1971

The Chemical, Toxic, And Transforming Activities Of N-methyl-n-nitroso Compounds

Carolyn Jane Miller

Follow this and additional works at: https://ir.lib.uwo.ca/digitizedtheses

Recommended Citation
https://ir.lib.uwo.ca/digitizedtheses/478

This Dissertation is brought to you for free and open access by the Digitized Special Collections at Scholarship@Western. It has been accepted for inclusion in Digitized Theses by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.
The author of this thesis has granted The University of Western Ontario a non-exclusive license to reproduce and distribute copies of this thesis to users of Western Libraries. Copyright remains with the author.

Electronic theses and dissertations available in The University of Western Ontario’s institutional repository (Scholarship@Western) are solely for the purpose of private study and research. They may not be copied or reproduced, except as permitted by copyright laws, without written authority of the copyright owner. Any commercial use or publication is strictly prohibited.

The original copyright license attesting to these terms and signed by the author of this thesis may be found in the original print version of the thesis, held by Western Libraries.

The thesis approval page signed by the examining committee may also be found in the original print version of the thesis held in Western Libraries.

Please contact Western Libraries for further information:
E-mail: libadmin@uwo.ca
Telephone: (519) 661-2111 Ext. 84796
Web site: http://www.lib.uwo.ca/
Chemical, Toxic, and Transforming Activities of
N-Alkyl-N-Nitroso Compounds

BY

CARYOLYN THATCHER MILLER
Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
February, 1971
This work was supported by
the National Cancer Institute of Canada
ACKNOWLEDGEMENTS

The co-operation of Dr. P.D. Lawley and Dr. I.G. Walker is gratefully acknowledged. Mrs. Ingrid Beauchamp provided skilled technical assistance.
# TABLE OF CONTENTS

Acknowledgements.................................................................................................. iv  
Abstract......................................................................................................................... vi  
List of Abbreviations and Definitions.................................................................. viii  
List of Figures........................................................................................................... xv  
List of Tables......................................................................................................... xxiv  

INTRODUCTION.............................................................................................................. 1  
MATERIALS AND METHODS................................................................................... 8  

CHAPTER 1. The activation of methyl-nitroso compounds in vitro.  
Results......................................................................................................................... 25  
Figures......................................................................................................................... 38  
Discussion..................................................................................................................... 71  

CHAPTER 2. The reactions of methyl-nitroso compounds in vivo.  
Results......................................................................................................................... 79  
Figures......................................................................................................................... 97  
Discussion..................................................................................................................... 120  

CHAPTER 3. The toxicity of methyl-nitroso compounds.  
Results......................................................................................................................... 126  
Figures......................................................................................................................... 133  
Discussion..................................................................................................................... 150  

CHAPTER 4. The role of methyl-nitroso compounds in the induction of genetic change.  
Results......................................................................................................................... 156  
Figures......................................................................................................................... 167  
Discussion..................................................................................................................... 211  

BIBLIOGRAPHY......................................................................................................... 214  
Vita.............................................................................................................................. 222
ABSTRACT

The historic discovery that cancer could be induced by pure chemicals of known structure was greeted with optimism. It was hoped that these chemicals, synthesized from radioactive materials and reacted with living cells, would quickly indicate the cellular targets responsible for the carcinogenic transformation. However, the early promise of the discovery of chemical carcinogenesis was not fulfilled. First, evidence accumulated indicating that the agent itself was often inactive and was only a precursor of some still undefined carcinogen. Second, the cancer-inducing agents were soon shown to react not with a single cellular target, but with a great many different cell systems. Which of these was involved in the carcinogenic, rather than the lethal, effects of the agent remained obscure.

In order to isolate chemical reactions, and relate each to its biological effect, a group of compounds was selected which, although chemically similar, had quite different biological properties. Two of the agents (MNUA and MNNG) were potent carcinogens while two (DMS and MNTS) were very weak carcinogens. On the other hand, when killing potential was measured, MNNG and MNTS proved to be far more lethal than MNUA or DMS.

Study of chemical reactivity under conditions designed to parallel the cell interior as closely as possible revealed that the compounds could decompose in two ways. One decomposition route resulted in oxidation of thiols, while the other gave rise to a methylating species shown to attack DNA. The extent of each of these reactions was different for each of the agents tested. Study of the reactions of the agents in living cells provided good evidence that
activity in vivo did in fact follow closely the pattern observed with the purely chemical system.

It was then established that the two extremely toxic agents were the two that reacted extensively by the thiol-oxidizing route. Further, if treatment was followed with a thiol-reducing agent, the extent of killing was reduced. A first correlation between chemical reactivity (thiol oxidation) and biological effect (loss of colony-forming ability) could therefore be made.

The two carcinogenic agents (MNUA and MNNG), but not the others, gave rise to an unusual product via the alternative methylating decomposition route. This was identified as $^6\text{O}$-methylguanine, and could be derived from either DNA or RNA. However, this was only one of a number of products, all produced by the carcinogen. The question remained: "Is it involved in carcinogenesis?"

Some $^6\text{O}$-methylguanine was synthesized, made radioactive, and fed to cultures of mammalian cells. It was possible to demonstrate that the enzyme systems of the cells could incorporate very small amounts of the unusual base into its nucleic acid without toxic effect. Further, when given to primary hamster embryo cultures, $^6\text{O}$-methylguanine induced the disorganized growth characteristic of potential tumour cells. Since these changes occurred after incorporation of $^6\text{O}$-methylguanine only, in the absence of the other chemical reactions of the original carcinogens, a second correlation between chemical reactivity and biological effect could be made. Methylation at the 6-oxygen atom of the nucleic acid component guanine can disrupt normal control of genetic expression.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine, a base found in nucleic acid.</td>
</tr>
<tr>
<td>A_{260}</td>
<td>Absorption of light in optical density units, at the wavelength indicated in the subscript.</td>
</tr>
<tr>
<td>AHE</td>
<td>Newly activated hamster embryo cells. See &quot;growth cycle&quot;</td>
</tr>
<tr>
<td>AME</td>
<td>Newly activated mouse embryo cells.</td>
</tr>
<tr>
<td>AL</td>
<td>Newly activated L cells.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine, a base found in nucleic acid.</td>
</tr>
<tr>
<td>^{14}C</td>
<td>A radioactive isotope of carbon.</td>
</tr>
</tbody>
</table>

**Cell cycle, or replication cycle.** The progression of a cell or a culture of cells once through the phases of asexual reproduction. Cells first enter $G_1$, the first gap in DNA synthesis, then enter S-phase during which DNA is replicated to give the 2N complement of genetic material, then enter $G_2$, the second gap in DNA synthesis, and then finally divide by mitosis into two daughter cells.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHE</td>
<td>Confluent hamster embryo cells.</td>
</tr>
<tr>
<td>Confluent</td>
<td>See &quot;contact inhibition&quot;.</td>
</tr>
</tbody>
</table>

**Contact inhibition.** The normal interaction of adjacent cells causing growth to stop when a continuous layer one cell deep has been produced. Cells in such a monolayer are termed confluent.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP</td>
<td>Cytidine triphosphate.</td>
</tr>
</tbody>
</table>
Dimethylsulphate.

Deoxyribonucleic acid phosphorus. In DNA and RNA there is one phosphorus atom for each nucleotide residue in the polymer. The average molecular weight of the nucleotide residues is 309. Therefore 309 grams of DNA is equivalent to one gram mole DNAP.

5,5'-Dithio-bis-(2-nitrobenzoic acid), the reagent used in Ellman's (1959) colorimetric estimation of thiol content.

Dithiothreitol, or Cleland's reagent (1964). This compound reacts specifically with oxidized S-S groups. In the course of the two-step reaction the thiol groups of DTT become oxidized or bonded to each other, with the concomitant reduction of other S-S groups. Thus the total number of -SH groups in the mixture remains constant.

Example: DTT = R-SH Oxidized glutathione = GSSG

R-SH + GSSG → R-SG + GSH R-SG + GSH → R-S + 2GSH

Extinction, used as a synonym of A, absorption of light in O.D. units.

ε The molar extinction coefficient of a compound. ε equals the absorption in optical density units of a one molar solution.

Exponential phase. A synonym for logarithmic phase. See "growth cycle".

FUdR Fluorodeoxyuridine, an analog of thymidine, which competes for the methylating enzyme of dUMP. Since the cellular pool of dTMP is small, cells treated with FUdR are unable to continue DNA synthesis. Other phases of the cell cycle are not inhibited and the
FUdR (cont'd.) cells collect at the beginning of S-phase. The inhibition of the now synchronous culture may be reversed by adding a 1000-fold excess of thymidine over FUdR.

G
Guanine, a base found in nucleic acid.

G₁,G₂
See "cell cycle".

GdR
Guanosine, or guanine deoxyribonucleoside.

Growth cycle
When cells become crowded interactions between cells cause cell division to stop. Dilution of such a stopped or "stationary phase" culture activates a new growth cycle. The cells are first in a "lag" phase during which metabolic activity is high but little cell division occurs. Then cells enter the "logarithmic" growth phase during which several cycles of cell division occur. As the cells again become crowded division stops and stationary phase is reached. See figures 32 and 37.

GSH
Glutathione, a tripeptide of glycine, cysteine, and glutamic acid.

GSSG
Oxidized glutathione.

GTP
Guanosine triphosphate.

³H
Tritium, a radioactive isotope of hydrogen.

³H-TdR
Tritiated thymidine.

HE
Hamster embryo cells.

IAM
Iodoacetamide.

L cells
A subline of L-60-T cells derived from Earle's (1943) mouse fibroblast cultures. These cells have now been continuously cultured in vitro for at least 28 years. They will grow in suspension and produce tumours when injected subcutaneously into animals. L-60-T is a line
L cells (cont'd.) selected for the ability to grow without exogenous thymidine. The line originally averaged 60 chromosomes per diploid cell, but this number is variable.

"L" cells
Late logarithmic phase L cells. See "growth cycle".

Lag phase
See "growth cycle".

λ
Wave length, usually measured in nanometers.

Log phase
Logarithmic growth phase. See "growth cycle".

M
Mitosis, or asexual cell division.

MMS
Methylmethanesulphonate.

MNG
N-methyl-N'-nitroguanidine.

MNNG
N-methyl-N'-nitro-N-nitrosoguanidine.

MNTS
N-methyl-N-nitrosotoluenesulphonamide.

MNUA
N-methyl-N-nitrosourea.

MTS
N-methyltoluenesulphonamide.

1-MA
1-methyladenine.

3-MA
3-methyladenine.

6-MAP
6-methylaminopurine, a rearrangement product of 1-methyladenine.

3-MC
3-methylcytosine.

7-MG
7-methylguanine.

MW
Molecular weight.

N
Symbols for specific atoms in a compound are underlined to avoid ambiguity.

2N complement of DNA. The amount of DNA in a cell after replication. See "growth cycle".

NATCA
2-Nitraminothiazoline-4-carboxylic acid.

\( ^6\text{MeGdR} \)
Guanosine methylated on the 6-oxygen atom.

\( ^6\text{MG} \)
2-Amino-6-methoxypurine or \( ^6\text{methylguanine} \).
Oxidized thiol. The product of dehydrogenation of 2 thiol groups.

\[ 2 \text{RSH} \rightarrow \text{RSSR} + 2\text{H}^+ \]

poly(G,C) Synthetic RNA, containing residues of guanosine triphosphate and cytidine triphosphate in the same strand.

S-phase The period during which DNA is synthesized. See "cell cycle".

SH See "thiol".

SL cells A synchronized culture of L cells in S-phase.

\[ \text{SN}_1 \]

Substitution, nucleophilic, first order: a reaction mechanism defined by Ingold (1953). In a nucleophilic substitution an electron-rich reagent (nucleophile) takes the place of a leaving group of the substrate. However, if the reaction is \( \text{SN}_1 \), the reaction occurs in two steps. First, the substrate ionizes slowly (i.e., loses its leaving group). However, the ion produced is highly reactive and combines with the nucleophile instantaneously. Thus only the first (ionizing) step is rate limiting and the overall reaction will have first-order kinetics. Further, the differences in reactivity of various nucleophiles are insignificant compared to the reactivity of the ion. Therefore, in a mixture of nucleophiles, reaction will occur at random, and even weakly nucleophilic sites will take part.
SN₁ (cont’d.) Example: Two-step reaction with first order kinetics:

\[
\text{SN}_1 \quad \text{(a) } \text{CH}_3 \text{–N=NN} \xrightarrow{\text{slow}} \text{CH}_3^+ + N_2
\]

DIAZOMETHANE \hspace{1cm} \text{CARBONIUM}

ION \hspace{1cm} \text{(substrate)} \hspace{1cm} \text{(leaving group)}

\[
\text{(b) } \text{CH}_3^+ + \text{R-\text{OH}} \xrightarrow{\text{fast}} \text{R-O-CH}_3 + H^+
\]

(nucleophile)

SN₂

In the second order nucleophilic substitution mechanism the reaction occurs in one step, via a transition complex. Rate of reaction will depend on the concentration of both nucleophile and substrate. The nucleophile must be sufficiently powerful to compete with a leaving group that would not normally ionize. Thus in a mixture of nucleophiles, the extent of reaction with each will depend on how nucleus-loving it is. Example:

One-step reaction with second order kinetics: SN₂:

\[
\text{CH}_3\text{–O–S–O–CH}_3 + \text{R-SH} \rightarrow \text{[CH}_3\text{–O–S–O–CH}_3\text{]} \rightarrow \text{CH}_3\text{–O–S–O}^- + \text{RSCH}_3 + H^+
\]

DMS \hspace{1cm} \text{(Substrate)} \hspace{1cm} \text{(nucleophile)} \hspace{1cm} \text{(leaving group)}

\text{(transition comp.)}

SN character. The two mechanisms SN₁ and SN₂ define two extremes involving total ionization and no ionization. The truth generally lies somewhere between and the extent to which a given reaction approaches either of the extremes is called its SN character.

Sta L cells \hspace{1cm} \text{Stationary phase L cells. See "growth cycle".}

Sulphydryl \hspace{1cm} \text{See "thiol".}
Synchronous

A culture of cells in which a large proportion are simultaneously in the same phase of the cell cycle.

T

Thymine, a base of deoxyribonucleic acid.

t₁₂

The time required to reduce substrate concentration by one-half.

TCA

Trichloroacetic acid. If cells are lysed in cold TCA an arbitrary division of components is achieved. Low molecular weight compounds dissolve in TCA at 0°C while higher molecular weight substances including nucleic acids do not. On heating to 100°C nucleic acids form soluble products.

TdR

Thymidine, or deoxyribothymine.

TEAH

Tetraethylammoniumhydroxide.

Thiol, sulphydryl, or SH. All of these represent the chemical grouping -sulphur atom-hydrogen atom.

TMV

Tobacco Mosaic Virus.

Transformed

Cells which have lost control of cell division and will grow into tumours are termed transformed.

U

Uracil, a base of ribonucleic acid.

UTP

Uridine triphosphate.
RESULTS

LIST OF FIGURES

Chapter I : The activation of methyl-nitroso compounds \textit{in vitro}.

Section 1 : The kinetics of decomposition of methyl-nitroso compounds.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>The effect of pH on the decomposition of nitroso compounds.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 2</td>
<td>The effect of acid, alkali, or thiol on MNTS decomposition.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 3</td>
<td>The rate of decomposition of MNNG in the presence of equimolar thiol or amino groups</td>
<td>40</td>
</tr>
<tr>
<td>Figure 4</td>
<td>The effect of added amino acids on decomposition of MNNG.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 5</td>
<td>The second order kinetics of the reaction of MNNG with thiol.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 6</td>
<td>The second order kinetics of the reaction of glutathione with MNNG.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 7</td>
<td>The rate of reaction of MNNG with medium, or equimolar thiol and amino groups</td>
<td>42</td>
</tr>
<tr>
<td>Figure 8</td>
<td>The kinetics of decomposition of MNUA or MNNG $\pm$ GSH.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 9</td>
<td>The effect of GSH concentration on MNUA decomposition.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 10</td>
<td>The effect of glycine concentration on MNUA decomposition.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 11</td>
<td>The effect of GSH concentration on MNTS decomposition.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 12</td>
<td>The effect of glycine concentration on MNTS decomposition.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 13</td>
<td>The effect of MNTS concentration on its reaction with GSH.</td>
<td>48</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (continued)

Section 2 : The mechanism of decomposition of MNNG

Figure 14 : Column chromatography of the MNNG -- cysteine reaction mixture................................. 50

Figure 15 : Elementary analysis of the major MNNG -- cysteine product.......................... 51

Figure 16 : Ultraviolet absorption spectra of 2-nitraminothiazoline-4-carboxylic acid, acid range..................... 53

Figure 17 : Ultraviolet absorption spectra of 2-nitraminothiazoline-4-carboxylic acid, alkaline range............... 53

Figure 18 : Titration curve: 2-nitraminothiazoline-4-carboxylic acid.................. 55

Figure 19 : Irreversible change with time, pH 9.9, 37°C, in
2-nitraminothiazoline-4-carboxylic acid........................................ 55

Figure 20 : Electrophoresis of 2-nitraminothiazoline-4-carboxylic acid........ 56

Figure 21 : Absorption spectra of authentic N-methyl-N'-nitroguanidine........ 58

Figure 22 : Absorption spectra of MNNG -- cysteine minor product.............. 58

Figure 23 : Separation of MNNG--cysteine products by paper
chromatography.............................................................. 59

Figure 24 : The effect of pH on the reaction of MNNG and cysteine.............. 61

Figure 25 : The mechanisms of reaction of MNNG with cysteine.................... 62

Figure 26 : The reaction of MNNG with GSH in vitro: rate of thiol oxidation............................................. 64
LIST OF FIGURES (continued)

Section 3 : The mechanism of reaction of MNTS with glutathione

Figure 27 : Absorption spectra: reaction mixtures of MNTS in acid, alkali, or GSH solution............................................. 66
Figure 28 : Change of absorption spectra with time: reaction mixtures of MNTS and GSH....................................................... 66
Figure 29 : Change in concentration of components of a MNTS -- GSH reaction mixture......................................................... 68

Section 4 : In vitro methylation of DNA

Figure 30 : The effect of N-acetylcysteine on methylation of DNA by 14C-methyl MNNG................................................................. 70
Figure 31 : Competition of thiol in the methylation of DNA by MMS or DMS.............................................................................. 70

Chapter II : The reactions of methyl-nitroso compounds in vivo.

Section 1 : The growth characteristics of the tissue cultures used.

Figure 32 : The growth cycle of L cells............................................. 97
Figure 33 : The acid soluble thiol content of L cells.......................... 97
Figure 34 : The percentage of L cells in S-phase............................... 97
Figure 35 : Size distributions of L cells........................................... 97
Figure 36 : Factors influencing stationary phase in suspension culture. 99
LIST OF FIGURES (continued)

Figure 37 : The growth of HE cells in vitro. .......................................................... 101
Figure 38 : The growth and acid soluble thiol content of HE cultures........ 101
Figure 39 : The growth of HE cells and the percentage of cells synthesizing
DNA. .................................................................................................................. 103
Figure 40 : The rate of incorporation of $^3$H-TdR by HE cells................. 101

Section 2 : Reactions with thiol in vivo.

Figure 41 : The effect of MNNG on the acid soluble thiol content of
L cells. .............................................................................................................. 105
Figure 42 : The effect of DMS on the acid soluble thiol content of
L cells. .............................................................................................................. 105
Figure 43 : Separation of IAM complexes of normal cell thiols without
DTT reduction. ............................................................................................... 107
Figure 44 : Separation of IAM complexes of normal cell thiols and
disulphides after DTT reduction. ................................................................. 107

Section 3 : Methylation by MNNG in vivo.

Figure 45 : The incorporation of $^{14}$C-MNNG by L cells......................... 109
Figure 46 : The incorporation of $^{14}$C-MNNG by CHE cells.................... 109
Figure 47 : The binding of $^{14}$C-MNNG to acid insoluble cell fractions, of
various cell types. ............................................................................................ 111
Figure 48 : The methylation of DNA, RNA, and protein by $^{14}$C-MNNG in HE cells
Figure 49 : The methylation of DNA, RNA, and protein by $^{14}$C-MNNG in L cells
Figure 50 : The persistence of MNNG in cells and medium....................... 113
LIST OF FIGURES (continued)

Section 4 : Methylation by DMS in vivo.

Figure 51 : The effects of a non-toxic dose of DMS on L cells........... 115
Figure 52 : The binding of $^{14}$C-methyl DMS to acid insoluble cell
frations of various cell types................................. 117
Figure 53 : The relative extent of methylation of DNA, RNA, and protein
by DMS and MNNG.................................................. 117

Section 5 : Methylation by MNUA or MNTS in vivo.

Figure 54 : The percentage of $^{14}$C-methyl from MNNG bound to the
TCA precipitate...................................................... 119
Figure 55 : The percentage of $^{14}$C-methyl from DMS bound to the TCA
precipitate.......................................................... 119
Figure 56 : The percentage of $^{14}$C-methyl from MNUA incorporated into
acid soluble or precipitable cell fractions..................... 119
Figure 57 : The percentage of $^{14}$C-methyl from MNTS incorporated into
acid soluble or precipitable cell fractions..................... 119

The author regrets that due to revisions during typing figures 58 to
65 inclusive no longer exist.
Chapter III: The toxicity of methyl-nitroso compounds

Section 1: Factors affecting sensitivity

Figure 66: The effect of culture technique on toxicity of MNNG.... 133
Figure 67: The effect of time of exposure to MNNG (3 ug/ml) on suspended or attached L cells......................... 133
Figure 68: Growth of L cells........................................... 135
Figure 69: Variations in toxicity of MNNG with population density and culture technique................................. 135
Figure 70: Sensitivity of HE cells to MNNG......................... 137
Figure 71: Variations in toxicity of MNNG and thiol content of L cells throughout the growth cycle......................... 139
Figure 72: Variations in toxicity of MNNG and thiol content of L cells around the cell cycle................................. 141
Figure 73: The effect of population density of toxicity of DMS.... 143
Figure 74: The sensitivity of HE cells to DMS....................... 143
Figure 75: The toxicity of MNUA......................................... 145
Figure 76: The toxicity of MNTS........................................ 145
Figure 77: Dithiothreitol protection from MNNG.................... 147
LIST OF FIGURES (continued)

Section 2: The independence of toxicity of methyl-nitroso compounds and extent of methylation.

Figure 78: Toxicity related to incorporation into the TCA precipitate...... 149
Figure 79: Toxicity related to methylation of DNA......................... 149

Chapter IV: The role of methyl-nitroso compounds in the induction of genetic change.

Section 1: The sites of methylation of DNA by MNNA, MNNG, MNTS, and DMS in vitro and in vivo.

Figure 80: Absorption spectra of $\text{6}^6$-methylguanine......................... 174
Figure 81: Absorption spectra of $6$-ethoxy-2-aminopurine and 6-chloro-2-aminopurine.................................................. 176
Figure 82: Hydrolysis of $\text{6}^6$-methylguanosine........................................... 176
Figure 83: Demethylation of $\text{6}^6$-methylguanine........................................... 178
Figure 84: Absorption spectra of synthetic $\text{6}^6$-methylguanine and the product isolated after methylation of DNA............................................. 180
Figure 85: Chromatography of methylated purines from DNA treated with MNNG 182
Figure 86: Chromatography of methylated purines from DNA treated with MNNG 182
Figure 87: Chromatography of methylated purines from DNA treated with MNNG 184
Figure 88: Chromatography of methylated purines from denatured DNA treated with MNNG.......................................................... 186
Figure 89: Chromatography of methylated deoxynucleosides from DNA treated with MNNG......................................................... 188
LIST OF FIGURES (continued)

Figure 90: Chromatography of methylated purines from DNA treated with DMS ................................................................. 190

Figure 91: Dowex column chromatography of hydrolysates of DNA treated with MNNG ......................................................... 192

Figure 92: Dowex column chromatography of hydrolysates of DNA treated with DMS .......................................................... 192

Figure 93: Chromatography of methylated purines from the DNA of L cells treated with MNNG in vivo ...................................... 194

Figure 94: Chromatography of methylated purines from the DNA of L cells treated with MNNG or DMS in vivo ............................. 194

Figure 95: Chromatography of methylated purines from the DNA of L cells treated with MNUA in vivo ........................................ 196

Figure 96: Chromatography of methylated purines from the DNA of L cells treated with MNTS in vivo ....................................... 198

Section 2: A biological effect of $\text{O}^6$-methylguanine.

Figure 97: The growth of hamster embryo cells in tissue culture .......... 200

Figure 98: The colony forming ability of hamster embryo cells ............ 202

Section 3: The incorporation of 2-amino-6-methoxypurine ($\text{O}^6$-methylguanine) by L cells.

