SYNTHESIS OF MODIFIED PURINES.

A Thesis submitted by

NEIL FAIRHURST.

in partial fulfilments of the requirements
of the degree of Doctor of Philosophy
of the University of London.

Department of Chemistry,
Imperial College,
South Kensington,
London.
SW7 2AZ.

November 1989.

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Abstract.

The Thesis concerns the chemical aspects of alkylation of DNA bases by potentially carcinogenic N-nitrosopeptides and related compounds. The relevance of DNA alkylation to carcinogenic activity is discussed.

A major part of the Thesis concerns the development of a synthetic route to O^6-alkylated-2/-deoxyguanosines, containing relatively complicated groups, (eg O^6-carboxymethyl-2/-deoxyguanosine). This involved important modifications to existing literature procedures, which are rationalised and discussed and their limitations determined.

The new method was successfully applied to the synthesis of O^6-methyl-2/-deoxyguanosine, O^6-isopropyl-2/-deoxyguanosine and O^6-carboxymethyl-2/-deoxyguanosine. The identification and characterisation of these compounds by spectroscopic methods is also described.

The synthesis of N-(N/-acetyl-L-prolyl)-N-nitrosoglycine and its interaction with 2/-deoxyguanosine and DNA under physiological conditions *in vitro* is reported, together with assay procedures for the

quantitation of O^6-carboxymethyl-2/-deoxyguanosine, O^6-carboxymethylguanine and N^7-carboxymethylguanine. These assays involved the development of several reverse phase H.P.L.C. methods. Variations in the extent of 2/-deoxguanosine and DNA base (guanine) alkylation with changes in the concentration of alkylating agent, (N-nitrosopeptide), is also described.

Related synthetic procedures for the synthesis of O^6-carboxymethylguanosine are reported, to facilitate the development of an immunochemical assay for the detection of O^6-carboxymethyl-2/-deoxyguanosine in conjunction with the I.A.R.C. The selectivity and sensitivity of the immunochemical assay (ELISA) is discussed and evaluated.

Acknowledgements.

This work was carried out mainly at the Chemistry Department, Imperial College, University of London, partly at the Chemistry Department of the Open University and the immunochemical studies were performed at the I.A.R.C. in Lyon France. I am grateful to the S.E.R.C. and M.R.C., who provided the funding for my work in England, and the E.S.F., who funded my stay in France.

I would like to thank my supervisors Professor Brian Challis and Dr. David Shuker for their guidance, instruction and friendship during the course of this work and for their advice and suggestions in formatting this manuscript.

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Finally, help from all the technical staff of the Open University, Imperial College and the I.A.R.C., and interaction with colleagues has played an equally important part in completion of this work. Particularly,

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This Thesis is dedicated to my sister and my parents, who through their sacrifices, successfully created an environment conducive to educational advancement and intellectual stimulation.

ABBREVIATIONS.

APNG	=	N-(N/-Acetyl-L-prolyl)-N-nitrosoglycine.
BAP	=	Bacterial Alkaline Phosphatase.
DBU	=	1,8-Diazabicyclo[5,4,0]undec-7-ene.
DCM	=	Dichloromethane.
DES	=	Diethyl Sulphate.
DMH	=	1,2-Dimethylhydrazine.
DMF	=	N-N-Dimethyl Formamide.
DMN	=	N-N-Dimethylnitrosamine.
DMS	=	Dimethyl Sulphate.
DMSO	=	Dimethyl Sulphoxide.
dR	=	2/-Deoxyribose.
ELISA	=	Enzyme Linked Immunosorbent Assay.
EMS	=	Ethylmethane Sulphonate.
ENU	=	N-Ethyl-N-nitrosourea.
HFBA	=	Heptafluorobutyric acid.
m-BSA	=	Methylated bovine serum albumin.
MNNG	=	N-Methyl-N/-nitro-N-nitrosoguanidine.
MMS	=	Methylmethane Sulphonate.
NOC	=	N-Nitrosocompounds.
NOGC	=	N-Nitrosoglycocholic acid.
NOTC	=	N-Nitrosotaurocholic acid.
PBS	=	Phosphate Buffered Saline.
THF	=	Tetrahydrofuran.

CONTENTS.

		Page
Chapter 1.	Introduction.	1
1.1	Cancer the Disease.	2
1.2	Endogenous Formation of N-nitrosocompounds.	3
1.3	Biological Activity of N-nitrosocompounds.	10
1.4	Structure of DNA.	11
1.5	Interaction of Carcinogens with DNA.	15
1.6	The Role of DNA Alkylation in Carcinogenesis and Mutagenesis.	22
1.7	Synthesis of Alkylated Nucleosides.	25
1.7.1	O^6-Alkyl-2/-deoxyguanosines.	25
1.7.2	O^4-Alkylthymidines.	28
1.8	DNA Adduct Analysis.	32
1.9	Conclusion.	34
Chapter 2.	Synthetic Routes to O^6-alkylated Nucleosides. Results and Discussion.	36
2.1	Preview.	37

2.2.1	Synthesis of O^6-alkyl-2/-deoxyguanosines.	38
2.2.2	2/-Deoxyguanosine Protection.	41
2.2.3	Direct Displacement of the O^6-mesitylsulphonyl group.	42
2.2.4	Indirect Displacement of the O^6-mesitylsulphonyl group.	44
2.2.5	Glycolate Moiety.	48
2.2.6	Solvent.	49
2.2.7	Conversion of O^6-mesitylsulphonyl-3/-5/-di-O-methoxyacetyl-2/-deoxyguanosine to O^6-carboxymethyl-2/-deoxyguanosine.	51
2.2.8	Removal of the 2-methoxyacetyl groups and Purification.	54
2.2.9	Spectroscopic Characterisation of O^6-alkyl-2/-deoxyguanosines.	56
2.3.1	Synthesis of O^6-carboxymethyl-guanosine.	62
2.3.2	Guanosine Protection.	64
2.3.3	Conversion of O^6-mesitylsulphonyl-2/-3/-5/-tri-O-acetylguanosine to O^6-carboxymethylguanosine.	66
2.3.4	Removal of the acetyl Protecting groups.	67
2.3.5	Spectroscopic identification of O^6-alkylguanosines.	68
2.4	Synthesis of N-(N/-acetyl-L-prolyl)-N-nitrosoglycine (APNG).	72

Chapter 3. Alkylation Reaction of 2/-deoxyguanosine and DNA with APNG *in vitro*. 76

3.1 Reaction of N-(N/-acetyl-L-prolyl)-N-nitrosoglycine (APNG) with 2/-deoxyguanosine and DNA *in vitro*. 77

3.2 Analytical H.P.L.C. Procedures for 2/-deoxyguanosine reactions. 79

3.3 Reaction of APNG with 2/-deoxyguanosine. 87

3.4 Reaction of APNG with calf-thymus DNA. 95

3.5 Analytical H.P.L.C. Procedures for DNA alkylation reactions. 96

3.6 Hydrolytic Decomposition of DNA. 100

3.7 DNA alkylation reactions. 104

3.8 Conclusions. 108

Chapter 4. Immunochemical Analysis of DNA. 111

4.1 Introduction 112

4.2.1 ELISA Procedure 113

4.2.2	Non-Equilibrium cELISA Assay.	115
4.3	Cross-Reactivity of the Rabbit Antiserum CMGuo1.	116
4.4	H.P.L.C. ELISA Method.	123
4.5	Direct DNA Analysis.	130
4.6	Conclusion.	131

