (+)-Anti-BPDE binds predominantly to the exocyclic amino group of dG on DNA to form the carcinogenic adduct (17).34
The aminofluorenes are representative of carcinogenic aromatic and heteroaromatic amines present in tobacco smoke, automobile exhaust, protein-containing foods, and as by-products of industrial activity. The heteroaromatic amines (food mutagens) are pyrolysis products of proteins and amino acids. The aminofluorenes act by covalently binding to DNA, predominantly at the C8 position of dG via the nitrogen atom. Before this can occur, bioactivation is required and it is generally accepted that this is accomplished through oxidation, followed by O-esterification (likely sulfation or acetylation – as with TAM). Like TAM, the reaction proceeds via an S_N_1 mechanism. This results in an aryl nitrenium ion, which is the intermediate found to react with dG within DNA.

![Diagram](image_url)

**Figure 1.6.** Pathway leading to the formation of an aryl nitrenium ion and DNA adducts. R = -H (2-aminofluorene), -(C=O)CH₃ (N-acetylfluorene).
Many other carcinogenic compounds are metabolically activated to nitrenium ions. Benzidine, a known occupational carcinogen generates an arylnitrenium ion thereby leading to the formation of guanine C8 adducts as seen with other such electrophiles.\textsuperscript{39} As shown in Figure 1.7, this adduct arises by acetylation to N-acetylbenzidine, oxidation by cytochrome P450 to a hydroxylamine, followed by the formation of the arylnitrenium ion.\textsuperscript{40}

\textbf{Figure 1.7.} The formation of the arylnitrenium ion from benzidine.
1.4 Mutagenesis

The majority of carcinogenic compounds are believed to cause cancer because of their ability to interact and covalently bind to cellular DNA. This event results in the formation of adducts that potentially lead to frameshift or deletion mutations, which will be discussed in the next subsection of this chapter.

1.4.1 DNA Binding

In the cell, DNA is usually found very tightly packed in the nucleus. Although it has its millions of mass units wrapped around itself, its medium is quite fluid and is thereby allowed to move and shift into different conformations. Daune et al. studied the dynamics of DNA by looking at the reactions of two known carcinogens, 2-(N-hydroxyamino)fluorene (N-OH-AF) and 2-(N-acetoxy-N-acetylamino)fluorene (N-Aco-AAF), that form very similar DNA adducts.41

With N-OH-AF, they found that it was necessary for the DNA to be in its native B conformation, as this allowed the C8 position of guanine to be in close proximity to the anionic phosphate group. The phosphate helps to stabilize the transient nitrenium ion that is formed in the metabolism of N-OH-AF.

This native B conformation is a poor setup for N-Aco-AAF, as this leaves the acetyl group on the carcinogen too close to the phosphate group, to the point where they actually collide. With this in consideration, adducts are still observed with N-Aco-AAF and DNA. Therefore, the dynamics of the DNA are very fast, where it bends and kinks itself, allowing changes in its conformation, thereby exposing different positions of the bases. These researchers also suggest that the hydrogen exchange phenomenon also takes place during
these conformational changes of the DNA. This helps to stabilize the DNA in its different conformations.\textsuperscript{41}

Novak and Kennedy present data that shows that, \textit{in vivo}, double stranded DNA should be quite resistant to attack by the N-Aco-AAF nitrenium ion, suggesting that only cells undergoing DNA replication or transcription are likely to react efficiently with these nitrenium ions.\textsuperscript{42} This is in agreement with the fact that actively growing cells are more susceptible to anti-cancer drugs that attack DNA.\textsuperscript{43}

It has been established that DNA adduct formation is common amongst most potent carcinogens. that is, there is a covalent bond formed between the carcinogen and one of the DNA bases. Most carcinogens either alkylate, arylaminate or aralkylate the DNA. Dipple summarized carcinogen adduct formation by emphasizing features that are common to the different chemical classes.\textsuperscript{44}

In alkylation, there are numerous sites of substitution of DNA bases by alkyl residues. Most ring nitrogens and exocyclic oxygens are targeted, especially at the N7 position. However, alkylating agents do not effectively target the exocyclic nitrogens. In arylamination, the C8 atoms and the amino groups of the purines (especially of dG) are the major targets of arylaminating agents. but are unaffected by alkylating agents. In aralkylation, alkenyl benzenes undergo reactions that are similar to those of the arylaminating agents. Polycyclic aralkylating agents (typified by the dihydrodiol epoxides of polycyclic aromatic hydrocarbons) react with DNA primarily at the exocyclic amino groups of deoxyadenosine (dA) and of dG residues (N2 position).

Quinone methides (QM) also react with the exocyclic amino groups of dA and dG.\textsuperscript{45} and have already been shown to react with the N3 position of deoxycytosine (dC) by more
than 10 times in comparison to dA and dG. In double stranded DNA, guanine was the predominant target. whereas reaction with cytosine was suppressed because the N3 position is occupied by a hydrogen bond.

1.4.2 Miscoding Potential

Tumor cells grow uncontrollably because they have mutations in critical genes that are involved in growth control. Carcinogens often induce these mutations, which are initiated when a DNA polymerase encounters a carcinogen-DNA adduct during replication. These adducts can cause misincorporation or slippage by the DNA polymerase or the adduct can be recognized as a foreign entity and consequently misrepaired by cellular repair enzymes. One must of course consider that mutations may arise spontaneously due to replication errors by the DNA polymerase, although DNA synthesis is usually of extreme high fidelity.

There are several factors that affect the ability of an adduct to induce mutations: the orientation of the modifying group, the size and conformation of the group, the nature of the polymerase, the sequence context and rearrangement of the adduct due to tautomerization, ionization, rotation and wobbling.

Different DNA adducts have varying capabilities of inducing mutations. TAM has been shown to cause several different mutations. Styles et al. have seen G:C to T:A transversions, insertions of base pairs, and deletions of pairs of G:C base pairs. Shibutani et al. synthesized a 15-mer oligodeoxynucleotide with a single Z-TAMdG site (no other dG residues: 5'-TCCTCCTC-Z-TAMdG-CCTCTC) and investigated the mutagenic specificities of the adduct. They found that one epimer of the Z-TAMdG 15-mer showed targeted mutation of about 0.7-1.5%. The other epimer showed 9.6% G to T transversions.
and about 2.8% G to A transitions. From these results, they concluded that TAMdG adducts are mutagenic lesions in mammalian cells and may initiate endometrial cancer.

1.4.3 Summary

As shown previously, several different carcinogen-DNA adducts have been identified and characterized. Their effects during the replication of DNA are mutagenic, leading to possible base deletions (frameshift mutations), base insertions, or base transversions and transitions. The mutations caused by TAM have been directly associated with the tumors found in the endometrial tissues of patients who are administered TAM in the treatment of breast cancer.

All of these findings have prompted interest in the initial contact between the TAM carbocation and DNA. How does TAM associate with DNA? Is there an initial binding complex, and does it intercalate? This leads to further questions on reaction rates, salt effects and pH effects. These issues will be the main topic of discussion in this thesis.

The original plan was to see if DNA was able to quench TAM, and if there was a significant rate acceleration. The TAM carbocation and several nitrenium ions were observed in the presence of DNA by laser flash photolysis (LFP) and it was found that DNA does in fact quench TAM, but there is a saturation effect in the observed rate. This saturation implies a pre-association complex between the TAM carbocation and the DNA. This led to the study of the effect of salt, and its role in the binding complex.

Product studies on the HPLC were also conducted with TAM and DNA. These were mainly to observe the products of the reaction, as well as to compare the rates between HPLC and LFP.
Chapter Two:

Experimental Procedure
2.1 The Synthesis of α-Acetoxytamoxifen (TAMOAc)

The synthesis of α-hydroxytamoxifen (TAMOH) is a multi-step process that is outlined very clearly in C. Sánchez's Ph.D. thesis from the University of Toronto. Fortunately, several grams were synthesized of each isomer (cis/trans) and were available for use in future experiments. Osborne et al. outline the synthesis of α-acetoxytamoxifen (TAMOAc) from TAMOH. Minor changes resulted in the following procedure. 0.1 ml acetic anhydride (1 mmol. BDH Inc., Toronto, Ontario) were added to 0.1 g of α-hydroxytamoxifen (TAMOH. 0.26 mmol) in only 0.3 ml of anhydrous pyridine. The reaction mixture was stirred under argon gas for 16 hours at room temperature (22°C).

![Figure 2.1. The general synthesis of TAMOAc.](image)

TAMOAc was then extracted with 10 ml diethyl ether and washed with 30 ml water. The ether layer was collected and dried over magnesium sulfate. After the removal of the magnesium sulfate, ~20 mg of silica gel (130-270 mesh, 60 Å) was added.

