DETECTION OF DNA DAMAGE CAUSED BY
N-NITROSOINDOLES

Thesis submitted for the degree of Doctor of
Philosophy
at the University of Leicester

by

Lynda T. Lucas BSc

MRC Toxicology Unit
University of Leicester

August 2001
DETECTION OF DNA DAMAGE CAUSED BY N-NITROSOINDOLES

Lynda T. Lucas, Biomonitoring Section, MRC Toxicology Unit, Hodgkin Building,
University of Leicester, Leicester, LE1 9HN

ABSTRACT

N-Nitrosation frequently transforms innocuous nitrogen-containing compounds into toxic compounds. The endogenous formation of N-nitrosoindoles is of concern since humans are exposed to a variety of naturally occurring and synthetic indolic compounds. To evaluate the genotoxicity of N-nitrosoindoles, three model compounds, 1-nitrosoindole-3-acetonitrile (N1AN), 1-nitrosoindole-3-acetamide and 1-nitrosoindole-3-acetic acid methyl ester, were reacted with isolated purine nucleotides at physiological pH. The profile of reaction products was identical for each of the N-nitrosoindoles. The results indicated that N-nitrosoindoles can efficiently transfer the nitroso group to nucleophilic targets in isolated purine nucleotides, causing depurination, deamination coupled with depurination to afford hypoxanthine and xanthine, and formation of a novel deoxyguanosine monophosphate analogue, 2'-deoxyoxanosine monophosphate and its corresponding depurination product, oxanine. These pathways of modification were preserved at the macromolecular level in oligonucleotides and calf thymus DNA, with guanine residues appearing to be a primary site of reaction. The studies revealed an additional cross-linked product at CG residues in N1AN-treated duplex DNA. Pyrimidine residues were inactive toward nitroso transfer by N1AN. The ability of the nitroso group to exert damage at the nuclei was demonstrated in vivo in the glandular stomach of CD-1 mice via detection of abasic site damage, and in single cells in vitro as shown by the Comet assay. N1AN was mutagenic in the Ames II assay.

In contrast to many other genotoxic N-nitrosocompounds, which are known to alkylate DNA, the genotoxicity of N-nitrosoindoles arises via efficient transnitrosation to nucleophilic sites on the purine bases. All of the products resulting from transnitrosation by N-nitrosoindoles are potentially mutagenic if they occur in vivo. These findings reveal a new pathway for N-nitrosocompounds, exemplified by the N-nitrosoindoles, to exert genotoxicity. In the wider perspective, this pathway may be operative for many agents that release nitric oxide.
THESIS ASSOCIATED PUBLICATIONS

Abstracts


Papers


ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor David Shuker for his continued support and infectious enthusiasm throughout this project.

Also, many thanks to Glaxo Wellcome for the studentship and to my industrial supervisor Dr David Gatehouse and his colleagues in Genetic and Reproductive Toxicology, Medicines Safety and Evaluation Division of Glaxo Wellcome Research and Development, especially Dr Brian Burlinson, Leigh Penrose, Julia Mattison and David Wedd.

I would like to acknowledge Dr Don Jones at the Centre for Mechanisms of Human Toxicity at the MRC Toxicology Unit. We enjoyed a successful collaboration and many thanks go to his research group, especially Dr Zara Dodderidge and Lynda Dickinson.

Dr Toshinori Suzuki of Kyoto University, Japan, is thanked for a generous gift of 2'-deoxyoxanosine and for providing unpublished information.

Assistance with the recording of nmr and mass spectra by Bee Jukes and John Lamb is gratefully acknowledged.

I have thoroughly enjoyed every aspect of my research and this enjoyment is due to working in the Biomonitoring and Molecular Interactions (BMI) Section of the MRC Toxicology Unit under the direction of Professor Peter Farmer. I cannot acknowledge my colleagues and very dear friends in BMI and in the Unit enough. It is a very special place where I spent over three extremely happy years of my life.

To Brian, Kwame, Duma, Kafka and Powder, thank you.
CONTENTS

Title Page I
Abstract II
Thesis Associated Publications III
Acknowledgements IV
Contents V
Abbreviations XII

CHAPTER 1
INTRODUCTION 1
1.1 Indoles and human exposure 2
1.1.1 Endogenous sources 3
1.1.2 Dietary sources 4
1.1.3 Therapeutic potential 7
1.1.3.1 Naturally occurring indole alkaloids 7
1.1.3.2 Synthetic substituted indoles 9
1.1.4 Toxicity of indoles 11
1.2 Nitrosation 12
1.2.1 N-Nitrosation pathways in vitro 12
1.2.1.1 N-Nitrosation of amines and amides 13
1.2.1.2 N-Nitrosation of indole (aromatic secondary amine) 15
1.2.2 N-Nitrosation pathways in vivo "endogenous nitrosation" 16
1.2.2.1 The acid-catalysed reaction 17
1.2.2.2 The bacterially-mediated reaction 18
1.2.2.3 Activated-macrophage-mediated nitrosation 19
1.2.3 Catalysts and inhibitors of endogenous nitrosation 20
1.3 N-Nitroso compounds in human cancer 21
1.3.1 The possible role of NNOC in the etiology of cancer 21
1.3.2 Interaction of NNOC with biological systems 23
1.3.2.1 Chemical carcinogenesis 23
1.3.2.2 NNOC and the formation of DNA adducts 24
1.3.2.3 Are all alkylation products important for mutation induction? 28
1.3.2.4 Influence of DNA repair and sequence specificity 30
1.3.2.5 Denitrosation of NNOC and possible relevance for carcinogenesis

1.3.3 Approaches for the detection of genotoxic damage
   1.3.3.1 Bacterial and mammalian assays
   1.3.3.2 Biomarkers as indicators of exposure

1.3.4 The significance of N-nitrosation of drugs
   1.3.4.1 The cimetidine story

1.4 N-Nitrosoindoles: evidence for concern to their exposure
   1.4.1 Observed mutagenicity
   1.4.2 Nitrosated indoles as a risk factor for gastric cancer
      1.4.2.1 The jawa bean mutagen
   1.4.3 Proposed mechanism of DNA damage

1.5 Aims and scope of this thesis

CHAPTER 2
SYNTHESIS AND CHARACTERISATION OF A SERIES OF 3-SUBSTITUTED N-NITROSOINDOLES

2.1 Introduction

2.2 Experimental Procedures
   2.2.1 Materials
   2.2.2 Synthesis of 3-substituted N-nitrosoindoles
   2.2.3 Purification of N-nitrosoindoles
   2.2.4 Characterisation of N-nitrosoindoles

2.3 Results
   2.3.1 Synthesis and characterisation of NIAN
   2.3.2 Synthesis and characterisation of NIAM
   2.3.3 Synthesis and characterisation of NIAAME

2.4 Discussion
   2.4.1 Treatment of parent 3-substituted indoles with acidified nitrite affords N-nitrosoindoles as crystalline products

CHAPTER 3
REACTIONS OF A SERIES OF 3-SUBSTITUTED N-NITROSOINDOLES WITH PURINE NUCLEOTIDES AND NUCLEOSIDES

3.1 Introduction

3.2 Experimental Procedures
   3.2.1 Materials
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.2 Synthesis and characterisation of 3-substituted nitrosated indoles</td>
<td>64</td>
</tr>
<tr>
<td>3.2.3 Reactions of 2'-deoxyadenosine-3'- or 5'-monophosphate (dAp or dAP) with NIAN</td>
<td>64</td>
</tr>
<tr>
<td>3.2.4 Reactions of 2'-deoxyguanosine-3'- or 5'-monophosphate (dGp or dGP) with NIAN</td>
<td>64</td>
</tr>
<tr>
<td>3.2.5 Reactions of dP(A) and dP(G) with NIA(M) and NIA(M)E</td>
<td>65</td>
</tr>
<tr>
<td>3.2.6 Reactions of NIA(N), NIA(M) or NIA(M)E with 2'-deoxyguanosine (dGuo)</td>
<td>65</td>
</tr>
<tr>
<td>3.2.7 Reactions of NIA(N) with 2'-deoxyxanosine (dOxo)</td>
<td>65</td>
</tr>
<tr>
<td>3.2.8 HPLC analyses of reaction mixtures</td>
<td>65</td>
</tr>
<tr>
<td>3.2.9 Characterisation of reaction products by mass spectrometry</td>
<td>66</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>67</td>
</tr>
<tr>
<td>3.3.1 Reactions of nitrosated indoles with dP(A) and dAP</td>
<td>67</td>
</tr>
<tr>
<td>3.3.2 Reactions of nitrosated indoles with dP(G) and dGp</td>
<td>77</td>
</tr>
<tr>
<td>3.3.3 Reactions of nitrosated indoles with dGuo</td>
<td>93</td>
</tr>
<tr>
<td>3.3.4 Reactions of NIA(N) with dOxo</td>
<td>93</td>
</tr>
<tr>
<td>3.3.5 Decomposition of nitrosated indoles in the reaction system</td>
<td>101</td>
</tr>
<tr>
<td>3.4 Discussion</td>
<td>107</td>
</tr>
<tr>
<td>3.4.1 All pathways of modification can be rationalised by a transnitrosation mechanism</td>
<td>107</td>
</tr>
<tr>
<td>3.4.1.1 Transnitrosation to explain depurination</td>
<td>107</td>
</tr>
<tr>
<td>3.4.1.2 Transnitrosation to explain deamination</td>
<td>107</td>
</tr>
<tr>
<td>3.4.1.3 Transnitrosation to explain the formation of oxanine</td>
<td>108</td>
</tr>
<tr>
<td>3.4.1.4 Transnitrosation to explain the formation of N-acetyl adducts</td>
<td>109</td>
</tr>
<tr>
<td>3.4.1.5 Transnitrosation to explain decomposition of nitrosated indoles</td>
<td>110</td>
</tr>
</tbody>
</table>

CHAPTER 4

REACTIONS OF 1-NITROSOINDOLE-3-ACETONITRILE WITH OLIGONUCLEOTIDES, DUPLEX DNA AND CALF THYMUS DNA 111

4.1 Introduction 112

4.2 Experimental Procedures 114

4.2.1 Enzymes and general materials 114

4.2.2 Synthesis and characterisation of NIA(N) 114

4.2.3 Preparation of radiolabelled oligonucleotides 114

4.2.4 Reactions of NIA(N) with single-stranded oligonucleotides (Ia), (II), (III) and (IV) 115
4.2.4.1 Preparation of Maxam and Gilbert sequencing markers for single-stranded oligonucleotides (Ia), (II), (III) and (IV) modification reaction specific for guanine
4.2.4.2 Analysis of oligonucleotide fragment samples by denaturing polyacrylamide gel electrophoresis (DPAGE)
4.2.4.3 Data analysis and quantitation of strand breaks

4.2.5 Cross-linking reactions of NIA N and mechlorethamine hydrochloride with duplex DNA
  4.2.5.1 Preparation of radiolabelled duplex DNA
  4.2.5.2 Confirmation of duplex formation by non-denaturing polyacrylamide gel electrophoresis (PAGE)
  4.2.5.3 Cross-linking reactions of mechlorethamine hydrochloride with duplex DNA sequence (VI): measure of duplex availability
  4.2.5.4 Cross-linking reactions of NIA N with duplex DNA sequence (I)
  4.2.5.5 Analysis of cross-linked samples by DPAGE, data analysis and quantitation of strand breaks

4.2.6 Reactions of NIA N with single-stranded oligonucleotide (V) and calf thymus DNA
  4.2.6.1 HPLC analyses of digested reaction mixtures
  4.2.6.2 Characterisation of reaction products by mass spectrometry

4.3 Results
  4.3.1 NIA N reactions with single-stranded oligonucleotides (Ia), (II), (III) and (IV)
  4.3.2 Cross-linking reactions of mechlorethamine hydrochloride with duplex DNA: assessment of duplex availability
  4.3.3 Cross-linking reactions of NIA N with duplex DNA
  4.3.4 NIA N reactions with single-stranded oligonucleotide (V) and calf thymus DNA
    4.3.4.1 NIA N reactions with single-stranded oligonucleotide (V)
    4.3.4.2 NIA N reactions with calf thymus DNA

4.4 Discussion
  4.4.1 The spectrum of potentially mutagenic products is preserved at the macromolecular level
  4.4.2 Dose-dependent cross-links at CG residues in duplex DNA are induced by nitroso transfer by NIA N
CHAPTER 5
DETECTION OF APURINIC RESIDUES INDUCED BY
1-NITROSOINDOLE-3-ACETONITRILE IN CALF THYMUS DNA AND IN
THE GLANDULAR STOMACH OF CD-1 MICE BY 32P-POSTLABELLING

5.1 Introduction 156

5.2 Experimental Procedures 159

5.2.1 Enzymes and general materials 159

5.2.2 Synthesis and characterisation of NIAN 160

5.2.3 Reactions of NIAN with calf thymus DNA 160

5.2.4 Azide inhibition experiments 160

5.2.5 Animal study 160

5.2.5.1 Animals 161

5.2.5.2 Treatment of animals 161

5.2.5.3 DNA isolation from glandular stomach 161

5.2.5.4 Parity check of mouse stomach DNA by HPLC 162

5.2.6 Incubation of DNA samples with damage-recognising enzymes 162

5.2.6.1 Incubation of DNA samples with E.coli exonuclease III 162

5.2.6.2 Incubation of DNA samples with E.coli endonuclease IV 162

5.2.6.3 Preparation of 50 Gy irradiated DNA 163

5.2.7 Postlabelling assay 163

5.2.7.1 Enzymatic digestion of DNA samples 163

5.2.7.2 32P-Postlabelling of enzyme digested DNA 163

5.2.7.3 Analysis of postlabelled DNA samples by denaturing polyacrylamide gel electrophoresis (DPAGE) 164

5.2.7.4 Data analysis and lesion quantitation 164

5.3 Results 165

5.3.1 Postlabelling detection of apurinic residues in calf thymus DNA treated with NIAN at mM concentrations 165

5.3.2 Postlabelling detection of apurinic residues in calf thymus DNA treated with NIAN at μM concentrations 170

5.3.3 Inhibition of reaction product formation by azide ion 173

5.3.4 In vivo study 176

5.3.4.1 Quality check of mouse stomach DNA 176

5.3.4.2 Postlabelling detection of apurinic residues in DNA from the glandular stomach of CD-1 mice 177

5.3.5 Influence of damage-recognising enzymes 185
5.4 Discussion

5.4.1 The detection of abasic sites in calf thymus DNA induced by exposure to μmolar concentrations of NIAN 191
5.4.2 NIAN induces abasic site formation in vivo 191
5.4.3 NIAN-induced abasic sites are repaired in vivo 192

CHAPTER 6

ACTIVITY OF 1-NITROSOINDOLE-3-ACETONITRILE IN THE COMET AND AMES II ASSAYS

6.1 Introduction 195

6.2 Experimental Procedures

6.2.1 Materials 198
6.2.2 Synthesis and characterisation of NIAN 198
6.2.3 The Comet Assay 198

6.2.3.1 Cell culture 198
6.2.3.2 Treatment of cells with NIAN and the control chemical 199
6.2.3.3 Single-cell gel electrophoresis 199
6.2.3.4 Slide analysis and parameters measured 200
6.2.3.5 Data interpretation 201

6.2.4 The Ames II assay 201

6.2.4.1 Bacterial strains 201
6.2.4.2 Control chemicals 201
6.2.4.3 Test chemical (NIAN) 201
6.2.4.4 Preparation of tester strains 202
6.2.4.5 Chemical exposure 202
6.2.4.6 Prototrophic selection 202
6.2.4.7 Plate scoring and data interpretation 202

6.3 Results 204

6.3.1 Response of NIAN in the Comet assay 204
6.3.2 Response of NIAN in the Ames II assay 205

6.4 Discussion 211

6.4.1 In vitro genetic toxicology assays identify NIAN as a DNA-damaging agent and a mutagen 211
CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS 212

7.1 Transnitrosation: A new pathway for N-nitrosocompounds to exert genotoxicity 213

7.1.1 Transnitrosation may be operative for many agents which release nitric oxide 216

7.2 The significance and mutagenic implications of N-nitrosoindole-induced DNA damage 216

7.2.1 Deamination pathway of DNA modification 216

7.2.2 Depurination pathway of DNA modification 219

7.2.3 The significance of oxanine as a lesion in DNA 220

7.2.4 The significance of NIAN-induced cross-links 223

7.2.5 Influence of DNA sequence 223

7.3 Future work 224

7.4 Conclusions 226

REFERENCES 227


APPENDIX 3 Comet Assay Scores XVI

APPENDIX 4 Ames II Assay Scores XVII

APPENDIX 5 List of Schemes XVIII

List of Figures XVIII

List of Tables XVIII

XI
ABBREVIATIONS

Ac: acetyl
ACN: acetonitrile
Ado: adenine
AGT: O'-alkylguanine-DNA alkyltransferase
5azaC: 5-aza cytidine
Cl: 4-chloroindole
CMI: 4-chloro-6-methoxyindole
d-ACN: deuterated acetonitrile
dAp: 2'-deoxyadenosine-3'-monophosphate
dATP: deoxyadenosine triphosphate
dCp: 2'-deoxyctydine-3'-monophosphate
dGp: 2'-deoxyguanosine-3'-monophosphate
dGTP: deoxyguanosine triphosphate
dGuo: deoxyguanosine
DMSO: dimethyl sulphoxide
dOTP: deoxyoxanosine triphosphate
dOxo: deoxyoxanosine
dOp: 2'-deoxyoxanosine-3'-monophosphate
dpA: 2'-deoxyadenosine-5'-monophosphate
dpG: 2'-deoxyguanosine-5'-monophosphate
dpO: 2'-deoxyoxanosine-5'-monophosphate
DMN: dimethylnitrosamine
DPAGE: denaturing polyacrylamide gel electrophoresis
ESI-MS: electrospray mass spectrometry
ESI-MS-MS: electrospray tandem mass spectrometry
FAB: fast atom bombardment
Gua: guanine
HGPRT: hypoxanthine-guanine phosphoribosyltransferase
HPLC: high performance liquid chromatography
I33': 3,3'-diindolylmethane
I3AA: indole-3-acetic acid
I3C: indole-3-carbinol
I3CHO: indole-3-carboxaldehyde
IAAME: indole-3-acetic acid methyl ester
IAM: indole-3-acetamide
Abbreviations

IAN: indole-3-acetonitrile
MeOH: methanol
MMS: methyl methanesulphonate
MNNG: N-methyl-N'-nitro-N-nitrosoguanidine
MNU: methyl nitrosourea
N^2-AcGp: N^2-acetyldeoxyguanosine-3'-monophosphate
N^2-AcGMP: N^2-acetyldeoxyguanosine-5'-monophosphate
N^2-AcGu: N^2-acetylguanine
N^6-AcAd: N^6-acetyladenine
NCI: 4-chloro-2-hydroxy-N'-nitrosoindolin-3-one oxime
NCMI: 4-chloro-6-methoxy-2-hydroxy-N'-nitrosoindolin-3-one oxime
NIAAME: 1-nitrosourodo-3-acetic acid methyl ester
NIAAM: 1-nitrosourodo-3-acetamide
NIAAN: 1-nitrosourodo-3-acetonitrile
NMR: nuclear magnetic resonance
NNK: 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN: N^1-nitrosonornicotine
NNOC: N-nitroso compounds
NO: nitric oxide
4NQO: 4-nitroquinoline-N-oxide
Oxa: oxanine
PAGE: polyacrylamide gel electrophoresis
PhOH: phenol
SAP: shrimp alkaline phosphatase
SCN: thiocyanate ion
STN: streptonigrin
SVPD: snake venom phosphodiesterase
T4 PNK: T4 polynucleotide kinase
TK: thymidine kinase
Tp: thymidine-3'-monophosphate
UDS: unscheduled DNA synthesis
VBL: vinblastine
VCR: vincristine
Xan: xanthine
CHAPTER 1

INTRODUCTION
1.1 INDOLES AND HUMAN EXPOSURE

The study of the chemistry of indoles is an active area of heterocyclic chemistry, because naturally occurring indole alkaloids have demonstrated important physiological activity. Although natural product chemistry is still an active area of primarily academic research, increasing resources are targeted towards the preparation of indole derivatives as potential drug candidates.

Fusion of a benzene ring onto the C2/C3 positions of pyrrole formally produces the corresponding benzopyrrole, known as indole. Indole is a ten-\(\pi\) electron aromatic system and similarly to pyrrole, delocalisation of the lone pair of electrons from the nitrogen atom is necessary for aromaticity. The structure of indole is most completely described in Scheme 1.1 whereby the mesomeric representation contributes to the electronic structure of indole.

\[
\begin{array}{c}
\text{Scheme 1.1 Structure of indole.} \\
\end{array}
\]

As a consequence of the delocalisation of the lone pair on the nitrogen atom, indole is a weakly basic heterocycle as the lone pair is not available for protonation under moderately acidic conditions. Indole easily undergoes aromatic electrophilic substitution, being an "electron-rich" heterocycle, and selectively substitutes at the C-3 position.

The indole unit occurs naturally in a wide variety of structures, many of which are substituted at the C-3 position. More than a thousand indole alkaloids (both simple and complex bis-indole structures) are known and many of these possess interesting physiological activity.
**1.1.1 Endogenous Sources**

The main endogenous source of indoles is tryptophan and its metabolites, which include serotonin and tryptamine. Serotonin, or 5-hydroxytryptamine (5-HT), is widely distributed in nature but occurs only in low concentration. Hydroxylation of the benzenoid ring of tryptophan, followed by decarboxylation gives rise to serotonin and many simple alkaloids contain the indole-C₅N sub-unit derived from tryptophan. Most of the important indole alkaloids have more complex structures, but the aminoethyl side chain of tryptophan is still discernible in their structure. The structure of tryptophan and metabolites tryptamine and serotonin are shown in Scheme 1.2.

![Tryptophan](image1)  ![Tryptamine](image2)  ![Serotonin (5-Hydroxytryptamine, 5-HT)](image3)

**Scheme 1.2** Structures of tryptophan and metabolites tryptamine and serotonin.

Serotonin was first isolated from natural sources in 1948 and is now known to have a wide and complex range of pharmacological actions (Gilchrist 1996). These include the contraction of blood vessels by stimulation of smooth muscle and blood platelet aggregation. It has an essential function in the central nervous system as a neurotransmitter, where it is responsible, at least in part, for control of sleep patterns.
Introduction

(Mann 1987). Changes in serotonin levels have been implicated in migraine, depression, lack of appetite and even schizophrenia (Mann 1987).

1.1.2 Dietary Sources

Many indole derivatives that mimic or block the binding of serotonin to its receptors are closely related to serotonin in structure and are derived from vegetable sources.

These alkaloids differ from serotonin, primarily in the position of hydroxylation on the benzenoid ring and in the chemical modification of the side-chain. The result of the slight manipulation in the structure is that these indole derivatives interfere with the metabolism of serotonin in the brain and exert potent hallucinogenic effects.

Bufotenine is found in toadstools and is the N-N-dimethyl analogue of serotonin. Psilocin, a metabolite from the species of mushroom *Pseilocybe*, differs from bufotenine only by the positioning of the phenolic hydroxyl group, which is at the 4-position (Scheme 1.3). Both these compounds have been used as hallucinogens in Central and South America (Gilchrist 1996).

![Scheme 1.3 Structures of the hallucinogens bufotenine and psilocin.](image)

A major source of exposure of dietary indoles to humans is the consumption of cruciferous vegetables that contain indole glucosinolates.

Glucosinolates are mainly found in the plant family *Brassicaceae* (*Cruciferae*) which include broccoli, cabbage, kale, Brussel sprouts, cauliflowers, radishes, watercress and
turnips. Five indole glucosinolates have been characterised: glucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin and N-sulphoglucobrassicin (Vang and Dragsted 1996).

Glucosinolates are degraded chemically or enzymatically by myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) during food preparation, cooking and chewing. Glucosinolates and myrosinase are compartmentalised in plants but interact when tissue damage occurs. Myrosinase activity of intestinal bacteria may also contribute to the degradation of glucosinolates in vivo (Thorvald 1999).

Scheme 1.4 shows a summary of the autolytic products of glucobrassicin. Myrosinase catalyses the hydrolytic cleavage of the thioglucoside bond in the intact glucosinolates, yielding glucose and a resulting unstable thioglycosyl-O-sulphonate, which rearranges spontaneously with the release of sulphate to the corresponding unstable isothiocyanate compound, indole-3-methyl isothiocyanate (Bradfield and Bjeldanes 1987; Latxague et al. 1991; Vang and Dragsted 1996; Thorvald 1999). Indole-3-acetonitrile (IAN) and indole-3-acetic acid (I3AA) are also released as spontaneous rearrangement products (Bradfield and Bjeldanes 1987; Latxague et al. 1991; Vang and Dragsted 1996; Thorvald 1999). The unstable isothiocyanate compound releases thiocyanate ion (SCN) to afford a stabilised carbonium ion, which can react with different nucleophiles to form a wide variety of products. The carbonium ion can also form oligomeric products or react with vitamin C to produce ascorbigens (Vang and Dragsted 1996). Reaction of the carbonium ion with water affords indole-3-carbinol (I3C). I3C is the major metabolite generated when plant material is disrupted, but it is unstable in aqueous solutions and undergoes self-condensation to the dimer 3,3'-diindolylmethane (I33') or oxidation to the aldehyde indole-3-carboxaldehyde (I3CHO) (Bradfield and Bjeldanes 1987; Latxague et al. 1991).

The exact nature of the products formed can depend on the pH. Isolation of the indole glucosinolate autolysis products from plant material yields primarily nitriles and carboxaldehydes at neutral pH (Bradfield and Bjeldanes 1987). It has been demonstrated that at acidic pH greater than 99% of the autolysis product is IAN (Latxague et al. 1991).

Common cooking practices employed on cruciferous vegetables do not inactivate myrosinase activity to a significant extent, indicating that these autolytic products are likely to occur in cooked as well as raw vegetables (Bradfield and Bjeldanes 1987).
**Scheme 1.4** Summary of the autolytic products of glucobrassicin. Enzymatic hydrolysis yields primarily either the acetonitrile (IAN) or the alcohol (I3C). The resulting I3C is unstable in aqueous solutions and undergoes self-condensation to the dimer (I33') or oxidation to the aldehyde (I3CHO).
Overall, these observations suggest that IAN is an abundant indole glucosinolate autolysis product, in the acidic environment of the stomach, and is potentially of the most dietary importance in this environment. The relevance of IAN in the scope of this thesis is discussed in section 1.4 and subsequent chapters.

Humans are also exposed to some chlorine containing indole compounds. For example, in fava beans (Vicia faba), 4-chloro-6-methoxyindole has been identified (Yang et al. 1984).

The intake of cruciferous vegetables varies greatly in different populations and in different countries and the amounts of indole glucosinolates contained varies on the season, growing conditions and storage. It has been estimated that the average daily intake of cruciferous vegetables in the United Kingdom is 45 g, corresponding to an estimated glucobrassicin daily intake of 30 mg, which if totally converted to the indole products, represents a 10 mg intake (Yang and Dragsted 1996).

1.1.3 Therapeutic Potential

As many naturally occurring indole alkaloids have demonstrated biological activity they are, or are under development as, therapeutic agents. The ease by which indoles can be subtly manipulated in the laboratory, to achieve a desired modification in function, has been exploited in the laboratory for the development of synthetic indole derivatives as new pharmaceutical agents.

1.1.3.1 Naturally Occurring Indole Alkaloids

The consumption of higher levels of vegetables and fruit is associated consistently, although not universally, with a reduced risk of cancer at most sites, most notably for epithelial cancers (reviewed in Steinmetz and Potter 1991).

The chemoprotective effect of I3C, the major hydrolytic product derived from glucobrassicin as described in section 1.1.2, has been demonstrated on spontaneous mammary and endometrial tumours in rodent models (Bradlow et al. 1991; Malloy et al. 1997). I3C has reduced tumour incidence when administered prior to and during treatment with mammary carcinogens (Grubbs et al. 1995). As a result of these findings, I3C has
been suggested to be a good candidate for chemoprevention and is currently under study as a preventative agent for cancers of the breast and other organs (Riby et al. 2000).

The *Catharanthus* alkaloids (also commonly referred to as the *Vinca* alkaloids) are found in the plant *Catharanthus roseus*, a native plant of Madagascar which is now widely cultivated. Extracts of *C. roseus* were used as early as 1653 for hemostasis and for treating gingivitis and diabetes but it was not until 300 years later that the leukopenic and antitumour activities of such extracts were discovered (Sinha and Jain 1994). Two of the most important alkaloids isolated were vinblastine (VBL) and vincristine (VCR) and both these bis-indole compounds are today used mostly in combination chemotherapy regimens, in both the adjuvant and metastatic settings (El-Sayed and Cordell 1981; Van Tellingen et al. 1992; Rosazza et al. 1992; Kirsch-Volders and Parry 1996). The only structural difference between VBL and VCR is in the state of oxidation of the carbon atom on the indoline nitrogen. Their complex bis-indole structure is shown in Scheme 1.5.

![Scheme 1.5 Bis-indole structures of the potent antitumour agents vinblastine and vincristine from *Catharanthus roseus*.](image)

VBL and VCR represent just two out of over 90 alkaloids that have been isolated from *C. roseus*. The effects of small structural differences on the pharmacological activity of these compounds have directed research towards the development of synthetic derivatives with
the aim to improve the therapeutic index. From a wider perspective, the discovery of the vinca alkaloids contributed to the wider search for natural products and their analogues as anticancer drugs.

1.1.3.2 Synthetic Substituted Indoles

There are a variety of synthetic therapeutics available based on indolic structures. Sumatriptan and zolmitriptan for example, are synthetic derivatives of serotonin and are potent and selective 5-HT₁ receptor agonists marketed for the treatment of migraine (Owen et al. 1995; Zomig Product Monograph, AstraZeneca 2000).

Serotonin is believed to be a possible mediator of migraine because of its actions on blood vessels but its clinical utility is limited by side effects (Kimball et al. 1960). While serotonin is a primary amine (Scheme 1.2), sumatriptan and zolmitriptan (Scheme 1.6) are tertiary amines and have the same functionality at the C-3 position as the hallucinogens, bufotenine and psilocin (Scheme 1.3). However, rather than possessing a phenolic hydroxyl group and interfering with the metabolism of serotonin in the brain, both sumatriptan and zolmitriptan have much more complex functionality at the 5-position.

![Scheme 1.6 Sumatriptan and zolmitriptan, both related to serotonin in structure, are potent 5-HT₁ receptor agonists marketed for the treatment of migraine.](image-url)
As a result of this chemical modification, both sumatriptan and zolmitriptan achieve the selective effect of promoting activity at 5-HT$_1$ receptors affording vasoconstriction of blood vessels and alleviating migraine without the adverse effects seen with serotonin.

Interestingly, LSD (Scheme 1.7), the very potent Class A hallucinogenic drug, is the $N$-$N$-diethylamide synthetic derivative of lysergic acid, which is an ergot indole alkaloid produced by the fungus Claviceps purpurea, which can infect rye.

Scheme 1.7 Structure of the potent hallucinogen LSD, which is the $N$-$N$-diethylamide synthetic derivative of lysergic acid.

The development of a considerable number of synthetic derivatives of the Catharanthus roseus indole alkaloids, as chemotherapeutic agents, has been reported. These include vindesine, vinorelbine, vinzolidine and vintripitol (reviewed in Van Tellingen et al. 1992).

Ondansetron and tropisetron (Scheme 1.8), belong to a group of potent 5-HT$_3$ receptor antagonists and are used to treat nausea and vomiting caused by chemotherapy (Butcher 1993; Macor et al. 2001). Additional 5-HT$_3$ receptor antagonists, that are all based on indolic structures, include granisetron, dolasteron and ramosetron (Gaster and King 1997).
1.1.4 Toxicity of Indoles

The antitumourigenic activities of indoles in the diet have been studied for over 20 years, but despite this, much information is still lacking. No adverse effects of indoles have been reported at the level of normal dietary intakes (Vang and Dragsted 1996). At very high doses, I3C has been shown to promote tumourigenesis in the thyroid gland, colon, pancreas and liver of rodents when administered following treatment with a carcinogen (Pence et al. 1986; Bailey et al. 1987; Kim et al. 1997).

Recently, it has been reported that I3C exhibits tumour-promoting activity in the trout model in a range of concentrations, below the levels that are thought to be important to induce cancer protective effects (Organesian et al. 1999). Additional studies are necessary to enable general conclusions about the toxicity of indoles to be made. Furthermore, I3C-associated mechanisms of tumour-promotion and the relation to human cancer risk must be thoroughly understood, if I3C is to be developed further as a chemoprotective agent.

**Scheme 1.8** Structures of ondansetron and tropisetron, potent 5-HT3 antagonists used to treat vomiting and nausea caused by chemotherapy.
1.2 NITROSATION

The use of many indole-containing compounds in widely used drugs, and their abundance in our diet provides the impetus for an evaluation of the genotoxicity of this class of compounds due to their propensity for nitrosation.

$\psi$-Nitrosation frequently transforms innocuous nitrogen-containing compounds into toxic compounds (Bartsch 1991) and this is of concern since there are endogenous sources of nitrite and nitrosating agents. The role of $\psi$-nitroso compounds (NNOC) in human cancer and the relevance of $\psi$-nitrosoindoles, specifically, are discussed in sections 1.3 and 1.4, respectively.

Nitrosation chemistry has been studied extensively since the discovery in 1956 that nitrosamines are powerful rodent carcinogens (Magee and Barnes 1956) and many reactions are now well understood. Most of our understanding about endogenous nitrosation has arisen from studying this pathway in vitro.

1.2.1 N-Nitrosation Pathways In Vitro

The most widely used reagent in the laboratory for nitrosation is nitrous acid, generated by treating sodium nitrite with a strong mineral acid such as hydrochloric acid. Nitrous acid is an unstable species that exists in equilibrium with a number of other species, depending on the acidity of the solution and the other ions present. Nitrous acid is in equilibrium with its anhydride, dinitrogen trioxide, which in turn is in equilibrium with nitric oxide (NO) and nitrogen dioxide (Williams 1988). In strongly acidic solutions, nitrous acid is protonated and its conjugate readily loses water to generate the potent nitrosating agent, the nitrosonium ion (Scheme 1.9). Most of these reactive species contribute to nitrosation (Williams 1988), and can readily nitrosate nucleophilic nitrogen atoms in many nitrogen-containing compounds. Common catalysts of nitrosation include non-basic nucleophiles such as halide ion or SCN$^-$. Other nitrosating agents, many of which can be used in non-aqueous solvents, include nitrosonium salts such as NO$^+$BF$_4^-$, alkyl nitrites RONO and nitrosyl acetate NOOAC (which is probably the effective agent when sodium nitrite is dissolved in acetic acid).
Introduction

\[\text{Scheme 1.9} \text{ Nitrous acid exists in equilibrium with a number of other species, most of which contribute to afford nitrosation products.}\]

1.2.1.1 \textit{N-Nitrosation of Amines and Amides}

The first product in the \textit{N-nitrosation} of primary aliphatic and aromatic amines is the formation of a primary nitrosamine $\text{R NHNO}$ as outlined in equation (1) (Scheme 1.10). In most cases these nitrosamines are not stable and in a series of rapid reactions, including proton transfer and the loss of a water molecule, generate the diazonium ion $\text{RN}_2^+$ (and subsequent deamination products) as shown in equation (2) (Scheme 1.10). Aryl diazonium ions are much more stable than their aliphatic counterparts, since the positive charge on the nitrogen atom can be delocalised into the aromatic systems. Similarly, heterocyclic diazonium ions can be stabilised in the same way. Diazonium ions are reactive species and the consequences of diazonium ion formation from $\text{NNOC}$ are discussed in section 1.3.2.

Secondary amines, both aliphatic and aromatic, react readily with the usual range of nitrosating agents to give nitrosamines; equation (3) (Scheme 1.10). In contrast to primary
nitrosamines, secondary nitrosamines are relatively stable since there are no hydrogen atoms directly bonded to the nitrogen atom available for the necessary proton transfer reactions, leading to diazonium ion formation. Most reactions are quite rapid and some are significantly reversible (e.g. with N-acetyltryptophan, see section 1.2.1.2).

\[ \text{RNH}_2 + \text{H}_2\text{NO}_2^+ \rightarrow \text{RNH}_2\text{NO} \rightarrow \text{RNHNO} \] (1)

\[ \text{RNHNO} \leftrightarrow \text{RN}==\text{N}==\text{OH} \leftrightarrow \text{RN}==\text{N}==\text{OH}_2 \rightarrow \text{RN}_2^+ + \text{H}_2\text{O} \] (2)

\[ \text{R}_2\text{NH} + \text{H}_2\text{NO}_2^+ \rightarrow \text{R}_2\text{NNO} \] (3)

\[ 2\text{R}_2\text{NCHR}_2 + 4\text{HNO}_2 \rightarrow 2\text{R}_2\text{NNO} + 2\text{R}_2\text{CO} + \text{N}_2\text{O} + 3\text{H}_2\text{O} \] (4)

\[ \text{RCONH}_2 + \text{H}_2\text{NO}_2^+ \rightarrow \text{RCO}_2\text{H} + \text{N}_2 \] (5)

\[ \text{RCONHR'} + \text{H}_2\text{NO}_2^+ \rightarrow \text{RCON(NO)R'} \] (6)

**Scheme 1.10** Summary of the N-nitrosation pathways of amines and amides.

It was generally believed that tertiary amines were not reactive toward nitrous acid, but it had been reported as early as 1864 that triethylamine gave diethylnitrosamine with nitrous acid (Williams 1988). These reactions have not been studied as widely as the corresponding reactions of primary and secondary amines, but interest in this pathway has surfaced recently since there are a number of tertiary amine structures in pharmaceuticals. Nitrosamine formation can occur, along with the generation of a carbonyl compound and nitrous oxide; the stoichiometry of the reaction is given in equation (4) (Scheme 1.10), and kinetic studies have demonstrated the reaction to be around 10 000 times slower than the corresponding secondary amine reactions (Williams 1988). However, the reactions of some tertiary amines, containing other than simple alkyl groups, are much faster. This
Introduction

group includes a number of drugs such as aminopyrine and monocycline and is discussed in more detail in section 1.3.4.

Nitrosation of primary amides results in a deamination reaction giving the carboxylic acid and nitrogen as products; equation (5) (Scheme 1.10). These reactions are generally much slower than those of the corresponding amines, due to the presence of the strongly electron-withdrawing carbonyl group. Nitrosation of secondary amides afford nitrosamides in a reversible process and this reaction also occurs for the nitrosation of ureas; equation (6) (Scheme 1.10).

1.2.1.2 N-Nitrosation of Indole (Aromatic Secondary Amine)

Indole is a nucleophilic heterocycle and reacts easily with electrophiles such as nitrosating agents. The preferred site for electrophilic substitution is C-3. However, if C-3 has additional functionality, as is common with many of the natural and synthetic indolic compounds in the human environment, nitrosating agents react readily with the lone pair of electrons on the nitrogen atom to afford the nitrosation product at N-1 [Scheme 1.11 (A)]. However, an internal rate-limiting nitroso group rearrangement has been proposed as one of the pathways in the nitrosation of N-acetyltryptophan (Castro et al. 1986), and this mechanism may be quite general for all indole systems. The initial attack by the nitrosating agent in this mechanism occurs at C-3, and internal rearrangement of the nitroso group to the indole nitrogen occurs [Scheme 1.11 (B)] to yield the thermodynamically more stable N-nitroso product.

The advantage of the non-direct mechanism has been suggested to lie, at least in part, in the avoidance of the highly energetic intermediate formed as a result of direct nitrogen atom nitrosation; an extremely acidic N(NO)H⁺ moiety (Peña 1995).

These reactions are reversible and evidence exists for an equilibrium between tryptophan and its nitrosated form (Mellet et al. 1986). Denitrosation studies of N-acetyl-N'-nitrosotryptophan support the internal rearrangement mechanism (Castro et al. 1986).

It is likely that both direct nitrogen atom nitrosation and attack at C-3, are probably involved in the nitrosation and denitrosation of all 3-substituted indoles, leading to formation of the overall N-nitrosoindole product.
Scheme 1.11 Mechanisms for the formation of N-nitrosoindoles. (A) Direct nitrogen atom nitrosation and (B) internal rearrangement of the nitroso group from C-3 to N-1.

The pH optimum of the acid-catalysed N-nitrosation reaction is similar to that of the normal stomach (Hill 1996a). Consequently, endogenous synthesis of NNOC is possible since there are endogenous sources of nitrite, nitrosating agents and nitrosatable nitrogen.

1.2.2 N-Nitrosation Pathways In Vivo "Endogenous Nitrosation"

The process of endogenous nitrosation is important for human health risk assessment. Endogenous nitrosation occurs predominantly in the stomach, via the acid-catalysed route, leading to the synthesis of a range of NNOC, which may exert their carcinogenic action in gastric epithelium, or upon their appearance in the systemic circulation, in other target tissues. Any environment where a nitrosating agent and nitrosatable nitrogen exist, is a potential source of NNOC.
Endogenous nitrosation can occur in both acidic and neutral environments via these pathways: the acid-catalysed reaction and nitrosation under neutral conditions. Nitrosation in neutral environments can occur via the bacterially-mediated reaction and the activated-macrophage-mediated pathway.

1.2.2.1 The Acid-Catalysed Reaction

The pH optimum of the acid-catalysed reaction is around 2, but varies between nitrogen containing compounds and ensures that the only site in the body where this reaction can occur is the acid stomach (Hill 1996b).

The main source of endogenous nitrite is derived from dietary nitrate naturally present in vegetables and other food sources. Humans continually ingest nitrates, and to a much lesser extent nitrites, from vegetables. Nitrates are normally present in vegetables at concentrations up to several milligrams per kilogram of fresh weight (Vittozzi 1992). The general level of nitrate in vegetables over recent years has risen, mainly due to the increased use of nitrate-based fertilisers (Tenovuo 1986), although in future years, the levels are likely to decline with the introduction of organic farming methods. Nitrites and nitrates are also widely used as additives in sausages and cured meat and fish to prevent bacterial growth (Parke and Lewis 1992; Skovgaard 1992; Massey and Lees 1992). Nitrate is also added to certain continental cheeses such as Gouda and Edam as a preservative (Massey and Lees 1992) and has been found in increasing levels (up to 50 mg per litre) in drinking water (Møller et al. 1989).

Nitrate is absorbed from the upper small intestine and is secreted in all body secretions, particularly saliva and gastric juice (Bartholomew and Hill 1984). Salivary nitrate is reduced by bacterial action to nitrite and is swallowed in the saliva with food. Human saliva contains both nitrate and nitrite reductase activity which are likely to be of microbial origin; the optimum pH for reduction is 6-6.4 with no activity beyond pH 4 and pH 9 (Tenovuo 1986). The level of salivary nitrite is dependent on salivary nitrate and consequently, is dependent on the dietary intake of nitrate (Tenovuo 1986). Such nitrate-derived nitrite is the major source of exogenous nitrites for most humans, and it has been estimated from pharmacological studies that 10% of nitrate enters the stomach as salivary nitrite (Bartholomew and Hill 1986).
Introduction

Gastric juice is a rich source of nitrosatable precursors from both biological matrices (eg DNA, amino acids and proteins), food matrices (eg amino acids and glucosinolates) and other substances, including drugs. This has been demonstrated by numerous studies analysing NNOC present in gastric juice (Pignatelli et al. 1990; Xu and Reed 1993; reviewed in Reed 1996; Dallinga et al. 1996).

1.2.2.2 The Bacterially-Mediated Reaction

Similarly to many other types of chemical reaction, endogenous nitrosation can proceed at neutral pH catalysed by bacterial enzymes (Hawkesworth and Hill 1971; Calmels et al. 1983; Leach et al. 1985). Bacterial nitrosation can occur wherever in the body bacteria, nitrate or nitrite and nitrosatable nitrogen co-exist. The mouth, stomach, large bowel, bladder and vagina are all possible sites for bacterial nitrosation. Table 1.1 summarises the factors limiting this pathway indicating their likely relative importance at each site.

<table>
<thead>
<tr>
<th>Site</th>
<th>Mouth</th>
<th>Stomach</th>
<th>Large Bowel</th>
<th>Bladder</th>
<th>Vagina</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>6-8</td>
<td>5-8</td>
<td>5-8</td>
<td>4-8</td>
<td>6-8</td>
</tr>
<tr>
<td><strong>Flora</strong></td>
<td>Complex</td>
<td>Complex</td>
<td>Complex</td>
<td>?</td>
<td>Complex</td>
</tr>
<tr>
<td><strong>Nitrite conc.</strong></td>
<td>++</td>
<td>+++</td>
<td>?</td>
<td>+++</td>
<td>?</td>
</tr>
<tr>
<td><strong>N-Nitrosatable substrate</strong></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Incubation time (h)</strong></td>
<td>0-1</td>
<td>3-5</td>
<td>20-50</td>
<td>3-10</td>
<td>3-5</td>
</tr>
</tbody>
</table>

Table 1.1 Factors controlling bacterial N-nitrosation at various sites in the body (Hill 1996b).

The mouth has a rich and permanent bacterial flora and saliva contains both nitrate and nitrite reductase activity (Tenovuo 1986). However, salivary flow is considerable and greatly limits the time available in saliva for bacterially-catalysed N-nitrosation to occur.
The human stomach appears to be most likely organ to be exposed to NNOC, because not only does the acid-catalysed reaction contribute to endogenous nitrosation, but so does the bacterially-mediated reaction. It has been demonstrated that significantly higher NNOC concentrations were found at intragastric pH ranges of 1.13-2.99 and 6-8.42 compared with that at 3.00-5.99 (Xu and Reed 1993). The normal acidic stomach is essentially sterile and any intragastric nitrite found is largely of salivary origin. The acid-catalysed reaction predominates. It is likely that nitrite may be the first limiting substrate, because there is an excess of total nitrosatable substrates in gastric juice. Under achlorhydric conditions when gastric acid secretion is impaired (eg gastric surgery or natural atrophy) and the resting gastric pH rises consistently above 4, then overgrowth with nitrate-reducing bacteria occurs, leading to an increase in intragastric nitrite concentration. The study by Xu and Reed confirms that NNOC formation in the achlorhydric stomach depends primarily on the activity of intragastric bacterial flora. It appears that at an intermediate pH range of 3-5.99, both pathways seemed to be suppressed. The results of the Xu and Reed study also demonstrated that there were pronounced inter-individual variation in intragastric nitrite and NNOC concentrations under both acidic and achlorhydric conditions in both low and high pH samples. These findings indicate that both endogenous nitrosation pathways are markedly affected by factors other than intragastric pH and nitrite.

Although the urinary bladder is normally free of bacteria, bladder infections are common and NNOC have been identified (Hicks et al. 1977). Significant N-nitrosation would also be expected in the infected vagina but there has been no reported studies of nitrate secreted into the vagina. Similarly for the large bowel, the status of the colon with respect to nitrate concentration is unclear (Hill 1996c).

1.2.2.3 Activated-Macrophage-Mediated Nitrosation

Chronic infection and resulting tissue inflammation have long been recognised as risk factors for a variety of human cancers. Infection with hepatitis virus, for example, is a risk factor for the development of hepatocellular carcinoma (Palmer-Beasley 1988), human papilloma virus is a risk factor for cervical cancer (Herrero and Munoz 1998; Munoz 2000) and infection with Helicobacter pylori bacteria is a risk factor for stomach cancer (Pignatelli et al. 1998).
There is increasing evidence that overproduction of cellular NO by the inducible form of NO-synthase (type II), resulting as a consequence of activated macrophage, may play a role in carcinogenesis (Ohshima and Bartsch 1994; Wink et al. 1998; Pignatelli et al. 1998).

NO can react to form other reactive nitric oxide species (NOx), and three main processes that control the fate of NO in biological systems have been identified: diffusion, auto-oxidation to nitrous anhydride (dinitrogen trioxide, N₂O₃, Scheme 1.9) and reaction with superoxide ion (O₂⁻) to form peroxynitrite (ONOO⁻) (Burney et al. 1999). The formation of N₂O₃ as previously described in section 1.2.1, is a nitrosating agent and so can contribute to endogenous nitrosation and the formation of NNOC. Peroxynitrite, although not considered a direct nitrosating agent (Williams 1997) is involved in oxidative DNA damage (Burney et al. 1999; Wink et al. 1998).

Overall, activated-macrophage-mediated endogenous nitrosation can occur at many sites of the body and because chronic inflammation involving constant activation of macrophages can continue for several months and sometimes years, these NO-mediated pathways have been implicated in inflammation-related carcinogenesis (Ohshima and Bartsch 1994; Wink 1998).

1.2.3 Catalysts and Inhibitors of Endogenous Nitrosation

Catalysts and inhibitor effects are also likely to contribute to the inter-individual variations in NNOC. SCN⁻, a normal constituent of human saliva, is a powerful catalyst of nitrosation reactions (Boyland and Walker 1973). The average concentration of SCN⁻ in human saliva is about 1 mM and increases markedly in smokers to about 6 mM (Tenovuo 1986). Although swallowed saliva is diluted in the stomach, even these diluted concentrations of SCN⁻ can enhance nitrosation reactions up to 300-fold at pH 2 (Boyland and Walker 1973).

Inhibitors of endogenous nitrosation include vitamins C and E (reviewed in Mirvish 1996; Bartsch et al. 1988; Mackerness et al. 1989), green tea and its components, particularly polyphenolic catechins (Tanaka et al. 1998; Vermeer et al. 1999) and some sulphur containing compounds, such as thiols (Bartsch et al. 1988).
1.3 N-NITROSO COMPOUNDS IN HUMAN CANCER

Humans are exposed to NNOC via endogenous nitrosation of amino precursors in the body as previously described in sections 1.1 and 1.2. Humans can also be exposed to preformed NNOC. The highest exposure is in tobacco users, in particular from the tobacco-specific nitrosamines N-nitrosonornicotine (NNN), 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and dimethylnitrosamine (DMN) (Hoffman and Hecht 1985; Spiegelhalder and Bartsch 1996). Preserved meat and fish, already a source of nitrite and nitrate, is also a source of volatile nitrosamines, as is occupational exposure in certain industries such as the rubber, leather and metal industries (Forman et al. 1989).

1.3.1 The Possible Role of NNOC in the Etiology of Cancer

Concern has arisen over the exposure of humans to NNOC and the possible role of NNOC in human cancer, since the original discovery in 1956 that DMN is a powerful carcinogen in rodents (Magee and Barnes 1956). Since the discovery, this class of versatile carcinogens has been shown to induce tumours in 40 animal species in most organs of the body (Bartsch et al. 1988; Bartsch 1991). Both NNN and NNK, just two of more than 3800 compounds found in cigarette smoke, are potent and organ-specific carcinogens. The organospecificity of NNK for the lung supports the assumption that NNK plays an important role in tobacco carcinogenesis (Hoffman and Hecht 1985).

As a result, the interest in defining the etiological role of NNOC in many human cancers has persisted. Although definite proof that NNOC are carcinogenic to humans is still lacking, as a result of over 30 years of research in both experimental systems and humans, there is convincing evidence for concern over the exposure of humans to NNOC (Table 1.2, Bartsch 1991).

Precursors of endogenous nitrosation, whether derived from the diet or from other exposures, have been implicated in the etiology of certain human cancers, including gastric, esophageal, nasopharyngeal and bladder (Mirvish 1983; Bartsch and Ohshima 1988; Bartsch et al. 1990; Bartsch 1991; Tricker and Preussmann 1991; Mirvish 1995).
Many NNOC are multispecies and multiorgan carcinogens

Very similar cellular and molecular damage is produced in animal and human tissues.