Chapter 5. Experimental. 134

5.1	Synthesis.	135
5.1.1	Instrumentation.	135
5.1.2	Reagents and Chemicals.	135
5.2	Synthesis of Nucleosides.	136
5.2.1	N-Methylpyrrolidine.	136
5.2.2	Methoxyacetic anhidride.	137
5.2.3	3/-5/-Di-O-methoxyacetyl-2/-deoxyguanosine.	138
5.2.4	O^6-(2,4,6-Trimethylbenzene sulphonyl)-3/-5/-di-O-methoxyacetyl-2/-deoxyguanosine.	139
5.2.5	2/-3/-5/-Tri-O-methoxyacetyl-guanosine.	140
5.2.6	2/-3/-5/-Tri-O-acetylguanosine.	140
5.2.7	O^6-(2,4,6-Trimethylbenzene sulphonyl)-2/-3/-5/-tri-O-acetylguanosine.	141

5.2.8	O^6-(2,4,6-Trimethylbenzene sulphonyl) -2/-3/-5/-tri-O-methoxyacetyl guanosine.	142
5.2.9	O^6-Methyl-2/-deoxyguanosine.	144
5.2.10	N^6-pyrrolidyl-2/-deoxyguanosine.	145
5.2.11	General procedure for the synthesis of O^6-substituted-2/-deoxyguanosines.	147
5.2.11.1	O^6-Methyl-2/-deoxyguanosine.	148
5.2.11.2	O^6-Isopropyl-2/-deoxyguanosine.	149
5.2.11.3	O^6-Tert-butyl-2/-deoxyguanosine.	149
5.2.11.4	O^6-Carboxymethyl methyl ester-2/-deoxyguanosine.	150
5.2.11.5	O^6-Carboxymethyl methyl ester guanosine.	151
5.2.11.6	O^6-Carboxymethyl-2/-deoxyguanosine.	153
5.2.11.7	O^6-Carboxymethylguanosine.	154
5.3	Synthesis of an N-nitrosodipeptide.	155
5.3.1	N-Acetyl-L-proline.	155
5.3.2	N-Acetyl-L-prolylglycine benzyl ester.	156
5.3.3	N-(N/-Acetyl-L-prolyl)-N-nitrosoglycine benzyl ester.	157
5.3.4	N-(N/-Acetyl-L-prolyl)-N-nitrosoglycine.	158
5.4	Alkylation of 2/-deoxyguanosine. General Procedure.	159
5.4.1	Preparation of the stock 2/-deoxyguanosine solution.	159

5.4.2	Alkylation and Analysis.	159
5.4.3	Reaction of APNG with 2/-dG.	160
5.5	Alkylation of DNA.	
	General Procedure.	160
5.5.1	Preparation of the stock DNA	
	solution.	160
5.5.2	Alkylation and Analysis.	161
5.5.3	DNA Precipitation.	161
5.5.4	General Procedure for Acid	
	Hydrolysis of DNA.	162
5.6	Immunochemical Procedures.	162
5.6.1	Enzyme Linked Immunosorbent Assay	
	(ELISA) Methods.	162
5.6.1.1	Plate Preparation.	163
5.6.1.2	Room Temperature ELISA Procedure.	163
5.6.1.3	Non-equilibrium ELISA Assay	
	Procedure at 4°C.	166
5.6.2	DNA Enzyme Hydrolysis for the	
	ELISA Assay.	167
5.6.2.1	Ordinary Enzyme Procedure.	167
5.6.2.2	New Enzyme Method.	168
5.6.2.3	Hydrolysis of DNA for Comparative	
	Fluorescence, H.P.L.C. and ELISA	
	Analysis.	168
5.7	H.P.L.C. Methods.	169
5.7.1	Purification of the Synthesis	
	Products.	169

5.7.1.1	O^6-Carboxymethyl methyl ester-2/-deoxyguanosine.	170
5.7.1.2	O^6-Carboxymethyl methyl ester guanosine.	170
5.7.2	Analysis of Alkylated DNA bases.	171
5.7.2.1	Nucleosides.	171
5.7.2.2	O^6-Carboxymethyl-2/-deoxyguanosine.	171
5.7.2.3	Guanine Bases.	172
5.7.2.4	N^7-Carboxymethylguanine.	172
5.7.2.5	O^6-Carboxymethylguanine.	173
5.8	G.C. Methods.	174
5.8.1	Analysis of Pyrrolidine in N-Methylpyrrolidine.	174
5.8.2	Decomposition of Methylglycolate.	175
5.9	Addendum.	176
5.9.1	Preparation of the O^6-CMGuo conjugated with m-BSA.	176
5.9.2	Immunization of the rabbits and isolation of the rabbit antiserum CMGuo1.	177
5.9.3	Preparation of the O^6-CMGuo conjugated with ovalbumin.	178
	References	179

Chapter 1.

INTRODUCTION.

1.1 CANCER THE DISEASE.

Cancer has long been a major human disease and a major cause of death in the developed countries. It also effects people in under-developed countries but usually with a lower incidence. Cancer is a collective term for over a hundred different types of malignant tumours, which can be defined as abnormally proliferating cells arising in any part of the body and spreading from their site of origin. If the growth is localised and non-invasive, it is called a benign tumour . Cancer cells arise from normal cells, whose DNA has been permanently altered to induce rapid replication (mitosis). The lethal nature of cancer is associated with the ability of cancer cells to be invasive and to migrate throughout the body, rather than to be confined to specific organs (as normal cells are).

The majority of human cancers are now considered to be caused by environmental factors. Early evidence to this effect was the association of cancers with specific industries and exposure to individual substances, as for skin cancers among tar and cotton- mill workers[1]. The isolation of dibenz[a,h]anthracene[2] and benz[a]pyrene[3] in 1930-1933, and the production of tumours when these compounds were applied to mouse skin, was the first evidence that specific chemicals could cause cancer.

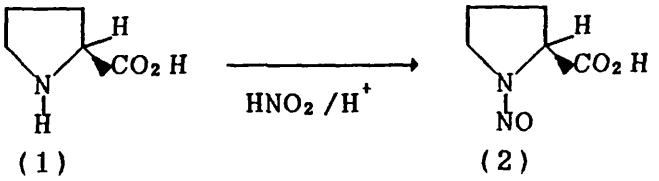
The first evidence that chemical carcinogens caused chemical changes *in vivo* was obtained by Magee and Farber in 1962[4]. They showed that the liver carcinogen N-nitrosodimethylamine produced N^7-methylguanine in the liver. Earlier work on the *in vitro* alkylation of nucleic acids and their constituents[5], had shown that the N^7-position of guanine was susceptible to alkylation by dimethyl sulphate[6,7] and mustard gas[8]. Subsequently, the reactions of many alkylating agents, such as N-nitroso compounds with DNA[9], *in vivo* and its components *in vitro* have shown that alkylation of DNA is a characteristic property of many carcinogens[10,11,12].

1.2 ENDOGENOUS FORMATION OF N-NITROSO COMPOUNDS.

The biological properties of N-nitroso compounds (NOC) have been widely examined and it is known that most are powerful carcinogens[10,13,14]. N-Nitrosodimethylamine has been shown to be carginogenic in over 40 animal species[15]. Nonetheless, the links between exposure to environmentally formed NOC and human cancer is tenuous[16], and only substantial evidence relates to exposure to some tobacco specific N-nitrosamines[17]. It has been suggested recently that formation of NOC *in vivo* (endogenous formation) may be more a important cause than exposure to

exogenous compounds[18,19]. The endogenous formation of NOC is feasible from dietary nitrate reacting with amino compounds in the acidic conditions of the stomach. Both nitrate and nitrite are present in the diet[20], the amount of nitrite present being increased by bacterial reduction of some of the nitrate in the mouth. It has been estimated that ca 5% of the dietary nitrate is reduced in this fashion[21], giving a varying level of nitrite in the healthy stomach of 1-10uM[22].