*Unless otherwise indicated, all chemicals were purchased from the Sigma-Aldrich Chemical Company in Oakville, Ontario.*
The ether was removed thereby putting the product on silica. This was loaded on the top of a 30 x 1 silica gel column to purify and the product eluted with a 8:11:1 diethyl ether: hexanes: triethylamine (ACP Chemicals Inc., Montreal, Quebec) solvent system (ratios by volume). The TAMOAc eluted first to yield an off white solid in 55% yield.

\[ ^1H\text{-NMR} \ (300 \text{ MHz, CDCl}_3) : \delta = 1.27 \text{ (d, } 3H, J = 7 \text{ Hz, CH}_3CH); 
1.90 \text{ (s, } 3H, O(C=O)CH); 
2.27 \text{ (s, 6H, N(CH}_3)_2); 
2.62 \text{ (t, 2H, } J = 6 \text{ Hz, CH}_2N); 
3.90 \text{ (t, 2H, } J = 6 \text{ Hz, OCH}_2); 
5.78 \text{ (q, } 1H, J = 7 \text{ Hz, } \alpha\text{-CH}); 
6.53 \text{ (d, 2H, } J = 9 \text{ Hz, ArH } \alpha \text{- to O);} 
6.79 \text{ (d, 2H, } J = 9 \text{ Hz, ArH } m \text{- to O);} 
7.1-7.4 \text{ (m, 10H, phenyl H).} \]

\[ \text{MS (EI, 70 eV)} : 429.2119 \text{ (M}^+\text{, 51%); 369.2130 (M}^+\text{ - acetic acid, 7.7%);} 
72.1176 \text{ ((CH}_3)_2N(CH}_2)_2^-, 41.2%); 
58.0939 ((CH}_3)_2NCH}_2^-, 100.0\%. \]

Please refer to Appendix A1 and Appendix A2 for the spectra.
2.2 The Hydrolysis of α-Acetoxytamoxifen

Stock solutions of TAMOAc were prepared in HPLC grade CH$_3$CN (Caledon Laboratories LTD, Georgetown, Ontario). A 51 μM solution of TAMOAc in 4 mM phosphate buffer (pH 6.7)* was prepared (from the 5.1 mM stock solution) and the rate of hydrolysis to TAMOH was measured using a Perkin Elmer Series 410 LC Pump High Performance Liquid Chromatography (HPLC) with an Applied Biosystems 785A Programmable Absorbance Detector set at λ = 260 nm. Turbochrom Navigator 4.1 was the software used to do the data analysis. The TAMOAc solutions (at 22°C and at 37°C) were injected immediately after preparation, and then continuously at regular intervals onto a Waters Symmetry® C$_{18}$ 5 μm column of dimensions 4.6 x 150 mm. An isocratic run of 50% CH$_3$CN and 50% 50 mM 1:1 Acetic Acid: Acetate Buffer yielded the following retention times: 2.80 min (trans-TAMOH), 3.10 min (cis-TAMOH) and 5.70 min (TAMOAc). The peak areas of the hydrolysis products were analyzed using GRAFIT™ (version 3.00), a data analysis and graphics program, to obtain the necessary rate constants.

*all pH measurements were performed using a Corning pH Meter 130 at room temperature, accurate ± 0.02 pH units
2.3 The Reaction of α-Acetoxytamoxifen with Guanosine and 1-Methylguanosine

1 x 10^{-4} M of TAMOAc was added to 2.5 x 10^{-4} M of guanosine (18) and 1-methylguanosine (19) in both phosphate and carbonate buffers (pH 6.8 and 10 respectively).

The four reaction mixtures were allowed to react overnight at room temperature, then injected onto a Waters 600E HPLC with a Waters 486 Tunable Absorbance Detector and recorded on a Waters 746 Data Module. The same Waters Symmetry® column as before was used, but now with the gradient presented in Table 2.1.

Table 2.1. Gradient program used for elution on the HPLC.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (ml/min)</th>
<th>% CH₃CN</th>
<th>% Buffer</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1</td>
<td>25</td>
<td>75</td>
<td>*</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>25</td>
<td>75</td>
<td>isocratic</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>85</td>
<td>15</td>
<td>linear gradient</td>
</tr>
<tr>
<td>4.5</td>
<td>1</td>
<td>85</td>
<td>15</td>
<td>isocratic</td>
</tr>
<tr>
<td>4.5</td>
<td>2</td>
<td>25</td>
<td>75</td>
<td>immediate step</td>
</tr>
</tbody>
</table>
The retention times of the notable peaks were: 10.80 min ($trans$-TAM-guanosine adduct), 12.10 min ($cis$-TAM-guanosine adduct), 13.10 min ($trans$-TAMOH), 13.74 min ($cis$-TAMOH) and 15.50 min (TAMOAc).
2.4 The Purification of DNA

The purification process was the same for both calf thymus and salmon testes DNA.\(^\text{10}\)

Approximately 1 mg of sodium citrate dihydrate was added to the minimum volume\(^*\) (in this case, 150 ml) of water and the pH was adjusted to 7.0 with sodium chloride. One gram of DNA was dissolved in the sodium citrate buffer by stirring it overnight at room temperature.

The DNA solution was then incubated for 30 minutes at 37°C with 50 mg of ribonuclease A. The same solution was then incubated for 60 minutes at 37°C with 100 mg of protease VI. Ten grams of phenol was added to the minimum volume of water to make a solution of water saturated phenol, and this was added to the DNA solution. The mixture was then shaken 30-60 times per minute at 4°C for 25 minutes, resulting in an emulsion. A minimum volume of chloroform-isoamyl alcohol (24:1) was added, and the reaction mixture was shaken for an additional 5 minutes.

The solution was then centrifuged at 8000 rpm for 15 minutes at 4°C, resulting in the formation of 2 separate phases. The aqueous layer (top) was pipetted off, ensuring no uptake of protein or chloroform. Twice the minimum volume of ice cold 95% ethanol was added to the viscous aqueous phase. The solution was then stirred twice to initiate the precipitation of the DNA, then let stand for 6 hours at -15°C.

The solution was again centrifuged at 10000 G for 20 minutes, and the supernatant was decanted off. Cold absolute ethanol was then added to the pellet, the DNA was filtered off and washed with additional cold absolute ethanol, then with diethyl ether (~100 ml of

\(^*\) Representative of the least amount of water necessary to completely dissolve the DNA.
each). The dry DNA sample was set on a watchglass and placed in a vacuum dessicator overnight.

The purity and concentration of the DNA were calculated by spectrophotometric methods on a Hewlett Packard 8452A Diode array Spectrophotometer with a 1 cm path length. This will be discussed in Chapter 4.
2.5 The Reaction of α-Acetoxytamoxifen with DNA

TAMOAc is both a solvolytic and photolytic precursor for the TAM carbocation. This means that laser flash studies with photochemical generation were possible for kinetic studies while product analysis could be performed using solvolysis.

![Reaction Diagram]

Figure 2.2. Solvolytic and photolytic generation of the TAM carbocation from its precursor, TAMOAc.

2.5.1 HPLC Experiments

Known concentrations of purified DNA were incubated with 50 μM of TAMOAc (at both 22°C and 37°C) in either a 4 mM phosphate buffer (pH 6.8) or a 50 mM Tris buffer (pH 8.0). The reaction mixtures were allowed to react overnight, and the resulting products were analyzed by HPLC. Before injecting the DNA samples onto the Waters Symmetry® column, the DNA needed to be digested\(^{28}\) to avoid the clogging of the column.

This was accomplished by the addition of DNA backbone digestion enzymes directly to the reaction mixtures. Firstly, 30 μl (20 units) of deoxyribonuclease I and 18 μl (0.02 units) of venom phosphodiesterase I were added to each sample and allowed to react for 16 hours at 37°C. Then 2 mg (3 units) of alkaline phosphatase was added and the solution was
incubated for a further 4 hours at 37°C. The resulting solutions were directly injected onto the HPLC.

The same gradient shown in Table 2.1 was used on the Waters HPLC to observe the results of the reaction of TAMOAc with the DNA, with analysis of the peak areas at the same identified retention times previously mentioned performed using GRAFIT™.

2.5.2 Laser Flash Photolysis Experiments

A nanosecond laser flash photolysis apparatus was used to generate the TAM carbocation. A Lumonics Excimer 510 Laser (with a 20 ns pulse width) was used to photolyze the carbocation precursor. The actinic flash (~150 ± 30 mJ / pulse) was set to 308 nm (XeCl emission) for all the experiments and a UV detector monitored the transients. A Tektronix Oscilloscope TDS-210 digitizer connected to a DX 386 computer digitized the signals from the detector. The computer software, TekReader 1.0, reads the data from the digitizer (changes in voltage with time), converts the voltage into absorbance using baseline level (I₀/V) and stores the data in ASCII format. This generates a change in optical density (ΔOD) versus time graph and GRAFIT™ was again used to determine rate constants, but now by directly fitting the data (ΔOD versus time traces).