There is widespread human exposure to exogenous preformed and endogenously formed NNOC.

Lifetime or chronic exposure to even low levels of certain carcinogenic NNOC may pose a significant cancer risk.

Endogenous NNOC synthesis can occur by multiple pathways and at different sites in the body.

Table 1.2 Evidence for concern over the exposure to humans of NNOC (Bartsch 1991).

Since the stomach is an environment in which all three pathways of endogenous nitrosation operate, it is not surprising that intragastric formation of NNOC from ingested precursors has been implicated in the etiology of human stomach cancer especially (Mirvish 1983). By monitoring N-nitrosoproline excreted in the urine (the NPRO test, Ohshima and Bartsch 1981) measurements of endogenous nitrosation were made possible in high- and low-risk areas for gastric cancer in Northern Japan. Endogenous nitrosation of proline was found to be greater in subjects living in the high-risk area, and nitrosation could be efficiently inhibited by the intake (100 mg doses) of vitamin C after each meal. In contrast, subjects living in low-risk areas showed no increased nitrosation after proline ingestion, and vitamin C had no inhibitory effect. These studies indicated that subjects in low-risk areas are protected against nitrosation by sufficient intake of inhibitory substances in the diet (Kamiyama et al. 1987).

The NPRO test has been applied to other human subjects in clinical and epidemiological studies and in most instances higher exposures to endogenous NNOC were found in high-risk subjects, but individual exposure was greatly affected by dietary modifiers or disease state (reviewed in Bartsch et al. 1990). These studies also supported Kamiyama’s findings (Kamiyama et al. 1987) that vitamin C efficiently lowered the body burden of intragastrically formed NNOC.

Specific foodstuffs containing nitrosatable precursors such as salted and preserved fish and meat and soy sauce have been implicated in the etiology of gastric cancer, especially in
Introduction

areas where these foodstuffs are a major dietary source (Shephard 1987; Wakabayashi et al. 1989; Shephard and Lutz 1989; Mirvish 1995).

In Colombia, where fava beans are a prominent component of the diet and intake of nitrate is high, there is a high incidence of gastric cancer in the population (Correa et al. 1975; Tannenbaum et al. 1979). It has been demonstrated that direct-acting mutagens (possibly gastric carcinogens) are formed after nitrosation of the fava bean (Yang et al. 1984; Montes et al. 1984). The precursor of the mutagen isolated was identified as 4-chloro-6-methoxyindole (Yang et al. 1984) and is discussed in further detail in section 1.4.2.1.

1.3.2 Interaction of NNOC with Biological Systems

1.3.2.1 Chemical Carcinogenesis

Chemical carcinogenesis is a multi-step process whereby a normal cell evolves into a cancer cell as a result of heritable changes in multiple, independent genes. The multi-step model is often separated into three stages, initiation, promotion and progression (Barret 1993), but it is clear that the process of carcinogenesis is extremely complex and current definitions are inadequate.

Initiation involves the induction of an irreversibly altered cell and is frequently equated with a mutational event. The proficiency of initiation is dependent on the rate of cell proliferation, triggered by the promoting agent, to transform a single, potential cancer cell into a multi-cellular tumour. Promotion is the experimentally defined process by which the initiated cell clonally expands into a visible tumour, often a benign lesion such as papilloma. A common feature of promotion is the formation of new blood vessels and the process is likely to involve epigenetic (non-genotoxic) factors, such as hormones, that selectively influence the proliferation of the initiated cell.

Cells within the benign lesion must undergo one or more additional irreversible changes, defined as progression, to result in a malignant neoplasm. Progression reflects multiple changes in growth regulatory mechanisms. These include altered sensitivity to adjacent cells, local growth factor production, changes in receptors that initiate signal transduction and alterations to the downstream transduction pathways (King 1996). The overall result of these events is autonomous cell growth, an ability to grow outside their normal environment and to metastasise to other parts of the body.
There have been major advances in the understanding of the target genes in carcinogenesis (Weinberg 1989; Fearon and Vogelstein 1990). The involvement of two classes of genes in the evolution of most, if not all cancers, has been identified; they are the proto-oncogenes and the tumour-suppressor genes. Proto-oncogenes are involved in cellular growth and differentiation and when activated by mutational mechanisms, result in positive proliferation for tumours. In contrast, tumour-suppressor genes block the neoplastic growth of cells. They are inactivated by mutational mechanisms and consequently, lost in tumour cells.

Identification of the genes involved in carcinogenesis and elucidation of the mechanisms of their activation or inactivation, enables a more thorough understanding of how chemical carcinogens influence the process that results in the progression of a cell toward the endpoint of malignancy. The findings of multiple genetic changes in activated proto-oncogenes and inactivated tumour-suppressor genes, lend support to initiation frequently being associated with a mutational event.

In addition to mutagenic mechanisms, chemicals may also heritably alter cells by non-genotoxic mechanisms, and overall mechanisms of initiation may vary in different tissues (Barrett 1993).

1.3.2.2 NNOC and the Formation of DNA Adducts

With the knowledge that NNOC produce tumours in 40 animal species, combined with their implicated role in the etiology of human gastric cancer, how do NNOC exert their carcinogenic effect and where in the multi-step model of carcinogenesis do NNOC fit?

NNOC are potent alkylating agents. Depending on the structure of the N-substituents, NNOC can decompose spontaneously, as is the case for the alkylnitrosoureas, to generate alkyl diazonium ions (Scheme 1.12 A), or do so after metabolic activation to α-hydroxy derivatives (Scheme 1.12 B). Alternatively, certain NNOC lose the NO group. This latter pathway has been less well studied and the significance for NNOC carcinogenesis is not as well established compared with alkylation reactions (see section 1.3.2.5).

The alkyl or aromatic diazonium ion generated by either decomposition pathway, is a reactive electrophile and can react at nucleophilic sites of various cellular constituents,
such as proteins and nucleic acids. In the latter case, DNA adducts are generated (e.g., reaction at nucleophilic sites in guanine residues, Scheme 1.12).

\[ \text{alkyl or aromatic diazonium ion} \]

\[ \text{O}^\text{6-alkyl, N}^\text{7-alkyl and C}^\text{8-aromatic guanine adducts} \]

**Scheme 1.12** Summary of \( \text{N} \)-nitrosation of amines and related compounds and decomposition pathways of the resulting \( \text{N} \)-nitroso compounds. The diazonium ion generated can readily react with DNA to afford DNA adducts.

The formation of DNA adducts has been studied widely as a probable mechanism by which NNOC exert biological effects. The misreplication or misrepair by a DNA polymerase, of genetic damage caused by carcinogen-DNA adducts, thus leading to mutation induction, is thought to be an early step in the initiation of cancer (Swenberg 1985; Hemminki 1993; Loechler 1996).
Studies by Singer (Singer 1976) of the reactions of ethynitrosourea with DNA have shown that most of the nucleophilic sites on DNA bases can be alkylated (Scheme 1.13).

\[\text{Scheme 1.13 Summary of the major sites on DNA bases susceptible to alkylation or interaction with aromatic diazonium ions.}\]

The alkylation reaction is usually described as involving either $S_N 1$ or $S_N 2$ nucleophilic substitution. The $S_N 2$ mechanism is a bimolecular substitution reaction, in which the attacking nucleophile displaces the leaving group via a transition intermediate (Swenson 1983). Alkylating agents reacting via the $S_N 2$ mechanism, such as the alkyl sulphates, usually prefer centres with high nucleophilic strength such as ring nitrogens.
Alkylation agents reacting via the S<sub>N</sub>1 mechanism, such as the alkyl nitrosoureas, react by unimolecular nucleophilic substitution mechanisms, and are usually less selective about nucleophilic strength, reacting with hydroxylic and exocyclic amino groups (Swenson 1983). However, it is known that most nucleophilic sites on the DNA bases can be targeted by such electrophiles (Singer 1976).

The N-7 position of guanine is frequently the site that is modified by nitrosamines most extensively since it is the most nucleophilic; the C-8 atoms and exocyclic amino groups are not effectively targeted by the simple alkylating nitrosamines, probably due to the inherent low nucleophilicity of these sites (Swenson 1983). In contrast, however, these sites appear to be more reactive toward agents with aromaticity in their structure. The C-8 atoms of the purine nucleosides, particularly of deoxyguanosine, are the major targets for the interaction with aromatic diazonium ions, (Scheme 1.13; Chin et al. 1981; Hung and Stock 1982). Electrophilic attack by the aromatic diazonium ion at the nitrogen atom of exocyclic amino groups can result in the formation of triazene products (Williams 1988).

In addition, the dediazoniation reaction of both primary aliphatic and aromatic diazonium ions, whereby the N<sub>2</sub><sup>+</sup> is replaced by a nucleophile, occurs readily for a range of nucleophiles including water, resulting in finally products of deamination (Williams 1988). This pathway represents a detoxifying pathway for such nitrosamines, effectively quenching the reactive intermediate and rendering the ion no longer capable of covalent interaction with DNA.

It is also important to note that ring nitrogen-substituted products are all unstable in certain environments and biological effects can be influenced by chemical transformations secondary to the initial DNA adduction. Examples include the facile depurination of N-7 substituted deoxyguanosine residues and the N-3 and N-7 substituted deoxyadenosines (Dipple 1995), leading to the formation of abasic sites, and hydrolysis of unstable triazene adducts which can lead to deamination of the initial exocyclic amino group (Brown et al. 1992).

The relative proportions of DNA adduction at nitrogen and oxygen atoms of both purine and pyrimidine bases depend on the chemical structure of the nitrosamine and the specificity of the P<sub>454</sub> activation in different tissues. Alkyl nitrosoureas are direct-acting, and are the exception in the fact that they do not require metabolic activation and can alkylate all tissues with the same efficiency (Lijinsky 1992).
Various tissues and cells from different species, including humans, have demonstrated the capability of metabolic transformation, especially of simple symmetrical dialkynitrosamines (Bartsch and Montesano 1984), but not all tissues are able to metabolise cyclic and asymmetrical dialkynitrosamines (Tricker and Preussman 1991). Liver usually shows a higher capacity than other tissues for metabolising nitrosamines but there are exceptions, including N-nitrosobenzyamine, a carcinogen specific for rat oesophagus, which is metabolised extensively by oesophagus mucosa in the rat (Hodgson et al. 1982; Labuc and Archer 1982). Interestingly, N-nitrosobenzyamine is metabolised hardly at all by human oesophagus cells in culture. In addition to species variation in metabolic capability of a given tissue, inter-individual variation exists, up to 150-fold, with human tissue specimens (Harris et al. 1982).

These findings indicate that, in the multi-stage process of carcinogenesis, tissue-specific metabolic activation is a necessary although not exclusive requirement for tumour induction.

1.3.2.3 Are all Alkylation Products Important for Mutation Induction?

It is unlikely that DNA adducts formed at all sites on DNA contribute to mutation induction. The problem in evaluating the spectrum of products afforded by alkylation, is deciding which of the alkylated products (or sites in the DNA macromolecule) are biologically important in terms of persistence in the absence of repair pathways.

The work of Loveless, and Swann and Magee, was instrumental in addressing which alkylation sites were important for mutation induction (Loveless 1969; Swann and Magee 1968). A distinction was drawn between those sites on DNA that were merely susceptible to alkylation (such as guanine N-7) and those that were biologically more important (such as guanine O') in the initiation of the carcinogenic process by nitrosamines in a specific tissue or cell.

Alkylation at the N-7 atom of guanine, the most readily alkylated position, shows little correlation with carcinogenic activity and is believed to play a minor role in the initiation of cancer by nitrosamines (Swann and Magee 1971).

The formation of O-alkythymine, in addition to O-alkylguanine, is considered to be a biologically important lesion in the initiation of tumours (Swenberg et al. 1984).
formation and persistence of O'-alkylguanine is generally believed to be the more critical modification (Pegg 1984) that results in miscoding during replication.

It has generally been assumed that O'-alkylguanine and O3'-alkylthymine miscode as a consequence of their stable mispairs with thymine and guanine respectively (Scheme 1.14).

![Scheme 1.14](image)

Scheme 1.14 O'-Methylguanine and O3'-methylthymine are proposed to form stable mispairs with thymine and guanine respectively.

Recent structural studies have shown that actually DNA polymerase might mistake O6'-alkylguanine for adenine and O3'-alkylthymine for cytosine, because of the physical similarity of these bases (Swann 1990). In addition, the mispairs may retain the Watson-Crick alignment with less distortion than if the natural base were inserted opposite (Swann 1990). Recent kinetic measurements have shown that the essential aspect of base selection in DNA synthesis is the ease of formation of the phosphodiester links both 3'- and 5'- to the incoming base. The Watson-Crick alignment of the alkylG:T and alkylT:G mispairs may facilitate formation of these phosphodiester links and thus it has been suggested that it is this alignment, rather than the strength of the base pairs, which is important in the ability of these lesions to miscode (Swann 1990). These observations are interesting in the fact that if this suggestion is true for these alkylated bases it is no doubt true for other adducted bases and normal bases, which will have an impact on mutational frequencies induced by chemical modification.

The significance of O6'-alkylguanine has been demonstrated in carcinogenesis studies involving the induction of mammary tumours in rats with methylnitrosourea (MNU). The
Introduction

mismatching O\textsuperscript{6}-methylguanine with thymine during DNA replication results exclusively in a single point mutation, a G:C to A:T transition (Loechler et al. 1984). It has been demonstrated that Ha-ras oncogenes present in mammary tumours induced by MNU in rats became activated by G:C to A:T transitions (Zarbl et al. 1985). The G:C to A:T transition mutation involved the second guanine of codon 12 of the Ha-ras oncogene (Zarbl et al. 1985). In human colon carcinomas and adenomas, G:C to A:T transition mutations in the K-ras gene at the 12\textsuperscript{th} codon, are often found (Bos et al. 1987). In fact, specific alterations in members of the ras gene family, either by point mutations or in amplification of the wild-type gene, can result in activation, and such activated oncogenes have been found in a significant proportion of all tumours (Bos 1988). These observations are consistent with the involvement of activated oncogenes in the initiation of carcinogenesis.

The alkylation of the O\textsuperscript{4} atom of thymine has been shown to mispair with guanine, resulting in a single point mutation, a T:A to C:G transition (Preston et al. 1986). However, whether the persistence of O-alkylpyrimidines in DNA contributes to carcinogenesis as a consequence of their mispairing properties during replication, is unclear.

Further strong evidence linking adducts, mutations and cancer, comes from the p53 gene, a tumour-suppressor gene. This gene contains mutations in about 50% of human tumours (Pfeifer and Denissenko 1998) and specific mutations appear to be present in hepatocellular carcinomas of populations assumed to ingest large quantities of aflatoxin B\textsubscript{1} (Hsu et al. 1991). Many bulky adducts, such as aflatoxin B\textsubscript{1}, form N-7-deoxyguanosine adducts and cause predominantly G:C to T:A transversions (Hsu et al. 1991; McConnell and Garner 1994).

1.3.2.4 Influence of DNA Repair and Sequence Specificity

The organotrophy of carcinogenesis by NNOC may be partially explained by the different metabolic capability in different tissues. However, the significance of certain DNA adducts in mutagenesis is also dependent on the different repair capabilities in different organs or different cell types within the same organ.

A significant proportion of alkylated DNA is repaired by 2 main processes: one is enzymic depurination or depyrimidation by a DNA glycosylase, and the other is O\textsuperscript{6}-
alkyltransferase, which transfers the alkyl group to a cysteine group in a receptor protein leaving an intact base. In general, DNA polymerases copy past small alterations such as methyl or ethyl groups with high efficiency and therefore are likely to produce mutations depending on the miscoding potential of the altered base. In contrast, bulky adducts generated from aromatic NNOC are not easily bypassed by normal cellular polymerases and are repaired by the nucleotide excision pathway. This pathway removes damaged DNA bases by introducing nicks 5' and 3' to the damage. After the oligonucleotide including the damaged base is removed, DNA polymerase fills in the gap and DNA ligase joins the ends. Errors can occur to afford mutagenesis, frequently involving error-prone DNA synthesis.

Repair of lesions at the O\textsuperscript{6} position occurs via the action of alkyltransferase. The alkyl group is removed intact by a protein called O\textsuperscript{6}-alkylguanine-DNA alkyltransferase (AGT) (Pegg and Dolan 1987) to leave guanine base. The amount of AGT present is dependent on the cell type and species. The protein is inactivated by the repair process, and consequently, the number of lesions repaired is dependent on the amount of protein present. There is a strong correlation between AGT level and resistance to the mutagenic effect of alkyl nitrosoureas and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Domoradzki et al. 1984; Dolan et al. 1986).

O\textsuperscript{3}-methylthymine is repaired by the same pathway in DNA in E.coli, although it has been suggested that several methyltransferases may exist (McCarthy et al. 1984).

DNA glycosylases display a broad substrate range and initiate the first event in base-excision repair by cleaving the N-glycosidic bond between the damaged base and the sugar, to result in the formation of an abasic site (Loeb and Preston 1986). AP (apurinic/apyrimidinic) endonucleases generate a strand break at the resulting abasic site. The nick is expanded into a gap, by excision of the AP site as a 3'- or 5'-deoxyribose phosphate by a cellular exonuclease. The gap is filled in by a DNA polymerase and the ends are rejoined by DNA ligase (Leob and Preston 1986). Class I AP endonucleases cleave 3' to the AP site, and Class II AP endonucleases cleave 5' to the AP site. Alkylated DNA products including 7-methylguanine, 3-methylguanine and 3-methyladenine are repaired by this pathway (Wyatt and Samson 2000).

It is clear that from the significance and persistence of certain DNA alkylation products, resulting in mutation induction, repair is insufficient and the miscoding properties of these
altered bases predominates. Although human cells generally have a higher capacity to repair O⁶-methylguanine than animal cells (especially in the liver which contains higher levels of AGT than extrahepatic tissues), human cells deficient in O⁶-methylguanine-DNA transferase activity in culture have been found (Yarosh et al. 1984; Scudiero et al. 1984).

Mutations induced by alkylating agents, especially at guanine residues, appear to be significantly influenced by the molecular environment surrounding the guanine. As previously mentioned, most mammary tumours induced by MNU have a G:C to A:T transition mutation involving the second but not the first guanine of codon 12 of the H-ras oncogene (Zarbl et al. 1985). In the E.coli lacI gene, it has been demonstrated that guanines preceded 5' by a purine base are 10 times more likely to be mutated than those preceded by a pyrimidine residue (Burns et al. 1987). These observations of non-random distribution of mutations, may reflect not only differences in the level of alkylation, but also differences in the rate of repair.

Studies have shown a slower rate of repair by AGT for sites in which a modified guanine such as O⁶-methylguanine and O⁴-ethylthymine, is preceded by a purine rather than a pyrimidine residue (Dolan et al. 1988; Burns et al. 1988). More specifically, it has been demonstrated that the rate of repair of O⁶-methylguanine in various positions in chemically synthesised DNA duplex having the H-ras sequence, varied 25-fold, depending on the sequence flanking the methylguanine; O⁶-methylguanine in position two of codon 12, was the least well repaired (Georgiadis et al. 1991a). Further studies indicated that the rate of repair is a reflection of the confirmation of the sequence containing the alkylated base (Georgiadis et al. 1991a). Furthermore, it has been shown that O⁴-alkylthymine causes a much greater effect on the flexibility of DNA than O⁶-methylguanine (Georgiadis et al. 1991b). These confirmational differences are likely to affect the accessibility of the lesions to alkyltransferase and consequently, the rate of repair. Sequence-dependent glycosylase repair has been reported (Hang et al. 1998; Wyatt and Samson 2000).

Despite the existence of repair pathways for damage induced by NNOC, these observations have at least, in part, explained the persistence and significance of certain lesions. What is clear is that the induction of tumours in a given organ is critically determined by a mutagenic event produced by an unrepaired DNA lesion during replication.
1.3.2.5 Denitrosation of NNOC and Possible Relevance for Carcinogenesis

The carcinogenic potential raised by endogenous nitrosation not only depends upon the extent of nitrosamine formation, but also upon the rates of relevant metabolic pathways. In addition to the metabolic activation of NNOC generally required for carcinogenic activity, nitrosamine denitrosation by rat liver microsomes has been demonstrated for various substrates and is thought to represent an important detoxification pathway for nitrosamines (Lorr et al. 1982; Appel et al. 1986).

There are a number of nitrosamines that are mutagenic, but not carcinogenic, and in these cases, the loss of the NO group is generally equated with detoxification. Nitrosocimetidine is such an example and undergoes rapid denitrosation in vivo. Nitrosocimetidine and denitrosation is discussed in detail in section 1.3.4.1.

Other NNOC demonstrate a particular tendency to lose the nitroso group. MNNG and related N-nitrosoguanidines, are potent and local-acting carcinogens, related to nitrosocimetidine in structure, and their major metabolic fate is denitrosation to generate the relatively innocuous parent guanidinium compound both in vitro and in vivo (Jensen et al. 1990a; Jensen et al. 1997). This efficient detoxification of MNNG and related compounds is likely to explain why their carcinogenicity is generally limited to regions local to the site of application. For both N-nitrosoguanidines and nitrosocimetidine, the fate and relevance to carcinogenesis of the NO moiety released, remains to be determined.

DMN, the potent hepatocarcinogen, is converted by α-hydroxylation to a methylation agent capable of reacting with DNA, however an additional metabolic fate of DMN is denitrosation (about 15-30% of DMN metabolised) to generate methyamine and nitrite in vitro and in vivo (Keefer et al. 1987; Streeter et al. 1990). These two pathways, the former a bioactivation pathway and the latter a presumed detoxification pathway, have been demonstrated to be separable and competitive (Heur et al. 1989). Consequently, it has been proposed that it may be possible to limit the damage from this carcinogen, by selectively inhibiting its activation and forcing metabolism along the denitrosation pathway (Heur et al. 1989). Further studies were carried out to evaluate the roles of metabolic denitrosation and α-hydroxylation in the hepatotoxicity of DMN, and it was suggested by the authors that denitrosation was an effective detoxification pathway; formation of the methanediazonium ion via α-hydroxylation was responsible for the toxicity observed (Lee et al. 1996).
It is clear that a number of NNOC undergo metabolic denitrosation and it is generally perceived that this pathway represents one of detoxification. However, the involvement and significance of the release of the NO group in possible DNA-damaging events has not been studied as widely as for alkylation.

1.3.3 Approaches for the Detection of Genotoxic Damage

1.3.3.1 Bacterial and Mammalian Assays

Molecular mutagenesis assays combined with assays to detect direct genotoxic damage, such as DNA adducts or strand breaks, or damage as a consequence of repair for example, contribute to the evaluation of the mutagenic and carcinogenic potential of DNA-damaging agents, including NNOC.

The study of molecular mutagenesis requires the development of sensitive assays in which usually selective conditions are established whereby the parent phenotype cannot grow, but the mutant can. The Ames assay, or the Salmonella Mutagenicity test, was instrumental in detecting mutagens and evaluating carcinogenic potential as a consequence of exposure to chemicals (Ames et al. 1975). Test data on more than 5000 chemicals has now been published using the sensitive and simple bacterial test, which is used extensively in genetic toxicology to screen chemicals and drugs. The tester strains that are used traditionally, such as TA1535, TA100 and TA98, are deficient in DNA repair and identify mutagens that induce reversion of specific base-pair substitution and small frameshift mutations in the his operon (Maron and Ames 1983). Since the majority of mutagens require metabolism to form the ultimate genotoxic species, a drug metabolising system (S9) derived from the liver homogenates of rats treated with an inducer of cytochrome P450, such as aroclor 1254, can be added to the assay. In order to be sensitive to a broad range of chemicals, the his strains chosen for routine use carry target sites that revert by numerous pathways of mutagenesis. The specific base mutation cannot be discerned without molecular analysis of the reverted cells. More recently, a set of six his mutant strains (TA7001 to TA7006) have been developed to revert by unique base-pair substitutions (Gee et al. 1994; the Ames II assay). These strains are described in more detail in chapter 6.
In addition to the Ames test, several *in vitro* mammalian cell mutation assays have been developed which are generally less sensitive than bacterial assays. These include the Chinese hamster ovary assay, the mouse lymphoma L5178Y assay and the V79 fibroblast assay, all of which identify chemicals as mutagens (Hsie *et al.* 1981; Ames 1984; Combes *et al.* 1995).

The development of transgenic mouse models such as BigBlue™ (Kohler *et al.* 1990; Kohler *et al.* 1991) and Muta™Mouse (Gossen *et al.* 1989) have enabled mutagenicity studies in whole animals to be carried out. The inductions and spectra of *lacZ* gene mutations (Muta™Mouse) and *lai* gene mutations (BigBlue™) can be evaluated in virtually any tissue. These assays have potentially useful applications in evaluating the carcinogenic potential of NNOC in the target organ, such as the stomach, thus enabling evaluations to be made on the potential of NNOC to act as gastric carcinogens.

Unscheduled DNA synthesis (UDS) involves the measurement of DNA repair as an indication of DNA damage. Rat liver primary cell cultures are often used for exposure to the genotoxic compound and the assay relies on the incorporation of radiolabelled thymidine into DNA (quantified by the number of grains on an autoradiography film) which results as a consequence of enzymatic repair following UDS (Williams 1977).

The Comet assay or single cell gel electrophoresis assay, is a rapid, simple, visual and sensitive technique for measuring DNA damage in the form of DNA strand breakage, in single cells (Östling and Johanson 1984). The Comet assay is described in more detail in chapter 6.

These assays previously discussed are just a number of tools available for the detection of genotoxic damage by chemicals. Overall, by evaluating the mutagenicity and DNA-damaging potential of agents in a variety of assays enables a more thorough understanding of how the chemical is acting, and enables structure-activity relationships and mutagenic and carcinogenic potential evaluations to be made. Because most NNOC are mutagenic to bacteria and are mutagenic in other test systems (Lijinsky 1992), the significant interaction of NNOC or their metabolites in cells is believed to be via formation of DNA adducts in the nucleus, predominantly by alkylation as demonstrated in section 1.3.2.2. Consequently, the sensitive detection of DNA adducts, which are often used as biomarkers, contribute to the evaluation of carcinogenic risk to humans of certain NNOC.
1.3.3.2 Biomarkers as Indicators of Exposure

Biomarkers are used to describe early measurable changes that have occurred in living organisms that could later lead to clinical disease, such as cancer, following exposure to carcinogens. These changes can include DNA adducts, protein adducts such as haemoglobin or albumin adducts, or secondary biological effects such as mutations. Consequently, biomarkers have an important role to play in cancer risk assessment (Perera 1988; Farmer 1994; Hemminki 1995; La and Swenberg 1996; Perera 1996; Perera 1988).

An analysis of biomarkers is increasingly being incorporated into epidemiological studies to gain greater knowledge of the risk factors and mechanisms responsible for the induction of cancer (Perera and Whyatt 1994; Farmer et al. 1996). This molecular epidemiological approach has the advantage of being directly relevant to human risk, unlike animal or other experimental models that require extrapolation to humans. Exposure assessment can be carried out at different stages in the process that can lead to the development of tumours (Fig 1.1).

Biomonitoring involves assessment of the internal dose of a toxic compound by measurement of the extent of chemical interaction of the compound with biological macromolecules; the biologically effective dose (Farmer 1994). Such measurements reflect individual differences in absorption, metabolism, distribution and excretion (individual susceptibility).

The development of analytical methods for the detection of DNA adducts, such as $^{32}$P-postlabelling have enabled sensitive detection of these biomarkers (1 adduct per $10^{10}$ nucleotides) and are applicable to structurally diverse classes of chemicals. The $^{32}$P-postlabelling assay was developed by Randerath et al. and enabled the determination of DNA adducts in experimental and human samples (Randerath et al. 1981; Gupta et al. 1982). Additional analytical approaches, with differing associated limits of detection, to detect DNA damage in biomonitoring studies include immunoassays, mass spectrometry, electrochemical detection and fluorescence (Farmer 1995; Hemminki 1995).

Knowledge that a particular NNOC produces a particular level of alkylation of DNA, in the absence of information that relates such alkylation of DNA to induction of tumours, is non-conclusive (Lijinsky 1992). However, incorporation of such data into additional studies, enables estimations of carcinogenic risk to humans to be made.
**Introduction**

**Figure 1.1** Biomonitoring exposure to genotoxic carcinogens.

### 1.3.4 The Significance of N-Nitrosation of Drugs

Many drugs contain primary, secondary or tertiary nitrogen atoms that are potentially nitrosatable by endogenous pathways, to afford the production of NNOC. As the outstanding biological activity of NNOC is carcinogenicity, evaluation of the propensity for nitrosation of drugs is extremely important in evaluating risk to humans.

The nitrosation assay procedure (the NAP test) was developed in the investigation of the potential hazards of nitrosatable drugs (Coulston and Dunne 1980). The NAP test was recommended as a means of ranking drugs in relation to their sensitivities to nitrosation under simulated, standardised conditions (40 mM nitrite at pH 3.0).
Many drugs have been subjected to the NAP test and were converted to $N$-nitroso derivatives with varying propensity (Gillatt et al. 1984). The highest yields were generally associated with structures containing secondary rather than tertiary amino groups. This is to be expected because as described in section 1.2.1.1, the nitrosation rate for tertiary amines is much lower compared with secondary amines and therefore such amines are considered of little relevance with regard to endogenous nitrosation. The variation in susceptibility to nitrosation was exemplified by the approximately 600-fold difference between piperazine with two secondary amino groups in the molecule, and cyclizine, in which each secondary amino group is replaced by a tertiary amino group (Scheme 1.15).

![Piperazine and Cyclizine](Diagram)

Scheme 1.15 Structures of piperazine and cyclizine.

However, there were two notable exceptions; aminopyrine and minocycline. Specific structural features can strongly increase the nitrosation rate of tertiary amines and both these drugs produced large amounts of DMN. The nitrosation of aminopyrine to afford DMN is shown is Scheme 1.16.

Subsequent studies with aminopyrine, an analgesic, have shown that by monitoring the urine in humans, in vivo formation of DMN occurs after intake of the nitrosatable drug (Spiegelhalder 1990). Under normal conditions, DMN formation in vivo could not be directly monitored in urine due to high metabolic conversion. However, an increased excretion rate was observed if ethanol was administered simultaneously. The amount excreted in urine appeared to be influenced by salivary nitrate concentrations. These findings are consistent with previous observations that ethanol alters the pharmacokinetics
of nitrosamine metabolism in the liver, and more of the carcinogen can reach extrahepatic tissues, such as the kidneys (Swann et al. 1987). As was demonstrated by Swann and co-workers, increased alkylation rates of rat kidney DNA are found following administration of DMN with ethanol (Swann et al. 1987).

\[
\begin{align*}
\text{Scheme 1.16 Nitrosation of aminopyrine yields DMN.}
\end{align*}
\]

Piperazine (Scheme 1.15) has been used for the treatment of worms in humans and animals. Nitrosation of piperazine gives rise to \(N\)-mononitrosopiperazine and \(N,N'\)-dinitrosopiperazine. The carcinogenic effects of the dinitroso derivative have been demonstrated in rats (Lijinsky and Kovatch 1993). The mononitroso derivative has been shown to form \textit{in vivo} in humans, after oral intake of piperazine and after inhalation exposure, and formation was increased by dietary intake of nitrate and decreased by intake of ascorbate (Bellander 1990).

As was demonstrated by Gillatt and colleagues, many drugs are capable of forming NNOC, but under standardised conditions their susceptibility to nitrosation varied (Gillatt et al. 1984). Since endogenous nitrosation is most likely to occur in the stomach, as it is within this environment that all pathways of endogenous nitrosation can occur, a selection of drugs were studied under simulated gastric conditions. The conditions simulated those within the normal fasting stomach at pH 2, where a nitrite concentration of 25 \(\mu\text{M}\) was continually replenished (Gillatt et al. 1985).

The susceptibility of the drugs to \(N\)-nitrosation varied widely but this variability was less than that described previously under standard conditions. Of the 46 drugs tested, 38 gave evidence of \(N\)-nitrosation, with the highest yield obtained with the benzathine salt of
penicillin G (Gillatt et al. 1985). The degree of N-nitrosation was consistently reduced when the initial nitrite concentration was not replenished during incubations and inhibition of nitrosation occurred for all drugs in the presence of ascorbic acid (Gillatt et al. 1985).

Studies such as these, combined with in vitro mutagenicity and DNA damage studies, play an important role in evaluating the susceptibility and significance of N-nitrosation of drugs. For the pharmaceutical industry, such procedures should enable structures particularly susceptible to N-nitrosation to be elucidated, so as to allow selection of new chemical entities with the least potential hazard. However, as studies with cimetidine have shown, in vitro procedures alone, often do not tell the whole story.

1.3.4.1 The Cimetidine Story

Cimetidine (Tagamet) is a drug used for the clinical treatment of gastrointestinal disorders (Brimblecombe et al. 1978). In 1979 Elder and his colleagues reported the occurrence of gastric cancer in a small number of patients who had been treated with cimetidine and suggested a causal role for cimetidine (Elder et al. 1979). They also drew attention to the similarity of the nitrosated derivative, nitrosocimetidine, with that of MNNG, one of the most potent carcinogens for induction of stomach cancer in animals. Indeed, MNU also shows structural similarities (Scheme 1.17).

It was subsequently demonstrated that nitrosocimetidine methylates DNA in vitro to generate the 7-methylguanine, O\(^\text{6}\)-methylguanine and 3-methyladenine lesions in the same relative proportions as MNNG and MNU (Jensen and Magee 1981). Similarly, alkylation products were seen with the reaction of these compounds with the DNA of cells in culture (Jensen 1981). Nitrosocimetidine also produced responses in cultured cells which reflected DNA damage such as sister chromatid exchanges and chromosomal aberrations, DNA strand breaks, DNA repair, mutation and cell transformation, with an effectiveness comparable to that of MNNG (Henderson et al. 1981; reviewed in Jensen and Magee 1990). These findings suggested that all three compounds shared a common reactive intermediate and that from the in vivo biological activity, nitrosocimetidine would be expected to be a potent chemical carcinogen.
However, nitrosocimetidine, in contrast to its structural relatives, does not produce pathological changes when administered to the rat at high doses (Lijinsky and Reuber 1984; reviewed in Jensen and Magee 1990b). Relatively low levels of rat organ DNA methylation are generated and nitrosocimetidine has proven to be a very weak or non-carcinogen in rats and mice treated orally (Lijinsky and Reuber 1984; reviewed in Jensen and Magee 1990b). In fact, it has been demonstrated that nitrosocimetidine undergoes rapid metabolic denitrosation and hence is inactivated as a methylating agent (Jensen 1983). The compound is effectively detoxified in vivo. The denitrosation of nitrosocimetidine has been shown to be strongly pH dependent and the rate of denitrosation is accelerated greatly under acidic conditions (Frank et al. 1988). The mechanisms of this denitrosation include interaction with haemoglobin (Jensen et al. 1987) and the enzymatic-mediated activity of cytosolic glutathione transferases (Jensen and Stelman 1987).

It is clear from the cimetidine story, that whole animal and pharmacokinetic studies on compounds which register positive in in vitro tests, but negative in long-term carcinogenicity studies, are extremely important.
Introduction

1.4 $N$-NITROSOINDOLES: EVIDENCE FOR CONCERN TO THEIR EXPOSURE

The endogenous formation of $N$-nitrosoindoles is of concern since humans are exposed to a variety of naturally occurring and synthetic indolic compounds, and $N$-nitrosation frequently transforms innocuous nitrogen-containing compounds into toxic compounds. What evidence is there for concern to exposure to the indole class of compounds, as a consequence of nitrosation via the endogenous nitrosation pathway?

1.4.1 Observed Mutagenicity

Among the many hundreds of nitrogenous compounds studied, a number of 3-substituted indoles have been found to produce mutagenic products upon treatment with nitrous acid (Wakabayashi et al. 1989).

IAN is a plant growth hormone present in various vegetables, notably Chinese cabbage, a common foodstuff in Japan, and is released upon enzymatic degradation as described in section 1.1.2. The mono-$N$-nitroso derivative, 1-nitrosoindole-3-acetonitrile (NIAN), is a direct-acting mutagen toward Salmonella typhimurium TA98 and TA100 and Chinese hamster lung cells (Wakabayashi et al. 1985a; Wakabayashi et al. 1985b; Wakabayashi et al. 1987). Two other indole compounds 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde have also been isolated from Chinese cabbage as nitrosatable mutagen precursors (Wakabayashi et al. 1986; Wakabayashi et al. 1987) (Scheme 1.18).

Further indole compounds derived from indole glucosinolates, including I3C, indole-3-acetamide (IAM) and I3AA have been identified to be active toward Salmonella strains, without S9 mix, after nitrite treatment (Sasagawa and Matsushima 1991).

In addition, there are around 20 naturally occurring indole compounds, mostly 3-substituted, including tryptophan and its metabolites tryptamine and 5-hydroxytryptamine, that have been demonstrated to be direct-acting mutagens toward Salmonella strains (Ohta et al. 1981; Gatehouse and Wedd 1983; Valin et al. 1985; Ochiai et al. 1986).
Introduction

![Chemical structures of the three mutagen precursors isolated from Chinese cabbage.](image)

Scheme 1.18 Structures of the three mutagen precursors isolated from Chinese cabbage.

1.4.2 Nitrosated Indoles as a Risk Factor for Gastric Cancer

The relevance of IAN and the mono-N-nitroso derivative NIAN, has been further demonstrated in \(^{32}\)P-postlabelling studies. Using this assay, Yamashita and co-workers, have demonstrated the formation of DNA adducts by NIAN in vitro with calf thymus DNA, and in the stomach of rats after intragastric administration of NIAN (Yamashita et al. 1988). The level of DNA adducts in both the forestomach and glandular stomach two hours after administration of 100 mg/kg body weight of the compound was one adduct per \(10^7\) nucleotides. The products were not characterised. Marked inductions (up to a 100-fold increase) in ornithine decarboxylase activity and increases (up to 10-fold) in DNA synthesis in rat stomach mucosa have been reported, after administration of NIAN at doses of 40 to 300 mg/kg body weight by gastric intubation (Furihata et al. 1987). Further in vivo studies demonstrated NIAN as inducing single-strand scission in the pyloric mucosa of rat stomach (Furihata et al. 1996).

These findings have suggested that NIAN has potential carcinogenic activity in the glandular stomach.
Stability studies on the nitrosated products of IAN, I3C and indole have demonstrated that these compounds, which are nitrosated rapidly, are more stable at pH 8 than at pH 2 and that the existence of an equilibrium between the nitrosated indole compound and free indole compound plus nitrite, was responsible for this stability (Tiedink et al. 1989). The existence of an equilibrium has also been shown between tryptophan and its nitrosated form (Mellet et al. 1986). These results suggest that the compounds, if nitrosated in the stomach, would be stable in the presence of a continual supply of the nitrite anion.

It has been suggested that from the mutation spectrum induced by N-acetyl-N'-nitrosotryptophan and its methyl ester, these compounds react with DNA to form excisable DNA adducts which cause mutation by error-prone repair (Venitt et al. 1980).

1.4.2.1 The Fava Bean Mutagen

Studies have been carried out on a Columbian population at high risk for gastric cancer, where nitrate intake is high, using urinary N-nitrosoproline levels, and urinary 3-methyladenine and 7-methylguanine, as biomarkers for gastric nitrosation and DNA methylation (Wishnok et al. 1993). Nitrosoproline excretion was highly correlated with nitrate excretion in the subpopulation with advanced gastric cancer, but not in control subpopulations. Neither urinary 7-methylguanine nor 3-methyladenine was strongly related to gastric pathology or to urinary nitrate or nitrosoproline levels.

Fava beans are a prominent component of the diet in Columbia and studies identified a nitrosatable precursor present in fava beans as 4-chloro-6-methoxyindole (CMI) (Yang et al. 1984). The nitrosation of CMI affords the major isomeric product 4-chloro-6-methoxy-2-hydroxy-N'-nitrosoindolin-3-one oxime (NCMI) (Scheme 1.19).

This was the first report of a stable α-hydroxy N-nitrosocomound. Mutation assay results indicated that NCMI is among the most potent mutagens known (four times more potent than MNNG) (Yang et al. 1984; Tiedink et al. 1991).

An analogous, equally active mutagen, 4-chloro-2-hydroxy-N'-nitrosoindolin-3-one oxime (NCI) is produced upon nitrosation of the synthetic, structurally similar, 4-chloroindole (CI) ( Büchi et al. 1986; Tiedink et al. 1991).
1.4.3 Proposed Mechanism of DNA Damage

Despite consistent evidence that N-nitrosoindoles are mutagenic in a range of test systems and some evidence that they are DNA-damaging agents, little attention has been paid to their mechanism of action. Most of the knowledge to date, has been gained through study of the fava bean mutagen NCMI and simpler analogues such as NCI, both of which are 4-substituted N-nitrosoindoles.

The direct-acting mutagenicity of N-nitrosoindoles, suggests that spontaneous decomposition of these compounds gives rise to DNA-reactive intermediates, possibly involving diazonium ion formation.

Although NCMI and NCI are relatively stable for α-hydroxy-N-nitrosamines, hydrolysis studies have indicated that they may decompose via a benzene diazonium ion intermediate (Brown et al. 1992). It has been proposed that it is this reactive intermediate which reacts with nucleophilic sites on cellular macromolecules to form unstable triazene adducts or subsequent deamination products (Brown et al. 1992). The formation of 6-hydrazinopurine has been demonstrated after reaction with NCI and subsequent reduction (Brown et al. 1992).

The reaction of this intermediate with the amino group of adenine is shown in Scheme 1.20.
Scheme 1.20 Proposed reaction mechanism of the fava bean mutagen NCM1 and simpler analogues such as NCI, with DNA.

An unstable triazene adduct has been identified in vitro for the oesophageal carcinogen N-nitroso-N-methylaniline which is metabolised to form a benzenediazonium ion (Koepke et al. 1990). The reactions of 4-chlorobenzenediazonium ion with model peptides gave rise to adducts of the 4-chlorobenzenediazo group (Tracey and Shuker 1997).

Aromatic diazonium ions can react at the C-8 position of purines, which has been demonstrated in vitro for 4-(hydroxymethyl)benzenediazonium ion, the gastric carcinogen derived from the mushroom Agaricus bisporus (Hiramoto et al. 1995).

Spontaneous decomposition of nitrosated indoles has been proposed as the likely pathway to generate reactive diazonium ion intermediates capable of covalent interaction with DNA (Tracey and Shuker 1997). However, attempts to unequivocally identify
characteristic DNA adducts of the fava bean mutagen NCMI, were unsuccessful (Brown et al. 1992).

The structural features of the 3-substituted N-nitrosoindoles are different to the 4,6-disubstituted fava bean mutagen, but hydrolytic attack at the C-2 position would afford the α-hydroxy function necessary for spontaneous decomposition to the benzene diazonium ion intermediate, which is capable of inducing DNA damage (Scheme 1.21).

The existence of this pathway may explain the observations of DNA adduct formation \textit{in vitro} and \textit{in vivo}, induced by the potent mutagen NIAN (Yamashita et al. 1988). The DNA adducts observed by Yamashita and co-workers were not characterised (Yamashita et al. 1988), and to date, there have been no reports of unequivocally identified characteristic DNA adducts of N-nitrosoindoles.

\begin{center}
\includegraphics[width=0.8\textwidth]{scheme1.21.png}
\end{center}

\textbf{Scheme 1.21} Proposed spontaneous decomposition of 3-substituted N-nitrosoindoles to afford benzenediazonium ion intermediate capable of covalent interactions with DNA.

If the proposed mechanism of action whereby spontaneous decomposition to afford reactive diazonium ion intermediate is an operating pathway for N-nitrosoindole-induced DNA damage, discrete covalent adducts would structurally depend on the nitrosated indole. Generic modification may also be induced whereby the diazonium ion generated reacts with amino functions on the DNA bases to afford deamination products. Alternatively, other DNA damage products may be characterised, which can be rationalised and supported by alternative reaction pathways, identifying a different operating chemistry to the one initially proposed.
1.5 AIMS AND SCOPE OF THIS THESIS

The overall aims of this thesis are to detect, characterise and evaluate the relevance of DNA damage induced by various model 3-substituted N-nitrosoindoles in a variety of different systems \textit{in vitro} and \textit{in vivo}, and be able to rationalise the biological activity by elucidation of possible mechanisms involved.

The aims are summarised below:

- Synthesis and characterisation of a series of model 3-substituted N-nitrosoindoles
- Detection and characterisation of damage induced by model N-nitrosoindoles at the level of isolated nucleotides
- Detection and characterisation of damage induced by NIAN at the macromolecular level \textit{in vitro}, in oligonucleotides and calf thymus DNA
- Identify DNA damaging pathways
- Identify the mechanism whereby N-nitrosoindoles induce DNA damage
- Examine the ability of NIAN to induce damage \textit{in vivo}, in mouse stomach DNA
- Examine the response of NIAN in the Ames II assay for identification as a mutagen
- Examine the response of NIAN as a DNA-damaging agent in the Comet assay

The results of the studies undertaken to achieve these aims are presented in chapter 2 to chapter 6.

The significance and implications of the DNA-damaging pathways and mechanisms identified for N-nitrosoindoles are discussed in chapter 7.
CHAPTER 2

SYNTHESIS AND CHARACTERISATION OF A SERIES OF 3-SUBSTITUTED N-NITROSOINDOLES
2.1 INTRODUCTION

Three indole compounds were selected for nitrosation in this study (Scheme 2.1).

![Scheme 2.1 Structures of the indole compounds selected for nitrosation.](image)

The basis for selection was three-fold: (1) indole compounds that could be easily nitrosated in the laboratory were selected, to afford mono-N-nitrosoindoles that could be fully characterised; (2) indole compounds differing in the nature of the 3-substituent were selected, so that any adducts formed could be characterised; (3) indole compounds and their nitrosated derivatives that had been previously studied were selected, so that any findings could be related to existing literature.

Consequently, indole-3-acetonitrile (IAN), indole-3-acetamide (IAM) and indole-3-acetic acid methyl ester (IAAME) were selected as the parent indole compounds for nitrosation by acidified nitrite.
2.2 EXPERIMENTAL PROCEDURES

**Caution:** NIAN is mutagenic and should be handled with extreme caution. NIAM and NIAAME are suspected mutagens and should be handled with extreme caution.

2.2.1 Materials

HPLC solvents were purchased from Fisher Scientific, U.K. IAN was purchased from Fluka Chemicals, U.K. All other chemicals were purchased from Sigma Chemical Co., U.K.

2.2.2 Synthesis of 3-Substituted N-Nitrosoindoles

IAN, IAM or IAAME dissolved in the minimum amount of acetonitrile (ACN), were reacted with a 15-fold molar excess of aqueous 50 mM nitrous acid (345 mg sodium nitrite in 100 ml of distilled water, adjusted to pH 3 with 6 M HCl) at 37°C in the dark for 3 h. Reaction mixtures were extracted three times with three volumes of dichloromethane and dried over anhydrous sodium sulphate. The resulting filtrates were dried in a stream of nitrogen.

2.2.3 Purification of N-Nitrosoindoles

The crude products were purified by HPLC using a Gilson-gradient controlled system equipped with either a dual-wavelength 116 Gilson UV detector or an Applied Biosystems Inc., 1000s diode array detector. Analyses were performed using a Hypersil C18 BDS, 5μ, 250 x 10 mm reverse-phase Shandon preparative column employing the following elution program: 0 min, 40% B, 20 min, 100% B, 25 min, 100% B, 30 min, 40% B (solvent A water; solvent B methanol) at a flow rate of 3 ml/min with UV detection at 260 and 328 nm. The nitrosated indole was dried in a stream of nitrogen to give a crystalline product in around 50% yield; the main impurity being unreacted starting material.

2.2.4 Characterisation of N-Nitrosoindoles

UV spectra were obtained using the Gilson gradient-controlled system equipped with an Applied Biosystems Inc., 1000s diode array detector. \(^1\)H NMR spectra were recorded on a Bruker ARX 250 MHz instrument. Mass spectra were recorded by using a VG 70-SEQ instrument. Microanalyses were provided by Butterworth Laboratories Ltd., U.K.
2.3 RESULTS

2.3.1 Synthesis and Characterisation of NIAN

NIAN was obtained as a yellow crystalline product. HPLC analysis of the pure product afforded a single peak with a retention time of 12.3 min. There is no evidence of the starting material IAN present (RT 8.8 min) in the pure NIAN sample [Fig. 2.1 (A)].

$\lambda_{\text{max}}$ (Methanol) 204, 260, 331 nm [Fig. 2.1 (B)].

**NMR** $\delta$ (CDCl$_3$) 2-H, 7.8 (major, s), and 8.4 (minor, t); 4-H, 5-H, and 6-H, 7.45-7.65; 7-H, 8.24 (major, d), and 8.35 (minor, s); -CH$_2$-, 3.8 (major, d) and 3.9 (minor, d) [Fig. 2.2 (A)].

**MS** $m/z$ (FAB positive ion) 186 [M+H]$^+$; 156 [M+H]$^+$ - 30, this is consistent with loss of the NO group; 130 [M+H]$^+$ - 56, this is consistent with a further loss of -CN [Fig. 2.2 (B)].

**Microanalysis results** $C_{10}H_7N_3O_2$ Calculated: C, 64.86; H, 3.78; N, 22.70; O, 8.64

Found: C, 65.05; H, 3.60; N, 22.25

2.3.2 Synthesis and Characterisation of NIAM

NIAM was obtained as a yellow crystalline product. HPLC analysis of the pure product afforded a single peak with a retention time of 10.4 min. There is no evidence of the starting material IAM present (RT 5.7 min) in the pure NIAM sample [Fig. 2.3 (A)].

$\lambda_{\text{max}}$ (Methanol) 207, 260, 329 nm [Fig. 2.3 (B)].

**NMR** $\delta$ (CD$_3$COCD$_3$) 2-H, 7.9 (major, s), and 8.4 (minor, m); 4-H, 5-H, and 6-H, 7.5-7.85; 7-H, 8.3 (major, d), and 8.45 (minor, s); -CH$_2$-, 3.75 (major, d) and 3.8 (minor, d); -NH$_2$, 7.2 and 6.5 (2 br. s) [Fig. 2.4 (A)].

**MS** $m/z$ (FAB positive ion) 204 [M+H]$^+$; 174 [M+H]$^+$ - 30, this is consistent with loss of the NO group; 130 [M+H]$^+$ - 74, this is consistent with a further loss of -CONH$_2$ [Fig. 2.4 (B)].

**Microanalysis results** $C_{10}H_9N_3O_2$ Calculated: C, 59.11; H, 4.43; N, 20.69; O, 15.76

Found: C, 59.5; H, 4.45; N, 19.9
Chapter 2: Synthesis and Characterisation of N-Nitrosoindoles

It was not possible to obtain satisfactory microanalytical results with NIAM and low nitrogen values were consistent with denitrosation (see section 2.4.1).

2.3.3 Synthesis and Characterisation of NIAAME

NIAAME was obtained as an orange crystalline product. HPLC analysis of the product after purification afforded a main peak with a retention time of 15.4 min. There is some evidence of the starting material IAAME present (RT 11.6 min) in the purified NIAAME sample [Fig. 2.5 (A)].

$\lambda_{\text{max}}$ (Methanol) 204, 261, 331 nm [Fig. 2.5 (B)].

**NMR** δ (CD$_2$COCD$_3$) 2-H, 7.9 (major, s), and 8.35 (minor, m); 4-H, 5-H, and 6-H, 7.45-7.75; 7-H, 8.24 (major, d), and 8.45 (minor, s); -CH$_2$-, 3.85 (major, d) and 3.95 (minor, d); -CH$_3$, 3.7 (major, d) and 3.75 (minor, d) [Fig. 2.6 (A)].