The sound evidence for occurrence of endogenous N-nitrosation of dietary materials in humans has been provided by Oshima and Bartsch[23]. They showed increased urinary levels of N-nitrosoproline (2) following the ingestion of proline (1) and carrot-juice containing added $NaNO_3$ (Figure 1.1). Tannenbaum and his colleagues[24] confirmed these observations using N^{15} labelled N-nitrosoproline.



(1) (2)

FIGURE 1.1 N-NITROSATION OF PROLINE IN GASTRIC CONDITIONS.

The biological importance of compounds and their derivatives with regard to endogenous nitrosation, are usually considered in terms of the daily consumption of their dietary precursors[18]. Thus, amides (ca 100,000mg/day) are generally considered to be the most important precursor class in the diet, and the highest potential source of N-nitroso derivatives. The average daily intake of several dietary sources of N-nitroso compounds are given in Table 1.1[18].

Another potential source of N-nitrosamides in the diet are the bile acids. Bile acids are normally present in human bile as amides of glycine or taurine[25]. Generally, 10-15g of the bile acid conjugates are excreted in the intestine daily[26]. Consequently, endogenous N-nitrosation of proteins (peptides) or bile acid conjugates is considered to be biologically more significant than reactions with dietary amines.

Endogenous nitrosation reactions with peptides may occur at either the terminal or peptide N-atoms (Figure 1.2), as well as at other sites in substrates containing cysteine, glutamine or lysine[27].

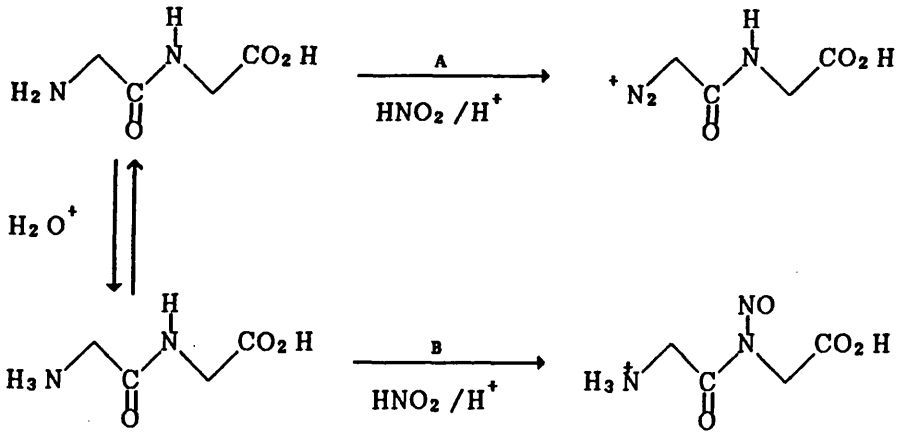


FIGURE 1.2 ACIDIC NITROSATION OF DIPEPTIDES

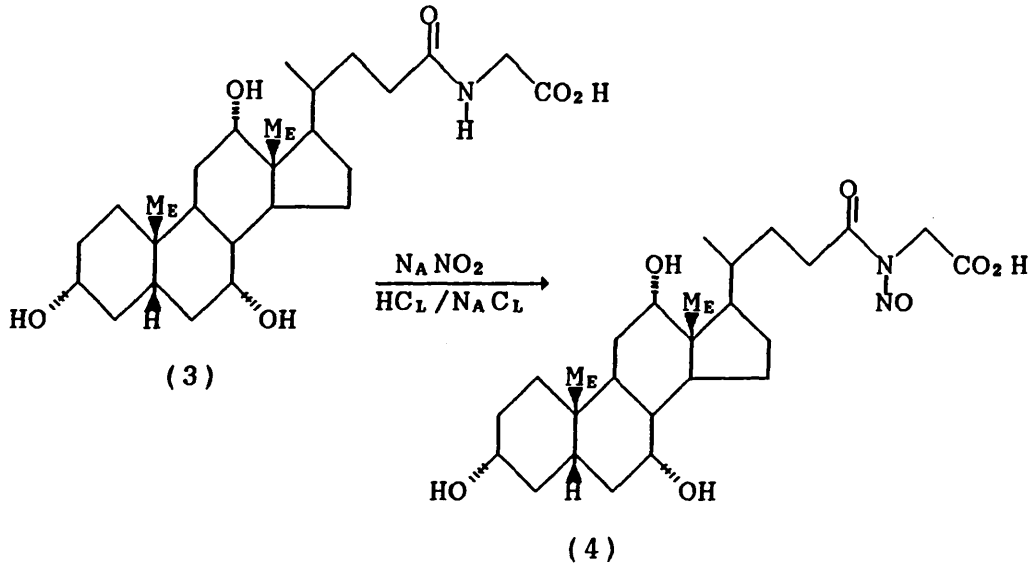
Challis and co-workers[27] observed the formation of both N-nitroso and diazopeptides under normal gastric conditions. For small peptides eg glycylglycine, the observed rate of diazotization was 20 times faster than the rate of peptide nitration. Thus, endogenous peptide diazotization appears to be the most likely outcome of gastric nitration of small proteins and peptides.

Nitrosation of bile acid conjugates has also been observed under simulated gastric conditions[26]. Thus, treatment of glycocholic acid (3) with $NaNO_2$ in HCl/NaCl buffer at 37°C, gave the nitrosated bile acid conjugate N-nitrosoglycocholic acid (NOGC) (4) (Figure 1.3).

Precursor Class	Compound	Daily Intake (mg)
Primary Amines[a]	Spermidine	35
	Tyramine	21
	Methylamine	3
Total		100
Secondary Amines[a]	Dimethylamine	1.7
	Pyrrolidine	0.6
Total		5
Primary Amino Acids[a]	Glutamic Acid	>3.2
	Glycine	>1.3
	Alanine	>0.4
Total		>10
Secondary Amino Acids[a]	Proline	>0.5
	Sarcosine	1
Total		>1.5
Amides[a]	Protein	92,000
	Carnosine	2,000
Total		100,000
Bile Acid Conjugates[b] Total		10,000-15,000

Table 1.1 Estimates of the daily intake of potential nitrosatable food precursors in the human diet.

a=ref18; b=ref26.



(3)

(4)

FIGURE 1.3 NITROSATION OF GLYCOCHOLIC ACID UNDER GASTRIC CONDITIONS.

N-Nitrosopeptides and N-nitrosobile acid conjugates are known to decompose by a similar mechanism in acidic aqueous media[26,28] (Figure 1.5). Hydrolysis of both N-nitrosopeptides and N-nitrosobile acid conjugates result in the formation of a diazo derivative, a potential alkylating agent. Consequently, recent biological studies on both N-nitrosopeptides and N-nitrosobile acid conjugates have been performed to evaluate their mutagenic and carcinogenic potential.

The N-nitrosobile acid conjugates N-nitrosotaurocholic acid (NOTC) and NOGC have been shown to be mutagenic in the absence of activation in both forward (Salmonella typhimurium) and reversion (Ames S

typhimurium) mutation assays[29]. Carcenogenicity studies in rats showed the formation of hepatocellular carcinoma and gastric tumours for both NOGC and NOTC[30]. The N-nitrosopeptide, N-(N/-acetyl-L-prolyl)-N-nitrosoglycine (APNG), has also been shown to be mutagenic in several bacterial assays[31,32]. In particular APNG has been shown to be a potent direct acting mutagen in the Ames Salmonella/mammalian mutagenicity test, which detect base-pair substitution mutation[31]. Studies of the in vivo genotoxic activity of APNG in mice, have shown that it produces an increase in late embryonic deaths in dominant lethal assays[33,34]. Mutagenicity testing of diazopeptides, another potential gastric carcinogen, have also proved positive, although the mutagenic activity varies greatly with the diazo peptide structure[35]. The high instability of diazopeptides in gastric media would probably decrease their effectiveness as gastric carcinogens. However, diazopeptides have been found to be stable in blood, and have significant potential as circulating carcinogens[36].