A small and consistent (in each individual experiment) amount of TAMOAc was added (from the stock solution) to a 10 ml volumetric flask to a final concentration between 5 x 10⁻⁵ M and 2 x 10⁻⁴ M, thereby bringing the optical density (per cm) between 0.5 and 2.0. A laser quality four-sided transparent quartz cuvette with dimensions 4 x 1 x 1 cm (Hellma Canada Limited) was used for all the laser experiments.

The TAMOAc precursor was added to a 4 mM phosphate buffer solution containing various nucleophiles: calf thymus DNA, salmon testes DNA, single stranded poly G or
double stranded poly C • poly G. The effects of varying the pH of the DNA solution and changing the ionic strength (with added sodium perchlorate) were further studies that were also performed.

All the rate constants were taken as the average of at least ten kinetic runs, where the solutions were flashed up to six times each. The rate of decay ($k_{obs}$, from the ΔOD versus time plot) at 460 nm ($\lambda_{max}$ for the TAM carbocation) allowed for the determination of the rate constants. Plots of $k_{obs}$ versus the concentration of added nucleophile were used to draw conclusions.
2.6 The Reaction of Nitrenium Ions with DNA

The same Lumonics laser flash photolysis apparatus used to generate the TAM carbocation was used to generate nitrenium ions from several different precursors, as shown in Figure 2.3.

\[
\begin{align*}
R-N-N=N^- & \xrightarrow{hv} R-N^+ \quad \text{(very fast)} \quad R-N-H \\
\text{H}_2\text{O} & \quad \text{OH}^- 
\end{align*}
\]

**Figure 2.3.** Photolytic generation of the nitrenium ion from the azide precursor.

The second step to form the nitrenium ion is too fast to see by nanosecond laser flash photolysis. Table 2.2 displays the different precursors studied, their final concentrations in 25 ml volumetric flasks and the wavelength the transient was detected at.

Salmon testes DNA was the only added nucleophile to the precursor solutions. An added salt effect was additionally examined with the azidofluorene precursor.

All the rate constants were taken as the average of at least five kinetic runs, where the solutions were flashed only once. The same plots were generated as those for the TAM carbocation and used to draw conclusions.
Table 2.2. The different precursors used in the kinetic studies of the nitrenium ions, and their experimental specifications.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Name</th>
<th>Final Conc.</th>
<th>Detection $a_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="2-Azidofluorene" /></td>
<td>2-Azidofluorene</td>
<td>$6 \times 10^{-6}$ M</td>
<td>450 nm</td>
</tr>
<tr>
<td><img src="image" alt="N-Acetyl-N-trimethylacetoxy-2-aminofluorene" /></td>
<td>N-Acetyl-N-trimethylacetoxy-2-aminofluorene</td>
<td>$2 \times 10^{-5}$ M</td>
<td>450 nm</td>
</tr>
<tr>
<td><img src="image" alt="4'-Methoxy4-azidobiphenyl" /></td>
<td>4'-Methoxy4-azidobiphenyl</td>
<td>$8 \times 10^{-5}$ M</td>
<td>490 nm</td>
</tr>
<tr>
<td><img src="image" alt="4'-N-Acetyl-N-methylamino)-4-azidobiphenyl" /></td>
<td>4'-N-Acetyl-N-methylamino)-4-azidobiphenyl</td>
<td>$8 \times 10^{-5}$ M</td>
<td>560 nm</td>
</tr>
<tr>
<td><img src="image" alt="4'-Methyl-4-azidobiphenyl" /></td>
<td>4'-Methyl-4-azidobiphenyl</td>
<td>$8 \times 10^{-5}$ M</td>
<td>470 nm</td>
</tr>
<tr>
<td><img src="image" alt="4-Azidobiphenyl" /></td>
<td>4-Azidobiphenyl</td>
<td>$8 \times 10^{-5}$ M</td>
<td>460 nm</td>
</tr>
</tbody>
</table>
2.7 The Synthesis of the Tamoxifen-dG Adduct

TamOAc was reacted with dG in a basic solution in the following concentrations: 0.01 M dG; 0.008 M NaOH; 0.001 M TAMOAc. These were added together in a 250 ml. 30% CH$_3$CN solution with a resulting pH ~10.5. The reaction proceeded overnight at 37°C. acetic acid was added. The adduct was extracted with chloroform, put on silica gel (130-270 mesh, 60 Å) and run down a 1 x 60 cm silica gel column. Table 2.3 presents the solvent system used, with monitoring on the Perkin Elmer HPLC.

**Table 2.3.** Solvent system used to purify and isolate the TAMdG adduct (ratios are by volume, except where percentages are used).

<table>
<thead>
<tr>
<th></th>
<th>Ethyl Acetate</th>
<th>Hexanes</th>
<th>Methanol</th>
<th>Triethylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>START</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>10%</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>FINISH</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

MS (ESI): 637 (MH$^+$, 56%)

*Please refer to Appendix A3 for spectrum.*
2.8 The Synthesis of an N-Methylated α-Acetoxytamoxifen

To N-methylate the ethyldimethylamino side chain of TAMOAc, a variation of Hunter and Ponce's methodology was applied. A small scale reaction of 9 mg (2.1 x 10^-5 mol) of TAMOAc in chloroform was reacted with 5.3 μl (8.4 x 10^-5 mol) of iodomethane, a four fold excess. The reaction was allowed to proceed overnight, although it seemed that it was over within 2 hours.

![Chemical structure](image)

**Figure 2.4.** The synthetic scheme to form an N-methylated TAMOAc.

^H-NMR (400 MHz, CDCl₃): δ = 1.27 (d, 3H, J = 7 Hz, CH₂CH); 1.90 (s, 3H, O(C=O)CH₃); 3.48 (s, 9H, (CH₃)₃N⁺); 4.16 (t, 2H, J = 5 Hz, CH₂N); 4.30 (t, 2H, J = 5 Hz, OCH₂); 5.73 (q, 1H, J = 7 Hz, α-CH); 6.53 (d, 2H, J = 9 Hz, ArH o- to O); 6.84 (d, 2H, J = 9 Hz, ArH m- to O); 7.1-7.4 (m, 10H, phenyl H).

*Please refer to Appendix A4 for the spectrum.*
Chapter Three:

Preliminary Work
3.1 Introduction

In Chapter 1, it is established that TAM was a carcinogen, with studies showing the formation of DNA adducts in the endometrium of women taking the drug to treat breast cancer. These TAM-DNA adducts have been isolated and characterized as a covalent linkage at the α-position of TAM to the exocyclic amine of dG. This is thought to occur mainly through a metabolic pathway involving the formation of a reactive carbocation intermediate. TAMOH, a known metabolite, leads to the formation of α-sulfate TAM (TAMOSO₃⁻, 26), a precursor to the TAM carbocation.

Although the in vivo mechanism involves TAMOSO₃⁻, all of the experimental in vitro work performed within the context of this project was done using TAMOAc. This was due to its slower rate of solvolysis ($t_{1/2} = ~90$ minutes vs. 8.7 seconds for TAMOSO₃⁻).³⁵ thereby rendering it much easier to handle. Sánchez showed, by the spectrum of the transient, the ratios of the rate constants, and by their quenching behaviour, that both TAMOAc and TAMOSO₃⁻ produce the same TAM carbocation and Shibutani et al. confirmed the formation of the same TAMdG product.²⁸ This indicates that TAMOAc is a suitable model for TAMOSO₃⁻ in all of the experiments performed.
A reaction through a carbocation intermediate implies an $S_N1$ mechanism. An added nucleophile had no effect on the rate of solvolysis of TAMOAc nor of TAMOSO$_3^-$, as the rate of disappearance of the precursor remained unaffected. This behaviour is classic evidence for an $S_N1$ mechanism, with a rate-determining ionization to form the carbocation. This is also evident in the hydrolysis of TAMOAc from both the trans- and cis- forms. Both trans- and cis- TAMOH are formed in the same ratio, whether from the trans- TAMOAc or from the cis- TAMOAc, as shown if Figure 3.1. This isomerization is consistent with an allylic cation intermediate that can undergo rotation prior to capture.

![Diagram of reaction](image)

**Figure 3.1.** The formation of both isomers of TAMOH (5) from either isomer of TAMOAc (2).
3.2 **Rates of Hydrolysis**

Ground state solvolysis studies on TAMOAc were performed on the Waters HPLC, monitoring the disappearance of the TAMOAc peak and the growth of the TAMOH peaks, as seen in Figure 3.2.