**MS** m/z (FAB positive ion) 219 [M+H]$^+$; 189 [M+H]$^+$ - 30, this is consistent with loss of the NO group; 130 [M+H]$^+$ - 89, this is consistent with a further loss of -COOCH$_3$ [Fig. 2.6 (B)].

**Microanalysis results** C$_{11}$H$_{10}$N$_2$O$_3$  Calculated: C, 60.5; H, 4.59; N, 12.84; O, 22.0

  Found: C, 60.68; H, 4.56; N, 12.50
Figure 2.1 (A) HPLC chromatogram of pure NIAN, showing the absence of IAN starting material. HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm.

(B) UV spectrum of NIAN, $\lambda_{\text{max}}$ (Methanol) 204, 260, 331 nm is shown.
Chapter 2: Synthesis and Characterisation of N-Nitrosoindoles

Figure 2.2 (A) $^1$H NMR spectrum of NIAN recorded in CDCl$_3$.
(B) Fast Atom Bombardment (positive ion) mass spectrum of NIAN.
Figure 2.3 (A) HPLC chromatogram of pure NIAM, showing the absence of IAM starting material. HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm.

(B) UV spectrum of NIAM, \( \lambda_{\text{max}} \) (Methanol) 207, 260, 329 nm is shown.
Figure 2.4 (A) $^1$H NMR spectrum of NIAM recorded in CD$_3$COCD$_3$.

(B) Fast Atom Bombardment (positive ion) mass spectrum of NIAM.
Chapter 2: Synthesis and Characterisation of N-Nitrosoindoles

Figure 2.5 (A) HPLC chromatogram of NIAAME after purification, showing the slight presence of IAAME starting material. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.

(B) UV spectrum of NIAAME, \( \lambda_{\text{max}} \) (Methanol) 204, 261 and 331 nm is shown.
Chapter 2: Synthesis and Characterisation of N-Nitrosoindoles

Figure 2.6 (A) $^1$H NMR spectrum of NIAAME recorded in CD$_3$COCD$_3$.

(B) Fast Atom Bombardment (positive ion) mass spectrum of NIAAME.
2.4 DISCUSSION

2.4.1 Treatment of Parent 3-Substituted Indoles with Acidified Nitrite Affords N-Nitrosoindoles as Crystalline Products

NIAN, NIAM and NIAAME (Scheme 2.2) were obtained as crystalline products by treatment of the parent compounds with acidified nitrite followed by HPLC purification.

Spectral data for NIAN agreed well with data previously published by Wakabayashi (Wakabayashi et al. 1985).

All three nitrosated indoles are isomeric compounds. The $E/Z$ isomerisation of the NO group is reflected in the nmr data, affecting principally the 2-H, 7-H and -CH$_2$-resonances; essentially the indole nucleus remains the same for the series of nitrosated indoles.
Chapter 2: Synthesis and Characterisation of \(N\)-Nitrosoindoles

Indoles. The ratio of the two isomers was consistent for all three compounds at approximately 2:1. The disappearance of the -NH signal (due to the production of -NNO) was evident in all nmr spectra.

Satisfactory microanalytical data could not be obtained for NIAM despite precautions taken to reduce denitrosation, especially in transit to the microanalytical laboratory. However, spectral data for NIAM were consistent with spectral data obtained for NIAN and NIAAME.

All three nitrosated indoles were stored in glass vials at -20°C protected from light. Prior to an experiment using the nitrosated indoles, a purity check was carried out, either by mass spectrometry and/or HPLC to ensure the quality of the nitrosated indole did not deteriorate upon storage. In studies involving the use of NIAN, NIAM or NIAAME, co-solvent was added to the nitrosated indole to obtain the required concentration, immediately prior to addition of the nitrosated indole to the reaction system.

In summary, three model \(N\)-nitrosoindoles were synthesised and fully characterised. Studies involving the use of these model compounds are described in subsequent chapters of this thesis.
CHAPTER 3

REACTIONS OF A SERIES OF 3-SUBSTITUTED N- NITROSOINDOLES WITH PURINE NUCLEOTIDES AND NUCLEOSIDES
3.1 INTRODUCTION

In this study, isolated purine nucleotides and nucleosides (Scheme 3.1) were selected for reaction with NIAN, NIAM and NIAAME with anticipation that the nucleophilic amino groups of the adenine and guanine bases are likely modification targets for any nitrosated indole-induced damage.

Scheme 3.1 Structures of the purine 5'-monophosphate nucleotides and 2'-deoxyguanosine used as reactive substrates in this study.

A simple monophasic reaction system was used to study the reactions.
3.2 EXPERIMENTAL PROCEDURES

**Caution:** NIAN is mutagenic and should be handled with extreme caution. NIAM and NIAAME are suspected mutagens and should be handled with extreme caution. Sodium azide is a hazardous chemical and care should be exercised in its handling.

### 3.2.1 Materials

HPLC solvents were purchased from Fisher Scientific, U.K. 2'-Deoxyoxanosine was a generous gift from Dr. Toshinori Suzuki of Kyoto University, Japan. All other chemicals were purchased from Sigma Chemical Co., U.K, unless stated otherwise.

### 3.2.2 Synthesis and Characterisation of 3-Substituted Nitrosated Indoles

NIAN, NIAM and NIAAME were synthesised by nitrous acid treatment of IAN (Fluka), IAM and IAAME respectively, and characterised by ^1^H-NMR, mass spectrometry and microanalysis as described in chapter 2.

### 3.2.3 Reactions of 2'-Deoxyadenosine-3'- or 5'-Monophosphate (dAp or dpA) with NIAN

NIAN at molar ratios ranging from 1:0.5 to 1:20 (dAp/dpA:NIAN, NIAN=0.14, 0.28, 0.56, 1.4, 2.8, 4.2 and 5.6 mg in 250 μl ACN) were incubated with solutions of dAp or dpA (0.5 mg: 1.5 μmol in 250 μl 10 mM Tris-HCl buffer, pH 7.4) in a water bath at 37°C for 6 h in the dark. The reaction mixtures were extracted 3 times with 3 volumes diethyl ether to remove unreacted NIAN, dried down in a centrifugal vacuum evaporator (DNA 110, Savant) and redissolved in 500 μl water prior to HPLC analysis. Control incubations were set up of NIAN, IAN or dAp/dpA alone. Diethyl ether extracts were also dried down and redissolved in 1500 μl ACN prior to HPLC analysis.

### 3.2.4 Reactions of 2'-Deoxyguanosine-3'- or 5'-Monophosphate (dGp or dpG) with NIAN

NIAN at molar ratios ranging from 1:0.5 to 1:20 (dGp/dpG:NIAN, NIAN=0.13, 0.27, 0.53, 1.33, 2.65, 3.98 and 5.3 mg in 250 μl ACN) were incubated with solutions of dGp or...
dpG (0.5 mg: 1.44 μmol in 250 μl 10 mM Tris-HCl buffer, pH 7.4) as described for reactions of dAp with NIAN.

3.2.5 Reactions of dpA and dpG with NIAM and NIAAME

NIAM (4.8 mg in 250 μl ACN) or NIAAME (4.65 mg in 250 μl ACN) were reacted with dpA or dpG (0.5 mg in 250 μl 10 mM Tris-HCl buffer pH 7.4) as described for reactions of dpA with NIAN. Both these concentrations correspond to a 15-fold molar excess of nitrosated indole over nucleotides.

3.2.6 Reactions of NIAN, NIAM or NIAAME with 2'-Deoxyguanosine (dGuo)

NIAN (5.2 mg in 250 μl ACN), NIAM at molar ratios ranging from 1:2 to 1:15 (dGuo:NIAM, NIAM=0.76, 1.9 and 5.7 mg in 250 μl ACN) or NIAAME at molar ratios ranging from 1:1 to 1:10 (dGuo:NIAAME, NIAAME=0.4, 1.0, 2.0, 3.0 and 4.0 mg in 250 μl ACN) were incubated with solutions of dGuo (0.5 mg: 1.87 μmol in 250 μl 10 mM Tris-HCl buffer, pH 7.4) as described for reactions of dpA with NIAN.

3.2.7 Reactions of NIAN with 2'-Deoxyoxanosine (dOxo)

NIAN at molar ratios ranging from 1:2 to 1:15 (dOxo:NIAN, NIAN=0.7, 1.73 and 5.2 mg in 250 μl ACN) were incubated with solutions of dOxo (0.5 mg: 1.87 μmol in 250 μl 10 mM Tris-HCl buffer, pH 7.4) as described for reactions of dpA with NIAN.

3.2.8 HPLC Analyses of Reaction Mixtures

HPLC-UV analyses were performed using a Hypersil C18 BDS, 5μ, 250 x 4.6 mm reverse-phase Shandon analytical column on the Gilson-gradient controlled system equipped with either a dual-wavelength 116 Gilson UV detector or to obtain UV spectra, an Applied Biosystems Inc., 1000s diode array detector. 100 μl injection volumes of aqueous reaction mixture were analysed at a flow rate of 1 ml/min. For the separation of NIAN and dpA, dAp and dGp reaction mixtures the following elution program was used: 0 min, 0%B, 15 min, 20%B, 25 min, 35%B, 30 min, 0%B [solvent A 50 mM ammonium formate, pH 5.4; solvent B methanol]. Column eluants were monitored at 260 and 290 nm.

For the separation of all other aqueous reaction mixtures, the following elution program was used: 0 min, 0%B, 25 min, 20%B, 35 min, 50%B, 40 min, 0%B [solvent A 50 mM ammonium formate, pH 5.4; solvent B methanol].
ammonium formate, pH 5.4; solvent B methanol]. Column eluants were monitored at 260 and 290 nm. For the separation of NIAN, NIAM or NIAAME extracts after reaction, 30 μl aliquots were analysed at a flow rate of 1 ml/min using the elution program described in chapter 2 for the purification of the nitrosated indoles. Column eluants were monitored at 260 and 328 nm. The fractions corresponding to reaction products were collected from multiple HPLC runs, pooled together and dried down in a centrifugal vacuum evaporator for further analysis by ESI-MS. Reaction products were identified from their UV spectra, obtained by diode array analysis, and retention times which were compared to those of the authentic standards. Further evidence for structural assignment was obtained from the ESI-MS and ESI-MS-MS spectra of the pooled fractions.

3.2.9 Characterisation of Reaction Products by Mass Spectrometry

Off-line ESI-MS and ESI-MS-MS characterisation of reaction products was carried out using a VG Autospec-Ultima Q. Dried fractions previously collected from the HPLC were resuspended in 50:50 ACN/water and inserted into the ESI via 20 μl ‘loop’ injection or continuous infusion, at a flow rate of typically 8 μl/min. The cone voltage was in the range 8-23 V and full scan mass spectra were obtained by scanning from m/z 1 650 to 50 at a scan speed of 10 s/decade. ESI-MS-MS product ion spectra were obtained by selecting the desired precursor ion with MS1 and allowing collision induced dissociation to occur in the collision cell using air as the target gas, typically at 10% transmission of the precursor ion with a collision energy of 48 eV. The resulting product ions were analysed in MS2.
3.3 RESULTS

3.3.1 Reactions of Nitrosated Indoles with dpA and dAp

Reaction of NIAN with dpA at pH 7.4 in buffered 50% aqueous acetonitrile yielded three products not seen in control incubations, hypoxanthine (Hyp), adenine (Ade) and N<sup>6</sup>-acetyladenine (N<sup>6</sup>-AcAde) with retention times of 7.7, 11.4 and 15.5 min, respectively [Fig. 3.1].

Identification of hypoxanthine was based on comparison of the retention time and UV spectrum with authentic hypoxanthine analysed under the same conditions. The UV spectrum of the HPLC fraction corresponding to hypoxanthine, λ<sub>max</sub> 248 nm (10% MeOH) [Figure 3.2 (B)] was analogous to that generated from hypoxanthine standard, λ<sub>max</sub> 248 nm (10% MeOH) [Figure 3.2 (A)]. When ESI-MS was performed on this fraction, a molecular ion with m/z 137 [M+H]<sup>+</sup> was observed [Fig. 3.2 (C)].

Identification of adenine was again based on comparison of the retention time and UV spectrum with adenine standard analysed under the same conditions. The UV spectrum of the HPLC fraction corresponding to adenine and dpA, λ<sub>max</sub> 217, 257 nm (15% MeOH) [Figure 3.3 (B)] was similar to that generated from adenine standard, λ<sub>max</sub> 216, 257 nm (15% MeOH) [Figure 3.3 (A)]. When ESI-MS was performed a main molecular ion with m/z 136 [M+H]<sup>+</sup> was observed. A molecular ion with m/z 332 [M+H]<sup>+</sup> was also observed suggesting the presence of unreacted dpA [Fig. 3.3 (C)]. Adenine and dpA co-elute under the HPLC conditions described.

The identification of reaction product N<sup>6</sup>-acetyladenine was based on ESI-MS results. A molecular ion with m/z 178 [M+H]<sup>+</sup> was observed for this fraction. When ESI-MS was performed under conditions promoting cone voltage induced dissociation, a fragment ion with m/z 136 corresponding to protonated adenine was observed, confirming the structure as an adenine adduct [Fig. 3.4 (A)]. When deuterated ACN was used as co-solvent in the reaction mixture, a molecular ion with m/z 181 [M+H]<sup>+</sup> was observed for the fraction, corresponding to N<sup>6</sup>-acetyl (d<sub>1</sub>)-adenine [Fig. 3.4 (B)].

Reaction of NIAN with dAp yielded the same products, not seen in control incubations, as described for reactions of NIAN with dpA [Figure 3.5].
All three reaction products increased in concentration with accompanying increases in the molar ratio of NIAN to dpA [Figure 3.6 (A and B)] or NIAN to dAp [Figure 3.6 (C and D)].

Analogous reaction products, not seen in control incubations, were seen with reactions of NIAM and dpA [Figure 3.7]. Other peaks in the reaction mixture chromatogram [Figure 3.7 (B)] are attributed to NIAM decomposition products that have remained in the aqueous phase after the extraction process; NIAM is the most hydrophilic of the nitrosated indoles used in this study.

Similarly, analogous reaction products, not seen in control incubations, were seen with reactions of NIAAME and dpA [Figure 3.8]. The small peaks after 30 min in the reaction mixture chromatogram [Figure 3.8 (B)] are again attributed to NIAAME decomposition products that have remained in the aqueous phase after the extraction process. The high yield of adenine produced from the reaction of NIAAME and dpA is likely to have swamped the $\text{M}^\text{+}$-acetyladenine signal.
Figure 3.1 HPLC separation of (A) Control reaction mixture and (B) NIAN and dpA reaction mixture (molar ratio 20:1). HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm.
Figure 3.2 UV spectra of (A) Hyp standard, $\lambda_{\text{max}}$ 248nm (10% MeOH) and (B) HPLC fraction corresponding to Hyp, $\lambda_{\text{max}}$ 248nm (10% MeOH) are shown. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Hyp. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.
Figure 3.3 UV spectra of (A) Ade standard, λ<sub>max</sub> 216, 257 nm (15% MeOH) and (B) HPLC fraction corresponding to Ade + dpA, λ<sub>max</sub> 217, 257 nm (15% MeOH) are shown. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Ade + dpA. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.

71
Figure 3.4 Mass spectrometric analyses of HPLC fraction corresponding to N⁶-AcAde. (A) ESI-MS spectrum with conditions used to promote cone voltage induced dissociation. (B) ESI-MS spectrum of fraction generated from reaction mixture using d₃-ACN as co-solvent. Chromatographic separation and mass spectrometric conditions are described under "Experimental Procedures".
Figure 3.5 HPLC separation of (A) Control reaction mixture and (B) NIAN and dAp reaction mixture (molar ratio 15:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Chapter 3: Reactions of $N$-Nitrosoidoles with Nucleotides and Nucleosides

Figure 3.6 The increase in the production of (A) Hyp and $N^6$-AcAde and (B) Ade + dpA with increasing mole equivalents of NIAN to dpA; (C) Hyp and $N^6$-AcAde and (D) Ade + dAp with increasing mole equivalents of NIAN to dAp. Yields as monitored by HPLC analysis.
Figure 3.7 HPLC separation of (A) Control reaction mixture and (B) NIAM and dpA reaction mixture (molar ratio 15:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Figure 3.8 HPLC separation of (A) Control reaction mixture and (B) NIAAME and dpA reaction mixture (molar ratio 15:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
3.3.2 Reactions of Nitrosated Indoles with dpG and dGp

Reaction of NIA with dpG at pH 7.4 buffered 50% aqueous acetonitrile yielded 6 products, guanine (Gua), xanthine (Xan), oxanine (Oxa), 2'-deoxyoxanosine-5'-monophosphate (dpO), N^2-acetyldeoxyguanosine-5'-monophosphate (N^2-AcdpG) and N^2-acetylguanine (N^2-AcGua), not seen in control incubations, with retention times of 7.8, 8.7, 11.4, 12.1, 19.6, and 20.1 min respectively [Figure 3.9].

Identification of reaction product guanine was based on comparison of retention time and UV spectrum with guanine standard analysed under the same conditions. The UV spectrum of the HPLC fraction corresponding to guanine, \( \lambda_{\text{max}} \) 245, 273 nm (6% MeOH) [Figure 3.10 (B)] was identical to that generated from guanine standard [Figure 3.10 (A)]. When ESI-MS was performed on this fraction, a molecular ion with m/z 152 [M+H]^+ was observed [Figure 3.10 (C)].

Similarly, the identification of reaction product xanthine was based on comparison of retention time and UV spectrum with xanthine standard analysed under the same conditions. The UV spectrum of the HPLC fraction corresponding to xanthine, \( \lambda_{\text{max}} \) 225, 265 nm (6.5% MeOH) [Figure 3.11 (B)] was identical to that generated from xanthine standard [Figure 3.11 (A)]. When ESI-MS was performed on this fraction, a molecular ion with m/z 153 [M+H]^+ was observed [Figure 3.11 (C)].

The identification of reaction product N^2-acetyldeoxynucleosine-5'-monophosphate was based on ESI-MS results. A molecular ion with m/z 388 [M-H]^- was observed for this fraction [Figure 3.12 (A)] and by exchanging the co-solvent to deuterated ACN, an increase of 3 units to m/z 391 [M-H]^- was observed for the molecular ion [Figure 3.12 (B)].

The identification of reaction product N^2-acetylguanine was again based on ESI-MS results and comparison of retention time and UV spectrum for authentic N^2-acetylguanine. The UV spectrum of the HPLC fraction corresponding to N^2-acetylguanine, \( \lambda_{\text{max}} \) 221, 257 nm (15.5% MeOH) [Figure 3.13 (B)] was identical to that generated from N^2-acetylguanine standard [Figure 3.13 (A)]. A molecular ion with m/z 194 [M+H]^+ ion was observed for the fraction corresponding to N^2-acetylguanine. When conditions were employed to promote cone voltage induced dissociation, a fragment ion with m/z 152 corresponding to protonated guanine was observed, confirming the structure as a guanine adduct [Figure 3.13 (C)]. Replacement of acetonitrile in the monophasic reaction system
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

by the deuterated solvent gave a molecular ion with m/z 197 [M+H]+ for this fraction confirming it as N(2)-(d1) acetylguanidine [Figure 3.13 (D)].

The concentration of reaction product xanthine increased with accompanying increases in the molar ratio of NIAN to dpG, whilst the concentration of guanine, after initially increasing, decreased as conversion to reaction products occurred [Figure 3.14 (A)]. These results indicate that guanine can be deaminated directly. The results also indicate that depurination occurs independently of the other pathways and is more evident at the higher molar ratios. This is illustrated by the dose-response behaviour of the N(2)-acetyl adducts.

The formation of N(2)-acetyldeoxyguanosine-5'-monophosphate increased with an increase in the molar ratio of NIAN to dpG up to a ratio of 10:1. As the molar ratio increased to 20:1, the concentration of the product decreased in the system, and an increase in the depurinated adduct N(2)-acetylguanine was observed [Figure 3.14 (B)].

The identification of reaction products 2'-deoxyoxanosine-5'-monophosphate and the depurination product oxanine, was based on comparison of retention time, UV spectra and ESI-MS results when compared to oxanine derived from the hydrolysis of the N-glycosidic bond of authentic 2'-deoxyoxanosine (dOxo). dOxo was hydrolysed as described by Suzuki (Suzuki et al. 1997b). dOxo (0.37 mM) was incubated in 0.1 M acetate buffer, pH 4.0 for 4 h at 70°C. At hourly intervals, 100 µl aliquots were injected onto the Gilson-gradient controlled HPLC system exactly as previously described for the analysis of aqueous reaction mixtures. As the dOxo peak (RT=19.9 min) decreased, a new peak (RT=11.4 min) appeared in the chromatogram [Figure 3.15], corresponding to oxanine, $\lambda_{\text{max}}$ 240, 287 nm [8% MeOH] [Figure 3.16 (B)]. The structure of oxanine is shown in Figure 3.16 (A). ESI-MS analysis of the HPLC fraction corresponding to oxanine derived from the hydrolysis of dOxo afforded a molecular ion with m/z 153 [M+H]+ and m/z 305 [2M+H]+ confirming the hydrolysis product as oxanine [Figure 3.16 (C)]. Thus the retention time of reaction product oxanine was identical (11.4 min) to the hydrolysis product of dOxo, as was the UV spectrum, $\lambda_{\text{max}}$ 240, 287 nm (8% MeOH) [Figure 3.17 (A)]. When ESI-MS was performed on this fraction, a molecular ion with m/z 153 [M+H]+ was observed [Figure 3.17 (B)].

The UV spectrum of the HPLC fraction corresponding to 2'-deoxyoxanosine-5'-monophosphate was very similar to that of oxanine; dpO, $\lambda_{\text{max}}$ 245, 288 nm (8.5% MeOH) [Figure 3.18 (B)]. The structure of dpO is shown in Figure 3.18 (A). Upon ESI-MS
analysis of the HPLC fraction corresponding to dpO, a molecular ion with m/z 347 [M-H]\(^-\) was observed [Figure 3.18 (C1)]. ESI-MS-MS analysis produced fragment ions with m/z 151, 79 (PO\(_3\)\(^-\)), 97 (H\(_2\)PO\(_4\))\(^-\) and 195 (C\(_6\)H\(_8\)PO\(_6\)) [Figure 3.18 (C2)]. These results alone would sensibly suggest the formation of 2'-deoxyxanthosine-5'-monophosphate, however the retention time and UV spectrum of the reaction product are not consistent with this suggestion. ESI-MS analysis on the fraction corresponding to oxanine resulted in a molecular ion with m/z 153 [M+H]\(^+\), corresponding to the same mass as xanthine and suggesting this product as the depurination product of dpO. The replacement of the N atom at N-1 of guanine with an O atom increases the relative molecular mass by one and coincidentally oxanine has the same mass as xanthine and 2'-deoxyoxanosine-5'-monophosphate has the same mass as 2'-deoxyxanthosine-5'-monophosphate.

Reaction of NIAN with dGp yielded the same profile of reaction products as described for the reactions of NIAN with dpG, guanine (Gua), xanthine (Xan), 2'-deoxyoxanosine-3'-monophosphate (dOp), oxanine (Oxa), N\(^2\)-acetyldioxynosine-3'-monophosphate (N\(^2\)-AcdGp) and N\(^2\)-acetylguanine (N\(^2\)-AcGua) not seen in control incubations, with retention times of 7.7, 8.4, 10.2, 10.4, 14.3 and 16.3 min, respectively [Figure 3.19]. Reaction products dOp and N\(^2\)-AcdGp are the corresponding 3'-monophosphate products. The positioning of the phosphate group on the ribose unit affects the retention time of the product. The peak corresponding to dGp elutes earlier in the analysis (0.6 min) than dpG under the HPLC conditions described. Consequently, the retention times of dOp and of N\(^2\)-AcdGp are affected accordingly when compared to the corresponding 5'-monophosphate reaction products. The elution of dOp before oxanine in the analysis, adds further confirmation of dOp as a monophosphate product. In addition, the retention times of all reaction products are slightly different to those described for NIAN reactions with dpG, due to a different elution program being used for the HPLC analysis as described under “Experimental Procedures”.

Analogous reaction products, not seen in control incubations, were seen with reactions of NIAM and dpG [Figure 3.20]. Again, other peaks in the reaction mixture chromatogram [Figure 3.20 (B)] are attributed to NIAM decomposition products as described for reaction of NIAM with dpA. Similarly, analogous reaction products, not seen in control incubations, were seen with reactions of NIAAME and dpG [Figure 3.21]. The small peaks after 30 min in the reaction mixture chromatogram [Figure 3.21 (B)] are attributed to NIAAME decomposition products.
Figure 3.9 HPLC separation of (A) Control reaction mixture and (B) NIAN and dpG reaction mixture (molar ratio 10:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Chapter 3: Reactions of \( N \)-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.10 UV spectra of (A) Gua standard, \( \lambda_{\text{max}} \) 245, 273 nm (6% MeOH) and (B) HPLC fraction corresponding to Gua, \( \lambda_{\text{max}} \) 245, 273 nm (6% MeOH) are shown. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Gua. Chromatographic separation and mass spectrometric conditions are described under "Experimental Procedures".
Chapter 3: Reactions of $N$-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.11 UV spectra of (A) Xan standard, $\lambda_{\text{max}}$ 225, 265 nm (6.5% MeOH) and (B) HPLC fraction corresponding to Xan, $\lambda_{\text{max}}$ 225, 265 nm (6.5% MeOH) are shown. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Xan. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.

82
Figure 3.12 Mass spectrometric analysis of HPLC fraction corresponding to N²-AcdpG.
(A) ESI-MS spectrum and (B) ESI-MS spectrum of fraction generated from reaction mixture using d₃-ACN as co-solvent. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.

83
Figure 3.13 UV spectra of (A) N²-AcGua standard, λ max 221, 257 nm (15.5% MeOH) and (B) HPLC fraction corresponding to N²-AcGua, λ max 221, 257 nm (15.5% MeOH) are shown. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to N²-AcGua with conditions used to promote cone voltage dissociation. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.

84
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.13 (D) Electrospray mass spectrometric analysis of HPLC fraction corresponding to N²-AcGua generated from reaction mixture using d₃-ACN as co-solvent. Chromatographic separation and mass spectrometric conditions are described under "Experimental Procedures".

Figure 3.14 Dose-response relationship of reaction products (A) Gua and Xan and (B) N²-AcdpG and N²-AcGua with increasing mole equivalents of NIAN to dpG. Yields as monitored by HPLC analysis.
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.15 HPLC separation of dOxo hydrolysis reaction mixture: (A) Control, (B) Incubation time 120 min and (C) Incubation time 180 min. HPLC conditions as described under "Experimental Procedures"; UV detection at 260 and 290 nm.

86
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

(A) The structure of oxanine. (B) UV spectrum of Oxa generated from the hydrolysis of dOxo, λ_max 240, 287 nm (8% MeOH) is shown. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Oxa generated from the hydrolysis of dOxo. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.

Figure 3.16
Figure 3.17 (A) UV spectrum of HPLC fraction corresponding to Oxa, λ_max 240, 287 nm (8% MeOH) is shown. (B) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Oxa. Chromatographic separation and mass spectrometric conditions are described under “Experimental Procedures”.
Figure 3.18 (A) The structure of dpO. (B) UV spectrum of HPLC fraction corresponding to dpO, $\lambda_{\text{max}}$ 245, 288 nm (8.5% MeOH) is shown. (C) Mass spectrometric analyses of HPLC fraction corresponding to dpO. 1, ESI-MS; and 2, ESI-MS-MS spectrum with precursor ion of $m/z$ 347 selected with MS1; resulting product ions analysed in MS2. Chromatographic separation and mass spectrometric conditions are described under "Experimental Procedures".
Figure 3.19 HPLC separation of (A) Control reaction mixture and (B) NIAN and dGp reaction mixture (molar ratio 15:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Figure 3.20 HPLC separation of (A) Control reaction mixture and (B) NIAM and dpG reaction mixture (molar ratio 15:1). HPLC conditions as described under "Experimental Procedures"; UV detection at 260nm.
Figure 3.21 HPLC separation of (A) Control reaction mixture and (B) NIAAME and dpG reaction mixture (molar ratio 15:1). HPLC conditions as described under "Experimental Procedures"; UV detection at 260nm.
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

3.3.3 Reactions of Nitrosated Indoles with dGuo

Reaction of NIAN with dGuo at pH 7.4 in buffered 50% aqueous acetonitrile yielded the same 4 products, guanine, xanthine, oxanine and N\(^2\)-acetylguanine, not seen in control incubations, with the same retention times as described for reactions of nitrosated indoles with dpG [Figure 3.22]. The absence of reaction products 2'-deoxyoxanosine-5'-monophosphate and N\(^2\)-acetyldeoxyguanosine-5'-monophosphate is expected, as these are nucleotide modification products.

Analogous reaction products, not seen in control incubations, were seen with reactions of NIAM and NIAAME with dGuo [Figures 3.23 and 3.24 respectively].

All four reaction products increased in concentration with accompanying increases in the molar ratio of NIAM to dGuo [Figure 3.25 (A, B and C)] or NIAAME to dGuo [Figure 3.26 (A, B and C)].

3.3.4 Reactions of NIAN with dOxo

Reaction of NIAN with dOxo at pH 7.4 in buffered 50% aqueous acetonitrile yielded two products not seen in control incubations, xanthine (Xan) and oxanine (Oxa) with retention times as previously described for the reactions of NIAN with dpG [Figure 3.27].

Identification of xanthine and oxanine was based on comparison of the retention times and UV spectra with the appropriate standards. The UV spectra of the HPLC fractions corresponding to oxanine, \(\lambda_{max} 240, 287 \text{ nm (8% MeOH)} \) [Figure 3.28 (A)] and xanthine, \(\lambda_{max} 225, 265 \text{ nm (6.5% MeOH)} \) [Figure 3.28 (B)] were identical to those generated for the corresponding standards as previously described for the identification of the reaction products of NIAN with dpG.

Both reaction products increased in concentration with accompanying increases in the molar ratio of NIAN to dOxo [Figure 3.29 (A and B)].
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.22 HPLC separation of (A) Control reaction mixture and (B) NIAN and dGuo reaction mixture (molar ratio 15:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Figure 3.23 HPLC separation of (A) Control reaction mixture and (B) NIAM and dGuo reaction mixture (molar ratio 15:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Figure 3.24 HPLC separation of (A) Control reaction mixture and (B) NIAAME and dGuo reaction mixture (molar ratio 15:1). HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm.
Figure 3.25 The increase in the production of (A) Gua and Xan, (B) Oxa and (C) N₂-AcGua with increasing mole equivalents of NIAM to dGuo. Yields as monitored by HPLC analysis.
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.26 The increase in the production of (A) Gua and Xan, (B) Oxa and (C) N2-AcGua with increasing mole equivalents of NIAAME to dGuo.
Figure 3.27 HPLC separation of (A) Control reaction mixture and (B) NIAN and dOxo reaction mixture (molar ratio 15:1). HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm.
Chapter 3: Reactions of \( \text{N} \)-Nitrosoindoles with Nucleotides and Nucleosides

![UV spectra of HPLC fractions](image)

**Figure 3.28** UV spectra of HPLC fractions corresponding to (A) Oxa, \( \lambda_{\text{max}} \) 240, 287nm (8\% MeOH) and (B) Xan, \( \lambda_{\text{max}} \) 225, 265nm (6.5\% MeOH) are shown. HPLC conditions as described under “Experimental Procedures”.

![Graph of peak area vs. molar ratio](image)

**Figure 3.29** The increase in the production of (A) Oxa and (B) Xan with increasing mole equivalents of NIAN to dOxo. Yields as monitored by HPLC analysis.
Chapter 3: Reactions of $N$-Nitrosoindoles with Nucleotides and Nucleosides

3.3.5 Decomposition of Nitrosated Indoles in the Reaction System

When diethyl ether extracts of NIAN reactions with nucleotides or nucleosides were analysed by HPLC, the major decomposition product was IAN [Figure 3.30 (B)]. Identification of IAN was based on comparison of the retention time and UV spectrum with IAN standard analysed under the same conditions [Figure 3.30 (A)]. The retention time and UV spectrum of the HPLC fraction corresponding to IAN, RT 8.8 min, $\lambda_{\text{max}}$ 219, 272 nm (MeOH) [Figure 3.30 (B)] was similar to that generated from IAN standard, RT 8.8 min, $\lambda_{\text{max}}$ 221, 272 nm (MeOH) [Figure 3.30 (A)].

Similarly, when diethyl ether extracts of NIAM reactions with nucleotides or nucleosides were analysed by HPLC, the major decomposition product was IAM [Figure 3.31 (B)]. The retention time and UV spectrum of the HPLC fraction corresponding to IAM, RT 5.7 min, $\lambda_{\text{max}}$ 220, 276 nm (MeOH) [Figure 3.31 (B)] was identical to that generated from IAM standard, RT 5.7 min, $\lambda_{\text{max}}$ 220, 276 nm (MeOH) [Figure 3.31 (A)].

Finally, the major decomposition product when diethyl ether extracts of NIAAME reactions with nucleotides or nucleosides were analysed by HPLC, was IAAME [Figure 3.32 (B)]. The retention time and UV spectrum of the HPLC fraction corresponding to IAAME, RT 11.6 min, $\lambda_{\text{max}}$ 220, 277 nm (MeOH) [Figure 3.32 (B)] was identical to that generated from IAAME standard, RT 11.6 min, $\lambda_{\text{max}}$ 220, 277 nm (MeOH) [Figure 3.32 (A)].

Thus, denitrosation was the major pathway of decomposition for all the nitrosated indoles within the reaction system.

All three $N$-nitrosoindoles gave a positive result in the Liebermann test (Furniss et al. 1989a) for a nitrosamine or $N$-nitroso compound. Under the usual conditions of the test, the nitrosamine is warmed with phenol and acid. The nitrosating species is liberated from the nitrosamine and nitrosates phenol to form $\rho$-nitrosophenol. Another molecule of phenol combines with $\rho$-nitrosophenol to form indophenol, which is red in colour. Under alkaline conditions, the red indophenol yields a blue indophenol anion [Scheme 3.1]. NIAN, NIAM or NIAAME [1 mg: 5.4, 4.9 or 4.6 μmol respectively in 250 μl of ACN containing phenol (0.94 mg: 10 μmol)] were incubated with 250 μl of 10 mM Tris-HCl buffer, pH 7.4 in a water bath at 37°C for 6 hours in the dark. On addition of alkali the
reaction solution turned blue. Thus, N-nitrosoindoles gave a positive result in this test, at pH 7.4 and at 37°C.

Scheme 3.2 Reaction mechanism of the Liebermann test to identify a nitrosamine or N-nitroso compound.

When reactions were repeated in the presence of azide ion, (azide ion: N-Nitrosoindole, 1:1 molar ratio), the formation of any reaction products was completely inhibited [Figure 3.33]. Azide ion behaves in many ways like a covalently bonded chlorine atom and is often referred to as a pseudo-halogen. It is a scavenger of nitrite and nitrosating agents (Williams 1988).
Figure 3.30 (A) HPLC chromatogram of IAN standard; RT IAN 8.8 min and UV spectrum of IAN standard, $\lambda_{max}$ 221, 272 nm (MeOH) are shown. (B) HPLC separation of NIAN and dpG reaction extract (molar ratio 5:1); RT of HPLC fraction corresponding to IAN 8.8 min and UV spectrum, $\lambda_{max}$ 219, 271 nm (MeOH) are shown. HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm.
Chapter 3: Reactions of N-Nitrosoindoles with Nucleotides and Nucleosides

Figure 3.31 (A) HPLC chromatogram of IAM standard; RT IAM 5.7 min and UV spectrum of IAM standard, $\lambda_{\text{max}}$ 220, 276 nm (MeOH) are shown. (B) HPLC separation of NIAM and dpG reaction extract (molar ratio 5:1); RT of HPLC fraction corresponding to IAM 5.7 min and UV spectrum, $\lambda_{\text{max}}$ 220, 276 nm (MeOH) are shown. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Figure 3.32 (A) HPLC chromatogram of IAAME standard; RT 11.6 min and UV spectrum of IAAME standard, $\lambda_{\text{max}}$ 220, 277 nm (MeOH) are shown. (B) HPLC separation of NIAAME and dpG reaction extract (molar ratio 10:1); RT of HPLC fraction corresponding to IAAME 11.6 min and UV spectrum, $\lambda_{\text{max}}$ 220, 277 nm (MeOH) are shown. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
Figure 3.33 HPLC separation of (A) NIA N and dpG reaction mixture (molar ratio 10:1) and (B) Azide, NIA N and dpG reaction mixture (molar ratio 10:10:1). HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm.
3.4 DISCUSSION

Identical nucleotide reaction products, which are structurally independent of the 3-substituted \(N\)-nitrosoindoles, were seen for reactions of nitrosated indoles with isolated nucleotides and dGuo. Modification pathways identified were depurination, deamination, and the formation of 2'-deoxyoxanosine monophosphate and the depurination product oxanine. Denitrosation was the major pathway of decomposition for all the nitrosated indoles within the reaction system, and \(N\)-nitrosoindoles gave a positive result in the Liebermann test, at pH 7.4, and at 37°C. \(N\)-Acetyl adducts were also observed, with the source of acetyl groups being the co-solvent used in the monophasic reaction system.

3.4.1 All Pathways of Modification can be Rationalised by a Transnitrosation Mechanism

The nitrosated indoles undergo \(N-N\) heterolytic cleavage, brought about by the solvent or nucleophiles present in the reaction system, and the free nitrosating agent is generally available to nitrosate any other nucleophilic species present. The evidence for this pathway is further strengthened by: denitrosation as the major decomposition pathway for the \(N\)-nitrosoindoles; a positive result in the Liebermann test for all \(N\)-nitrosoindoles; and lack of evidence for the formation of discrete adducts, structurally depending on the \(N\)-nitrosoindole.

3.4.1.1 Transnitrosation to Explain Depurination

Depurination is probably catalysed by \(N\)-nitrosation at the nucleophilic N-7 atom of guanine or adenine residues and/or the N-3 atom of adenine, imparting a destabilising positive charge on the purine ring system. Cleavage of the \(N\)-glycosidic bond neutralises this charge and gives the depurination products. The corresponding \(N\)-nitrosopurine is then rapidly hydrolysed to generate the observed base. Depurination to afford guanine is shown in Scheme 3.3.

3.4.1.2 Transnitrosation to Explain Deamination

The formation of deamination products can be explained by transnitrosation to exocyclic amino groups of purine bases. The nucleophilic amino group is a reactive target for the nitroso group transferred from the nitrosated indoles, leading to the formation of a diazonium ion.
The diazonium ion is unstable and contains a very good leaving group, a nitrogen molecule. Nitrogen is readily displaced from the purine diazonium ion generated, by nucleophiles present in the reaction mixture. In this case, simple hydrolysis affords the deamination product, namely hypoxanthine from adenine and xanthine from guanine. The formation of xanthine via deamination is shown in Scheme 3.4.

**3.4.1.3 Transnitrosation to Explain the Formation of Oxanine**

The mechanism for the formation of 2′-deoxyxanosine (dOxo) from dGuo by nitrous acid or nitric oxide has been reported (Suzuki et al. 1997a; Glaser and Son 1996). By using guanosine and its methyl derivatives, Suzuki and co-workers demonstrated that reaction at N-2 to give the diazonium ion is followed by cleavage of the N-1-C-6 bond and that the exocyclic amino nitrogen of dOxo originates from the imino nitrogen (N-1) of dGuo (Suzuki et al. 1997a). Consequently, the mechanism for the formation of dOp/dpO and oxanine would logically follow the same route starting with transnitrosation to N-2 of dGp/dpG or dGuo (Scheme 3.4).
Reaction of NIAN with dOxo yielded xanthine and oxanine, and the generation of both products increased with accompanying increases in the molar ratio of NIAN to dOxo. Depurination of dOxo to afford oxanine is probably catalysed by N-nitrosation at the nucleophilic N-7 atom of dOxo exactly as described in section 3.4.1.1 for guanine and adenine residues. Detection of xanthine from dOxo, lends further support to the mechanism described by Suzuki and co-workers for the formation of deamination products from guanine residues involving ring-opening (Suzuki et al. 1997a; Scheme 3.4), indicating that the reaction steps are reversible.

### 3.4.1.4 Transnitrosation to Explain the Formation of N-Acetyl Adducts

The transnitrosating potential of the three substituted nitrosoindoles is further highlighted by the formation of N-acetyl adducts. The replacement of ACN with d$_4$-ACN resulted in the formation of d$_4$-acyetylated products, thus confirming the co-solvent as the source of
acetyl groups. One possible explanation involves a slow hydrolysis step of acetonitrile to acetamide followed by transnitrosation to the amino nitrogen to produce reactive acetyldiazonium ion. However, when acetamide was added to the reaction mixtures at varying concentrations, no increase in the amount of acetylated products was observed, suggesting that this pathway may be at best, a minor pathway. Direct transnitrosation to the tertiary nitrogen atom of ACN followed by hydrolysis may be the more likely pathway and there is some evidence for this possibility (Crookes and Williams 1988).

3.4.1.5 Transnitrosation to Explain Decomposition of Nitrosated Indoles

When reactions were carried out using methanol and ethanol as co-solvents, no reaction products were seen (data not shown). It is likely that the free nitrosating agent was used up in the formation of volatile alkyl nitrites. Reaction of alcohol with nitrous acid is used as the route for their preparation (Furniss et al. 1989b). The procedure depends on the fact that the alkyl nitrite has a lower boiling point than the alcohol and can be distilled out from the equilibrium mixture. Similarly, when reactions were carried out in the presence of azide ion, the formation of any reaction products was completely inhibited. Azide is a scavenger of nitrite and nitrosating agents, resulting in the formation of nitrosyl azide, which rapidly and irreversibly decomposes to nitrogen and nitrous oxide. All three N-nitrosoindoles gave a positive result in the Liebermann test for a nitrosamine or N-nitrosocompound, at physiological pH and temperature. The positive result indicates transnitrosation from N-nitrosoindole to aromatic carbon.

In summary, it would appear that most, if not all, of the products formed by reaction of N-nitrosoindoles with isolated purine nucleotides and deoxyguanosine, are the result of transnitrosation. Transnitrosation is the ready transfer of the nitroso group to nucleophilic nitrogen atoms in the purines.

The results from this study demonstrate that, at neutral pH, N-nitrosoindoles transfer the nitroso group to nucleophilic sites on DNA bases resulting in depurination, deamination and the formation of the novel products dOp/dpO and oxanine. All of these processes are potentially mutagenic events if they occur in DNA.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

CHAPTER 4

REACTIONS OF 1-NITROSOINDOLE-3-ACETONITRILE
WITH OLIGONUCLEOTIDES, DUPLEx DNA
AND CALF THYMUS DNA

111
4.1 INTRODUCTION

At the isolated nucleotide level, 3-substituted nitrosated indoles induce depurination, deamination and formation of a novel nucleobase oxanine, in purine nucleotides via a transnitrosation mechanism as described in chapter 3. A summary of these pathways and the resulting reaction products formed, induced by nitroso transfer from NIAN to 2'-deoxyguanosine-5'-monophosphate (dpG), is shown in Scheme 4.1.

In this study, the ability of NIAN to transfer the nitroso group to nucleophilic target residues in oligonucleotides and calf thymus DNA is investigated, to ascertain whether the spectrum of potentially mutagenic products observed in isolated nucleotides is preserved at the macromolecular level in vitro.

As shown in Scheme 4.1, xanthine and oxanine are reaction products that are formed from hydrolysis and ring-opening reactions, respectively, of the purine diazonium ion. A further consequence of the formation of purine diazonium ion at C-2 of guanine in double-stranded DNA is that this may lead to the formation of interstrand cross-links. Displacement of nitrogen by the exocyclic amino group of guanine on the opposing strand would result in the formation of an interstrand cross-link and this pathway has been shown to be relevant for nitrous acid with duplex DNA (Kirchner et al. 1992). In this study, the relevance of this pathway for NIAN is also examined. In postlabelling studies such a G-G dimer would probably appear as a bulky adduct and this may explain the results of Yamashita (Yamashita et al. 1988) who observed adducts formed by reaction of NIAN with calf thymus DNA.
Scheme 4.1 Summary of NIAN-induced depurination, deamination and formation of oxanine via transnitrosation from NIAN to 2'-deoxyguanosine-5-monophosphate (dpG). (I) Transnitrosation to the N-7 atom of guanine followed by cleavage of the N-glycosidic bond and hydrolysis of the N-nitrosopurine affords depurination products. (II) Transnitrosation to the exocyclic amino group of guanine results in the formation of purine diazonium ion. Subsequent reactions give rise to deamination products and the formation of oxanine.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

4.2 EXPERIMENTAL PROCEDURES

Caution: NIAN and DMS are mutagenic. Mechlorethamine hydrochloride is mutagenic and carcinogenic. Acrylamide is a hazardous chemical. All chemicals must be handled with extreme caution.

4.2.1 Enzymes and General Materials

T4 polynucleotide kinase (T4 PNK, 10 units/μl) was purchased from Amersham; micrococcal nuclease (500 units/ml) was purchased from Sigma; calf spleen phosphodiesterase (2 units/mg) was purchased from Boehringer Mannheim. Unit definitions are as follows: T4 PNK, 1 unit catalyzes the transfer of 1 nmol of phosphate from ATP to polynucleotide in 30 min at pH 7.6 at 37°C; calf spleen phosphodiesterase, 1 unit releases 3’-mononucleotides equivalent to an increase in absorbance at 260 nm of 16 in 30 min at pH 6.5 at 37°C; micrococcal nuclease, 1 unit produces 1 μmol of polynucleotides from native DNA per min at pH 8.8 at 37°C. HPLC solvents were purchased from Fisher Scientific, U.K. All other chemicals were purchased from Sigma Chemical Co., unless otherwise stated.

4.2.2 Synthesis and Characterisation of NIAN

NIAN was synthesised by nitrous acid treatment of IAN (Fluka), purified by HPLC and fully characterised by 1H-NMR, mass spectrometry and microanalysis as described in chapter 2.

4.2.3 Preparation of Radiolabelled Oligonucleotides

The oligonucleotides used in this study [Table 4.1] were synthesised on a 40 nmol or 1 μmol scale using Applied Biosystems reagents on an Applied Biosystems Inc. 394 automated DNA/RNA synthesiser and purified using either reverse-phase HPLC or FPLC (Oswel DNA Research Products Laboratory, Southampton). Oligonucleotides were 5’-32P end-labelled using T4 PNK and [γ-32P]ATP. Typically, each phosphorylation reaction mixture (10 μl) contained kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂ and 10 mM 2-mercaptoethanol), oligonucleotide (100 pmol in distilled water), 1.67 pmol of [γ-32P]ATP (3 000Ci/mmol, Amersham) and 10 units of T4 PNK. After incubation for 1 h at
37°C, the end labelled oligomers were recovered by the addition of 1 µl of sodium acetate (2.5 M, pH 7) and 1 ml of absolute ethanol (-20°C). After 1 h at -20°C, the resulting precipitate was pelleted by centrifugation, the supernatant was discarded and the remaining solid was washed twice with 1 ml of 70% ethanol. The resulting pellets were dried in a centrifugal vacuum evaporator (DNA 110, Savant) and resuspended in water ready for use in the following reactions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>(T)₈CGGT(T)₈</td>
</tr>
<tr>
<td>Ib</td>
<td>(A)₈GGC(A)₈</td>
</tr>
<tr>
<td>II</td>
<td>(T)₇CCGGGG(T)₇</td>
</tr>
<tr>
<td>III</td>
<td>(T)₇CGCGCG(T)₇</td>
</tr>
<tr>
<td>IV</td>
<td>(T)₄CCGG(T)₄CCGG(T)₄</td>
</tr>
<tr>
<td>V</td>
<td>CTGACCGGATTACCGGAGTC</td>
</tr>
<tr>
<td>VIa</td>
<td>(T)₄GGCC(T)₄</td>
</tr>
<tr>
<td>VIb</td>
<td>(A)₄CCGG(A)₄</td>
</tr>
</tbody>
</table>

Table 4.1 Oligonucleotide sequences used in this study

4.2.4 Reactions of NIAN with single-stranded Oligonucleotides (Ia), (II), (III) and (IV)

Radiolabelled oligonucleotide (20 pmol in 20 µl of 0.5 mM Tris-HCl buffer, pH 7.4) was incubated at 37°C in the dark, with solutions of NIAN dissolved in acetonitrile such that the total NIAN concentration ranged from 0.2 to 2 mM in a total volume of 25 µl. Reactions were stopped at varying timepoints (6, 12, 18, 24 and 30 h) by extraction of NIAN twice with 2 volumes of diethyl ether. Reaction mixtures were dried down in a centrifugal vacuum evaporator (DNA 110, Savant) and resuspended in 100 µl of water.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Control incubations were set up that consisted of the oligonucleotide in buffer with acetonitrile only.

An aliquot of 50 µl from control and treated samples was dried in a centrifugal vacuum evaporator and resuspended in 4 µl of denaturing gel-loading buffer [95% formamide, 0.02% bromophenol blue and 0.02% xylene cyanol in TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.0)]. The remaining 50 µl was incubated with 50 µl of freshly prepared 0.1 M piperidine at 90°C for 30 min in a sealed microfuge tube. After cooling to room temperature, samples were dried in a centrifugal vacuum evaporator and lyophilized twice from 40 µl of water. Resulting pellets were resuspended in 4 µl of denaturing gel-loading buffer.

4.2.4.1 Preparation of Maxam and Gilbert Sequencing Markers for single-stranded Oligonucleotides (Ia), (II), (III) and (IV): Modification Reaction Specific for Guanine

Radiolabelled oligonucleotides were also specifically modified at guanine residues using DMS according to an established protocol for the preparation of Maxam-Gilbert sequencing markers (Sambrook et al. 1989). Typically, synthetic, radiolabelled oligonucleotide (20 pmol in 10 µl of water) was added to 190 µl of DMS buffer (50 mM sodium cacodylate, pH 7.0 and 1 mM EDTA, pH 8.0) and 4 µl of sonicated calf thymus DNA (1 mg/ml). The mixture was cooled to 0°C on ice before 1 µl of neat DMS was added. The sample was vortexed briefly. After incubation for 10 min at 30°C, 50 µl of ice chilled DMS “stop solution” [1.5 M sodium acetate, pH 7.0, 1 M β-mercaptoethanol and yeast tRNA (250 µg/ml)] was added and the solution was vortexed. 750 µl of absolute ethanol (-20°C) was added and the solution was placed on dry ice/IMS for 5 min. The supernatant was removed after centrifugation for 10 min and the resulting oligonucleotide fragments were precipitated by the addition of 300 µl of sodium acetate (0.3 M, pH 7.0) and 900 µl of absolute ethanol (-20°C). DNA recovery was performed as described for the preparation of radiolabelled oligonucleotides. Resulting pellets were resuspended in 50 µl of water before incubation with aqueous piperidine as described above. The resulting Maxam and Gilbert sequencing marker pellets were resuspended in 4 µl of denaturing gel-loading buffer.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

4.2.4.2 Analysis of Oligonucleotide Fragment Samples by Denaturing Polyacrylamide Gel Electrophoresis (DPAGE)

DPAGE was performed using the Model SA adjustable sequencing gel electrophoresis system (Gibco BRL, Life Technologies), using 17 cm (width) x 32 cm (long) glass plates, 0.4 mm spacers and a 20 tooth comb. 20% denaturing polyacrylamide gels containing 7 M urea were prepared by dissolving 31.5 g of urea in 40 ml of acrylamide/bisacrylamide solution (Accugel 19:1, National Diagnostics) and 8 ml of 10xTBE buffer. To induce polymerisation, 1.6 ml of 3% aqueous ammonium persulphate and 25 μl of tetramethylethylenediamine (TEMED) were added and the gel was poured. After at least 2 h, the gel was pre-run in TBE buffer at 1 500V for 30 min before samples (2 μl from 4 μl stock) were loaded. Gels were run until the bromophenol blue marker had migrated 20-22 cm into the gel (4 h). Once electrophoresis was complete, the gel was fixed for 1 h [methanol 40%, glycerol 3% and glacial acetic acid (10% in water)], transferred onto DE81 filter paper (Whatman), covered with clingfilm and dried on a Hoefer SE 1160 slab gel drier at 70°C for 1 h. Once dry, the gel was stored in a light-proof cartridge prior to data analysis.