The causative link between endogenous nitrosation and gastric cancer has been highlighted by the work of Kyrtopoulos et al[37], who demonstrated that normal-gastric juice after nitrosation contains alkylating agents that can form adducts with DNA compounds.

1.3 BIOLOGICAL ACTIVITY OF N-NITROSO COMPOUNDS.

N-Nitroso compounds are thought to exert their carcinogenic effect by acting as alkylating agents of DNA[10]. In order to act as alkylating agents, N-nitrosamines have to be activated metabolically. This activation is believed to involve enzymatic hydroxylation in the liver[38,39] which leads to dealkylation and generation of a diazoalkane moiety (Figure 1.4). Because N-nitrosamines require metabolic activation, they usually induce tumours in specific organs (the site of activation) remote from the site of administration[38].

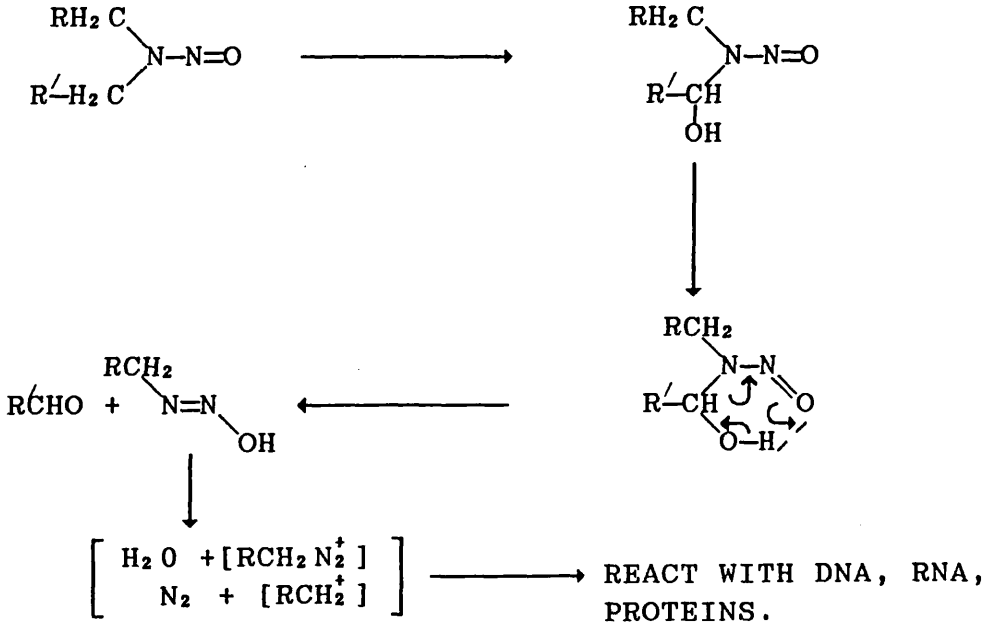


FIGURE 1.4 METABOLIC ACTIVATION OF DIALKYLNITROSAMINES

N-Nitroso compounds which also contain an α-acyl

moiety act as alkylating agents without prior metabolic activation. these compounds include N-nitrosamides, N-nitrosoureas, N-nitrosoguanidines, and significantly N-nitrosopeptides and N-nitrosobile acid conjugates. Nucleophilic interaction at the α-acyl groups releases a diazoalkane moiety, as exemplified for N-nitrosopeptides in Figure 1.5. Thus, N-nitrosamides, usually but not always, induce tumours at their site of application[40].

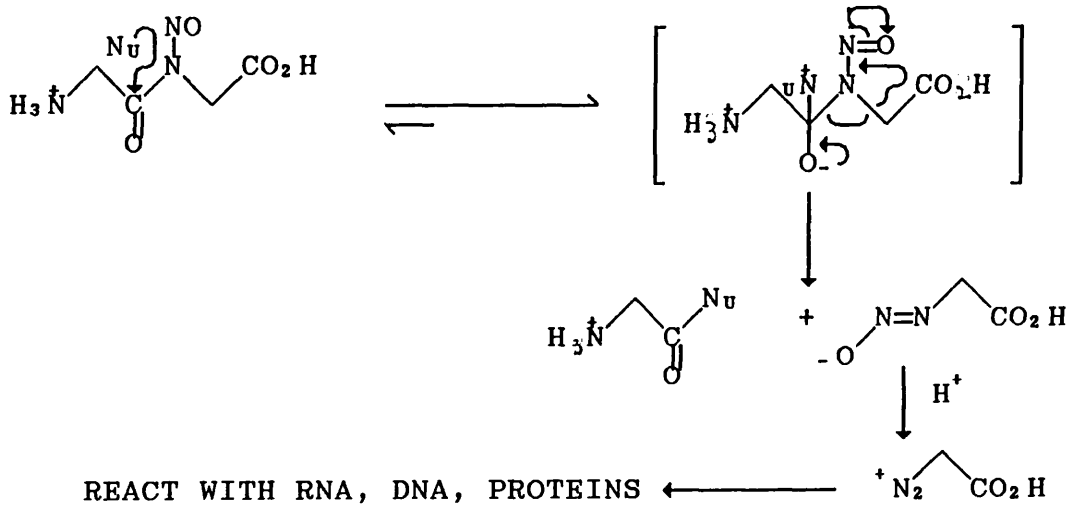
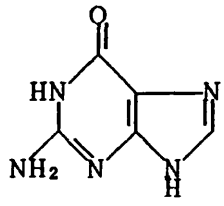


FIGURE 1.5

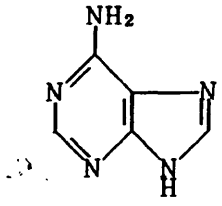
1.4 STRUCTURE OF DNA.

In the late 1940's and early 1950's one of the major areas of scientific research centred on the mechanism by which the genetic code was transferred from cell to cell during replication. Of particular interest was the role of deoxyribonucleic acid (DNA), a compound found in all living cells mainly in the nucleus, DNA being an

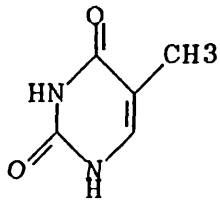
STRUCTURES OF THE PYRIMIDINE AND PURINE NUCLEIC ACID BASES.



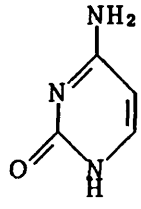
GUANINE (G)



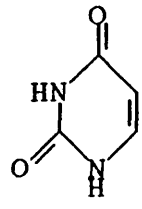
ADENINE (A)



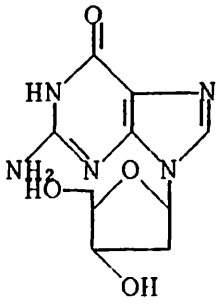
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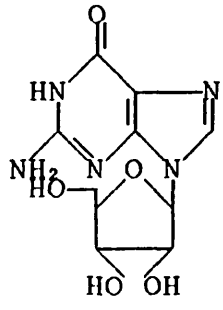
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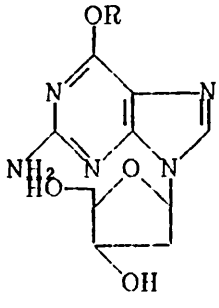
URACIL (U)



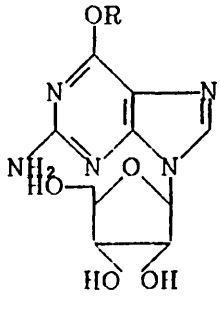
2′-DEOXYGUANOSINE (2′-DG)



GUANOSINE (G_{UO})



O^6-ALKYL-2′-$_D$G



O^6-ALKYLG_{UO}

FIGURE 1.6

essential constituent of the chromosomes. Evidence indicated that it may be the carrier of the genetic specificity of the chromosomes, although the manner by which the genetic information was transferred, ie DNA could undergo exact self duplication, was unknown.