![Chromatograms](image)

**Figure 3.2.** Four chromatograms illustrating the gradual decrease in TAMOAc and increase in the two TAMOH isomers. A: t = 51 min.: B: t = 105 min.: C: t = 157 min.: D: t = 1544 min.
Repeat injections containing 51 μM of TAMOAc allowed the measurement of the rate constants, as displayed in Figure 3.3, representative of a typical solvolysis experiment.

![Figure 3.3](image)

**Figure 3.3.** Peak area vs. Time (s) graph showing the single exponential decrease and increase of TAMOAc and TAMOH (both isomers combined) respectively in 4 mM phosphate buffer (1:1, pH ~6.8) at 22°C.

These experiments were conducted at 22°C and at 37°C, while minimizing the amount of CH3CN in the aqueous solution without jeopardizing the solubility of TAMOAc. Below 5% CH3CN, solubility concerns were a factor in the rate of hydrolysis. All plots produced data showing an exponential disappearance of TAMOAc and the first order rate constants were obtained by fitting the data to equation 3.1.

\[
[A] = [A]_0 e^{-kt} + \text{offset} \tag{3.1}
\]
Table 3.1 displays the first order rates of hydrolysis. Besides obtaining these first order rates, the total time necessary for complete reaction was noted and taken into consideration for future experiments. The hydrolysis experiment of greatest interest was to measure this rate in the presence of DNA, to observe the possibility of a DNA catalyzed mechanism. This objective was not achieved because a protocol could not be devised that would allow full length DNA to be injected directly onto a HPLC column.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Rate (k, s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>$1 \times 10^{-4}$</td>
</tr>
<tr>
<td>37</td>
<td>$4.2 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

Closer analysis of peak areas shows that not all of the TAMOAc gets hydrolyzed to TAMOH. The difference in the peak areas was about 5% less in the end than what there was at the start, i.e. the two TAMOH isomers only accounted for 95% of the hydrolysis products. The missing 5% was concluded by Sánchez et al. to be cyclic indene products, derived from intramolecular cyclization.⁵⁵
Figure 3.4. The formation of the E- (or trans-) and Z- (or cis-) indene products formed from the TAM carbocation where Ar is the \(-\text{O(CH}_2\text{)}_2\text{N(CH}_3\text{)}_2\) substituted phenyl ring.

They stated that 4.5% Z-indene was derived from cyclization into the aromatic ring with the ethoxy-dimethylamine sidechain, and 2.5% E-indene from cyclization with the phenyl ring (for a total of 7% indene products).
3.3 Free Guanosine and 1-Methylguanosine Nucleophiles

The reaction of guanosine (18) and its derivative, 1-methylguanosine (19), with TAMOAc was conducted at pH 7 and pH 10. The chromatograms were analyzed for the presence of adducts. At pH 7, 1.3% TAM-guanosine adducts and no TAM-1-methylguanosine adducts were detected. At pH 10, 43.6% TAM-guanosine adducts and no TAM-1-methylguanosine adducts were detected.

From the data, one can conclude that the N1 methyl group of 1-methylguanosine must be playing a part to inhibit the formation of adducts at the neighbouring N2 position. At pH 10, the N1 position of guanosine is partially ionized, yet adducts still form at the exocyclic amino group.

![Figure 3.5](image)

**Figure 3.5.** The anionic character of the exocyclic amino group of guanosine formed with assisted proton transfer by a water molecule.

This is thought to be due to the assistance by a water molecule, transferring a proton from N2 to N1, thereby making the N2 position more nucleophilic, and hence, more adducts resulting.\(^{56}\) In the 1-methylguanosine reaction, this is not a factor as there is a methyl group at N1 instead of hydrogen, therefore no ionization can occur. So, at both pH 7 and 10, very few (if any) TAM-1-methylguanosine adducts are observed.
Guanosine is a component of RNA, similar to dG except for the absence of the 2’ hydroxy group. It was previously mentioned that TAM binds to dG in DNA to form carcinogenic adducts. The following chapter will present the results of several different in vitro experiments, to better understand the interaction of TAM with DNA.
Chapter Four:

The First Model
The end of Chapter 1 proposed several questions, all dealing with the interaction between the TAM carbocation and DNA. Two such questions are: Is the TAM carbocation quenched by DNA and does it disrupt normal Watson-Crick base pairing? Is the TAMdG adduct wedged in the minor groove of DNA, as seen with the benzopyrene adducts?\textsuperscript{34, 57}

Laser flash photolysis (LFP) was used for the direct observation of the TAM carbocation to study the kinetics in the presence of DNA. HPLC analysis of a solvolysis mixture was used to observe products formed from the TAM carbocation in the presence of DNA. This, however, required DNA digestion workup before product analysis.

After purification, a DNA stock solution was prepared and its purity and concentration were verified. DNA's UV/Visible spectra identified \( \lambda = 260 \) nm as the maximum wavelength of absorption and its molar extinction coefficient (\( \varepsilon \)) at 260 nm is 6000.\textsuperscript{58a}

Osborne \textit{et al.} determined a standard, average MW of 314.9 g/mol per base for salmon testes DNA.\textsuperscript{10} This was used to prepare a stock solution. Equation 4.1 was then used to check the purity.

\[
A = \varepsilon c l
\]  

where \( A \) is the absorbance (A.U.), \( c \) is the concentration (mol/L), \( l \) is the path length (cm), and \( \varepsilon = 6000 \). For example, 7.20 mg of purified salmon testes DNA was dissolved in 200 ml of buffer. This corresponds to a \( 1.14 \times 10^{-4} \) M solution, which by equation 4.1 should have an absorbance of 0.68 A.U. The experimental absorbance measurement is 0.67, and thus the efficacy of the DNA purification can be checked: \( 0.67/0.68 = 0.98 \); therefore, the
DNA is 98% pure. Rearrangement of equation 4.1 allows for the determination of the concentration of a prepared DNA stock solution. In such a case the absorbance of a DNA solution is measured and the known molar extinction coefficient at 260 nm and cell path length applied. To put into terms of concentration of dG in DNA, the DNA concentration is multiplied by 0.206, which is the percentage composition of dG in both salmon testes and calf thymus DNA (i.e. 41.2% dG \cdot dC base pair).
4.1 Salmon Testes vs. Calf Thymus DNA

There are many different natural sources where DNA may be extracted from to be made commercially available, for example, from rat bone marrow, yeast, *E. coli*, calf thymus, or from salmon testes. Calf thymus DNA is very common and widely used in research. Salmon testes DNA is also quite common, but is utilised to a greater extent due to its cost effectiveness. These two types of DNA are similar in their base composition, 41-42% dG•dC base pairs, but have differences as well. Calf thymus DNA is more soluble in aqueous media than salmon testes which makes it easier to work with. This seems a little surprising as calf thymus DNA has a much higher molecular weight, being of about 15 million Daltons, while salmon testes DNA is only approximately 1 million Daltons making it much shorter than the calf thymus type. A few experiments were carried out to determine if there was any difference in the kinetics when different types of DNA were added.

Products studied on the HPLC found that both types of DNA formed the same TAMdG adduct, as displayed by a peak at the same retention time. LFP experiments produced some interesting results. When TAMOAc was added to the two types of DNA, the first order observed rates slightly differed, where calf thymus values were higher, indicating a slightly faster quenching (for a given [DNA]). Although the observed rates were greater for calf thymus DNA, the $k_{\text{int}}^{\text{DNA}}/k_{\text{w}}$ selectivity ratio, showing the preference of reaction of the TAM carbocation with DNA or water, was greater for salmon testes DNA. At 22°C, salmon testes DNA gave a $k_{\text{int}}^{\text{DNA}}/k_{\text{w}}$ value of $3.13 \times 10^4$ M$^{-1}$ in phosphate buffer, whereas calf thymus DNA resulted in a $k_{\text{int}}^{\text{DNA}}/k_{\text{w}}$ value of $2.08 \times 10^3$ M$^{-1}$ in the same buffer. This indicates that TAMOAc binds faster to salmon testes DNA than to calf
thymus DNA. This is an interesting finding, as the base compositions of the two types are quite similar.
4.2 The First Kinetic Model

The data acquired from the experiments outlined in Chapter 2 were analyzed. Figure 4.1 presents a typical graph obtained when the observed rate ($k_{obs}$) is plotted against the concentration of dG in DNA ([dG]).

![Graph](image)

**Figure 4.1.** Typical kinetic rate vs. concentration plot identifying rate constants conducted in 4 mM phosphate buffer (1:1, pH 6.8) at 22°C.