4.2.4.3 Data Analysis and Quantitation of Strand Breaks

Radiolabelled oligomer products were visualised by direct contact autoradiography using either Kodak X-Omat AR or LS film, and by phosphorimager analysis (Molecular Dynamics Model 425E, using ImageQuant™ software version 3.2). The bands were assigned by reference to Maxam and Gilbert G sequencing marker lanes. The yields of strand break products were calculated by determining the fraction of the lane’s total radioactivity present in each strand break band and correcting for any alkali-lability of the corresponding guanine residue in the control oligonucleotide.

4.2.5 Cross-linking Reactions of NIAN and Mechlorethamine Hydrochloride with Duplex DNA

4.2.5.1 Preparation of Radiolabelled Duplex DNA

Duplex DNA (8 pmol) was prepared for oligo sequence (I) and (VI) by heating a mixture of the two complementary strands (1, 2, 5 or 10-fold excess of unlabelled strand) in 100 μl of water at 90°C for 2 min and slowly cooling to 37°C over 3 h.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

4.2.5.2 Confirmation of Duplex Formation by Non-Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

To assess duplex formation, non-denaturing sequencing PAGE was performed using the Model SA adjustable sequencing gel electrophoresis system as described for DPAGE analysis using a 20% non-denaturing polyacrylamide gel. The gel was prepared by mixing 37.5 ml of acrylamide/bisacrylamide solution (Accugel, 19:1), 5.6 ml of 10xTBE buffer and water to give a final volume of 50 ml. To induce polymerization, 350 µl of 10% aqueous ammonium persulphate and 17.5 µl of TEMED was added and the gel was poured and left to set for at least at 4 h. 2 µl from each stock duplex sample was added to an equal volume of non-denaturing gel-loading buffer (30% sucrose and 0.1% bromophenol blue in 1xTBE). Half of the sample was loaded onto the gel. The gel was run in TBE buffer at 500 V either overnight or until the bromophenol blue marker had migrated at least 4 cm into the gel. Gels were then fixed, dried and autoradiographed or subjected to phosphorimager analysis as described for reactions of NIAN with single-stranded oligonucleotides.

4.2.5.3 Cross-linking Reactions of Mechlorethamine Hydrochloride with Duplex DNA Sequence (VI): Measure of Duplex Availability

To assess duplex stability at 37°C within the 10 mM phosphate buffered reaction system and to assess the effect of the introduction of organic solvent to the reaction system, mechlorethamine hydrochloride was used as an internal standard.

Radiolabelled duplex (VI) (1 pmol in 25 µl 10 mM phosphate buffer, pH 7.4) was incubated at 37°C in the dark with solutions of mechlorethamine hydrochloride in water such that the total mechlorethamine hydrochloride concentration ranged from 0.3 to 12 µM in a total volume of 50 µl. Control incubations were set up of the duplex in buffer only. Reactions were stopped after 18 h by extraction twice with 2 volumes of either n-butyl alcohol or diethyl ether. Reaction mixtures were dried down in a centrifugal vacuum evaporator and resuspended in 4 µl of denaturing gel-loading buffer. To assess organic solvent effects on duplex stability, 10, 25 or 50 µl of acetonitrile was added to the 50 µl reaction mixture prior to the incubation stage.
4.2.5.4 Cross-linking Reactions of NIAN with Duplex DNA Sequence (I)

Radiolabelled duplex (1 pmol in 50 μl 5 mM phosphate buffer, pH 7.4) was incubated with 1 μl of NIAN dissolved in acetonitrile at 37°C in the dark, such that the total NIAN concentration ranged from 5 to 160 μM. Control incubations were set up of the duplex in buffer with acetonitrile only. Reactions were stopped after 18 h by extraction of NIAN twice with 2 volumes of diethyl ether. Reaction mixtures were dried down in a centrifugal vacuum evaporator and resuspended in 4 μl of denaturing gel-loading buffer.

4.2.5.5 Analysis of Cross-linked Samples by DPAGE, Data Analysis and Quantitation of Cross-links

Samples were not heated at 90°C prior to electrophoresis, to minimize thermally-promoted decomposition of the cross-linked DNA. Half of the sample volume of mechlorethamine hydrochloride or NIAN treated duplex DNA was analysed on denaturing 20% polyacrylamide gels followed by autoradiography or phosphorimager analysis as described for NIAN reactions with single-stranded oligonucleotides.

The yield of cross-links was calculated by determining the fraction of the lane’s total radioactivity present in each cross-linked product band.

4.2.6 Reactions of NIAN with single-stranded Oligonucleotide (V) and Calf Thymus DNA

Single-stranded oligonucleotide sequence (V) or calf thymus DNA (50 μg in 50 μl 0.5 mM Tris-HCl buffer, pH 7.4) was incubated at 37°C in the dark, with solutions of NIAN dissolved in acetonitrile such that the total NIAN concentration ranged from 0 to 10 mM in a total volume of 100 μl. Control incubations were set up without NIAN but inclusive of acetonitrile. After 6 or 24 h, reactions were stopped by extraction of NIAN 3 times with 3 volumes of diethyl ether, and dried down in a centrifugal vacuum evaporator. Control and treated DNA samples were incubated overnight at 37°C, with 1.75 units of micrococcal nuclease and 0.03 units of calf spleen phosphodiesterase in 100 μl of digestion buffer (10 mM sodium succinate, 5 mM calcium chloride, pH 6.0). Water was added to the digested samples to give a final volume of 200 μl prior to HPLC analysis.

Calibration curves for the quantitation of nucleotides and reaction products were determined by preparing stock solutions of the base or 3’-monophosphate (typically 0.5
mg dissolved in 1 ml of 0.1 M KOH or water). Dilutions were made so that appropriate injection volumes contained a known amount of product (typically 0.05 to 6 μg). Injections onto the HPLC were then performed according to the program described below.

**4.2.6.1 HPLC Analyses of Digested Reaction Mixtures**

HPLC-UV analyses were performed using a Hypersil C18 BDS, 5μ, 250x4.6 mm reverse-phase analytical column (Shandon) on a Gilson gradient-controlled system equipped with either a dual-wavelength 116 Gilson UV detector or to obtain UV spectra, an Applied Biosystems Inc., 1000s diode array detector. Aliquots of 100 μl of digested aqueous reaction mixture were analysed at a flow rate of 1 ml/min, using the following elution program: 0 min, 0%B, 15 min, 0%B, 40 min, 35%B, 45 min, 0%B (solvent A 0.1 M triethylammonium acetate, pH 5.0; solvent B methanol). Column eluants were monitored at 260 and 290 nm. The fractions corresponding to reaction products were pooled from multiple HPLC runs and dried down in a centrifugal vacuum evaporator. Resulting product fractions were lyophilised twice from 100 μl water/methanol (50:50, v/v) prior to analysis by electrospray mass spectrometry (ESI-MS). Reaction products were further identified from their UV spectra, obtained by diode array analysis and retention times, which were compared with those of the authentic standards.

**4.2.6.2 Characterisation of Reaction Products by Mass Spectrometry**

Off-line ESI-MS characterisation of reaction products was carried out using a VG Autospec-Ultima Q. Dried fractions previously collected from the HPLC, were resuspended in acetonitrile/water (50:50, v/v) and inserted into the mass spectrometer interface via 20 μl “loop” injection or continuous infusion, at a flow rate of typically, 8 μl/min. The cone voltage was in the range 8-23 V and full scan mass spectra were obtained by scanning from m/z 1 650 to 50 at a scan speed of 10 s/decade.
4.3 RESULTS

4.3.1 NIAN Reactions with single-stranded Oligonucleotides (Ia), (II), (III) and (IV)

NIAN-induced immediate and alkali-labile strand break products of 5’-32P-end labelled single-stranded oligonucleotides, were examined using high resolution DPAGE. The products were assigned by reference to the products of the Maxam and Gilbert sequencing reaction for specific modification at guanine residues.

NIAN (2 mM) treatment of radiolabelled oligo (III) for 18 h, resulted in 3 latent (piperidine treated) strand break products 8, 10 and 12 nucleotides (G9, G11 and G13 respectively) in length [Figure 4.1]. Treatment of radiolabelled oligo (IV) with 2 mM NIAN resulted in 4 latent strand break products 6, 7, 14 and 15 nucleotides in length (G7, G8, G15 and G16 respectively). Similarly, treatment of radiolabelled oligo (II) resulted in 3 latent strand break products 10, 11 and 12 nucleotides in length (G11, G12 and G13 respectively), and 2 latent strand break products 10 and 11 nucleotides in length (G11 and G12) resulted from 2 mM NIAN treatment of oligo (I) [Figure 4.1]. Co-migration of the resulting fragments with the products of the Maxam and Gilbert sequencing marker specific for reaction at guanine residues, combined with observations that immediate strand breakage did not occur, indicates that NIAN treatment was responsible for the generation of alkali-labile lesions at guanine residues within the radiolabelled oligonucleotide.

The yield of alkali-labile lesions at guanine residues induced by NIAN increased with NIAN concentration. Treatment of radiolabelled oligo (I) for 18 h with increasing NIAN concentrations (0.2 mM, 1 mM and 2 mM) resulted in a clear increase in intensity of the strand break bands at guanine residues G11 and G12 [Figure 4.2 (A)]. The yield of total strand break products ranged from 1.2 % (0.2 mM) to 19.6 % (2 mM) [Figure 4.2 (B)].

Treatment of oligo (II) with increasing NIAN concentration resulted in a clear increase in intensity of the strand break bands at guanine residues G11, G12 and G13 [Figure 4.3 (A)]. DPAGE analysis revealed a further minor product, the least electrophoretically mobile component of the reaction mixture in lane (N3-) [2 mM NIAN treatment, no piperidine treatment; Figure 4.3 (A)]. This higher molecular weight band is alkali-labile; the band disappears on piperidine treatment. The band may represent some kind of
oligonucleotide conformational structure that is resistant to the denaturing conditions employed. The yield of total strand break products ranged from 1.4% (0.2 mM) to 25.8% (2 mM) [Figure 4.3 (B)].

Similarly, treatment of oligo (III) with increasing NIAN concentration resulted in an increase in intensity of the strand break bands at guanine residues G9, G11 and G13 [Figure 4.4 (A)]. Samples in lanes (N3-) and (N3+) have run quite diffusely due to inconsistencies in the wells that resulted when the comb was removed from the gel. The yield of total strand break products ranged from 2.2% (0.2 mM) to 20.6% (2 mM) [Figure 4.4 (B)].

Finally, treatment of oligo (IV) with increasing NIAN concentration resulted in an increase in intensity of the strand break bands at guanine residues G7, G8, G15 and G16 [Figure 4.5 (A)]. As described for oligo (II) there is a higher molecular weight band than the band corresponding to single strand in lane (N3-), that disappears upon piperidine treatment. The yield of total strand break products ranged from 0.3% (0.2 mM) to 21.2% (2 mM) [Figure 4.5 (B)].

By analyzing the yield of strand breaks at each guanine residue it is evident that at NIAN concentrations of 2 mM, [G11]>[G12] in oligo (I) and [G11]>[G12]>[G13] in oligo (II). Yields in oligo (III) and (IV) follow the same pattern: [G9]>[G11]>[G13] and [G7]>[G8], [G15]>[G16] respectively.

An increase in intensity of the strand break bands at guanine residues G9, G11 and G13 in oligo (III) also resulted as incubation time increased [Figure 4.6 (A)]. Again, a higher molecular weight band as previously mentioned is evident, which disappears upon piperidine treatment. The yield of strand breaks in radiolabelled oligo (III) and induced by 2 mM NIAN, rose with time out to the longest timepoint measured, 30 h, reaching almost 31% at that time [Figure 4.6 (B)].
Figure 4.1 NIAN-induced strand break products of 5'-$^{32}$P-end labelled oligonucleotides. Products were resolved on a 20% denaturing polyacrylamide gel: lane (G), products of the Maxam and Gilbert sequencing reaction for cleavage at guanine sites; lane (-p), immediate strand break products due to 2 mM NIAN treatment for 18 h; lane (+p), strand break products upon piperidine treatment.
Figure 4.2 (A) Dose-dependent formation of strand break products at guanine residues G11 and G12 in NIAN treated oligo (I) for 18h: lanes (C), controls; lanes (N1), 0.2mM NIAN; lanes (N2), 1mM NIAN; lanes (N3), 2mM NIAN; (-) immediate strand break products, (+) strand break products upon piperidine treatment. (B) % single strand breakage at guanine residues G11 and G12 in NIAN treated oligo (I) for 18h; yields determined by phosphorimager analysis.
Figure 4.3 (A) Dose-dependent formation of strand break products at guanine residues G11, G12 and G13 in NIAN treated oligo (II) for 18 h: lanes (C), controls; lanes (N1), 0.2 mM NIAN; lanes (N2), 1 mM NIAN; lanes (N3), 2 mM NIAN; (-) immediate strand break products, (+) strand break products upon piperidine treatment. (B) % single strand breakage at guanine residues G11, G12 and G13 in NIAN treated oligo (II) for 18 h; yields determined by phosphorimager analysis.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Figure 4.4 (A) Dose-dependent formation of strand break products at guanine residues G9, G11 and G13 in NIAN treated oligo (III) for 18 h: lanes (C), controls; lanes (N1), 0.2 mM NIAN; lanes (N2), 1 mM NIAN; lanes (N3), 2 mM NIAN; (-) immediate strand break products; (+) strand break products upon piperidine treatment. (B) % single strand breakage at guanine residues G9, G11 and G13 in NIAN treated oligo (III) for 18 h; yields determined by phosphorimager analysis.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Figure 4.5 (A) Dose-dependent formation of strand break products at guanine residues G7, G8, G15 and G16 in NIAN treated oligo (IV) for 18 h; lanes (C), controls; lanes (N1), 0.2 mM NIAN; lanes (N2), 1 mM NIAN; lanes (N3), 2 mM NIAN; (-) immediate strand break products, (+) strand break products upon piperidine treatment. (B) % single strand breakage at guanine residues G7, G8, G15 and G16 in NIAN treated oligo (IV) for 18 h; yields determined by phosphorimager analysis.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Figure 4.6 (A) Strand break products of radiolabelled oligo (III) incubated with 2 mM NIAN for 6, 12, 24 and 30 h: lanes (C), controls; (-) immediate strand break products; (+) strand break products upon piperidine treatment. (B) Yield of total strand break products as a function of time. Yields determined by phosphorimager analysis.
4.3.2 Cross-Linking Reactions of Mechlorethamine Hydrochloride with Duplex DNA: Assessment of Duplex Availability

Duplex formation for oligo (VI) was examined by using high resolution non-denaturing sequencing PAGE. The difference in electrophoretic mobility of the double-stranded duplex compared to the single strand oligonucleotide was confirmation of successful duplex formation. Solutions containing differing ratios of $^{32}$P-labelled oligo (V Ia) to “cold” counter strand (V Ib) (1:0, 1:1, 1:5 and 1:10) were prepared and analysed. Duplex formation was successful at ratios of 1:1 and above [Figure 4.7 (A)]. For all cross-linking reactions, duplex prepared at a ratio of 1:1 were used.

The stability of the duplex at 37°C within a 10 mM phosphate buffered reaction system was assessed with the use of an internal cross-linking standard. Reaction of mechlorethamine hydrochloride with radiolabelled duplex (VI) containing a GGCC motif at physiological pH and temperature yielded cross-linked products that migrated slower through the gel when compared to unmodified single strands; monoalkylated single strands were also evident [Figure 4.7 (B)]. Lane (1) represents a control of the duplex in water alone. Lane (2) represents a control of the duplex in 10 mM phosphate buffer. Lanes (3-8) show the appearance of cross-linked product bands and monoalkylation product bands as mechlorethamine hydrochloride is introduced into the reaction system at concentrations ranging from 0.3 to 12 μM. All reaction product bands increased in intensity with increasing mechlorethamine hydrochloride concentration.

The generation of cross-linked material confirmed the availability of duplex substrate and verified that the duplex/single strand equilibria of duplex (VI) [complementary 12-mer oligonucleotides containing 2 GC base pairs] and hence any duplex prepared with at least 12-mer complementary oligonucleotides with a GC content of at least 33.3%, did reside predominantly on the side of the duplex under the conditions of physiological pH, temperature and ionic strength.

To assess the effects of NIAN co-solvent on duplex availability, 10, 25 or 50 μl of acetonitrile was added to the reaction of mechlorethamine hydrochloride (3, 6 or 9 μM) with duplex (VI) prior to incubation at 37°C for 18 h. To examine the effects of the extraction process on the stability of any cross-links formed, unreacted mechlorethamine hydrochloride was extracted with either n-butyl alcohol or diethyl ether after reaction.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Products were examined by high resolution denaturing PAGE and the phosphorimage of the resulting gel is shown in Figure 4.8.

Lane (1) represents a control of the duplex in water alone. Lanes (2, 3) represent a control of the duplex in the buffered reaction conditions with extraction with n-butyl alcohol and diethyl ether, respectively. Lanes (4-6) represent mechlorethamine hydrochloride treatment (3, 6 or 9 µM respectively) with n-butyl alcohol extraction. Lanes (7-9) represent mechlorethamine hydrochloride treatment (3, 6 or 9 µM respectively) with diethyl ether extraction. Examination of lanes (4-9) demonstrates that extraction of unreacted mechlorethamine hydrochloride with diethyl ether does not affect the stability of cross-links formed, as the intensity of cross-linked and monoalkylation bands in lanes (7-9) are analogous to those present in lanes (4-6). Consequently, it was deemed acceptable to remove any unreacted NIAN with diethyl ether after reaction as the process should not disrupt the stability of any cross-links formed.

Lanes (10-12) represent mechlorethamine hydrochloride treatment (3, 6 or 9 µM, respectively) with the addition of 10 µl of acetonitrile to the reaction system. Lanes (13-15) and lanes (16-18) represent equivalent mechlorethamine hydrochloride treatments (3, 6 or 9 µM) with the addition of 25 µl or 50 µl respectively. Lanes (10-18) show no cross-link formation but monoalkylation product bands are still evident. It is clear that the introduction of organic solvent into the reaction system had a severe effect upon the yield and hence availability of duplex substrate for cross-linking. The duplex/single strand equilibria appears to have shifted predominantly to the side of single strand. As a result, it was deemed necessary to use the minimum amount of co-solvent for NIAN when performing cross-linking reactions of NIAN with duplex DNA.
Figure 4.7 (A) Confirmation of duplex formation for sequence VI at ratios of 1:1, 1:5 and 1:10 [radiolabelled strand (VIa): “cold” counter strand (VIb)], as determined by high resolution non-denaturing PAGE. (B) Formation of mechlorethamine hydrochloride induced cross-linked DNA: lanes (1,2) controls; lanes (3-8) duplex (VI) exposed to mechlorethamine hydrochloride (0.3, 1, 3, 6, 9 and 12µM) as determined by high resolution denaturing PAGE.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Figure 4.8 Effect of co-solvent and diethyl ether extraction on the formation and stability of mechlorethamine hydrochloride induced cross-linked DNA: lanes (1-3) controls; lanes (4-6) mechlorethamine hydrochloride exposed duplex (VI) with n-butyl alcohol extraction; lanes (7-9) mechlorethamine hydrochloride exposed duplex (VI) with diethyl ether extraction; lanes (10-12), (13-15) and (16-18), mechlorethamine hydrochloride exposed duplex (VI) with the addition of 10, 25 and 50μl ACN respectively in the reaction system.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

4.3.3 Cross-Linking Reactions of NIAN with Duplex DNA

Duplex formation for oligonucleotide sequence (I) was examined by using high resolution non-denaturing PAGE. The difference in electrophoretic mobility of the double-stranded duplex compared to the single strand oligonucleotide was confirmation of successful duplex formation. Solutions containing differing ratios of 5′-32P-end-labelled oligonucleotide (Ib) to “cold” counter strand (Ia) (1:0, 1:1, 1:2, 1:5 and 1:10) were prepared and analysed. Duplex formation was successful at ratios of 1:1 and above [Figure 4.9 (A)].

Reaction of NIAN with radiolabelled duplex (I) containing a CCGG motif at physiological pH and temperature yielded cross-linked product that migrated more slowly through the gel than the unmodified single strands [Figure 4.9 (B)]. The cross-linked product had roughly half the electrophoretic mobility of the unmodified single strands. Lane (1) represents a control of the duplex in water alone. Lane (2) represents a control of the duplex in the reaction system used for cross-linking, comprising phosphate buffer and co-solvent. The cross-linking reaction system resulted in a minute amount of residual duplex being present after electrophoresis. Lanes (3-8) show a clear dose-dependent increase in the intensity of the slower migrating band as NIAN is introduced into the cross-linking system at concentrations ranging from 5 to 160 μM; the yields have been corrected for the residual duplex remaining in the control. Smearing or tailing of the single strands is also evident as NIAN is introduced into the reaction system, and is inversely proportional to NIAN concentration.

The yield of cross-linked duplex (I) exposed to NIAN rose with a roughly first order dependence on NIAN concentration in the range of 5 to 20 μM [Figure 4.9 (C)]. Above 20 μM, the yield continued to rise to almost 0.35%, but with an order in NIAN less than one.

For NIAN to be reactive, full solubilisation must be achieved. By increasing the concentration of NIAN in the reaction, but keeping the level of co-solvent (i.e acetonitrile) constant, at some point saturation of NIAN will occur. Increasing the volume of co-solvent in the cross-linking reaction system was not feasible due to destabilisation of the duplex, as described for cross-linking reactions with mechlorethamine hydrochloride (measure of duplex availability).
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Figure 4.9 (A) Confirmation of duplex formation for sequence I at ratios of 1:1, 1:2, 1:5 and 1:10 [radiolabelled strand (lb): 'cold' counter strand (la)], as determined by high resolution non-denaturing PAGE. (B) Formation of NIAN-induced cross-linked DNA: lanes (1,2) controls; lanes (3-8) duplex I exposed to NIAN (5, 10, 20, 40, 80 and 160 μM) as determined by high resolution denaturing PAGE. (C) Yield of interstrand cross-linked DNA as a function of NIAN concentration.
4.3.4 NIAN Reactions with Single-Stranded Oligonucleotide (V) and Calf Thymus DNA

Calibration curves of reaction products were determined by injecting known amounts of the bases onto the HPLC. The resulting curves for reaction products guanine, xanthine, oxanine and adenine [Figure 4.10 (A), (B), (C) and (D) respectively] were used to determine the quantity of reaction product after NIAN incubation with oligonucleotide or calf thymus DNA.

Calibration curves of the four 3'-monophosphates were determined by injecting known amounts of the 3'-monophosphates onto the HPLC. The resulting curves for dGp, dAp, thymidine-3'-monophosphate (Tp) and 2'-deoxycytidine-3'-monophosphate (dCp) [Figure 4.11 (A), (B), (C) and (D) respectively] were used to determine the quantity of each 3'-monophosphate remaining after NIAN incubation with oligonucleotide or calf thymus DNA.

4.3.4.1 NIAN Reactions with Single-Stranded Oligonucleotide (V)

Digestion of oligo (V) into 3'-monophosphates produced the four expected nucleotide peaks, dCp, dGp, Tp and dAp with retention times of 9.5, 22.8, 25.5 and 28.4 min respectively [Figure 4.12 (A)]. Enzymatic digestion with calf spleen phosphodiesterase resulted in a further peak in the chromatogram with a retention time of 9.0 min. This peak is a CT dinucleotide as the enzyme does not digest polynucleotides having 5'-monoester end groups. Thus one-quarter of T’s and one-sixth of C’s within the sequence of oligonucleotide (V) elute as a CT dinucleotide rather than the individual mononucleotides.

Reaction of NIAN with oligonucleotide (V) at pH 7.4 in buffered 50% aqueous acetonitrile for 6 h, followed by enzymatic digestion into 3'-monophosphates, yielded 3 products not seen in control incubations, guanine, xanthine and adenine with retention times of 7.0, 7.6 and 14.1 min, respectively [Figure 4.12 (B) and (C)]. Identification of guanine, xanthine and adenine were all based on comparison of retention times and UV spectra with those of authentic standards analysed under the same conditions, exactly as described for the identification of reaction products of nitrosated indoles with purine nucleotides in chapter 3. When ESI-MS was performed on the fraction corresponding to guanine, a molecular ion with m/z 152 [M + H]^+ was observed. When ESI-MS was
performed on the fractions corresponding to xanthine and adenine, molecular ions with m/z 153 [M + H]^+ and m/z 136 [M + H]^+ respectively, were observed.

After 24 h incubation, a further reaction product oxanine appeared in the chromatogram with a retention time of 11.8 min [Figure 4.13 (B) and (C)]. This product was not present in control incubations [Figure 4.13 (A)]. Identification of this reaction product was based on comparison of retention time, UV spectrum and ESI-MS results when compared with oxanine derived from the hydrolysis of the N-glycosidic bond of authentic 2'-deoxyoxanosine, exactly as described for the identification of oxanine observed in the reactions of purine nucleotides with nitrosated indoles in chapter 3.

Peaks eluting in the chromatograms after 28.4 min (6 or 24 h incubation) are likely to represent unreacted NIAN and NIAN decomposition products and possible N-acetyl adducts.

The yield of reaction products resulting from NIAN exposed oligo (V) increased with NIAN concentration and with time [Figures 4.12 (B) and (C) and Figures 4.13 (B) and (C)]. HPLC analysis was used to assess the reactivity of the residues within the macromolecule upon treatment with NIAN over a range of concentrations (0 to 10 mM).

A gradual loss of the parent nucleotides dGp and dAp was observed with increasing NIAN concentration after 6 h incubation [Figure 4.14 (A)]. Reaction products guanine, xanthine and adenine increased in concentration with the accompanying decrease in concentration of dGp and dAp [Figure 4.14 (B)]. After 10mM NIAN treatment for 6 h, the yield of guanine, xanthine and adenine was 2.4, 2.8 and 1.8 nmoles respectively. The yields of nucleotides dCp and Tp remained relatively constant after 6 h incubation regardless of the NIAN concentration used in the treatment [Figure 4.14 (C)].

After 24 h incubation with NIAN, the loss of parent nucleotides dGp and dAp is no longer gradual but decreases rapidly [Figure 4.15 (A)]. In control incubations, the amount of dGp and dAp present after enzymatic digestion was 39.9 and 26.1 nmoles respectively. Upon 10 mM NIAN treatment for 24 h, the amount of dGp and dAp remaining had decreased to 0.9 and 1.9 nmoles respectively. Consequently, reaction products guanine, xanthine and adenine increased in concentration rapidly with increasing NIAN concentration [Figure 4.15 (B)]. The yield of reaction products guanine, xanthine and adenine was 15.1, 3.3 and 13.9 nmoles respectively after 10 mM NIAN treatment for 24 h.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

The formation of oxanine in NIAN treated oligo (V) increased with increasing NIAN concentration after 24 h incubation up to 8 mM [Figure 4.15 (C)] with a yield of 3.6 pmoles. The yields of oxanine were over 1 000 times less than those observed for depurination and deamination products. At 10 mM however, the yield had decreased to 2.2 pmoles. This reduction in yield is most likely due to general degradation of the products within the reaction mixture after 24 h incubation and this degradation is supported by the dose-response of the pyrimidine nucleotides. After 24 h incubation with NIAN, the yields of dCp and Tp no longer remained relatively constant as was observed after 6 h incubation, but gradually decreased with increasing NIAN concentration [Figure 4.15 (D)] with dCp seemingly more sensitive to the reaction conditions than Tp.

4.3.4.2 NIAN Reactions with Calf Thymus DNA

Control incubations of calf thymus DNA at pH 7.4 in buffered 50% aqueous acetonitrile, followed by enzymatic digestion into 3'-monophosphates produced the 4 expected nucleotide peaks: dCp, dGp, Tp and dAp with retention times of 9.0, 22.3, 25.2 and 28.2 min respectively [Figure 4.16 (A)].

Analogous reaction products, with analogous dose-responses to those described for reactions of NIAN with oligo (V), were seen with reactions of NIAN with calf thymus DNA. Reaction of NIAN with calf thymus DNA at pH 7.4 in buffered 50% aqueous acetonitrile for 6 h, followed by enzymatic digestion into 3'-monophosphates, yielded 3 products not seen in control incubations, guanine, xanthine and adenine with retention times of 6.6, 7.2 and 13.6 min, respectively [Figure 4.16 (B) and (C)].

As described for the reactions of NIAN with oligo (V), identification of all reaction products were based on comparison of retention times, UV spectra and ESI-MS results with those of authentic standards analysed under the same conditions.

After 24 h incubation, a further reaction product oxanine appeared in the chromatogram with a retention time of 12.4 min [Figure 4.17 (B) and (C)]. This product was not seen in control incubations [Figure 4.17 (A)].

Peaks eluting in the chromatograms after 28.2 min are likely to be due to impurities present in the calf thymus DNA or unreacted NIAN and NIAN decomposition products.

The yield of reaction products resulting from NIAN exposed calf thymus DNA increased with NIAN concentration and with incubation time [Figures 4.16 (B) and (C) and Figures

137
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

4.17 (B) and (C). Loss of the parent nucleotides dGp and dAp was observed with increasing NIAN concentration after 6 h incubation as conversion to guanine, xanthine and adenine occurred [Figure 4.18 (A) and (B)]. After 10 mM NIAN treatment for 6 h, the yield of guanine, xanthine and adenine was 3.0, 1.7 and 4.8 nmoles respectively. The yield of pyrimidine nucleotides dCp and Tp decreased very gradually over the 10 mM NIAN treatment range after 6 h incubation [Figure 4.18 (C)].

After 24 h incubation, the loss of parent nucleotides dGp and dAp, is especially marked at NIAN concentrations of 4 mM and above [Figure 4.19 (A)]. In control incubations, the amount of dGp and dAp present after enzymatic digestion was 15.4 and 19.1 nmoles, respectively. NIAN treatment of 10 mM for 24 h resulted in 0.5 nmoles of dGp and 0.8 nmoles of dAp only, remaining. Consequently, reaction products guanine, xanthine and adenine increased rapidly in concentration at NIAN treatments of 4 mM and above [Figure 4.19 (B)]. As the supply of active sites becomes exhausted (as represented by the nmoles of dGp and dAp remaining) the yield of reaction products changes very little at the higher NIAN treatments.

The formation of oxanine in NIAN treated calf thymus DNA increased with increasing NIAN concentration after 24 h incubation up to 8 mM [Figure 4.19 (C)] with a yield of 1.3 pmoles. The yields of oxanine were again 1 000 times less than those observed for depurination and deamination products and less than half the yields observed for NIAN reactions with oligo (V). At 10 mM, the yield of oxanine had decreased to 1.1 pmoles and as previously described for NIAN reactions with oligo (V), this decrease is most likely due to general degradation of the products within the reaction mixture after 24 h incubation. The dose-response behaviour of the pyrimidine nucleotides again supports this degradation, because after 24 h incubation, the yields of dCp and Tp gradually decreased with increasing NIAN concentration [Figure 4.19 (D)] with dCp seemingly more sensitive to the reaction conditions than Tp.
Figure 4.10 Calibration curves for the quantitation of reaction products in NIAN treated oligonucleotide and calf thymus DNA: (A) Guanine, (B) Xanthine, (C) Oxanine and (D) Adenine; HPLC conditions as described under "Experimental Procedures".
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

(A)

Peek Area

1500000
1000000
500000

y = 262293x
R² = 0.9964

0 2 4 6
ug (dGp)

(B)

Peek Area

2000000
1500000
1000000
500000

y = 321595x
R² = 0.9964

0 1 2 3 4 5
ug (dAp)

(C)

Peek Area

1500000
1000000
500000

y = 217056x
R² = 0.9925

0 2 4 6
ug (Tp)

(D)

Peek Area

1000000
800000
600000
400000
200000

y = 148994x
R² = 0.9999

0 2 4 6
ug (dCp)

Figure 4.11 Calibration curves for the quantitation of dNps in NIAN treated oligonucleotide and calf thymus DNA: (A) dGp, (B) dAp, (C) Tp and (D) dCp; HPLC conditions as described under “Experimental Procedures”.

140
Figure 4.12 HPLC separation of NIAN and oligo (V) reaction mixture after 6h incubation and enzymatic digestion to 3'-monophosphates. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm. (A) Control; (B) 4 mM NIAN treatment; (C) 8 mM NIAN treatment. The box shows a magnified part of the chromatogram (11.5-13.5 min).
Figure 4.13 HPLC separation of NIAN and oligo (V) reaction mixture after 24 h incubation and enzymatic digestion to 3'-monophosphates. HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm. (A) Control; (B) 4 mM NIAN treatment; (C) 8 mM NIAN treatment. The box shows a magnified part of the chromatogram (10.5-12.5 min).
Figure 4.14 Quantitation of parent nucleotides and reaction products in NIAN treated oligo (V) for 6 h, as a function of NIAN concentration as monitored by HPLC analysis: (A) loss of dGp and dAp; (B) increase in the production of Gua, Xan and Ade; (C) no change in dCp or Tp.
Figure 4.15 Quantitation of parent nucleotides and reaction products in NIAN treated oligo (V) for 24 h, as a function of NIAN concentration as monitored by HPLC analysis: (A) loss of dGp and dAp; (B) increase in the production of Gua, Xan and Ade; (C) production of Oxa and (D) gradual loss of dCp and Tp.
Figure 4.16 HPLC separation of NIAN and calf thymus DNA reaction mixture after 6 h incubation and digestion to 3'-monophosphates. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm. (A) Control; (B) 4 mM NIAN treatment; (C) 8 mM NIAN treatment. The box shows a magnified part of the chromatogram (11.5-13.5 min).

145
Figure 4.17 HPLC separation of NIAN and calf thymus DNA reaction mixture after 24 h incubation and enzymatic digestion to 3'-monophosphates. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm. (A) Control; (B) 4 mM NIAN treatment; (C) 8 mM NIAN treatment. The box shows a magnified part of the chromatogram (11.5 to 13.5 min).
**Figure 4.18** Quantitation of parent nucleotides and reaction products in NIAN treated calf thymus DNA for 6 h, as a function of NIAN concentration as monitored by HPLC analysis: (A) loss of dGp and dAp; (B) increase in the production of Gua, Xan and Ade; (C) slight loss of dCp and Tp.
Chapter 4: Reactions of NIAN with Oligomers, Duplex and CT DNA

Figure 4.19 Quantitation of parent nucleotides and reaction products in NIAN treated calf thymus DNA for 24 h, as a function of NIAN concentration as monitored by HPLC analysis: (A) loss of dGp and dAp; (B) increase in the production of Gua, Xan and Ade; (C) production of Oxa and (D) gradual loss of dCp and Tp.
4.4 DISCUSSION

Guanine residues within both single-stranded oligomers and calf thymus DNA were reactive nucleophilic targets for transnitrosation by NIAN resulting in alkali-labile lesions, which increased with NIAN concentration and with incubation time. HPLC analysis confirmed these lesions as apurinic sites due to the detection of the bases released. Modification at guanine residues via depurination, deamination to form xanthine and the formation of oxanine were apparent, with the former pathway predominating.

Similarly, adenine residues were also reactive nucleophilic targets for nitroso group transfer, although to a lesser extent than guanine residues, with modification via depurination. It is likely that formation of the deamination product of adenine, hypoxanthine, also occurred but was at the limits of detection under the HPLC conditions employed.

4.4.1 The Spectrum of Potentially Mutagenic Products is Preserved at the Macromolecular Level

The results demonstrate that the reactivity of the purine residues towards nitroso transfer by NIAN is preserved at the macromolecular level, both in single-stranded oligonucleotides and calf thymus DNA. No appreciable differences in reaction product yields (with the exception of the formation of oxanine) were observed for reactions of NIAN with single-stranded oligonucleotides and calf thymus DNA.

Due to NIAN causing depurination, which was the major reactive pathway, especially at guanine residues, it was not feasible to study NIAN reactions with duplex oligonucleotides, other than for the detection of cross-links. Depurination results in a loss of Watson-Crick base-pairing and consequently, throughout reactions of NIAN with duplex, the equilibrium of the duplex will gradually shift to the single stranded form. As a result, the reactivity of the single strand would contribute to the observed reactivity of the double-stranded macromolecule, giving inconclusive results. Furthermore, to react NIAN at concentrations of 0.2 mM to 2 mM with the duplex for comparison with single strand, an increase in the amount of co-solvent would be necessary to achieve NIAN solubilisation. This was not feasible due to > 1% co-solvent within the reaction system destroying the stability of the duplex.
Cytosine residues, along with thymine residues, appear to be inactive residues for nitroso transfer by NIAN. Reactions of single-stranded oligonucleotides with NIAN and subsequent piperidine treatment indicated no modification to cytosine residues had occurred. In addition, HPLC analysis of NIAN treated calf thymus DNA or oligonucleotide showed no evidence of uracil formation. These observations suggest that transnitrosation to purine bases in DNA by nitrosated indoles may be mediated by intercalation, whereby the nitroso group is most efficiently transferred when the indole and nucleophilic site are in close proximity.

It has previously been shown that nitric oxide-induced deamination of cytosine and guanine residues in double-stranded oligomers was 10-fold less than for single-stranded oligomers (Caulfield et al. 1998). The lack of accessibility of the exocyclic amino groups, combined with base-pairing when these residues are in the internal position of the double helix, would contribute to the decrease in reactivity of these groups. However, in this study, the yield of xanthine was not appreciably different in single-stranded oligonucleotides compared to calf thymus DNA, suggesting that deamination of the guanine base can occur directly after depurination events.

A further possible fate of xanthine and hypoxanthine is depurination to form an abasic site. Evidence of this event, combined with depurination of guanine and adenine residues directly, is presented in this study. Nitroso group transfer from NIAN to the N-7 atom of guanine and adenine residues to induce depurination is clearly the most dominant pathway. The yield of depurination products is not significantly lower in single-stranded oligonucleotides compared to calf thymus DNA, as the N-7 position of the purine residues is exposed within the major groove of the double-helix. Any protective effects due to base-pairing within the double-stranded macromolecule will have little influence on the reactivity of these positions in the purine residues.

At NIAN concentrations greater or equal to 1 mM, guanine residues nearest the 5'-end of the oligomers appeared to be more reactive and this reactivity decreased from the 5'- to 3'-end. However, this is likely to be an artefact of the experimental procedure as oligomers containing two or more depurination sites will only be detected as a single strand-break product with a bias toward the site nearest the 5'-end label.

The formation of oxanine was detected in both NIAN treated single-stranded oligonucleotide and calf thymus DNA, with yields more than 1000 times smaller than
those observed for deamination and depurination products. In addition, the yields of oxanine observed in calf thymus DNA were less than half the yields observed for NIAN reactions with single-stranded oligonucleotide. These observations suggest that the ring-opening reaction (as described in section 3.4.1.3) is somewhat quenched in the double-stranded macromolecule.

Since the yield of xanthine is not appreciably different in both environments, and the formation of xanthine and oxanine is influenced by nitroso transfer to the exocyclic amino groups, then either: deamination of guanine to form xanthine mainly occurs directly (and this pathway to form oxanine directly is not favoured), or the ring-opening reaction is quenched, when the guanine is in the conformation provided by the double-stranded macromolecular environment coupled with any intercalation afforded by NIAN prior to nitroso transfer. The isolation and characterisation of 2'-deoxyxanosine from 2'-deoxyguanosine, oligodeoxynucleotide and calf thymus DNA treated with nitrous acid and nitric oxide, has been reported (Suzuki et al. 1996). In the study by Suzuki, interestingly, the yield of 2'-deoxyxanosine was not appreciably different between oligonucleotide and DNA, and much higher (around 25 to 30% of total products) compared with the yields observed for NIAN reactions.

4.4.2 Dose-Dependent Cross-Links at CG residues in Duplex DNA are Induced by Nitroso Transfer by NIAN

Diazotisation of the N-2 atom of guanine is considered to be the key intermediate process for the formation of xanthine and the ring-opened product oxanine (Suzuki et al. 1997a). A further consequence of purine diazonium ion in double-stranded DNA is that formation of interstrand cross-links may result. In the investigation for cross-link formation, mechlorethamine hydrochloride was used as an internal standard to ensure duplex formation and reaction conditions were such that cross-links could be identified if this pathway was relevant for NIAN. Mechlorethamine [bis(2-chloroethyl)methylamine] belongs to a class of compounds known as the nitrogen mustards that are bifunctional alkylating agents, used extensively in cancer chemotherapy, which induce interstrand cross-links in DNA, most abundantly at the sequence 5'-GNC (Ojwang et al. 1989; Grueneberg et al. 1991; Rink et al. 1993).

Cross-link formation was demonstrated to be a relevant pathway for NIAN. Dose-dependent cross-links at CG residues in duplex DNA were induced by NIAN. The effects
of the depurination pathway will influence the yield of cross-links; depurination would have resulted in a loss of Watson-Crick base pairing and consequently a loss of duplex substrate, and more specifically, a loss of guanine residues available for cross-linking.

Cross-link formation between deoxyguanosine residues at the sequence 5'-CG induced by nitrous acid in duplex DNA has been reported (Kirchner et al. 1992), whereby the covalent nucleus of the linkage results from the presence of a residue in which the original deoxyguanosyl residues on opposite strands are linked through a single N-2 atom common to both. The structure of this cross-link is shown in Scheme 4.2.

![Scheme 4.2 Covalent structure of the dG-to-dG cross-link at the sequence 5'-CG induced by nitrous acid (Kirchner et al. 1992). Original deoxyguanosyl residues on opposite strands are cross-linked through a single N-2 atom common to both.](image)

The cross-link induced by NIAN may be structurally identical. The detection of NIAN-induced cross-links suggests that an additional fate of purine diazonium ion in double-stranded DNA, is displacement of the ion by the exocyclic amino group of guanine on the opposing strand as described by Kirchner and co-workers (Kirchner et al. 1992), resulting in the same cross-link covalent structure (Scheme 4.3).

However, it is possible that the cross-link may also be derived from ring opened guanine intermediates following deazioniation of the guanine diazonium ion. Since none of the DNA base diazonium ions have been observed or characterised directly, theoretical studies of DNA base deamination have shown pyrimidine ring cleavage upon guanine ion deazioniation (Glaser and Son 1996; Glaser et al. 1999). Alternatively, the NIAN-induced cross-link may be derived from the final product oxanine, which is chemically reactive (Suzuki et al. 2000). Suzuki and co-workers have identified and characterised a stable ring-opened adduct between 2'-deoxyoxanosine and glycine (Suzuki et al. 2000).
Oxanine has an $O$-acylisourea six-membered ring structure present in the base moiety which can react with various primary amines affording ring-opened adducts linked by an amide bond. An alternative mechanism for cross-linking via ring-opened intermediates is shown in Scheme 4.4.

In postlabelling studies, the cross-linked dimer would probably appear as a bulky adduct and this may explain the observation by Yamashita and co-workers of DNA adducts formed by reaction of NIAN with calf thymus DNA (Yamashita et al. 1988).

Scheme 4.3 Proposed mechanism for formation of NIAN-induced cross-links at CG residues: direct diazonium ion chemistry; displacement of the diazonium ion by the exocyclic amino group of guanine on the opposing strand.
Scheme 4.4 Alternative reaction mechanism for formation of NIAN-induced cross-links at CG residues: via ring-opened guanine or oxanine intermediates.

In summary, at physiological pH and temperature, the transnitrosating ability of NIAN to modify purine residues is preserved at the macromolecular level, with guanine residues appearing to be a primary site of reaction. By using a more complex reaction system, in addition to modification via depurination, deamination and the formation of oxanine, DNA damage in the form of interstrand cross-links induced by NIAN were detected.

The results indicate that N-nitrosoindoles are efficient transnitrosating agents causing a variety of DNA damage products, of which all are potentially mutagenic if they occur in vivo.
CHAPTER 5

DETECTION OF APURINIC RESIDUES INDUCED BY 1-NITROSOINDOLE-3-ACETONITRILE IN CALF THYMUS DNA AND IN THE GLANDULAR STOMACH OF CD-1 MICE BY $^{32}$P-POSTLABELLING
5.1 INTRODUCTION

In chapters 3 and 4, it has been demonstrated that \(N\)-nitrosoindoles induce depurination, deamination and the formation of a novel nucleobase oxanine, in isolated purine nucleotides, oligonucleotides and calf thymus DNA via a transnitrosation mechanism.

In this study, the ability of NIAN to transfer the nitroso group to nucleophilic purine nitrogen atoms in DNA to induce depurination (which appears to be the major modification pathway), and hence the formation of abasic sites, at low levels of NIAN, is investigated. The depurination pathway, demonstrating the equilibrium nature of the abasic site formed is described in Scheme 5.1.

![Scheme 5.1](image)

**Scheme 5.1** The depurination pathway induced by nitroso transfer from NIAN to the nucleophilic \(N\)-7 atom of guanine. The abasic site generated is not a chemically unique species.
Chapter 5: \(^{32}\)P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

As previously described in section 3.4.1.1, depurination is probably catalysed by \(N\)-nitrosation at the N-7 atom of guanine or adenine residues and/or the N-3 atom of adenine, imparting a destabilising positive charge on the purine ring system. Cleavage of the \(N\)-glycosidic bond neutralises this charge to induce depurination and the formation of an abasic site. The corresponding \(N\)-nitrosopurine is then rapidly hydrolysed to generate the observed base. The resulting abasic site is not a chemically unique species but is an equilibrium mixture of \(\alpha\)-(I) and \(\beta\)-(II) hemi-acetals that are 2-deoxy-D-erythropentofuranoses of aldehyde (III) and its hydrated form (Scheme 5.1).

A \(^{32}\)P-postlabelling approach based on a method described by Weinfeld and co-workers (Weinfeld et al. 1990) for the quantitation of abasic sites in DNA was used to quantify base damage induced by NIAN. The assay is based on previous observations that certain lesions in DNA, such as abasic sites, prevent the hydrolysis by snake venom phosphodiesterase (SVPD) and DNase I of adjacent 5'-internucleotide phosphodiester linkages (Liuzzi et al. 1989; Weinfeld et al. 1989). Consequently, digestion of damaged DNA with these enzymes and shrimp alkaline phosphatase (SAP) generates lesion-containing “dinucleoside” monophosphates (dNpS) where the abasic site (S) is 3' to a normal nucleoside 3’-monophosphate moiety (dNp-). As this moiety is unmodified, these dimers are good substrates for T4 polynucleotide kinase (T4 PNK) and [\(\gamma\)-\(^{32}\)P]ATP, allowing for their exclusive labelling and detection as 5’-\(^{32}\)P end-labelled dimer species (d-\(^{32}\)pNpS). The remaining undamaged bases are recovered as mononucleosides, which are not phosphorylated by this kinase. The labelled dimer species are easily resolved and quantified by DPAGE. The strategy for this postlabelling assay is summarised in Scheme 5.2.

As the formation of depurination products is mediated by transfer of the nitroso group to nucleophilic nitrogen atoms on the purine bases, the influence of azide ion, a scavenger of nitrosating agents, on inhibiting this reaction pathway, was investigated.

Evidence to suggest that this reaction pathway is relevant for inducing DNA damage in vivo, in mouse stomach, the most likely organ to be affected by endogenous nitrosation of indole compounds, is also presented in this chapter.
The final objective of this study was to examine the influence of damage-recognising enzymes (*E. coli* exonuclease III and *E. coli* endonuclease IV) on the NIAN-induced damage observed both in NIAN treated calf thymus DNA and in the DNA of the glandular stomach of CD-1 mice treated with NIAN. Both *E. coli* exonuclease III and *E. coli* endonuclease IV are class II AP endonucleases which initiate repair of apurinic residues in double stranded substrates by cleaving the phosphodiester bond immediately 5' to the AP site generating a normal 3'-hydroxyl group (Singer and Hang 1998; Ramotar and Demple 1998).
Chapter 5: $^3$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

5.2 EXPERIMENTAL PROCEDURES

Caution: NIAN is mutagenic and should be handled with extreme caution. Acrylamide and sodium azide are hazardous chemicals and care should be exercised in their handling.

5.2.1 Enzymes and General Materials

Exonuclease III (100 units/μl) and endonuclease IV (nfo protein; 10 units/μl) were purchased from Trevigen Inc.; DNase I (10 000 units/ml), snake venom phosphodiesterase (SVPD, Crotalus atrox, type IV, 10 units/g), micrococal nuclease (500 units/ml), proteinase K (9.8 units/mg), RNase A (2 470 Kunitz units/ml) and RNase T1 (1 250 units/μl) were purchased from Sigma; shrimp alkaline phosphatase (SAP, 1 unit/μl) and T4 polynucleotide kinase (T4 PNK, 10 units/μl) were purchased from Amersham; calf spleen phosphodiesterase (2 units/mg) was purchased from Boehringer Mannheim. Unit definitions are as follows: exonuclease III, 1 unit produces cleavage of the AP-site oligonucleotide at the rate of 1 nmol per 30 min at pH 7 at 37°C; endonuclease IV, 1 unit produces cleavage of the AP-site oligonucleotide at the rate of 100 fmol per h at pH 7.4 at 37°C; proteinase K, 1 unit will hydrolyse casein to produce colour equivalent to 1.0 μmole (181 μg) of tyrosine per min at pH 7.5 at 37°C; RNase A, activity is the general degradation of RNA; RNase T1, 1 unit will produce acid soluble oligonucleotides equivalent to a change in absorbance at 260 nm of 1.0 in 15 min per ml at pH 7.5 at 37°C; micrococcal nuclease, 1 unit produces 1.0 μmol of polynucleotides from native DNA per minute at pH 8.8 at 37°C; DNase 1, 1 unit increases the absorbance of calf thymus DNA solution (50 μg/ml) at a rate of 0.001 A260 units per min per ml at pH 5.0 at 25°C; SVPD, 1 unit hydrolyses 1.0 μl of bis(p-nitrophenyl) phosphate per minute at pH 8.8 at 37°C; calf spleen phosphodiesterase, 1 unit releases 3'-mononucleotides equivalent to an increase in absorbance at 260 nm of 16 in 30 min at pH 6.5 at 37°C; SAP, 1 unit hydrolyses 1 μmol of p-nitrophenyl phosphate per min at pH 10.7 at 37°C; T4 PNK, 1 unit catalyses the transfer of 1 nmol of phosphate from ATP to polynucleotide in 30 min at pH 7.6 at 37°C. HPLC solvents were purchased from Fisher Scientific, U.K. All other chemicals were purchased from Sigma Chemical Co., unless otherwise stated.
Chapter 5: \(^{32}\text{P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA}\)

5.2.2 Synthesis and Characterisation of NIAN

NIAN was synthesized by nitrous acid treatment of IAN (Fluka), purified by HPLC and fully characterised by \(^1\text{H-NMR, mass spectrometry and microanalysis as described in chapter 2.}\)

5.2.3 Reactions of NIAN with Calf Thymus DNA

Calf thymus DNA (20 µg in 40 µl 5 mM Tris-HCl buffer, pH 7.4) was incubated at 37°C in the dark, with solutions of NIAN dissolved in DMSO or acetonitrile such that the total NIAN concentration ranged from 12.5 to 800 µM, or 2 to 8 mM in a total volume of 80 µl. Reactions were stopped after 16 h by addition of 8 µl of sodium acetate solution (2.5 M, pH 7), followed by 1 ml of absolute ethanol (-20°C). After 3 h at -20°C, the resulting precipitate was pelleted by centrifugation and the supernatant was discarded. To ensure that all traces of NIAN had been removed, pellets were washed twice with 1 ml of 70% ethanol. The resulting pellets were dried in a centrifugal vacuum evaporator (DNA 110, Savant) and resuspended in 20 µl of water prior to postlabelling. Control incubations were set up of calf thymus DNA in buffer with NIAN co-solvent only.