Watson and Crick[41] in 1953 proposed the now universally accepted model of the structure of DNA, Figure 1.7, based mainly on the X-ray work of Franklin and Gosling[42] and co-workers[43]. Their proposed structure consisted of two polynucleotide chains coiled around a common axis, but running in opposite directions. The two chains of DNA are held together by hydrogen bonds between the bases. However, only certain pairs of bases are capable of forming hydrogen bonds, that is a purine-pyrimidine hydrogen bond. In particular, adenine (A) base pairs with thymine (T) and guanine (G) base pairs with cytosine (C) (Figure 1.7). The proposed hydrogen bonding model was confirmed by analytical data, which indicated that for a variety of sources of DNA, the amount of A and T and G and C were similar[44].

The genetic code of DNA is associated with the sequence of bases in DNA[41]. The successful transfer of genetic information during mitosis, the ability of DNA to

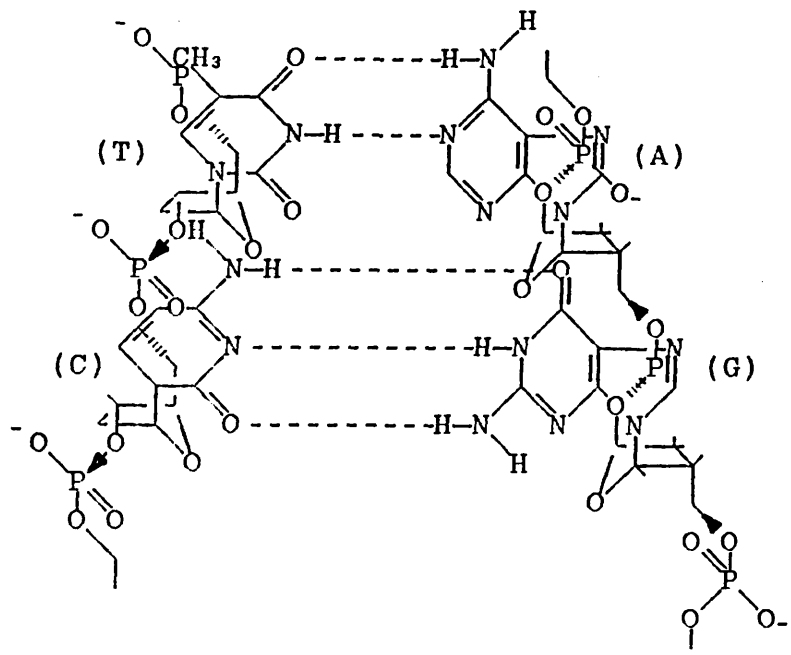


FIGURE 1.7 A SEGMENT OF THE DNA DOUBLE HELIX, SHOWING THE HYDROGEN BONDING BETWEEN BASE PAIRS ACROSS THE AXIS OF THE HELIX.

copy itself exactly, is governed by the specific hydrogen bonding interactions of the bases of DNA. Thus, as DNA uncoils during replication, each strand of DNA automatically dictates the structure of the new strand. Generally, DNA replication is an error free process. However, modifications to the structure of DNA by carcinogens, (discussed in detail in Sections 1.5 and 1.6), may result in mispairing of the DNA bases (Figure 1.8) and a permanent alteration of the genetic sequence. It is the alteration in the genetic sequence of a cell that is associated with the formation of cancer.

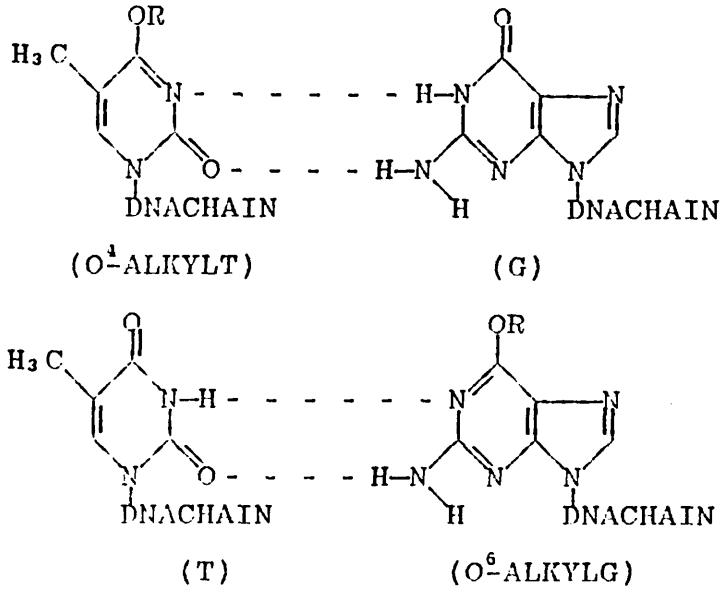
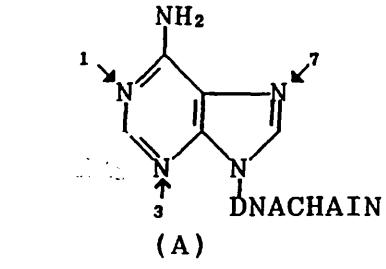


FIGURE 1.8 MISPAIRING OF O^4-ALKYLT WITH G AND O^6-ALKYLG WITH T

1.5 INTERACTION OF CARCINOGENS WITH DNA.

For the most part, the covalent interaction of carcinogens with DNA (and its components) under physiological conditions *in vitro* or *in vivo*, involves the purine and pyrimidine bases. Interaction with phosphodiester linkages does occur for some carcinogens but this leads to rapid cleavage of the phosphate link (strand breaking), which is not a carcinogenic event. Alkylation has been shown to occur on at least 12 sites in DNA which are summarised in Figure 1.9[12].

Alkylation proceeds at most of the heterocyclic N-atoms of the constituent DNA bases. For reactions *in vivo* this includes the 1-position of A, the 7-position of

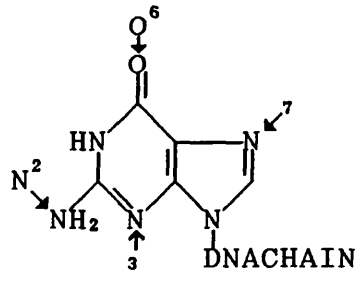


(A)

N^1- ENU, MNU, MNNG

N^3- ENU, MNU, MNNG

N^6- BENZO[A]PYRENE



(G)

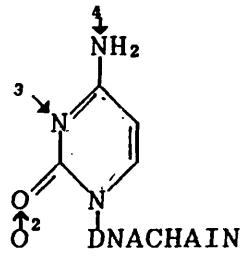
N^2- BENZO[A]PYRENE

N^3- ENU, MNU

O^6- N-HYDROXY-1-NAPTHYLAMINE

N^7- DMN, ENU, MNU

C^8- N-HYDROXY-2-NAPTHYLAMINE

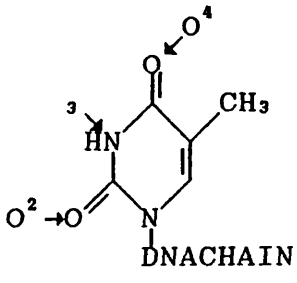


(C)

O^2- ENU

N^3- ENU, MNU, MNNG

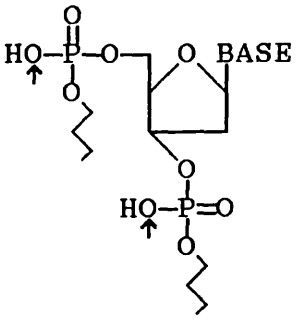
N^4- BENZ[A]PYRENE



(T)

O^2- ENU

O^4- ENU, MNU



PHOSPHATE GROUPS

FIGURE 1.9 SHOWING THE SITES OF IN VIVO ALKYLATION CAUSED BY CARCINOGENS.