From this plot, one can see that at lower concentrations of DNA (the first few data points), the TAM carbocation is quenched by DNA. The plot begins to curve quite early because the rate constants plateau very quickly, but at lower concentrations of DNA (less than $8 \times 10^{-9}$) there is a short linear segment of the graph. The slope of this line ($k_{init}^{DNA}$) is the apparent second order rate constant. At higher concentrations of DNA, the observed rate constants level off, displaying a saturation effect. This suggests a model (Model I) where the TAM carbocation forms a non-covalent association complex with the DNA before the reaction occurs.
Model I

With \( \text{TAM}^- \) as the limiting species in this equation, once all the carbocations are completely bound to the DNA, the observed rate constant levels off and a saturation effect is observed. In this Model I, it is proposed that \( k_{\text{DNA}} \) is the first order rate constant for the reaction of the TAM carbocation bound to the DNA and \( K_{\text{ass}} \) is an equilibrium constant representing the association of the TAM carbocation and the DNA. It should be noted that this model may also be applied to other cationic intermediates (e.g. nitrenium ions). All the equations and terms of this model will be derived and discussed in Chapter 5: section 5.2.
4.3 The Carbocation-DNA Complex

From Model I, it is proposed that the TAM carbocation initially binds in a non-covalent manner to the DNA. However, it is unclear whether intercalation (slipping between stacked base pairs) or electrostatic attraction with the DNA phosphate backbone occurs. Intercalation is an event generally undergone by planar aromatic compounds like ethidium, acridine orange and proflavin (which are all cationic species). The TAM carbocation is not a planar molecule, but takes on a more "propeller-like" conformation, as shown by some preliminary AM1 and ab initio calculations by McClelland. Hogan et al. also rule out any intercalation of the bound benzopyrene diol epoxide, which forms a similar N2-dG adduct as TAM, in between the base pairs in DNA.

![Figure 4.2. The approximate proposed structure of the "propeller-like" TAM.](image)

Zimmermann, in a review on the binding of specific cationic dyes to DNA and organelles, suggests that studying binding in the presence of an added salt will determine whether a compound intercalates or binds "externally". The addition of salt will allow for direct competition of the positive ionic species (e.g. Na⁺) and the carbocation for negatively charged phosphate moieties on the DNA backbone. If there is competition, then the
association constant ($K_{ass}$) for the TAM carbocation should decrease with increasing salt concentration. Figure 4.2 shows the results of LFP studies of TAMOAc with differing concentrations of calf thymus DNA, as well as varying concentrations of salt.

![Graph](image)

**Figure 4.3.** Graph of rate vs. $dG$ concentration showing the effect of different concentrations of added salt to the reaction of TAMOAc with DNA.

Table 4.1 presents the numerical data from Figure 4.2. While $k_w$ and $k_{DNA}^{\text{int}}$ remain relatively constant, there is a definite decrease in $K_{ass}$ and $k_{int}^{DNA}$ with increasing sodium perchlorate concentration. This, as well as the obvious decrease in curvature in the above graph, is clear evidence that increasing the Na$^+$ concentration results in a decrease in the binding of the TAM carbocation to DNA. There is a direct competition for DNA binding sites between Na$^+$ and the TAM carbocation.
Table 4.1  The effect of added salt on kinetic rates of the quenching of the TAM carbocation by DNA.

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>4 mM</th>
<th>8 mM</th>
<th>18 mM</th>
<th>38 mM</th>
<th>108 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_w (s^{-1})$</td>
<td>2.50e4</td>
<td>2.81e4</td>
<td>2.28e4</td>
<td>2.42e4</td>
<td>2.38e4</td>
</tr>
<tr>
<td>$k_{DNA} (s^{-1})$</td>
<td>5.92e4</td>
<td>7.09e4</td>
<td>6.12e4</td>
<td>5.45e4</td>
<td>3.28e4</td>
</tr>
<tr>
<td>$K_{ass} (M^{-1})$</td>
<td>9.93e3</td>
<td>5.96e3</td>
<td>4.19e3</td>
<td>1.74e3</td>
<td>5.30e2</td>
</tr>
<tr>
<td>$k_{int}^{DNA} (M^{-1}s^{-1})$</td>
<td>5.87e8</td>
<td>4.23e8</td>
<td>2.56e8</td>
<td>9.49e7</td>
<td>1.74e7</td>
</tr>
</tbody>
</table>

These results have been confirmed by other findings. Daune et al. have claimed that the phosphate group on DNA stabilizes the transient nitrenium ion during the reaction of 2-(N-hydroxyamino)fluorene with guanine.\textsuperscript{41} Rokita et al. conducted studies with permanganate, an anionic oxidant, that oxidizes thymine residues on DNA producing thymine glycol and barbituric acid derivatives. The oxidation reaction was somewhat inhibited due to electrostatic repulsion of the two components. They found that adding salt into the reaction mixture accelerated the reaction by neutralizing the anionic phosphate DNA backbone, thus lowering the entropic barriers.\textsuperscript{53} Conversely, if one had a cationic substrate, there should be better quenching at lower concentrations of the added salt.

To proceed beyond the binding of the transients, there seems to be binding of the adducts to the DNA. Patel et al. established by NMR that the S-cis TAMdG adduct is accomodated within a bent and widened minor groove without the disruption of the Watson-Crick TAMdG*C base pair and any neighbouring Watson-Crick base pairs.\textsuperscript{64} The same group also saw this with benzopyrene. Their NMR and computational studies show minor groove binding of (+)-trans-anti-benzopyrene diol epoxide-N2-dG lesion positioned opposite dC in double stranded DNA.\textsuperscript{34}
4.4 Polyguanylic Acid

To further study the effect of binding the entire DNA polymer plays on its reaction with the TAM carbocation, a single stranded polyguanylic acid (poly (G)) polymer was employed. This single stranded poly (G) consists of a guanylic acid repeating unit.

![Polyguanylic Acid Repeating Unit](image)

**Figure 4.4.** Polyguanylic acid repeating unit. MW = 382 g/mol.

When dissolved in water, poly (G) transfers to a linear form from a globular form. In a neutral solution, both forms are almost always in a four-stranded helical conformation.\(^{65a}\) By decreasing the pH, the poly (G) transforms to a double-stranded form, with the bases being protonated.\(^{65b}\) Although a double-stranded form would have been preferred over a four-stranded helix, it was necessary to avoid the protonation of the bases.
LFP studies of TAMOAc with poly (G) in 4 mM phosphate buffer were performed at 22°C and the results are presented in the graph below.

![Graph of the reaction of TAMOAc with poly (G) and with calf thymus DNA.](image)

**Figure 4.5.** Graph of the reaction of TAMOAc with poly (G) and with calf thymus DNA.

When the poly (G) graph is directly compared to a plot of TAMOAc in the presence of double stranded DNA, one observes a significant difference between the two: there is no quenching seen with the poly (G). The average \( k_{\text{obs}} \) is \( 2.83 \times 10^4 \, \text{s}^{-1} \) which is similar to \( k_w \) normally obtained in these calculations. The average \( k_{\text{obs}} \) value also lies within one standard deviation of each data point on the poly (G) graph. This is a good indication that the complementary strand of DNA plays an important part in the formation of TAMdG adducts, probably due to the role of the neighbouring DNA bases.
4.5 Poly Cytidylic Acid • Poly Guanylic Acid

To follow up on the findings in section 4.4, a double stranded poly cytidylic acid • poly guanylic acid (poly (C) • poly (G)) homopolymer was introduced. This polymer consists of two different strands, a poly (C) strand and a poly (G) strand. Each individual strand consists of repeating units of that particular component. The two strands spontaneously come together to form Watson-Crick base pairs, thus taking a double helical conformation. The melting temperature of this duplex is assumed to be higher than that of DNA due to the significant increase in G • C base pairs.

![Poly Cytidylic Acid Poly Guanylic Acid Homopolymer](image)

**Figure 4.6.** Poly cytidylic acid • poly guanylic acid homopolymer. MW = 694 g/mol.

The results of the LFP experiments of TAMOA with this homopolymer in 4 mM phosphate buffer at 22°C are presented in Figure 4.6.
One can see that the data better support a linear fit rather than a fit to Model I as they do not seem to saturate, at least, not as quickly as the calf thymus DNA. Furthermore, the homopolymer shows significantly less quenching relative to that of calf thymus DNA. With the incorporation of an added salt (sodium perchlorate), almost no quenching was observed. This salt effect proves that the TAM carbocation is binding to this homopolymer, but this binding is presumably decreased. Although the binding is evident, the reason behind the decrease in quenching is not, and must be further looked into.
4.6 Dication vs. Monocation

In Chapter 1, it was noted that the dimethylamino ethoxy sidechain was one of the components of TAM necessary for biological activity. The $pK_a$ of the protonated amino group ($pK_a = 7.9$) is very important, as it is near physiological pH.

$$\text{Ph} \quad \text{Ph} \quad + \quad \text{Ph} \quad \text{Ph}$$

Figure 4.8. The TAM dication deprotonating to form the monocation.