Figure 99: Chromatography of $^3\text{H}$-$\text{O}^6$-methylguanine .................. 204
LIST OF FIGURES (continued)

Figure 100: Chromatography of medium, TCA supernatant of cells, and hydrolysates of DNA and RNA, following treatment of L cells with $^3$H-0$^6$-methylguanine in vivo for 10 minutes........... 206

Figure 101: Chromatography of medium, TCA supernatant of cells, DNA hydrolysates, and RNA hydrolysates after treatment of L cells with $^3$H-0$^6$-methylguanine in vivo for 120 minutes.......... 208

Figure 102: Chromatography of $^3$H-0$^6$-methylguanine in various solvent systems.............................................................. 210
| Table 1 | Characteristics of absorption of MNNG and related compounds... | 31 |
| Table 2 | Factors altering the rate of decomposition of MNNG and the proportions of products | 32 |
| Table 3 | The DNA content of L cells | 80 |
| Table 4 | The DNA content of HE cells | 82 |
| Table 5 | Thiol content of L cells after reaction with methyl-nitroso compounds \textit{in vivo} | 85 |
| Table 6 | Extent of methylation of cellular constituents of cultured mammalian cells by MNUA or MNITS | 94 |
| Table 7 | Spectral properties of guanine derivatives | 157 |
| Table 8 | Products from methylation of DNA by $^{14}\text{C-MNNG}$ or $^{14}\text{C-DMS}$ \textit{in vitro} | 160 |
| Table 9 | Products from methylation of DNA in L cells treated \textit{in vivo} with $^{14}\text{C-MNNG}$, $^{14}\text{C-DMS}$, or $^{14}\text{C-MNUA}$ | 162 |
| Table 10 | The incorporation of $^3\text{H-}O^6$-methylguanine by L cells | 169 |
INTRODUCTION

The three compounds MNUA, MNNG, and MNTS* have in common the \(N\)-methyl-\(N\)-nitroso group, a source of the carcinogen and alkylating agent diazomethane. McKay (1950) demonstrated that MNNG is an efficient laboratory reagent for diazomethane; Garrett (1965) discussed the mechanism by which MNUA gives rise to diazomethane; and McCalla (1968) found that MNTS methylates DNA in vitro with a pH dependence similar to other diazomethane precursors. It has been postulated that diazomethane and related alkylating mutagens and carcinogens function by reacting directly with the bases of nucleic acid (Brookes and Lawley, 1964). More recently, evidence has accumulated which suggests that some alkylating agents (the diazomethane precursors were not included) first attack the phosphate esters of nucleic acid, and that modification of the bases results from secondary transalkylation (Alexander, 1969). By either direct- or trans-alkylation, modification of nucleic acid bases could induce heritable changes by altering coding properties, or promoting depurination and chain scission.

\[
\begin{align*}
\text{MNUA} & \quad \text{MNNG} & \quad \text{MNTS} \\
\text{CH}_3-N-C-NH_2 & \quad \text{CH}_3-N-C-NHNO_2 & \quad \text{CH}_3-N-S-C-\text{CH}_3 \\
\end{align*}
\]

A fourth methylating agent, DMS, was also used:

\[
\text{CH}_3-O-S-O-\text{CH}_3
\]
However, the postulate that base alkylation is involved in the induction of genetic changes has recently been criticized, since not all carcinogens have been shown to alkylate DNA (Lijinsky, 1969) and since agents yielding the same alkylated product (7-alkylguanine) are not all mutagenic (Loveless, 1969). Alternative postulates are that methylation of the phosphate of the polynucleotide backbone may be more important than methylation of the bases (Mizrahi, 1964); that carcinogenesis may require a synergistic action by polyfunctional agents affecting protein thiol and nucleic acid simultaneously (Schoental, 1966; Harington, 1967); and that quantitatively minor alkylation products in DNA can be carcinogenic (Loveless, 1969; Lawley and Thatcher, 1970). Clearly, detailed knowledge of the reactions of the methyl-nitroso compounds with cells is required if the basis of the carcinogenic action of these compounds is to be understood.

Three \( N\)-methyl-\( N\)-nitroso compounds which differ considerably in biological effect were selected for this study. Of the three, MNUA is a potent carcinogen (Druckrey, 1966) although Frei (1970) finds it less toxic than methylnitrosourethane, or MNTS, in mice. MNNG is "the most potent known bacterial mutagen" (Magee, 1967). It is also a carcinogen (Schoental, 1966), but unlike MNUA it is also extremely toxic, both in the whole animal and in tissue cultures (this thesis). With MNTS, however, the situation is reversed, and although highly toxic, MNTS is hardly carcinogenic or mutagenic at all (Druckrey, 1967; Marquardt, 1964; McCalla, 1966; Frei, 1970). It seems, therefore, that extent of toxicity and genetic effects are inversely related. In the series MNTS, MNNG, MNUA, carcinogenicity increases in this order, but
toxicity decreases. It is hoped that study of the chemical and biological effects of these three chemically related, yet biologically distinct compounds will provide insight into the events which initiate the various cellular changes.

Mechanism of activation of the methylating species:

MNNG and MNTS have marked stability in aqueous solution, yet DNA is rapidly methylated by these agents in vivo. Treatment of Euglena with MNTS gives rise to small amounts of 7-methylguanine (Allan, 1967), and the present work demonstrates methylation of DNA in mammalian cells treated in vivo with MNNG. Some feature of the intracellular condition must therefore catalyse decomposition of the parent compound, even at neutral pH. That this activation is chemical rather than enzymatic was first suggested when Schoental (1967) demonstrated that tissue thiol catalyse nitrosomethylurethane decomposition. However, although methylation of DNA in vitro is increased by the addition of thiol if MNNG is the methyl source, the presence of thiol inhibits methylation by MNTS (McCalla, 1968). The need to study the involvement of cellular thiol in the in vivo activation of the nitroso compounds is strongly indicated, and the first part of this study is devoted to the exposition of the chemical mechanisms leading to the production of an active methylating species.

The nature of the methylated products in nucleic acid:

The second approach to the elucidation of the reaction mechanism of the selected compounds is through study of the sites in nucleic acid
modified by each nitrosomethyl agent. Lawley (1964) proposed that genetic changes could result from methylation of the 7-nitrogen of guanine, since the acidity at the $N\text{-}1$ atom would be increased, promoting ionization at physiological pH. As a result, 7-methylguanine could mispair with thymine. An alternative mechanism proposed by Nagata et al. (1963) suggests that after methylation at $N\text{-}7$ of guanine, proton transfers create the anomalous tautomers of both guanine and cytosine, causing mispairing in both DNA strands. Such theoretical mechanisms are consistent with the observed mutations induced by MNNG in bacteria (Baker and Tessman, 1968). In addition, methylation promotes depurination, and could lead to the GC deletions observed by Oeschger and Hartman (1970).

On the other hand, MNUA is highly mutagenic in bacteriophage T2, while MMS is inactive, although the 7-$N$ of guanine is the major extensively methylated site with both agents (Loveless and Hampton, 1969). The mechanism by which methylating agents alter genetic properties can therefore not be based solely on the alkylation of guanine at the 7-$N$ position.

Friedman (1965) demonstrated that diazomethane (but not DMS or the nitrogen mustards) methylates the 6-oxygen atom of guanosine 

\textit{in vitro} \textit{in addition to the 7-nitrogen position}. Further, a correlation between agents with the theoretical capacity for $O$-methylation and agents with carcinogenic potency has been enunciated by Loveless (1969). This alkylation of an oxygen atom rather than a nitrogen atom can be invoked to explain the difference between MNUA and MMS as mutagens and carcinogens. In theory, whereas MMS would form a transition complex with a nucleophilic group in DNA, and react almost exclusively by
Ingold's (1953) SN2 mechanism at strongly nucleophilic sites such as \(N-7\), the alkylnitrosamines or alkylnitrosamidines could react either as diazoalkyl cations by the SN2 mechanism, or as carbonium ions by the SN1 mechanism. Since the SN1 reagents attack nucleophilic groups at random, independent of their relative nucleophilicity, agents with SN1 character would be expected to alkylate weakly nucleophilic sites, untouched by SN2 agents, such as the \(O-6\) atom of guanine. Methylation at \(O-6\) would promote mispairing of guanine with thymine even more strongly than would methylation at \(N-7\). It is therefore of interest to compare the DNA-derived products after alkylation (in vitro and in vivo) by a simple non-carcinogenic SN2 agent (DMS), and after alkylation by other reagents having some SN1 character (MNNG and MNUA). If the theoretical considerations implicating \(O\)-methylation in carcinogenesis are valid, \(O\)-methyl products should be found in greater abundance after reaction with the carcinogenic agents.

The relationship between chemical events and biological effects:

The third aim of this study is to correlate chemical events with biological effects. Although carcinogenic, mutagenic, and toxic events are often initiated by a single chemical agent, there is evidence to suggest that, for nitroso-alkyl agents, these biological endpoints result from different reactions within the cells. In the series MNUA, MNNG and MNTS, toxicity increases in this order, while carcinogenicity decreases. In addition, there is no correlation between extent of methylation of DNA and toxicity (present results) and one is led to suggest that the alkyl-nitrosamide or alkylnitrosamide exerts at least two chemical effects - one toxic and one carcinogenic.
Rosenkranz and Carr (1970) provide support for the view that even closely related nitroso-alkyl agents interact differently in vivo, since they find no cross-resistance of the various strains used to MNUA and methylnitrosourethane.

A third type of evidence supporting polyfunctional action by MNNG is provided by Cerda-Olmedo and Hanawalt (1968) who show that the lethal action, but not the mutagenic action on E. Coli is subject to repair.

It seems reasonable, therefore, to anticipate more than one mode of action for nitroso-alkyl agents. Chemical studies in this laboratory (Lawley and Thatcher, 1970) and the work of Schulz and McCalla (1969) define at least two activities for nitroso-methyl compounds: (1) methylation following nucleophilic attack and release of diazomethane, and (2) thiol oxidation following denitrosation, both of which occur in neutral aqueous medium. In an attempt to separate toxic from carcinogenic events, the present work delineates correlations between (1) extent of methylation, (2) interaction with thiol, and (3) toxicity, of the selected alkyl-nitroso compounds.

Finally, an attempt is made to associate the qualitative difference in DMS and MNNG alkylation sites with carcinogenic events. Theoretical considerations implicate methylation at the 6-oxygen atom of guanine in carcinogenesis, and this postulated methylation product does occur following treatment of mammalian cells with MNUA and MNNG, but not DMS or MNTS (Lawley and Thatcher, 1970, and present results). However, evaluation of the importance of 0-6 methylation is complicated by the simultaneous production of a wide range of other effects by these agents. To avoid this ambiguity, a study of
the incorporation of O-6 methylguanine, and its effects on growth, colony formation, and transformation of tissue cultures was undertaken. The results indicate that this unusual base is incorporated into nucleic acid in vivo. Further, it induces an increase in plating efficiency and in life span of hamster embryo cells in tissue culture, as well as several morphological changes previously reported in cells transformed with dimethylnitrosamine (Huberman et al., 1968).

Studies of the relationship between chemical activities and biological effects of the selected methyl-nitroso compounds suggest that interaction with cellular thiol markedly increases the toxicity of an agent, while the ability to methylate the O-6 position of guanine is strongly implicated in carcinogenic potency.
MATERIALS AND METHODS

Radioactive Compounds:

$^{14}$C-methyl-$N'$-nitro-$N$-nitrosoguanidine (0.3 mCi/mmole) was prepared from $^{14}$C-methylamine (The Radiochemical Centre, Amersham, Bucks, U.K.) by a modification of the methods of McKay & Wright (1947) and McKay (1950). To a small flask of ice containing 0.125 ml 40% aqueous $^{14}$C-methylamine, 0.6 ml water, and 2.0 ml ether, crystalline MNNG (210 mg) was slowly added. The white precipitate was evaporated to dryness, and then dissolved in 0.15 ml conc HNO$_3$ and 0.5 ml water. (A small amount of the white intermediate $^{14}$C-methyl-$N$-nitroguanidine was removed for use as a marker.) After chilling on ice, 0.075 g NaN$_3$ in 0.12 ml water were added. The yellow precipitate was washed with ice-cold water until the filtrate was neutral. The product was desiccated.

$^{14}$C-methyl-$N$-nitrosourea (1.01 mCi/mmole) was kindly supplied by Dr. J. V. Frei, and $^{14}$C-methyl-$N$-nitrosotoluenesulphonamide (1.08 mCi/mmole) was purchased from Amersham-Searle, Des Plains, Illinois, USA. Tritiated $^6$-methylguanine ($^6$-MG, 5.9 mCi/mg) was prepared by New England Nuclear Corporation, 575 Albany Street, Boston, Mass., USA 02118, as follows: 20 mg of $^6$-MG prepared by the author were dissolved in 0.2 ml glacial acetic acid containing 10 curies of tritiated water and 25 mg pre-reduced platinum catalyst. The reaction mixture was heated to 50°C overnight. The labile tritium was removed with 10 ml water twice, and the solvent was removed by vacuum distillation. The labelled compound was dissolved in water and refrigerated. Prior to experimental use, an aliquot was chromatographed in solvent (6) and the material at $R_f$ 0.62 was eluted into 3 ml water. This eluate was the starting material for all experiments.
$^{14}\text{C}$-Methylmethanesulphonate (45.2 mCi/mmol) was obtained from the Radiochemical Centre, and di-$^{14}\text{C}$-methyl sulphate was supplied by the New England Nuclear Corporation, Boston, Mass., USA. Both were stored in ether solution at $-20^\circ\text{C}$.

Assay of Radioactivity:

A Nuclear Chicago 720 or a Beckman LS 150 liquid scintillation counter was used. Initially, for assay of radioactivity of solutions, 0.1–0.2 ml in 10 ml of the following phosphor was used: naphthalene (100 g), 2,5-diphenyloxazole (PPO, 4 g), 1,4-bis-(5-phenyloxazol-2-yl)-benzene (POPOP, 100 mg), methanol (100 ml), and dioxane to 1 litre. Paper chromatograms were cut into sequential 1 x 4 cm wide strips which were placed in vials with 0.5 ml of the following phosphor: PPO (4 g), POPOP (50 mg), in 1 litre toluene. For later experiments, a third phosphor was substituted for use with solutions or paper (10 ml/vial). It contained PPO (5 g), naphthalene (100 g), and dioxane (900 ml). Efficiency was about 75% for $^{14}\text{C}$ and 25% for $^3\text{H}$ as determined with internal standards in toluene.

DNA (about 1 mg) was dissolved in 0.3 ml 5% TCA at 100$^\circ\text{C}$ and RNA was dissolved in water. Nucleic acid concentration was established from $A_{260}$ of a 1/100 dilution, assuming $A_{260} = 26$ O.D. units at 1 mg DNA/ml. Protein samples or TCA precipitates of cells were dissolved at 4 mg/ml in tetraethylammonium hydroxide (TEAH).

Special Chemicals:

The 2-amino-6-methoxypurine ($O^6$-methylguanine, $O^6$-MG) was prepared as described by Balsiger and Montgomery (1960). Sodium metal (550 mg) was broken into small, clean pieces under ether, and then transferred to a 50 ml flask under a reflux condenser. Cold methanol
(20 ml) was added through the top of the condenser which was then covered with a tube of loosely packed drierite. When the sodium had reacted completely 500 mg 6-chloro-2-aminopurine were added to the flask. After refluxing 18 hours the product was cooled to room temperature, neutralized with 1.2 ml glacial acetic acid, and evaporated to dryness. The white residue was dissolved by slowly heating with 10 ml water to 80°C. The crystals obtained after cooling the filtrate to 0°C were collected in a Buchner funnel and sucked dry. This $6^6$-MG product was stored in a desiccator. After one such preparation ultraviolet spectroscopy revealed a contaminant of the parent 6-chloro-2-aminopurine which was removed by a second recrystallization from water.

MNUA and MNNG were purchased from K&K Laboratories, Plainview, N.Y., USA. MNTS was obtained from Eastman Organic Chemicals, Rochester, N.Y., USA. $6^6$-Methyldeoxyguanosine and marker bases 7-methylguanine, 3-methyladenine, and 3-methylcytosine were supplied by Dr. P. D. Lawley. Mann Research Laboratories, New York, N.Y., USA, supplied salmon sperm DNA.

Paper chromatography:

Whatman 3 MM paper was used. The solvents were: (1) propan-2-ol: conc. HCl: water (170:41:39); (2) butan-1-ol: acetic acid: water (2:1:1); (3) butan-1-ol: aqueous NH$_3$ (sp.gr.0.88): water (43:2:5); (4) propan-1-ol: water (7:3); (5) butan-1-ol: water (43:7); (6) propan-2-ol: aqueous NH$_3$ (sp.gr.0.88): water (7:1:2). Ultraviolet-absorbing spots were detected with light at 254 nm, and amino groups were detected after dipping in 0.25% ninhydrin solution in 95% ethanol and drying.
Rate of decomposition of MNUA, MNNG, and MNTS:

Decomposition of MNNG and MNUA was measured as loss of absorption in the visible range due to the nitroso group. One-tenth volume of the stock solution (50-100 mM in methanol) was added to the other components of the reaction mixtures in a thermostatically controlled cell housing (Gilford or Cary 15 spectrophotometer) and absorption at 390 nm, 395 nm, or from 460 to 340 nm was recorded at intervals. Since the low solubility of MNTS did not permit study of this compound in the visible range, decomposition of 0.1 mM or 0.2 mM solutions in 10% methanol (and for comparison, MNNG and MNUA) in the presence of buffer or catalyst was followed as loss of absorption in the ultraviolet range (350-220 nm). Decomposition under the following conditions was examined: (1) 37°C and pH 2 in glycine-HCl-glycine, 200 mM; pH 4 in acetic acid-Na acetate, 100 mM; pH 5.4 in acetic acid-Na acetate, 200 mM; pH 7 in KH$_2$PO$_4$-Na$_2$HPO$_4$, 100-200 mM; pH 8 in tris-HCl, 200 mM; pH 10 in NaHCO$_3$-Na$_2$CO$_3$, 200 mM; or as stated in the text; (2) 0.1 N HCl or 0.1 N NaOH; (3) neutral aqueous solutions (equimolar unless otherwise stated) of sodium thiosulphate, cysteine, N-acetylcysteine, GSH, glycine, histidine, and glutamic acid; (4) growth medium CMRL 1066 with or without serum (Parker, 1961).

Reaction of MNNG with cysteine:

Components of the reaction mixture at 37°C were combined in the following order: 3.0 ml neutralized 2 M cysteine; 24 ml 35 mM Na$_2$HPO$_4$-30 mM NaH$_2$PO$_4$, pH 7.0; 3.0 ml 2 M-MNNG in NN-dimethylformamide. After 1 hour 0.01 ml aliquots were applied to 3 MM paper and chromatographed in solvent (5). After standing overnight at 24°C, the white precipitate was removed on high-retention filter paper in a Buchner funnel. The precipitate was washed with cold water and dried in a desiccator. A
small amount was dissolved in N HCl for chromatography in solvents (4) and (5). The infrared spectra of the dried crystalline product were studied in Nujol mull by the Department of Chemistry, UWO.

The filtrate was applied to a Dowex 50- H\(^+\) form column (30 cm x 4 cm) and eluted with water to yield 160 fractions of 10 ml. The ultraviolet absorption at 280 nm and 260 nm of each fraction was determined, and revealed two absorbing peaks. The first, consisting of fractions 58-65, was evaporated to dryness and recrystallized from cold water. The yield was 280 mg of blue crystals with m.p. 190° C (decomp.).

Elementary analysis was performed by A. G. Gygli, 329 St. George St., Toronto 5, Ontario, Canada. Absorption spectra of aqueous solutions (0.1 mM) at various pH were recorded and e calculated. Values of pK were calculated from \(A_{330}\) of 100 μg/ml solutions of different pH,

\[
pK_a = \text{pH} - \log \frac{A - A_{\text{acid}}}{A_{\text{base}} - A}
\]

Electrophoresis was performed on 17 cm x 2.5 cm Whatman 3MM paper in 0.05 I glycine-HCl pH 2.4, 0.05 M sodium phosphate, pH 6, and 0.05 I Na\(_2\)CO\(_3\) -NaHCO\(_3\), pH 11.9, with a current of 2 mA for 0.5 hours. The thiazoline derivative was applied as a hair line of saturated water solution at the centre of the paper, and its ultimate position was detected by its absorption under the ultraviolet lamp.

The second chromatographic peak consisting of fractions 120-143 was evaporated to dryness and recrystallized from propan-2-ol:ether. Aliquots of a water solution (7.4 μg/ml) were adjusted to various pH values for spectral analysis in a Cary 15 recording spectrophotometer. \(R_f\) in solvent (5) was determined.
Reaction of MNNG with GSH:

Aliquots of reaction mixtures of MNNG and GSH (7 mM each) in 100 mM sodium phosphate pH 7.4 were chromatographed in duplicate in solvent (5). Ultraviolet-absorbing spots were eluted into 5 ml water for spectral analysis. Duplicates were dipped in ninhydrin to detect amino groups.

Reaction of MNTS in acid, alkali, or GSH:

To enable detection of small amounts of product after reaction with the relatively insoluble compound MNTS, solutions of $^{14}\text{C}\text{-MNTS}$ were prepared by addition of 0.01 ml of 8.25 mM $^{14}\text{C}\text{-MNTS}$, (1.08 mCi/mmol) to 1.0 ml of 2.0 mM MNTS in methanol. The $^{14}\text{C}\text{-MNTS}$ (0.3 ml of 2 mM solution in methanol) was added to 2.4 ml water and 0.3 ml N HCl or 0.3 ml N NaOH in a spectrophotometer cell. The spectrum was scanned at intervals, and after completion of the reaction 0.2 ml was applied to paper and chromatographed in solvent (4). Markers of $^{14}\text{C}\text{-MTS}$ and MTS were chromatographed in parallel. In some reaction mixtures equimolar GSH was added in the buffer.

Thiol measurement:

For measurement of thiol loss during in vitro reaction with MNNG or MNTS, 0.5 ml reaction mixture (0.6 to 6.0 mM in thiol originally) were removed at intervals and added to 2.5 ml 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (1 mM, in 200 mM phosphate buffer pH 7.8) at 37°C, and thiol concentration was calculated from the O.D. at 410 nm assuming $\varepsilon = 13,000$ (Ellman 1959).

Total reduced + oxidized thiol in reaction mixtures was estimated after addition of sodium borohydride (10 mg) to 0.5 ml reaction mixture.
The tube was sealed with parafilm for 30 minutes at 37°C. Excess borohydride was then removed with 0.2 ml 5 N HCl. The solution was adjusted to pH 7.8 with 3 N NaOH (about 0.3 ml) and flushed with nitrogen. Thiol in 0.5 ml was estimated after addition of 2.5 ml DTNB solution (Cavallini, Graziani, and Dupré, 1966).

To measure acid soluble cellular thiol at least 2 x 10⁷ cells were collected by centrifugation, washed in cold PBS, suspended in 0.7 ml ice-cold 5% TCA for 20 minutes, and centrifuged at 2500 rpm for 5 minutes. Thiol was estimated as above with 0.5 ml TCA-soluble cell extract substituted for reaction mixture.

Cellular thiol and disulphide were also measured by complexing with ¹⁴C-IAM as described by Walker and Smith (1969). Typical reaction mixtures contained 0.25 ml water (blank) or 0.25 ml 2 mM GSH or GSSG (standards), or 0.25 ml cell extract; 0.05 ml THAM buffer 0.3 M, pH 8; 0.05 ml dithiothreitol, 20 mM (for oxidized plus reduced thiol) or 0.05 ml water (for reduced thiol only); and 0.15 ml ¹⁴C-IAM, 50 mM, sp.act.0.1 mCi/μmol. After 20 minutes at 24°C, 20 μl reaction mixture were spotted on Whatman 3MM paper and chromatographed 16 hours in solvent (4). Papers were cut into 1 cm strips and radioactivity assayed in liquid phosphor. Standard GSH reaction mixtures containing 0.005 μmoles SH/paper gave 500 cpm in the GSH peak.

In the above preparation of TCA cell extracts, any mixed disulphides formed between small, soluble molecules and protein-bound SH groups formed during reaction in vivo, were discarded with the precipitate. To avoid this thiol loss, the following modification was adopted: PBS-washed cells were lysed in 0.6 ml 4 M urea, and 0.25 ml urea lysate were substituted for cell extract in the ¹⁴C-IAM complexing reaction. After reduction and complexing of disulphides was complete, the reaction
mixture was acidified with 0.1 volume 50% TCA and cleared by centrifugation. The supernatant was chromatographed as before. The precipitate was washed in ice-cold 5% TCA, ethanol, and ether, dried at 37°C, and dissolved in 0.25 ml TEAH. Radioactivity in 0.1 ml was assayed in liquid phosphor.

The in vitro methylation of DNA:

In a typical experiment salmon sperm DNA dissolved at 500 µg/ml in 2.5 mM sodium acetate was used directly, or was first denatured by heating at 100°C for 10 minutes followed by rapid cooling in ice. Temperature was restored to 37°C before reaction. DNA solution (4.0 ml) was buffered with 0.4 ml 500 mM sodium phosphate pH 7.4. (In one experiment tris-HCl, 100 mM pH 7.4 was substituted.) When desired, thioles such as N-acetylcysteine or GSH were added to the solution in crystalline form to yield a thiol concentration of about 10 mM. ¹⁴C-MNNG (1-2 mg) was added in 0.1 ml methanol. Dose (1.25-3.5 mM) was calculated from the radioactivity in 0.1 ml reaction mixture, after mixing. After 20 minutes (or as indicated) at 37°C, DNA was precipitated with 1.5 volumes ethoxyethanol, and was then washed with ethanol and ether. Radioactivity was assayed as described previously.

To prove that no radioactivity from the remaining reagent was occluded with the DNA, ¹⁴C-MNNG and GSH (1 mM of each) were first reacted in buffer for 40 minutes at 37°C before addition of the DNA. After precipitation as described above, no radioactivity was detectible in the DNA.