A and G, and the 3-position of T,C,G and A. Alkylation *in vivo* at the N^2-position of G appears to be the most important for polycyclic hydrocarbons, *eg* benzo[a]pyrene[45]. Alkylation *in vitro* proceeds at the above N-atoms but also at the 1-position of G and the exo amino group N^4-C and N^6-A[46,47]. Alkylation *in vivo* proceeds at all the ring O-atoms and adducts therefore form at the O^6-position of G, the O^4-T, and the O^2-T and C. As mentioned above phosphate O-alkylation is also prominent, but is a non-carcinogenic event.

The extent of alkylation at each site of DNA is dependent on the structure of the carcinogen and therefore of the alkyl group[11,46-50]. The total extent of alkylation, however, appears to be dependent mainly on the size of the alkylating agent. Thus, with increasing size alkylation decreases. For this reason, N-methyl-N-nitrosourea (MNU), reacts with DNA by an order of magnitude greater than N-ethyl-N-nitrosourea (ENU).

Factors governing the extent of alkylation at specific sites of DNA are complex, but the extent of O-alkylation appears to be inversely related to the Swain Scott constant *s*[49,51]. Ethylating agents give lower *s* values than methylating agents, and consequently produce larger proportion of O-alkyl products as shown in Table 1.2.

PERCENTAGE OF TOTAL ALKYLATION BY

Product	NDM MNU DMH	NDE ENU	MMS	EMS	DMS
1-alkylA	0.7	0.3	1.2	1.7	1
3-alkylA	8	4	11	4.2	16
7-alkylA	1.5	0.4	1.9	1.9	2
3-alkylC	0.5	0.2	*	0.4	*
O^2-alkylC	0.1	0.3	*	0.3	*
3-alkylG	0.8	0.6	0.7	0.3	1
7-alkylG	68	12	83	58	79
O^6-alkylG	7.5	8	0.3	2	0.2
3-alkylT	0.3	0.8	*	*	*
O^2-alkylT	0.1	7	*	*	*
O^4-alkylT	0.4	4	*	*	*
alkylphosphates	12	53	1	12	1

*=not quantified; MMS=Methylmethane sulphonate

EMS=Ethylmethane sulphonate; DMS=Dimethyl sulphate

Table 1.2 Relative Proportions Of Alkylation Products From DNA *In Vitro*[12].

Experiments with single-stranded and double-stranded DNA *in vitro* have shown that the extent of alkylation at specific sites is not strongly dependent on the DNA

structure (Table 1.3)[52,53,54]. The exception appears to be N^1-A and the N^3-C, where ethylation occurs to a much greater extent in single-stranded DNA. These differences can be explained by hydrogen bonding interactions in double-stranded DNA which shield the N^1-A and N^3-C sites from reaction. Significantly, the alkylation of O-atoms is unaffected by the type of DNA, because for both single-stranded and double-stranded DNA, the O-lone pair electrons are available for interaction with the alkylating agent.

Early studies with dimethyl sulphate and methylmethane sulphonate, showed extensive formation of N^7-G[55,56], and it was assumed that formation of this adduct was critical for the induction of cancer. Subsequent work, however, showed that dimethyl sulphate and methylmethane sulphonate were only weak carcinogens[57]. The relevance of N^7-G alkylation to carcinogenicity was further questioned when ethylmethane sulphonate was found to be more carcinogenic than methylmethane sulphonate, but produced less N^7-G alkylation *in vivo*[58]. The uncertainty remained until Loveless[59] indicated the formation of O^6-MedG in low yield from the reaction of 2/-deoxyguanosine with MNU *in vitro*, and postulated the importance of O^6-G alkylation

to carcinogenesis. Subsequently, many alkylating agents have been shown to form O^6-alkylG adducts on reaction with DNA *in vitro* and *in vivo*[60-63].

Table 1.3 Extent of Ethylation of Nucleic Acid Bases by Diethylsulphate (DES) and N-ethyl-N-nitrosourea (ENU) *In Vitro*.

Derivative	Single-Stranded		Double-Stranded	
	DES	ENU	DES	ENU
N-ethyl				
1-ethylA	11	2	2	~0.1
3-ethylA	3	1.2	10	4.3
7-ethylA	3	0.6	1.5	0.2
3-ethylG	2	0.5	0.9	0.6
7-ethylG	62	10	67	11
3-ethylC	11	1.7	0.7	0.2
3-ethylT	*	~0.5	*	0.8
O-ethyl				
O^6-ethylG	1-2	6	*	7
O^2-ethylC	1-2	7	*	4
O^2-ethylT	1-2	6	*	7
O^4-ethylT	1	4	*	4
Phosphate groups	*	50-60	16	60

*=not quantified;

Following the discovery of O-alkylation of purines, the relevance of similar alkylations in pyrimidines to carcinogenis has also been examined. Singer[54] obtained the first evidence for the O-alkylation of pyrimidines, by showing the formation of O^2-EtdC,O^2-EtdT and O^4-EtdT in the reaction of single-stranded and double-stranded DNA with N-ethyl-N-nitrosourea (ENU), and N-ethyl-N/-nitro-N-nitrosoguanidine (ENNG) in vitro. Subsequent work has shown that many other alkylating agents interact with O-atoms of pyrimidines in vitro and in vivo[64,65]. As in the case of purine alkylations, the yield of O-alkylated pyrimidines is dependent on the type of carcinogen and structure of the alkylating species, as shown in Table 1.2.

To summarize, S_n2 alkylating agents (eg alkyl sulphonates, mustards and dialkyl sulphates), predominantly react with double-stranded DNA at the N^7-G and N^3-A.The S_n1 type reagents (eg N-nitroso compounds, dimethylhydrazine), give more extensive reaction at O-atoms, which is dependent on the nature of the alkyl group. Methylation of DNA occurs primarily on N-atoms (80%), whereas ethylation occurs primarily on O-atoms (80%).

1.6 THE ROLE OF DNA ALKYLATION IN CARCINOGENESIS AND MUTAGENESIS.

The most widely accepted theory concerning the mutagenic properties of alkylating agents, is that their reaction with DNA leads to a modification of the normal Watson and Crick hydrogen bonding pattern. During normal DNA replication, the A and G of the parent strand hydrogen bond (base pair) with the T and C respectively of the daughter strand (Figure 1.7)[41]. Miscoding could arise if an abnormal alkylated nucleic acid base of the parent strand engages in atypical pairing with a nucleic acid base of the daughter strand.

The original proposal that the zwitterionic form of N^7-MedG would be present in DNA and that it could mispair with T[66], has not been confirmed using either DNA or RNA polymerising assays[67]. There is good evidence, however, that the modified base O^6-alkylG induces miscoding both *in vitro* and *in vivo*[46,67,68]. Both O^6-MeG and O^6-EtG lead to aberrant nucleotide incorporation during RNA and DNA synthesis[69]. *In vitro* DNA experiments indicate that O^6-Pr^{n}dG and O^6-Bu^{n}dG are also promutagenic[69].