In all of the previous experiments, this was not a concern as all the experiments were performed in consistent conditions, all in a phosphate buffer (pH ca. 6.8). To see the difference in reaction kinetics between the monocation and the dication, LFP studies of TAMOAc with salmon testes DNA were conducted within a short pH range (Figure 4.9). The pHs were low and high enough to get 100% dication and 100% monocation respectively, while keeping the ionic strength constant at 16 mM (with NaClO₃).
The association constant is the most important term. As the pH increases, TAM changes from the dication to the monocation, and there is a decrease in $K_{\text{ass}}$, hence showing a decrease in the binding of the TAM monocation. The TAM dication displays greater binding to DNA, thereby increasing the observed rate ($k_{\text{obs}}$). This is assumed to be a consequence of having two positive charges to associate with the negative DNA phosphate backbone: leading to favourable competition with other ions for binding sites on the DNA.

Table 4.2. The effect of varying pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>6.11</th>
<th>6.80</th>
<th>7.44</th>
<th>8.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_w$ ($s^{-1}$)</td>
<td>3.00e4</td>
<td>2.28e4</td>
<td>2.14e4</td>
<td>1.24e4</td>
</tr>
<tr>
<td>$k_{\text{DNA}}$ ($s^{-1}$)</td>
<td>6.73e4</td>
<td>6.12e4</td>
<td>5.59e4</td>
<td>6.41e4</td>
</tr>
<tr>
<td>$K_{\text{ass}}$ ($M^{-1}$)</td>
<td>2.75e3</td>
<td>4.19e3</td>
<td>4.04e3</td>
<td>5.93e2</td>
</tr>
<tr>
<td>$k_{\text{int, DNA}}$ ($M^{-1}s^{-1}$)</td>
<td>1.85e8</td>
<td>2.56e8</td>
<td>2.25e8</td>
<td>3.78e7</td>
</tr>
</tbody>
</table>

The TAM dication is more reactive than the monocation, thereby having a shorter half-life. Therefore, as the pH increases, $k_w$ and $k_{\text{int, DNA}}$ should decrease. There was great concern in moving to even higher pHs because a pH range above 9 would result in the
deprotonation of the dG residue, along with an increase in hydroxide ion concentration. Both these species are much better nucleophiles and would result in elevated $k_w$ and $k_{int}^{DNA}$ values.

The greater reactivity of the TAM dication has also been studied with other electrophilic carcinogens. The 4-biphenylnitrenium ion, the 2-fluorenylnitrenium ion and some 4-alkoxyphenynitrenium ions all showed a more reactive dication.\textsuperscript{40} McClelland \textit{et al.} made an odd finding where the benzidine and N, N-dimethylbenzidine dications were actually less reactive towards water than the monocations.\textsuperscript{40} This is odd because, as previously mentioned, one would expect to see an increase in electrophilicity with protonation. The $k_w$ values of the TAM dication are greater than those of the monocation, but cannot be directly compared as they were taken in the presence of DNA. This is the reason why Section 2.8 outlines the synthesis of a permanent TAM dication, an N-methylated TAMOAc. With this compound, all the experiments previously conducted, including hydrolysis, can be redone for direct comparison. This will definitely indicate whether the increase in reactivity with DNA is due to binding, or if there are other factors involved.
4.7 Summary

With knowledge that TAM is carcinogenic and that the formation of TAM-DNA adducts is the ultimate origin of the cancer, further investigation into their formation is necessary. In vivo, these adducts, as well as the mechanism of their formation, has been observed and established (Chapter 1). The specifics of the events following the formation of the TAM carbocation were still unclear.

The in vitro reaction of TAMOAc with standard B form DNA (calf thymus or salmon testes) by LFP at room or physiological temperature and around physiological pH. produced a characteristic $k_{obs}$ vs. [dG in DNA] graph. This plot suggested a model (Model I) where the TAM carbocation forms an association complex with DNA prior to their reaction together. Further studies indicated that this binding was due to electrostatic attraction rather than intercalation and that reaction with the DNA duplex produced higher rates of quenching than with poly G and poly C • poly G strands.

* Rates taken at high concentrations of dG in DNA (> 1 mM) were omitted as their rates were uncharacteristically lower than the rate at saturation, probably due to an increase in ionic strength and an increase in the viscosity of the solution.
Chapter Five:

The Second Model
5.1 Competition Kinetics in the Ground State

This section describes the product studies of the solvolysis of TAMOAc in the presence of DNA. The objective is to obtain product ratios (by HPLC) that can be directly compared to those determined by laser flash photolysis.

Product analyses of the reaction of TAMOAc with DNA were conducted at both 22°C and 37°C. These ground state experiments could not be immediately analyzed after the reaction was complete, as the DNA backbone needed to be digested. After this extensive procedure was completed, the solution of nucleotides, TAMOH products, and TAM-nucleotide adducts was injected onto the HPLC column. The specific product peaks were identified by their retention times and quantified by integrating the area underneath the peaks (done by the HPLC).

At the different concentrations of dG in DNA, the amount of TAMOH (both isomers) and the amount of TAMdG adducts were recorded. It has been suggested that a minor adduct is formed along with the TAMdG adduct (major adduct); an N6-TAM-
deoxyadenosine (TAMdA, 27 where dR = deoxyribose sugar) adduct is said to contribute a very small amount, about 7% of the total adducts formed.\textsuperscript{28,67}

\[
\text{TAMOAc} \\
\downarrow \text{ionization} \\
\text{TAM carbocation} \\
\downarrow \text{DNA}_{\text{aq.}} \\
\text{TAMOH + TAM-DNA adducts} \\
\downarrow \text{enzymatic hydrolysis to nucleotides} \\
\text{TAMdG (major product) + TAMdA (minor product)}
\]

**Figure 5.1.** Ground state scheme leading to the formation of TAMdG and TAMdA products.

These TAMdA adducts were not detected on the HPLC chromatograms as a protocol was not devised for their identification because of the minute quantities formed. It was extremely difficult to identify and quantify the TAMdG adducts from the DNA due to the low amounts formed, so to identify the TAMdA peak, which is less than 10% of the
TAMdG peak would have been very problematic. By itself, the TAMdA adduct was observed when TAMOAc was reacted with dA in 30% CH₃CN at pH 11, but when put on a scale next to the TAMdG adducts and the TAMOHs, it is not detected. The TAMdG adduct was also synthesized in 30% CH₃CN at pH 11 (for characterization purposes). The adduct was isolated, but the four isomers could not be separated. For this reason, the NMR spectra obtained were complex, but the ES-MS trace was quite clear with the MH⁺ peak at m/z = 637, as MS does not distinguish between the different isomers.

The HPLC product analysis of TAMOH and TAMdG resulted in the graph presented as Figure 5.2. DNA adducts are observed at low DNA concentrations, but at saturation, the majority of the products were still mainly TAMOH. Further calculations predicted that at high concentrations of dG in DNA, only 22% adducts are expected at 22°C and only 33% adducts are expected at 37°C.

![Figure 5.2](image.png)

**Figure 5.2.** Percentage of products formed in the ground state competition kinetic experiments (4 mM, 1:1 phosphate buffer at 22°C).

Whalen et al. had similar findings with the reactive benzopyrene diol epoxide. They found that in the presence of aqueous DNA solutions, greater than 90% of the diol
epoxide undergoes hydrolysis to tetrols and less than 10% covalently binds to DNA. From these results, they propose a scheme (Figure 5.3) where there is an association of the diol epoxide to the DNA.

![Figure 5.3. Kinetic scheme devised by Whalen and coworkers incorporating a diol epoxide-DNA association complex leading to the formation of two separate products where DE = diol epoxide. $k_d$: rate constant for the formation of products in the absence of DNA. $K_e$: equilibrium constant for the formation of the DE-DNA complex. $k^{\text{cat}}_{\text{cat}}$: pH dependent rate constant. $k^0_{\text{cat}}$: spontaneous, pH independent rate constant.][1]

From all this data, the evidence suggests that the kinetic Model I could be modified, since the products require that the TAM carbocation-DNA (TAM$^+$-DNA) complex react both with water and with DNA so that $k^{\text{DNA}}$ contains more than one term. This has resulted in a new kinetic model, Model II, where $k^{\text{DNA}} = k^{\text{addDNA}} + k^{\text{wDNA}}$. Thus at saturation where all the cation is associated with the DNA, there is still significant TAMOH formation since the associated cation still reacts with water.
Model II

\[ \text{TAM}^+ + \text{DNA} \xrightarrow{k_{\text{ass}}} \text{TAM}^+ \cdot \text{DNA} \]

\[ \text{TAMOH} \xrightarrow{k_{\text{w}}} \text{TAMOH} \]

\[ \text{TAMOH} \xrightarrow{k_{\text{DNA}}} \text{DNA-Adduct} \]

\[ \text{DNA} \xrightarrow{k_{\text{add}}} \text{DNA-Adduct} \]
5.2 Kinetic Analysis

From the LFP experiments and the product studies, the DNA quenching plots exhibit saturation kinetics. Referring to Model II, this section will concentrate on the derivation of the expressions used for kinetics and products for the reaction of the TAM carbocation in the presence of DNA.