5.2.4 Azide Inhibition Experiments

Solutions of calf thymus DNA (20 µg in 20 µl of water) and sodium azide dissolved in 20 µl of 10 mM Tris-HCl, pH 7.4 were prepared such that the total azide concentration ranged from 0 to 800 µM in a total aqueous volume of 40 µl. These solutions were incubated at 37°C in the dark, with solutions of NIAN dissolved in DMSO, such that the NIAN concentration remained constant at 800 µM in the final reaction volume of 80 µl. Control incubations were set up of calf thymus DNA in buffer with azide ion only. Reactions were stopped after 16 h and the DNA recovered by ethanol precipitation as described for the reactions of NIAN with calf thymus DNA. Resulting pellets were resuspended in 20 µl of water prior to postlabelling.

5.2.5 Animal Study

The housing of animals, animal dosing, animal sacrifice and animal stomach removal was carried out at GlaxoWellcome Research and Development, Ware, UK, under the direction of the Genetic and Reproductive Toxicology Group within the division of Medicines.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Safety and Evaluation. DNA isolation from the glandular stomach and subsequent treatments and postlabelling were carried out at Leicester.

5.2.5.1 Animals

Six-week-old male CD-1 mice were supplied by Charles River UK Ltd., (Kent). The mice were housed in plastic cages and kept at standard conditions (20-22°C, 45-70% relative humidity, 12 h light/dark cycle) prior to treatment. Standard diet and drinking water was given to the animals.

5.2.5.2 Treatment of Animals

NIAN was prepared as a heavy suspension in 10% DMSO/water just before administration. The selection of the dosage (100 mg/kg) was based on results of a previous adduct study carried out by Yamashita and co-workers (Yamashita et al. 1988). A control group of 5 male mice received the vehicle alone. Twenty male mice received a single dose of NIAN (100 mg/kg) orally by gavage. The dosing volume was 10 ml/kg. Ten of the NIAN treated animals were killed after 3 h post dosing by cervical dislocation. The remaining ten NIAN treated animals and control animals were killed 24 h post dosing by cervical dislocation.

5.2.5.3 DNA Isolation from Glandular Stomach

The stomach was promptly isolated, snap frozen in liquid nitrogen and stored at −80°C until DNA isolation. Stomachs were defrosted, the contents washed away using phosphate buffered saline and the mucosa of the glandular stomach was gently removed using a spatula. DNA was isolated from the mucosa of the glandular stomach as described in the manufacturer’s manual for DNA isolation from tissue using 500/G Maxi tips (Qiagen 1997) with the following modifications.

After the tissue was homogenized, 318 units of proteinase K, 800 units of RNase A and 2500 units of RNase T₁ were added. The homogenates were incubated at 45°C for 2 h.

After the DNA recovery step, the resulting DNA pellets were dissolved in water and quantified by measuring the absorbance at 260 nm using a Gene Quant spectrophotometer (Pharmacia Biotech). DNA was stored at −80°C at a concentration of 1 μg/μl prior to postlabelling. To check the effect of the DNA isolation procedure on the quality of the resulting DNA, 350 μg of calf thymus DNA (1 μg/μl in water) was put through the Qiagen DNA isolation procedure from the homogenisation stage of the protocol (Qiagen 1997).
5.2.5.4 Purity Check of Mouse Stomach DNA by HPLC

Control calf thymus DNA, calf thymus DNA that had been through the Qiagen DNA isolation procedure and mouse stomach DNA (10 μg) were incubated overnight at 37°C, with 0.35 units of micrococcal nuclease and 0.006 units (6 mU) of calf spleen phosphodiesterase in 20 μl of digestion buffer (10 mM sodium succinate, 5 mM calcium chloride, pH 6.0). Water was added to the digested samples to give a final volume of 100 μl prior to HPLC analysis.

HPLC-UV analyses were performed using a Hypersil C18 BDS, 5μ, 250x4.6 mm reverse-phase analytical column (Shandon) on a Gilson gradient-controlled system equipped with a dual-wavelength 116 Gilson UV detector. Digested aqueous samples (100 μl) were analysed at a flow rate of 1 ml/min, using the following elution program: 0 min, 0% B, 10 min, 0% B, 28 min, 25% B, 30 min, 0% B (solvent A: 0.1 M triethylammonium acetate, pH 5.0; solvent B methanol). Column eluants were monitored at 260 nm.

5.2.6 Incubation of DNA Samples with Damage-Recognising Enzymes

5.2.6.1 Incubation of DNA Samples with E.coli Exonuclease III

Control or NIAN treated calf thymus DNA, mouse stomach DNA or 50 Gy irradiated DNA (10 μg in 10 μl of water) was incubated for 1 h at 37°C with 200 units of exonuclease III in 90 μl of buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM MgCl₂ and 1 mM EDTA. Controls were incubated in the absence of the enzyme. Samples were dried down in a centrifugal vacuum evaporator (DNA 110, Savant) and resuspended in 10 μl of water prior to postlabelling.

5.2.6.2 Incubation of DNA Samples with E.coli Endonuclease IV

Control or NIAN treated calf thymus DNA, mouse stomach DNA or 50 Gy irradiated DNA (10 μg in 10μl of water) was incubated for 16 h at 37°C with 50 units of endonuclease IV in 20 μl of buffer containing 10 mM HEPES-KOH (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]-KOH) (pH 7.4) and 100 mM KCl. Controls were incubated in the absence of the enzyme. DNA was recovered by ethanol precipitation, as described for reactions of NIAN with calf thymus DNA, and resuspended in 10 μl of water prior to postlabelling.
5.2.6.3 Preparation of 50 Gy Irradiated DNA

Irradiated DNA (50 Gy, 1 mg/ml) was prepared by Mrs Lynda Langford, Department of Oncology, Hodgkin building, University of Leicester using the following protocol. Calf thymus DNA (0.5 mg/ml) dissolved in air-saturated 10 mM sodium phosphate buffer (pH 7.4) was irradiated with γ-rays at room temperature in a $^{60}$Co Vickers Vickrad at a dose rate of 4 Gy/min as determined by Fricke dosimetry. The DNA was then precipitated by addition of one-tenth the volume of 2.5 M sodium acetate (pH 7) and 2 volumes of ethanol (-20°C), collected by centrifugation, washed twice with 70% ethanol and redissolved in water to a concentration of 1 mg/ml.

5.2.7 Postlabelling Assay

Samples were postlabelled using a method for the detection of apurinic sites in DNA (Weinfeld et al. 1990).

5.2.7.1 Enzymatic Digestion of DNA Samples

10 μg of control and treated DNA samples were incubated overnight at 37°C with 0.4 units of DNase 1, 0.044 units of snake venom phosphodiesterase and 0.4 units of shrimp alkaline phosphatase in 30 μl of digestion buffer (10 mM Tris, pH 7.4, 6 mM MgCl$_2$ and 1 mM EDTA, pH 8.0). The enzymes were precipitated by the addition of 3 volumes of ice-cold ethanol. After 1 h at -20°C, the enzymes were removed by centrifugation (15 000 rpm, 15 min, 4°C). Aliquots of the supernatants (90 μl) were dried down in a centrifugal vacuum evaporator, dissolved in 75 μl of water and heated at 100°C to inactivate any residual nuclease and phosphatase activity. The digested DNA samples were stored at -20°C prior to postlabelling.

5.2.7.2 $^{32}$P-Postlabelling of Enzyme Digested DNA

Each phosphorylation reaction mixture (10 μl) contained kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl$_2$ and 10 mM 2-mercaptoethanol), 5 μl of digested DNA, 1.67 pmol of [γ-$^{32}$P] ATP (3 000Ci/mm, Amersham), 7.5 units of T4 PNK and 0.05 pmoles of oligo (dT)$_{16}$ (acting as an internal standard during labelling). The samples were incubated for 1 h at 37°C and then the bulk of the excess ATP was consumed by incubation for a further 30 min with 1 μl of oligo (dT)$_{16}$ (5 $A_{260}$ units/ml, Pharmacia) and 3.75 units of T4 PNK. An equal volume of denaturing gel-loading buffer [95% formamide, 0.02%
Chapter 5: 12P-Postlabelling Detection of AP Sites Induced by NIA in CT and Mouse Stomach DNA

Bromophenol blue and 0.02% xylene cyanol in TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.0) was added to the samples and stored at -20°C prior to electrophoresis.

5.2.7.3 Analysis of Postlabelled Samples by Denaturing Polyacrylamide Gel Electrophoresis (DPAGE)

DPAGE was performed using the Model S2 sequencing gel electrophoresis system (Gibco BRL, Life Technologies), using 30 cm (width) x 40 cm (long) glass plates, 0.8 mm spacers and a 32 tooth comb. 20% denaturing polyacrylamide gels containing 7 M urea were prepared by dissolving 78.75 g of urea in 100 ml of acrylamide/bisacrylamide solution (Accugel 19:1, National Diagnostics) and 20 ml of 10xTBE buffer. To induce polymerisation, 4 ml of 3% aqueous ammonium persulphate and 67.5 μl of tetramethylethylenediamine (TEMED) were added and the gel was quickly poured. After at least 2 h, but preferably overnight, the gel was pre-run in TBE buffer at 1200V for 30 min before samples were loaded. 5 μl of postlabelled digested DNA was loaded on to the gel and the gel was run until the bromophenol blue marker had migrated 11-12 cm (2-3 h) into the gel. Once electrophoresis was complete, the gel was covered with clingfilm and stored in a light proof cartridge prior to data analysis.

5.2.7.4 Data Analysis and Lesion Quantitation

Radiolabelled digested DNA products were visualized by direct contact autoradiography using Kodak X-Omat AR film, and by phosphorimager analysis (Molecular Dynamics Model 425E, using ImageQuant™ software version 3.2).

Electrophoresis conditions are such that all radioactivity is retained on the gel. The molar quantity of detectable damage can be quantified by determining the fraction of the lane’s total radioactivity present in the bands of interest, and multiplying this by the molar quantity of ATP used in the phosphorylation reaction (3 333 fmol) and any dilution factor if relevant.
Chapter 5: \textsuperscript{32}P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

5.3 RESULTS

5.3.1 Postlabelling Detection of Apurinic Residues in Calf Thymus DNA treated with NIAN at mM Concentrations

Apurinic residues induced by NIAN in calf thymus DNA at mM levels of NIAN were examined using a \textsuperscript{32}P-postlabelling approach for the detection and quantitation of apurinic residues in DNA (Weinfeld \textit{et al.} 1990).

Calf thymus DNA treated with NIAN at concentrations ranging from 2 to 8 mM at physiological pH and temperature in buffered 50% aqueous DMSO or acetonitrile was enzymatically digested. NIAN treated digested samples were diluted in water by a factor of 500 prior to postlabelling. Gel electrophoresis and the resulting phosphorimage of the end-labelled compounds demonstrated the appearance, with increasing NIAN concentration, of several distinct bands (bands 1-3, and band X) [Figure 5.1]. Lanes (1-5) show the products of CT DNA treated with NIAN (0, 2, 4, 6 and 8 mM respectively) dissolved in acetonitrile. Similarly, lanes (6-10) show the products of CT DNA treated with NIAN (0, 2, 4, 6 and 8 mM respectively) dissolved in DMSO. It is clear that the intensity of bands (1-3) and band (X) is greater in the samples where the co-solvent used was acetonitrile.

The labelled oligo (dT)\textsubscript{16} band reflects excess \textit{[\textgamma;\textsuperscript{32}P]}ATP remaining after the labelling of the damage-containing dimer species and can be seen at the top of the gel. The oligo is included in the phosphorylation reaction so as to reduce background radioactivity in the vicinity of the small products, especially band (3); excess ATP that is not consumed migrates slightly faster through the gel than the products of interest. The labelled oligo (dT)\textsubscript{8} band is used as an internal control to check the efficiency of the labelling procedure. Bands (A-C) represent inorganic phosphate breakdown products from ATP. These 3 bands containing inorganic mono-, di- and triphosphate, migrate fastest through the gel.

All subsequent phosphorimages described in this chapter will not show bands (A-C) and will focus on the small products in the area of interest, however the full phosphorimages demonstrated the same overall pattern of bands.

The profile of bands (1-3) is identical to that described by Weinfeld and co-workers for the postlabelling detection of apurinic residues (Weinfeld \textit{et al.} 1990). Further support that bands (1-3) did indeed contain the labelled apurinic residue containing dinucleotides was
Chapter 5: \(^3\)P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

achieved by comparing bands (1-3) with those derived from control CT DNA that had been boiled for 30 min to induce depurination [Figure 5.2 (A) and (B)].

Band (1), the slowest migrating band represents the dimer species d-pGpS whereby the nucleotide 5' to the abasic site (S) contains a guanine residue. Similarly, band (2) represents the dimer species whereby the nucleotide 5' to the abasic site contains either an adenine or a thymine residue; both d-pApS and d-pTpS migrate at the same rate. Finally band (3) represents the dimer species d-pCpS whereby the nucleotide 5' to the abasic site contains a cytosine residue. The profile of these bands generated from CT DNA treated with 6 mM NIAN dissolved in acetonitrile (lane 1) is identical to the profile of bands generated from CT DNA that has been boiled for 30 min (Control) [Figure 5.2 (A)]. Similarly, the profile of these bands generated from CT DNA treated with 8 mM NIAN dissolved in DMSO (lane 2) is identical to the profile of bands generated from CT DNA that has been boiled for 30 min (Control) [Figure 5.2 (B)].

NIAN treatment of CT DNA results in a slight increase in a further product obtained by digestion and phosphorylation and subsequent gel electrophoresis. This end-labelled damage-containing dimer species (band X) migrates slightly slower through the gel than the dimer species d-pGpS. The lesion in band (X) has yet to be identified but may contain dimer species whereby the nucleotide 5' to the abasic site contains a xanthine, hypoxanthine or oxanine residue. NIAN also induces deamination products and the formation of oxanine as described in chapters 3 and 4.

The controls for this experiment [Figure 5.1, lanes 1 and 6) contain damage that is quantifiable using this assay (bands 1-3 and band X); the detection of abasic site damage is consistent with this endogenous type of damage being present in untreated DNA.

Electrophoresis conditions are such that all radioactivity is retained on the gel. This is illustrated in the phosphorimage shown in Figure 5.1 and damage was quantified as described in section 5.2.7.4. Damage due to the generation of abasic sites (bands 1-3) ranged from 8.8 to 124.5 pmoles per µg of DNA over the NIAN concentration range of 2-8 mM where the NIAN co-solvent was acetonitrile. Where DMSO was used as NIAN co-solvent, the level of damage due to the generation of abasic sites was 10.2 to 60.2 pmoles over the same concentration range [Figure 5.2 (C)]. Total detectable damage (bands 1-3 +X) ranged from 11.7 to 151.2 pmoles per µg of DNA where the NIAN co-solvent was
acetonitrile and 14.4 to 71.6 pmol per µg of DNA where the NIAN co-solvent was DMSO over the NIAN concentration range of 2 to 8 mM [Figure 5.2 (D)].

Control samples where the CT DNA was incubated with buffer and acetonitrile or DMSO only, had detectable levels of damage due to the generation of abasic sites of 14.7 and 20.1 fmol per µg of DNA respectively; total detectable damage was 21.0 and 28.0 fmol per µg of DNA respectively. The controls for this experiment show levels of damage which are consistent with this spontaneous endogenous type of damage being present in untreated DNA, however it is clear from the data that NIAN co-solvent is affecting the levels of damage present. Where acetonitrile is used as co-solvent the levels of damage at a NIAN concentration of 8 mM are more than double the levels where DMSO is used as co-solvent. This result is unsurprising since N-acetyl adducts generated from transnitrosation by NIAN to acetonitrile as discussed in chapter 3, will influence the level of depurination resulting from NIAN treated CT DNA. Consequently, to eliminate solvent effects as far as possible, DMSO was chosen as the co-solvent for use in subsequent NIAN reactions with CT DNA. DMSO was also the solvent used for NIAN, prior to intragastric administration in the in vivo study (section 5.3.4), and in the Ames II and Comet assays (chapter 6).
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Figure 5.1 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN (0-8 mM) dissolved in either ACN (lanes 1-5) or DMSO (lanes 6-10). Lanes (1,6) controls; lanes (2-5) and lanes (7-10) CT DNA exposed to 2, 4, 6 and 8 mM NIAN respectively. Bands (1-3), contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified. Inorganic phosphate breakdown products from ATP are shown in bands (A-C). Samples were diluted 1 in 500 prior to end-labelling.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Figure 5.2 (A) and (B) Comparison of bands (1-3) generated from CT DNA treated with NIAN [6 mM dissolved in ACN (lane 1), or 8 mM dissolved in DMSO (lane 2)] with control CT DNA containing apurinic residues, following enzymatic digestion and phosphorylation of the products. Control DNA has been boiled for 30 min. NIAN treated samples were diluted 1 in 500 prior to end-labelling.

(C) and (D) plots of detectable damage [bands (1-3) or bands (1-3) + X] from 1 ug of DNA vs NIAN dose.
5.3.2 Postlabelling Detection of Apurinic Residues in Calf Thymus DNA Treated with NIAN at $\mu$M Concentrations

NIAN at $\mu$M concentrations was used to induce damage in calf thymus DNA to evaluate the sensitivity of the assay towards the detection of abasic site damage. Detectable damage at the low concentration of NIAN suggests that abasic site damage induced in vivo, in the glandular stomach of mice, is likely to be detected by this assay.

Calf thymus DNA treated with NIAN at concentrations ranging from 12.5 to 800 $\mu$M at physiological pH and temperature in buffered 50% aqueous DMSO, was postlabelled and subjected to denaturing PAGE. Gel electrophoresis of the end-labelled compounds demonstrated the appearance, with increasing NIAN concentration, of several distinct bands (bands 1-3, and band X) [Figure 5.3]. The profile of bands (1-3) is identical to that described by Weinfeld and co-workers (Weinfeld et al. 1990) for the detection of apurinic residues and as described in section 5.3.1. Similarly, to confirm that bands (1-3) did contain the labelled apurinic residue containing dinucleotides, the profile of bands resulting from CT DNA treated with NIAN at 800 $\mu$M [Figure 5.4 (A), lane 1] was compared with the profile of bands resulting from control CT DNA that had been boiled for 30 min to induce depurination [Figure 5.4 (A), lane Control]. The resulting profiles are identical, supporting that bands (1-3) contain the labelled apurinic residue containing dinucleotides. As previously described in section 5.3.1, the lesion in band (X) has yet to be identified.

Damage due to the generation of abasic sites (bands 1-3) increased linearly with NIAN concentration and ranged from 107.4 to 393.4 fmoles damage per $\mu$g DNA over the NIAN concentration range of 12.5 to 800 $\mu$M [Figure 5.4 (B)]. The yield of total detectable damage (bands 1-3 + X) also increased linearly with NIAN concentration and ranged from 140 to 490 fmoles damage per $\mu$g DNA over the same NIAN concentration range.

Control samples where the CT DNA was incubated with buffer and NIAN co-solvent only had detectable levels of damage due to the generation of abasic sites of 80.4 fmoles damage per $\mu$g DNA; total detectable damage was 105.1 fmoles damage per $\mu$g DNA. Again, these levels of damage are consistent with this endogenous type of damage being present in untreated DNA coupled with any induction caused by the experimental system (ie solvent effects).
Figure 5.3 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN (0-800μM). Bands (1-3), contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified. Oligo (dT)$_{16}$ was used to consume the bulk of the excess ATP, thereby reducing the background radioactivity in the vicinity of the products of interest.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Figure 5.4 (A) Comparison of bands (1-3) generated from CT DNA treated with NIAN (800 µM, lane 1) with control CT DNA containing apurinic residues, following enzymatic digestion and phosphorylation of the products. Control DNA has been boiled for 30 min.

(B) Plot of detectable damage [bands (1-3) or bands (1-3)+X] from 1 µg of DNA vs NIAN dose.

- d-pGpS
- d-pApS+d-pTpS
- d-pCpS

**Figure 5.4 (A)** Comparison of bands (1-3) generated from CT DNA treated with NIAN (800 µM, lane 1) with control CT DNA containing apurinic residues, following enzymatic digestion and phosphorylation of the products. Control DNA has been boiled for 30 min.

**Figure 5.4 (B)** Plot of detectable damage [bands (1-3) or bands (1-3)+X] from 1 µg of DNA vs NIAN dose.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

5.3.3 Inhibition of Reaction Product Formation by Azide Ion

When reactions of calf thymus DNA with NIAN at a concentration of 800 µM, were repeated in the presence of sodium azide and postlabelled, a gradual decrease in the formation of the apurininc residue containing dinucleotides (bands 1-3) and band (X) was observed with increasing azide concentration [Figure 5.5]. The resulting phosphorimage of the polyacrylamide gel demonstrates the decrease in radioactivity, with increasing azide concentration ranging from 25 to 800 µM, of the damage containing bands. The control for this experiment (lane C) was an incubated mixture containing calf thymus DNA, cosolvent and sodium azide at a concentration of 800 µM.

The yield of damage due to the generation of abasic sites (bands 1-3) was 480.9 fmoles per µg of DNA where the DNA was exposed to 800 µM NIAN [Figure 5.6]. As sodium azide was gradually introduced into the system this level decreased. Where the molar ratio of NIAN to sodium azide was equal (800 µM), the level of damage was 123.2 fmoles per µg of DNA and was at control levels, indicating that the formation of reaction products was completely inhibited. Similarly, the yield of total detectable damage (bands 1-3 + X) was 602 fmoles per µg of DNA where the DNA was exposed to 800 µM NIAN and in the presence of increasing concentrations of azide ion, this level decreased. Where the molar ratio of NIAN to sodium azide was equal, the level of total detectable damage was at control levels.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

**Figure 5.5** Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN (800 μM) in the presence of sodium azide (0-800 μM). Bands (1-3) contain the labelled apurinic residue containing dinucleotides; band (X) has yet to be identified. Lane C represents endogenous damage present in control CT DNA.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

![Graph](image)

**Sodium azide conc. (μM)**

**KEY**
- • Abasic site damage
- ■ Total damage
- --- Control levels [Total endogenous damage]
- --- Control levels [Abasic site endogenous damage]

**Figure 5.6** Plot of detectable damage [bands (1-3) or bands (1-3) + X] from 1 μg of CT DNA treated with NIAN (800 μM) vs sodium azide dose. Endogenous levels of damage present in control CT DNA are shown.
Chapter 5: \textsuperscript{3}P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

5.3.4 In Vivo Study

The ability of NIAN to induce depurination via transnitrosation \textit{in vivo} in the glandular stomach of CD-1 mice, using the postlabelling method previously described was investigated.

5.3.4.1 Quality Check of Mouse Stomach DNA

DNA was isolated from the mucosa of the glandular stomach of control CD-1 mice and CD-1 mice following a single dose of NIAN at 100 mg/kg using a Qiagen DNA isolation procedure described in section 5.2.5.3. Five animals were in the control group, received the vehicle alone and were sacrificed after 24 h. Ten animals received the single dose of NIAN and were sacrificed after 3 h. Ten animals received the single dose of NIAN and were sacrificed after 24 h.

The resulting DNA was dissolved in water and quantified by measuring the absorbance at 260 nm. The ratio of the absorbance at 260 nm to 280 nm was in the region of 1.5 to 1.7 for all the stomach DNA samples, compared to a ratio of 1.6 for batch standard calf thymus DNA (Sigma). The yields of DNA obtained from the glandular stomachs of the mice ranged from 89 \(\mu\text{g}\) to 392 \(\mu\text{g}\) [Table 5.1].

All mouse stomach DNA samples and control calf thymus DNA were enzymatically digested into 3'-monophosphates and analysed by HPLC to check the purity of the DNA. Enzymatic digestion of calf thymus DNA produced the four expected nucleotide peaks, dCp, dGp, Tp and dAp with retention times of 10.2, 19.8, 21.7 and 24.3 min respectively [Figure 5.7 (A)]. Enzymatic digestion of calf thymus DNA that had been through the DNA isolation procedure using the Qiagen protocol produced the same 4 peaks, but it is clear from the chromatogram that the isolation procedure resulted in slight degradation of the DNA as shown by the minor peaks at retention times of 9.8, 22.5 and 26.2 min [Figure 5.7 (B)]. The profile of nucleotide peaks resulting from the enzymatic digestion of mouse stomach DNA was almost identical for all the samples. Representative chromatograms from a sample in the control group, the 3 h group and the 24 h group are shown in Figure 5.8 (A), (B) and (C) respectively. The peaks in addition to the nucleotide peaks can be attributed to the DNA isolation procedure as described for calf thymus DNA. The quality of the DNA from mouse number 21 (24 h group) was deemed unsatisfactory for analysis as shown by the intensity of the degradation product peaks [Figure 5.8 (D)].
<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Group</th>
<th>Yield of Glandular Stomach DNA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>215</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>392</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>190</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>244</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>256</td>
</tr>
<tr>
<td>6</td>
<td>3 h</td>
<td>*</td>
</tr>
<tr>
<td>7</td>
<td>3 h</td>
<td>206</td>
</tr>
<tr>
<td>8</td>
<td>3 h</td>
<td>346</td>
</tr>
<tr>
<td>9</td>
<td>3 h</td>
<td>263</td>
</tr>
<tr>
<td>10</td>
<td>3 h</td>
<td>263</td>
</tr>
<tr>
<td>11</td>
<td>3 h</td>
<td>149</td>
</tr>
<tr>
<td>12</td>
<td>3 h</td>
<td>89</td>
</tr>
<tr>
<td>13</td>
<td>3 h</td>
<td>270</td>
</tr>
<tr>
<td>14</td>
<td>3 h</td>
<td>204</td>
</tr>
<tr>
<td>15</td>
<td>3 h</td>
<td>210</td>
</tr>
<tr>
<td>16</td>
<td>24 h</td>
<td>268</td>
</tr>
<tr>
<td>17</td>
<td>24 h</td>
<td>116</td>
</tr>
<tr>
<td>18</td>
<td>24 h</td>
<td>175</td>
</tr>
<tr>
<td>19</td>
<td>24 h</td>
<td>261</td>
</tr>
<tr>
<td>20</td>
<td>24 h</td>
<td>180</td>
</tr>
<tr>
<td>21</td>
<td>24 h</td>
<td>167</td>
</tr>
<tr>
<td>22</td>
<td>24 h</td>
<td>196</td>
</tr>
<tr>
<td>23</td>
<td>24 h</td>
<td>283</td>
</tr>
<tr>
<td>24</td>
<td>24 h</td>
<td>284</td>
</tr>
<tr>
<td>25</td>
<td>24 h</td>
<td>184</td>
</tr>
</tbody>
</table>

* DNA was not recovered from mouse stomach number 6.

**Table 5.1** Yields of DNA isolated from the glandular stomach of CD-1 mice.

### 5.3.4.2 Postlabelling Detection of Apurinic Residues in DNA from the Glandular Stomach of CD-1 Mice

DNA isolated from the mucosa of the glandular stomach of control CD-1 mice and CD-1 mice following a single dose of 100 mg/kg NIAN, was subjected to the postlabelling assay. DNA was not recovered or deemed of sufficient quality from one animal in both the 3 and 24 hour groups. The end-labelled compounds were resolved by gel electrophoresis and the same profile of bands (bands 1-3 and band X) were detected autoradiographically.
as described for the reactions of calf thymus DNA with NIAN, sections 5.3.1 and 5.3.2 [Figure 5.9 (A) and Figure 5.10 (A)].

Interestingly, band (3) which represents the dimer species d-pCpS whereby the nucleotide 5’ to the abasic site (S) contains a cytosine residue, is much more intense than band (3) derived from control calf thymus DNA or calf thymus DNA treated with NIAN. Moreover, this band is much more intense than band 2, which contains 2 abasic site containing dinucleotides. There appears to be a further species within the digested mouse stomach DNA, which is labelled via this assay, which has identical mobility to d-pCpS [ie band (3)]. Consequently, this band has been omitted from the damage quantitation analysis.

Levels of detectable damage (bands 1-2 + X) as shown by the rectangular box on the autoradiograph were quantified using phosphorimaging as previously described [Figure 5.9 (B) and Figure 5.10 (B)] and are expressed as median levels of damage for each of the animal groups [Figure 5.11]. The median level of damage in the control group of animals was 43 fmoles per µg of DNA (range 24 – 61 fmoles, n = 5). The median level of damage detected in the group of animals that were killed 3 h and 24 h post dosing was 56 fmoles per µg of DNA (range 43 – 76 fmoles, n = 9) and 36 fmoles per µg of DNA (range 28 – 49 fmoles, n = 9) respectively.

The data were analysed using Kruskal Wallis ANOVA with post hoc median comparison by the Mann Whitney U-test (one tailed). The Kruskal Wallis test returned a significance level of p = 0.013 (H = 8.8, d.f. = 2). Post hoc analysis using Mann Whitney showed DNA from the animals killed at 3 h post dosing had a significantly higher level of damage than DNA from the control group (p = 0.016). There was no significant difference in the level of damage between the control group and the group of mice killed after 24 h post dosing (p = 0.4).

Consequently, the level of damage observed, had increased significantly at 3 h compared to background levels observed in the control group. At 24 h, the level of damage had decreased to background level.
Figure 5.7 HPLC separation of CT DNA after digestion to 3'-monophosphates. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm. (A) control CT DNA and (B) control CT DNA that has been through the DNA isolation procedure using the Qiagen protocol.
Figure 5.8 HPLC separation of mouse stomach DNA, after digestion to 3’-monophosphates. DNA has been isolated from the glandular stomach of CD-1 mice using the Qiagen protocol. HPLC conditions as described under “Experimental Procedures”; UV detection at 260 nm. (A) control DNA from mouse no. 3 and (B) DNA from mouse no. 8 that has been treated with NIAN and sacrificed after 3 h.
Figure 5.8 HPLC separation of mouse stomach DNA, after digestion to 3'-monophosphates. DNA has been isolated from the glandular stomach of CD-1 mice using the Qiagen procedure. HPLC conditions as described under "Experimental Procedures"; UV detection at 260 nm. (C) DNA from mouse no. 22 that has been treated with NIAN and sacrificed after 24 h and (D) DNA from mouse no. 21 that has been treated with NIAN and sacrificed after 24 h.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

(A) Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CD-1 mouse stomach DNA; mice (1-3) controls, mice (7-11) treated with NIAN and sacrificed after 3 h, mice (16-19) treated with NIAN and sacrificed after 24 h. Control DNA represented by NIAN treated CT DNA (200 μM). Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified.

(B) Quantitation of detectable damage (bands 1, 2 and X) as shown by the rectangular box in (A), as determined by phosphorimaging.

Figure 5.9 (A) Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CD-1 mouse stomach DNA; mice (1-3) controls, mice (7-11) treated with NIAN and sacrificed after 3 h, mice (16-19) treated with NIAN and sacrificed after 24 h. Control DNA represented by NIAN treated CT DNA (200 μM). Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified.

(B) Quantitation of detectable damage (bands 1, 2 and X) as shown by the rectangular box in (A), as determined by phosphorimaging.
Figure 5.10 (A) Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CD-1 mouse stomach DNA; mice (4,5) controls, mice (12-15) treated with NIAN and sacrificed after 3 h, mice (20-25) treated with NIAN and sacrificed after 24 h. The quality of DNA from mouse no. 21 was deemed unsatisfactory for analysis. Control DNA represented by NIAN treated CT DNA (200 μM). Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified.

(B) Quantitation of detectable damage (bands 1, 2 and X) as determined by phosphorimaging.
Figure 5.11 Dependence on time of exposure to NIAN on the accumulation of damage detected in the glandular stomach of CD-1 mice using a $^{32}$P-postlabelling assay for the detection of apurinic residues in DNA.

Male mice were dosed orally with NIAN at 100 mg/kg (18 animals in total). Nine animals were sacrificed 3 h after dosing and nine animals were sacrificed 24 h after dosing. The control group (5 animals) were sacrificed 24 h after receiving the vehicle alone. Stomachs were removed and DNA was extracted from the mucosa of the glandular stomach.

Median values are shown for each animal group with range indicated by lines.

Significance level of $p < 0.025$ is indicated by ** where the level of damage detected in the 3 h group was significantly higher than in the control group.
5.3.5 Influence of Damage-Recognising Enzymes

Both *E. coli* exonuclease III and *E. coli* endonuclease IV are class II AP endonucleases that initiate repair of apurinic residues in double-stranded substrates by cleaving the phosphodiester bond immediately 5' to the AP site generating a normal 3'-OH group. Both enzymes possess 3'-diesterase activity and remove 3'-blocking damages, such as phosphoglycolate residues to create normal 3'-OH ends (Singer and Hang 1998; Ramotar and Demple 1998). Endonuclease IV has no confounding exonuclease III or associated DNA-glycosylase activity and is unable to cleave the N-glycosydic bond of several pyrimidine ring saturation products, such as thymine glycol (Singer and Hang 1998; Ramotar and Demple 1998).

Calf thymus DNA and mouse stomach DNA exposed to NIAN and the appropriate untreated controls were incubated with *E. coli* exonuclease III and *E. coli* endonuclease IV enzymes individually and subjected to the postlabelling assay. To assess the activity and specificity of these enzymes and to ensure that the enzymes were working selectively, irradiated DNA containing phosphoglycolate residues and thymine glycols was used as an external standard. Samples were also incubated in the appropriate buffers without enzyme.

Calf thymus DNA treated with NIAN (50, 200 and 800 μM) was incubated with *E. coli* exonuclease III prior to enzymatic digestion and phosphorylation. The phosphorimage of the resulting polyacrylamide gel demonstrates clearly that the enzyme acted so as to reduce the counts in bands (1-3) and band (X) [Figure 5.12]. Incubation of the DNA with the enzyme led, as expected, to a sharp reduction in the radioactivity associated with the abasic site containing dimers present after NIAN treatment and present in the control. Cleavage of the phosphodiester bond immediately 5' to the abasic site has effectively digested the 5'-internucleotide phosphodiester linkage and, as a result, enzyme treatment has removed the abasic sites as substrates for postlabelling. Control and treated mouse stomach DNA was incubated with *E. coli* exonuclease III prior to enzymatic digestion and phosphorylation. Similarly, the phosphorimage of the resulting polyacrylamide gel demonstrates that the enzyme acted so as to reduce the counts in bands (1-3) and band (X) [Figure 5.13].

Calf thymus DNA treated with NIAN (800 μM) and irradiated DNA (50 Gy) were incubated with *E. coli* exonuclease III and *E. coli* endonuclease IV prior to postlabelling. The phosphorimage of the resulting polyacrylamide gel is shown in Figure 5.14. From a
comparison of exonuclease III treated DNA exposed to NIAN (lane B) and endonuclease IV treated DNA exposed to NIAN (lane C) with the control (lane A), both enzymes have acted so as to reduce the counts in (bands 1-3) and band (X). Incubation of the DNA with both enzymes, led as expected, to a sharp reduction in the radioactivity associated with the abasic site containing dinucleotides. Endonuclease IV treatment results in cleavage of the phosphodiester bond immediately 5' to the abasic site as previously described for exonuclease III treatment.

Radioactivity associated with bands (4-6) and bands (10-12) in irradiated (50Gy) DNA (lane D), is consistent with the formation of thymine glycols and phosphoglycolates respectively, induced by λ irradiation as previously as described by Weinfeld and co-workers (Weinfeld et al. 1991). The lesions in bands (7-9) have yet to be identified (Weinfeld et al. 1991). Exonuclease III treatment of irradiated DNA (lane F) has reduced radioactivity in bands (4-6) and bands (10-12) compared to its control (lane E). However, endonuclease IV treatment (lane H), when compared to its control (lane G) results in a reduction in the radioactivity associated with bands (10-12) but is unable to reduce the counts in bands (4-6). More specifically, endonuclease IV treatment is able to remove phosphoglycolate residues but is unable to remove thymine glycol damage. These observations are consistent with the activity and specificity of these enzymes previously described (Singer and Hang 1998; Ramotar and Demple 1998). These results lend further support to the observations that both E.coli exonuclease III and E.coli endonuclease IV were acting selectively upon apurinic residues induced by NIAN in DNA.

DNA from the glandular stomach of mouse number 14 (treated with NIAN and sacrificed after 3 h) was incubated with both enzymes prior to digestion and phosphorylation. The phosphorimagine of the resulting polyacrylamide gel demonstrated, as expected, a reduction in the radioactivity associated with bands (1-3) and band (X) [Figure 5.15]. Interestingly, from a comparison of exonuclease III treated DNA (lane B) and endonuclease IV treated DNA (lane D), endonuclease IV is unable to remove all of the damage associated with band (3). As described previously in the in vivo results (section 5.3.4.2), there appears to be a further labelled dimeric species within the digested mouse stomach DNA that has identical mobility to d-pCpS and hence is contained within band (3). This species appears to be resistant to cleavage by endonuclease IV and consequently this species is not an apurinic residue containing dinucleotide, but some other species that is a ready substrate for the postlabelling assay.
Chapter 5: $^{32}$P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Figure 5.12 Influence of repair enzymes. Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN (50, 200 and 800 μM); lane C represents control CT DNA. Lanes (+) refer to DNA that was incubated with *E. coli* exonuclease III prior to digestion and end-labelling. Lanes (-) represent the corresponding incubated controls.

Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified.
Chapter 5: \(^{32}\)P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Mouse Number

<table>
<thead>
<tr>
<th>Mouse Number</th>
<th>(+)</th>
<th>(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>2</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>3</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>4</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>5</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>6</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>7</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>8</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>9</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>10</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>11</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>12</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>13</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>14</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>15</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>16</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>17</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>18</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>19</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>20</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>21</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>22</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>23</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>24</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>25</td>
<td>(+)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Figure 5.13 Influence of repair enzymes. Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CD1 mouse stomach DNA; mice (1, 4) controls, mice (7, 14) treated with NIAN and sacrificed after 3h, mice (23, 25) treated with NIAN and sacrificed after 24h. Lanes (+) refer to DNA that was incubated with E.coli exonuclease III prior to digestion and end-labelling. Lanes (-) represent the corresponding incubated controls.

Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified.
Figure 5.14 Influence and selectivity of repair enzymes. Calf thymus DNA exposed to NIAN (800 μM) was incubated with E. coli exonuclease III (lane B) and E. coli endonuclease (IV) (lane C). Lane A represents the incubated control. Irradiated (50 Gy) DNA (lane E) was incubated with exonuclease III (lane F) and endonuclease IV (lane H). Lanes D and G represent the incubated controls. All samples were digested and end-labelled after incubations with or without enzyme. Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified. Bands (4-6) contain the labelled thymine glycol-containing dinucleotides; the lesions in bands (7-9) have yet to be identified. Bands (10-12) contain the labelled phosphoglycolate molecules.
Figure 5.15 Influence and selectivity of repair enzymes. Mouse stomach DNA from mouse number 14 (treated with NIAN and sacrificed after 3 h) was incubated with *E.coli* exonuclease III (lane B) or *E.coli* endonuclease IV (lane D) prior to digestion and end-labelling. Lanes A and C represent the incubated controls. Bands (1-3) contain the labelled apurinic residue containing dinucleotides; the lesion in band (X) has yet to be identified.
5.4 DISCUSSION

5.4.1 The Detection of Abasic Sites in Calf Thymus DNA Induced by Exposure to μmolar Concentrations of NIAN

Using the postlabelling approach described by Weinfeld and co-workers (Weinfeld et al. 1990) it was possible to detect apurinic residues as postlabelled dinucleotides in calf thymus DNA exposed to μmolar concentrations of NIAN, at physiological pH and temperature. These apurinic residues increased in yield with increasing concentrations of NIAN and the assay was sensitive enough to detect endogenous formation of abasic sites in control samples. A further DNA damage product was detected in this study (band X) which has yet to be characterised. This end-labelled damage-containing dinucleotide migrated more slowly through the gel than the other abasic site containing dimers. Since NIAN induces deamination products and the formation of oxanine as described in previous chapters, it is possible that the additional product is a dimer species whereby the nucleotide 5' to the abasic site contains a xanthine, hypoxanthine or oxanine residue.

A progressive reduction in the formation of abasic sites was observed when reactions of calf thymus DNA exposed to NIAN were repeated in the presence of increasing concentrations of sodium azide. Azide is a scavenger of nitrosating agents and nitrite, and these observations add further confirmation that the mechanism by which NIAN exerts genotoxicity is via transnitrosation.

Incubation of damaged DNA with AP endonucleases led to a sharp reduction in the radioactivity associated with these dinucleotides confirming the enzymes’ selective action upon apurinic residues induced by NIAN in DNA.

5.4.2 NIAN Induces Abasic Site Formation In Vivo

Loss of bases, primarily purines, from DNA occurs spontaneously in living cells and it has been calculated that approximately $10^4$ apurinic/apyrimidinic sites arise in each mammalian cell per day (Lindahl and Nyberg 1972). The process is enhanced by low pH, elevated temperature and by exposure to DNA-damaging agents, which may generate certain base modifications, such as alkylation, that destabilise the $N$-glycosidic bond leading to a faster rate of hydrolytic release (reviewed in Loeb and Preston 1986).
Since the endogenous nitrosation of indoles could predominantly take place in the stomach, where a low pH is suitable for the nitrosation reaction, DNA from the mucosa of the glandular stomach in control CD-1 mice and CD-1 mice exposed to NIAN was evaluated for the presence of abasic sites. A background level of damage in mouse stomach DNA was detected which is consistent with the spontaneous formation of abasic sites. The level of damage from mouse stomach exposed to NIAN for 3 hours increased significantly, suggesting that the nitroso group was efficiently transferred to the DNA bases to exert genotoxic damage. These findings are consistent with those previously reported by Furihata and co-workers who demonstrated DNA single-strand scission, upon alkaline lysis, in the mucosa of rat stomach induced by NIAN (Furihata et al. 1996).

The transnitrosation-inhibitory effect of azide ion has been demonstrated in this study in vitro. Similarly, it is likely that in vivo inhibitors of nitrosation will also play an important role in influencing the level of damage induced by nitrosated indoles. Inhibitors of nitrosation, such as vitamins C and E (reviewed in Mirvish 1996; Bartsch et al. 1988; Mackerness et al. 1989) are likely to influence the availability of the transnitrosation species/pathway to exert genotoxic damage. Perhaps more importantly, inhibitors will act as competitors for the indole compound that serves as a substrate for the nitrosating species, thus reducing the total NNOC contribution provided by nitrosated indoles.

5.4.3 NIAN-Induced Abasic Sites are Repaired In Vivo

The level of damage detected in DNA from mouse stomach exposed to NIAN for 24 hours was not significantly different to the level detected in the control group of animals, whereas levels at 3 hours were significantly raised, suggesting that repair events had occurred. These findings may explain the observations of Furihata and co-workers who reported marked inductions in ornithine decarboxylase activity and increases in DNA synthesis in rat stomach mucosa after administration of NIAN at doses of 40 to 300 mg/kg body weight by gastric intubation (Furihata et al. 1987).

The findings by Lindahl and Nyberg that depurination of DNA occurs at a physiologically significant rate under in vivo conditions (Lindahl and Nyberg 1972), suggested that such lesions introduced in this fashion must be repaired. Moreover, these lesions are repaired in vivo because of their potential as mutagenic lesions (Loeb and Preston 1986).

Repair of abasic sites is initiated by AP endonucleases, ubiquitous in both prokaryotes and eukaryotes (Friedberg et al. 1995). Class II AP endonucleases, specifically E. coli
Chapter 5: \(^{32}\)P-Postlabelling Detection of AP Sites Induced by NIAN in CT and Mouse Stomach DNA

Exonuclease III and *E. coli* endonuclease IV, and the major human AP endonuclease, recognise any AP site and repair is carried out through the base excision repair pathway (Singer and Hang 1998). The AP endonuclease cleaves the phosphodiester bond immediately 5' to the AP site. Since exonuclease III and endonuclease IV both possess 3'-repair diesterase activity (Ramotar and Demple 1998), the enzymes produce normal 3'-hydroxyl termini that can be used by DNA polymerase for DNA repair synthesis. By cleaving the phosphodiester bond, an entry point for exonucleases is provided, and the nick is expanded into a gap, by excision of the AP site as 5'-deoxyribose phosphate. In the base-excision-repair system, the single-stranded gap is resynthesised by DNA polymerase and sealed with ligase.

Both *E. coli* exonuclease III and *E. coli* endonuclease IV treatment of NIAN-induced abasic sites, both with calf thymus DNA and with mouse stomach DNA *in vitro*, cleaved the phosphodiester bond immediately 5'-to the abasic site, thus digesting the 5'-internucleotide linkage. Consequently, the abasic sites were removed as substrates for detection by the postlabelling method. These results demonstrate that both AP endonucleases were acting selectively upon apurinic residues induced by NIAN in DNA. The fact that NIAN-induced abasic site formation *in vivo* appeared to be repaired at 24 hours suggests that the repair machinery in the cell was efficient at repairing such induced damage. The mutagenic potential of unrepaired AP sites is explored in chapter 7.

In summary, the results from this study have demonstrated that NIAN is able to efficiently transfer the nitroso group to DNA bases to induce depurination at low levels of NIAN in calf thymus DNA, and in the glandular stomach of CD-1 mice. These results support the fact that NIAN, or *N*-nitrosoindoles in general, demonstrate possible activity in carcinogenesis in rat stomach mucosa. The fact that DNA depurination has been induced *in vivo* by NIAN confirms the *in vitro* results, which suggested a major role for transnitrosation by *N*-nitrosoindoles. Since *N*-nitrosoindole-induced DNA base damage comprises not only depurination, but deamination, the formation of oxanine and interstrand cross-links, the results presented in this chapter imply that these additional pathways may also be active *in vivo*. It is therefore important to evaluate the mutagenic potential of the different types of NIAN-induced DNA damage, and this is the subject of the next chapter.
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

CHAPTER 6

ACTIVITY OF 1-NITROSOINDOLE-3-ACETONITRILE IN THE COMET AND AMES II ASSAYS
6.1 INTRODUCTION

In this final study, NIAN was evaluated in two genetic toxicology assays that are employed to test new chemical entities in the pharmaceutical industry: the Comet and Ames II assays.

Since NIAN has been demonstrated to induce depurination in a variety of systems described in previous chapters, the ability of NIAN to induce DNA damage in single cells, as evaluated by the Comet assay, was investigated.

The single cell gel electrophoresis assay, more commonly known as the Comet assay, is a rapid, visual and quantitative method for measuring DNA strand breaks and alkali-labile sites. The Comet assay was first introduced by Östling and Johanson in 1984 as a microelectrophoretic technique for the direct visualisation of DNA damage in individual cells (Östling and Johanson 1984). The assay was further developed by Singh, who used alkaline electrophoresis to analyze DNA damage from treatments with X-rays and hydrogen peroxide (Singh et al. 1988). The assay enables rapid and sensitive detection of chemically induced damage in cells or in tissues from which viable cell preparations can be maintained. The use of the assay has increased significantly in the past few years, with different protocols and different applications emerging (McKelvey-Martin et al. 1993; Fairbairn et al. 1995).

In essence, cell suspensions isolated from any tissue or in vitro-treated cell lines are embedded in agarose gel on glass microscope slides. The cells are lysed by detergent and high salt solution to rupture the cell membranes, extract the nuclear proteins and leave the supercoiled DNA in a nucleus-type structure; the nucleoid. Subsequently, the supercoiled DNA is left to relax and ‘unwind’ in a strongly alkaline buffer; any alkali labile lesions, or abasic sites resulting from the chemical insult, will result in a DNA strand break at the site of damage. The non-supercoiled loops and single strand fragments migrate toward the anode on electrophoresis. If DNA strand breaks occur directly as a result of the chemical insult, the open loops or fragments migrate further into the gel.

Following electrophoresis, the slides are neutralised before staining with propidium iodide. The cells are visualised using fluorescence microscopy as having the characteristic shape of a comet; a brightly fluorescent head (nucleoid) containing undamaged DNA and the tail containing fragments of DNA. The strategy of the Comet assay used to evaluate
the DNA damaging ability of NIAN in the presence or absence of an exogenous metabolising system (S9) is summarised in Figure 6.1.

**Figure 6.1** Summary of the methodology used in the Comet assay.

Results from previous chapters in this thesis have identified NIAN as a transnitrosating agent resulting in a variety of DNA damage at the nucleophilic nitrogen atoms on the purine bases. Consequently, NIAN would be expected to induce damage both at G:C base pairs and at A:T base pairs.

To determine the mutation spectrum induced by NIAN, the compound was evaluated in a set of six his mutant Salmonella strains that were developed to revert by unique base-pair substitutions; the Ames II assay (Gee et al. 1994).
The six strains, TA 7001, TA 7002, TA 7003, TA 7004, TA 7005 and TA 7006, each detect one, and only one, of six possible base substitutions. Strains TA 7001 to TA 7003 detect base changes at A:T base pairs, while strains TA 7004 to TA 7006 detect base changes at G:C base pairs. The bacterial strains used and the specific base-pair substitution detected are summarised in Table 6.1.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Mutation Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 7001</td>
<td>A:T → G:C</td>
</tr>
<tr>
<td>TA 7002</td>
<td>A:T → T:A</td>
</tr>
<tr>
<td>TA 7003</td>
<td>A:T → C:G</td>
</tr>
<tr>
<td>TA 7004</td>
<td>G:C → A:T</td>
</tr>
<tr>
<td>TA 7005</td>
<td>G:C → T:A</td>
</tr>
<tr>
<td>TA 7006</td>
<td>G:C → C:G</td>
</tr>
</tbody>
</table>

*Table 6.1* Bacterial strains used in the Ames II assay. Each strain detects one, and only one, of six possible base substitutions.

Because the reversion pathway for each strain is restricted to a specific base change, the spontaneous reversion frequencies are low (from less than one to about 25 revertants per plate) and the ability of these strains to detect mutagens has been evaluated in a large validation study of over 30 chemicals (Gee et al. 1998).

The response of NIAN toward these base-specific Salmonella tester strains was evaluated, the results of which will enable discussion in relation to the *in vitro* pathways of DNA damage identified.
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

6.2 EXPERIMENTAL PROCEDURES

The Comet and the Ames II assays were undertaken at GlaxoWellcome Research and Development, Ware, UK, under the direction of the Genetic and Reproductive Toxicology Group within the division of Medicines Safety and Evaluation.

**Caution:** NIAN is mutagenic and should be handled with extreme caution. Methyl methanesulphonate (MMS), streptonigrin (STN), 5-aza cytidine (5azaC) and 4-nitroquinoline-N-oxide (4NQO) are mutagens and should be handled with extreme caution. 1-Methyl-3-nitro-1-nitroso-guanidine (MNNG) is carcinogenic and extreme care should be exercised in its handling.

6.2.1 Materials

L5178Y cells were obtained from GlaxoWellcome Research and Development, Ware, UK. RPMI 1640 Glutamax-1 medium, L-glutamine, HEPES, streptomycin sulphate, benzylpenicillin and donor horse serum were purchased from Gibco Life Technologies.