Hydrolysis of DNA or RNA:

Several mg of DNA previously treated with the radioactive agent were dissolved in 0.5 to 1.0 ml water, and the pH was adjusted to 7.0 or
7.4 with 1/10 volume of 0.01 M sodium phosphate buffer. After heating at 100°C for 20 minutes the solution was acidified with 1/10 volume N HCl, centrifuged, and the supernatant chromatographed in solvent (1), (3), or (6). This "neutral" supernatant contained a large proportion of the 7-methylguanine and 3-methyladenine and a small proportion of the O₆-methylguanine. The precipitate was further hydrolyzed overnight in 0.3 ml of 0.1 N HCl at 37°C prior to chromatography with markers (~50 µg) of 7-methylguanine, 3-methyladenine, guanine, adenine or O₆-methylguanine. This dilute acid hydrolysis realeased the balance of the 7-methylguanine and 3-methyladenine, most of the O₆-methylguanine, and the normal purines adenine and guanine. The preliminary removal of the bulk of the 7-methylguanine and 3-methyladenine allowed improved separation of the minor product O₆-methylguanaine from the neighbouring 3-methyladenine. Alternatively, the DNA solution could be hydrolyzed directly in dilute acid (0.1 N HCl) for 16 hours at 37°C as was the RNA. This procedure released all the methylated and normal purines together.

For complete hydrolysis of DNA to its constituent bases, 15 µl of aqueous 72% (w/v) HClO₄ was added for each ml of aqueous DNA solution (2-4 mg DNA/ml). The tube was sealed and heated at 100°C for one hour. The hydrolysate was diluted with 3 volumes of water, and centrifuged to remove charred sugars prior to chromatography.

DNA was enzymatically hydrolyzed by the method of Hall (1967), employing spleen DNAase, snake venom phosphodiesterase, and alkaline phosphatase, to yield nucleosides.

Dowex chromatography of hydrolysates:

The cleared perchloric acid hydrolysate was added to a Dowex 50 column, 13 x 1 cm, H + form and eluted with a gradient from 0.75 to 2.0
N HCl. Fractions of 10 ml were collected, ultraviolet absorption spectra were recorded, and 0.1 ml of each fraction was assayed for radioactivity.

Tissue culture: A. L cells.

L-60-T cells (Till et al., 1963), a subline of Earle's L-cells (1943-1944) were grown in suspension at 37°C in medium CMRL 1066 (Parker, 1961) lacking coenzyme and thymidine, and supplemented with 5% bovine serum (Grand Island Biological Co., Inc., Grand Island, New York), 10 IU/ml streptomycin sulphate (Streptolin '33', GlaxoAllenburys (Canada) Ltd., Weston, Ontario), and 100 mg/ml penicillin (Penicillin G, Eli Lilly & Co. (Canada) Ltd., Toronto). Cells were maintained between $0.5 \times 10^5$ and $5.0 \times 10^5$ cells/ml. For plating experiments, the medium was supplemented with 10% bovine serum.

Tissue culture: B. Hamster embryo cells (HE cells).

Embryos from randomly bred hamsters (High Oaks Ranch, Downsviue, Ontario) 11-13 days pregnant, were decapitated, minced, and washed in 10 ml trypsin solution for each embryo (1 vial Difco Bacto-Trypsin in 400 ml sterile 0.015 M Na citrate, 0.15 M KCl) for 10 minutes. The supernatant was discarded, and after further digestion of the tissue for 40 minutes in 10 ml trypsin solution for each embryo, clumps were filtered out through sterile gauze, and the digestion was stopped with 1.0 ml fetal calf serum for each 20 ml trypsin. Cells were then collected by centrifugation at 2000 rpm for 5 minutes, and suspended in growth medium 1066 supplemented with 10 µg of each of guanosine, uridine, cytidine, and adenosine; 100 IU penicillin; 0.1 ml streptomycin sulphate; and 12.5 units Mycostatin, (E. R. Squibb & Sons Inc., New York) per ml medium; and 10% by volume fetal calf serum (Highland Div. Travenol
Laboratories Inc., Los Angeles, California). The cells in the resulting suspension were termed "primary." To establish secondary cultures at least $10^7$ primary cells were seeded in an 800 ml glass bottle with 50 ml medium. After 3-4 days the confluent monolayer of cells was resuspended with 10 ml trypsin solution, and then cultured on glass a second time, with an initial inoculum of at least $10^6$ cells. The resulting monolayer was suspended to provide "secondary" cells.

Estimation of the probability of survival of single cells by colony assay (Puck, Marcus, and Cieciura, 1956):

L cells were treated in suspension under normal growing conditions, or, where indicated, after attachment to plates. H.E. cells were removed from the glass with trypsin, washed, and suspended in growth medium prior to treatment. Alkylating agents were added as methanol solutions, while guanine derivatives were added as aqueous solutions. The volume of drug solution added was no more than 1/100 of the volume of the cell culture. After adding drug, cell cultures were rolled at $37^\circ$C for 40 minutes.

L cells were treated in the medium in which they were grown without changing the population density. After treatment the cells were collected by centrifugation and resuspended in growth medium, and a cell count was made. The cultures were then diluted and plated in quadruplicate by adding 1.0 ml cell suspension to Petri dishes containing 8 ml plating medium. Feeder cells (non-dividing but metabolizing cells) prepared by treating cells with 2 $\mu$g/ml sulphur mustard for one hour at $37^\circ$C were added to bring the total number of cells in each dish to $10^5$. The plates were incubated at $37^\circ$C in 5% $\text{CO}_2$ until colonies became visible to the eye (about 7 days for controls, and up to 14 days for treated
cultures). The medium was then decanted, and the plates rinsed with PBS. After 20 minutes in ethanol, the plates were stained for 10 minutes with 0.5% aqueous methylene blue.

Time of exposure of suspension cultures was limited by centrifugation of treated cultures, followed by resuspension in fresh medium. For treatment of attached cells, the plates were prepared with medium and the appropriate number of cells and feeders, and allowed 1–3 hours for cells to adhere. The test agents (1/100 volume) were then added to the medium in the plates. Time of exposure was limited by replacing the treated medium with drug-free medium.

Induction of synchrony in L cells:

DNA synthesis was inhibited in L cells by incubating suspension cultures in the presence of $10^{-7}$ M fluorodeoxyuridine (FUDR, Stanners and Till, 1960) for 14 hours, at which time 90% of the cells were in S-phase (Walker and Thatcher, 1968). DNA synthesis was reinitiated by addition to the cell suspension of 1/1000 volume of 1% thymidine. The resulting S-phase lasted about 4 hours.

Determination of cell cycle parameters:

Population density and cell size distribution were determined with a Coulter electronic cell counter.

To determine % S-phase cells, mitotic index, and rate of TdR incorporation, plated cells, seeded at $10^5$ cells/5 cm plate, were used. The plated cells were washed with PBS, and 5 ml of medium without thymidine were added. Plates were incubated 1 hour in the presence of 1.25 or 2.5 μCi/ml of $^3$H-TdR (thymidine-(methyl)-$^3$H, specific activity 3.47 Ci/mmol, Schwartz BioResearch Inc., Orangeburg, New York) and then radioactivity was diluted by adding 0.1 ml 1% TdR solution. The cells
were either autoradiographed to determine the % cells synthesizing DNA and the mitotic index, or were suspended with trypsin to determine the extent of $^3$HTdR incorporation. Plates to be autoradiographed were washed in PBS, and the cells were swollen with 1:1 PBS:water, fixed with 95% ethanol:glacial acetic acid, 3:1, and stained with orcein. The fixed plated cells were then coated with 2 ml Kodak NTB film emulsion diluted 1:1 with water. Plates were developed after 1 week and the number of labeled cells or mitotic figures in 1,000 cells was scored.

For measurement of $^3$HTdR incorporation, cells were resuspended, washed with PBS, and resuspended in cold 5% TCA. After 20 minutes acid-insoluble material was collected on a Millipore filter (0.45 μ), washed with 5% TCA and absolute ethanol, and dried. The radioactivity on the filter was then counted in 5 ml phosphor solution.

DNA content of cells was determined by the colorimetric method of Burton, 1956, on about 5 x $10^6$ cells. DNA concentration was estimated by comparison with the absorption at 600 nm of standards containing 1 to 8 μg DNAP/ml.

Rate of methylation in vivo:

Prior to reaction L cells were concentrated by centrifugation, or HE cells (figure 46) were removed from the glass with trypsin and washed. In a typical experiment 4 litres of L cells grown to 5 x $10^5$ cells/ml, were centrifuged, and resuspended to give 16 ml at 1.4 x $10^8$ cells/ml. The suspension was maintained at 37°C and agitated with a magnetic stirrer. For SH assay, 0.1 ml cell suspension was removed. About 0.6 mg $^{14}$C-MNNG in 0.2 ml methanol were added. The actual dose determined by radioactivity assay was 0.273 mM. At various times from 0.5 - 270 minutes, 0.25 ml suspension were withdrawn and added to 15 ml
ice-cold PBS. Cells were separated by centrifugation and the radioactivity in the medium was measured. The cells were suspended in 0.7 ml cold 5% TCA for 20 minutes, and the supernatant was cleared by centrifugation. Radioactivity in 2 x 0.2 ml samples of supernatant was counted. The precipitate was washed twice with cold 5% TCA, once each with ethanol and ether, and then was assayed in TEAH. Isolation of DNA, RNA, and protein, and assay of the extent of methylation of each macromolecular fraction required 1-5 ml of cell suspension.

Isolation of DNA, RNA, and protein:

A modification of the method of Kirby (1957) was used. At least $10^8$ treated cells were suspended in 10 ml p-amino-salicylic acid (5%), lysed with 1.0 ml 10% sodium dodecylsulphate, and protein was extracted with 10 ml phenol:cresol:8-OH quinoline (500 ml:50 ml:0.55 g). After separation of phases at 5000 rpm for 20 minutes, the upper layer was removed and DNA was precipitated from it with 1.5 volumes ethoxyethanol. After the DNA was dissolved in 10 ml 0.4% sodium acetate, 0.5 ml 4 M NaCl and 0.4 ml 2.5 M Na-acetate were added. The solution then was incubated at 37°C with 0.2 ml boiled RNAase (500 μg/ml) for 20 minutes. The phenol extraction, ethoxyethanol precipitation, and dissolution in salt were repeated, and the solution was cleared of carbohydrate at 35,000 rpm for 0.5 hours in a Beckman model L centrifuge. The DNA was finally precipitated with 1.5 volumes ethoxyethanol, washed in ethanol and ether, and desiccated.

RNA was precipitated from the liquid phase after precipitation of DNA by adding two volumes of ethanol. The RNA precipitate was washed twice with 70% ethanol-2% aqueous sodium acetate, and once each with ethanol and ether. Protein was precipitated from the original
phenol lower phase by addition of 80 ml methanol. The precipitate was washed twice with methanol, and once with ether, and ground to a powder under a stream of hot air. Radioactivity in the DNA, RNA, or protein was determined as described under "Assay of Radioactivity."

Relationship between toxicity and extent of methylation:

With $^{14}C$-MNNG, sp. act. 0.3 mCi/mmol, detectible radioactivity could not be introduced at doses permitting measurable survival. In order to express toxicity in terms of extent of binding to TCA precipitates or DNA, the linear relationship between dose and extent of binding was exploited. Figures 48 and 49 show that this relationship is linear, and extrapolates back to the origin. Toxicity of MNNG was measured using the necessary very low doses, and extent of binding was estimated from back-extrapolation of the available data. Survival curves relating dose of MNUA or MNTS (abscissa) to toxicity were also established with unlabelled agents, and the abscissa converted to extent methylation on the basis of the values of the binding constants "K". These constants were determined after treatment of cultures with extremely toxic doses of labelled compound. However, with MNUA and MNTS the specific activity of the corresponding labelled compound was high enough to determine both survival and extent of methylation using only one suspension of $^{14}C$-methylated cells, as described below. Directly measured and calculated points were coincident.

In a typical experiment, 2 l. of L cells grown from 0.5 to 4.2 x $10^5$ cells/ml, were centrifuged at 2000 rpm for 5 minutes, and re-suspended at 8.8 x $10^7$ cells/ml in 12 ml growth medium at 37°C. One ml of the suspension was set apart in a 5 ml sterile plastic tube and treated with 0.01 ml methanol, as control. To the remainder, $^{14}C$-MNUA
was added in 0.1 ml methanol to give a concentration of 3.05 mM. One ml of the treated suspension was also transferred to a plastic tube and all three suspensions were rolled gently for 40 minutes at 37°C. The 1.0 ml aliquots of treated and control cells were diluted and plated for colony assay. Surviving fractions averaged 0.8 for control cells, and $3 \times 10^{-4}$ for treated cells, in agreement with toxicity data obtained with unlabelled MNUA.

One ml of the treated cell suspension was centrifuged, washed in PBS, and resuspended in cold 5% TCA for 20 minutes. Acid-soluble and acid-insoluble cell fractions were assayed for radioactivity as previously described. The balance of the treated culture was used for isolation of DNA, RNA, and protein, and assay of the extent of methylation of each.

Assay of growth potential in tissue culture:

To study the biological effect of $\mathbf{O}_6$-MG, primary hamster embryo cells were seeded at 1.0 to $10.0 \times 10^5$ cells/5 cm diameter plastic Petri dish and allowed 3-24 hours to adhere. The $\mathbf{O}_6$-MG was dissolved in water at 5 mg/ml by heating to 100°C. Two treatment techniques were tested: (1) after removal of the medium, 0.1 ml $\mathbf{O}_6$-MG solution were pipetted directly onto the attached cells, followed immediately by addition of 10 ml medium, as suggested by Kuroki and Sato (1968); (2) 0.1 ml $\mathbf{O}_6$-MG solution were added to 10 ml medium in the culture dish containing the cells. Controls received 0.1 ml water by the corresponding route. Every four days the cells were harvested by addition of 2.0 ml trypsin solution for 2-5 minutes, and washing the surface twice with 2.0 ml medium. After resuspension with a pipette, cell density in 1.0 ml of suspension was determined on a Coulter electronic cell counter; the balance was centrifuged, resuspended in 5.0 ml medium, and replated at $10^5$ cells/plate.
Total progeny, corrected for dilutions, was plotted against time. At the time of each subculture, one plate of each treated or control series was rinsed with PBS and stained with methylene blue for photography.

Assay of incorporation and distribution of $^{3}\text{H}-\overset{6}{\text{O}}\text{MG}$ in vivo:

L cells in suspension culture initiated at $0.5 \times 10^5$ cells/ml were grown to about $4 \times 10^5$ cells/ml, harvested by centrifugation, and resuspended in 8.0 ml medium. $^{3}\text{H}-\overset{6}{\text{O}}\text{MG}$ was added in 1.0 ml water (about 15 $\mu$Ci/ml) with an equal volume of double-strength 1066 medium. For adjustment of specific activity additional unlabelled $\overset{6}{\text{O}}\text{MG}$ was included. After rolling the suspension at 37°C for 10, 20, or 120 minutes, cells were collected by centrifugation and washed with PBS. One-tenth of the cells were lysed in 0.6 ml cold 5% TCA. After 20 minutes on ice the supernatant was collected, and 2 x 0.1 ml were assayed for radioactivity in liquid phosphor in a Beckman scintillation counter. The balance of the TCA supernatant was chromatographed in solvent (2). The TCA precipitate was washed in cold 5% TCA (15 ml), ethanol, and ether, and dissolved in 0.3 ml TEAH. Radioactivity was assayed in 2 x 0.1 ml of the TEAH solution. From the balance of the cells DNA, RNA, and protein were isolated and their radioactivities were assayed.
RESULTS

Chapter I.  The activation of methyl-nitroso compounds in vitro.

Section 1.  The kinetics of decomposition of methyl-nitroso compounds.

Loss of absorption of visible or ultraviolet light due to decomposition of the nitroso compound is followed spectrophotometrically by rapidly scanning the absorption spectrum at intervals as reaction takes place in the cuvette.  The logarithm of the time required to reduce the concentration by one-half is plotted against pH in figure 1.  Decomposition rate of MNNG has a minimum at about pH 5.5, and increases as the solution is made either more alkaline or more acid.  The minimum rate of reaction of MNTS occurs at identical pH; however, increasing alkalinity is less effective on MNTS.  The minimum reaction rate of MNUA is not reached until pH is lowered to 3, after which further increase in acidity causes pronounced acceleration of decomposition.

Of the three methyl-nitroso compounds, at physiological pH, MNUA is most reactive ($t_{1/2} = 16$ minutes) MNNG is of intermediate stability ($t_{1/2} = 90$ minutes) and MNTS is most stable ($t_{1/2} = 10^3$).

In figure 2 the catalytic effect of excess thiol on MNTS decomposition is compared to that of acid or alkali.  In the presence of 0.5 mM glutathione (GSH), a neutral aqueous solution of MNTS at 37°C exhibits a half-life of 50 minutes, while the same solution without thiol is stable.  Thiol also catalyzes the decomposition of MNNG, and the higher solubility of MNNG permits study of spectra in the visible range.
where absorption is due to the nitroso group (figure 3). At equimolar concentrations of MNNG and the basic amino acid histidine, the half-life of MNNG is 70 minutes, but N-acetylcysteine reduces the half-life to two minutes. Since the amino group of the cysteine is blocked, the reaction must be with the sulphydryl moiety. Figure 4 shows the MNNG absorption at a single wave length (401 nm) plotted against time. The rate of loss of the nitroso group is only slightly greater than the rate observed in buffer alone, when either acidic (glutamic) or basic (histidine, lysine) amino acids are present at equimolar concentrations. The rate of reaction with a thiol (cysteine) is much more vigorous, and is clearly dependent on the thiol concentration.

Second order kinetics prevail (figure 5) for the reaction between MNNG and a thiol-containing compound, suggesting an SN$_2$ or bimolecular nucleophilic substitution mechanism. The kinetics remain unchanged when the amino group of cysteine is blocked by amide formation, and are confirmed over a 10-fold concentration range (figure 6) when glutathione is substituted for cysteine, or when loss of the thiol group rather than the nitroso group is measured. (The method of Ellman (1959) is used to measure thiol.) Figure 7 emphasizes the slow reaction of MNNG with amino groups, or complete growth medium, rich in amino acids. Addition of bovine serum albumin has negligible effect on MNNG decomposition, indicating relatively little reaction with protein side chains.

Figure 8 compares the kinetics of decomposition of MNNG and MNUA. Unlike MNNG, MNUA does not undergo second order nucleophilic substitution by thiol, but instead retains first-order kinetics. A slight inhibition of MNUA hydrolysis occurs in the presence of GSH.

In figures 9 through 12 the effect of thiol (GSH) or amino groups
(glycine) on MNUA is compared to that on MNTS. The hydrolytic rate of MNUA is unaffected by a 5-fold excess of thiol or amino groups. The slight recovery of absorption between 1 and 2 hours of reaction in GSH solution may result from the decomposition of a small amount of MNUA-GSH complex, which would account for the slight inhibition already noted. Unlike the situation with MNUA the extent of MNTS decomposition depends on the concentration of glutathione (figure 11). It is noteworthy that with equimolar concentrations of GSH and MNTS, the reaction stops well before the MNTS is completely degraded. As observed also by Schulz and McCalla (1969), analysis of the reaction mixture with Ellman's reagent shows that virtually all of the free thiol is removed, suggesting a catalytic rather than a strictly stoichiometric role for MNTS during reaction with thiol. Figure 13 shows the atypical (neither first nor second order) kinetics. The more dilute the solution, the longer it takes for the reaction to begin; it would appear that before decomposition can occur a prior slow reaction must take place. MNTS is remarkably stable in buffered glycine solutions (figure 12).

Section 2: The mechanism of decomposition of MNNG

Acid hydrolysis of MNNG releases nitrous acid, as shown by the superposition of the peaks characteristic of nitrous acid on the absorption of other products (table 1). The major product of MNNG-HCl reaction co-chromatographs with methylnitroguanidine (MNG) ($R_f = 0.4$, $\lambda_{max} = 267$ nm) in solvent (5). In contrast, chromatography of the final reaction mixture of MNNG from pH 6-12 shows only the salt of nitrocyanamide ($\lambda_{max} = 263$ nm). Melting point determinations and elementary
analysis of the recrystallized sodium or potassium salts confirm their identity with nitrocyanamide.

Elution with water from Dowex-50 (H\(^+\) - form) of the soluble reaction products of MNNG with equimolar cysteine gives two ultraviolet absorbing products, in fractions 50-75 and 115-155 (figure 14). These peaks contain 54\% and 35\% of the total E\(_{260}\) units and 83\% and 15\% of the E\(_{280}\) units respectively. The first peak is not homogeneous, since fractions 66-75 contain material with E\(_{280}/E_{260}\) less than that observed for the main peak (3.1). The second peak is homogeneous, E\(_{280}/E_{260}\) = 0.76. Fractions 58-65 yield 2-nitraminothiazoline-4-carboxylic acid (NATCA) as pale blue-green prisms (melting point = 190 (decomp.)) on recrystallization from water. Elementary analysis (figure 15) is consistent with the above-designated structure.

Absorption spectra of the thiazoline derivative in the acid range (figure 16) and the alkaline range (figure 17) indicate the presence of two titratable groups. Figure 18 shows these to have pK values of 2.5 and 9.0. In alkali (pH 9.9) an irreversible change occurs on standing at 37\(^\circ\) C (figure 19). The presence of two titratable groups is further confirmed by electrophoretic mobility in 0.05 M phosphate buffer (figure 20).

The minor ultraviolet-absorbing, soluble reaction product of MNNG and cysteine has absorption spectra identical to those of MNNG at the seven pH values tested (figures 21 and 22).

The reaction of MNNG with cysteine also yields a white precipitate, sparingly soluble in water, but soluble in 1.0 M HCl; examination by paper chromatography with solvents (2) and (4) detected no ultraviolet absorbing spots and the sole ninhydrin positive spot had R\(_{f}\) values identical to those of cystine (figure 23). Examination of the
precipitate by infrared spectroscopy in Nujol mull confirmed its identity to that of authentic cystine. The reaction of MNNG with cysteine is highly sensitive to pH (figure 24), the rate decreasing rapidly below pH 7.

The mechanisms of reaction of MNNG with cysteine, as elucidated here, are summarized in figure 25.

The reaction of MNNG with glutathione (GSH), rather than cysteine, as the thiol source, is more complex, producing several chromatographically distinct products in addition to MNG and GSSG — the only other ultraviolet absorbing product having $R_f = 0.16$, solvent (5), and $\lambda_{\text{max}} = 261.5 \text{ nm}$, $\lambda_{\text{min}} = 238.0 \text{ nm}$. The spectrum of this compound is quite similar to that of MNG ($\lambda_{\text{max}} = 262.5 \text{ nm}$, $\lambda_{\text{min}} = 243.0 \text{ nm}$, under these conditions of elution), although its $R_f$ is much slower, suggesting that this spot may be a stable MNNG-GSH complex analogous to the straight chain precursor of NATCA formed after reaction with cysteine.

As with cysteine, free SH groups disappear concomitant with MNNG decomposition (figure 26). There follows, however, a slow increase in SH concentration, with no reappearance of MNNG, as was seen after reaction of GSH with MNUA (figure 9), indicating that with MNNG the nitroso group is not retrievable. Of the thiol lost during reaction 94% can be recovered by reducing the reaction mixture with sodium borohydride. Although most of the thiol so recovered represents GSSG in the reaction product, the MNNG-GSH complex may also be reduced by borohydride, making quantitative estimate of thiol oxidation by this method impractical.

It is clear that MNNG can react with thiol by two distinct mechanisms: (a) the acid-type reaction leading to thiol oxidation, following denitrosation; or (b) the basic-type reaction leading to the
release of a methylating species following attack on the guanidine carbon. Reactions (a) and (b) produce different ultraviolet-absorbing products after reaction with cysteine -- MNG and NATCA respectively. These can be separated by paper chromatography and eluted for ultraviolet absorption measurement (table 1), and thus serve as quantitative markers for the extent of each reaction, despite the fact that they occur simultaneously. The effects of various pH, buffers, and nucleophilic reagents on the proportions of reactions (a) and (b) are shown in Table 2. The rates of decomposition of MNNG shown in figure 1 represent the sum of reactions (a) and (b) and reach a minimum at pH 5.5. As pH is reduced, the reaction becomes faster, until at pH of 1.28 only mechanism (a) is operative, the yield of ultraviolet absorbing product being 100% MNG. At physiological pH, 10% of the MNNG decomposes by route (b) yielding nitrocyanamide (and a methylating species). As pH is further increased into the alkaline range, the proportion of the MNNG decomposing by route (b) increases until at pH 12, 100% of the ultraviolet absorbing product is nitrocyanamide and reaction is again rapid.

As also shown by McCalla, Reuvers, and Kitai (1968), phosphate catalyzes the decomposition of MNNG. The \( t_{1/2} \) in 0.22 M sodium phosphate (pH 7.40) is 87 minutes, but \( t_{1/2} \) in 0.44 M sodium phosphate (pH 7.25) is shorter (50 minutes), despite the decrease in pH, which would normally lengthen the half-life.