The miscoding properties of these bases arises because O^6-alkylG prefers to hydrogen bond to T, which is

introduced in place of C and therefore leads to a permanent alteration in the genetic sequence (Figure 1.8). The extent of this mispairing is related to the nature of the alkyl group. Thus, Lawley et al[70] have shown that O^6-EtG incorporates only 36% of T and 64% of C, and other work has shown that O^6-alkylG incorporates both C, (the normal base), and T, (the abnormal base), during DNA synthesis[67,71].

The only other known carcinogenic lesion in DNA, is the formation of O^4-alkylT adducts. Experiments with RNA and DNA polymerases have shown that O^4-alkylT base pairs with G, (whereas T prefers to base pair with A)[72,73]. Further, O^4-alkylT does not appear to incorporate the normal base T during DNA synthesis[74].

Alkylation of the other sites of DNA do appear to be significant from a carcinogenic stand point. Thus, formation of N^3-alkylG occurs but in vitro miscoding experiments have not indicated that this leads to mispairing. Excision of N^3-alkylG from DNA proceeds readily in vivo, which implies that the lesion is potentially harmful[75].Both N^1 and N^7-alkylA are removed enzymatically from DNA[76]. N^3-alkylA is very unstable, and enzymes have been shown to remove this adduct from DNA in vitro and in vivo[76]. The major alkylation product of C, N^3-alkylC allows non-complementary incorporation of

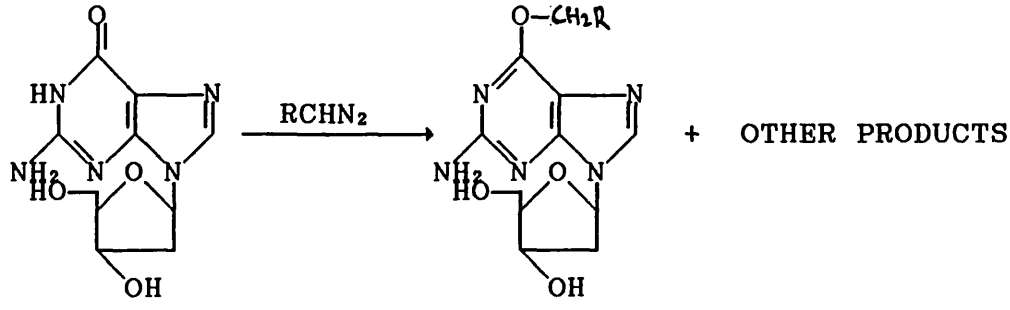
UMP (uracil monophosphate) and AMP (adenosine monophosphate) during polyribonucleotide synthesis[77,78], but N^3-alkylC does not appear to miscode in a DNA polymerase system[71]. No evidence has so far been obtained to suggest that O^2-alkylC induces mispairing[79].

To summarize, when alkyl modifications in DNA are considered in terms of their promutagenic properties, and location of the alkyl group around the base, three classes can be recognized. The first consists of modifications which alter the tautomeric form of the base and lead to base pairing with non-complementary bases, eg O^6-alkylG and O^4-alkylT[74]. The second consists of modifications involving steric interference with the base pairing, with or without tautomeric change, eg N^1-alkyl purines and N^3-alkyl pyrimidines. These do not give significant errors during DNA synthesis[67,72], but can give base pairing errors during RNA synthesis[74,80]. The third group are adducts that do not involve hydrogen bonding positions, such as N^7 and N^3-alkyl purines and miscoding is not involved[67,72].

1.7 SYNTHESIS OF ALKYLATED NUCLEOSIDES.

1.7.1 O^6-ALKYL-2/-DEOXYGUANOSINE.

The classical synthesis of O^6-alkyl-2/-deoxyguanosines (6) involves the reaction between the unblocked nucleoside (5) and an active alkyl moiety generated from the appropriate N-alkyl-N-nitroso compound[81-83], as shown in Figure 1.10.



(5) (6)

FIGURE 1.10 DIRECT ALKYLATION OF $2'_D$G BY N-ALKYL-N-NITROSOCOMPOUNDS.

There are two fundamental problems with this approach, formation of regio isomers, and rearrangement of the alkyl chain often occurs. The ambiguous nature of direct alkylation reactions of 2/-dG, results in the formation of several products whose yields vary with the reaction conditions. Typically, three adducts are generally obtained by direct alkylation. Thus, N-methyl-

N-nitrosourea in CH_3OH gave N^1-methyl-2/-dG (19%), O^6-methyl-2/-dG (22%) and N^7-methyl-2/-dG (10%)[84]. The alkylation reaction proceeds through generation of an alkyl carbocation[85], and when the reaction is carried out in aqueous solution rearrangement of the alkyl group can occur. For example, Park et al observed the formation of O^6-isopropyl-2/-dG (39%) when N-n-propyl-N-nitrosourea was reacted with 2/-dG in phosphate buffer pH=7.4, and Saffhill et al[87] observed the formation of n-butyl and sec-butyl-2/-dG derivatives with N-n-butyl-N-nitrosourea in Tris buffer pH=7.2. The extent of rearrangement is dependent on the reaction conditions, solvent polarity appears to be important[81,88].

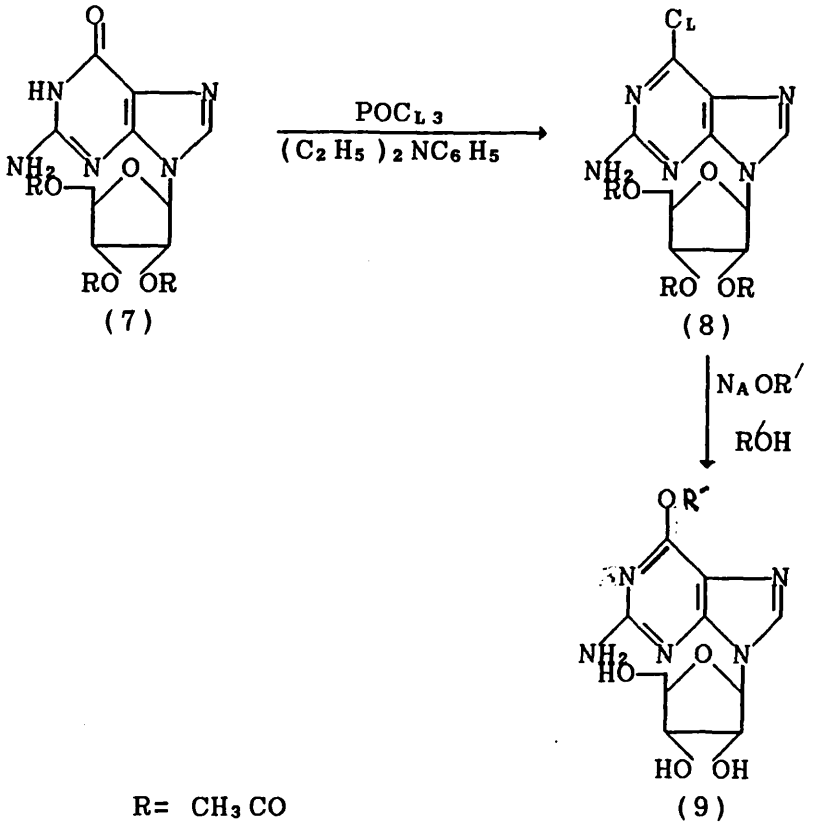
As an alternative to direct alkylation of 2/-dG, activation of 2/-dG followed by addition of alkoxide ions is used to synthesize O^6-alkyl-2/-dG compounds by a regio specific route.