The six Ames II tester strains were obtained from Xenometrix (Ames II™ Mutagenicity Assays by Xenometrix Inc., Boulder, Colorado). STN, 5azaC, 4NQO and MNNG were purchased from Aldrich Chemical Co. All solutions used for the mutagenicity assay were prepared and handled in a sterile manner. All other chemicals were purchased from Sigma Chemical Co., unless otherwise stated.

6.2.2 Synthesis and Characterisation of NIAN

NIAN was synthesized by nitrous acid treatment of IAN (Fluka), purified by HPLC and fully characterized by ¹H-NMR, mass spectrometry and microanalysis as described in chapter 2.

6.2.3 The Comet Assay

6.2.3.1 Cell Culture

L5178Y cells (mouse lymphoma cells) were grown in RPMI 1640 Glutamax-1 medium containing 3 mM L-glutamine and 25 mM HEPES, supplemented with 200 µg/ml sodium pyruvate, 50 µg/ml streptomycin sulphate and 50 IU/ml benzylpenicillin and 10% (v/v) heat inactivated donor horse serum at 37°C in a 5% CO₂ atmosphere.
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

6.2.3.2 Treatment of Cells with NIAN and the Control Chemical

On the day of use, the cells were measured using a Coulter counter (Coulter Electronic Ltd, UK) and an appropriate dilution was made in RPMI medium containing 3% donor horse serum, to give a final cell density of 1x10^5 cells per ml. The concentration of donor horse serum was reduced from 10% to 3% for the treatment so as to minimise any binding of the test chemical to the proteins in the serum.

Stock solutions of NIAN were prepared in neat DMSO at 100x the required concentrations. Aliquots of 25 μl of each NIAN stock solution was added to 2.5 ml of confluent cells giving final doses of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 125 and 150 μg/ml in 1% DMSO. Concurrent vehicle and positive controls were also set up, using DMSO and 10 μg/ml of MMS respectively. Treatments were carried out in 6-well plates (35 mm well, Falcon), protected from light and incubated for 3 h at 37°C on a shaking incubator (New Brunswick) at 75 rpm. After this time, the cultures were dispensed into test tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellets were resuspended in 0.25 ml of phosphate buffered saline (PBS)

6.2.3.3 Single-Cell Gel Electrophoresis

Twin frosted-end glass microscope slides were dipped in 0.5% normal melt agarose (Type I) and left to air dry prior to the addition of the cell suspension layer. For each treatment group 175 μl of the cell suspension was mixed with 175 μl of 0.5% low melt agarose (Flowgen). Immediately after mixing, 75 μl of the suspension was pipetted on to the agarose-coated slide, covered with a cover slip (22x32 mm) and left to set at room temperature. Once set, the cover slip was carefully removed and a further 75 μl of 0.5% low melt agarose was pipetted over the cell layer and covered with a clean cover slip. Two slides per treatment group were prepared.

Once the agar had set, the cover slips were carefully removed and the slides were immersed in freshly prepared chilled lysis solution [2.5 M sodium chloride, 0.1 M EDTA (disodium salt) and 10mM Tris, pH 10] containing 1% Triton X-100 and 10% DMSO in a light proof box for at least 1 h at 4°C.

Alkaline electrophoresis was carried out in a horizontal electrophoresis unit (35x21 cm, Amersham Pharmacia Biotech Ltd.). Slides were placed onto the platform of the unit and the buffer reservoir was filled with chilled electrophoresis buffer [1 mM EDTA (disodium
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

salt) and 0.3 M sodium hydroxide, pH 13]. Slides were left to unwind for 20 min at 4°C, followed by electrophoresis for 20 min at 25 V and 300mA (0.8V/cm).

Once electrophoresis was complete, the alkaline samples were neutralised in 0.4 M Tris, pH 7.5. The slides were rinsed in distilled water, air dried and stored in lightproof boxes with moistened tissues to prevent agar evaporation, at 4°C prior to slide analysis.

6.2.3.4 Slide Analysis and Parameters Measured

DNA was stained with 45 µl of propidium iodide (20 µg/ml) and the slides were allocated random numbers and scored “blind” to prevent operator bias. The comets were examined using an Olympus BH2 fluorescence microscope fitted with a green excitation filter (515-560 nm) and a barrier filter of 590 nm. This was linked via a CCD camera to a computer and comets were scored using Perceptive Instruments COMET II™ image analysis software. Fifty morphologically normal cells were scored per slide to give a total number of 100 cells per treatment group.

The parameters measured are described below.

**Head Length**: The measurement from the point of lowest intensity on the left of the comet head, to the point of highest intensity within the head. This value is then doubled to give a value for the entire head length. Head length is an important parameter because it gives a measure of the homogeneity of the cell population and consequently acts as a control to keep experimental conditions constant throughout subsequent assays.

**Tail Length**: The measurement from the point of greatest intensity within the head to the end of the tail.

**Head/Tail Intensity**: The intensity of the fluorescence detected by image analysis of the head and tail. The value is proportional to the amount of DNA that has moved from the head region into the comet tail.

**Tail Moment**: This measurement is defined as the product of the tail length and the fraction of total DNA in the tail, i.e % DNA in the tail x tail length. The tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed broken pieces (as represented by the intensity of DNA in the tail) and was used as the parameter to express the results in this study.
6.2.3.5 Data Interpretation

A positive response was judged as a two-fold increase in the value of the tail moment over the average control value. The slides were also examined visually for the incidence of apoptotic cells and background debris, which is indicative of toxicity. After completion of a slide, the data was transferred to the Excel component of COMET II™ software, to collect the data for each parameter and to automatically calculate the tail moment.

6.2.4 The Ames II Assay

6.2.4.1 Bacterial Strains

Each of the six base-specific Ames II tester strains (TA 7001-7006) carry a target missense mutation in the histidine biosynthetic operon that reverts to prototrophy by base-substitution events unique to each strain. Strains TA 7001, TA 7002 and TA 7003 were developed to detect point substitutions at A:T base pairs while TA 7004, TA 7005 and TA 7006 detect base changes at G:C base pairs.

An aliquot of 100 µl of a freshly thawed frozen stock of each strain was inoculated into 10 ml of nutrient broth (Oxoid Broth No. 2, UnipathOxoid, Basingstoke, UK). The cultures were grown overnight (10 hours) at 37°C in an environmental shaker (New Brunswick Innova 4000) at 220 rpm in the presence of 25 µg/ml ampicillin.

6.2.4.2 Control Chemicals

STN was used as a positive control chemical for tester strains TA 7001, TA 7002 and TA 7003 at a concentration of 10 ng/ml dissolved in DMSO. MNNG was used as a positive control chemical for tester strain TA 7004 at a concentration of 1 µg/ml dissolved in DMSO. 4NQO was used as a positive control chemical for tester strain TA 7005 at a concentration of 0.5 µg/ml dissolved in DMSO. 5azaC was used as a control chemical for tester strain TA 7006 at a concentration of 2 µg/ml dissolved in water. The final concentration of solvent in each experiment was 2% in a final exposure volume of 0.5 ml.

6.2.4.3 Test Chemical (NIAN)

NIAN was evaluated in all six tester strains at concentrations of 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 µg/ml dissolved in DMSO. The final concentration of solvent in each experiment was 2% in a final exposure volume of 0.5 ml.
6.2.4.4 Preparation of Tester Strains

The overnight cultures of each tester strain were pelleted by centrifugation and resuspended in 10 ml of Micro F solution [15 mM potassium phosphate (dibasic), 10 mM potassium phosphate (monobasic), 10 mM ammonium sulphate, 1 mM trisodium citrate and 0.5 mM magnesium sulphate (heptahydrate)]. Aliquots of 650 µl (1xN) or 1.3 ml (2xN) of each bacterial suspension was added to 12.25 ml or 11.6 ml of Exposure Media [Micro F solution containing biotin (16 µg/ml), histidine (10 µg/ml) and 0.8% glucose] respectively.

6.2.4.5 Chemical Exposure

Aliquots of 10 µl of the appropriate concentrations of NIAN and control chemicals, were dispensed into individual wells of 24-well microtiter plates (Falcon, Becton Dickinson) containing 490 µl of the appropriate prepared tester strain. Seven wells were dedicated to the vehicle control (DMSO) and the eighth well was used for the positive control. For NIAN, one well for each concentration was used. The 24-well plates were incubated at 37°C for 4 h, with shaking at 250 rpm.

6.2.4.6 Prototrophic selection

The 24-well plates were removed from the incubator to room temperature and an aliquot of 2.5 ml of bromocresol purple indicator media [Micro F solution containing bromocresol purple (25 µg/ml), biotin (8 µg/ml) and 0.8% glucose] was added to each well. The histidine deficient indicator medium that selects for prototrophic reversion was mixed gently by pipetting and dispensing the volume in place several times. When adequately mixed, the contents of each well of a 24-well microtiter plate were distributed in 50 µl aliquots over 48 wells of a 384-well microtiter plate (Nunc™) using an 8-channel pipette (Anachem). Each column (4 wells) of the 24-well plate was transferred into one-half of a 384-well plate, effectively dividing each sample among 48 wells of the plate. The 384-well microtiter plates were placed in a sealed plastic box on tissues moistened with 2% Hycolin (a wide spectrum disinfectant) to prevent evaporation and incubated at 37°C for 3 days.

6.2.4.7 Plate Scoring and Data Interpretation

Bromocresol purple turns yellow as the pH drops as catabolites accumulate from the metabolic activity of revertant cells, which grow in the absence of histidine. The number
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

of positive wells out of a total of 48 wells is an indication of the frequency of reversion per replicate per dose, and was compared to the number of spontaneous revertant wells obtained in the solvent control sections. Each 48-well section of the 384-well plates was scored for the number of revertant wells (yellow) manually.

For the purposes of evaluating NIAN in this assay, the response of NIAN in each tester strain was judged mutagenic (+), weakly mutagenic (+w), non-mutagenic (-) or equivocal (?). A mutagenic response was judged as a two-fold increase in the number of positive wells over the mean control values. A weakly mutagenic response was judged as a low level increase over background observed in both 1x normal (1xN) and 2x normal (2xN) inoculum treatments. NIAN responses were judged equivocal where the positive control chemical response was low (less than 10 revertant wells per 48 well section) and the NIAN response was ambiguous.
6.3 RESULTS

6.3.1 Response of NIAN in the Comet Assay

A single cell suspension of mouse lymphoma L5178Y cells was prepared and treated with NIAN at doses ranging from 0.1 to 150 μg/ml. Cells were also treated with the vehicle alone (DMSO) and a positive control chemical (MMS) at a dose of 10 μg/ml. The treated cells were embedded in agarose gel on glass microscope slides and lysed by detergent and high salt solution. The resulting supercoiled DNA was left to relax and “unwind” in a strongly alkaline buffer. Following electrophoresis, comets were analysed using the COMET II™ image analysis system (Perseptives).

Fifty morphologically normal cells were scored per slide to give a total number of 100 cells per treatment group. The appearance of the type of cells scored and comets generated after NIAN treatment are shown in Figure 6.2. A summary of the results is shown in Table 6.2 and slide analysis scores from a representative number of slides are given in Appendix 3.

The actual comet images induced by NIAN were not available for inclusion into this thesis. However, example comet shapes demonstrating the appearance of cells after treatment with a test chemical are shown in Figure 6.2; these images were provided by the Glaxo laboratory.

Alkaline conditions resulted in comets induced by NIAN, with a relatively dispersed tail due to the generation of single strand breaks at alkali-labile sites. These comets resembled the images shown in Figure 6.2 (A) and (B). An undamaged cell is shown in Figure 6.2 (C). Comet shapes (A), (B) and cell shape (C) were seen in all NIAN treatment groups. Comet shape (A) resulted most often from cells treated with NIAN at doses of 1 or 2.5 μg/ml. Comet shape (B) resulted most often from cells treated with NIAN at doses of 0.1 and 0.5 μg/ml. The appearance of an undamaged cell, cell shape (C), was seen most often in the vehicle control group. Cells scored as apoptotic were those where DNA was neither concentrated in the head nor tail region of the comet, but was widely dispersed around a central region indicating toxic doses. Examples of comet shapes where the cells are judged as apoptotic are shown in Figure 6.2 (D) and (E). Cells from NIAN treatment groups
where the dose was in excess of 2.5 μg/ml, were judged as apoptotic and the NIAN dose was judged as toxic as they mostly resembled the appearances of cells (D) and (E).

NIAN, in the absence of an exogenous metabolising system, induced a dose-dependent positive response in the Comet assay over the dose range of 0.1 to 2.5 μg/ml as determined by tail moment [Table 6.2]. The absolute values for the average tail moment were 2.44, 3.57, 4.44 and 4.60 for doses 0.1, 0.5, 1.0 and 2.5 μg/ml respectively. The vehicle induced comets with an average tail moment of 1.15 and the positive control chemical, MMS at a dose of 10 μg/ml, induced comets with an average tail moment of 4.67.

As shown in Table 6.2, NIAN at doses in excess of 2.5 μg/ml, induced apoptotic responses in the cells and the doses were judged as toxic. Consequently, the slides were rejected.

### 6.3.2 Response of NIAN in the Ames II Assay

To verify the mutability of the tester strains, four positive control chemicals were used. STN was used to induce reversions at A:T base pairs and was used as the positive control chemical for strains TA 7001, TA 7002 and TA 7003. MNNG, 4NQO and 5azaC were used to induce reversions at G:C or base pairs and were used as positive control chemicals for tester strains TA 7004, TA 7005 and TA 7006 respectively.

A summary of the results from the Ames II assay are presented in Table 6.3 and scores for the number of revertant (+) wells in each 48-well section of the 384-well plates are given in Appendix 4.

Strains TA 7001, TA 7002 and TA 7003 which have A:T base pairs as their target mutations, responded weakly to the control chemical STN, as determined by the low level number of revertants per 48 wells (6 revertants on average for TA 7001, 5 revertants on average for TA 7002 and 1.5 revertants on average for TA 7003). Consequently, although NIAN induced revertants in strains TA 7001 and TA 7002 (both when 1x normal inoculum and 2x normal inoculum were prepared for the assay) and the number of spontaneous revertants was zero, the NIAN response was judged equivocal. NIAN failed to induce revertants in strain TA 7003 when 1x normal inoculum was prepared for the assay, but revertants were induced by NIAN when 2x normal inoculum was prepared for the assay. Although the mean number of induced revertants per section (0.5) was higher than the spontaneous number (0.3), the NIAN response in this strain was judged equivocal due to the very weak response of the control chemical as previously described.
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

In contrast, strains TA 7004 and TA 7005 which have G:C base pairs as their target mutations, responded strongly to their control chemicals, MNNG and 4NQO respectively, as determined by the reversion of all 48 wells in the section of the plate.

NIAN induced reversions of almost 3-fold over spontaneous reversions in strain TA 7004, when 1x normal inoculum was prepared for the assay. Hence the NIAN response was judged mutagenic. When 2x normal inoculum was prepared for the assay, NIAN induced reversions of almost 2-fold over spontaneous reversions and the NIAN response was judged weakly mutagenic. Overall, NIAN was judged mutagenic in this strain and is consequently able to induce G:C\rightarrow A:T transitions.

The average number of reversions induced by NIAN in strain TA 7005 was greater than the average number of spontaneous reversions when both 1x normal inoculum and 2x normal inoculum was prepared in the assay. NIAN was judged as being weakly mutagenic in this strain as the number of reversions were less than 2-fold over spontaneous reversions and consequently NIAN is able to induce G:C\rightarrow T:A transversions.

Strain TA 7006 which has G:C base pairs as the target mutation responded weakly to the control chemical 5azaC, as determined by the low level number of revertants per 48 wells (5.5 revertants on average). However, the response of NIAN was identical to the control chemical when 2x normal inoculum was used for the assay and the number of reversions were more than 3-fold over spontaneous reversions. When 1x normal inoculum was used for the assay, the number of reversions was less than 2-fold over spontaneous reversions. Overall, NIAN was judged weakly mutagenic in this strain and consequently is able to induce G:C\rightarrow C:G transversions.
Figure 6.2 Example comet shapes and appearance of cells after treatment with a test chemical (images supplied by Genetic and Reproductive Toxicology Group, Medicines Safety and Evaluation, GlaxoWellcome Research and Development, Ware, UK).

(A) Comet with a high tail moment value, (B) comet with a medium tail moment value, (C) undamaged cell, (D) apoptotic cell and (E) severely apoptotic cell.
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

<table>
<thead>
<tr>
<th>Group No.</th>
<th>NIAN dose (µg/ml)</th>
<th>Slide No.</th>
<th>Tail Length</th>
<th>Average Tail Length</th>
<th>Tail Moment</th>
<th>Average Tail Moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO</td>
<td>4</td>
<td>38.72</td>
<td>36.96</td>
<td>1.04</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>35.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>13</td>
<td>58.19</td>
<td>56.12</td>
<td>2.25</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>54.04</td>
<td></td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>5</td>
<td>68.06</td>
<td>76.46</td>
<td>3.07</td>
<td>3.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>84.85</td>
<td></td>
<td>4.06</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>14</td>
<td>86.79</td>
<td>94.25</td>
<td>4.11</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23</td>
<td>101.71</td>
<td></td>
<td>4.77</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>1</td>
<td>86.12</td>
<td>87.36</td>
<td>5.21</td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>88.59</td>
<td></td>
<td>3.98</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>15</td>
<td></td>
<td>Slides rejected due to high levels of background debris and increased incidence of apoptotic cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7.5</td>
<td>22</td>
<td></td>
<td>No cells present</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>75</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>125</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>150</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>MMS (10)</td>
<td>21</td>
<td>103.78</td>
<td>106.89</td>
<td>4.33</td>
<td>4.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>110.00</td>
<td></td>
<td>5.01</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2 Results summary of the Comet assay as determined by Comet II™ analysis software. Complete Comet assay scores are given for a representative number of slides in Appendix 3.
Chapter 6: Activity of NIAN in the Comet and Ames II Assays

Figure 6.3 Activity of NIAN in the Comet Assay, in the absence of an exogenous metabolising system.

MMS was used as a positive control chemical at a concentration of 10 µg/ml.

Tail Moment is defined as the product of the tail length and the fraction of total DNA in the tail, as determined by Comet II™ analysis software.
### Table 6.3 Responses of base-specific Salmonella tester strains for identifying NIAN as a mutagen, in the absence of an exogenous metabolising system.

<table>
<thead>
<tr>
<th>Ames II Tester Strain</th>
<th>Mutation Detected</th>
<th>Inoculum Prepared</th>
<th>Positive Control Chemical</th>
<th>Mean Control Value (no. of + wells per section)</th>
<th>Mean NIAN Value (+ wells per section)</th>
<th>NIAN Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 7001</td>
<td>A:T → G:C</td>
<td>1 x N</td>
<td>STN (8)</td>
<td>0</td>
<td>0.3</td>
<td>?</td>
</tr>
<tr>
<td>TA 7001</td>
<td>A:T → G:C</td>
<td>2 x N</td>
<td>STN (4)</td>
<td>0</td>
<td>0.3</td>
<td>?</td>
</tr>
<tr>
<td>TA 7002</td>
<td>A:T → T:A</td>
<td>1 x N</td>
<td>STN (5)</td>
<td>0</td>
<td>0.6</td>
<td>?</td>
</tr>
<tr>
<td>TA 7002</td>
<td>A:T → T:A</td>
<td>2 x N</td>
<td>STN (5)</td>
<td>0</td>
<td>0.1</td>
<td>?</td>
</tr>
<tr>
<td>TA 7003</td>
<td>A:T → C:G</td>
<td>1 x N</td>
<td>STN (2)</td>
<td>0.1</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>TA 7003</td>
<td>A:T → C:G</td>
<td>2 x N</td>
<td>STN (1)</td>
<td>0.3</td>
<td>0.5</td>
<td>?</td>
</tr>
<tr>
<td>TA 7004</td>
<td>G:C → A:T</td>
<td>1 x N</td>
<td>MNNG (48)</td>
<td>4.3</td>
<td>12.3</td>
<td>+</td>
</tr>
<tr>
<td>TA 7004</td>
<td>G:C → A:T</td>
<td>2 x N</td>
<td>MNNG (48)</td>
<td>8.1</td>
<td>15.5</td>
<td>+w</td>
</tr>
<tr>
<td>TA 7005</td>
<td>G:C → T:A</td>
<td>1 x N</td>
<td>4NQO (48)</td>
<td>9.7</td>
<td>11.4</td>
<td>+w</td>
</tr>
<tr>
<td>TA 7005</td>
<td>G:C → T:A</td>
<td>2 x N</td>
<td>4NQO (48)</td>
<td>15.1</td>
<td>21.1</td>
<td>+w</td>
</tr>
<tr>
<td>TA 7006</td>
<td>G:C → C:G</td>
<td>1 x N</td>
<td>5azaC (5)</td>
<td>1.9</td>
<td>3.1</td>
<td>+w</td>
</tr>
<tr>
<td>TA 7006</td>
<td>G:C → C:G</td>
<td>2 x N</td>
<td>5azaC (6)</td>
<td>1.6</td>
<td>5.5</td>
<td>+</td>
</tr>
</tbody>
</table>

(-), negative; (+), positive; (+w), weakly positive; (?), equivocal. N = normal inoculum; (1xN) and (2xN) refer to 650 and 1300 μl of the prepared bacterial suspension respectively, as described under “Experimental Procedures”. Scores for the number of revertant (+) wells in each 48-well section of the 384-well plates are given in Appendix 4.
6.4 DISCUSSION

6.4.1 In Vitro Genetic Toxicology Assays Identify NIAN as a DNA-Damaging Agent and a Mutagen

NIAN, in the absence of an exogenous metabolising system, induced a dose-dependent positive response in the Comet assay over the dose range of 0.1 to 2.5 μg/ml as determined by tail moment. Consequently, NIAN was identified as a DNA-damaging agent able to generate lesions in DNA, which upon alkali treatment result in strand breaks. Although the precise nature of these lesions were not identified, since NIAN is predominantly able to induce depurination in DNA \textit{in vitro} and \textit{in vivo}, it is likely that these lesions were abasic sites. These results are consistent with previous \textit{in vivo} dose-dependent DNA single-strand scission reports, induced by NIAN, in the mucosa of rat stomach (Furihata et al. 1996).

The NIAN response in the Ames II strains TA 7001 to TA 7003 was judged equivocal due to the lack of mutability shown by these strains, and hence no conclusions regarding the specific base-substitutions at A:T base pairs induced by NIAN can be made. Further investigation is necessary.

Since NIAN induces DNA damage at guanine residues, NIAN would be expected to be judged mutagenic in the Ames strains that detect mutations at G:C base pairs. NIAN was judged mutagenic and weakly mutagenic in both TA 7004 and TA 7005 strains respectively, and is thus able to induce G:C to A:T transitions and G:C to T:A transversions respectively. Overall, NIAN was judged weakly mutagenic in strain TA 7006 and consequently is able to induce G:C to C:G transversions.

The results from the Ames II assay (in the absence of an exogenous metabolising system) are consistent with previous mutagenicity studies that demonstrated NIAN as a direct-acting mutagen toward the Salmonella strains TA98 and TA100 and Chinese hamster lung cells (Wakabayashi et al. 1985a; Wakabayashi et al. 1985b; Wakabayashi et al. 1987).

In summary, NIAN was identified as a DNA-damaging agent and a mutagen as demonstrated by the results of \textit{in vitro} genetic toxicology assays. The significance of the pathways of damage induced by NIAN, in relation to the mutations detected in the Ames II assay, is explored in chapter 7.
CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS
7.1 Transnitrosation: A New Pathway for N-Nitrosocompounds to Exert Genotoxicity

\(N\)-Nitrosoureas can efficiently transfer the nitroso group to nucleophilic targets in isolated purine nucleotides, causing depurination, deamination, and the formation of a novel guanine analogue, oxanine. The transnitrosating ability of NIAN to modify purine residues is preserved at the macromolecular level, with guanine residues appearing to be a primary site of reaction. An additional interstrand cross-linked product between guanine residues in duplex DNA was also detected. The relevance of the depurination pathway induced by NIAN was demonstrated \textit{in vivo}, in the glandular stomach of CD-1 mice and in single cells \textit{in vitro} as shown by the Comet assay.

The fact that \textit{in vivo} damage was demonstrated, in the form of abasic site damage, suggests the ability of the nitroso group to exert damage in cell nuclei. Consequently, the detection of abasic site damage, just one of the pathways of damage induced by \(N\)-nitrosoureas, indicates that the additional pathways of damage induced by NIAN may also be operative \textit{in vivo}.

\(N\)-Nitrosoureas can be associated with an efficient transnitrosation system, reacting with specific biological targets such as DNA bases. All the DNA modification pathways detected for \(N\)-nitrosourea are potentially mutagenic, as demonstrated \textit{in vitro} by the Ames II assay results.

Despite consistent evidence that \(N\)-nitrosoureas are mutagenic in a range of test systems and some evidence that they are DNA-damaging agents, little attention had previously been paid to their mechanism of action. It had been proposed that \(N\)-nitrosoureas may exert their DNA damaging potential via spontaneous decomposition to generate benzenediazonium ion intermediates (Tracey and Shuker 1997) based on mechanistic observations of the decomposition of the fava bean mutagen (Brown \textit{et al.} 1992). The benzene diazonium ion intermediate generated from spontaneous decomposition was postulated to be the reactive intermediate, which reacts with nucleophilic sites on cellular macromolecules to form adducts or deamination products.

The results presented in this thesis, suggest that the previously proposed operating chemistry for \(N\)-nitrosoureas (Tracey and Shuker 1997) may, at best, be a minor operating chemistry. No adducts, structurally related to the \(N\)-nitrosourea, were detected.
in any of the reaction systems employed. Attempts to unequivocally identify characteristic DNA adducts of the fava bean mutagen were unsuccessful (Brown et al. 1992). Yamashita observed adducts formed by reaction of NIAN with calf thymus DNA (Yamashita et al. 1988) but the adducts were not characterised. In postlabelling studies, the cross-linked dimer detected by NIAN reaction with duplex DNA, would probably appear as a bulky adduct, and may explain these observations.

All of the pathways of DNA modification identified for N-nitrosoindoles in this thesis can be rationalised and supported by an initial transnitrosation step; that is, the ready transfer of the nitroso group to nucleophilic nitrogen atoms in the purines.

These results suggest that, in contrast to many other genotoxic N-nitrosocompounds, which are known to alkylate DNA, via the generation of diazonium ions either spontaneously, or after metabolic activation, the genotoxicity of N-nitrosoindoles is likely to arise through efficient transnitrosation. These observations represent a new pathway for N-nitrosocompounds, exemplified by the N-nitrosoindoles, to exert genotoxicity as mediated by transfer of the nitroso group to DNA bases.

Transnitrosation has been invoked, without any direct evidence, to explain direct-acting mutagenicity of N-nitrosoindoles, specifically N-acetyl-N'-nitrosotryptophan and its methyl ester in bacteria (Venitt et al. 1980). The mechanism of denitrosation of nitrosotryptophan under weakly acidic or neutral conditions (pH 4-7) has been studied and may involve intramolecular transfer of the nitroso group to C-3 of the indole, prior to transnitrosation of weakly basic amines and other nucleophiles (Bonnett and Holleyhead 1974; Meyer et al. 1982; Castro et al. 1986). Additional studies revealed that, in fact, an equilibrium exists between tryptophan and its nitrosated form (Mellot et al. 1986) and subsequently, because of this equilibrium, the authors proposed that nitrosated tryptophan could participate in transnitrosation reactions; nitrite could be transferred from one site to another site, depending on the reactivity of the accepting substrate.

The transnitrosating ability of nitrosamines has been studied under mild conditions in dilute aqueous acid, the results of which gave rise to the Liebermann test for the detection of a nitrosamine or N-nitrosocompound (Furniss et al. 1989a). This test demonstrates transnitrosation to aromatic carbon in the form of phenol. The reactions of nitrosoureas and nitrosoguanidines in the participation of transnitrosation to piperidine have been studied (Singer and Cole 1981). In these studies, the ability of N-nitrosocompounds to act
as nitrosating agents was demonstrated (albeit at low pH), however, the alkylating chemistry of the \textit{N}-nitrosocompounds to induce genotoxic effects predominated.

Many \textit{N}-nitrosocompounds are susceptible to loss of the NO group and this denitrosation pathway is generally equated with detoxification. The fate and relevance of the NO moiety released remains to be determined. The results presented in this thesis suggest that \textit{N}-nitrosoindoles are involved in transfer of the nitroso group to nucleophilic macromolecular sites under physiological conditions. The mechanism of this class of \textit{N}-nitrosocompounds to exert genotoxic effects is in contrast to the alkylation chemistry associated with \textit{N}-nitrosocompounds in general. Whether other classes of NNOC, are also associated with similar nitroso transfer capabilities, in addition to alkylation, remains to be determined.

Transnitrosation from aromatic \textit{N}-nitrosocompounds such as \textit{N}-nitrosoureas, \textit{N}-nitrosoamides and \textit{N}-nitrosamines, to aromatic amines or ureas has been observed under non-acidic conditions and at room temperature (Tanno \textit{et al.} 1994). Later studies demonstrated that the NO-generating ability of the aromatic \textit{N}-nitrosoureas and the aromatic \textit{N}-nitrosamides was greater than that of the aromatic \textit{N}-nitrosamines (Tanno \textit{et al.} 1997). In contrast, NO production from aliphatic \textit{N}-nitrosocompounds was not observed. However, it has been postulated that aliphatic \textit{N}-nitrosamines with certain structures enhance N-NO bond cleavage and can act as potential NO donors under physiological conditions (Miura \textit{et al.} 2000). These results suggest structure-activity relationships are extremely important in considering the potential transnitrosating capability of certain NNOC.

What is clear, however, is that the involvement and significance of the release of the NO group from certain NNOC in possible DNA-damaging events has not been studied as widely as for alkylation. The possibility of a NNOC-associated transnitrosation chemistry in addition to alkylation chemistry in contributing to the overall DNA-damaging potential of certain NNOC cannot be ruled out. Interestingly, very recently, it has been reported that base deamination \textit{via} transnitrosation from \textit{N}-nitroso-2-hydroxymorpholine, an oxidative activation product of \textit{N}-nitrosodiethanolamine, in addition to adduct formation, is an operating pathway of genomic alteration and must be considered as a possible mechanism of carcinogenesis for \textit{N}-nitrosodiethanolamine (Park and Loeppky 2000). These results support the fact that with regard to NNOC, a transnitrosation chemistry in inducing DNA damage is not exclusive to \textit{N}-nitrosoindoles, and is likely to contribute to the overall DNA-damaging potential of certain NNOC.
7.1.1 Transnitrosation may be Operative for many Agents that Release Nitric Oxide

In the wider perspective, the existence of a transnitrosation system that facilitates delivery of the nitroso group to specific biological targets has been suggested to explain the biological effects induced by nitrosated tryptophan residues in serum albumin and model dipeptides (Zhang et al. 1996). How extracellular N-nitrosotryptophan causes intracellular biological effects has not been investigated, and the authors suggest that because the model dipeptides cannot freely diffuse through cellular membranes, the NO-like biological effects induced suggests that a (membrane-associated) transnitrosation system may be operating.

Similarly, it has been assumed that S-nitroso compounds, such as S-nitrosothiols, induce NO-like biological effects via the ability of these compounds to donate the NO group under physiological conditions (Arnelle and Stamler 1995; Singh et al. 1996) and undergo transnitrosation reactions at the cell surface (Liu et al. 1998; Zai et al. 1999).

7.2 The Significance and Mutagenic Implications of N-Nitrosoindole-Induced DNA Damage

7.2.1 Deamination Pathway of DNA Modification

The mutations that may ultimately result from the deamination of guanine and adenine residues are shown in Table 7.1.

Xanthine has no known repair mechanism and because xanthine is able to base-pair with thymine (Kamiya et al. 1992), deamination of guanine to xanthine will lead to a G:C → A:T transition mutation upon replication.

In the Ames II assay, NIAN was identified as a mutagen in strain TA 7004 which identifies such a transition mutation. Consequently, it can be suggested from these observations, that N-nitrosoindoles induce G:C to A:T transitions via the deamination of guanine to xanthine.

This type of mutation has frequently been observed as the primary type of mutation involved in nitric oxide-induced mutagenesis, via a mechanism probably involving deamination of cytosine and 5-methyl cytosine residues (Wink et al. 1991; Routledge et
General Discussion and Conclusions


<table>
<thead>
<tr>
<th>Conversion</th>
<th>Type of Mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>guanine → xanthine</td>
<td>G:C → A:T</td>
<td>Kamiya <em>et al.</em> 1992</td>
</tr>
<tr>
<td>adenine → hypoxanthine</td>
<td>A:T → G:C</td>
<td>Karran and Lindahl 1980</td>
</tr>
</tbody>
</table>

*Table 7.1* Mutations that potentially arise from deamination of the purine bases.

It has been generally recognised that nitrite under acidic conditions can afford nitrosating agents (Williams 1988) which can react with amines and other nucleophiles. Similarly, the autoxidation of NO can lead to the formation of reactive nitrogen species, including dinitrogen trioxide, or in the presence of superoxide, peroxynitrite, which all can react with DNA to afford a variety of lesions and mutations, including deamination and the formation of cross-links (reviewed in Burney *et al.* 1999; reviewed in Routledge 2000).

Deamination of cytosine to uracil or 5-methyl cytosine to thymine also occurs spontaneously, resulting in the same transition mutation and providing a significant source of spontaneous mutations (Duncan and Miller 1980; Coulondre *et al.* 1978).

Interestingly, cytosine residues, along with thymine residues, appear to be inactive residues for nitroso transfer by *N*-nitrosoidoines, under the reaction systems employed, and methods of detection employed throughout this thesis. Although the damage detected via transnitrosation by *N*-nitrosoindoles is similar compared with the pathways of damage identified by nitric oxide or nitrous acid, as would be expected with the nitroso group reacting with nucleophilic sites on the DNA bases, the absence of cytosine deamination is in contrast to NO-mediated damage. These observations perhaps suggest that transnitrosation to purine bases may be mediated by intercalation, whereby the nitroso group is most efficiently transferred when donating and accepting environments are in close proximity. Alternatively, the amino group of the guanine residues may simply be more reactive toward the nitroso group released, as purine bases are more easily deaminated by nitrous acid than pyrimidine bases (Caulfield *et al.* 1998). These results
General Discussion and Conclusions

strongly suggest that N-nitrosoindoles are associated with an efficient transnitrosation system whereby delivery of the nitroso group is only facilitated in certain environments.

As xanthine has no known repair mechanism, this lesion induced by N-nitrosoindole nitroso transfer may contribute to the overall generation of G:C to A:T mutations. The involvement of G:C to A:T transitions in the activation of ras oncogenes in tumours has been demonstrated (Zarbl et al. 1985; Bos et al. 1987).

Deamination of adenine to hypoxanthine will lead to an A:T to G:C transition mutation upon replication (Karran and Lindahl 1980). In contrast to xanthine, there is an enzyme that specifically catalyses the cleavage of hypoxanthine residues in DNA. Hypoxanthine-DNA glycosylase efficiently removes hypoxanthine residues that lie in complementary positions to either cytosine or thymine (Karran and Lindahl 1980). Hypoxanthine is also a substrate base for 3-methyladenine-DNA glycosylase removal (Wyatt and Samson 2000). These enzymes are consequently important in DNA repair by cleaving the glycosidic bond of hypoxanthine base and deoxyribose, thus initiating base-excision repair via AP endonuclease action upon the resulting abasic site.

In the Ames II assay, the response of NIAN in the strain that identified A:T to G:C transitions, strain TA 7001, was equivocal, mainly due to a lack of response by the control chemical in this strain, indicating a lack of mutability shown by this strain. Further investigation as to the ability of this pathway to induce such mutations is necessary.

Deamination of adenine residues to hypoxanthine can occur spontaneously in DNA under physiological conditions, and the rate is 40 times slower than that of hydrolytic cytosine deamination (Karran and Lindahl 1980); pyrimidine bases in DNA are more susceptible to spontaneous deamination than are the purine bases. However, the fact that a specific mammalian repair enzyme exists for this lesion, suggests that the spontaneous reaction to form hypoxanthine occurs in vivo. Nitroso transfer from N-nitrosoindoles to adenine residues to induce deamination and the formation of hypoxanthine may contribute to levels of background damage or hypoxanthine generated via alternative pathways. Due to the existence of a repair system, it is unlikely that the presence of hypoxanthine in DNA is as persistent a lesion as xanthine, unless cells are deficient in any aspect of the base-excision repair pathway for this lesion.
7.2.2 Depurination Pathway of DNA Modification

Repair of AP sites, initiated by AP endonuclease via the base-excision-repair pathway, is suggested to be robust, since the spontaneous generation of AP sites from DNA occurs in living cells and has been calculated at approximately $10^4$ apurinic/apyrimidinic sites in each mammalian cell per day (Lindahl and Nyberg 1972). The in vivo study, in the glandular stomach of CD-1 mice demonstrated that repair of apurinic sites induced by NIAN was efficient, in the sense that after 24 hours, the level of damage was at control or endogenous levels.

These damaged sites must be repaired, because such non-coding lesions represent gaps in the templates used by DNA polymerases. Consequently, should any cells defective or diminished in AP endonuclease activity exist, there is the potential for mutation induction.

Mechanisms exist whereby abasic sites may induce mutations (Leob and Preston 1986; Table 7.2).

<table>
<thead>
<tr>
<th>Conversion</th>
<th>Type of Mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>guanine → xanthine → apurinic site</td>
<td>G:C → T:A</td>
<td>Loeb and Preston 1986</td>
</tr>
<tr>
<td>adenine → hypoxanthine → apurinic site</td>
<td>A:T → T:A</td>
<td>Loeb and Preston 1986</td>
</tr>
</tbody>
</table>

Table 7.2 Mutations that potentially arise from depurination of the purine bases.

DNA polymerases can copy past AP sites and are characteristically error-prone. The DNA strand synthesised most frequently (but not always) contains an adenine residue opposite an abasic site (Loeb and Preston 1986). As a result, depurination of guanine and adenine residues in DNA, will lead to G:C to T:A and A:T to T:A transversion mutations respectively.

In the Ames II assay, the result of NIAN in strain TA 7002, which identifies A:T to T:A transversions was equivocal due to the lack of mutability shown by this strain. However, NIAN was judged mutagenic in Strain TA 7005 which identifies G:C to T:A transversions. Thus it can be suggested that NIAN induces such mutations via the depurination pathway.
Although it is suggested that adenine is preferentially incorporated opposite AP sites there is no consensus as to the frequency at which other nucleotides are incorporated (Loeb and Preston 1986). In bacteriophage for example, the second most frequently incorporated nucleotide opposite the abasic site contains a guanine residue. It has been demonstrated that Watson-Crick hydrogen bonding was not a necessary requirement for insertion opposite an abasic site (Matray and Kool 1999). A non-hydrogen-bonding triphosphate derivative of a pyrene nucleoside was efficiently and specifically inserted by DNA polymerases opposite abasic sites, confirming that steric complementarity is an additional and important factor in the fidelity of DNA synthesis. Also, the frequency of misincorporation by DNA polymerase is governed by the ratio of incorrect to correct deoxynucleoside triphosphate substrates. This ratio will be influenced by DNA-damage induced in the intracellular nucleotide pool and the misincorporation of such nucleotides in vivo, may be enhanced as a result. Furthermore, a distortion of the structure of DNA induced by an AP site may cause misreading of the nucleotide sequences in the template (Wang et al. 1997).

All of these factors suggest that misincorporation and miscoding can result as a consequence of abasic site formation. The possibility of alternative misincorporations, other than adenine opposite the abasic site, by DNA polymerase may explain the weak mutagenicity observed by NIAN in the Ames II strain TA 7006; this strain identified G:C to C:G transversions.

7.2.3 The Significance of Oxanine as a Lesion in DNA

In the case of guanine, transnitrosation results in formation of a diazonium ion at C-2. The solvolysis product of this diazonium ion to give xanthine is a well-known reaction in purine chemistry but it is only recently that a more profound consequence of this pathway has been discovered. Suzuki and co-workers demonstrated that treatment of dGuo, oligodeoxynucleotides and DNA with nitrous acid and nitric oxide resulted in the formation of dOxo (Suzuki et al. 1996), due to a rearrangement of the C-2-diazonium ion (Suzuki et al. 1997a).

Oxanine and dOp were detected as products of nitroso transfer by NIAN in isolated dGp and dGuo. When a more complex reaction system was investigated for oxanine formation, the novel nucleobase was detected in single-stranded oligonucleotide and calf thymus
DNA. Since \textit{in vivo} damage was induced by NIAN in the form of abasic sites, in the glandular stomach of CD-1 mice, it is possible that NIAN also induced formation of this novel nucleobase \textit{in vivo}. The biological significance of oxanine as a lesion in DNA is unknown and elucidation of the genotoxic effects is an important subject of future studies.

The presence of an oxygen atom in place of N-1 of guanine is likely to have a profound effect on Watson-Crick base pairing. Recent results on the misincorporation of 2'-deoxyoxanosine triphosphate (dOTP) into oligonucleotides by DNA polymerases, suggest that it is likely to be a potent mutagenic lesion (Suzuki \textit{et al.} 1998). dOTP substituted for dGTP and to a lesser extent for dATP. As a result, dOTP was incorporated opposite both template C and T. The proposed base-pairing schemes for oxanine with cytosine and thymine are shown in Scheme 7.1.

![Scheme 7.1 Proposed base pairs between oxanine and cytosine, and oxanine and thymine.](image)

When the structure of oxanine is considered, both the O:C and O:T pairs can adopt the Watson Crick geometry in duplex DNA without introducing any steric perturbation.

The generation of O:C base pairs is not likely to induce mutation since cytosine is the natural base to be incorporated opposite guanine, from which oxanine is derived. However, the generation of O:T base pairs is likely to elevate the mutation frequency since the mispairing of oxanine with thymine during replication will result in a single point mutation, a G:C to A:T transition mutation. Interestingly, the response of NIAN in the Ames II TA 7004 strain, which detects G:C to A:T transition mutations was judged mutagenic. Consequently, any oxanine formed in DNA as a consequence of nitroso transfer by \textit{N}-nitrosoindoles either directly, or via formation in the intracellular nucleotide
pool and subsequent misincorporation by DNA polymerase, is likely to elevate the mutation frequency and contribute to the generation of G:C to A:T transitions via this pathway.

Interestingly, the $N$-glycosidic bond of dOxo is as stable as that of dGuo and hydrolysed 44-fold more slowly than that of 2'-deoxyxanthosine (Suzuki et al. 1997b). These findings suggest that oxanine formation in DNA is likely to be a persistent lesion in the absence of a specific repair pathway. No specific repair enzymes have been identified for xanthine in DNA as previously described, so it is possible that xanthine is released spontaneously by nonenzymatic hydrolysis and the resultant abasic site is repaired by AP endonucleases via base excision repair. Ultimately, the formation of oxanine in DNA could result in more serious mutagenic events than that of xanthine formation in DNA.

In addition to the possible genotoxic effects of oxanine formation, oxanine is likely to react with intracellular nucleophiles under physiological conditions since it has an $O$-acylisourea structure. It has recently been reported that a ring-opened adduct between dOxo and glycine is formed under physiological conditions (Suzuki et al. 2000). The adduct was very stable, and also was produced when dOxo in oligonucleotide was reacted with glycine. The six-membered ring of dOxo ($O$-acylisourea; 2-amino-4,5-benz-6-oxo-1,3-oxazine), in the base moiety, reacted with the amino group of glycine under physiological conditions, resulting in the adduct via formation of an amide bond.

These results suggest that an additional fate of oxanine in DNA is reaction with glycine and other free amino acids, indeed other intracellular nucleophiles possessing an amino function, to afford a variety of dOxo adducts as demonstrated in Scheme 7.2. These observations lend further support to an alternative mechanism for cross-linking induced by NIAN, whereby the dG to dG cross-link is derived from ring-opened products.

It is likely that since the structure of oxanine and any oxanine adducts are quite different, should these lesions exist in DNA in vivo, distinctly different repair systems may be required to eliminate these lesions. Consequently, the generation of oxanine in DNA may represent an important mechanism of genomic alteration.

Interestingly, the riboside of oxanine, oxanosine, is a novel nucleoside antibiotic isolated from the culture filtrate of *Streptomyces capreolus* MG265-CF3 and characterised by X-ray crystallography (Shimada et al. 1981; Nakamura et al. 1981). Oxanosine has been shown to possess important biological activity including inhibition of ras-transformation
and activity toward tumour cells (Hori et al. 1989; Itoh et al. 1989; Watanabe et al. 1994; Sugita and Ohtani 1997).

Scheme 7.2 Reaction of dOxo with a primary amine affords a ring-opened adduct.

7.2.4 The Significance of NIAN-Induced Cross-Links

It has been estimated that for every four deaminations of deoxyguanosine, one interstrand cross-link is formed and they are generally believed to be toxic if not lethal to cells by blocking DNA replication (Ojwang et al. 1989; Grueneberg et al. 1991).

Complete cross-link repair requires excision of one of the cross-linked bases by a glycosylase or endonuclease and subsequent removal of the adducted base in the opposite strand, as well as re-synthesis by DNA polymerase and ligation in both strands (Bohr 1994). Presumably, this process is made much more challenging by the lack of an intact template in both strands. The potential for error-prone synthesis and possible deletions exist, affording a wide variety of mutations including base substitutions at both G:C and A:T base pairs, deletions and possible chromosomal rearrangements (reviewed in Povirk and Shuker 1994).

7.2.5 Influence of DNA Sequence

As GC rich regions can be preferred sites of damage for many DNA-damaging agents (Mattes et al. 1988), damage at these sites may elevate the mutation frequency. Studies in single-stranded oligonucleotides as shown in section 4.3.1, demonstrated efficient nitroso transfer to induce depurination at guanine residues preceded by a guanine residue or
alternating with a cytosine residue. Such damage induced by \(N\)-nitrosoindoles at guanine residues in close proximity either on the same strand or on opposite strands, is likely to influence the mutation frequency and rate of repair.

A potential consequence of the repair of abasic sites located in close proximity on opposite strands is the formation of double-strand breaks, which is considered to contribute significantly to cytotoxicity (Ward 1988, Illakis 1991). Chaudry has demonstrated that single strand scission by AP endonuclease at closely positioned abasic sites induces double-strand breaks and consequently, could have serious deleterious consequences in the cell (Chaudry and Weinfeld 1997). Since AP sites are the intermediates in base-excision repair, it is likely that the formation of double-strand breaks is not limited to the formation of abasic sites directly, but as a consequence of the repair also of other damages in close proximity. The biological consequences of other types of multiple damages, requires further investigation and will depend on the recognition of the lesions by the initial damage-recognition enzymes.

These observations suggest that multiple lesions, not only abasic sites, but deamination products, the formation of oxanine and cross-links, within GC rich regions induced by nitrosated indoles, may be critical lesions in DNA as they present an additional challenge to the repair machinery of the cell.

### 7.3 Future Work

From the above discussion, it is clear that further investigation of the potential role of nitrosated indoles and transnitrosation pathways in human disease is required to more fully understand the relevance and biological significance of the induced DNA damage.

The ability of other \(N\)-nitrosocompounds to exert similar effects via a transnitrosation pathway would be extremely interesting to investigate in the reaction systems employed in this thesis, to elucidate a possible prediction of a structure-activity relationship for \(N\)-nitrosocompound induced DNA damage via transnitrosation.

An immediate area of further research, highlighted by the results presented in this thesis, is to investigate the biological significance of oxanine as a lesion in DNA. This lesion is particularly important because it may be a useful marker for transnitrosation-induced and nitrosative DNA damage. Site-specific mutagenesis to investigate the effect of the oxanine
lesion, by incorporating the lesion into an oligonucleotide at a known location in the DNA sequence, could be used to study the misincorporation of bases opposite the lesion by different polymerases in vitro. The effect of sequence context within which the lesion is placed, and the influence of DNA repair on mutagenesis may also be studied. The development of sensitive methods to detect this lesion in DNA, such as LC-MS or via the generation of a specific antibody, would enable oxanine to be used as a biomarker for this type of damage, which can also be generated via nitrous acid and nitric oxide (Suzuki et al. 1997a).

The mutation spectrum induced by N-nitrosoindoles may also be studied in the supF forward mutation assay, which will enable comparisons with spectra induced by other reactive nitrogen oxide species (Routledge 2000). Studies such as these, combined with further mechanistic studies, possibly involving intercalation, may shed more light as to why pyrimidine residues, or cytosine residues in particular, appear to be inactive targets for nitroso transfer by N-nitrosoindoles.

Detecting gene mutations in vivo, using the Muta™Mouse model (Gossen et al. 1989), would be useful to investigate the potential for mutation induction in the target organ of the stomach and other organs. Results of such studies, combined with perhaps a long-term in vivo study would be useful in assessing the significance of N-nitrosoindole-induced damage in possible carcinogenesis.

The potentially cytotoxic DNA cross-links also formed via transnitrosation by N-nitrosoindoles have not been characterised. By improving the 32P-postlabelling method for the detection of these lesions, by designing oligonucleotides that have greater melting temperatures with an increased number of possible cross-linking sites containing both guanine and oxanine residues, should facilitate improved detection and enable elucidation about the sequence specificity. The possibility of dG to dA cross-links could also be explored. By increasing the yield of such lesions, their characterisation via LC-MS may be investigated, the results of which would be extremely interesting, given the possibility of ring-opened adducts. Again the sensitive detection of these lesions would enable them to be used as biomarkers for N-nitrosoindole-induced damage and nitrosative damage in general, and should enable further studies as to the repair and significance of such lesions in vitro and in vivo.
General Discussion and Conclusions

The results of such studies in the longer term may facilitate a greater understanding in the role and significance of N-nitrosoindole-induced damage in human disease. Moreover, the results of investigating other N-nitrosocompounds, for inducing similar pathways of damage via transnitrosation would again be extremely interesting given the historical view that loss of the NO group is equated with detoxification.

7.4 Conclusions

A series of three model N-nitrosoindole compounds were synthesised and fully characterised in order to examine the DNA-damaging pathways induced by N-nitrosoindoles. At physiological pH and temperature, N-nitrosoindoles induce DNA damage in vitro via distinct reaction pathways. These were (I) depurination to the corresponding purine bases, (II) deamination coupled with depurination to give hypoxanthine and xanthine, (III) the formation of a novel nucleobase oxanine and (IV) cross-linked DNA. N-Nitrosoindoles induce these pathways of DNA damage via a transnitrosation mechanism. The ability of the nitroso group to exert damage in nuclei via the formation of abasic sites was demonstrated in vivo, in the glandular stomach of CD-1 mice. NIAN induced single-strand breaks in vitro as shown by the results of the Comet assay. NIAN was mutagenic in the Ames II assay. In contrast to many other genotoxic N-nitrosocompounds, which are known to alkylate DNA, these findings reveal a new pathway for N-nitrosocompounds, exemplified by N-nitrosoindoles, to exert genotoxicity. In the wider perspective, DNA damage mediated by efficient transfer of the nitroso group to DNA bases, may be operative for many agents that release nitric oxide.
REFERENCES


Bellander, T. (1990) Nitrosation of piperazine after oral intake or inhalation exposure. In The Significance of 
N-Nitrosation of Drugs (Eisenbrand, G., Bozler, G., and Nicolai, H.v., eds) pp. 213-233, Gustav Fischer 
Verlag, Stuttgart, New York.