Increasing the concentration of thiosulphate also accelerates the decomposition of MNNG. The reaction occurs 91% by route (a) at pH 6.6, in agreement with the trend indicated by the phosphate series of varying pH. The only anomaly occurs with tris-HCl as buffer. Here, the alkaline-type reaction dominates, giving 100% nitrocyanamide even at pH 7.15. As already shown (figure 4), lysine has a slight catalytic
<table>
<thead>
<tr>
<th>Solvent or pH</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$E_{\text{max}}$</th>
<th>$E_{280}/E_{260}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNNG</td>
<td>7</td>
<td>402</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>278</td>
<td>17600</td>
<td>1.6</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>419</td>
<td>142</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>402</td>
<td>179</td>
<td>—</td>
</tr>
<tr>
<td>Nitrous acid</td>
<td>5% tri-chloroacetic acid</td>
<td>385</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>371</td>
<td>53</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>356</td>
<td>51</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>346</td>
<td>37</td>
<td>—</td>
</tr>
<tr>
<td>N-Methyl-N'-nitroguanidine</td>
<td>2-10</td>
<td>267</td>
<td>14500</td>
</tr>
<tr>
<td>0.2M-NaOH</td>
<td>252</td>
<td>8000</td>
<td>0.74</td>
</tr>
<tr>
<td>10M-HCl</td>
<td>225</td>
<td>8900</td>
<td>0.55</td>
</tr>
<tr>
<td>MNNG: acid hydrolysis product</td>
<td>2-10</td>
<td>267</td>
<td>—</td>
</tr>
<tr>
<td>MNNG: neutral hydrolysis product</td>
<td>7</td>
<td>263</td>
<td>—</td>
</tr>
<tr>
<td>MNNG: alkaline hydrolysis product (nitrocyanamide salt)</td>
<td>0-12</td>
<td>263</td>
<td>19200</td>
</tr>
<tr>
<td>MNNG: cysteine, purified product (2-nitraminothiazoline-4-carboxylic acid)</td>
<td>0</td>
<td>280</td>
<td>16900</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>282</td>
<td>17200</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>269</td>
<td>11300</td>
</tr>
</tbody>
</table>
Table 2. Factors altering the rate of decomposition of MNNG and the proportions of products

<table>
<thead>
<tr>
<th>pH</th>
<th>BUFFER</th>
<th>REAGENT</th>
<th>*t1/2 min</th>
<th>U.V. ABSORBING PRODUCTS</th>
<th>**APPROXIMATE YIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A MAX</td>
<td>A_{280/A_{260}}</td>
</tr>
<tr>
<td>1.28</td>
<td>0.09 M HCl</td>
<td>none</td>
<td>62</td>
<td>267</td>
<td>0.72</td>
</tr>
<tr>
<td>6.15</td>
<td>0.18 M Na-phosphate</td>
<td>none</td>
<td>990</td>
<td>263</td>
<td>-</td>
</tr>
<tr>
<td>6.95</td>
<td>0.18 M Na-phosphate</td>
<td>none</td>
<td>220</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.25</td>
<td>0.44 M Na-phosphate</td>
<td>none</td>
<td>50</td>
<td>262</td>
<td>0.50</td>
</tr>
<tr>
<td>7.40</td>
<td>0.22 M Na-phosphate</td>
<td>none</td>
<td>87</td>
<td>262</td>
<td>0.50</td>
</tr>
<tr>
<td>7.85</td>
<td>0.18 M Na-phosphate</td>
<td>none</td>
<td>48</td>
<td>263.5</td>
<td>0.63</td>
</tr>
<tr>
<td>9.95</td>
<td>0.18 M Na-phosphate</td>
<td>none</td>
<td>0.4</td>
<td>263.5</td>
<td>0.63</td>
</tr>
<tr>
<td>12</td>
<td>0.05 M NaOH</td>
<td>none</td>
<td>very small</td>
<td>263.5</td>
<td>0.65</td>
</tr>
<tr>
<td>3.43</td>
<td>0.1 M Na-acetate</td>
<td>none</td>
<td>855</td>
<td>267</td>
<td>0.72</td>
</tr>
<tr>
<td>5.50</td>
<td>0.25 M Na-acetate</td>
<td>none</td>
<td>3300</td>
<td>263</td>
<td>0.62</td>
</tr>
<tr>
<td>7.50</td>
<td>0.25 M Na-acetate</td>
<td>none</td>
<td>550</td>
<td>263.5</td>
<td>0.65</td>
</tr>
<tr>
<td>6.6</td>
<td>0.25 M Na-acetate</td>
<td>0.03 M thiosulphate</td>
<td>44</td>
<td>266</td>
<td>0.69</td>
</tr>
<tr>
<td>6.6</td>
<td>0.25 M Na-acetate</td>
<td>0.02 M thiosulphate</td>
<td>71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.6</td>
<td>0.25 M Na-acetate</td>
<td>0.007M thiosulphate</td>
<td>216</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.15</td>
<td>0.18 M tris-HCl</td>
<td>none</td>
<td>460</td>
<td>263.5</td>
<td>0.63</td>
</tr>
<tr>
<td>7.15</td>
<td>0.17 M tris-HCl</td>
<td>0.007M thiosulphate</td>
<td>138</td>
<td>266</td>
<td>0.66</td>
</tr>
<tr>
<td>7.15</td>
<td>0.16 M tris-HCl</td>
<td>0.010M lysine</td>
<td>88</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.5</td>
<td>0.23 M Na-acetate</td>
<td>6.8 mM cysteine</td>
<td>22</td>
<td>279</td>
<td>1.74</td>
</tr>
<tr>
<td>6.05</td>
<td>0.16 M Na-phosphate</td>
<td>6.8 mM cysteine</td>
<td>9.8</td>
<td>279</td>
<td>1.65</td>
</tr>
<tr>
<td>6.95</td>
<td>0.16 M Na-phosphate</td>
<td>6.8 mM cysteine</td>
<td>1.0</td>
<td>279</td>
<td>1.73</td>
</tr>
<tr>
<td>7.79</td>
<td>0.16 M Na-phosphate</td>
<td>6.8 mM cysteine</td>
<td>0.2 approx</td>
<td>279</td>
<td>1.89</td>
</tr>
</tbody>
</table>

*Rate of decomposition of MNNG, 6.8mM in 10% v/v ethanol, measured by the loss of absorption at 400 nm.

**MNG = N-methyl-N'-nitroguanidine  X = nitrocyanamide  Y = 2-nitraminothiazoline-4-carboxylic acid
effect.

Cysteine accelerates the decomposition of MNNG, and increases the proportion of route (b) above that occurring in buffer. In the physiological pH range, 67% to 80% of the MNNG decomposes to yield the thiazoline derivative (and its partner, the methylating species).

Section 3: The mechanism of reaction of MNTS with glutathione.

In agreement with the mechanism elucidated for MNNG decomposition, thiol accelerates MNTS decomposition (figure 11). However, the kinetics of reaction are not second order, and do not show mole SH / mole MNTS stoichiometry. Absorption spectra of reaction mixtures containing 0.5 mM MNTS in the presence of acid, alkali, water, or buffered GSH are shown in figure 27. The mixtures contain a common product (nitrite) producing a number of peaks at low wavelengths, but only reaction with GSH gives rise to the product absorbing at 315 nm.

Chromatography (solvent 4) of the acid hydrolysis product of $^{14}$C-methyl MNTS yields a single radioactive peak at the solvent front with MTS, indicative of the usual denitrosation reaction. Alkaline hydrolysis yields only a small amount of radioactive product, which runs at the front with MTS, and of the total radioactivity chromatographed, 90% evaporates readily, suggesting extensive formation of methylating species, lost as methanol. After reaction with GSH, 0.1% of the radioactivity runs at the position of S-methyl GSH (origin, solvent 4), indicating very little formation of methylating species, while 90% chromatographs as MTS, in agreement with the mechanism outlined by Schulz and McCalla (1969) for cysteine reaction.
However, unlike the reaction of MNNG with GSH, reaction of MNTS with GSH does not proceed with second order kinetics and quantitative production of MTS and GSSG. Some of the radioactivity from $^{14}$C-methyl MNTS can be recovered from an additional sharp peak ($R_f = 0.32$, solvent 4) containing approximately 2% of the radioactivity chromatographed. Further evidence for the formation of products in addition to MTS, GSSG, and methanol can be obtained from study of changes in the absorption spectrum of the reaction mixture with time. As the MNTS absorption decays the absorption at 305 nm increases, due to the formation of the new compound (figures 27 and 28) which must involve at least part of the GSH molecule, since it is not formed during acid or alkaline hydrolysis. This complex, when first formed, is not stable since $\lambda_{max}$ shifts from 325 nm, to 312 nm, to 327 nm with time (figure 29). After 3 hours, the changes in both $A_{305}$ and $\lambda_{max}$ indicate that a rearrangement has occurred. However, even this final product cannot be MTS, which does not absorb at 305 nm at all.

Aliquots of MNTS + GSH reaction mixture added to DTNB at intervals show a smooth decline in available SH groups (figure 29). However, even after the decay of MNTS absorption and the increase in $A_{305}$ are complete, free SH content of the reaction mixture continues to drop, suggesting a catalytic role for the new complex in GSSG production. Such a role is analogous to that already shown (Schulz and McCalla, 1969) for reaction of MNTS with cysteine, which yields on a molar basis, more cystine than MTS. Further aliquots of the MNTS + GSH reaction mixture were reduced with dithiothreitol (DTT), complexed with $^{14}$C-IAM, and chromatographed in solvent 4, to provide an estimate of the total GSH + GSSG. The resultant counts-per-minute measurement in the GSH peak, plotted on the right-hand scale of figure 29C, indicates that initially, all of the thiol lost can be recovered by reduction. However, coincident
in time with the postulated rearrangement of the intermediate complex, some of the S becomes resistant to DTT reduction, providing further evidence that the stable complex found at R_f 0.32 after chromatography does in fact contain at least a sulphur-containing fraction of the GSH, in addition to an ultraviolet absorbing moiety from the MNTS.

To summarize: MNTS can lead to GSH oxidation through the denitrosation mechanism elucidated by Schulz and McCalla (1969) for reaction with cysteine. However, this cannot be the only mechanism operative, since it would allow complete recovery of GSH after reduction, and show mole SH/mole MNTS stoichiometry and second order kinetics. None of these conditions prevail, and the unusual kinetics can be attributed to the formation of an ultraviolet-absorbing sulphur-containing complex between MNTS and GSH which slowly rearranges to a stable form.

Section 4: The \textit{in vitro} methylation of DNA.

In neutral aqueous solution at 37^\circ C, ^{14}C-methyl MNNG slowly methylates DNA (figure 30). The addition of 8 mM N-acetylcysteine increases the rate of methylation, although not the final extent. Rates of methylation of DNA were determined by precipitation of the DNA from aliquots of reaction mixture with cold 2-ethoxyethanol for assay of radioactivity, at various times. Extent of methylation is expressed by the quantity

\[
K = \frac{\text{umoles methyl / gram DNA}}{\text{concentration MNNG (mM)}}
\]

The extent of methylation of DNA achieved within 40 minutes in the presence of 8 mM N-acetylcysteine is as high as that achieved in 21 hours
in the absence of thiol at the same pH. If GSH is substituted for 
N-acetylcysteine, reaction rate is rapid, but the extent of methylation 
is lower, and is decreased by increased GSH concentration. These 
observations suggest that GSH promotes decomposition of MNNG, but with 
a greater proportion of the thiol oxidizing route relative to the methy-
lating route, than does cysteine.

In contrast to methylation by MNNG, extent of methylation of DNA 
by dimethylsulphate (DMS) or methylmethanesulphonate (MMS) decreases if 
thiol is added to the buffered system (figure 31). The kinetics of MMS 
and DMS methylation are similar to those of the hydrolysis of these 
methylation agents. (Time of half-reaction of MMS is about 5 hours, and 
of DMS is about 16 minutes.) Thus, the presence of thiol activates 
methylation by MNNG, but competes for the methyl groups of MMS and DMS.
Figure 1. The effect of pH on the decomposition of nitroso compounds.

Initial concentration = 6.8 mM MNNG ● (or 0.2 mM)
0.2 mM MNUA ○
0.2 mM MNTS ○

Temperature = 37°C

The time required for half completion of the reaction ($t_\frac{1}{2}$) was determined from the first order kinetic plot of the change in optical density with time. Absorption was observed at 390 nm for 6.8 mM solutions, or at 245 nm for 0.2 mM solutions.

Figure 2. The effect of acid, alkali, or thiol on MNTS decomposition.

■ 0.2 mM MNTS in water
● 0.2 mM MNTS + 0.5 mM GSH
○ 0.2 mM MNTS + 0.1 N HCl
× 0.2 mM MNTS + 0.1 N NAOH

Temperature = 37°C
Figure 3. Rate of decomposition of MNNG in the presence of equimolar thiol or amino groups.

\[ \text{pH} = 6.8, \text{37°C} \]

Numbers in the figure represent minutes elapsed.

Figure 4. The effect of added amino acids on decomposition of MNNG.

Concentration \( \text{MNNG} = 10 \text{ mM} \)

\[ \text{pH} = 7 \]

Temperature = 37°C
Figure 5. Second order kinetics of the reaction of MNNG with thiol.

\[ \begin{align*}
\Delta & \text{ cysteine 10 mM} \\
\circ & \text{ N-acetylcysteine 10 mM} \\
\bullet & \text{ N-acetylcysteine 10 mM} \\
\square & \text{ GSH 10 mM}
\end{align*} \]

pH 6.95, 37°C
pH 6.6, 28°C

Figure 6. Second order kinetics of the reaction of glutathione with MNNG.

GSH 6.7 mM 28°C pH 6.6
MNNG 6.7 mM
Rate determined by following the loss of absorption in the visible range due to the nitroso group of MNNG.

GSH 0.6 mM 22°C pH 7.0
Rate determined by following the concentration of thiol using Ellman's reagent.

Figure 7. The rate of reaction of MNNG with medium, or equimolar thiol and amino groups.

\[ \begin{align*}
\circ & \text{ N-acetylcysteine 6.8 mM 27.5°C pH 6.6} \\
\circ & \text{ Glycine 4.3 mM 37°C, pH 7.0} \\
\square & \text{ Medium + serum, 37°C, pH 7.4} \\
\blacksquare & \text{ Medium - serum, 37°C, pH 7.4} \\
\dagger & \text{ Water, 37°C}
\end{align*} \]
Figure 8. The kinetics of decomposition of MNUA or MNNG + GSH.

A. ○ MNNG (10 mM) in 0.2 M phosphate buffer, pH 7.0, 37°C.
    ● MNNG (10 mM) + GSH (10 mM) in 0.2 M phosphate buffer, pH 7.0, 37°C.

Insert: Second order kinetic plot.

B. ○ MNUA (10 mM) + GSH (10 mM) in 0.2 M phosphate buffer, pH 7.0, 37°C.
    ● MNUA (10 mM) + GSH (10 mM) in 0.1 M phosphate buffer, pH 7.0, 37°C.
    ○ MNUA (10 mM) only in 0.2 M phosphate buffer, pH 7.0, 37°C.

† MNUA (5 mM) + GSH (5 mM) in 0.05 M phosphate buffer, pH 7.0, 37°C.
    □ MNUA (5 mM) only in 0.05 M phosphate buffer, pH 7.0, 37°C.
Figure 9. The effect of GSH concentration on MNUA decomposition.

- 0.2 mM MNUA
- 0.2 mM MNUA + 0.2 mM GSH  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNUA + 0.5 mM GSH  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNUA + 1.0 mM GSH  \[ \text{50 mM phosphate} \]

Figure 10. The effect of glycine concentration on MNUA decomposition.

- 0.2 mM MNUA
- 0.2 mM MNUA + 0.2 mM glycine  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNUA + 0.5 mM glycine  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNUA + 1.0 mM glycine  \[ \text{50 mM phosphate} \]

Figure 11. The effect of GSH concentration on MNTS decomposition.

- 0.2 mM MNTS
- 0.2 mM MNTS + 0.2 mM GSH  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNTS + 0.5 mM GSH  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNTS + 1.0 mM GSH  \[ \text{50 mM phosphate} \]

Figure 12. The effect of glycine concentration on MNTS decomposition.

- 0.2 mM MNTS
- 0.2 mM MNTS + 0.2 mM glycine  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNTS + 0.5 mM glycine  \[ \text{pH} = 7.0, \ 37^\circ C \]
- 0.2 mM MNTS + 1.0 mM glycine  \[ \text{50 mM phosphate} \]
Figure 13. The effect of MNTS concentration on its reaction with GSH.

Reaction took place in 0.2 M phosphate buffer, pH 7, 37°C.
Figure 14. Column chromatography of the MNNG + cysteine reaction mixture.
Figure 15. Elementary analysis of the major MNNG - cysteine product

**POSTULATED STRUCTURE:**

\[
\begin{align*}
\text{HOOC} & \quad \text{CH} \quad \text{N} \\
\text{CH}_2 & \quad \text{C} \quad \text{NHNO}_2 \\
\text{S} & \\
\end{align*}
\]

\[C_4H_5N_3O_4S\]

**PERCENT COMPOSITION**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>O</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THEORETICAL COMPOSITION:</strong></td>
<td>25.1</td>
<td>2.6</td>
<td>22.0</td>
<td>33.5</td>
<td>16.8</td>
</tr>
<tr>
<td><strong>ELEMENTARY ANALYSIS:</strong></td>
<td>25.07</td>
<td>2.42</td>
<td>21.86</td>
<td>17.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 16. Ultraviolet absorption spectra of 2-nitraminothiazoline-4-carboxylic acid; acid range.

Figure 17. Ultraviolet absorption spectra of 2-nitraminothiazoline-4-carboxylic acid; alkaline range.
Figure 18. Titration curve: MNNG -- cysteine major product.

concentration = 100 µg/ml

Figure 19. 2-nitraminothiazoline-4carboxylic acid; irreversible change with time, at pH 9.9, 37°C.
Figure 20. Electrophoresis of 2-nitraminothiazoline-4-carboxylic acid

<table>
<thead>
<tr>
<th>pH (0.05 M buffer)</th>
<th>migration towards +ve pole under 2 ma/mm</th>
<th>dominant ionic species</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>4</td>
<td>I</td>
</tr>
<tr>
<td>6.8</td>
<td>21</td>
<td>II</td>
</tr>
<tr>
<td>11.8</td>
<td>35</td>
<td>III</td>
</tr>
</tbody>
</table>

Ionization of 2-nitraminothiazoline-4-carboxylic acid.
Figure 21. Absorption spectra of the MNNG--cysteine minor product.

Figure 22. Absorption spectra of methylnitroguandine.
Figure 23. Chromatography of MNNG + cysteine reaction products


<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>$R_f$ ON PAPER</th>
</tr>
</thead>
<tbody>
<tr>
<td>cysteine</td>
<td>0</td>
</tr>
<tr>
<td>2-nitroaminothiazoline-4-carboxylic acid</td>
<td>0.03</td>
</tr>
<tr>
<td>nitrocyanamide</td>
<td>0.12</td>
</tr>
<tr>
<td>methyl nitroguanidine</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Figure 24. The effect of pH on the reaction of MNNG and cysteine.

- 6.8 mM cysteine
- 6.8 mM MNNG
- 37°C, 0.16 M Na phosphate pH 6.97

- 6.8 mM cysteine
- 6.8 mM MNNG
- 37°C, 0.16 M Na phosphate pH 6.07

- 6.8 mM cysteine
- 6.8 mM MNNG
- 37°C, 0.225 M Na acetate pH 5.55

- 6.8 mM cysteine
- 6.8 mM MNUN (methylnitrosourethane)
- 37°C, 0.225 M Na acetate pH 5.55
FIGURE 25

MECHANISMS FOR REACTION OF MNNG WITH THIOL

\[
\text{MNNG} + \text{HS-CH}_2-\text{CH-COOH} \rightarrow \text{PRODUCTS}
\]

Methylation route

\[
\left[ \text{CH}_3-\text{N}=\text{NOH} \right] + \left[ \text{HO}_2\text{C-CH-NH}_2 \right] \rightarrow \text{CH}_3\text{N}^+ + \text{N}_2
\]

Oxidizing route

\[
\left[ \text{FREE RADICALS} \right] + \text{CH}_3-\text{NH}-\text{C}^\cdot-\text{NHNO}_2 \rightarrow \text{MNG}
\]

\[
\text{R-S-S-R} + \text{NH}_3 \rightarrow \text{CYSTINE}
\]
Figure 26. The reaction of MNNG with glutathione in vitro: rate of thiol oxidation

- O Thiol concentration
- △ MNNG concentration
- ● Total SH + SS (Borohydrde reducible)
- ◆ Oxidized thiol (Total - SH)
Figure 27. Absorption spectra: reaction mixtures of MNTS in water, acid, alkali, or GSH.

"H₂O" 0.5 mM MNTS in 10% v/v aqueous methanol
"+HCl" 0.5 mM MNTS in 0.1 N HCl + 0.1 volumes methanol
"+NaOH" 0.5 mM MNTS in 0.1 N NaOH + 0.1 volumes methanol
"+GSH" 0.5 mM MNTS in 0.5 mM GSH and 0.2 M Na phosphate buffer pH 7, + 0.1 volumes methanol

Absorption spectra were recorded after 2 hours reaction, 37°C.

"pH 10 (20 h)"
0.5 mM MNTS at pH 10 in Na bicarbonate-Na carbonate buffer, 0.2 M, + 0.1 volumes methanol

Absorption spectrum was recorded after 20 hours reaction, 37°C.

Figure 28. Change of absorption with time of reaction mixture of MNTS in the presence of GSH.

MNTS - 0.2 mM
GSH - 0.2 mM
Buffer - 0.2 M Na phosphate pH 7, 37°C.

The numbers in the figure represent minutes elapsed.
Figure 29. Change in concentrations of components of an MNTS-GSH reaction mixture.

The data in A. and B. were taken directly from rapid scans of the absorption spectrum of the reaction mixture. The shifts in $\lambda_{\text{max}}$ of the absorption peak developed during reaction are shown. The data in C. were obtained by pipetting 0.5 ml reaction mixture into 2.5 ml DTNB reagent at $37^\circ\text{C}$, and reading $A_{410}$ immediately (left-hand side); and by reducing 0.25 ml reaction mixture with DTT, complexing with ($^{14}$C) - IAM, and assaying radioactivity in the GSH peak after chromatography (right-hand side).
Figure 30. The effect of N-acetylcysteine on methylation of DNA by \(^{14}\text{C}\)-methyl MNNG.

\[
\begin{align*}
0.25 \text{ mM } \left(^{14}\text{C}\right)\text{-MNNG} & \quad \bigcirc \text{ no thiol} \\
11.4 \text{ mM DNA polymerase (salmon sperm)} & \quad \bigcirc + 8\text{mM N}-\text{acetylcysteine} \\
15 \text{ mM sodium phosphate} & \quad \Delta + 8\text{mM GSH} \\
\text{pH 7.0, } 37^\circ\text{C} & \quad \Delta + 16\text{mM GSH}
\end{align*}
\]

\[
K = \frac{\mu\text{mole CH}_3/\text{g. DNA}}{\mu\text{mole MNNG/ ml medium}}
\]

Figure 31. Competition of thiol with methylation of DNA by MMS and DMS.

A. \(9.7 \text{ mM DNA P (Salmon sperm)}\) \(\bigcirc\) DNA isolated after 2.8 h.
\[
\begin{align*}
15 \text{ mM sodium phosphate buffer, pH 7} & \\
0.09 \text{ mM } \left(^{14}\text{C}\right)\text{-methylmethanesulphonate} & \quad \bigcirc \text{ DNA isolated after 17 h.}
\end{align*}
\]

B. \(6.6 \text{ mM DNA P (salmon sperm)}\) \(\bigtriangleup\bigtriangleup\) DNA isolated after 2 h.
\[
\begin{align*}
50 \text{ mM disodium EDTA buffer, pH 7.6} & \\
1.4 \text{ mM di}(^{14}\text{C})\text{-methyl sulphate} & \quad \bigtriangleup\bigtriangleup\bigtriangleup
\end{align*}
\]

\[
K = \frac{\mu\text{mole CH}_3/\text{g. DNA}}{\mu\text{mole methylating agent/ ml}}
\]
DISCUSSION

Chapter 1. The activation of methyl-nitroso compounds \textit{in vitro}.

Whereas chemical carcinogens such as dimethylnitrosamine and the aromatic amines and amides have been shown (Miller, 1969) to require enzymatic modification before the active species is released, the three methyl-nitroso compounds selected for this study -- MNUA, MNNG, and MNTS -- are all sufficiently reactive under physiological conditions as demonstrated \textit{herein}, to decompose without enzyme catalysts. The dependence of reactivity on pH (figure 1) shows that all three compounds contain at least two labile sites -- one attacked by acid, and one by alkali, although at a given pH, reactivity (half-life) is different for each compound.

\begin{align*}
\text{acid-labile site of MNNG} & \quad \text{alkali-labile site of MNNG} \\
\begin{array}{c}
\text{CH}_3-\text{N}-\text{C}=\text{NH} \\
\text{\textbullet}-\text{N}=\text{C}=\text{NHNO}_2
\end{array} & \quad \begin{array}{c}
\text{CH}_3-\text{N}-\text{N}\text{H} \\
\text{\textbullet}-\text{N}=\text{C}=\text{NHNO}_2
\end{array}
\end{align*}

However, at pH 7.4, only MNUA decomposes at a significant rate ($t_\frac12 = 16$ minutes) without the presence of nucleophilic thiol groups. This decomposition involves almost exclusively the alkali-labile site; the acid-labile site of MNUA requires very highly acidic conditions (pH < 3). Details of the mechanism for hydroxyl ion catalysed solvolysis of MNUA have been discussed by Garrett \textit{et al}. (1965), and whether the mechanism involves direct participation of the hydroxyl group, or involves charge transfer, the net result is the release of the highly
active precursor of the methylating agent diazomethane:

$$\text{CH}_3 - \text{N} - \overset{\text{O}}{\text{C}} - \text{NH}_2 \xrightarrow{\text{OH}^-} [\text{CH}_3 - \overset{\text{N}}{\text{N}} - \text{N} = 0] + \text{CO}_2 + \text{NH}_3$$

MNUA

$$[\text{CH}_3 - \overset{\text{N}}{\text{N}} - \text{N} = 0] \xrightarrow{+} \text{CH}_3 - \overset{\text{N}}{\text{N}} \equiv \text{N}$$

diazomethane

In the case of $N$-methyl-$N$-nitrosoacetamide, diazomethane is produced from the ester tautomer (Cramm and Hammond, 1964, p. 252). By analogy the alkaline hydrolysis of MNUA might proceed as follows:

$$\text{CH}_3 - \overset{\text{O}}{\text{N}} - \overset{\text{C}}{\text{C}} - \text{NH}_2 \xrightarrow{\text{CH}_3 - \overset{\text{N}}{\text{N}} - \text{N} = 0} \xrightarrow{\text{OH}^-} [\text{CH}_3 - \overset{\text{N}}{\text{N}} - \text{N} = 0] + \text{CO}_2 + \text{NH}_3$$

ester tautomer

It may be that the equilibrium between the nitroso and ester tautomers of MNUA accounts for the differences in reactions of this compound and its relatives, MNNG and MNTS. Both of the activators RSH or HPO$_4^{-}$ have an affinity for the nitroso group, as shown by their ability to catalyze the acidic type of decomposition of MNNG. With MNUA however, the pH dependence (figure 1) shows that the nitroso group is loath to leave. Thus, if a small amount of intermediate is formed between MNUA and RSH or HPO$_4^{-}$, as postulated for MNNG and MNTS, this intermediate would not decompose, but simply stabilize the nitroso tautomer, leaving less ester tautomer available for alkaline hydrolysis. In this way RSH and HPO$_4^{-}$ would inhibit rather than accelerate MNUA decomposition, as seen in figure 9. Whatever the mechanism, at physiological pH, MNUA decomposes spontaneously to release an active methylating species.