5.1.1 Laser Flash Photolysis Studies

The expression for the rate constant for the decay of the cation is equation 5.1. This is under conditions where the concentration of DNA is in excess of the carbocation, and where the association equilibrium is established quite rapidly.

\[ k_{obs} = \frac{k_w^{DNA} + k_{sw}^{DNA} K_{av}[DNA]}{1 + K_{av}[DNA]} \]  

(5.1)

Note that [DNA] may be replaced by [dG in DNA] and that at low concentrations of DNA where not many TAM-DNA complexes have formed ([TAM] > [TAM-DNA]), the denominator goes to one and the expression takes the form of a linear equation (as mentioned in section 4.2).

\[ k_{obs} = k_w^{DNA} + k_{sw}^{DNA} K_{av}[DNA] \]  

(5.2)

Remember that \( k_w^{DNA} K_{ass} \) is defined as \( k_{init}^{DNA} \), the same expression that is used in the simple competition kinetics in the next section.

5.2.2 HPLC Studies

The expression for the product dependence in DNA is given in equation 5.3. The following is the derivation:

\[ \frac{d[TAMdG]}{dt} = k_{sw}[TAM^* - DNA] \]
\[
\frac{d[TAMOH]}{dt} = k_\text{w}[TAM^+] + k_{\text{DNA}}^{\text{DNA}}[TAM^+ \cdot DNA]
\]

\[
[TAM^+ \cdot DNA] = K_{\text{add}}[DNA]
\]

Note that product analysis does not distinguish this model from a simple competition kinetic model, so it can only measure the selectivity ratio \(k_{\text{mut}}^{\text{DNA}}/k_\text{w}\). If this is expressed in terms of fraction of products, for the dG product:

\[
\frac{[TAMdG]}{[TAMOH] + [TAMdG]} = \frac{k_{\text{DNA}}^{\text{DNA}} K_{\text{add}}[DNA]}{k_\text{w} + k_{\text{DNA}}^{\text{DNA}} K_{\text{add}}[DNA] + k_{\text{add}}^{\text{DNA}} K_{\text{add}}[DNA]}
\]

\[
= \frac{k_{\text{add}}^{\text{DNA}} K_{\text{add}}[DNA]}{k_\text{w} + (k_{\text{DNA}}^{\text{DNA}} + k_{\text{add}}^{\text{DNA}}) K_{\text{add}}[DNA]}
\]

Remember \(k_{\text{DNA}}^{\text{DNA}} = k_\text{w}^{\text{DNA}} + k_{\text{add}}^{\text{DNA}}\)

\[
= \frac{\begin{pmatrix} k_{\text{add}}^{\text{DNA}} \\ k_\text{w}^{\text{DNA}} \end{pmatrix} \begin{pmatrix} k_{\text{DNA}}^{\text{DNA}} \\ k_\text{w}^{\text{DNA}} \end{pmatrix} K_{\text{add}}[DNA]}{1 + \left(\frac{k_{\text{DNA}}^{\text{DNA}}}{k_\text{w}^{\text{DNA}}}\right) [DNA]}
\]

\[
f = \frac{k_{\text{DNA}}^{\text{DNA}}}{k_\text{w}^{\text{DNA}} + k_{\text{add}}^{\text{DNA}}}
\]

\[
f = \frac{f\left(\frac{k_{\text{mut}}^{\text{DNA}}}{k_\text{w}^{\text{DNA}}}\right) [DNA]}{1 + \left(\frac{k_{\text{mut}}^{\text{DNA}}}{k_\text{w}^{\text{DNA}}}\right) [DNA]}
\]

Note that \([TAMOH] + [TAMdG]\) is constant and equal to the initial \([TAMOAc]\). Let \(\text{Area}_{\text{e}}\) equal the area of the TAMdG adduct at high concentrations, i.e. the area when the entire product is TAMdG adduct. In this case, this is equal to \([TAMOH] + [TAMdG]\) so that the expression for Area at smaller concentrations becomes:
Area (TAMdG adduct) = \frac{([TAMOH] + [TAMdG]) \left( \frac{k_{\text{intDNA}}}{k_w} \right) [DNA]}{1 + \left( \frac{k_{\text{intDNA}}}{k_w} \right) [DNA]}

\begin{align*}
\text{Area} & \left( \frac{k_{\text{intDNA}}}{k_w} \right) [DNA] \\
& = \frac{\text{Area} \left( \frac{k_{\text{intDNA}}}{k_w} \right) [DNA]}{1 + \left( \frac{k_{\text{intDNA}}}{k_w} \right) [DNA]} \quad \text{(5.3)}
\end{align*}

This was the equation used to fit all the HPLC data, giving the $k_{\text{intDNA}}/k_w$ ratio and Area. This model can also be expressed in terms of the fraction of TAMOH products, but this was not used in these studies, so its derivation is not included. Equations 5.1 and 5.3 now allow for direct comparison of the rates between the LFP and the HPLC data.
5.3 The Kinetics of α-Acetoxytamoxifen with DNA

Now that the kinetic expressions have been established, the results of the LFP and HPLC experiments can be compared. It is understood that this can only be accomplished using the $k_{\text{init}}^{\text{DNA}}/k_w$ ratio. Below are the results displayed in Table 5.1.

**Table 5.1.** Comparison of the HPLC and LFP selectivity ratios of TAMOAc in the presence of calf thymus DNA at both 22°C and 37°C.

<table>
<thead>
<tr>
<th></th>
<th>22°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{init}}^{\text{DNA}}/k_w$</td>
<td>HPLC</td>
<td>LFP</td>
</tr>
<tr>
<td></td>
<td>7.31 x 10^3</td>
<td>2.76 x 10^4</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>LFP</td>
</tr>
<tr>
<td></td>
<td>3.75 x 10^3</td>
<td>1.87 x 10^4</td>
</tr>
</tbody>
</table>

The HPLC and LFP results should give the same value at the indicated temperature. The values are relatively close, within a factor of 5 difference, but they are not the same and the differences in these rates need to be examined further. One possible explanation may be the formation of either unstable products or ones that are stable but undetectable under the HPLC conditions employed. Dipple *et al.* found that dG reacts with the paramethoxybenzyl cation to form an unstable O6 adduct. If it is the case that the TAM carbocation forms a similar adduct, then it would go undetected. It would not show up in the HPLC chromatograms as those reaction mixtures are left to react overnight. However, the trapping of the carbocation by DNA to form this adduct will increase the LFP measured $k_{\text{init}}^{\text{DNA}}$, resulting in an elevated $k_{\text{init}}^{\text{DNA}}/k_w$ ratio. This explanation can also be applied to the possible minor TAMdA adduct that is not accounted for by HPLC.

Looking at the numbers in Table 5.1, one notices that the ratio is greater at 22°C than at 37°C. This indicates that at the higher temperature, the TAM carbocation preferentially reacts with water rather than with DNA. This coincides with the expected amount of
adduct formation in Section 5.1. Recall that at 22°C, only 22% adducts are expected at saturation. From this result, one would expect that this value should more than double at 37°C (because of the increase in temperature), but the calculation only predicts 33% adducts. Therefore, as the temperature increases, the TAM carbocation tends more and more to react with water over the DNA. This raises an interesting thought regarding the role of DNA in the reaction of the TAM carbocation with water, and if it is catalyzing the formation of these hydrolysis products.

5.3.1 Other Cationic Intermediates

The rates of several different nitrenium ions in the presence of salmon testes DNA were measured by LFP and are displayed in Table 5.2. Their rates should not be directly compared to those of TAMOAc because they form different DNA adducts; they form a covalent bond with dG at the C8 position.70 Interestingly, the lifetime of the nitrenium ion generated from 2-azidofluorene (AF. 20) is 30μs, very similar to that of the TAM carbocation (22μs).5,71

Table 5.2. The $k_{init}^{DNA}/k_w$ ratios of a few nitrenium ions. The structures of these compounds are depicted in Table 2.2.