Identification and Biological Significance (Hemminki, K., Dipple, A., Shuker, D.E.G., Kadlubar, F.F., and 


Bradlow, H.L., Michnovicz, J.J., Telang, N.T., and Osborne, M.P. (1991) Effects of dietary indole-3-
carbinol on estradiol metabolism and spontaneous mammary tumours in mice. Carcinogenesis 12, 1571-
1574.

Brimblecombe, R.W., Duncan, W.A.M., Durant, G.J., Emmett, J.C., Ganellin, C.R., Leslie, G.B., and 
Pearsons, M.E. (1978) Characterisation and development of cimetidine as a histamine H2-receptor 
antagonist. Gastroenterology 74, 339-347.


References


References


References


References


References


References


References


References


References


References


References


References


241
References


References


References


References


SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
APPENDIX 1

LUCAS, L. T., GATEHOUSE, D., and SHUKER, D.E.G.

J. BIOL. CHEM. 1999, 274, 18319-18326
SPECIAL NOTE

This item is tightly bound and while every effort has been made to reproduce the centres force would result in damage.
Efficient Nitroso Group Transfer from N-Nitrosoindoles to Nucleotides and 2'-Deoxyguanosine at Physiological pH

A NEW PATHWAY FOR N-NITROSOCOMPOUNDS TO EXERT GENOTOXICITY

Lynda T. Lucas, David Gatehouse, and David E. G. Shuker

From the Biomonitoring and Molecular Interactions Section, MRC Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester LE1 9HN, United Kingdom and Genetic and Reproductive Toxicology, Medicines Safety and Evaluation Division, Glaxo Wellcome Research and Development, Park Road, Ware, Hertfordshire SG12 0DP, United Kingdom

The endogenous formation of N-nitrosoindoles is of concern since humans are exposed to a variety of naturally occurring and synthetic indolic compounds. As part of a study to evaluate the genotoxicity of N-nitrosoindoles, the reactions of three model compounds with purine nucleotides and 2'-deoxyguanosine at physiological pH were investigated. The profiles of reaction products were identical for each of the N-nitrosoindoles and three distinct pathways of reaction could be discerned. These pathways were: (i) depurination to the corresponding purine bases, (ii) deamination, coupled with depurination, to give hypoxanthine and xanthine, and (iii) formation of the novel nucleotide 2'-deoxyxanthosine monophosphate and its corresponding depurination product oxanine in reactions with 2'-deoxyguanosine monophosphate. 2'-Deoxyoxanines and oxanines were observed in reactions with 2'-deoxyguanosine. Further studies showed that formation of all of these products could be rationalized by an initial nitrosation step. These results suggest that, in contrast to many other genotoxic N-nitrosocompounds which are known to alkylate DNA, the genotoxicity of N-nitrosoindoles is likely to arise through transfer of the nitroso group to nucleophilic sites on the purine bases. All of the products resulting from transnitrosation by N-nitrosoindoles are potentially mutagenic. These findings reveal a new pathway for N-nitrosocompounds to exert genotoxicity.

N-Nitrosation frequently transmutes innocuous nitrogen-containing compounds into toxic compounds (1). Depending on the structure of the N-substituents the resulting N-nitrosocompounds can either decompose spontaneously to give alkylating intermediates or do so after metabolic activation to α-hydroxy derivatives (Fig. 1). Alkylation agents such as alkylazonium ions react with DNA to give adducts which can be mutagenic upon replication. Among the many hundreds of nitrogenous compounds that have been studied a number of 3-substituted indoles have been found to produce mutagenic products upon treatment with nitric acid (2). 3-Substituted indoles occur widely in nature as natural products, such as tryptophan, or the plant growth hormone, indole-3-acetic acid, and this has lead to a concern that endogenous nitrosation of indoles in the acidic environment of the stomach could contribute to the risk of gastric cancer.

Indole-3-acetonitrile (IAN)1 is a plant growth hormone that is present in various vegetables, notably Chinese cabbage, a common foodstuff in Japan. The mono-N-nitroso derivative, 1-nitrosoindole-3-acetonitrile (NIAN), is a direct acting mutagen toward Salmonella typhimurium TA98 and TA100 and Chinese hamster lung cells (3, 4). 32P-Postlabeling has shown that DNA adducts are formed in vivo and in the gastrointestinal tissues of rats treated with NIAN (5), but no attempt was made to characterize the products. Marked inductions of ornithine decarboxylase and DNA synthesis in rat stomach mucosa have been reported, after administration of NIAN, suggesting that NIAN also has potential tumor promoting activity in carcinogenesis in the glandular stomach (6). Two other indole compounds, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde have also been isolated from Chinese cabbage as nitrosatable mutagen precursors (7, 8). In addition there are around 20 naturally occurring indole compounds, mostly 3-substituted, that have been demonstrated to be mutagenic toward Salmonella strains without S9 mix after nitrite treatment (9–12).

Despite consistent evidence that N-nitrosoindoles are mutagenic in a range of test systems and some evidence that they are DNA-damaging agents, little attention has been paid to their mechanism of action. The direct acting mutagenicity of N-nitrosoindoles suggested that spontaneous decomposition of these compounds gives rise to DNA-reactive intermediates, possibly involving diazonium ions. In this study, reactions of a series of 3-substituted nitrosated indoles with 2'-deoxyadenosine-3'-' and 5'-monophosphates (dAp and dpA), 2'-deoxyguanosine-3'-' and 5'-monophosphates (dpG and dpG') and 2'-deoxyguanosine (dGuo) were investigated. The results suggest that, unlike many other mutagenic N-nitroso compounds, N-

1 The abbreviations used are: IAN, indole-3-acetonitrile; ACN, aceto-nitrile; Ade, adenine; dA, ACN, deuterated acetonitrile; dA, 2'-deoxyadenosine-3'-monophosphate; dGp, 2'-deoxyguanosine-3'-monophosphate; dGuo, 2'-deoxyguanosine; dGlo, 2'-deoxyguanosine; dOp, 2'-deoxyadenosine-3'-monophosphate; dPhA, 2'-deoxyadenosine-5'-monophosphate; dGp, dGp' 2'-deoxyguanosine-5'-monophosphate; dOp, 2'-deoxyguanosine-5'-monophosphate; ESI-MS-MS, electrospray tandem mass spectrometry; FAB, fast atom bombardment; Gua, guanine; HPCL, high performance liquid chromatography; IAAME, indole-3-acetic acid methyl ester; MeOH, methanol; N2-AcGp, N2-acetyldihydroxyguanosine-5'-monophosphate; N2-AcGu, N2-acetylguanine; NIAAME, 1-nitrosoindole-3-acetic acid methyl ester; NIAM, 1-nitrosoindole-3-acetamide; NIAN, 1-nitrosoindole-3-acetonitrile; NO, nitric oxide; Ac, acetyl.
Nitroso Transfer from N-Nitrosodioxoles to DNA Bases

Nitrosodioxoles react with DNA bases primarily as potent transnitrosation agents. The evidence for this surprising conclusion is presented in this paper.

EXPERIMENTAL PROCEDURES

Caution—NIA is mutagenic and should be handled with extreme caution. 1-Nitrosodiole-3-acetamide (NIAM) and 1-nitrosodiole-3-acetic acid methyl ester (NIAAME) are suspected mutagens and should be handled with extreme caution.

Synthesis of 3-Substituted Nitrosated Indoles—IAN (Fluka), indole-3-acetamide (Sigma), or indole-3-acetic acid methyl ester (IAAME) (Sigma), dissolved in the minimum amount of acetonitrile (ACN), were reacted with a 15-fold molar excess of aqueous 50 mM nitrous acid (pH 3) at 37 °C for 3 h. Reaction mixtures were extracted 3 times with 3 volumes of dichloromethane and dried over anhydrous sodium sulfate. The resulting filtrates were dried in a stream of nitrogen.

Purification and Characterization of Nitrosated Indoles—The crude products were purified by HPLC using a Gilson-gradient controlled system equipped with either a dual-wavelength 116 Gilson UV detector or an Applied Biosystems Inc., 1000s diode array detector. Analyses were performed using a Hypersil C18 BDS, 5 μ, 250 × 10-mm reverse-phase Shandon preparative column employing the following elution program: 0 min, 40% B, 20 min, 100% B, 25 min, 100% B, 30 min, 40% B (solvent A: water; solvent B: methanol) at a flow rate of 3 ml/min with UV detection at 260 and 328 nm. The pure nitrosated indole was dried in a stream of nitrogen to give yellow crystalline product in around 50% yield; the main impurity being unreacted starting material. 1H NMR spectra were recorded on a Bruker ARX 250 MHz instrument. Mass spectra were recorded by using either a VG 70-SEQ or a VG Autospec Ultima-Q. Microanalyses were provided by Buttersworth Laboratories Ltd., Middlesex, United Kingdom.

Characterization of NIA—NIA (Methanol) 204, 260, 331; NMR δ (CDCl₃) 2-H, 7.8 (major, s), and 8.4 (minor, t); 4-H, 5-H, and 6-H, 7.45-7.65; 7-H, 8.24 (major, d), and 8.35 (minor, s); -CH₂-, 3.8 (major, d) and 3.85 (minor, d); -NH₂, 7.2 and 6.5 (2 br. s).; MS m/z (electron impact) 185 (M+); 155 (M+-30), this is consistent with loss of the NO group.

Calculated: C, 59.5 H, 4.45 N, 19.9

Found: C, 59.5 H, 4.45 N, 19.9

It was not possible to obtain satisfactory microanalytical results with NIAAM and low nitrogen values were consistent with denitrosation (see below).

Characterization of NIAAME—NIAAME (Methanol) 204, 261, 331; NMR δ (CD₂COCD₃) 2-H, 7.9 (major, s), and 8.4 (minor, m); 4-H, 5-H, and 6-H, 7.5-7.85; 7-H, 8.3 (major, d), and 8.45 (minor, s); -CH₂-, 3.75 (major, d) and 3.8 (minor, d); -NH₂, 7.2 and 6.5 (2 br. s).; MS m/z (FAB positive ion) 219 (M+); 174 (M+ - 30), this is consistent with loss of the NO group; 130 (M+ - 89), this is consistent with a further loss of -CONH₂.

Calculated: C₁₁H₁₆N₂O₃

Found: C, 60.65 H, 4.59 N, 12.84 O, 22.0

Reactions of 2'-Deoxyadenosine-3'- or 5'-Monophosphate (dAp or dpmP) with NIA—NIA at molar ratios ranging from 1:0.5 to 1:20 (dAp/dpN: NIA, NIA = 0.14, 0.28, 0.56, 1.4, 2.8, 4.2, and 5.6 mg in 250 μl of ACN) were incubated with solutions of dAp or dpmP (0.5 mg; 1.5 μmol in 250 μl of 10 mM Tris-HCl buffer, pH 7.4) in a water bath at 37 °C for 6 h in the dark. The reaction mixtures were extracted 3 times with 3 volumes of diethyl ether to remove unreacted NIA, dried down in a centrifugal vacuum evaporator (DNA 110, Savant), and redissolved in 500 μl of water prior to HPLC analysis. Control incubations were set up of NIA, IAN, or dAp/dpA alone. Diethyl ether extracts were also dried down and redissolved in 1500 μl of ACN prior to HPLC analysis.

Reactions of 2'-Deoxyguanosine-3'- or 5'-Monophosphate (dGp or dGpG) with NIA—NIA at molar ratios ranging from 1:0.5 to 1:20 (dGp/dGpG: NIA, NIA = 0.133, 0.265, 0.53, 1.325, 2.65, 3.975 and 5.3

Fig. 1. Summary of N-nitrosation of amines and related compounds, and decomposition pathways of the resulting N-nitroso compounds.

Fig. 2. Structures of 3-substituted nitrosated indoles described in this study.
mg in 250 μl of ACN) were incubated with solutions of dGp or dpG (0.5 mg: 1.44 μmol in 250 μl of 10 mM Tris-Cl buffer, pH 7.4) as described for reactions of dpA with NIA.

Reactions of dpA and dpG with NIA and NIAAME—NIA (4.8 mg in 250 μl of ACN) or NIAAME (4.65 mg in 250 μl of ACN) were reacted with dpA or dpG (0.5 mg in 250 μl of 10 mM Tris-Cl buffer, pH 7.4) as described for reactions of dpA with NIA. Both these concentrations correspond to a 15-fold excess of nitrosated indole over nucleotides.

Reactions of NIA, NIAAME, or NIA with 2'-Deoxyguanosine (dGuo)—NIA, NIAAME, or NIA at molar ratios ranging from 1.1 to 1.15 (dGuo: nitrosated indole) were incubated with solutions of dGuo (0.5 mg: 1.87 μmol in 250 μl of 10 mM Tris-Cl buffer, pH 7.4) as described for reactions of dpA with NIA.

HPLC Analyses of Reaction Mixtures—HPLC-UV analyses were performed using a Hypersil B 18 BDS, 5 μm, 250 × 4.6-mm reverse-phase Shandon analytical column on the Gilson gradient-controlled system equipped with a diode-array 110 Gilson UV detector or to obtain UV spectra, an Applied Biosystems Inc., 1000s diode array detector. 100-μl injection volumes of aqueous reaction mixture were analyzed at a flow rate of 1 ml/min using the following elution program: 0 min, 0% B; 25 min, 20% B; 35 min, 50% B; 40 min, 0% B (solvent A: 50 mM ammonium formate, pH 5.4; solvent B: methanol). Column eluants were monitored at 260 and 290 nm. For the separation of NIA, NIAAME, or NIA, a different injection volume of reaction mixture was analyzed at a flow rate of 1 ml/min using the elution program described for the purification of the nitrosated indoles. Column eluants were monitored at 260 and 290 nm. Fractionating and drying onto a centrifugal vacuum evaporator for further analysis by ESI-MS. Reaction products were identified from their UV spectra, obtained by diode array analysis, and retention times which were compared with those of the authentic standards. Further evidence for structural assignment was obtained from the ESI-MS and ESI-MS-MS spectra of the pooled fractions.

Characterization of Reaction Products by Mass Spectrometry— Off-line ESI-MS and ESI-MS-MS characterization of reaction products was carried out using a VG Autospec Ultima Q. Dried fractions previously collected from the HPLC were resuspended in 50:50 ACN/water and inserted into the VG via 20-μl "loop" injection or continuous infusion, at a flow rate of typically 8 μl/min. The cone voltage was in the range 8–23 V and full scan mass spectra were obtained by scanning from m/z 1550 to 50 at a scan speed of 10 s/decade. ESI-MS product ion spectra were obtained by selecting the desired precursor ion with MSI and allowing collision-induced dissociation to occur in the collision cell using air as the target gas, typically at 10% transmission of the precursor ion, with a collision energy of 48 eV. The resulting product ions were analyzed in MS2.

RESULTS

Synthesis and Characterization of a Series of 3-Substituted Nitrosated Indoles—NIA, NIAAME, and NIAAME (Fig. 2) were obtained as crystalline products by treatment of the parent compounds with acidified nitrite followed by HPLC purification. Spectral data agreed well with data previously published by Watabayashi et al. (3). NIA, NIAAME, and NIAAME are isomeric compounds. The E/Z isomerization of the NO group is reflected in the nmr data, affecting principally the 2-H, 7-H, and -CH2- resonances; essentially the indole nucleus remains the same for the series of nitrosated indoles. The ratio of the two isomers was consistent for all three compounds at approximately 2:1. The disappearance of the -NH signal (as -NNO is produced) is evident in all nmr spectra. Satisfactory microanalytical data could not be obtained for NIA, despite repeated preparations, with precautions taken to reduce denitrosation. However, spectral data for NIA were consistent with that obtained for NIA and NIAAME.

Reaction of Nitrosated Indoles with dAp and dpA—Reaction of NIA with dpA at pH 7.4 in buffered 50% aqueous acetonitrile yielded 3 products not seen in control incubations, hypoxanthine, adenine (Ade), and N2-acetyl adenine with retention times of 7.7, 11.4, and 15.5 min, respectively (Fig. 3). Identification of hypoxanthine was based on comparison of the retention time and UV spectrum with authentic hypoxanthine analyzed under the same conditions. When ESI-MS was performed on this fraction, a molecular ion with m/z 137 (M + H)+ was observed. Identification of adenine was again based on comparison of the retention time and UV spectrum with adenine standard. When ESI-MS was performed a main molecular ion with m/z 136 (M + H)+ was observed. A molecular ion with m/z 352 (M + H)+ was also observed suggesting the presence of unreacted dpA. Adenine and dpA co-elute under the HPLC conditions described. The identification of reaction product N2-acetyl adenine was based on ESI-MS results. A molecular ion with m/z 178 (M + H)+ was observed for this fraction. When ESI-MS was performed under conditions promoting cone voltage-induced dissociation, a fragment ion with m/z 136 corresponding to protonated adenine was observed, confirming the structure as an adenine adduct. When deuterated ACN was used as co-solvent, a molecular ion with m/z 181 (M + H)+ was observed for the fraction, corresponding to N2-acetyl (d2)-adenine. All 3 reaction products increased in concentration with accompanying increases in the molar ratio of NIA to dpA.

Analogue reaction products with similar dose-related responses were seen with reactions of NIA and NIAAME with dpA and NIA and NIA with dpA (data not shown).

Reactions of Nitrosated Indoles with dGp and dpG—Reaction of NIA with dGp at pH 7.4 in buffered 50% aqueous acetonitrile yielded 6 products, guanine (Gua), xanthine, oxanine, 2'-deoxyoxanosine-5'-monophosphate (dOP), N2-acetyldexoxyguanosine-5'-monophosphate (N2-AcdGp), and N2-acetylguanine (N2-AcGua), not seen in control incubations, with retention times of 7.8, 8.7, 11.4, 12.1, 19.6, and 20.1 min, respectively (Fig. 4). Identification of reaction products guanine and xanthine was based on comparison of retention times, UV spectra, and ESI-MS results compared with standards analyzed using the same systems and conditions. The identification of reaction products N2-acetyldexoxyguanosine-5'-monophosphate and N2-acetylguanine was based on ESI-MS results and comparison of retention time and UV spectrum for authentic N2-acetylguanine. A molecular ion with m/z 194 (M + H)+ was observed for the fraction corresponding to N2-acetylguanine. When conditions were employed to promote cone voltage-induced dissociation, a fragment ion with m/z 152 corresponding to protonated guanine was observed, confirming the structure as a guanine adduct. Replacement of acetonitrile in the reaction mixture by the deuterated solvent gave a molecular ion with m/z 197 (M + H)+ for this fraction confirming it as N2-(d2)-acetylguanine. Similarly, ESI-MS analysis for the fraction corresponding to N2-acetyldexoxyguanosine-5'-monophosphate afforded a molecular ion with m/z 388 (M + H)+ and again by exchanging the solvent as described above, an increase of 3 units to m/z 391 (M + H)+ was observed for the molecular ion.

The concentration of xanthine increased with accompanying increases in the molar ratio of NIA to dpG, while the concentration of guanine decreased as conversion to reaction products occurred. The results indicate that depurination occurs independently of the other pathways and is more evident at the higher molar ratios. This is illustrated by the dose-response behavior of the N2-acetyl adducts. The formation of N2-acetyldexoxyguanosine-5'-monophosphate increased with an increase in the molar ratio of NIA to dpG up to a ratio of 10:1. As the molar ratio increased to 20:1, the concentration of the product decreased in the system, and an increase in the depurinated adduct N2-acetylguanine was observed.

The identification of reaction products 2'-deoxyoxanosine-5'-monophosphate and the depurination product oxanine (Fig. 5) was based on comparison of retention time, UV spectra, and ESI-MS results when compared with oxanine derived from the hydrolysis of the N-glycosidic bond of authentic 2'-deoxy-oxanosine-5'-monophosphate. The formation of N2-acetyldexoxyguanosine-5'-monophosphate increased with an increase in the molar ratio of NIA to dpG up to a ratio of 10:1. As the molar ratio increased to 20:1, the concentration of the product decreased in the system, and an increase in the depurinated adduct N2-acetylguanine was observed.

The identification of reaction products 2'-deoxyoxanosine-5'-monophosphate and the depurination product oxanine (Fig. 5) was based on comparison of retention time, UV spectra, and ESI-MS results when compared with oxanine derived from the hydrolysis of the N-glycosidic bond of authentic 2'-deoxy-
Nitroso Transfer from N-Nitrosoindoles to DNA Bases

anosine (dOxo, 13). dOxo was hydrolyzed as described by Suzuki et al. (14). dOxo (0.37 mM) was incubated in 0.1 M acetate buffer at pH 4.0 for 4 h. At hourly intervals, 100-μl aliquots were injected onto the Gilson gradient-controlled HPLC system exactly as described previously for the analysis of aqueous reaction mixtures. As the dOxo peak (retention time = 19.9 min) decreased, a new peak (retention time = 11.4) appeared in the chromatogram, corresponding to oxanine; \( \lambda_{\text{max}} \) 240, 287 nm (8% MeOH). The UV spectra of the novel products are very similar (Fig. 4): oxanine, \( \lambda_{\text{max}} \) 240, 287 nm (8% MeOH); dO, \( \lambda_{\text{max}} \) 245, 288 nm (8.5% MeOH). Thus the retention time and UV spectrum of the hydrolysis product of dOxo was identical to that of reaction product oxanine. Upon ESI-MS analysis of the HPLC fraction corresponding to dO, a molecular ion with \( m/z \) 347 (M + H)\(^+\) was observed (Fig. 6a). ESI-MS-MS analysis produced fragment ions with \( m/z \) 151, 79 (PO₄\(^-\)), 97 (H₂PO₄\(^-\)) \(^-\), and 195 (C₆H₈PO₆)\(^-\) (Fig. 6b). These results alone would sensibly suggest the formation of 2'-deoxyxanthosine-5'-monophosphate, however, the retention time and UV spectrum of the reaction product are not consistent with this suggestion. ESI-MS analysis on the fraction corresponding to oxanine resulted in a molecular ion with \( m/z \) 153 (M + H)\(^+\), corresponding to the same mass as xanthine and suggesting this product as the depurination product of dO. The replacement of the N atom at N-1 of guanine with an O atom increases the relative molecular mass by one and coincidentally oxanine has the same mass as xanthine and 2'-deoxyxanthosine-5'-monophosphate has the same mass as 2'-deoxyoxanosine-5'-monophosphate. The dose-response behavior of these novel reaction products was similar to that found for the N-acetyl adducts previously described, lending further support to their identification. Analogous reaction products with similar dose-related responses were seen with reactions of NIAM and NIAAME with dpG and NIAN with dGp (data not shown).

Reaction of NIAN, NIAM, or NIAAME with dGuo at pH 7.4 yielded the same 4 products, guanine, xanthine, oxanine, and \( N₂ \)-acytelyguanine with the same retention times as described for reactions of nitrosated indoles with dpG or dGp. The absence of reaction products 2'-deoxyoxanosine-5'-monophosphate and \( N₂ \)-acytelydeoxyxanosine-5'-monophosphate is expected, as these are nucleotide adducts. All products exhibited analogous dose-response relationships to those described above.

Decomposition of Nitrosated Indoles in the Reaction System—When diethyl ether extracts of reaction mixtures were analyzed by HPLC, denitrosation was the major pathway of decomposition for all the nitrosated indoles. N-Nitrosoindoles gave a positive result in the Liebermann test (15) for a nitrosamine or \( N \)-nitroso compound, without the addition of acid and at room temperature. Under the usual conditions of the test, the nitrosamine is warmed with phenol and acid. The nitrosating species is liberated from the nitrosamine and nitrosates phenol to form \( \rho \)-nitrosophenol. Another molecule of phenol combines with \( \rho \)-nitrosophenol to form indophenol which is colored red. Under alkaline conditions, the red indophenol yields a blue indophenol anion. When reactions were repeated in the presence of azide ion, a scavenger of nitrosating agents (azide ion: \( \text{AT-nitrosoindole, 1:1 molar ratio} \)), the formation of any reaction products was completely inhibited (data not shown).
Nitroso Transfer from N-Nitrosoindoles to DNA Bases

**DISCUSSION**

Identical nucleotide reaction products, which are structurally independent of the 3-substituted nitrosated indoles, were seen for reactions of nitrosated indoles with nucleotides and dGuo. Modification via depurination, deamination, and the formation of 2'-deoxyoxanosine monophosphate and the depurination product oxanine were apparent. N-Acetyl adducts were also observed, with the source of acetyl groups being the co-solvent used in the monophasic reaction system. All these pathways of modification can be rationalized by a transnitrosation mechanism.

Depurination is probably catalyzed by N-nitrosation at the N-7 atom of guanine or adenine residues and/or the N-3 atom of adenine, imparting a destabilizing positive charge on the purine ring system. Cleavage of the N-glycosidic bond neutralizes this charge and gives the depurination products. The corresponding N-nitrosopurine is then rapidly hydrolyzed to generate the observed base (Fig. 7).

The formation of deamination products can be explained by transnitrosation to exocyclic amino groups of purine bases. Nitrogen is readily displaced from the purine diazonium ion generated via transnitrosation. In this case, simple hydrolysis affords the deamination product, namely hypoxanthine from adenine and xanthine from guanine (Fig. 8).

The mechanism for the formation of dOxo from dGuo by nitrous acid or nitric oxide has been reported (14, 16). By using guanosine and its methyl derivatives, Suzuki et al. (14) demonstrated that reaction at N-2 to give the diazonium ion is followed by cleavage of the N-1-C6 bond and that the exocyclic amino nitrogen of dOxo originates from the imino nitrogen (N-1) of dGuo. Consequently, the mechanism for the formation of dOp/dpO and oxanine would logically follow the same route starting with transnitrosation to N-2 of dGp/dpG or dGuo (Fig. 8).

The transnitrosating potential of the three substituted nitrosoindoles is further highlighted by the formation of N-acetyl adducts. The replacement of ACN with d3-ACN resulted in the formation of d3-acetylated products, thus confirming the co-solvent as the source of acetyl groups. One possible explanation involves a slow hydrolysis step of acetonitrile to acetamide followed by transnitrosation to the amino nitrogen to produce reactive acetyldiazonium ion. However, when acetamide was
Nitroso Transfer from N-Nitrosoindoles to DNA Bases

added to the reaction mixtures at varying concentrations, no increase in the amount of acetylated products was observed, suggesting that this pathway may be at best, a minor pathway. Direct transnitrosation to the tertiary nitrogen atom of ACN followed by hydrolysis may be the more likely pathway and there is some evidence for this possibility (17).

When reactions were carried out using methanol and ethanol as co-solvents, no reaction products were seen. The rate of denitrosation was slightly higher in alcohols than in ACN, and it is likely that the free nitrosating agent was used up in the formation of volatile alkyl nitrates. Reaction of alcohol with nitrous acid is used as the route for their preparation (18). The procedure depends on the fact that the alkyl nitrite has a lower boiling point than the alcohol and can be distilled out from the equilibrium mixture.

It would appear therefore, that most, if not all of the products formed by reaction of N-nitrosoindoles with isolated purine nucleotides and deoxyguanosine, are the result of transnitro-
sation, that is, the ready transfer of the nitroso group to nucleophilic nitrogen atoms in the purines. In the case of guanine this results in formation of a diazonium ion at C-2. The solvolysis product of this diazonium ion to give xanthine is a well-known reaction in purine chemistry but it is only recently that a more profound consequence of this pathway has been discovered.

The interesting observations by Suzuki et al. (13) that treatment of dGuo, oligodeoxynucleotides, and DNA with nitrous acid resulted in the formation of dOxo due to a rearrangement of the C-2-diazonium ion (14) (Fig. 8), have important mutagenic implications. The presence of an oxygen atom in place of N-1 of guanine is likely to have a profound effect on Watson-Crick base pairing and recent results on the misincorporation of a G-G dimer would probably appear as a bulky adduct and this may explain the results of Yamashita et al. (14) following transnitrosation from 1-nitrosoureas.

A further consequence of the formation of purine diazonium ion in double-stranded DNA is that this may lead to the formation of interstrand cross-links by displacement of nitrogen by the exocyclic amino group of guanine on the opposing strand (23). Current studies are directed at investigating the relevance of this pathway for N-nitrosoureas. In postlabeling studies such a G-G dimer would probably appear as a bulky adduct and this may explain the results of Yamashita et al. (5) who observed adducts formed by reaction of NNI with calf thymus DNA.

Transnitrosation has been invoked, without any direct evidence, to explain direct-acting mutagenicity of N-nitrosoureas (24). The mechanism of denitrosation of N-nitrosoureas under weakly acidic or neutral conditions (pH 4–7) has been studied and may involve intramolecular transfer of the nitroso group to C-3 of the indole prior to transnitrosation of weakly basic amines and other nucleophiles (25–27). The results presented in this paper demonstrate that, at neutral pH, N-nitrosoureas transfer the nitroso group to nucleophilic sites on DNA bases resulting in depurination, deamination, and the formation of the novel products dOdp/dOp and oxanine. All of these processes are potentially mutagenic events if they occur in DNA (28, 29). These observations represent a new pathway for N-nitrosocompounds, exemplified by the N-nitrosoureas, to exert genotoxicity mediated by transfer of the nitroso group to DNA bases. In the wider perspective, this pathway may be operative for many agents which release nitric oxide.

Acknowledgments—Assistance with the recording of nmr and mass spectra by Bec Jukes and John Lamb is gratefully acknowledged. Dr. Toshinori Suzuki of Kyoto University, Japan, is thanked for a generous gift of 2-deoxyoxanosine and for providing unpublished information.

REFERENCES


FIG. 8. Reaction mechanism for the formation of deamination product, deoxyxanthosine, and dOdp, based on mechanisms described by Suzuki et al. (14) following transnitrosation from 1-nitrosoureas.
Nitroso Transfer from N-Nitrosoindoles to DNA Bases


SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
APPENDIX 2

LUCAS, L.T., GATEHOUSE, D., JONES, G.D.D.,
AND SHUKER, D.E.G.

CHEM. RES. TOXICOL. 2001, 14, 158-164
Characterization of DNA Damage at Purine Residues in Oligonucleotides and Calf Thymus DNA Induced by the Mutagen 1-Nitrosoidole-3-acetonitrile

Lynda T. Lucas,1 David Gatehouse,2 George D. D. Jones,5 and David E. G. Shuker*1,4

Biomonitoring and Molecular Interactions Section and Centre for Mechanisms of Human Toxicity, MRC Toxicology Unit, Hodgkin Building, University of Leicester, Leicester LE1 9HN, U.K., and Genetic and Reproductive Toxicology, Medicines Safety and Evaluation Division, Glaxo Wellcome Research and Development, Ware, Hertfordshire SG12 6DP, U.K.

Received August 17, 2000

Published on Web 01/05/2001


158

Introduction

A number of 3-substituted indoles have been found to produce mutagenic products upon nitrosic acid treatment (1). Indoles substituted at the 3-position occur widely in nature as natural products, such as tryptophan, serotonin, or the plant growth hormone indole-3-acetic acid. This has led to concern that endogenous nitrosation of indoles in the acidic environment of the stomach could contribute to the risk of gastric cancer. 1-Nitrosoidole-3-acetonitrile (NIAN) is the mono-N-nitroso derivative of indole-3-acetonitrile, a plant growth hormone found in various vegetables, notably, Chinese cabbage, a common foodstuff found in Japan. NIAN is a direct-acting mutagen toward Salmonella typhimurium TA98 and TA100 and Chinese hamster lung cells (2, 3). 32P-postlabeling has shown DNA adduct formation in vitro and in the gastrointestinal tissues of rats treated with NIAN (4), but these products were not characterized. Marked inductions of ornithine decarboxylase and DNA synthesis in rat stomach mucosa have been reported after administration of NIAN, suggesting that NIAN has potential tumor promoting activity in carcinogenesis in the glandular stomach (5).

We have previously shown that a number of 3-substituted N-nitrosoidoles, including NIAN, induce depurination, deamination, and formation of a novel nucleobase, oxanine, in isolated purine nucleotides via a transnitrosation mechanism (6). N-Nitrosation at the N-7 atom of guanine or adenine residues and/or the N-3 atom of cytosine and thymine residues was inert toward NIAN. Further studies revealed an additional product in NIAN-treated duplex DNA containing a CCGG motif that was characterized as an interstrand cross-link, the yield of which increased with increasing NIAN concentration. These results indicate that the transnitrosating ability of NIAN to modify purine residues is preserved at the macromolecular level, with guanine residues appearing to be a primary site of reaction. All of these modification processes are potentially mutagenic if they occur in vivo.
Guanine results in the formation of purine diazonium ion, guanosine 5'-monophosphate. (I) Transnitrosation to the N7 atom of guanine followed by cleavage of the N-glycosidic bond and hydrolysis of the N-nitrosopurine affords depurination products. (II) Transnitrosation to the exocyclic amino group of guanine results in the formation of purine diazonium ion. Subsequent reactions give rise to deamination products and the formation of oxime.

Via ring opening [Figure 1 (II)]. This transfer of the nitroso group to specific biological targets has also been suggested to explain the NO-like biological effects induced by nitrosated tryptophan residues in serum albumin and model dipeptides [7]. In this study, we examine the ability of NIAN to transfer the nitroso group to nucleophilic target residues in oligonucleotides and calf thymus DNA, to ascertain whether the spectrum of potentially mutagenic products observed in isolated nucleotides is preserved at the macromolecular level. A further consequence of purine diazonium ion at C2 of guanine, in double-stranded DNA, is that this may lead to the formation of interstrand cross-links by displacement of nitrogen by the exocyclic amino group of guanine on the opposing strand. We also examine the relevance of this pathway for NIAN.

Materials and Methods

Caution: NIAN and dimethyl sulfate (DMS) are mutagenic and should be handled with extreme caution. Acrylamide is a hazardous chemical, and care should be exercised in its handling.

Synthesis of NIAN. NIAN was synthesized by nitrous acid treatment of IAN (Huka), purified by HPLC, and characterized by 1H NMR, mass spectrometry, and microanalysis as described by Lucas et al. [6].

Enzymes. T4 polynucleotide kinase (T4 PNK, 10 units/μL) was purchased from Amersham. Micrococcal nuclease (500 units/ml) was purchased from Sigma. Calf spleen phosphodiesterase (2 units/mg) was purchased from Boehringer Mannheim. Unit definitions are as follows. For T4 PNK, 1 unit catalyzes the transfer of 1 μmol of phosphate from ATP to

polymer in 30 min at pH 7.6 and 37 °C. For calf spleen phosphodiesterase, 1 unit releases 1 μmol of 4-nitrophenol per minute at pH 6.0 and 25 °C. For micrococcal nuclease, 1 unit produces 1 μmol of nucleotides from native DNA per minute at pH 8.8 and 37 °C.

Preparation of Radiolabeled Oligonucleotides. Synthetic oligonucleotides were prepared and HPLC purified by the Oswel DNA Research Products Laboratory (Southampton, U.K.). Oligonucleotides were 5'-32P end-labeled using T4 PNK and [γ-32P]ATP. Typically, each phosphorylation reaction mixture (10 μL) contained kinase buffer [50 mM Tris-Cl (pH 7.6), 10 mM MgCl2, and 10 mM 2-mercaptoethanol], oligonucleotide (100 pmol), 1.67 pmol of [γ-32P]ATP (3000 Ci/mmol, Amersham), and 10 units of T4 PNK. After incubation for 1 h at 37 °C, the end-labeled oligomers were recovered by ethanol precipitation.

Reactions of NIAN with Single-Stranded Oligonucleotides I–IV (Table 1). Radiolabeled oligonucleotide [20 pmol in 20 μL of 0.5 mM Tris-HCl buffer (pH 7.4)] was incubated at 37 °C in the dark, with solutions of NIAN dissolved in acetonitrile such that the total NIAN concentration ranged from 0.2 to 2 mM in a total volume of 25 μL. Reactions were stopped at varying time points (6, 12, 18, and 30 h) by extraction of NIAN twice with 2 volumes of diethyl ether. Reaction mixtures were dried down in a centrifugal vacuum evaporator (DNA 110, Savant) and resuspended in 100 μL of water. Control incubations were set up and consisted of the oligonucleotide in buffer with acetonitrile only. An aliquot of 50 μL from control and treated samples was dried in a centrifugal vacuum evaporator and resuspended in 4 μL of denaturing gel-loading buffer (95% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanol in TBE buffer [89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0)]). The remaining 50 μL was incubated with 50 μL of freshly prepared 0.1 M piperidine at 30 °C for 30 min in a sealed microfuge tube. After cooling to room temperature, samples were dried in a centrifugal vacuum evaporator and lyophilized twice from 40 μL of water. Radiolabeled oligonucleotides were also specifically modified at guanine residues using DMS according to an established protocol for the preparation of Maxam–Gilbert sequencing markers [8]. Resulting sample pellets were resuspended in 4 μL of denaturing gel-loading buffer. Samples (2 μL from 4 μL of stock) were loaded onto 20% denaturing polyacrylamide gels (PAGE) containing 7 M urea in TBE buffer. Following electrophoresis, gels were subjected to phosphorimager analysis (Molecular Dynamics).

Cross-Linking Reactions of NIAN with Duplex DNA. Duplex DNA (8 pmol) was prepared for oligo sequence I (Table 1) by heating a mixture of the two complementary strands (1.25, 2.5, and 10-fold excess of unlabeled strand) in 100 μL of water at 90 °C for 2 min and slowly cooling to 37 °C for the course of 3 h. To confirm duplex formation, 2 μL from the stock duplex sample was added to an equal volume of nondenaturating gel-loading buffer (30% sucrose and 0.1% bromophenol blue in TBE buffer), and 2 μL was loaded onto 20% nondenaturing polyacrylamide gels (37.5 mL of an acrylamide/bisacrylamide solution [Axxcel 19:1, National Diagnostics], 5.6 mL of 10× TBE, and 7.15 mL of distilled water). Polymerization was induced by adding 350 μL of 10% aqueous ammonium persulfate and 17.5 μL of tetramethylethylenediamine (TEMED). Following electrophoresis, gels were subjected to phosphorimager analysis. Radiolabeled duplex (1 pmol in 50 μL of 5 mM phosphate buffer (pH 7.4)) was incubated with 1 μL of NIAN dissolved in acetonitrile at 37 °C in the dark such that the total NIAN concentration ranged from 5 to 160 μM. Control incubations of

Table 1. Oligonucleotide Sequences Used in This Study

<table>
<thead>
<tr>
<th>Oligonucleotide sequence</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>(TgCCCGGT)8</td>
</tr>
<tr>
<td>Ib</td>
<td>(AaGGCCCA)8</td>
</tr>
<tr>
<td>II</td>
<td>(TgCCCGGGGT)7</td>
</tr>
<tr>
<td>III</td>
<td>(TgCCGGGT)9</td>
</tr>
<tr>
<td>IV</td>
<td>(TgCCGGT)10</td>
</tr>
<tr>
<td>V</td>
<td>CTGACGCGATTACC GGAGTC</td>
</tr>
</tbody>
</table>

Figure 1. Scheme of NIAN-induced depurination, deamination, and formation of oxime via transnitrosation from 2'-deoxyguanosine 5'-monophosphate. (I) Transnitrosation to the N7 atom of guanine followed by cleavage of the N-glycosidic bond and hydrolysis of the N-nitrosopurine affords depurination products. (II) Transnitrosation to the exocyclic amino group of guanine results in the formation of purine diazonium ion. Subsequent reactions give rise to deamination products and the formation of oxime.

DNA Damage from 1 Nitrosaindle-3-acetonitrile

the duplex in buffer with acetonitrile only were set up. Reactions were stopped after 18 h by extraction of NIAN twice with 2 volumes of diethyl ether. Reaction mixtures were dried down in a centrifugal vacuum evaporator and resuspended in 4 µL of denaturing gel-loading buffer. Samples were not heated at 90 °C prior to electrophoresis to minimize the thermally promoted decomposition of the cross-linked DNA. Half of the sample volume was analyzed on denaturing 20% polyacrylamide gels followed by phosphorimager analysis as described earlier.

Reactions of NIAN with Calf Thymus DNA and Single-Stranded Oligonucleotide V. Calf thymus DNA or oligo V [50 µg in 50 µL of 0.5 mM Tris-Cl buffer (pH 7.4)] was incubated at 37 °C in the dark, with solutions of NIAN dissolved in acetonitrile such that the total NIAN concentration ranged from 0 to 10 mM in a total volume of 100 µL. Control incubations were set up without NIAN but with acetonitrile. After 6 or 24 h, reactions were stopped by extraction of NIAN three times with 3 volumes of diethyl ether, and the mixtures were dried down in a centrifugal vacuum evaporator. Control and treated DNA samples were incubated overnight at 37 °C, with 1.75 units of micrococcal nuclease and 0.03 unit of calf spleen phosphodiesterase in 100 µL of digestion buffer [10 mM sodium succinate and 5 mM calcium chloride (pH 6.0)]. Water was added to the digested samples to give a final volume of 200 µL prior to HPLC analysis.

HPLC Analyses of Digested Reaction Mixtures. HPLC/UV analyses were performed using a Hypersil C18 BDS, 5 µm, 250 mm x 4.6 mm reverse-phase analytical column (Shandon) on a gradient-controlled system (Gilson) equipped with either a dual-wavelength 116 Gilson UV detector or, to obtain UV spectra, a diode array detector (Applied Biosystems Inc.). Aliquots of 100 µL of digested aqueous reaction mixture were analyzed at a flow rate of 1 mL/min, using the following elution program: 0% B at 0 min, 0% B at 15 min, 35% B at 40 min, 0% B at 45 min (solvent A, 0.1 M triethylammonium acetate (pH 5.0); solvent B, methanol). Column eluants were monitored at 260 and 290 nm. The fractions corresponding to reaction products were pooled from multiple HPLC runs and dried down in a centrifugal vacuum evaporator. Resulting product fractions were lyophilized twice with 2 mM NIAN for 18 h; and lane +p, strand break products due to piperidine treatment.

Characterization of Reaction Products by Mass Spectrometry. Off-line ESI-MS characterization of reaction products was carried out using a VG Autospec-Ultima Q instrument. Dried fractions previously collected from the HPLC system were resuspended in acetonitrile/water/methanol mixture (50:50, v/v) and inserted into the mass spectrometer interface via 20 µL “loop” injection or continuous infusion, at a flow rate of typically 8 µL/min. The cone voltage was in the range of 8—23 V, and full scan mass spectra were obtained by scanning from m/z 65 to 1650 at 50 to 1 scan speed of 10 s/decade.

Results

NIAN Reactions with Oligonucleotides I—IV. NIAN-induced immediate and alkali-labile strand break products of 5'-32P end-labeled single-stranded oligonucleotides were examined using high-resolution denaturing PAGE. The products were assigned by reference to the products of the Maxam—Gilbert sequencing reaction for specific modification at guanine residues. NIAN (2 mM) treatment of radiolabeled oligo III for 18 h resulted in three latent (piperidine-treated) strand break products 8, 10, and 12 nucleotides (G9, G11, and G13, respectively) in length (Figure 2). Treatment of radiolabeled oligo IV with 2 mM NIAN resulted in four latent strand break products 6, 7, 14, and 15 nucleotides in length (G7, G8, G15, and G16, respectively). Similarly, treatment of radiolabeled oligo II resulted in three latent strand break products 10, 11, and 12 nucleotides in length (G11, G12, and G13, respectively), and two latent strand break products 10 and 11 nucleotides in length (G11 and G12, respectively) resulted from 2 mM NIAN treatment of oligo I. Conformation of the resulting fragments with the products of the Maxam—Gilbert modification reaction specific for guanine, combined with observations that immediate strand breakage did not occur, indicates that NIAN treatment is responsible for the generation of alkali-labile lesions at guanine residues within the radiolabeled oligonucleotide.

The yield of alkali-labile lesions induced by NIAN increased with NIAN concentration and with incubation time. Treatment of radiolabeled oligo II with 0.2, 1, and 2 mM NIAN for 18 h produced a level of total strand break products ranging from 1.4 to 25.8% (Figure 3A). Treatment of radiolabeled oligo III with the same three NIAN concentrations produced a level of total strand break products ranging from 2.2 to 20.6% (Figure 3B). As all radioactivity is retained on the gel, the percentage of strand break products is calculated by determining the fraction of the lane’s total radioactivity present in the strand break band. By analysis of the yield of strand breaks at each guanine residue, it is evident that at NIAN concentrations of 1 and 2 mM, [G11] > [G12] > [G13] in oligo II, and similarly, yields in oligo III follow the same pattern ([G9] > [G11] > [G13]) (Figure 3A,B). Similar profiles of strand break products with increasing NIAN concentration were seen with oligos I and IV. The yield of strand breaks in radiolabeled oligonucleotides III and IV induced by 2 mM NIAN increased linearly with...
show a clear dose-dependent increase in the intensity of duplex being present after electrophoresis. Tracks 3—8 represent a control of the duplex strands. Track 1 represents a control of the duplex in the reaction system used for cross-linking, comprising the electrophoretic mobility of the unmethylated single strands. NIA N treated oligo III and oligo IV as a function of time. Yields were determined by high-resolution denaturing PAGE and phosphorimager analysis.

time out to the longest time point measured, 30 h, reaching almost 80% at that time for oligo IV (Figure 3C).

Cross-Linking Reactions of NIA N with Duplex DNA. Confirmation of duplex formation for oligonucleotide sequence I was achieved by using high-resolution non-denaturing PAGE. The difference in electrophoretic mobility of the double-stranded duplex macromolecule compared to the single-strand oligonucleotide confirmed that the duplex—single strand equilibria of the oligonucleotides studied did reside predominantly on the side of duplex under the conditions used for cross-linking. Solutions containing differing ratios of 5'-32P end-labeled oligonucleotide (lb) to "cold" counter strand (la), as determined by high-resolution non-denaturing PAGE. (A) Percentages of single-strand break products. The yield of total strand break products of 2 mM NIA N-treated oligo III and oligo IV as a function of time. Yields have been corrected for any residual duplex due to the cross-linking reaction system remaining in the control. The cross-linked product had roughly half the electrophoretic mobility of the unmodified single strands. Track 1 represents a control of the duplex in water alone. Track 2 represents a control of the duplex in the reaction system used for cross-linking, comprising phosphate buffer and cosolvent. The cross-linking reaction system resulted in a minute amount of residual duplex being present after electrophoresis. Tracks 3—8 show a clear dose-dependent increase in the intensity of the slower migrating band as NIA N is introduced into the cross-linking system at concentrations ranging from 5 to 160 μM; the yields have been corrected for the residual duplex remaining in the control and are calculated as previously described for the percentage of strand break products. The yield of cross-linked duplex I exposed to NIA N increased with a first-order dependence on NIA N concentration in the range of 5—20 μM (Figure 4C). At >20 μM, the yield continued to increase to almost 0.35%, but with an order in NIA N of less than 1. For NIA N to be reactive, full solubilization must be achieved. By increasing the concentration of NIA N in the reaction, but keeping the level of cosolvent (i.e., acetonitrile) constant, we found it was clear that at some point saturation of NIA N will occur. Increasing the volume of cosolvent in the cross-linking reaction system was not feasible due to destabilization of the duplex (data not shown).

NIA N Reactions with Calf Thymus DNA and Single-Stranded Oligonucleotide V. Control incubations of calf thymus DNA at pH 7.4 in buffered 50% aqueous acetonitrile, followed by enzymatic digestion into 3'-monophosphates, produced the four expected nucleotide peaks: 2'-deoxyadenosine 3'-monophosphate (dAp), 2'-deoxyguanosine 3'-monophosphate (dGp), thymidine 3'-monophosphate (Tp), and 2' deoxyadenosine 3'-monophosphate (dAp) (Figure 5A). Reaction of NIA N with calf thymus DNA at pH 7.4 in buffered 50% aqueous acetonitrile for 6 h yielded three products not seen in control incubations, guanine (Gua), xanthine (Xan), and adenine (Ade) with retention times of 6.6, 7.2, and 13.6 min, respectively (Figure 5B). Identification of guanine, xanthine, and adenine was all based on comparison of retention times and UV spectra with those of authentic standards analyzed under the same conditions. When
Figure 5. HPLC separation of the NIAN and calf thymus DNA reaction mixture after enzymatic digestion to 3'-monophosphates. HPLC conditions were as described in Materials and Methods, with UV detection at 260 nm: (A) control, (B) 4 mM NIAN treatment, with an incubation time of 6 h, and (C) 4 mM NIAN treatment, with an incubation time of 24 h.

ESI-MS was performed on the fraction corresponding to guanine, a molecular ion with \( m/z \) 152 \((M + H)^+\) was observed. When ESI-MS was performed on the fractions corresponding to xanthine and adenine, molecular ions with \( m/z \) 153 \((M + H)^+\) and \( m/z \) 136 \((M + H)^+\), respectively, were observed.

After incubation for 24 h, a further reaction product, oxanine (Oxa), appeared in the chromatogram with a retention time of 12.4 min (Figure 5C). Identification of this reaction product was based on comparison of retention time, UV spectrum, and ESI-MS results with those of oxanine derived from the hydrolysis of the N-glycosidic bond of authentic 2'-deoxyxanosine [dOxo (9)] as described by Suzuki et al. (10). Oxanine is a guanine analogue where the N-1 atom is replaced with an O atom. This replacement increases the relative molecular mass by 1, and consequently, oxanine has the same mass as xanthine, which was confirmed by ESI-MS results.

Figure 6. (A and B) Loss of parent nucleotides compared to the increase in the level of base release in NIAN-treated calf thymus DNA as a function of NIAN concentration as monitored by HPLC analysis: (A) incubation for 6 h and (B) incubation for 24 h. (C) Increase in the production of oxanine from NIAN-treated calf thymus DNA after incubation for 24 h. Legend for panels A–C: (■) dGp, (▲) dAp, (●) Gua, (●) Xan, (+) Ade, and (♦) Oxa.

The yield of reaction products resulting from NIAN-exposed calf thymus DNA increased with NIAN concentration and time. HPLC analysis was used to assess the reactivity of the residues within the macromolecule upon treatment with NIAN over a range of concentrations (0–10 mM). Loss of the parent nucleotides dGp and dAp was observed with increasing NIAN concentrations after incubation for 6 h as conversion to guanine, xanthine, and adenine occurred (Figure 6A). The yield of reaction products increases almost linearly with NIAN concentration from 4 to 10 mM. After incubation for 24 h, however, this linear relationship between reaction product yield and NIAN concentration no longer exists for the products guanine and adenine, as the supply of active sites, as represented by the concentration of the parent nucleotides, becomes exhausted (Figure 6B). The yield of oxanine in NIAN-treated calf thymus DNA increases with increasing NIAN concentration after incubation for 24 h, with yields more than 1000 times smaller than...
those observed for depurination and deamination products (Figure 6C).

Regardless of the concentration of NIAN used to treat calf thymus DNA, there was no significant loss of nucleotides dCp and Tp within the reaction system, suggesting the unreactivity of cytosine and thymine residues toward NIAN.

Analogous reaction products with similar dose-related responses and with no appreciable differences in reaction product yields were observed for reactions of NIAN with single-stranded oligonucleotide V (data not shown).