MNNG decomposition is catalyzed by either acidic or basic
conditions, with both routes producing biologically active products.

Acidic conditions (pH 1) result in decomposition of MNNG by route (a) --
denitrosation of the molecule, with production of methyl-nitroguanidine
and nitrous acid:

(a) CH₃-N\text{NH}_2\text{C-NHNO}_2 + 2H^+ \rightarrow CH₃-N\text{NH}_2\text{C-NHNO}_2 + HNO

MNNG                             MNG                             nitrous acid

Zimmerman, Schwaier, and Laer (1965) have shown that at pH 2 MNNG acts
as nitrous acid in the mutagenesis of yeast, while above pH 6 MNNG
activity corresponds to that of diazomethane. As described by McKay,
Ott, Taylor, Buchanan, and Crooker (1950), basic conditions (pH 12)
result in decomposition by route (b) -- attack at the imino carbon, with
production of diazomethane and the salt of nitrocyanamide:

(b) CH₃-N\text{NH}_2\text{C-NHNO}_2 + \text{OH}^- \rightarrow [CH₃-N=\text{N-OH}] + \text{N}=\text{C-N-NO}_2 + \text{H}_2\text{O}

MNNG                             nitrocyanamide

[CH₃-N=\text{N-OH}] \rightarrow CH₃-N\equiv N

diazomethane

Reaction routes (a) and (b) have both been verified by the in vitro
studies reported in the Results section of this thesis (Chapter I,
Section 2).

Although both nitrous acid and diazomethane are highly effective
mutagens, their participation in MNNG mutagenesis has been questioned.
The rate of decomposition of MNNG is at a minimum at pH 5.5, but mut-
ations in bacteria are still induced by MNNG in solid medium at this pH (Mandell and Greenberg, 1960). Because of the stability of both acid- and alkali-labile sites at pH 5.5, these authors attribute mutagenicity to the whole MNNG structure rather than to a breakdown product. However, it seems unlikely that the intra-cellular pH would drop to pH 5.5, and despite the acidity of the environment, after absorption the MNNG would be subject to neutral intra-cellular conditions. The only effect of adjusting the medium to pH 5.5 would be to ensure minimum decomposition before absorption and thus maximum contact between cellular components and the active species after decomposition.

Unlike MNUA, MNNG at pH 7.4 is relatively slow to hydrolyze \((t_\frac{1}{2} = 90 \text{ minutes})\), while the second-order reaction with physiological concentrations of thiol proceeds readily \((t_\frac{1}{2} = 2 \text{ minutes})\). The mode of action of MNNG in the presence of thiol is here elucidated in detail. Cysteine at concentrations in the range estimated from thiol content of mammalian L or hamster embryo cells and cell volume, attacks MNNG at both the acid- and alkali-labile sites. The reaction products include MNG and cystine following denitrosation, route (a, SH), and a methylating species (diazomethane) and 2-nitraminothiazoline-4-carboxylic acid (NATCA) following attack at the imino carbon, route (b, SH). Details of the experimental evidence supporting these reaction mechanisms are presented in the Results section of this thesis (Chapter I, Section 2), and are supported by the work of Schulz and McCalla (1969).

\[
\begin{align*}
(a, \text{SH}) \quad \text{CH}_3 - \text{N} - \text{C} - \text{NHNO}_2 + \text{HSR} \quad & \quad \text{CH}_3 - \text{N} - \text{C} - \text{NHNO}_2 + [\text{RS} - \text{N} = 0] \\
\text{MNNG} \quad & \quad \text{cysteine} \quad \text{MNG}
\end{align*}
\]
Reaction of MNNG with GSH proceeds by similar mechanisms and promotes both production of a methylating species and oxidation of thiol. However, studies using DNA as methyl acceptor indicate that the proportion of methylating species released from MNNG is lower after GSH activation than after cysteine activation.

The ability of thiol to activate the methylation of DNA by MNNG (Schoental; 1966; McCalla, 1968; Craddock, 1969) is verified, and compared to the intervention of thiol in other methylating reactions. While the presence of thiol promotes release of the methylating species from MNNG, resulting in greatly increased rate of DNA methylation, the presence of thiol competitively inhibits the methylation of DNA by the simple, labile agents MMS and DMS (figures 30 and 31).

The proportions of routes (a, SH) and (b, SH) as determined
in vitro in the physiological pH range (table 2) are sensitive to changes in environment, making extrapolation to a complex in vivo situation problematical, but the data suggest that 70% to 80% of the MNNG could be expected to react via the methylation route (b, SH). The remaining 20% to 30% of the MNNG reacts in vitro by route (a, SH) leading to thiol oxidation. In vivo, then, methylation should not be the only effect with physiological significance. One would also expect loss of thiol through oxidation, in addition to that lost during activation of the methylating species by route (b, SH) and subsequent direct methylation of free SH.

The presence of thiol alters not only the rate at which MNNG releases its methylating species, but also the extent to which MNNG decomposes into a methylation agent rather than into the stable denitrosated MNG (table 2). Clearly, if cellular components such as nucleic acid or protein are to be reacted with MNNG in vitro as models of the in vivo situation, a physiological concentration of thiol must be included. Singer and Fraenkel-Conrat (1969) have reported a wide discrepancy in mutagenicity when purified TMV-RNA is reacted with MNNG in vitro, as opposed to reaction with RNA in the intact virus. Interpretation of such results would be greatly facilitated if the thiol content of the intact virus could be simulated in vitro, so that the mode of activation of the drug would more closely approximate that occurring in vivo.

Blocking the amino group of cysteine as in N-acetylcysteine has no detectable effect on reaction rate with MNNG (figures 4, 5, 6), consistent with the observation of Ross (1958) that the thiol group is 1000 times more nucleophilic than the amino group. Further, since none
of glycine, histidine, lysine, glutamic acid, or bovine serum albumin has much catalytic effect (figures 4 and 11) relative to N-acetylcysteine, it seems improbable that reaction of MNNG with protein amino groups would account for any significant proportion of the reaction in vivo, where thiol is ubiquitous. The biological relevance of the labelling of protein by reaction of amino groups with $^{14}$C-guanido MNNG as discussed by Sugimura, Fujimura, Nagao, Yokoshina, and Hasegawa (1968) is therefore in jeopardy.

Although MNTS is even more stable than MNNG at neutral pH, physiological concentrations of GSH again produce decomposition of the methyl-nitroso compound (figure 2). The reaction is not second order (figure 11) and results in greater depletion of GSH than MNTS (figures 13 and 29). The formation of a new ultraviolet absorbing product in the presence of GSH, but not acid or alkali (figure 27) confirms the production of a GSH-MNTS complex, which could play a catalytic role in thiol oxidation. The postulated intermediate is itself unstable, as demonstrated by the shifts in $A_{\text{max}}$ with time (figure 29). Unlike MNNG, only a small proportion of the MNTS gives rise to methanol. MTS is the major product and very little $^{14}$C-methyl is transferred to the S-methyl GSH position after paper chromatography. This weak methylating potential of MNTS is in sharp contrast to the extensive methylating ability of methyl nitrosourethane reported by Schoental and Rive (1965). In the presence of cysteine the major products of the latter agent were S-methylcysteine and its methyl ester. McCalla (1968) has shown that while cysteine increases the extent of methylation by MNNG, it decreases methylation by MNTS, again demonstrating that thiol catalyses the thiol-oxidizing route of MNTS decomposition but not the production.
of the methylating species. Thus, the biological effects of MNTS can be
ascribed to effects on cellular thiol. MNTS would be expected to de-
plete free cellular thiol extensively as a result of both thiol oxidation
and the production of a stable complex. Labelling of cellular protein
by \(^{14}\)C-methyl MNTS would probably be due more to the formation of RSH-MNTS
complexes than to actual methylating reactions.

In summary, decomposition of all three methyl-nitroso compounds
is activated by simple chemical reaction under conditions consistent with
those found in thiol-containing cells. However, MNUA decomposes almost
entirely into methylating species, independent of cellular thiol; MNNG
undergoes both thiol-activated decomposition to a methylating species,
and denitrosation with concomitant thiol oxidation; and MNTS decomposes
almost entirely by the thiol-oxidizing route, with very little formation
of methylating species. However, after exerting a catalytic effect on
thiol oxidation, a stable MNTS-GSH complex persists.
RESULTS

Chapter II: The reactions of methyl-nitroso compounds in vivo.

Section 1: The growth characteristics of the tissue cultures used.

The growth of suspension cultures of L cells is characterized by three phases of different growth rate -- lag phase, exponential phase, and stationary phase. Although the transitions between phases are gradual, arbitrary divisions termed newly activated or AL and late exponential or "L" may be selected as shown in figure 32. These segments of exponential phase correspond to periods of high (AL) or low ("L") acid soluble thiol content (figure 33). Throughout the exponential growth phase, including both AL and "L" segments, the doubling time (figure 32), the percent of cells in S-phase (figure 34), and the amount of deoxyribonucleic acid (DNA) / cell (table 3) are fairly constant. However, newly activated (AL) cells differ from "L" cells in size (figure 35) and thiol content (figure 33).

Study of the effects of population density (figure 36) indicates that medium depletion is not the only factor that inhibits growth. Cells diluted into medium previously conditioned by the continuous growth of 0.5 X 10^5 cells / ml up to 7.2 X 10^5 cells / ml are able to double at least twice. The doubling time, however, is extended from the normal 16 hours to 38 hours. Further, cells suspended at high population density in fresh medium are unable to double. Thus, at least two factors influence stationary phase: (1) medium depletion, and (2) interaction between crowded cells in a manner analogous to the contact inhibition of
Table 3

The DNA content of L cells.

<table>
<thead>
<tr>
<th>Population density (cells x 10^5 / ml)</th>
<th>Hours after dilution</th>
<th>μg DNA / 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.82 diluted to 0.71</td>
<td>0</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>1.06</td>
</tr>
<tr>
<td>5.51 diluted to 0.98</td>
<td>0</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean =</td>
<td></td>
<td>1.10</td>
</tr>
</tbody>
</table>

Standard deviation = ± 0.06
fixed tissue cultures. Since cells labelled "L" in this work are obtained from the intersection between exponential phase and stationary phase, the changes involved in the induction of stationary phase are already underway in a large proportion of "L" cells, and may influence the interaction of nitroso-methyl agents with these cells.

Following subculture, hamster embryo (HE) cells grow rapidly, after a short lag period (figure 37), until, when a continuous monolayer of cells covers the plate, contact inhibition occurs. Repeated change of medium causes only a slight increase in the number of cells after confluence has been reached. Again, segments of the growth cycle termed newly activated (AHE) or confluent (CHE) may be defined, and correspond to high and low thiol content respectively (figure 38).

During the initial four-day period of rapid growth the proportion of HE cells synthesizing DNA decreases steadily as cells drop out of the replication cycle (figure 40). The rate of tritiated thymidine (\(^{3}\text{H}-\text{TdR}\)) incorporation into acid-precipitable material rapidly rises through days zero to two, suggesting the induction of the enzyme systems required in the replication cycle (figure 39). Subsequently, incorporation in one hour by \(10^5\) cells drops somewhat faster than the decrease in proportion of cells synthesizing DNA, indicating a true drop in rate of nucleic acid synthesis. During the long period of contact inhibition very little \(^{3}\text{H}-\text{TdR}\) is incorporated during a one-hour exposure. The average DNA content of HE cells reaches a maximum 24 hours after re-plating confluent cultures (table 4). Then, as cells begin to divide, average DNA content decreases until, by day four, half the maximum content remains. These observations suggest the following sequence of events after resuspension: (1) DNA replication is completed, and cells advance through the pre-mitotic gap
Table 4

DNA content of HE cells.

<table>
<thead>
<tr>
<th>Days after seeding</th>
<th>µg DNAP / $10^6$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.83</td>
</tr>
<tr>
<td>2</td>
<td>0.57</td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
</tr>
<tr>
<td>4</td>
<td>0.39</td>
</tr>
<tr>
<td>30</td>
<td>0.75</td>
</tr>
</tbody>
</table>
in DNA synthesis \((G_2)\); (2) cells enter mitosis in a fairly synchronous manner; (3) the cell cycle is completed an average of three to four times; (4) the cells, after the final division before contact inhibition, do not double their DNA, but come to rest again in the post-mitotic gap in DNA synthesis \((G_1)\). However, during a long period of contact inhibition the cells proceed slowly from \(G_1\) into the DNA-synthetic phase \((S)\), and are largely in \(G_2\) by day 30 (figure 40 and table 4).

The cells termed AHE are therefore largely \(G_2\) cells with the 2N complement of DNA, and are poised for entry into mitosis. Cells termed CHE are either \(G_1\) cells or cells slowly replicating the complementary DNA. As is the case with AL and "L" cells, AHE and CHE differ markedly in their thiol content (figure 38).

Section 2: Reactions with thiol in vivo.

After treatment with \(N\)-methyl-\(N'\)-nitro-\(N\)-nitrosoguanidine (MNNG), the acid-soluble thiol content of L cells, as assayed with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), is decreased, the extent of thiol loss being greater for larger doses (figure 41). However, doses of more than 0.2 mM (10 times the dose required to reduce survival three logarithms) must be given before a permanent detectable decrease in thiol occurs. Further, even with quite large doses, there is a pronounced recovery of acid-soluble thiol between 10 and 15 minutes after
addition of the agent. On the other hand, if the simple direct alkylating agent dimethylsulphate (DMS) is used, changes in acid-soluble thiol are slight (figure 42).

During the *in vitro* chemical reactions of nitroso-methyl agents, some of the thiol is converted to the oxidized disulphide form. To assay for a comparable oxidation *in vivo* the method described by Sparkes and Walker (1966) can be used. Buffered cell lysates are reacted with $^{14}$C-iodoacetamide ($^{14}$C-IAM) to form a labelled S-carboxamido derivative of any SH-containing compounds present, which, after precipitation of acid-insoluble material with trichloroacetic acid (TCA), can be separated by chromatography. The disulphides in a second aliquot of buffered cell lysate are reduced with dithiothreitol (DTT) prior to $^{14}$C-IAM labelling, to provide a measure of the total content of reduced and oxidized thiol. Paper chromatography in solvent (4) separates markers of glutathione (GSH) and cysteine from unreacted DTT and IAM. The typical distribution of label obtained after chromatography of L cell extracts, without DTT reduction (figure 43) or with DTT reduction (figure 44) displays four peaks coincident with the IAM-complexed markers GSH, cysteine, DTT, and unreacted IAM. (The material in the DTT position in figure 43 was shown to be a degradation product of $^{14}$C-IAM.) Reduction of the lysate of normal cells with DTT releases very little additional thiol, indicating that the equilibrium

$$2\text{RSH} \rightleftharpoons \text{RS-SR} + 2\text{H}^+$$

is normally shifted far to the left within the cell.

Treatment of L cells with 0.8 mM MNNG causes $\sim 50\%$ depletion of cellular thiol (table 5). Reduction of the cell extract with DTT restores the SH, indicating that $\sim 50\%$ had been oxidized to the disulphide form. As in the acid soluble fraction, oxidized thiol groups
### Table 5

**SH content of L cells after reaction in vivo**

<table>
<thead>
<tr>
<th>DRUG</th>
<th>MNNG</th>
<th>MNTS</th>
<th>MNTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOSE</td>
<td>0.8 mM</td>
<td>0.1 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>EXPOSURE TIME (minutes)</td>
<td>40</td>
<td>40</td>
<td>120</td>
</tr>
</tbody>
</table>

**Radioactivity (% of solvent-treated control)**

<table>
<thead>
<tr>
<th></th>
<th>TCA soluble SH</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>54</td>
<td>23</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>100</td>
<td>35</td>
<td>32</td>
<td>After reduction</td>
</tr>
<tr>
<td>(3)</td>
<td>92</td>
<td>38</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>107</td>
<td>105</td>
<td>***</td>
<td></td>
</tr>
</tbody>
</table>

*** = not measured

1. Cells were lysed in 4M urea, complexed with $^{14}$C-IAM for 20 minutes, acidified to 5% TCA, and centrifuged; after 6 hours at room temperature the clear supernatant was spotted on paper, chromatographed overnight, and the radioactivity in the GSH + cysteine peaks assayed.

2. Cells were lysed in 4M urea, reduced with DTT, and thiol measured as in (1).

3. The precipitate of the acidified extract in (1) was washed in 5% TCA, ethanol, and ether, dissolved in TEA, and assayed for radioactivity.

4. The precipitate of the reduced, acidified extract in (2) was washed and assayed as in (3).
associated with acid precipitable material are recoverable by reduction. MNNG must be blocking thiol groups during the primary activation of its reactive sites, since otherwise, thiol would be a major target for subsequent methylation, and become reduction resistant. However, very little reduction-resistant bound thiol is produced, and oxidation of cellular thiol occurs to a significant extent.

The affinity of MNTS for cellular thiol is much greater in vivo than that of either MNUA or MNNG. MNTS at 0.1 mM causes more extensive depletion of thiol than does 0.8 mM MNNG (Table 5). If the in vivo reaction time is extended from 40 minutes to 120 minutes no further depletion of thiol occurs, indicating that the in vivo events are completed within the initial period. Only a small part of the acid-soluble thiol lost is restored by reduction with DTT. The situation is analogous to the in vitro case, where a stable MNTS-GSH complex was demonstrated. Acid-precipitable groups also react extensively with MNTS, but these, unlike the acid-soluble groups, are completely restored by reduction. MNTS therefore both binds to and oxidizes cellular thiol.

Section 3: Methylation by MNNG in vivo.

As a measure of the extent of incorporation of the $^{14}$C-methyl group of MNNG into cells, the quantity $K'$ is used, where

$$
K' = \frac{\mu \text{moles methyl bound}}{10^4 \text{ cells}} \div \text{dose MNNG (mM)}
$$

MNNG incorporation is rapid, and the amount incorporated reaches a maximum before two minutes (figure 45). Once inside the cell the drug is activated, and methylation of cellular constituents is complete after 10 minutes. Since the half-life of MNNG in growth medium is ~ 90 minutes,
the abrupt halt of incorporation is surprising. Ample drug should remain in the medium, but after the initial confrontation, the cells apparently develop the ability to exclude this remainder. In order to guarantee a supply of unreacted MNNG outside the cell, the drug can be added as a suspension in water, instead of the usual methanol solution, and the slow dissolution of the MNNG causes a steady rise in the external concentration. (Before assaying radioactivity in the medium the undissolved drug is filtered out with the TCA precipitable material.) Even with increasing amounts of MNNG in the medium (figure 46 A) incorporation by low thiol CHE cells halts abruptly before 2 minutes (figure 46 B), and subsequent binding of $^{14}C$-methyl is complete in 10 minutes (figure 46 C). Some of the $^{14}C$-methyl is returned to the medium from the acid soluble cell fraction, probably as methanol (figures 46 A and B).

The ability of MNNG to methylate TCA precipitable material varies widely with metabolic state (high-thiol AL or AHE as opposed to low-thiol "L" or CHE, figure 47). Although methylation of "L" and CHE cells stops after 10 minutes, further increase in extent of methylation occurs in AL cells. The cessation of methylation of low thiol cells may result not only from insufficient thiol to activate what MNNG is incorporated, but also from a self-induced block to the initial incorporation itself, as seen at 2 minutes in "L" and CHE cells. AL cells, on the other hand, bind more $^{14}C$-methyl to acid precipitable material alone than "L" cells incorporate into both cell fractions (figure 45), indicating that passage of MNNG through the membrane is much more extensive in high thiol cells.

The affinity of methyl groups for DNA, RNA, and protein during incubation with MNNG in vivo is expressed as μmoles $^{14}C$-methyl bound per gram of cellular constituent at various doses (figures 49 and 53). Since
the relationship of binding to dose is linear, the binding constant "K" is the slope of the line produced:

\[ K = \frac{\text{umoles methyl bound / gram cellular constituent}}{\text{dose MNNG (mM)}} \]

After MNNG treatment, nucleic acids are preferentially methylated relative to protein. It is reasonable that RNA binds more than DNA, since DNA is confined to the nucleus, remote from the drug, while the low affinity for protein is attributed to the prior reaction of susceptible SH groups at the activation step, leaving them blocked either by the residual guanido moiety of the MNNG, or by disulphide formation.

In addition to the difference in the affinity of each type of macromolecule for methylation, differences in the binding constants are apparent for various cell types. All three macromolecules of AHE cells are more extensively methylated than those of low thiol CHE cells (figure 48). However, the three binding constants \( K_{\text{DNA}} \), \( K_{\text{RNA}} \), and \( K_{\text{protein}} \) are all increased in AHE cells by the same fraction. Thus the ratios of \( K_{\text{DNA}} \), \( K_{\text{RNA}} \), \( K_{\text{protein}} \) are the same for AHE and CHE cells (3.8 : 7.4 : 1). It therefore seems highly unlikely that the increased extent of methylation in AHE cells could be due to alteration of the number or vulnerability of target groups, since any such alteration would have to occur to exactly the same extent in molecules as diverse as nucleic acid and protein. Rather, the increased methylation of AHE cells must reflect more extensive incorporation of the agent and a higher intracellular dose.

On the other hand, the ratios of \( K_{\text{DNA}} \), \( K_{\text{RNA}} \), \( K_{\text{protein}} \) are not identical for AL and "L" cells. (The ratios are 11 : 17 : 1 for AL cells, and 3.6 : 10 : 1 for "L" cells, figure 49.) Since in AL cells methylation of DNA is increased by a factor of three, while RNA methylation is
increased by less than two, some change other than cell permeability promotes methylation of DNA preferentially. It is possible that methylation could be directed towards DNA by increased numbers of activating SH groups in close proximity with DNA.

An indication that MNNG is activated in vivo by reaction with cellular thiol is the directly measured loss of thiol groups (figure 41). Further evidence can be obtained by chromatographic analysis of the products of reaction in vivo. While MNNG itself decomposes during chromatography, its denitrosated product MNG does not. Thus to measure residual MNNG, the test extract is heated to convert MNNG quantitatively to MNG, and the sum of MNNG and MNG appears as a single spot after chromatography. The amount of MNNG + MNG in the medium at 0.5 minutes after treatment decreases to a residue of 54% by 80 minutes reflecting the known hydrolytic rate, but during the same time interval, the intracellular concentration falls to 25% (figure 50). Decomposition of MNNG, other than denitrosation to MNG, is therefore more extensive within the cells than in the surrounding medium, and reaction of MNNG in vivo is not identical to simple hydrolysis in the medium.
Section 4: Methylation by DMS \textit{in vivo}.

One of the most critical results of the study of MNNG interaction with cells is the demonstration of the importance of the activation step. Many features of MNNG interaction, such as extent of incorporation, extent of methylation, and distribution of methylation, are attributed to the fact that MNNG in aqueous medium is relatively stable, and must be activated by reaction with the highly nucleophilic thiol group before extensive methylation occurs. In order further to verify the importance of the activation step, a number of the experiments are here repeated with DMS substituted for MNNG. DMS is a methylating agent of comparable molecular weight (MW MNNG = 147, MW DMS = 126) but differs in that it is a labile, direct methylating agent which requires no prior activation.

After $^{14}$C-DMS treatment radioactivity in the medium is depleted concomitant with incorporation of label into the two cell fractions (figure 51). Incorporation continues for 40 minutes despite the 16 minute half-life of DMS. In contrast, the previous results show that incorporation of MNNG is abruptly halted within 10 minutes, despite the fact that MNNG persists in the medium much longer than DMS. Clearly, the reduction of permeability induced by MNNG does not occur after DMS treatment.

After incorporation of $^{14}$C-DMS, radioactivity is either transferred to the precipitate or else is returned to the medium. Not all of the methyl groups returned to the medium represent elimination of methanol formed during simple hydrolysis, since the amount of radioactivity actually bound in the cell precipitate also decreases with time. Active excision of methyl groups or methylated products must be occurring and approaches completion in 17 hours.
The drop in SH content at exactly the time at which binding of methyl groups is reversed circumstantially implicates thiol metabolism in the recovery process; subsequently thiol content returns to normal. Untreated L cells retain a constant acid soluble thiol level under these incubation conditions.

As with MNNG, the final extent of methylation by DMS may be influenced by the rate of incorporation of the highly labile drug. However, since DMS does not have the affinity of MNNG for thiol (figure 42) chemical effects on the membranes are limited to direct methylation, and are unlikely to be extensive. Predictably, the differences between cell types are much smaller with DMS than with MNNG, and the acid-precipitable material of AL and "L" cells is methylated to about the same extent (figure 52), despite the marked difference in thiol content.