<table>
<thead>
<tr>
<th>Structure #</th>
<th>$k_{init}^{DNA}/k_w (M^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>$2.93 \times 10^4$</td>
</tr>
<tr>
<td>21</td>
<td>$2.14 \times 10^4$</td>
</tr>
<tr>
<td>22</td>
<td>$1.89 \times 10^5$</td>
</tr>
<tr>
<td>23</td>
<td>$1.20 \times 10^4$</td>
</tr>
<tr>
<td>24</td>
<td>$3.64 \times 10^4$</td>
</tr>
<tr>
<td>25</td>
<td>${3.01 \times 10^5}^8$</td>
</tr>
</tbody>
</table>

* This is the lifetime of the TAM dication, the major form of TAM at the pH of the conducted experiments.
The ionic strength effect was also studied with AF. Figure 5.4 exhibits the same findings as found with TAMOAc. With increasing salt, there is less quenching observed as well as a decrease in the initial slope and curvature of the graph. This is due to the competition for DNA binding sites on the phosphate backbone between the nitrenium ion and Na⁺.

![Graph displaying the ionic strength effect on AF with salmon testes DNA.](image)

The $k_{int}^{DNA}/k_w$ ratio for AF is $2.93 \times 10^4$ M$^{-1}$, slightly higher than that of TAMOAc, $2.76 \times 10^4$ M$^{-1}$. Although these ratios are quite close, the quenching is very different. Firstly, AF takes longer to saturate and secondly, the AF's $k_{obs}$ values are much higher, showing a greater rate acceleration in binding to the DNA than TAMOAc.

In searching for TAM derivatives to use for direct comparison with TAM, it was discovered that very few in vitro studies have been conducted. However, there were a few in vivo experiments comparing TAM to toremifene (TOR 15). Pitot et al. found that TAM
induced high levels of DNA adducts in the livers of rats, whereas TOR did not.\textsuperscript{72} They found that in the results of chronic administration of the drugs, TAM's results were about 50 fold greater than TOR's, and that these levels were dose-dependent. Kuramochi used semiempirical and density functional methods to calculate the stability of the allylic carbocation of some TAM derivatives.\textsuperscript{33} Results showed that the TOR carbocation was less stable than that of TAM. This indicated that TOR was less frequently activated than TAM and that there was a close relationship between the stability of the intermediate and the amount of DNA adducts formed. These results suggest that TOR may be less genotoxic than TAM and may be a safer drug.
5.4 Rate Acceleration by DNA

Now that the kinetics for the reaction of DNA with the TAM carbocation have been established, it would be interesting to compare them with those for the reaction of the dG monomer with the same carbocation. These LFP studies would help investigate the effectiveness of dG to trap the TAM carbocation within DNA as compared to the nucleoside alone. Sánchez had previously conducted the experiments with the monomer and some results presented here were taken from those studies. Figure 5.5 displays a remarkable difference between the quenching by dG within DNA and on its own, and this is obvious in the selectivity ratios. For the dG monomer, $k_{\text{int}}^{dG}/k_w = 2.96 \times 10^{4} \text{ M}^{-1}$ while the DNA's was $k_{\text{int}}^{dG \text{ in DNA}}/k_w = 2.76 \times 10^{4} \text{ M}^{-1}$, representing an approximate 900-fold increase in selectivity for DNA as compared to dG alone. This is also observed in product studies. When TAMOAc is reacted with 0.05 mM dG only 0.02% TAMdG adducts are observed. but when reacted with 0.05 mM dG in DNA, 10% TAMdG adducts are formed.

![Figure 5.5](image)

**Figure 5.5.** Graph comparing the difference in observed rates between DNA and the dG monomer with the TAM carbocation.
These findings are surprising when compared to another electrophile that was previously mentioned. AF. McClelland et al. found that the nitrenium ion formed from AF had a $k_{int}^{dG}/k_w$ value of $2.81 \times 10^4 \text{ M}^{-1}$ when reacted with the dG monomer. This value is very similar to that of the TAM carbocation with DNA ($2.76 \times 10^4 \text{ M}^{-1}$). It is interesting how two electrophiles react so differently: nitrenium ions react extremely well with monomers yet carbocations do not.

Therefore, the TAM data suggests that DNA is a much better nucleophile than dG alone, and this may be due to several factors. Firstly, the carbocation is forming a complex with the DNA (pre-reaction complex), which may support their relative conformations within and increase their reactivities with each other. Other nucleotides (other than dG) may also be trapping the TAM carbocation (dA has been shown to, and possibly also dC). As previously mentioned, the carbocation may be forming unstable adducts with the DNA that would result in elevated selectivity ratios. Lastly, there is the possibility of general base catalysis by the base paired cytosine. This final point is likely the major contributor to explain the rate acceleration by DNA.

Dannenberg and Tomasz have conducted density functional theory calculations at the B3LYP/D95** level to study the mechanism of alkylation of the exocyclic nitrogen of guanine in G•C base pairs. This alkylation occurs as a result of hydrogen bond (H-bond) base catalysis (HBBC) which is a new variation of base catalysis; there is a catalytic loan of basicity of the cytosine to the guanine through one of the H-bonds. Referring to Figure 5.6, the mechanism is as follows: the H-bonded G•C base pair is attacked by the electrophile, TAM*, at the N2 position of guanine (the H-bond donor) which transfers the H-bonding proton to cytosine. Guanine loses a different proton while the H-bonding proton is returned
to it by cytosine. The H-bond remains intact throughout, but the donor/acceptor relationships are reversed twice. In HBBC, since the base is associated by a H-bond before the reaction, there is no kinetic dependence on base concentration. Therefore, HBBC would go undetected by kinetics if its concentration was held constant.

**Figure 5.6.** The first step in hydrogen bond base catalysis: the attack by the TAM carbocation and the H-bonded proton transfer from guanine to cytosine.
5.5 Conclusions

In regards to the earlier questions put forth, the TAM carbocation is quenched by DNA and forms covalent products. It and other electrophiles from carcinogens form non-covalent association complexes with DNA at physiologically relevant DNA concentrations. This binding is based on electrostatic attraction between the positively charged electrophile and the negatively charged DNA phosphate backbone. The TAM\textsuperscript{+}-DNA complex reacts with water to form the TAMOH alcohol products (DNA-catalysis of hydration) and reacts to form DNA adducts (predominantly TAMdG). At saturation, the majority of the products were TAMOH, suggesting that Model I needed to be modified to allow the TAM\textsuperscript{+}-DNA complex to react both with water and with DNA, hence kinetic Model II.

When the kinetic rates were measured for TAMOAc in the presence of DNA, the \( \frac{k_{\text{int, DNA}}}{k_w} \) ratio from products was lower than that measured by LFP. Although these results should give the same values, the elevated LFP values may be due to the formation of products that are not detected in the HPLC experiments. Such products may be the formation of unstable adducts, like the O6-dG adduct seen with the para-methoxybenzyl cation,\textsuperscript{69} or possibly minor adducts (TAMdA)\textsuperscript{28,67} that do not form in significant quantities to show up on the same scale as the major products. These same experiments conducted in the presence of an added salt (NaClO\textsubscript{4}) to measure the effect of the ionic strength of the solution showed that there was a decrease in the observed rate. This was concluded to be due to the decrease in the TAM carbocation binding to the DNA, as the different cations would compete for DNA binding sites. In the pH studies, a decrease in pH resulted in an increase in the observed rate as a result of the TAM dication's increased binding to DNA. The pK\textsubscript{a} of the ethoxyamine sidechain of TAM is 7.9, so at lower (more acidic) pHs, the
sidechain would be protonated, giving the TAM an extra positive charge to increase its binding to DNA leading to an increase in the rate of reaction.

The kinetic rates of the reaction with DNA were compared to those with dG alone. Figure 5.5 presents obvious differences, such as the observed rates are significantly greater in the reaction of the TAM carbocation with DNA and that the DNA data exhibits a saturation effect, whereas with dG a linear relationship is observed. DNA is a much better nucleophile than poly G and dG alone due to the complex formation with DNA and hydrogen bond base catalysis, where the absence of a base pairing cytosine residue results in a decrease in quenching of the TAM carbocation.
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Appendix A1

α-Acetoxytamoxifen

$^1$H-NMR

300 MHz

CDCl$_3$
α-Acetoxytamoxifen
EI-MS
70 eV
Appendix A3

Tamoxifen-deoxyguanosine Adduct

ESI-MS

[Chemical structure image]

MH$^+$

637

[m/z graph with peaks at various m/z values]
Appendix A4

N-methyl α-Acetoxymoxifen

$^1$H-NMR 400 MHz CDCl$_3$