**Discussion**

Guanine residues within a single-stranded oligomer were reactive nucleophilic targets for transnitrosation by NIAN, resulting in alkali-labile lesions, the level of which increased with NIAN concentration and with incubation time. HPLC analysis confirmed these lesions as apurinic sites due to the detection of the released bases. Modification at guanine residues via depurination, deamination to form xanthine, and the formation of oxanine were apparent, with the former pathway predominating. Similarly, adenine residues were also reactive nucleophilic targets for nitroso group transfer, although to a lesser extent than guanine residues, with modification via deamination. It is likely that formation of the deamination product of adenine, hypoxanthine, was also formed but was at the limits of detection of the HPLC conditions that were employed. The mutations that may ultimately result from the deamination and deamination of guanine and adenine residues shown in Table 2. Xanthine has no known repair mechanism, and because xanthine is able to base pair with thymine (11), deamination of guanine to xanthine will lead to a G-C → A-T transition mutation upon replication. This type of mutation has frequently been observed as the primary type of mutation involved in nitric oxide-induced mutagenesis via a mechanism probably involving deamination of cytosine and 5-methylcytosine residues (14, 15) and deamination of guanine to xanthine (16). Deamination of cytosine to uracil or 5-methylcytosine to thymine occurs spontaneously, resulting in the same transition mutation and providing a significant source of spontaneous mutations (17, 18). Interestingly, cytosine residues, along with thymine residues, appear to be inactive targets for nitroso transfer by NIAN. Reactions of single-stranded oligonucleotides with NIAN and subsequent piperdine treatment indicated no modification to cytosine residues had occurred. In addition, HPLC analysis of NIAN-treated calf thymus DNA or oligonucleotide showed no evidence of uracil formation. Our observations suggest that transnitrosation to purine bases in DNA by nitrosated indoles may be mediated by intercalation, whereby the nitroso group is most efficiently transferred when the indole and nucleophilic site are in proximity. Current studies are directed at investigating this hypothesis.

It has previously been shown that the level of nitric oxide-induced deamination of cytosine and guanine residues in double-stranded oligomers was 10-fold lower than for single-stranded oligomers (19). The lack of accessibility of the exocyclic amino groups, combined with base pairing when these residues are in the interior position of the double helix, would contribute to the decrease in the reactivity of these groups. However, in our studies, the yield of xanthine was not appreciably different in single-stranded oligonucleotides and calf thymus DNA, suggesting that deamination of the guanine base can occur directly after deamination events.

Deamination of adenine to hypoxanthine will lead to an A-T → G-C transition mutation upon replication (12). In contrast to xanthine, there is an enzyme that specifically catalyzes the cleavage of hypoxanthine residues in DNA. Hypoxanthine-DNA glycosylase efficiently removes hypoxanthine residues that lie in positions complementary to either cytosine or thymine (12). The enzyme is consequently important in DNA repair in initiating a base excision repair event, and it is unlikely that hypoxanthine in DNA is as persistent a lesion as xanthine.

A further possible fate of xanthine and hypoxanthine is depurination to form an abasic site. Evidence of this event, combined with depurination of guanine and adenine residues directly, is presented in this paper. Nitroso group transfer from NIAN to the N-7 atom of guanine and adenine residues to induce depurination is clearly the most dominant pathway. The yield of depurination products is not significantly lower in single-stranded oligonucleotides than in calf thymus DNA, as the N-7 position of the purine residues is exposed within the major groove of the double helix. Any protective effects due to base pairing within the double-stranded macromolecule will have little influence on the reactivity of these positions in the purine residues. A number of mechanisms exist whereby abasic sites may induce mutations (13). The abasic site can also be cleaved by an endonuclease or by base catalysis to yield a single-strand break that may be toxic to cells (20).

At NIAN concentrations of ≥ 1 mM, guanine residues nearest the 5'-end of the oligomers appeared to be more reactive and this reactivity decreased from the 5'- to 3'-end. However, this is likely to be an artifact of the experimental procedure as oligomers containing two or more depurination sites will only be detected as a single-strand break product with a bias toward the site nearest the 5'-end label.

The formation of oxanine was detected in both NIAN-treated single-stranded oligonucleotide and calf thymus DNA. The biological significance of oxanine as a lesion in DNA is unknown. 2'-Deoxyoxanosine (dOxo) has been isolated from the reaction of 2'-deoxyguanosine (dGuo), oligonucleotide, and calf thymus DNA with nitrous acid and nitric oxide (9). The presence of an oxygen atom in place of the N-1 atom of guanine is likely to have a profound effect on Watson-Crick base pairing, and formation of oxamine in DNA is likely to have important mutagenic implications. It has been shown that 2'-deoxyxanosine 5'-triphosphate (dOTP) is misincorporated by DNA polymerases in oligonucleotides (21), suggesting that formation of dOTP in the intracellular nucleotide pool would result in elevation of the mutation frequency. These observations combined with the fact that the N-glycosidic bond of dOxo has been shown to be
as stable as that of dGuo (10) suggest that oxanine in DNA is likely to be a persistent lesion in the absence of a specific repair pathway. In addition to the possible genotoxic effects of oxanine formation, oxanine is likely as stable as that of dGuo (10).

A specific repair pathway. In addition to the possible reparation of DNA is likely to be a persistent lesion in the absence of a specific repair pathway. Oxanilide might be derived from ring-opened guanine intermediates following dediazoniation of the guanine diazonium ion (24, 25) or from the final product, oxanine, which is chemically reactive (22). In postlabeling studies, the cross-linked dimer would probably appear as a bulky adduct, and this may explain the observation by Yamashita et al. (4) of adducts formed by reaction of NIA with calf thymus DNA.

In conclusion, we have demonstrated that at physiological pH and temperature, the ability of 1-nitroso-indole-3-acetonitrile to transfer the nitroso group to nucleophile targets on the purine bases, particularly guanine, is preserved at the macromolecular level. These results suggest that N-nitrosoureas are efficient transnitrosating agents causing a variety of DNA damage products, of which all are potentially mutagenic if they occur in vivo.

Acknowledgment. L.T.L. gratefully acknowledges the award of a Glaxo Wellcome studentship. John Lamb is thanked for assistance with the recording of mass spectra, and Zara Doddridge is thanked for her expertise in polyacrylamide gel electrophoresis.

References


SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
## Appendix 3: Comet Assay Scores

**Scores for Slide Number 1: NIAN treatment (2.5 μg/ml)**

<table>
<thead>
<tr>
<th>No.</th>
<th>Head Length</th>
<th>Tail Length</th>
<th>Head Intensity</th>
<th>Tail Intensity</th>
<th>Tail Moment</th>
<th>Total Area</th>
<th>Total Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max</td>
<td>82.39</td>
<td>152.67</td>
<td>100.00</td>
<td>41.89</td>
<td>26.02</td>
<td>2254.27</td>
<td>338882</td>
</tr>
<tr>
<td>Min</td>
<td>22.62</td>
<td>13.73</td>
<td>58.11</td>
<td>0.00</td>
<td>0.00</td>
<td>700.10</td>
<td>130475</td>
</tr>
<tr>
<td>Mean</td>
<td>35.70</td>
<td>68.12</td>
<td>83.09</td>
<td>16.91</td>
<td>5.21</td>
<td>1142.72</td>
<td>203086.24</td>
</tr>
<tr>
<td>SD</td>
<td>13.58</td>
<td>14.00</td>
<td>14.00</td>
<td>4.97</td>
<td>2.99</td>
<td>310.68</td>
<td>45740.46</td>
</tr>
</tbody>
</table>

1. 43.62 32.31 85.79 14.21 3.44 1727.08 295563
2. 30.69 42.31 94.12 5.88 1.00 1153.56 222214
3. 30.69 106.62 92.28 7.72 2.99 925.85 173000
4. 27.46 107.43 98.11 11.19 4.07 1388.62 233429
5. 35.54 123.59 88.81 16.91 5.21 1142.72 203086.24
6. 35.54 123.59 88.81 16.91 5.21 1142.72 203086.24
7. 27.46 152.67 64.99 11.19 4.07 1388.62 233429
8. 30.69 123.59 92.28 7.72 2.99 925.85 173000
9. 30.69 123.59 92.28 7.72 2.99 925.85 173000
10. 27.46 143.78 87.95 12.05 5.74 929.11 163479
11. 30.69 149.43 82.97 17.03 5.30 925.85 173000
12. 30.69 149.43 82.97 17.03 5.30 925.85 173000
13. 30.69 149.43 82.97 17.03 5.30 925.85 173000
14. 30.69 149.43 82.97 17.03 5.30 925.85 173000
15. 30.69 149.43 82.97 17.03 5.30 925.85 173000
16. 30.69 149.43 82.97 17.03 5.30 925.85 173000
17. 30.69 149.43 82.97 17.03 5.30 925.85 173000
18. 30.69 149.43 82.97 17.03 5.30 925.85 173000
19. 30.69 149.43 82.97 17.03 5.30 925.85 173000
20. 30.69 149.43 82.97 17.03 5.30 925.85 173000
21. 30.69 149.43 82.97 17.03 5.30 925.85 173000
22. 30.69 149.43 82.97 17.03 5.30 925.85 173000
23. 30.69 149.43 82.97 17.03 5.30 925.85 173000
24. 30.69 149.43 82.97 17.03 5.30 925.85 173000
25. 30.69 149.43 82.97 17.03 5.30 925.85 173000
26. 30.69 149.43 82.97 17.03 5.30 925.85 173000
27. 30.69 149.43 82.97 17.03 5.30 925.85 173000
28. 30.69 149.43 82.97 17.03 5.30 925.85 173000
29. 30.69 149.43 82.97 17.03 5.30 925.85 173000
30. 30.69 149.43 82.97 17.03 5.30 925.85 173000
31. 30.69 149.43 82.97 17.03 5.30 925.85 173000
32. 30.69 149.43 82.97 17.03 5.30 925.85 173000
33. 30.69 149.43 82.97 17.03 5.30 925.85 173000
34. 30.69 149.43 82.97 17.03 5.30 925.85 173000
35. 30.69 149.43 82.97 17.03 5.30 925.85 173000
36. 30.69 149.43 82.97 17.03 5.30 925.85 173000
37. 30.69 149.43 82.97 17.03 5.30 925.85 173000
38. 30.69 149.43 82.97 17.03 5.30 925.85 173000
39. 30.69 149.43 82.97 17.03 5.30 925.85 173000
40. 30.69 149.43 82.97 17.03 5.30 925.85 173000
41. 30.69 149.43 82.97 17.03 5.30 925.85 173000
42. 30.69 149.43 82.97 17.03 5.30 925.85 173000
43. 30.69 149.43 82.97 17.03 5.30 925.85 173000
44. 30.69 149.43 82.97 17.03 5.30 925.85 173000
45. 30.69 149.43 82.97 17.03 5.30 925.85 173000
46. 30.69 149.43 82.97 17.03 5.30 925.85 173000
47. 30.69 149.43 82.97 17.03 5.30 925.85 173000
48. 30.69 149.43 82.97 17.03 5.30 925.85 173000
49. 30.69 149.43 82.97 17.03 5.30 925.85 173000
50. 30.69 149.43 82.97 17.03 5.30 925.85 173000
### Appendix 3: Comet Assay Scores

#### Scores for Slide Number 4: Vehicle control (DMSO)

<table>
<thead>
<tr>
<th>No.</th>
<th>Head Length</th>
<th>Tail Length</th>
<th>Head Intensity</th>
<th>Tail Intensity</th>
<th>Tail Moment</th>
<th>Total Area</th>
<th>Total Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max</td>
<td>53.31</td>
<td>67.04</td>
<td>99.98</td>
<td>17.86</td>
<td>4.33</td>
<td>1847.79</td>
<td>413761</td>
</tr>
<tr>
<td>Min</td>
<td>30.69</td>
<td>16.16</td>
<td>82.14</td>
<td>0.02</td>
<td>0.00</td>
<td>693.57</td>
<td>131995</td>
</tr>
<tr>
<td>Mean</td>
<td>38.09</td>
<td>38.72</td>
<td>95.16</td>
<td>4.84</td>
<td>1.04</td>
<td>1215.42</td>
<td>246383.14</td>
</tr>
<tr>
<td>SD</td>
<td>6.70</td>
<td>15.08</td>
<td>4.94</td>
<td>4.94</td>
<td>1.07</td>
<td>297.61</td>
<td>59380.99</td>
</tr>
</tbody>
</table>

1. 50.08 39.58 99.33 0.67 0.19 1847.79 413761
2. 30.69 45.23 92.57 7.43 1.56 1215.42 246383.14
3. 33.93 23.42 95.16 0.80 0.17 1515.03 320110
4. 35.54 51.70 99.98 12.50 2.51 225110 472127
5. 38.77 25.85 97.25 0.25 0.08 1847.79 413761
6. 30.69 38.77 99.75 1.34 0.10 1043.30 230798
7. 32.31 38.77 99.72 0.28 0.01 1043.30 230798
8. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
9. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
10. 32.31 38.77 99.75 0.28 0.01 1207.72 289009
11. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
12. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
13. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
14. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
15. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
16. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
17. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
18. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
19. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
20. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
21. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
22. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
23. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
24. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
25. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
26. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
27. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
28. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
29. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
30. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
31. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
32. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
33. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
34. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
35. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
36. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
37. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
38. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
39. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
40. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
41. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
42. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
43. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
44. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
45. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
46. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
47. 38.77 38.77 99.75 0.25 0.06 1207.72 289009
48. 32.31 38.77 99.75 0.25 0.06 1207.72 289009
49. 35.54 38.77 99.72 0.22 0.07 1193.52 277074
50. 42.00 32.31 98.06 1.94 0.44 1382.58 272681
### Appendix 3: Comet Assay Scores

**Scores for Slide Number 9: NIAN treatment (0.1 μg/ml)**

<table>
<thead>
<tr>
<th>No.</th>
<th>Head Length</th>
<th>Tail Length</th>
<th>Head Intensity</th>
<th>Tail Intensity</th>
<th>Tail Moment</th>
<th>Total Area</th>
<th>Total Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max</td>
<td>45.23</td>
<td>71.08</td>
<td>100.00</td>
<td>35.60</td>
<td>8.05</td>
<td>1555.48</td>
<td>279142</td>
</tr>
<tr>
<td>Min</td>
<td>24.23</td>
<td>13.73</td>
<td>64.40</td>
<td>0.00</td>
<td>0.00</td>
<td>653.77</td>
<td>144107</td>
</tr>
<tr>
<td>Mean</td>
<td>31.83</td>
<td>54.04</td>
<td>89.29</td>
<td>10.71</td>
<td>2.62</td>
<td>1065.47</td>
<td>200363.24</td>
</tr>
<tr>
<td>SD</td>
<td>4.55</td>
<td>14.87</td>
<td>9.04</td>
<td>9.04</td>
<td>2.20</td>
<td>228.59</td>
<td>33660.52</td>
</tr>
</tbody>
</table>

1. 29.08 67.85 78.27 21.73 5.62 1270.35 211209
2. 29.08 47.66 88.31 11.69 2.27 931.07 184795
3. 29.08 49.27 90.95 9.05 1.61 894.53 176992
4. 29.08 64.62 73.04 2.04 0.38 653.77 153160
5. 38.77 20.19 100.00 0.00 0.00 1075.27 258769
6. 31.69 55.54 35.60 12.16 4.56 1010.02 167788
7. 27.46 38.77 74.71 17.98 2.61 1047.86 228367
8. 33.93 65.43 86.03 13.97 3.39 809.06 153573
9. 27.46 46.85 81.78 18.22 5.46 1301.67 224738
10. 38.77 37.16 74.91 25.09 4.05 896.49 192430
11. 30.69 62.20 90.09 9.91 2.64 1070.70 166055
12. 30.69 45.23 79.83 20.17 4.56 1010.02 167788
13. 27.46 58.16 91.86 8.14 2.43 1294.50 243807
14. 29.08 65.43 76.91 23.09 5.41 1294.50 198235
15. 27.46 37.16 74.91 17.98 2.61 1047.86 228367
16. 33.93 63.55 98.27 1.73 0.45 939.55 179399
17. 27.46 49.27 95.63 4.37 1.13 874.31 166841
18. 29.08 71.08 100.00 35.60 8.05 1555.48 279142
19. 30.69 60.58 90.06 9.94 1.93 1141.82 206585
20. 33.93 25.04 96.72 3.28 0.56 1060.26 214385
21. 32.31 16.96 98.62 1.38 0.10 774.48 175014
22. 30.69 62.20 99.44 8.14 2.43 1294.50 243807
23. 30.69 62.20 90.09 9.91 2.64 1070.70 166055
24. 27.46 37.16 74.91 25.09 4.05 896.49 192430
25. 32.31 65.43 99.21 0.79 0.15 939.55 179399
26. 30.69 45.23 79.83 20.17 4.56 1010.02 167788
27. 27.46 58.16 91.86 8.14 2.43 1294.50 243807
28. 24.23 45.23 82.02 17.98 2.61 1047.86 228367
29. 37.16 63.81 97.46 2.53 0.53 1103.97 219952
30. 37.16 35.54 99.89 0.51 0.11 874.31 166841
31. 32.31 16.96 98.62 1.38 0.10 774.48 175014
32. 30.69 62.20 99.44 8.14 2.43 1294.50 243807
33. 33.93 63.55 98.27 1.73 0.45 939.55 179399
34. 32.31 65.43 99.21 0.79 0.15 939.55 179399
35. 32.31 65.43 99.21 0.79 0.15 939.55 179399
36. 32.31 65.43 99.21 0.79 0.15 939.55 179399
37. 32.31 65.43 99.21 0.79 0.15 939.55 179399
38. 32.31 65.43 99.21 0.79 0.15 939.55 179399
39. 32.31 65.43 99.21 0.79 0.15 939.55 179399
40. 32.31 65.43 99.21 0.79 0.15 939.55 179399
41. 32.31 65.43 99.21 0.79 0.15 939.55 179399
42. 32.31 65.43 99.21 0.79 0.15 939.55 179399
43. 32.31 65.43 99.21 0.79 0.15 939.55 179399
44. 32.31 65.43 99.21 0.79 0.15 939.55 179399
45. 32.31 65.43 99.21 0.79 0.15 939.55 179399
46. 32.31 65.43 99.21 0.79 0.15 939.55 179399
47. 32.31 65.43 99.21 0.79 0.15 939.55 179399
48. 32.31 65.43 99.21 0.79 0.15 939.55 179399
49. 32.31 65.43 99.21 0.79 0.15 939.55 179399
50. 32.31 65.43 99.21 0.79 0.15 939.55 179399
Appendix 3: Comet Assay Scores

Scores for Slide Number 12: NIA N treatment (0.5 µg/ml)

<table>
<thead>
<tr>
<th>No.</th>
<th>Head Length</th>
<th>Tail Length</th>
<th>Head Intensity</th>
<th>Tail Intensity</th>
<th>Tail Moment</th>
<th>Total Area</th>
<th>Total Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max</td>
<td>75.93</td>
<td>128.43</td>
<td>100.00</td>
<td>62.49</td>
<td>15.97</td>
<td>1957.40</td>
<td>290108</td>
</tr>
<tr>
<td>Min</td>
<td>12.92</td>
<td>17.77</td>
<td>37.51</td>
<td>0.00</td>
<td>0.00</td>
<td>606.14</td>
<td>111901</td>
</tr>
<tr>
<td>Mean</td>
<td>33.60</td>
<td>84.85</td>
<td>86.05</td>
<td>13.95</td>
<td>4.06</td>
<td>1112.87</td>
<td>192484.20</td>
</tr>
<tr>
<td>SD</td>
<td>9.93</td>
<td>28.46</td>
<td>13.23</td>
<td>13.23</td>
<td>3.72</td>
<td>276.15</td>
<td>38666.29</td>
</tr>
</tbody>
</table>

1  29.08  47.66   85.98   14.02   3.06   981.96   150878
2  30.69  24.23   99.50   0.50   0.09   619.84   111901
3  22.62  17.77   78.96   21.04   2.55   606.14   126264
4  21.00  31.50   82.10   17.90   2.60   677.91   114928
5  35.54  67.85   94.99   5.01   1.42   1152.26  226636
6  25.85  71.89   83.51   16.49   5.60   1972.81  360460
7  35.54  92.89   94.99   5.01   1.42   1152.26  226636
8  30.69  102.58  79.73   20.27   6.71   1222.72  244762
9  35.54  92.89   94.99   5.01   1.42   1152.26  226636
10 32.31  101.78  94.83   5.17   1.84   1061.56  174519
11 37.16  87.24   96.70   3.30   0.93   1019.15  181482
12 32.31  80.78   97.75   2.25   0.76   820.15   166242
13 38.77  56.54   74.03   25.97   7.13   1346.04  252200
14 35.54  97.74   89.63   10.37   3.06   981.96   170472
15 17.77  86.43   69.33   30.67   9.96   728.15   137115
16 32.31  103.39  91.14   8.86   3.51   1485.02  233052
17 24.23  109.05  64.73   35.27   9.69   1380.62  204150
18 24.23  88.85   82.73   17.27   3.91   769.26   150711
19 30.69  46.85   96.55   3.45   0.73   692.27   156945
20 33.93  97.74   90.18   9.82   3.57   1186.84  205126
21 29.08  100.97  81.68   18.32   6.95   933.68   142558
22 38.77  90.47   98.36   1.64   0.53   1076.57  206150
23 33.93  88.05   97.81   2.19   0.85   934.99   197318
24 38.77  128.43  86.50   13.50   6.98   1657.92  244762
25 33.93  100.97  82.91   17.09   4.56   927.16   163797
26 40.39  125.20  92.30   7.70   3.42   1396.28  225220
27 37.16  124.39  97.02   2.98   1.47   856.04   154773
28 40.39  17.77   100.00  0.00   0.00   1008.06  230975
29 42.00  114.70  98.69   1.31   0.57   1233.82  290108
30 38.77  25.04   86.25   13.75   2.22   1137.25  205466
31 58.16  75.12   99.19   0.81   0.34   1002.19  197044
32 75.93  84.81   73.64   26.36   15.12   1957.40  212149
33 30.69  84.81   92.01   7.99   2.32   1000.23  182048
34 37.16  101.78  98.08   1.92   0.84   1061.56  203235
35 21.00  112.28  81.26   18.74   4.84   970.87   172752
36 40.39  57.35   98.76   1.24   0.40   1197.28  244210
37 27.46  97.74   78.02   21.98   5.68   1162.70  195719
38 29.08  92.89   95.79   4.21   1.26   816.24   146755
39 45.23  42.81   97.73   2.27   0.60   1058.95  215889
40 37.16  104.20  86.83   13.17   6.39   1291.23  208299
41 42.00  96.93   95.57   4.43   2.33   1284.06  239919
42 37.16  61.99   61.99   38.01   15.97   1258.61  223798
43 38.77  100.16  97.88   2.12   0.75   1316.68  257578
44 27.46  55.74   85.10   14.90   3.97   989.79   163676
45 25.85  109.05  93.54   6.46   2.87   1059.61  194440
46 12.92  113.09  37.51   62.49   10.60   1289.28  206734
47 32.31  100.16  90.04   9.96   3.46   1094.19  192711
48 37.16  89.66   94.50   5.50   2.40   1331.03  243372
49 24.23  91.28   61.30   38.70   11.25   1072.66  149567
50 22.62  104.20  62.76   37.24   8.42   995.01   160596
Appendix 3: Comet Assay Scores

Scores for Slide Number 21: MMS control (10 μg/ml)

<table>
<thead>
<tr>
<th>No.</th>
<th>Head Length</th>
<th>Tail Length</th>
<th>Head Intensity</th>
<th>Tail Intensity</th>
<th>Tail Moment</th>
<th>Total Area</th>
<th>Total Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75.93</td>
<td>142.97</td>
<td>99.95</td>
<td>39.53</td>
<td>14.10</td>
<td>2573.98</td>
<td>361539</td>
</tr>
<tr>
<td>2</td>
<td>60.47</td>
<td>18.58</td>
<td>696.18</td>
<td>148279</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>103.83</td>
<td>88.71</td>
<td>1127.99</td>
<td>225731.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>37.16</td>
<td>117.12</td>
<td>84.19</td>
<td>2573.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>33.93</td>
<td>131.66</td>
<td>4.33</td>
<td>3.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>117.93</td>
<td>84.77</td>
<td>1959.52</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>32.31</td>
<td>97.28</td>
<td>1522.86</td>
<td>229600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>38.77</td>
<td>116.32</td>
<td>5.37</td>
<td>5.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>75.93</td>
<td>114.70</td>
<td>120.00</td>
<td>177308</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>112.28</td>
<td>85.53</td>
<td>154.08</td>
<td>230686</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>21.00</td>
<td>142.97</td>
<td>4.33</td>
<td>4.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>130.05</td>
<td>95.64</td>
<td>154.08</td>
<td>230686</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>142.97</td>
<td>85.53</td>
<td>154.08</td>
<td>230686</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>32.31</td>
<td>117.93</td>
<td>84.77</td>
<td>1959.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>32.31</td>
<td>84.77</td>
<td>1959.52</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>116.32</td>
<td>97.28</td>
<td>1522.86</td>
<td>229600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>70.27</td>
<td>98.61</td>
<td>1387.86</td>
<td>219123</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>109.05</td>
<td>86.19</td>
<td>1387.86</td>
<td>219123</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>109.85</td>
<td>92.77</td>
<td>1689.89</td>
<td>256972</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>112.78</td>
<td>93.21</td>
<td>1689.89</td>
<td>256972</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>91.30</td>
<td>87.00</td>
<td>1659.88</td>
<td>249213</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>131.66</td>
<td>60.47</td>
<td>1456.31</td>
<td>227753</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>88.09</td>
<td>11.91</td>
<td>1188.14</td>
<td>220298</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>132.47</td>
<td>94.37</td>
<td>1695.11</td>
<td>345234</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>117.93</td>
<td>63.62</td>
<td>1830.17</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>126.01</td>
<td>83.81</td>
<td>1682.06</td>
<td>276348</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>112.78</td>
<td>93.21</td>
<td>1143.12</td>
<td>237991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>117.93</td>
<td>63.62</td>
<td>1830.17</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>91.30</td>
<td>87.00</td>
<td>1659.88</td>
<td>249213</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>131.66</td>
<td>60.47</td>
<td>1456.31</td>
<td>227753</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>88.09</td>
<td>11.91</td>
<td>1188.14</td>
<td>220298</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>132.47</td>
<td>94.37</td>
<td>1695.11</td>
<td>345234</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>117.93</td>
<td>63.62</td>
<td>1830.17</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>126.01</td>
<td>83.81</td>
<td>1682.06</td>
<td>276348</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>112.78</td>
<td>93.21</td>
<td>1143.12</td>
<td>237991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>117.93</td>
<td>63.62</td>
<td>1830.17</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>126.01</td>
<td>83.81</td>
<td>1682.06</td>
<td>276348</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>117.93</td>
<td>63.62</td>
<td>1830.17</td>
<td>290280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>126.01</td>
<td>83.81</td>
<td>1682.06</td>
<td>276348</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>109.85</td>
<td>94.90</td>
<td>1346.69</td>
<td>243578</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Max 75.93 142.97 99.95 39.53 14.10 2573.98 361539
Min 21.00 18.58 60.47 0.05 0.00 696.18 148279
Mean 39.00 103.83 88.71 11.29 4.33 1307.79 225731.80
SD 11.59 33.78 9.91 3.31 368.76 50126.58
### Appendix 3: Comet Assay Scores

#### Scores for Slide Number 23: NIAN treatment (1.0 μg/ml)

<table>
<thead>
<tr>
<th>No.</th>
<th>Head Length</th>
<th>Tail Length</th>
<th>Head Intensity</th>
<th>Tail Intensity</th>
<th>Tail Moment</th>
<th>Total Area</th>
<th>Total Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max</td>
<td>58.16</td>
<td>144.59</td>
<td>98.82</td>
<td>58.37</td>
<td>22.16</td>
<td>2909.35</td>
<td>280827</td>
</tr>
<tr>
<td>Min</td>
<td>19.39</td>
<td>34.73</td>
<td>41.63</td>
<td>1.18</td>
<td>0.52</td>
<td>723.59</td>
<td>134287</td>
</tr>
<tr>
<td>Mean</td>
<td>31.53</td>
<td>101.71</td>
<td>85.67</td>
<td>14.33</td>
<td>4.77</td>
<td>1190.10</td>
<td>194990.06</td>
</tr>
<tr>
<td>SD</td>
<td>6.75</td>
<td>29.20</td>
<td>13.10</td>
<td>13.10</td>
<td>4.30</td>
<td>383.29</td>
<td>35222.40</td>
</tr>
</tbody>
</table>

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  

Appendix 3: Comet Assay Scores

Scores for Slide Number 23: NIAN treatment (1.0 μg/ml)
SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
APPENDIX 4

AMES II ASSAY SCORES
### Appendix 4: Ames II Assay Scores

**Scores for Tester Strain TA 7001**

<table>
<thead>
<tr>
<th>TA 7001 (1xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>Mean = 0</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>STN</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7001 (1xN) NIAM (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>0</td>
<td>Mean = 0.3</td>
</tr>
<tr>
<td>0.625</td>
<td>0</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7001 (2xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>Mean = 0</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>STN</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7001 (2xN) NIAM (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>0</td>
<td>Mean = 0.3</td>
</tr>
<tr>
<td>0.625</td>
<td>1</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

N = normal inoculum. (1xN) and (2xN) refer to 650 µl and 1300 µl of the prepared bacterial suspension respectively (as described under “Experimental Procedures”).
### Appendix 4: Ames II Assay Scores

**Scores for Tester Strain TA 7002**

<table>
<thead>
<tr>
<th>TA 7002 (1xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>Mean = 0</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>STN</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7002 (1xN) NIAR (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>1</td>
<td>Mean = 0.6</td>
</tr>
<tr>
<td>0.625</td>
<td>2</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7002 (2xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>Mean = 0</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>STN</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7002 (2xN) NIAR (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>0</td>
<td>Mean = 0.1</td>
</tr>
<tr>
<td>0.625</td>
<td>0</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

N = normal inoculum. (1xN) and (2xN) refer to 650 µl and 1300 µl of the prepared bacterial suspension respectively (as described under “Experimental Procedures”).
## Scores for Tester Strain TA 7003

<table>
<thead>
<tr>
<th>TA 7003 (1xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>Mean = 0.1</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>STN</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7003 (1xN) NIAN (μg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>0</td>
<td>Mean = 0</td>
</tr>
<tr>
<td>0.625</td>
<td>0</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7003 (2xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>Mean = 0.3</td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>STN</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7003 (2xN) NIAN (μg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>0</td>
<td>Mean = 0.5</td>
</tr>
<tr>
<td>0.625</td>
<td>2</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

N = normal inoculum. (1xN) and (2xN) refer to 650 μl and 1300 μl of the prepared bacterial suspension respectively (as described under "Experimental Procedures").
### Appendix 4: Ames II Assay Scores

**Scores for Tester Strain TA 7004**

<table>
<thead>
<tr>
<th>TA 7004 (1xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>4</td>
<td>Mean = 4.3</td>
</tr>
<tr>
<td>DMSO</td>
<td>4</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7004 (1xN) NIAN (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>21</td>
<td>Mean = 12.3</td>
</tr>
<tr>
<td>0.625</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7004 (2xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>13</td>
<td>Mean = 8.1</td>
</tr>
<tr>
<td>DMSO</td>
<td>9</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7004 (2xN) NIAN (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>14</td>
<td>Mean = 15.5</td>
</tr>
<tr>
<td>0.625</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

N = normal inoculum. (1xN) and (2xN) refer to 650 µl and 1300 µl of the prepared bacterial suspension respectively (as described under “Experimental Procedures”).
### Appendix 4: Ames II Assay Scores

**Scores for Tester Strain TA 7005**

<table>
<thead>
<tr>
<th>TA 7005 (1xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>11</td>
<td>Mean = 9.7</td>
</tr>
<tr>
<td>DMSO</td>
<td>7</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>4NQO</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7005 (1xN) NIA N (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>17</td>
<td>Mean = 11.4</td>
</tr>
<tr>
<td>0.625</td>
<td>12</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7005 (2xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>14</td>
<td>Mean = 15.1</td>
</tr>
<tr>
<td>DMSO</td>
<td>14</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>4NQO</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7005 (2xN) NIA N (µg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>21</td>
<td>Mean = 21.1</td>
</tr>
<tr>
<td>0.625</td>
<td>25</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

N = normal inoculum. (1xN) and (2xN) refer to 650 µl and 1300 µl of the prepared bacterial suspension respectively (as described under "Experimental Procedures").
### Appendix 4: Ames II Assay Scores

**Scores for Tester Strain TA 7006**

<table>
<thead>
<tr>
<th>TA 7006 (1xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>2</td>
<td>Mean = 1.9</td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5azaC</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7006 (1xN) NIA N (μg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>3</td>
<td>Mean = 3.1</td>
</tr>
<tr>
<td>0.625</td>
<td>3</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7006 (2xN) Control</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>Mean = 1.6</td>
</tr>
<tr>
<td>DMSO</td>
<td>2</td>
<td>n = 7</td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5azaC</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TA 7006 (2xN) NIA N (μg/ml)</th>
<th>No. of + wells</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.078</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>0.312</td>
<td>3</td>
<td>Mean = 5.5</td>
</tr>
<tr>
<td>0.625</td>
<td>8</td>
<td>n = 8</td>
</tr>
<tr>
<td>1.25</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

N = normal inoculum. (1xN) and (2xN) refer to 650 μl and 1300 μl of the prepared bacterial suspension respectively (as described under "Experimental Procedures").
SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN
APPENDIX 5

LIST OF SCHEMES
LIST OF FIGURES
LIST OF TABLES
Appendix 5: List of Schemes

LIST OF SCHEMES

Chapter 1: Introduction

1.1 Structure of indole. 2
1.2 Structures of tryptophan and metabolites tryptamine and serotonin. 3
1.3 Structures of the hallucinogens bufotenine and psilocin. 4
1.4 Summary of the autolytic products of glucobrassicin. 6
1.5 Bis-indole structures of the potent antitumour agents, vinblastine and vincristine from Catharanthus roseus. 8
1.6 Sumatriptan and zolmitriptan, both related to serotonin in structure, are potent 5-HT$_1$ receptor agonists marketed for the treatment of migraine. 9
1.7 Structure of the potent hallucinogen LSD, which is the N-N-diethylamide synthetic derivative of lysergic acid. 10
1.8 Structures of ondansetron and tropisetron, potent 5-HT$_3$ antagonists used to treat vomiting and nausea caused by chemotherapy. 11
1.9 Nitrous acid exists in equilibrium with a number of other species, most of which contribute to afford nitrosation products. 13
1.10 Summary of the N-nitrosation pathways of amines and amides. 14
1.11 Mechanisms for the formation of N-nitrosoindoles. (A) Direct nitrogen atom nitrosation and (B) internal rearrangement of the nitroso group from C-3 to N-1. 16
1.12 Summary of N-nitrosation of amines and related compounds and decomposition pathways of the resulting N-nitroso compounds. The diazonium ion generated can readily react with DNA to afford DNA adducts. 25
1.13 Summary of the major sites on DNA bases susceptible to alkylation and interaction with aromatic diazonium ions. 26
1.14 O$^\delta$-Methylguanine and O$^\gamma$-methylthymine are proposed to form stable mispairs with thymine and guanine respectively. 29
1.15 Structures of piperazine and cyclizine. 38
1.16 Nitrosation of aminopyrine yields DMN. 39
1.17 Structural similarities of cimetidine, nitrosocimetidine, MNNG and MNU. 41
1.18 Structures of the three mutagen precursors isolated from Chinese cabbage. 43
1.19 Nitrosation of CI affords the fava bean mutagen NCMI. 45
1.20 Proposed reaction mechanism of the fava bean mutagen NCMI and simpler analogues such as NCI, with DNA. 46
1.21 Proposed spontaneous decomposition of 3-substituted N-nitrosoindoles to afford benzenediazonium ion intermediates, and possible subsequent covalent interaction with DNA. 47
Appendix 5: List of Schemes

Chapter 2: Synthesis and Characterisation of a Series of 3-Substituted N-Nitrosoindoles

2.1 Structures of the indole compounds selected for nitrosation. 50
2.2 Structures of the 3-substituted nitrosated indoles described in this thesis. 60

Chapter 3: Reactions of a Series of 3-Substituted N-Nitrosoindoles with Purine Nucleotides and Nucleosides

3.1 Structures of the purine 5'-monophosphate nucleotides and 2'-deoxyguanosine used as reactive substrates in this study. 63
3.2 Reaction mechanism of the Leibermann test to identify a nitrosamine or N-nitroso compound. 102
3.3 Transnitrosation to the N-7 atom of guanine followed by cleavage of the N-glycosidic bond and hydrolysis of the N-nitrosopurine affords depurination products. 108
3.4 Reaction mechanism for the formation of deamination product, xanthine and dOp, based on mechanisms described by Suzuki (Suzuki et al. 1997a) following transnitrosation from N-nitrosoindoles. 109

Chapter 4: Reactions of 1-Nitrosoindole-3-acetonitrile with Oligonucleotides, Duplex DNA and Calf Thymus DNA

4.1 Summary of NIAN-induced depurination, deamination and formation of oxanine via transnitrosation from NIAN to dpG. 113
4.2 Covalent structure of the dG-to-dG cross-link at the sequence 5'-CG induced by nitrous acid. 152
4.3 Proposed mechanism for formation of NIAN-induced cross-links at CG residues: direct diazonium ion chemistry. 153
4.4 Alternative reaction mechanism for formation of NIAN-induced cross-links at CG residues: via ring-opened guanine or oxanine intermediates. 154

Chapter 5: Detection of Apurinic Residues induced by 1-Nitrosoindole-3-acetonitrile in Calf Thymus DNA and in the Glandular Stomach of CD-1 Mice by 32P-Postlabelling

5.1 The depurination pathway induced by nitroso transfer from NIAN to the nucleophilic N-7 atom of guanine. The abasic generated is not a chemically unique species. 156
5.2 Strategy of the postlabelling assay for the detection of abasic sites in DNA. 158

Chapter 7: General Discussion and Conclusions

7.1 Proposed base pairs between oxanine and cytosine, and oxanine and thymine. 221
7.2 Reaction of dOxo with a primary amine affords a ring-opened adduct. 223
LIST OF FIGURES

Chapter 1: Introduction
1.1 Biomonitoring exposure to genotoxic carcinogens. 37

Chapter 2: Synthesis and Characterisation of a Series of 3-Substituted N-Nitrosoindoles
2.1 (A) HPLC chromatogram of pure NIAN and (B) UV spectrum of NIAN. 54
2.2 (A) $^1$H NMR spectrum of NIAN recorded in CDCl$_3$ and (B) Fast Atom Bombardment (positive ion) mass spectrum of NIAN. 55
2.3 (A) HPLC chromatogram of pure NIAM and (B) UV spectrum of NIAM. 56
2.4 (A) $^1$H NMR spectrum of NIAM recorded in CD$_3$COCD$_3$ and (B) Fast Atom Bombardment (positive ion) mass spectrum of NIAM. 57
2.5 (A) HPLC chromatogram of NIAAME after purification and (B) UV spectrum of NIAAME. 58
2.6 (A) $^1$H NMR spectrum of NIAAME recorded in CD$_3$COCD$_3$ and (B) Fast Atom Bombardment (positive ion) mass spectrum of NIAAME. 59

Chapter 3: Reactions of a Series of 3-Substituted N-Nitrosoindoles with Purine Nucleotides and Nucleosides
3.1 HPLC separation of (A) Control reaction mixture and (B) NIAN and dpA reaction mixture (molar ratio 20:1). 69
3.2 UV spectra of (A) Hyp standard and (B) HPLC fraction corresponding to Hyp. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Hyp. 70
3.3 UV spectra of (A) Ade standard and (B) HPLC fraction corresponding to Ade + dpA. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Ade + dpA. 71
3.4 Mass spectrometric analyses of HPLC fraction corresponding to $N^6$-AcAde. 72
3.5 HPLC separation of (A) Control reaction mixture and (B) NIAN and dAp reaction mixture (molar ratio 15:1). 73
3.6 The increase in the production of (A) Hyp and $N^6$-AcAde and (B) Ade + dpA with increasing mole equivalents of NIAN to dpA; (C) Hyp and $N^6$-AcAde and (D) Ade + dAp with increasing mole equivalents of NIAN to dAp. 74
3.7 HPLC separation of (A) Control reaction mixture and (B) NIAM and dpA reaction mixture (molar ratio 15:1). 75
3.8 HPLC separation of (A) Control reaction mixture and (B) NIAAME and dpA reaction mixture (molar ratio 15:1). 76
3.9 HPLC separation of (A) Control reaction mixture and (B) NIAN and dpG reaction mixture (molar ratio 10:1). 80
Appendix 5: List of Figures

3.10 UV spectra of (A) Gua standard and (B) HPLC fraction corresponding to Gua.  
(C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Gua.
81

3.11 UV spectra of (A) Xan standard and (B) HPLC fraction corresponding to Xan.  
(C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Xan.
82

3.12 Mass spectrometric analysis of HPLC fraction corresponding to N²-AcdpG.
83

3.13 UV spectra of (A) N²-AcGua standard and (B) HPLC fraction corresponding to N²-AcGua. (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to N²-AcGua with conditions used to promote cone voltage induced dissociation.
84

3.13 (D) Electrospray mass spectrometric analysis of HPLC fraction corresponding to N²-AcGua generated from reaction mixture using d₅-ACN as co-solvent.
85

3.14 Dose-response relationship of reaction products (A) Gua and Xan and (B) N²-AcdpG and N²-AcGua with increasing mole equivalents of NIAN to dpG.
86

3.15 HPLC separation of Doxo hydrolysis reaction mixture.
87

3.16 (A) The structure of oxanine, (B) UV spectrum of Oxa generated from the hydrolysis of Doxo and (C) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Oxa generated from the hydrolysis of Doxo.
88

3.17 (A) UV spectrum of HPLC fraction corresponding to Oxa and (B) Electrospray mass spectrometric analysis of HPLC fraction corresponding to Oxa.
89

3.18 (A) The structure of dpO, (B) UV spectrum of HPLC fraction corresponding to dpO and (C) Mass spectrometric analyses of HPLC fraction corresponding to dpO.
90

3.19 HPLC separation of (A) Control reaction mixture and (B) NIAN and dGp reaction mixture (molar ratio 15:1).
91

3.20 HPLC separation of (A) Control reaction mixture and (B) NIAM and dpG reaction mixture (molar ratio 15:1).
92

3.21 HPLC separation of (A) Control reaction mixture and (B) NIAAME and dpG reaction mixture (molar ratio 15:1).
93

3.22 HPLC separation of (A) Control reaction mixture and (B) NIAN and dGuo reaction mixture (molar ratio 15:1).
94

3.23 HPLC separation of (A) Control reaction mixture and (B) NIAM and dGuo reaction mixture (molar ratio 15:1).
95

3.24 HPLC separation of (A) Control reaction mixture and (B) NIAAME and dGuo reaction mixture (molar ratio 15:1).
96

3.25 The increase in the production of (A) Gua and Xan, (B) Oxa and (C) N²-AcGua with increasing mole equivalents of NIAM to dGuo.
97

3.26 The increase in the production of (A) Gua and Xan, (B) Oxa and (C) N²-AcGua with increasing mole equivalents of NIAAME to dGuo.
98
Appendix 5: List of Figures

3.27 HPLC separation of (A) Control reaction mixture and (B) NIAN and dOxo reaction mixture (molar ratio 15:1). 99

3.28 UV spectra of HPLC fractions corresponding to (A) Oxa and (B) Xan generated from the reaction of NIAN and dOxo. 100

3.29 The increase in the production of (A) Oxa and (B) Xan with increasing mole equivalents of NIAN to dOxo. 100

3.30 (A) HPLC chromatogram of IAN standard and (B) HPLC separation of NIAN and dpG reaction extract (molar ratio 5:1). UV spectra of IAN standard and HPLC fraction corresponding to IAN are shown. 103

3.31 (A) HPLC chromatogram of IAM standard and (B) HPLC separation of NIAM and dpG reaction extract (molar ratio 5:1). UV spectra of IAM standard and HPLC fraction corresponding to IAM are shown. 104

3.32 (A) HPLC chromatogram of IAAME standard and (B) HPLC separation of NIAAME and dpG reaction extract (molar ratio 10:1). UV spectra of IAAME and HPLC fraction corresponding to IAAME are shown. 105

3.33 HPLC separation of (A) NIAN and dpG reaction mixture (molar ratio 10:1) and (B) Azide, NIAN and dpG reaction mixture (molar ratio 10:10:1). 106

Chapter 4: Reactions of 1-Nitrosoindole-3-acetonitrile with Oligonucleotides, Duplex DNA and Calf Thymus DNA

4.1 NIAN-induced strand break products of 5'-32P-end labelled oligonucleotides. 123

4.2 (A) Dose-dependent formation of strand break products at guanine residues G11 and G12 in NIAN treated oligo (I) and (B) % single strand breakage at guanine residues G11 and G12 in NIAN treated oligo (I). 124

4.3 (A) Dose-dependent formation of strand break products at guanine residues G11, G12 and G13 in NIAN treated oligo (II) and (B) % single strand breakage at guanine residues G11, G12 and G13 in NIAN treated oligo (II). 125

4.4 (A) Dose-dependent formation of strand break products at guanine residues G9, G11 and G13 in NIAN treated oligo (III) and (B) % single strand breakage at guanine residues G9, G11 and G13 in NIAN treated oligo (III). 126

4.5 (A) Dose-dependent formation of strand break products at guanine residues G7, G8, G15 and G16 in NIAN treated oligo (IV) and (B) % single strand breakage at guanine residues G7, G8, G15 and G16 in NIAN treated oligo (IV). 127

4.6 (A) Strand break products of radiolabelled oligo (III) incubated with 2mM NIAN for 6, 12, 24 and 30h and (B) Yield of total strand break products as a function of time. 128

4.7 (A) Confirmation of duplex formation for sequence VI and (B) Formation of mechlorethamine hydrochloride induced cross-linked DNA. 131
Appendix 5: List of Figures

4.8 Effect of co-solvent and diethyl ether extraction on the formation and stability of mechlorethamine hydrochloride induced cross-linked DNA. 132

4.9 (A) Confirmation of duplex formation for sequence I, (B) Formation of NIAN induced cross-linked DNA and (C) Yield of interstrand cross-linked DNA as a function of NIAN concentration. 134

4.10 Calibration curves for the quantitation of reaction products in NIAN treated oligonucleotide and calf thymus DNA. 139

4.11 Calibration curves for the quantitation of dNps in NIAN treated oligonucleotide and calf thymus DNA. 140

4.12 HPLC separation of NIAN and oligo (V) reaction mixture after 6h incubation and enzymatic digestion to 3'-monophosphates. (A) Control; (B) 4mM NIAN treatment; (C) 8mM NIAN treatment. 141

4.13 HPLC separation of NIAN and oligo (V) reaction mixture after 24h incubation and enzymatic digestion to 3'-monophosphates. (A) Control; (B) 4mM NIAN treatment; (C) 8mM NIAN treatment. 142

4.14 Quantitation of parent nucleotides and reaction products in NIAN treated oligo (V) for 6h, as a function of NIAN concentration. 143

4.15 Quantitation of parent nucleotides and reaction products in NIAN treated oligo (V) for 24h, as a function of NIAN concentration. 144

4.16 HPLC separation of NIAN and calf thymus DNA reaction mixture after 6h incubation and enzymatic digestion to 3'-monophosphates. (A) Control; (B) 4mM NIAN treatment; (C) 8mM NIAN treatment. 146

4.17 HPLC separation of NIAN and calf thymus DNA reaction mixture after 24h incubation and enzymatic digestion to 3'-monophosphates. (A) Control; (B) 4mM NIAN treatment; (C) 8mM NIAN treatment. 146

4.18 Quantitation of parent nucleotides and reaction products in NIAN treated calf thymus DNA for 6h, as a function of NIAN concentration. 147

4.19 Quantitation of parent nucleotides and reaction products in NIAN treated calf thymus DNA for 24h, as a function of NIAN concentration. 148

Chapter 5: Detection of Apurinic Residues induced by 1-Nitrosoindole-3-acetonitrile in Calf Thymus DNA and in the Glandular Stomach of CD-1 Mice by $^{32}$P-Postlabelling

5.1 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN (mM) dissolved in ACN or DMSO. 168
Appendix 5: List of Figures

5.2 (A) and (B) Comparison of bands (1-3) generated from CT DNA treated with NIAN (mM), with control CT DNA containing apurinic residues. (C) and (D) Plots of detectable damage from 1μg of DNA vs NIAN dose. 169

5.3 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA with NIAN (μM). 171

5.4 (A) Comparison of bands (1-3) generated from CT DNA treated with NIAN, with control CT DNA containing apurinic residues. (B) Plot of detectable damage from 1μg of DNA vs NIAN dose. 172

5.5 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN in the presence of sodium azide. 174

5.6 Plot of detectable damage from 1μg of CT DNA treated with NIAN vs sodium azide dose. 175

5.7 HPLC separation of CT DNA after digestion to 3’-monophosphates. (A) Control CT DNA; (B) Control CT DNA that has been through the Qiagen DNA isolation procedure. 179

5.8 HPLC separation of mouse stomach DNA, after digestion to 3’-monophosphates. (A) DNA from mouse no. 3 (control); (B) DNA from NIAN mouse no.8 (NIAN treated and sacrificed after 3h). 180

5.8 HPLC separation of mouse stomach DNA, after digestion to 3’-monophosphates. (C) and (D) DNA from mouse nos. 22 and 21 respectively (NIAN treated and sacrificed after 24h). 181

5.9 (A) Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of control and NIAN treated CD-1 mouse stomach DNA and (B) Quantitation of detectable damage as determined by phosphorimaging. 182

5.10 (A) Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of control and NIAN treated CD-1 mouse stomach DNA and (B) Quantitation of detectable damage as determined by phosphorimaging. 183

5.11 Dependence on time of exposure to NIAN on the accumulation of damage detected in the glandular stomach of CD-1 mice. 184

5.12 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of CT DNA treated with NIAN and incubated with *E. coli* exonuclease III. 187

5.13 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of control and NIAN treated CD-1 mouse stomach DNA incubated with *E. coli* exonuclease III. 188
Appendix 5: List of Figures

5.14 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of NIAN treated and 50 Gy irradiated DNA incubated with \textit{E. coli} exonuclease III and \textit{E. coli} endonuclease IV. 189

5.15 Gel electrophoresis of the end-labelled compounds obtained by digestion and phosphorylation of DNA from mouse number 14 (NIAN treated and sacrificed after 3h) incubated with \textit{E. coli} exonuclease III and \textit{E. coli} endonuclease IV. 190

Chapter 6: Activity of \textit{1-Nitrosoindole-3-acetonitrile in the Comet and Ames II Assays}

6.1 Summary of the methodology used in the Comet assay. 196

6.2 Example comet shapes and appearance of cells after treatment with a test chemical. 207

6.3 Activity of NIAN in the Comet assay, in the absence of an exogenous metabolising system. 209
Appendix 5: List of Tables

LIST OF TABLES

Chapter 1: Introduction

1.1 Factors controlling bacterial N-nitrosation at various sites in the body. 18
1.2 Evidence for concern over the exposure to humans of NNOC. 22

Chapter 4: Reactions of 1-Nitrosoindole-3-acetonitrile with Oligonucleotides, Duplex DNA and Calf Thymus DNA

4.1 Oligonucleotide sequences used in this study. 115

Chapter 5: Detection of Apurinic Residues induced by 1-Nitrosoindole-3-acetonitrile in Calf Thymus DNA and in the Glandular Stomach of CD-1 Mice by $^{32}$P-Postlabelling

5.1 Yields of DNA isolated from the glandular stomach of CD-1 mice. 177

Chapter 6: Activity of 1-Nitrosoindole-3-acetonitrile in the Comet and Ames II Assays

6.1 Bacterial strains used in the Ames II assay. Each strain detects one, and only one, of six possible base substitutions. 197
6.2 Results summary of the Comet Assay as determined by Comet II™ analysis software. 208
6.3 Responses of base-specific Salmonella tester strains for identifying NIAN as a mutagen, in the absence of an exogenous metabolising system. 210

Chapter 7: General Discussion and Conclusions

7.1 Mutations that potentially arise from deamination of the purine bases. 217
7.2 Mutations that potentially arise from depurination of the purine bases. 219