Unlike MNNG, DMS does not preferentially methylate nucleic acid relative to protein (figure 53). While with MNNG a large proportion of the nucleophilic SH groups is removed from the competition for methylation during the prior activation step, DMS requires no activation, and the free SH groups are available for methylation, making $K_{protein}$ high.
The distribution of methyl groups among the three types of macromolecules differs very little among the various cell types, indicating that methylation by DMS is independent of thiol concentration. The only exception to this uniformity is the elevated binding to the protein fraction of CHE cells. These cells grow as a monolayer, embedded in a protein matrix, which, since it coats the cell exterior, can
trap methyl groups before they even reach the cell interior. No such effect is seen after MNNG treatment, since the methylating species is released only after the MNNG has entered the cell, and contacted cellular thiol.

Thus none of the phenomena attributed to the interaction of MNNG with cellular thiol occur after treatment with the simple labile methylating agent DMS. Membrane permeability of low thiol cells is not reduced by DMS; high thiol cells are not more extensively labelled than low thiol cells; and nucleic acid is not preferentially methylated relative to protein.

Section 5: Methylation by MNUA or MNTS in vivo.

Like DMS, MNUA methylates without interaction with thiol (Section 2) while MNTS has even greater affinity for thiol than MNNG. MNUA and MNTS can therefore be used to confirm the role of cellular thiol in determining extent and distribution of the methylating reactions. In the experiments reported here, the cell concentration was adjusted to $2 \times 10^7$ cells/ml before addition of the methylating agent, and extent of binding is expressed as a percentage of the dose:

$$\% \text{ dose incorporated} = \frac{\mu \text{moles methyl bound/ml cell suspension}}{\mu \text{moles agent/ml cell suspension}} \times 100$$

For comparison, data obtained with MNNG and DMS on less dense cell suspensions are arithmetically adjusted for cell number, and expressed as percentage of dose incorporated in figures 54 and 55.

A major chemical difference between AL and "L" cells is in the amount of GSH in the acid-soluble fraction. When cells are treated with
$^{14}\text{C}$-methyl MNNUA, about twice as much radioactivity becomes associated with the cells that have double the thiol content (figure 56), and this observation suggests direct methylation of the more abundant GSH. This difference in extent of methylation does not result from a self-induced block to incorporation, since there is no abrupt cessation of accumulation of $^{14}\text{C}$ from MNNUA even in low thiol cells. In addition, no extensive increase of methylation of the acid precipitate is seen in high thiol relative to low thiol cells, as was the case with MNNG. In fact, the retention of label in the soluble fraction of high thiol cells is balanced by a somewhat lower extent of methylation of the cell precipitate.

MNNTS, on the other hand, reacts readily with thiol, although the reaction produces very little methylating species. Like MNNG, incorporation of MNNTS is rapidly terminated, although MNNTS is the most persistent in the medium, suggesting again that agents reactive towards thiol alter membrane permeability (figure 57). MNNTS is sufficiently reactive to induce this effect even in high thiol cells, which, after MNNG treatment, were protected from the reduction in permeability. More radioactivity from $^{14}\text{C}$-methyl MNNTS becomes associated with the acid precipitate of high-thiol cells than low, but the additional binding is reversed with time. Thus one would suggest that the label is present in loose complexes rather than in actual methylated products.

The distribution of $^{14}\text{C}$ derived from $^{14}\text{C}$-methyl MNNUA or MNNTS among DNA, RNA, and protein is shown in table 6. Following MNNUA treatment RNA is again methylated more extensively than DNA, but there is little increase in methylation of nucleic acids in high thiol cells, since the difference in the extent of incorporation induced by MNNG does
Table 6

EXTENT OF METHYLATION OF CELLULAR CONSTITUENTS OF CULTURED MAMMalian cells by MNU or MNTS

<table>
<thead>
<tr>
<th>AGENT</th>
<th>MNUA</th>
<th>MNUA</th>
<th>MNUA</th>
<th>MNUA</th>
<th>MNUA</th>
<th>MNTS</th>
<th>MNTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>0.07mM</td>
<td>0.15mM</td>
<td>0.74mM</td>
<td>2.46mM</td>
<td>5.05mM</td>
<td>0.083mM</td>
<td>0.15mM</td>
</tr>
<tr>
<td>minutes, 37°</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>cell type</td>
<td>AL</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>AL</td>
</tr>
<tr>
<td>$K_{DNA}$</td>
<td>0.78</td>
<td>0.94</td>
<td>0.87</td>
<td>1.1</td>
<td>0.94</td>
<td>0.29</td>
<td>0.28</td>
</tr>
<tr>
<td>$K_{RNA}$</td>
<td>1.97</td>
<td>1.27</td>
<td>1.46</td>
<td>1.48</td>
<td>1.23</td>
<td>0.25</td>
<td>0.32</td>
</tr>
<tr>
<td>$K_{protein}$</td>
<td>4.67</td>
<td>0.59</td>
<td>0.20</td>
<td>0.21</td>
<td>0.25</td>
<td>0.07</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$K = \frac{\text{umole methyl bound / gram constituent}}{\text{umole agent / ml culture}}$
not occur. More significantly, while the level of methylation of protein by MNG is much lower than that of nucleic acid, MNUA, which does not block thiol in an activation step, is able to methylate the protein of high thiol cells extensively.

As noted in vitro, MNTS has less tendency to decompose into an active methylating species than its relatives, and extent of methylation of nucleic acid in vivo is correspondingly low. That the label associated with acid-precipitable fractions is indeed in the form of loose complexes rather than methylated products is affirmed by the very low residuum of radioactivity in the protein fraction after phenol extraction and repeated washing in organic solvents.

The conclusions drawn from in vitro studies of the methyl-nitroso compounds remain valid in vivo: (1) The order of reactivity with thiol is MNTS > MNG >> MNUA; (2) MNUA decomposes extensively and spontaneously into a methylating species, which alkylates nucleophilic sites at random; (3) MNG is activated by reaction with thiol prior to alkylation of residual nucleophilic sites, primarily of nucleic acids; (4) a significant proportion of the MNG decomposes by the thiol oxidizing route; (5) MNTS reacts extensively with thiol, giving rise to fairly stable intermediates and thiol oxidation, but little methylating species. Further, the ability of MNG and MNTS, but not MNUA or DMS to alter membrane permeability, deplete free cellular thiol, and preferentially methylate nucleic acid relative to protein is ascribed to their reaction with thiol.
exponential growth phase cells, $1.6 \times 10^5$ cells/ml.
stationary phase cells, $6.6 \times 10^5$ cells/ml.
The arbitrary threshold settings of the Coulter counter were standardized using a suspension of Ragweed pollen of mean diameter = $19.5\mu$.
Volume ($\mu^3$) per threshold division =
mean particle volume = 145.25
mean particle threshold
The volume range shown corresponds to $1.45 \times 10^3 \mu^3$ (threshold 20) to $14.5 \times 10^3 \mu^3$ (threshold 100).
Although the distribution of Stal cells is noticeably skewed to the left, the weighted average thresholds differ very little.
For Stal cells $\frac{\Sigma \text{Threshold} \times \text{frequency}}{100} = 37.5$
For AL cells $\frac{\Sigma \text{Threshold} \times \text{frequency}}{100} = 38.7$
Therefore, in aliquots of the cell suspensions, the volume actually occupied by cells differs only slightly.
Figure 36: Factors influencing stationary phase in suspension culture.

- young cells + new medium
- young cells + old medium
- old cells + new medium

"young" cells = grown to $3.3 \times 10^5$ cells/ml.
"old" cells = grown to $7.2 \times 10^5$ cells/ml.
"old" medium = medium in which cells had been grown from $0.5 \times 10^5$ cells/ml up to $7.2 \times 10^5$ cells/ml.
"new" medium = regular 1066 medium supplemented as usual.
Figure 37: The growth of HE cells \textit{in vitro}.

Figure 38: The acid soluble thiol content of HE cultures.

Figure 39: The rate of incorporation of $^3$H-TdR by HE cells.
FIGURE 37

CELLS x 10^3/PLATE

DAYS

AHE
CHE

FIGURE 38

µMOLES SH/10^6 CELLS

DAYS

FIGURE 39

cpm per 10^5 CELLS/HOUR

DAYS
Figure 40: The growth of HE cells and the percentage of cells synthesizing DNA.
FIGURE 40A
PERCENT OF CELLS SYNTHESIZING DNA

FIGURE 40B
GROWTH OF HE CELLS
Figure 41: The effect of MNNG on the acid soluble thiol content of L cells.

- AL cells, dose = 0.69 mM
- "L" cells, control, ethanol only
- "L" cells, dose = 0.218 mM
- "L" cells, dose = 0.94 mM
- "L" cells, dose = 0.62 mM
- "L" cells, dose = 1.89 mM
- "L" cells, dose = 2.82 mM
- "L" cells, dose = 3.23 mM

Figure 42: The effect of DMS on the acid soluble thiol content of L cells.

- AL cells, dose = 0.076 mM
- "L" cells, dose = 0.67 mM
Figure 43: Separation of $^{14}$C-IAM complexes of normal cell thiols without DTT reduction.

Figure 44: Separation of $^{14}$C-IAM complexes of normal cell thiols after DTT reduction.
Figure 45: The incorporation of $^{14}$C-MNNG by L cells.

- whole cells
- TCA precipitable fraction

$K' = \frac{\text{\mu moles methyl}}{\text{dose MNNG (mM)}} / 10^4 \text{ cells}$

Dose = 0.051 mM

Population density = $5 \times 10^5$ cells/ml

Thiol content = 83 \mu moles SH / $10^{10}$ cells

Figure 46: The incorporation of $^{14}$C-MNNG by CHE cells.

A. \mu moles methyl in medium, after removal of TCA-precipitable serum protein

B. \mu moles methyl in TCA-soluble cell fraction

C. \mu moles methyl in TCA-precipitable cell fraction
Figure 47: The binding of $^{14}$C-MNNG to acid insoluble cell fractions, of various cell types.

- △ AL cells, SH content 187 μmoles/10$^{10}$ cells
  Dose = 0.62 mM
- □ AL cells, SH content 108 μmoles/10$^{10}$ cells
  Dose = 0.69 mM
- △ CHE cells, SH content 30 μmoles/10$^{10}$ cells
  Dose = 0.19 mM
- ○ "L" cells, SH content 49 μmoles/10$^{10}$ cells
  Dose = 0.27 mM
- ○ "L" cells, SH content 34 μmoles/10$^{10}$ cells
  Dose = 0.55 mM
- ■ Sta L cells, SH content 65 μmoles/10$^{10}$ cells
  Dose = 0.021 mM

Figure 48: The methylation of DNA, RNA, and protein by $^{14}$C-MNNG in hamster or mouse embryo cultures.

- X RNA
- ○ DNA
- ○ protein

Figure 49: The methylation of DNA, RNA, and protein by $^{14}$C-MNNG in L cells.

- X RNA
- ○ DNA
- ○ protein
Figure 50: The persistence of $^*_\text{MNNG}$ in cells and medium.

- 0.5 minutes after treatment
- 80 minutes after treatment

$^{14}\text{C}$-methyl-MNNG was used.

All peaks shown occurred at the position of MNNG marker.
Figure 51: The effects of a non-toxic dose of DMS on L cells.

\[ K' = \frac{\mu\text{moles methyl bound} / 10^4 \text{ cells}}{\text{dose DMS (mM)}} \]
Figure 52: The binding of $^{14}\text{C-}\text{DMS}$ to acid insoluble cell fractions of various cell types.

- ▲ CHE cells, dose = 0.02 mM
- ■ AL cells, dose = 0.076 mM
- □ "L" cells, dose = 0.02 mM
- △ AHE cells, dose = 1.5 mM
- ○ "L" cells, dose = 0.67 mM

Figure 53: The relative extent of methylation of DNA, RNA, and protein by DMS and MNNG.

$$K = \frac{\text{µmoles methyl bound / gram cellular constituent}}{\text{dose (mM)}}$$
Figure 54: The percentage of $^{14}$C-methyl from MNNG bound to the TCA precipitate.

- AL (high thiol) cells
- "L" (low thiol) cells

Figure 55: The percentage of $^{14}$C-methyl from DMS bound in the TCA precipitate.

- AL (high thiol) cells
- "L" (low thiol) cells

Figure 56: The percentage of $^{14}$C-methyl from MNU incorporated into acid soluble or precipitable cell fractions.

- AL (high thiol) cells
- "L" (low thiol) cells

Figure 57: The percentage of $^{14}$C-methyl from MNTS incorporated into acid soluble or precipitable cell fractions.

- AL (high thiol) cells
- "L" (low thiol) cells
DISCUSSION

Chapter II: The reactions of methyl-nitroso compounds \textit{in vivo}.

The reactions \textit{in vivo} of the three methyl-nitroso compounds MNUA, MNNG, and MNTS correspond well to their chemical activities \textit{in vitro}. MNUA decomposes, releasing a methylating species, without prior reaction with cellular thiol. MNNG depletes cellular thiol although only at severely toxic doses. This is not surprising since glutathione reductase is an ubiquitous enzyme (Wendell, 1970), and related enzymes for the specific reduction of disulphides in higher-molecular-weight proteins have been reported (Tietze, 1970). The very small amount of disulphide normally present in the L cells of this study confirms the displacement of the equilibrium \[ 2 \text{RSH} \rightleftharpoons \text{RS-SR} + 2\text{H}^+ \] far to the left. It may be that depletion of thiol after MNNG treatment cannot be detected until these enzymes have been completely saturated and are no longer able to keep up with the drug-induced destruction. Thus, although the irreversible depletion of thiol caused by reaction at the alkali-labile site of MNNG would immediately affect the cell, gross depletion of acid-soluble thiol through disulphide formation would not become detectable until very high doses had been administered. The eventual depression of free cellular thiol with concomitant production of MNG, indicates that a considerable proportion of the MNNG-thiol interaction must occur via the
denitrosating, thiol-oxidizing route. The extensive methylation of macromolecules makes it clear that decomposition to a methylating species is also occurring, and accounts for that part of the thiol loss that is not due to disulphide formation.

MNHS, however, forms quite stable complexes with GSH \textit{in vitro} in addition to promoting disulphide formation. The observation that thiol depletion of cells with this agent is rapid, extensive, and is only partially reversed by reducing agents confirms the similarity of the chemical reactions \textit{in vitro} and \textit{in vivo}. Extent of methylation of cellular components is low, consistent with the small proportion of the methylating decomposition route \textit{in vitro}. However, the small amount of methylation that does occur is associated with the nucleic acids, even more selectively than with MNNG, since the nucleophilic groups of protein are obliterated in the initial reaction with the whole molecule.

Comparison of the extent of methylation and the distribution of methyl groups after reaction with thiol-activated MNNG, and direct reaction with DMS, supports the view that many of the effects seen after MNNG treatment can be attributed to its initial reaction with thiol. MNNG, when presented to cells of different thiol content, is much less extensively incorporated by lower thiol cells, which seem to develop a loss of permeability in the presence of thiol-depleting agents.* However, thiol content \textit{per se} may not be solely responsible for the altered permeability. The low thiol cells of this study are in, or close to, the stationary or contact-inhibited phase of the growth cycle. It is

*The fact that on the average AHE cells contain twice as much DNA as CHE cells does not contribute to the higher extent of methylation of nucleic acid in AHE cells, since incorporation is expressed per gram of DNA.
quite conceivable that the membranes of these cells differ from those of their high thiol relatives, quite apart from the difference in thiol content. Nonetheless, MNNG does affect membrane function, an observation that should not be ignored in evaluating the various chemical activities of an active carcinogen.

Commensurate with reduced incorporation, levels of methylation by MNNG of the acid precipitate of low thiol cells, or of DNA, RNA, or protein individually, are depressed well below levels in high thiol cells. DMS, on the other hand, is incorporated by, and binds to, both cell types to similar extents. Further, MNNG preferentially methylates nucleic acids over protein, since nucleophilic groups in protein are consumed during the initial activation step, while DMS is free to methylate protein extensively.

Comparison of the effects of MNNG and DMS is complicated by the lack of complete identity between the ultimate methylating species from MNNG and DMS. The former has some $\text{SN}_1$ character, while the latter reacts through $\text{SN}_2$ mechanisms. Chapter 4 will show that the two agents actually do produce qualitatively different methylated products. In order to eliminate the possibility that the differences between MNNG and DMS result from the qualitative difference in their ultimate methylating species, the comparison is extended to include MNUA and MNTS, agents which contain an $N$-methyl-$N$-nitroso precursor of diazomethane identical to that in MNNG. However, since MNUA has little affinity for thiol, and methylates without prior thiol activation, while MNTS reacts readily with thiol (Chapter I), the influence of reaction with cellular thiol can still be observed.

As might be predicted from the results with DMS, the thiol-
independent agent MNUA is incorporated with no sharp discontinuity by both high and low thiol cells and has little effect on thiol level. On the other hand, incorporation of MNTS, which like MNNG has a strong affinity for thiol, is abruptly halted after a brief initial reaction time. Although the period of incorporation is short, depletion of cellular thiol is nonetheless extensive. This congruity of action of MNUA with DMS, and MNTS with MNNG is reflected in methylation studies as well. MNTS methylates less efficiently than MNNG, but the same preferential affinity for methylation of nucleic acids relative to protein is expressed. However, nucleic acids are methylated by MNUA to about the same extent in high and low thiol cells, and, in high thiol cells, nucleic acids are not preferentially methylated relative to protein. Thus only in the cases involving thiol-depleting agents is cell permeability altered, is the extent of methylation dependent on growth phase, or is distribution of methylation directed preferentially towards nucleic acids. These phenomena are therefore attributed to reaction of the agent with thiol.

In vivo studies confirm the conclusion of chapter I that methylation by MNNG is activated through reaction with thiol. Measurement of the rate of decomposition of MNNG in cells as opposed to rate of decomposition in the surrounding medium, and direct assay of cellular thiol, indicate that intracellular MNNG decomposition is accelerated above its normal hydrolytic rate, with concomitant loss of thiol groups. This requirement for chemical activation could confer upon MNNG an unusual capacity for directed rather than random methylation. Nucleophilic targets relatively close to a thiol-rich site would be
preferentially methylated, since the methylating species, once released, is too reactive to travel far in an aqueous environment.

The promotion of activation of the methylating species of MNNG by thiol might well explain some of the unique physiological properties of this agent. MNNG preferentially mutagenizes the replication point of bacteria, with a specificity sufficient to allow mapping of the chromosome according to the time of appearance of particular mutants after re-initiation of DNA replication in synchronized cells (Cerda-Olmedo and Hanawalt, 1968). Since protein is attached to bacterial DNA specifically at the replicating point (Smith, 1967), one might expect to find enhanced activation of the methylating species at that point. Since the diazomethane so released is a highly active electrophile, it would rapidly methylate DNA at the location of its production, accounting for the unique specificity of MNNG for mutagenizing the replicating center.

It has also been reported that MNNG mutagenizes the intact tobacco mosaic virus (TMV), but is inactive toward isolated TMV-RNA (Singer and Fraenkel-Conrat, 1969). It is possible, first, that without a thiol catalyst, as in the in vitro reaction with purified RNA, the overall extent of methylation is too low, while in the intact virus activation and subsequent methylation can proceed. Second, it is possible that spatial proximity of target nucleotides and thiol groups could direct methylation so that sensitive sites in the RNA would be selectively attacked if intact TMV were methylated. If purified RNA were used the whole RNA polymer would be randomly attacked. The potential for selection of specific sites in vivo might also explain the unusually large proportion of methylated cytosine reported after reaction of MNNG with intact TMV. Finally, one cannot completely ignore
the possibility that mutagenesis might be favoured by the synergistic action of the thiol-oxidizing and methylating capacities of MNNG.
RESULTS

Chapter III: The toxicity of methyl-nitroso compounds.

Section 1: Factors affecting sensitivity.

From preliminary studies it soon became clear that the toxic action of MNNG was determined by several variables and experiments were performed to delineate them.

Variations in toxicity due to culture technique:

MNNG is more toxic if cells are treated in suspension before plating than it is if added to attached plated cells (figure 66), although the time of exposure to the drug is limited to 40 minutes in each case. The efficacy of increasing the time of exposure to MNNG reflects the hydrolytic decomposition of the agent in the medium, and as the first order decay of MNNG proceeds, additional time of exposure has diminishing effect (figure 67). Treatment of cells after attachment is again less toxic than treatment of suspension cultures.

Variations in toxicity due to the metabolic state of the cells:

The culture represented in figures 68 and 69 was initiated by dilution of cells from $2 \times 10^5$ cells/ml to $0.5 \times 10^5$ cells/ml and began to enter stationary phase at a population density of $5 \times 10^5$ cells/ml, after about three cycles of exponential growth with a normal doubling time of about 16 hours. The aliquots of cells treated in suspension before plating (figure 69, ▲) demonstrate increasing sensitivity until
a population density of 1.5 x 10^5 cells/ml is reached, 26 hours after diluting the culture. The suspension culture then becomes increasingly resistant, the surviving fraction increasing by almost 1½ logarithms. The late logarithmic phase "L" cell membrane is relatively impermeable to MNNG and "L" cells incorporate less MNNG than do AL cells (figure 47). This fact could explain the resistance of suspension-treated cells, as the culture moves towards stationary phase.

Combined effects of culture technique and metabolic state:

The survival of cells treated 2 or 3 hours subsequent to settling in the plates (figure 69, 0) fluctuates at low population densities, but increases to a peak at about 4 x 10^5 cells/ml, 54 hours from "activation." Then, unlike the suspension cultures, cells treated after attachment rapidly become more sensitive. This sensitivity of high population density cells treated after attachment is not unexpected, since at the time of plating the cells are diluted. Such dilution of high population density low thiol cultures very rapidly induces metabolic changes including thiol accumulation (figure 71) and a burst of DNA synthesis (table 3b). At the same time sensitivity to MNNG changes from that of the high population density resistant cells ("L") to that of the low population density AL state. The cells treated only one hour after plating demonstrate sensitivity intermediate between cells treated in suspension and cells treated after attachment, as would be expected. The plating efficiency of the controls is close to 100% at all population densities.

Figure 70 demonstrates that CHE cells are less sensitive to MNNG than AHE, in accord with the observation that CHE cells incorporate and bind a smaller proportion of the dose (figure 53A).
The foregoing data demonstrate that both culture technique and population density significantly affect the sensitivity of the cells to MNNG. Several repetitions of these experiments revealed that the population density had to be controlled for as much as five days prior to treatment with MNNG in order to obtain reproducible results. For example, the culture represented in figure 71 was in stationary phase at a population density of $6.4 \times 10^5$ cells/ml prior to dilution to $0.5 \times 10^5$ cells/ml at the beginning of the experiment. The cells required 12 hours to reach maximum thiol level. The culture represented in figure 69, however, was obtained from a stock at about $2 \times 10^5$ cells/ml and therefore already had maximum thiol content at the beginning of the experiment. The time of lowest survival for the culture of figure 71 is 12 hours later than for the culture of figure 69, a time lag which corresponds to the time required to accumulate thiol. Thus, to reproduce a given toxicity estimate it is necessary to control not only culture technique and population density, but also the prior history of the cells from the preceding stationary phase.

Although the above observations might tend to suggest some relationship between thiol metabolism and toxicity, the correlation is not quantitative. During early logarithmic growth (6-36 hours), thiol content and resistance rise together, suggesting a protective role for thiol groups. However, the subsequent minimum in survival occurs before the drop in thiol content, suggesting that sensitivity is the result of some metabolic transition, and that the decrease in thiol content is also a result and not a cause of this transition.

Sensitivity to MNNG shows fluctuations through the cell cycle in addition to those described for the growth cycle. After release of FUdR
inhibition by the addition of thymidine, synchronized cells progress around the cell cycle (figure 72). Although increase in thiol content precedes mitosis, the correlation between thiol content and toxicity is poor. However, the changes in survival do seem to correspond to cell cycle phase at the time of treatment. An increase in sensitivity to MNNG is evident during S-phase, followed by greater resistance as cells progress to G₂, mitosis, and G₁.

Comparison of MNNG with other methylating agents:

The diversity of toxicity seen after MNNG treatment of L cells cannot be observed when the various cell types are treated with DMS. The only variation is the introduction of a small shoulder into the survival curve of mid-exponential phase L cells (figure 73). Overall, with L or HE cells (figure 74), DMS is less toxic than MNNG by a factor of almost 20.

As in the study of chemical effects, the qualitative as well as the quantitative difference between the methylating species of MNNG, and DMS must be considered in interpreting differences in biological effect. However, comparison of MNNG with MNUA and MNTS provides assurance that, with respect to toxicity, the character of the methylating species is far less relevant than reactivity towards thiol. MNUA, which like DMS, has little affinity for thiol, is approximately 100 times less toxic to "L" cells than MNNG (figure 75), although both release diazomethane. MNTS, which binds to and oxidizes cellular thiol, is slightly less toxic than MNNG, which both oxidizes thiol and methylates, but is ~ 10 times more toxic than MNUA (figure 76).

The augmented killing potential of MNNG and MNTS cannot be attributed to the denitrosated products MNG and MTS since these, when
added directly in purified form, are totally harmless (e.g. figure 76).

In vitro chemical studies indicated that thiol oxidation occurs as a fairly slow reaction, and continues for some time after its initiation by the nitroso agent. In order to determine whether thiol oxidation is itself a toxic event, the thiol-reducing agent DTT was used. Cells were first treated in suspension with MNNG for 40 minutes, and after removal of MNNG, the treated cells were plated into medium free of thiol except for 10^{-5} M DTT. After two hours the DTT medium was replaced with normal medium. If the imposition of thiol oxidation is toxic, then DTT reduction should alleviate the damage. The data of figure 77 support the conclusion that thiol oxidation contributes to MNNG toxicity since DTT has marked palliative influence on MNNG killing.

Section 2: The independence of the toxicity of methyl-nitroso compounds and extent of methylation.

Interpretation of the toxicity data is complicated by the variations observed in the proportion of the drug that actually enters the cells. When judging the efficacy of a compound, the dose incorporated is a more meaningful parameter than dose in the external medium. However, the sum of radioactivity detectable in the TCA-soluble and -precipitable cell fractions is also misleading, since a great deal of the radioactivity in the soluble fraction represents relatively harmless methanol. Toxicity has therefore been correlated to radioactivity bound to the TCA precipitate only, and to DNA, since DNA is considered a critical target of carcinogens.

When toxicity is related to dose actually incorporated (figure 78),
the pronounced sensitivity of cells to both thiol-active compounds (MNTS and MNNG) as opposed to the resistance of cells to the thiol-indifferent compounds (DMS and MNUA) is manifest. MNTS and MNNG are 10 to 100 times more toxic than DMS or MNUA at equivalent extents of binding.

The acute toxicity of the two thiol-active agents, relative to the agents which methylate only, is again clear when loss of colony-forming ability is expressed in terms of methylation of DNA (figure 79). Cells can tolerate a 100-fold increase in DNA methylation if MNUA is the methyl source, rather than MNTS. Although methylation of nucleic acid may well be requisite for carcinogenesis and mutagenesis by these agents, methylation cannot quantitatively account for the destruction of colony-forming ability.
Figure 66: The effect of culture technique on toxicity of MNNG.

- treated after attachment to the plates.
- treated in suspension.

The cells had been grown from 0.5 to about $1.0 \times 10^5$ cells/ml before treatment and colony assay.

Figure 67: The effect of time of exposure to MNNG (3 ug/ml) on suspended and attached cells.

- treated after attachment; initial suspension was grown to $1.8 \times 10^5$ cells/ml.
- treated in suspension after growing to $1.2 \times 10^5$ cells/ml.
Figure 68: Growth of L cells.

Figure 69: Variations in toxicity of MNNG with population density and culture technique.

- cells plated 1 hour before treatment.
- cells plated 2 or 3 hours before treatment.
- cells treated in suspension, before plating.

Dose = 3 ug/ml.

The culture used was diluted at zero time from a stock of high thiol content at about 2 x 10^5 cells/ml.
GROWTH OF L CELLS

![Growth of L Cells Graph](image)

VARIATIONS IN TOXICITY OF MNNG WITH POPULATION DENSITY & CULTURE TECHNIQUE

![Variations in Toxicity Graph](image)
Figure 70: Sensitivity of HE cells to MNNG.

0       CHE

●       AHE

Each point represents the average of 4 replicates. Cultures from two hamsters are shown.
FIGURE 70

SURVIVING FRACTION

10^0

10^-1

10^-2

10^-3

0.005 0.01 0.015 0.02 0.025

DOSE MNNG (mM)
Figure 71: Variations in toxicity of MNNG and thiol content of L cells throughout the growth cycle.

The culture used was diluted at zero time from a stock in stationary phase at $6.4 \times 10^5$ cells/ml.
Figure 72: Variations in toxicity of MNNG and thiol content of L cells around the cell cycle.

DNA synthesis was blocked with FUdR at -14 hours. After addition of thymidine at time "0", 90% of the cells proceeded immediately into a 4-hour S-phase. There followed a short G2 period and a burst of cell division. Synchrony decayed during the second cycle. (Walker and Thatcher, 1969)
FIGURE 72
VARIATIONS IN TOXICITY OF MNNG & THIOL CONTENT OF L CELLS AROUND THE CELL CYCLE

GROWTH OF SYNCHRONIZED L CELLS

ACID SOLUBLE THIOL

TOXICITY OF MNNG 2 μg/ml
Figure 73: The effect of population density on toxicity of DMS.

- 1.05 x 10^5 cells/ml
- 3.3 x 10^5 cells/ml

Figure 74: The sensitivity of HE cells to DMS.

- CHE
- AHE
Figure 75: The toxicity of MNUA.
- AL, $1.3 \times 10^5$ cells/ml.
- "L", $4.83 \times 10^5$ cells/ml.

Figure 76: The toxicity of MNTS.
- AL, $1.3 \times 10^5$ cells/ml.
- "L", $4.83 \times 10^5$ cells/ml.
- treated with the denitrosated product of MNTS, methyltoluenesulphonamide (MTS), AL or "L" cells.

* Population density to which the cultures had grown at the time of treatment.
Figure 77: Dithiothreitol protection from MNNG.

- $10^{-5}$ M DTT in thiol-free medium for 2 hours subsequent to reaction with MNNG for 40 minutes.

- no DTT.
FIGURE 77

DTT "PROTECTION" FROM MNNG

![Graph showing survival fraction against μg MNNG/mL]
Figure 78: Toxicity related to incorporation into the TCA precipitate.

- AL cells
- "L" cells

Figure 79: Toxicity related to methylation of DNA.

- AL cells
- "L" cells
DISCUSSION

The study of the toxicities of the four methylating agents MNUA, MNNG, MNTS, and DMS provides two striking observations. First, the relationships between toxicity and extent of methylation summarized in figure 78 indicate clearly the importance of the source of the methyl groups in determining toxicity. Methylation of cells by DMS or MNUA is 10 to 100 times less toxic than methylation by MNNG or MNTS. Thus, extent of methylation alone cannot explain the toxicity of these agents. A second feature of the toxicity studies is the dependence of sensitivity to a single agent on the precise condition of the cells at the time of treatment.

With regard to the first of these variations in toxicity, a simple correlation is immediately apparent. The two highly toxic agents react readily with thiol groups both in vitro and in vivo (Chapter 2), while the less toxic pair of methylating agents are relatively inactive towards thiol. It seems reasonable therefore, to attribute the excessive loss of colony-forming ability caused by MNNG and MNTS to interaction with cellular thiol.

Two possible explanations for the enhanced toxicity of the thiol-reactive agents will be discussed here. The first is based on the potential of thiol-activated agents for non-random methylation. While MNUA decomposes spontaneously under aqueous conditions and would therefore methylate at random, MNNG and MNTS decompose through reaction with cellular thiol groups. However, once released the methylation species is highly reactive, and will methylate either a cellular component or water
in the immediate vicinity of its generation. Methylation by thiol-activated agents will therefore be directed towards any thiol-rich areas of the cells. If it so happens that the sites selectively methylated because of their proximity to thiol groups are also especially sensitive sites, where methylation is more likely to cause loss of colony-forming ability, then thiol-activated agents would have enhanced toxicity.

However, the chemical studies of Chapters 1 and 2 indicate that MNNG and MNTS react with thiol not only at the alkali-labile site to release a methylating species, but also at the acid-labile site. The latter reaction results in the oxidation of thiol. That this thiol oxidation is itself the cause of the excessive toxicity of MNNG and MNTS seems a much simpler explanation.

The former postulate, based on non-random methylation, requires the assumption that the selected sites are also the most sensitive ones -- a fortuitous association for which there is no evidence. The postulate also requires the distribution of thiol groups to be asymmetric, so as to favour methylation of certain areas. In bacteria, there is evidence for such asymmetry, since the DNA is associated with protein specifically at the replicating point (Cerda-Olmedo and Hanawalt, 1968). As discussed earlier, this could account for the specificity of MNNG for mutagenizing at the replicating point. In the studies of methylation of RNA in intact TMV (Singer and Fraenkel-Conrat, 1969) there is also some evidence to indicate asymmetric distribution of activating thiol groups since the quantitative extent of methylation of cytosine far exceeds both that predicted by the relative chemical reactivity of cytosine, and that observed when the RNA is treated in purified form. However, for the mammalian system of the present work there is as yet no evidence for asymmetric distribution of thiol groups along the DNA. The methylation
studies of Chapter 2 do suggest that the high thiol level of AL cells directs methylation to DNA rather than protein. However, there is nothing to suggest that certain parts of the DNA are selectively methylated. Further, the quantitative studies of Chapter 4 will show that the proportions of the various methylated bases are similar for MNNG and MNUA, and are quite in accord with chemical reactivities, unlike the findings of Singer (1969) for TMV. Therefore, both the assumptions underlying the postulate that thiol-activated agents are more toxic because they methylate specific sensitive sites remain unsubstantiated.

There is, however, considerable evidence to support the idea that thiol-oxidation accounts for the excess toxicity of thiol-activated agents. First, it is shown in Chapter 2 that MNNG and MNTS do in fact oxidize thiol in vivo. Second, the known importance of thiol and disulphide groups in the functional and structural integrity of protein (Mahler and Cordes, 1966), in permeability (Sutherland, et al, 1967), and the operation of the replication cycle (Ord and Stocken, 1968) make it quite plausible to suggest that thiol-oxidation could be toxic. Third, figure 78 shows that AL cells with a higher thiol content are less prone to the toxic effects of MNNG than lower thiol cells. This observation is consistent with the view that a high thiol level simply buffers the detrimental thiol-oxidation. The protection observed here is not due to inactivating or "mopping up" of the alkylating agent, since toxicity is expressed relative to extent of cell methylation, and not to the external concentration of agent. Further, the protection offered by thiol groups cannot be due to some interaction which alleviates the effects of methylation, since high thiol cells are not protected from the effects of MNUA.

Fourth, the presence of the thiol-reducing agent DTT after reaction with MNNG alleviates the toxicity. Again, the protection
cannot be ascribed to inactivation of the methylating agent since both the activation of the methylating species and its subsequent reaction with the cell are complete before the first contact with DTT.

Finally, study of the nature of methylated products of $^{14}$C-methyl MNTS suggests that thiol-oxidation and not directed methylation must be the cause of the added toxicity of thiol-activated agents. Chapters 1 and 2 indicate that MNTS reacts extensively by the thiol-oxidizing route, and very little by the methylating route. Evidence to be presented in Chapter 4 indicates that unlike MNNG and MNUA, there is no detectible methylation of the purine bases of DNA after treatment with $^{14}$C-methyl MNTS. The radioactivity associated with the DNA is apparently in a much more labile form, although the experimental techniques may have failed to detect very small amounts of the methylated bases. However, if one assumes that the added toxicity of MNNG and MNTS over DMS and MNUA is due to thiol-directed methylation, why should a very small amount of methylation caused by MNTS be just as toxic as the equally directed but extensive methylation caused by MNNG? The only simple and consistent explanation of all the observations is that thiol-oxidation is itself a toxic event.

Vulnerable cellular SH sites:

Since the $2\text{SH} \rightleftharpoons \text{S-S}$ equilibrium is shifted far to the left, oxidation of the major SH components is rapidly reversed by the cell (figure 41) until the reducing system is saturated. However, a super-toxic dose of MNNG is required to achieve saturation. Nonetheless, if there is even a very small number of SH sites for which there are no reducing enzymes, these would be sensitive to low doses, despite the fact that overall cellular thiol would not be measurably diminished. Presumably additional thiol would competetively protect these sites as well as the "bulk" SH sites, and reduce toxicity.
Reduction of MNNG toxicity was observed with only $10^{-5}$ M DTT. It is unlikely that the tiny amount of cysteine or GSH produced from cystine or GSSG could possibly have any biological effect when added to the approximately $6 \text{ mM}$ SH already in the cell. I therefore attribute DTT protection to the direct reduction of specific sites in the cell, presumably those that do not have an enzymatic reducing system.

The DNA polymerase shown (Anderson and Burdon, 1970) to be inhibited by MNNG in a manner reversible by cysteine may be one sensitive site. However, the dose of MNNG shown to inhibit polymerase in vitro was far greater than a lethal dose in vivo. Either a tiny amount of polymerase inactivation is lethal, or polymerase is only one of a number of SH-containing systems which would be affected by MNNG. I think the latter most likely.

SH groups have been implicated in the control of cell replication (Ørd and Stocken, 1968). However, a lot more will have to be known about the nature of the SH groups involved in "turning on" cells before anything can be said about the effect of MNNG on these. Nonetheless, in the light of the marked dependence of MNNG toxicity on the metabolic state of the cells, it is reasonable to suggest that these SH groups might be highly sensitive to MNNG.

Segregation of the causes of lethal and heritable changes:

Assignment of a lethal role to oxidation of cellular thiol is not intended to imply that this single chemical reaction is responsible for all of the biological effects of methyl-nitroso compounds. Rather, I suggest that toxic and carcinogenic reactions are different. Thiol-oxidation largely accounts for the toxicity of an agent, while ability to methylate is correlated to carcinogenic potency (see Introduction).
This assignment of different biological effects to different chemical reactions can also be applied to the fungal system studied by Delić, Hopwood, and Friend, (1970). These authors report that mutagenesis of \textit{Streptomyces coelicolor} increased as the decomposition of MNNG was promoted by increasing alkalinity. The genetic alterations induced by MNNG are therefore proportional to the production of methylating species. However, at acid pH (2.5) mutagenesis was weak, while lethality was high. Since at pH 2.5 MNNG decomposes almost entirely by the thiol-oxidizing route, the increased toxicity at acid pH might again be due to thiol-oxidation.
RESULTS

Chapter IV: The role of methyl-nitroso compounds in the induction of genetic change.

Section 1: The sites of methylation of DNA by MNua, MNNG, MNTS, and DMS in vitro and in vivo.

\( \text{O}^6 \)-MG was synthesized by the method of Balsiger and Montgomery (1960) and the characteristic absorption spectra of the product at three pH values are shown in figure 80. Figure 81 reproduces the spectra of the \( \text{O}^6 \)-ethylguanine (\( \text{O}^6 \)-EG) and the parent 6-chloro-2-aminopurine at neutral pH. The spectral properties and chromatographic behaviour of the three compounds are summarized and compared to published results in table 7. Although the methyl and ethyl derivatives cannot be distinguished on the basis of their ultraviolet spectra, 6-chloro-2-aminopurine has a different spectrum. A clean separation of all three is achieved by paper chromatography.

Conditions allowing retention of the methyl group while at the same time liberating the free base from DNA must be used in order to estimate the extent of occurrence of the \( \text{O}^6 \)-methylated product. At pH 0.93 and 24°C the riboside bond is slowly hydrolyzed, yielding the free base (figure 82). The methyl group is more resistant to acid hydrolysis. In 0.1 N HCl at 97°C demethylation of \( \text{O}^6 \)-MG to yield guanine proceeds with a half-life of about 1.4 hours; increase of acidity to 1.0 N HCl markedly accelerates demethylation (figure 83); reduction of the
TABLE 7

Spectral properties of guanine derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>λ_max</th>
<th>λ_min</th>
<th>ε x 10^{-3}</th>
<th>LITERATURE *</th>
<th>λ_max</th>
<th>ε x 10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>6'-methylguanine</td>
<td>1.0</td>
<td>286</td>
<td>252</td>
<td>11.0</td>
<td>286</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>240,280</td>
<td>257</td>
<td>7.8,7.8</td>
<td>240,280</td>
<td>7.88,7.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.0</td>
<td>282</td>
<td>257</td>
<td>7.9</td>
<td>284</td>
<td>7.86</td>
<td></td>
</tr>
<tr>
<td>6'-ethylguanine</td>
<td>7.0</td>
<td>240,280</td>
<td>256</td>
<td>-</td>
<td>240,280</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6-chloro-2-aminopurine</td>
<td>7.0</td>
<td>240,306</td>
<td>260</td>
<td>-</td>
<td>241,308</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>


R_f on paper in isopropanol:ammonia:water, 7:1:2

<table>
<thead>
<tr>
<th>Compound</th>
<th>R_f</th>
</tr>
</thead>
<tbody>
<tr>
<td>6'-methylguanine</td>
<td>0.64</td>
</tr>
<tr>
<td>6'-ethylguanine</td>
<td>0.72</td>
</tr>
<tr>
<td>6-chloro-2-aminopurine</td>
<td>0.55</td>
</tr>
</tbody>
</table>
temperature improves stability of the compound, and at pH 1.0 and 37°C the compound is stable for at least 50 hours. Lawley (1966) has shown that hydrolysis at pH 7.0, 100°C, for 20 minutes releases 7-methylguanine (7-MG) and 3-methyladenine (3-MA). To improve the separation between 06-MG and the other methylated purines during chromatography, DNA can first be subjected to the neutral hydrolytic conditions, and then acidified. Hydrolysis of the resulting precipitate in 0.1 N HCl, at 37°C for 16 hours releases the residual 7-MG and 3-MA and the bulk of the 06-MG. Alternatively, DNA can be hydrolysed in 0.1 N HCl directly.

Reaction of salmon sperm DNA with MNNG in the presence of 5 mM cysteine, and mild acid hydrolysis of the methylated DNA gives a blue fluorescent spot of Rf identical with that of 06-MG, and ultraviolet absorption spectra identical with those of the authentic base (figure 84). When 14C-methyl MNNG is used, a peak of radioactivity at the Rf of 06-MG is consistently observed in hydrolysates of 14C-methylated DNA produced under neutral or mild acid conditions (figures 85, 86, 87).

More vigorous hydrolysis with perchloric acid at 100°C, which is known to demethylate the base (Friedman et al, 1965) also destroys the 06-MG residues in 14C-methylated DNA (figure 91).

Denaturation of DNA by heating to 100°C and rapidly cooling prior to treatment with 14C-MNNG in the presence of cysteine (figure 88) alters only the amount of 6-methylaminopurine (6MAP), a rearrangement product from 1-methyladenine, which is more exposed to the methylating agent after destruction of the hydrogen-bonding in the native double-stranded structure (Lawley, 1963). The proportion of 7-MG, 3-MA, and 06-MG are similar after treatment of native or denatured DNA.

It was noted in Chapter I that decomposition of MNNG in tris rather than phosphate buffer occurred entirely at the base-catalysed
site yielding diazomethane and nitrocyanamide. In a solution at pH 7.4, 37°C, containing salmon sperm DNA (4 mg/ml), MNNG (3.6 mM), N-acetylcysteine (5 mM), but tris-HCl (10 mM) instead of phosphate buffer, the reaction which in phosphate buffer is complete in 20 minutes, is surprisingly slow. Some of the yellow colour of the MNNG persists 24 hours and assay for free thiol with DTNB remains positive. However, overall extent of methylation of DNA is about the same ($K_{DNA} = 5.3$) as in phosphate buffer ($K_{DNA} = 6$), and assay of the methylated purines from the DNA indicates a similar distribution of label, but with slightly more methylation at the $O^6$-position of guanine. Apparently, as it did in vitro, tris buffer permits reaction of MNNG at the alkali-catalysed site only.

Enzymatic hydrolysis (Hall, 1967) of DNA methylated with $^{14}C$-MNNG yields the deoxyribonucleoside of $O^6$-MG ($O^6$-MGdR, figure 89) at an early stage in the digestion. After 3 hours digestion, most of the radioactivity remains at the origin, but the peak at the position of the $O^6$-MGdR marker is already pronounced. Samples digested for 16 hours contain large amounts of the other methylated deoxyribonucleosides in addition, and the $O^6$-MGdR is the minor product. This early release of the $O^6$-MGdR indicates the relative lability of the deoxyribosyl bond of this product to enzymatic hydrolysis. On the other hand, this bond is more stable during chemical hydrolysis than that linking 7-MG or 3-MA to the sugar-phosphate backbone.

When DNA is methylated in vitro with di-$^{14}C$-methylsulphate paper chromatography of hydrolysates obtained at pH 7.0, with 0.1 N HCl, or with perchloric acid shows the presence of $^{14}C$-labelled 7-MG and 3-MA as products but not $O^6$-MG (figures 90, 92, and table 8). The proportion of 3-MA in DNA methylated by dimethylsulphate is consistently higher than that in DNA methylated by MNNG (table 8).
Products from methylation of DNA by $^{14}$C-MNNG or $^{14}$C-DMS in vitro. Proportions of the bases in DNA hydrolysates are given as percentages of the total radioactivity recovered after chromatography.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Extent of methylation (mmol CH$_3$/mol DNAP)</th>
<th>Method for hydrolysis</th>
<th>Method for chromatography</th>
<th>MNNG</th>
<th>DMS</th>
<th>$R_f$ solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HC$_2$O$_4$</td>
<td>HC$_2$O$_4$</td>
<td>pH1</td>
<td>pH1</td>
</tr>
<tr>
<td>7-methylguanine</td>
<td></td>
<td></td>
<td>a</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>3-methyladenine</td>
<td></td>
<td></td>
<td>3</td>
<td>11</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>7-MG + 3-MA</td>
<td></td>
<td></td>
<td>83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_6$-methylguanine</td>
<td></td>
<td></td>
<td>&lt;0.2</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>3-methylcytosine</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-methyladenine</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Salmon sperm DNA was treated at neutral pH with GSH added for the reaction with MNNG, and the DNA was isolated as described in the Methods section. The DNA was hydrolysed with 72% w/v perchloric acid for one hour at 100°C, or with 0.1 N HCl at 37°C for 16 hours (denoted as pH1), or by treatment at pH7 at 100°C for 20 minutes, followed by precipitation of the polynucleotide residue with cold 0.1 N HCl. Hydrolysates were chromatographed with Dowex 50 H$^+$ form (denoted a), or on paper with solvent 1, 3, or 6.
Other differences in proportion of methylated products from MNNG and DMS occur. In the Dowex 50 column chromatography of perchloric acid hydrolysates of DNA (figures 91 and 92) more unidentified minor peaks are found with $^{14}$C-MNNG than with $^{14}$C-DMS. One is eluted near thymine; one is eluted near cytosine, and is not identical with the known product 3-methylcytosine; and another is eluted near guanine, separated from the single main peak of 7-MG and 3-MA, and distinct from 1-methyladenine, which precedes adenine.

Methylation by MNNG or DMS (figures 93 and 94) of L cells in vivo followed by isolation, neutral or mild acid hydrolysis, and paper chromatography of the DNA, yields the same proportions of the methylated purines as the in vitro experiments (table 9). MNNG, but not DMS, gives rise to $^6$-MG. Further, if MNUA is substituted for MNNG, the proportion of radioactivity occurring in the $^6$-MG position is equivalent to that obtained from MNNG. However, recovery of $^6$-MG is improved if the DNA is hydrolysed at pH 7.4, 100°C for 20 minutes (figure 92 and table 9). A product "X" released by hydrolysis at pH 7.4, 100°C is apparent at $R_f = 0.8$ in figure 95. Further liberation of "X" occurs on hydrolysis of the neutral residue with 0.1 N HCl at 37°C, but no radioactivity occurs in the position of "X" if the DNA is hydrolysed in 0.1 N HCl directly. The production of "X" occurs with concomitant decrease in the proportion of 7-MG. Slow ring fission of 7-MG at pH 7.4, 37°C has been reported (Kriek and Emmelot, 1964), and "X" is therefore assumed to be such a rising-opened product.

After addition of $^{14}$C-MNNTS to a suspension of L cells radioactivity becomes associated with nucleic acids ($K_{DNA} = 0.3$). However, after subjecting this DNA to the same hydrolytic conditions used for liberation of methylated purines after MNNG or MNUA treatment, all of the radioactivity released, even in 0.1 N HCl at 37°C, travels with the
TABLE 9

Products from methylation of DNA in L cells treated in vivo with $^{14}$C-MNNG, di-$^{14}$C-methylsulphate, or $^{14}$C-MNUA.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>MNNG</th>
<th>DMS</th>
<th>MNUA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extent of methylation&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.3</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Method of hydrolysis</td>
<td>HC10&lt;sub&gt;4&lt;/sub&gt; HC10&lt;sub&gt;4&lt;/sub&gt; pH1 pH1 pH7</td>
<td>HC10&lt;sub&gt;4&lt;/sub&gt; pH1 pH1</td>
<td>pH1 pH7.4</td>
</tr>
<tr>
<td>Method for chromatography</td>
<td>a 6 6 6 6</td>
<td>6 3 6</td>
<td>6 6</td>
</tr>
<tr>
<td>7-methylguanine</td>
<td>70 71</td>
<td>84 80 71</td>
<td>70 61</td>
</tr>
<tr>
<td>3-methyladenine</td>
<td>7 7 7 7</td>
<td>15 15 11</td>
<td>8 7</td>
</tr>
<tr>
<td>7-MG + 3-MA</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G^6$-methylguanine</td>
<td>&lt;0.6 6 6</td>
<td>&lt;0.5 &lt;1 &lt;1</td>
<td>7 14</td>
</tr>
<tr>
<td>3-methylcytosine</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-methyladenine</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After treatment of L cells suspended in growth medium at 37°C for 40 minutes, DNA was isolated, and hydrolysed either with 72% w/v perchloric acid for 1 hour at 100°C, or with 0.1 N HCl at 37°C for 16 hours (denoted as pH1), or by treatment at pH7 at 100°C for 20 minutes, followed by precipitation of the poly-nucleotide residue with cold 0.1 N HCl. Methods for chromatography of $^{14}$C-methylated bases were with Dowex 50, H<sup>+</sup> form (2), or by descending paper chromatography with solvents (1), (3), or (6).

Proportions of the bases in the hydrolysates are given as percentages. *mmol CH<sub>3</sub> / mole DNAP
solvent front in either solvent (6) or (4). There is no evidence of formation of 7-MG, 3-MA, or $\text{O}^6$-MG (figure 96). Thus, as was observed in the TCA precipitate of cells after MNTS treatment, the label seems to be present in loose complexes rather than as actual methylated products.

The evidence presented here supports the view that the ability to methylate such weakly nucleophilic sites as the 6-oxygen atom of guanine may be involved in carcinogenesis, since both powerful carcinogens (MNNG and MNUA) give rise to $\text{O}^6$-MG in vivo, but neither DMS nor MNTS, both very weakly or non-carcinogenic agents, yields the $\text{O}^6$-methylated product.

Section 2: Some biological effects of $\text{O}^6$-methylguanine.

The ability of MNNG to methylate weakly nucleophilic sites, in particular $\text{O}^6$ of guanine, distinguishes MNNG from the less carcinogenic methylating agent DMS. In previous experiments the $\text{O}^6$-MG occurs after methylation of the preformed DNA macromolecule. Here, some biological effects of treating cells with $\text{O}^6$-MG as a free base are reported.

In order for hamster embryo cells to establish growth in tissue culture at least $10^6$ cells/5 cm diameter plate are required. With lower seeding the culture rapidly dies out, although secondary cultures which have been resuspended from successfully established primary cells will grow from a much lower seeding. In figure 97 A and B the inability of the control primary cells to establish continued growth after low seeding is apparent. The culture treated with a dose of MNNG known to leave 50% survivors (0.5 µg/ml) is also incapable of growth. After treatment with 50 µg/ml $\text{O}^6$-MG however, growth begins immediately, and
is maintained by subculture every 4 days, the time required for normal secondary cultures to approach confluence. The fact that the growth curve is very nearly exponential from day zero indicates that capacity for replication and division is conferred upon a large fraction of the cells. Further, the growth rate is identical to that observed for established secondary cultures (figures 37 and Chapter II).

If the size of the initial inoculum of cells is increased (figures 97 C, D, and E), the ability of control cultures to multiply improves, until at a seeding of $10^6$ cells/5 cm diameter surface, control cultures with capacity for many generations of growth, and tolerance toward regular trypsin suspension, dilution, and replating are established. However, none of the cultures, treated or control, are capable of indefinite growth, although treated cultures persist much longer than controls. Both treated and control cells show contact inhibition at the mass culture level (figure 97 E, days 14 to 35), although microscopic examination shows that treated cells tend to cross one another more frequently. The major effect of the $6^\text{th}$-MG appears to be during the initial adjustment of the cells to the new \textit{in vitro} conditions. During this period, treatment with the modified base may somehow confer upon the cells the ability to utilize what genetic information is necessary to adapt to the challenge of the \textit{in vitro} environment.

The results represented in figures 97 C and D are essentially the same, although the experimental conditions differed in four ways. First, in case C highly differentiated embryos were used one day before the parturition date, while in case D, less developed 11 day embryos were used; second, the initial inoculum was $2 \times 10^5$ cells for case C and $5 \times 10^5$ cells for case D; third, in case C, the agent was added directly
to the drained cells, which were then immediately flooded with medium, as recommended by Kuroki and Sato (1968), while in case D, the agent was added to the medium; finally, in case C, the cells were in contact with the agent for only 3 days, while in case D, the agent was present for 14 days. It appears that these differing conditions do not materially change the results, although the prolonged exposure in case D may have suppressed growth of this culture initially.

In Case E, although both treated and control cultures grow after an initial seeding of $10^6$ cells/plate, and both display contact inhibition when left without subculture, microscopic examination of stained duplicates reveals morphological differences similar to those seen in case C. Treated cells are larger, more irregular in shape, and do not display the orderly orientation into parallel streams characteristic of normal hamster embryo cultures (plates 1 and 2).

Assay of colony-forming ability of single cells supports the conclusion that $\text{O}^6$-MG has its effect during the initial challenge of the in vitro environment. The plating efficiency of $\text{O}^6$-MG treated primary HE cells is elevated 40 percent relative to that of the solvent-treated controls (figure 98).

Study of various other systems verifies the uniqueness of $\text{O}^6$-MG in elevating plating efficiency. First, treatment of established secondary cultures causes no increase in plating efficiency; second, the malignant L cell line, which has $0.9 \pm 0.1$ plating efficiency normally, is not affected by $\text{O}^6$-MG; and finally, the corresponding ethyl derivative, $\text{O}^6$-ethylguanine, does not "salvage" primary cells, but is actually toxic to both primary and secondary cells (figure 98).

It is noteworthy that while dimethylnitrosamine (Huberman, Salzberg, and Sachs 1968) and $\text{O}^6$-MG induce the increase in cloning
Plate 1: Control culture, figure 1E.

Primary hamster embryo cells were seeded at $10^6$ cells/plate, and were treated with 0.1 ml water added to 10 ml medium at the time of seeding. After 24 hours the medium was removed, the plates were washed with PBS, and untreated medium was substituted. The culture was subcultured five times over a period of 44 days, and the photograph was taken on day 48 four days after the last subculture. Note the few giant cells, and the high degree of orderly "streaming".

Plate 2: $O_6$-methylguanine treated culture, figure 1E.

The culture was manipulated as for plate 1, but was treated with 0.1 ml of a water solution of $O_6$-MG (5 mg/ml) in 10 ml medium at the time of seeding. Note that many of the cells are larger than in the control culture, that cells cross each other and pile up, and that "streaming" is much less pronounced.

Magnification x200
efficiency and the increase in duration of viability which are diagnostic of the early stages of transformation, MNNG, a potent carcinogen in the rat does not. The difference may be due to the added toxicity of MNNG which has been ascribed to its interaction with cellular thiol groups (Chapter III). The probability of observing a transforming event with MNNG is considerably reduced since the event must not just occur, but occur in one of the cells which escapes lethal damage.

Section 3: The incorporation of 2-amino-6-methoxypurine by L cells.

That MNUA, MNNG, and DMS all methylate pre-formed macromolecules is clearly demonstrated in section 1. However, the observance of a biological effect induced by addition of the free base $\text{O}^6$-MG to the medium surrounding the cells indicates that not only could methylation of the nucleic acid polymer induce genetic changes, but so also could the methylation of small soluble precursors. However, it is necessary to determine whether the $\text{O}^6$-MG survives the steps leading to its incorporation into high molecular weight polynucleotides.

A sample of 20 mg of $\text{O}^6$-MG was labelled with a specific activity of 5.9 mCi/mg by tritium exchange. The resulting product is slightly charred, but chromatography in solvent (6) shows a single main peak at the $R_f$ of the original material, with only a slight contaminant at the position of guanine (figure 99). The material at $R_f = 0.58$, after elution into water, contains 0.15 mCi/ml, and is the starting material for all experiments.

The distribution of radioactivity after treatment of L cells with tritiated $\text{O}^6$-MG ($^3\text{H}-\text{O}^6$-MG) is summarized in table 10. With increasing time of exposure increasing amounts of radioactivity are
TABLE 10

The incorporation of $^3$H-$\text{O}^6$-methylguanine by L cells.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Dose (ug/ml)</th>
<th>Specific Activity (mc/mmole)</th>
<th>Incubation Time (minutes)</th>
<th>% Dose Incorporated</th>
<th>Distribution of $^3$H Incorporated</th>
<th>Relative Radioactivity</th>
<th>Extent of Binding *mole $\text{O}^6$-MG/$10^8$ mole DNAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCA</td>
<td>SN</td>
<td>TCA ppt</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>91</td>
<td>10</td>
<td>2.0</td>
<td>98</td>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>91</td>
<td>20</td>
<td>-</td>
<td>83</td>
<td>17</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.6</td>
<td>120</td>
<td>4.7</td>
<td>78</td>
<td>22</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>0.1</td>
<td>120</td>
<td>1.6</td>
<td>94</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

*In making this calculation, it was assumed that all radioactivity detectable was in the form of $\text{O}^6$-MG.
incorporated by the cells. After 10 minutes exposure two percent of the radioactivity is associated with acid precipitable material, but incorporation into this macromolecular fraction rises rapidly from 10 to 20 minutes, with some further increase from 20 to 120 minutes. Isolation of DNA, RNA, and protein from the treated cells and assay of radioactivity per gram of component indicates preferential labelling of nucleic acid relative to protein, the difference being greater when a short pulse of high specific activity material is used. Because of the high degree of fidelity associated with DNA replication it is surprising to note that the proportion of radioactivity associated with DNA is higher than that in RNA after a short exposure time. Subsequently, the RNA becomes more extensively labelled than the DNA.

After 10 minutes incubation in vivo with $^3$H-$^6$-MG, and removal of the cells by centrifugation, chromatography of the medium in solvent (6) reveals only a single radioactive peak, coincident with marker $^6$-MG (figure 100 A) indicating that extracellular degradation of the $^6$-MG does not occur. Further, $^6$-MG is the only detectible labelled product in the TCA supernatant of the cells (figure 100 B) indicating little intracellular metabolism of the base as well. On the other hand, neutral or mild acid hydrolysis of the purified DNA and RNA reveals considerable metabolic activity (figures 100 C and D). After 10 minutes incubation in vivo, radioactivity from dilute acid hydrolysates of either DNA or RNA co-chromatographs with the blue fluorescence of $^6$-MG marker indicating incorporation of the base. However, a larger amount of radioactivity travels closer to the solvent front at $R_f = 0.81$. This fast-moving derivative is not an artifact induced during hydrolysis, or by the chromatography solvents, since none of solvents 4, 6 or 7 shows
transfer of more than one percent of the label from authentic $^3\text{H}-0^6\text{-MG}$ to $R_f$ 0.81 (figure 102). Further, the $0^6\text{-MG}$ is stable during heating to $100^\circ\text{C}$ for 20 minutes at pH 7.4. Its stability under acidic conditions was demonstrated in Section 1. Since there is a significant amount of the $R_f$ 0.81 material after 10 minutes incubation time, when background in the TCA-soluble cell fraction remains low, it seems unlikely that this product could represent low molecular weight material occluded with the nucleic acids during isolation. One can therefore conclude that the material at $R_f$ 0.81 results from metabolism of $^3\text{H}-0^6\text{-MG}$ after its incorporation into nucleic acid. Since, unlike guanine, the $R_f$ 0.81 product is readily released during hydrolysis at pH 7.4, $100^\circ\text{C}$, the ribosyl bond must be highly labile. Small amounts of radioactive guanine are present in nucleic acid hydrolysates as well.

The distribution of radioactivity after 2 hours incubation in vivo with a much larger dose of $^3\text{H}-0^6\text{-MG}$ is shown in figure 101. Again, a single radioactive peak co-incident with marker $0^6\text{-MG}$ is obtained from chromatography of the medium. However, a high background of radioactivity is present in the TCA cell supernatant. Although the peak at the position of unchanged $0^6\text{-MG}$ is prominent, considerable metabolism of the labelled base must have occurred.

Mild acid hydrolysis of DNA and RNA isolated after 2 hours incubation in vivo again demonstrates that label is no longer confined to its $^3\text{H}-0^6\text{-MG}$ source. After hydrolysis of DNA, radioactivity at the $R_f$ of $0^6\text{-MG}$ is indistinguishable from background, but significant amounts of radioactivity occur both at the fast-moving $R_f$ 0.81 position, and at the position of guanine. In the RNA however, not only is the $0^6\text{-MG}$ absent, but very little of the $R_f$ 0.81 product is apparent. The major source of radioactivity is guanine.
The results obtained after incubation for 10 minutes and for two hours cannot be compared to give precursor-product relationships since different doses were used (table 10). However, the data encourage speculation that after the initial mis-incorporation of $\text{O}^6$-MG, the error can be metabolically corrected by demethylation to guanine, or some other modification yielding the more easily depurinated $R_f$ 0.81 product.

The effects of $\text{O}^6$-MG on control of genetic expression, and the incorporation of the base into nucleic acid support the hypothesis of Loveless (1969) that O-methylation plays a role in carcinogenesis. Further, the data suggest that carcinogenesis may not require alteration of preformed macromolecules, but could result from incorporation of modified precursors.
Figure 80: Absorption spectra of $\text{O}^6$-methylguanine.

Spectra at pH 1.0, pH 7.0, and pH 13.0 are shown. The pK values of the two isosbestic points were calculated from the absorption of more concentrated solutions.
Figure 81: Absorption spectra of 6-ethoxy-2-aminopurine and 6-chloro-2-aminopurine.

Figure 82: Hydrolysis of $\text{O}^6$-methylguanosine.

The time elapsed at pH 0.93 and $24^\circ\text{C}$ is shown.  (minutes)
Figure 83: Demethylation of $\text{O}^6$-methylguanine.

The time elapsed at 85°C in 1.0 N HCl is shown. (minutes)
Figure 84: Absorption spectra of synthetic $\text{O}^6$-methylguanine and the product isolated after methylation of DNA.

---

**DNA**

0, $\Delta$

DNA was methylated *in vitro* with MNNG and hydrolysed in 0.1 N HCl, 37°C, 16 hours. The hydrolysate was chromatographed on paper in solvent (6), and the spot at the $R_f$ of synthetic $\text{O}^6$-methylguanine was eluted with water for examination of the absorption spectrum.
Figure 85: Chromatography of methylated purines from DNA treated with $^{14}\text{C-MNNG}$.

DNA source: salmon sperm DNA

Treatment: 3 mM $^{14}\text{C-MNNG}$ in the presence of 3 mM N-acetylcysteine, in 50 mM phosphate buffer, 37°C, 40 minutes.

Hydrolysis: neutral, 100°C, 20 minutes.

Figure 86: Chromatography of methylated purines from DNA treated with $^{14}\text{C-MNNG}$.

DNA source: salmon sperm DNA

Treatment: Same as (85)

Hydrolysis: the acid precipitate from (85) was further hydrolysed in 0.1 N HCl, 37°C, 16 hours.

The positions of markers on chromatograms are indicated as follows: 7-MG = 7-methylguanine; 3-MA = 3-methyladenine; $^{6}\text{MG} = ^{6}\text{methylguanine}$; 6-MAP = 6-methylaminopurine; G = guanine; A = adenine; T = thymine; C = cytidine.
Figure 87: Chromatography of methylated purines from DNA treated with $^{14}$C-MNNG.

DNA source: salmon sperm DNA

Treatment: Same as (85)

Hydrolysis: 0.1 N HCl, 37°C, 16 hours, directly, without prior neutral hydrolysis.
Figure 88: Chromatography of methylated purines from denatured DNA treated with $^{14}$C-MNNG.

DNA source: salmon sperm DNA, heat denatured

Treatment: 1.7 mM $^{14}$C-MNNG, 37°C, 40 minutes, in the presence of 2 mM N-acetylcysteine

A. Hydrolysis: neutral, 100°C, 20 minutes

B. Hydrolysis: 0.1 N HCl added to the acid precipitate of (A), for 16 hours at 37°C.
FIGURE 88

A

7MG

3MA

$^6$MG

6 MAP

B

7MG

3MA

$^6$MG

6 MAP

c.p.m.

centimeters from origin
Figure 89: Chromatography of methylated deoxynucleosides from DNA treated with $^{14}$C-MNNG.

DNA source: salmon sperm DNA

Treatment: 3 mM $^{14}$C-MNNG + 3 mM $\text{N}$-acetylcysteine, 37° C, for 40 minutes

Hydrolysis: Enzymatic. Spleen DNAase followed by snake venom phosphodiesterase and alkaline phosphatase, under the conditions of Hall(1967).

A. Chromatography of the digest after 3 hours incubation with the enzymes.

B. Chromatography of the digest after 16 hours incubation with the enzymes.

$\overset{6}{\text{Me}}\text{GdR} = \overset{6}{\text{methyldeoxyguanosine}}$
Figure 90: Chromatography of methylated purines from DNA treated with 14C-DMS.

DNA source: salmon sperm DNA

Treatment: 0.22 mM 14C-DMS, for final extent of methylation of 0.3 mmol CH₃ / mol DNAP

A. Hydrolysis: neutral, 100°C, 20 minutes

B. Hydrolysis: acid precipitate of (A) + 0.1 N HCl, 37°C, for 16 hours.
FIGURE 90

[Graph showing cpm measurements for 7MG and 3MA at different centimeters from the origin.]
Figure 91: Dowex column chromatography of hydrolysates of DNA treated with $^{14}\text{C-MNNG}$. 

DNA source: salmon sperm DNA 

Treatment: 0.65 mM $^{14}\text{C-MNNG}$, for a final extent of binding of 1.5 mmol CH$_3$ / mol DNAP 

Hydrolysis: perchloric acid, 100°C, 1 hour 

Eluant: 0.75–2.0 N HCl, 10 ml fractions 

Upper: ultraviolet absorption profile 
Lower: radioactivity (cpm) profile 

Figure 92: Dowex column chromatography of hydrolysates of DNA treated with $^{14}\text{C-DMS}$. 

DNA source: salmon sperm DNA 

Treatment: $^{14}\text{C-DMS}$ to give 3.0 mmol methyl /mmol DNAP 

Hydrolysis: perchloric acid, 100°C, 1 hour. 

Eluant: 0.75–2.0 N HCl, 10 ml fractions. 

Upper: ultraviolet absorption profile 
Lower: radioactivity (cpm) profile
Figure 93: Chromatography of methylated purines from the DNA of L cells treated with $^{14}$C-MNNG in vivo.

Treatment: 0.61 mM $^{14}$C-MNNG in cell suspension, 37°C, for 40 minutes.

A. neutral hydrolysate
B. 0.1 N HCl hydrolysate of the acid precipitate of (A)

Figure 94: Chromatography of methylated purines from the DNA of L cells treated with $^{14}$C-MNNG or $^{14}$C-DMS in vivo.

A. DNA of $^{14}$C-MNNG treated cells, 0.1 N HCl hydrolysate
B. DNA of $^{14}$C-DMS treated cells, 0.1 N HCl hydrolysate
Figure 95: Chromatography of methylated purines from the DNA of L cells treated with $^{14}$C-MNUA \textit{in vivo}.

Dose: 0.5 mM MNUA \hspace{1cm} K_{DNA} = 0.94

A. Hydrolysis: pH 7.4, 100°C, 20 minutes

B. Hydrolysis: 0.1 N HCl + the acid precipitate of (A) for 16 hours at 37°C

C. Hydrolysis: 0.1 N HCl directly, without prior treatment.
FIGURE 95

A

B

C

CPM
$^{14}$C

CENTIMETERS

$O_{6}^{6}$MG marker

$O_{6}^{6}$MG marker

$O_{6}^{6}$MG carrier
Figure 96: Chromatography of methylated purines from the DNA of L cells treated with $^{14}$C-MNTS in vivo.

Dose: 0.13mM MNTS \hfill K_{DNA} = 0.28

Hydrolysis: 0.1 N HCl directly
Figure 97: The growth of hamster embryo cells in tissue culture.

- \( \overline{6}-\)methylguanine, 50 ug/ml
- MNNG, 0.5 ug/ml
- control
- time of exposure to the agent
- cells disintegrating

A. The culture was seeded at \( 2 \times 10^5 \) cells / plate and the cells were drained before treatment. The culture was treated twice.

B. The cells were seeded at \( 5 \times 10^5 \) cells / plate, and were drained before treatment.

C. The embryos were advanced in development (19 days) and the culture was seeded at \( 2 \times 10^5 \) cells / plate. The cells were drained before treatment.

D. Embryos were used after 11 days pregnancy, and were seeded at \( 5 \times 10^6, 1 \times 10^5, \) and \( 5 \times 10^5 \) cells / plate. Only the cultures at the highest seeding grew. The agent was added to the medium in the culture dishes.

E. Embryos were used after 11 days pregnancy, and were seeded at \( 10^6 \) cells / plate. The agent was added to the medium in the culture dishes.
LOGARITHM OF TOTAL PROGENY

A

B

C

D

E

FIGURE 97
Figure 98: The colony forming ability of hamster embryo cells.

- $O_6^-$-methylguanine on primary HE cells
- $O_6^-$-methylguanine on L cells
- $O_6^-$-ethylyguanine on primary HE cells
- $O_6^-$-methylguanine on secondary HE cells
- $6$-chloro-$2$-aminopurine on secondary HE cells
- $O_6^-$-ethylguanine on secondary HE cells

At 25 µg $O_6^-$-MG/ml the difference between the treated primary HE culture and its solvent-treated control is significant at the $P < 0.001$ level.
FIGURE 98

FRACTION OF CONTROL SURVIVING

DOSE µg/ml

0 0.01 0.05 0.1 0.5 1.0 2.0

0 20 40 60
Figure 99: Chromatography of tritiated $^6$-methylguanine.

Solvent (6): isopropanol: ammonia: water (7:1:2)
FIGURE 99

CPM x 10^{-2}

CENTIMETERS

G

O^6_{MG}
Figure 100: Chromatography of medium, TCA supernatant of cells, and hydrolysates of DNA and RNA following treatment of L cells with $^3\text{H}-\text{O}^6$-methylguanine \textit{in vivo} for 10 minutes.

A. medium
B. TCA supernatant of cells
C. 0.1 N HCl hydrolysate of DNA
D. 0.1 N HCl hydrolysate of RNA
Figure 101: Chromatography of medium, TCA supernatant of cells, DNA hydrolysates, and RNA hydrolysates after treatment of L cells with $^3$H-$\text{O}^6$-methylguanine in vivo for 120 minutes.

A. medium

B. TCA supernatant of cells

C. pH 7.4 hydrolysate of DNA

D. 0.1 N HCl hydrolysate of the residue from (C)

E. 0.1 N HCl hydrolysate of RNA
Figure 102: Chromatography of tritiated $^{6}$-methylguanine in various solvent systems.

A. n-propanol:water 7:3
B. isopropanol:ammonia:water 7:1:2
C. methanol:HCl:water 7:2:1
D. $^{3}$H-$^{6}$-methylguanine was incubated for 20 minutes at 100°C, pH 7.4, and then chromatographed in isopropanol:ammonia:water as in (B)
DISCUSSION

Chapter IV: The role of methyl-nitroso compounds in the induction of genetic change.

MNNG and MNUA but not DMS methylate nucleic acid at the 6-oxygen position of guanine, both in vitro and in vivo. This result provides support for the view that the differences in the ultimate methylating species of MNNG or MNUA and DMS are reflected in the ability of MNNG and MNUA to methylate a wider spectrum of sites than can DMS. This difference may well be due to the significant contribution of the methylcarbonium ion to the mode of action of the diazomethane precursors (Lawley, 1968). Further, the fact that the two agents (MNNG and MNUA) which produce $O^6$-MG are carcinogenic, while the two that do not alkylate at $O^6$ in vivo (DMS and MNTS) are relatively poor carcinogens, adds to the evidence indicating that SN1 character, and the consequent O-methylation may be involved in the carcinogenic potency of alkylnitroso compounds.

Guanine could mispair with thymine provided there is no proton at the N-1 site. Whereas alkylation at N-7 of guanine would increase the acidity at N-1, and hence increase the proportion of the proton-deficient form, alkylation at $O^6$ guarantees that the methylated molecule must remain in the enol form, with no proton at N-1:
Normal tautomerism of guanine.

$^6\text{-methylguanine cannot tautomerize.}$

$^6\text{-alkylguanine mispairs with thymine.}$

The probability that $^6$-alkylation would lead to transition from a GC to an AT pair is even greater than the probability of the same event occurring after $\text{N-7}$ alkylation. Thus all of the mutations reported to involve GC to AT transitions or GC deletions could be explained by $^6$-alkylation just as easily as by $\text{N-7}$ alkylation.

Ludlum (1970) has shown that $7\text{-methylguanine}$ does not, in fact, promote miscoding in a cell-free RNA-dependent RNA polymerase system. When synthetic poly (7-MG,U) is substituted for poly (G,U) as template and CTP and ATP are used as substrate nucleotides, just as much C is incorporated with the normal as with the methylated template. Therefore, 7-MG can code for C in a normal fashion. When poly (7-MG,C) is substituted for poly (G,C) as template, and GTP and UTP are the substrate nucleotides, there is some abnormal incorporation of U but the error is
just as great with the normal template. It would be of great interest to repeat these coding experiments using O-6 MG in place of 7-MG.

The modification of growth characteristics in tissue culture by O\(^6\)-MG provides positive evidence that this methylated base can interfere in the normal control of genetic expression. A greater proportion of primary hamster embryo cells are capable of initiating growth after O\(^6\)-MG treatment; treated cultures persist longer than their controls; and treated cells differ morphologically from control cells. All of these effects were observed during the early stages of transformation observed by Huberman et al (1968) after treatment of primary hamster embryo cells with the carcinogen dimethylnitrosamine (DMNA). However, unlike DMNA, which is known to methylate pre-formed macromolecules, the O\(^6\)-MG in the present work is available to the cells as a free base. If the pre-transformation events induced by O\(^6\)-MG involve alteration of nucleic acid, the base must be linked to a sugar molecule, phosphorylated, and incorporated into high molecular weight material. Evidence is provided that these (on first consideration unlikely) events do occur.

Although corrective mechanisms also exist in the cell, if DNA replication precedes correction of the erroneous incorporation of O\(^6\)-MG, genetic mistakes might well be induced through mis-pairing with thymidine, and the effects of O\(^6\)-methylguanine as free base would be identical to the effects of methylation of the 6-oxygen atom of guanine in the polynucleotide.

The enhancement of the induction and persistence of growth in tissue culture, as well as the morphological changes produced by O\(^6\)-MG in hamster embryo cells may well represent initial steps in the carcinogenic process. That alkylation at the 6-oxygen position of guanine is a critical step in carcinogenesis by alkylating agents is strongly indicated.
BIBLIOGRAPHY


Stammers, C.P. and Till, J.E. (1960). DNA synthesis in individual

Sugimura, T., Fujimura, S., Nagao, M., Yokoshima, T., and Hasegawa, M.
(1968). Reaction of N-methyl-N'-nitro-N-nitrosoguani

membrane sulfhydryl groups and cation permeability.

for the presence of non-specific nucleotide-dependent disulphide
reductase and GSH-disulphide transhydrogenase activities in the

acid synthesis in individual L-strain mouse cells II. Effects

effects of sulfur mustard on dividing mammalian cells.

against the toxicity of sulfur-mustard.


of pH on the mutagenicity in yeast of N-methylnitrosamides and