Initially activation of the nucleoside involved conversion of the nucleoside to its 6-chloro derivative. Synthetic routes to 6-chloropurines were originally developed to enable successful synthesis of purine nucleosides to be performed using the coupling of an appropriately blocked halosugar with the metallic salt of a selected purine derivative[89-91]. In particular, the

use of 6-chloropurines enabled hydroxy, mercapto and aminopurines to be synthesized by the coupling procedure[92,93]. However, due to contamination with mercury and the low yields obtained with the chloro-mercury coupling procedure, the possibility of direct chlorination of nucleosides was considered[94].

Gerster et al[95] obtained 6-chloro-2/-3/-5/-tri-O-acetylguanosine (8) from 2/-3/-5/-tri-O-acetylguanosine (7) using $POCl_3$ and N-N-diethylaniline under refluxing conditions (Figure 1.11). N-N-Diethylaniline prevented loss of the ribose moiety through acidic hydrolysis. The Gerster procedure has been used to synthesize 6-chloro-3/-5/-di-O-acetyl-2/-deoxyguanosine[96], but in greatly reduced yield. Conversion of the 6-chloro nucleoside to the corresponding O^6-alkyl derivative (9), generally proceeds in high yields[96], although forcing reflux conditions are often required[97,98].

Due to difficulties in the synthesis of 6-chloro nucleosides, particularly 6-chloro-2/-deoxyguanosines, alternative activated nucleosides have been developed. Modern synthetic procedures use O^6-sulphonyl derivatives as the activated nucleoside to synthesize O^6-alkyl nucleosides[99]. This synthetic route is discussed in Chapter 2.



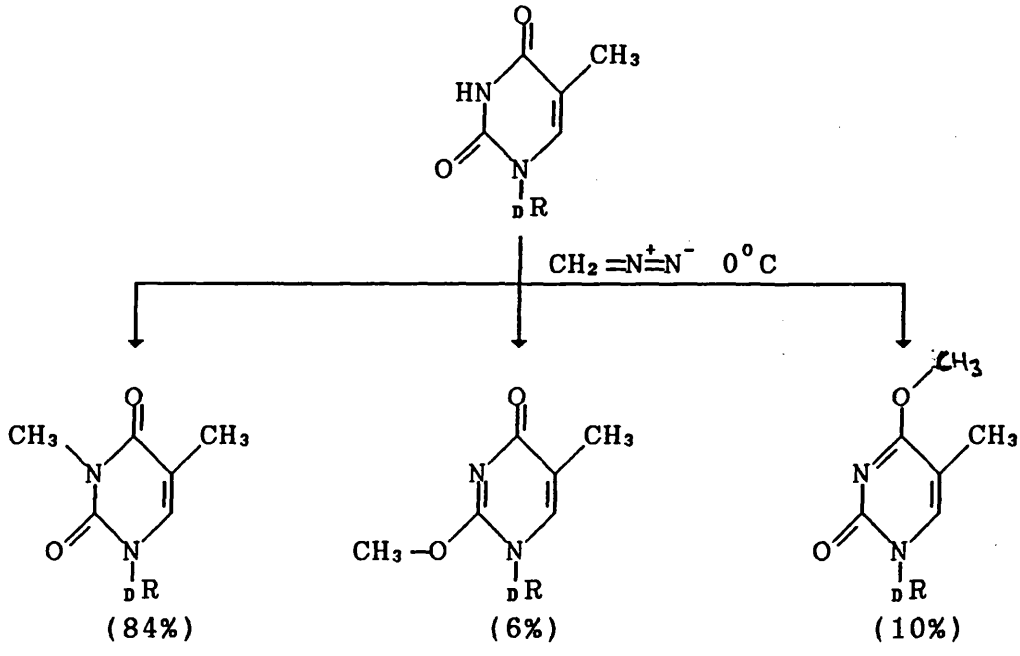
(7) (8) (9)

FIGURE 1.11 SYNTHESIS OF O^6-ALKYLNUCLEOSIDES VIA THE 6-CHLORO DERIVATIVE.

1.7.2 O^4-alkylthymidines.

Synthesis of alkylthymidines dates back to 1934 with the reaction of thymidine with diazoalkanes[100]. However, this approach leads to low yield reactions and mixtures of O and N-alkylthymidine derivatives, under a variety of conditions employed[65,83,101]. Generally, longer chain diazoalkanes give predominantly more O-alkyl adducts[102], but in all cases exhaustive purification techniques are required to isolate the pure product[65]. A typical

example of the reaction of diazoalkanes with thymidine is given in Figure 1.12.



DR= 2/-DEOXYRIBOSE

FIGURE 1.12

O^4-Alkylation of thymidine has also been achieved by reaction of a suitably protected thymidine with alkylhalides in the presence of a silver catalyst[103]. Thus, Kiriasis[104] reacted 3/-5/-di-O-acetylthymidine (10) with an alkylhalide and silver carbonate in toluene at 40-50°C. After deprotection, the expected O^4-alkylthymidine (11) and 2,4-di-O-alkylthymine (12) were isolated after chromatographic separation (Figure 1.13).

Recently developed methods for the synthesis of O^4-alkylthimidines also use amide activation but via chemical modification, using intermediary triazoles[104,105] and imidazoles[106], followed by nucleophilic displacement by the corresponding alcohols

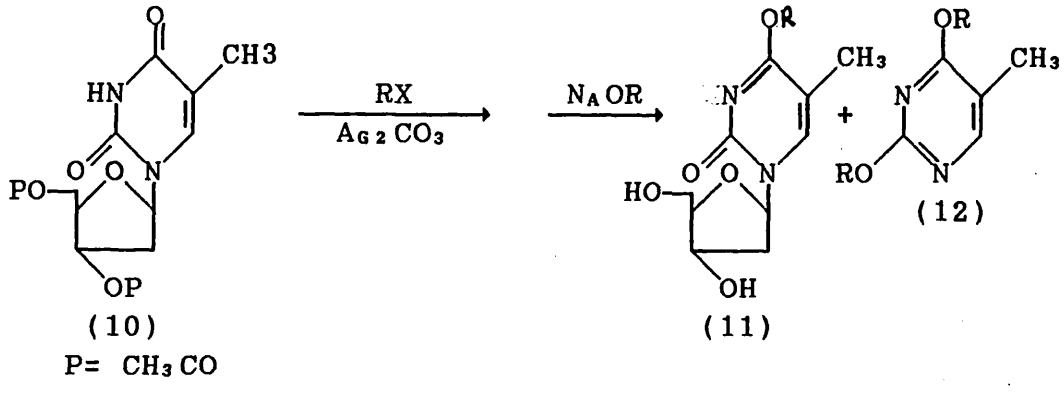


FIGURE 1.13

and alkoxides respectively. A typical example is the procedure reported by Li and Swann (Figure 1.14)[107,108]. The suitably protected thymidine (13) is converted to the O^4-phosphoryl derivative (14), followed by displacement of the phosphonate with 3-nitro-1,2,4,triazole to give a nitrotriazole intermediate (15). The nitrotriazole is then displaced from (15) by the appropriate alkoxide ion. Removal of the protecting groups results in isolation of O^4-alkylthymidines in good yields.

The procedure is similar to the Gaffney and Jones synthetic method for O^6-2/-deoxyguanosines[99]. However, the synthesis of O^4-alkylthymidines proceeds as a one pot synthesis starting with the protected thymidines and only isolating the final product. The isolated yields are again dependent on the nature of the alkoxide ion.

A MODERN ROUTE TO O^4-ALKYLTHYMIDINES.

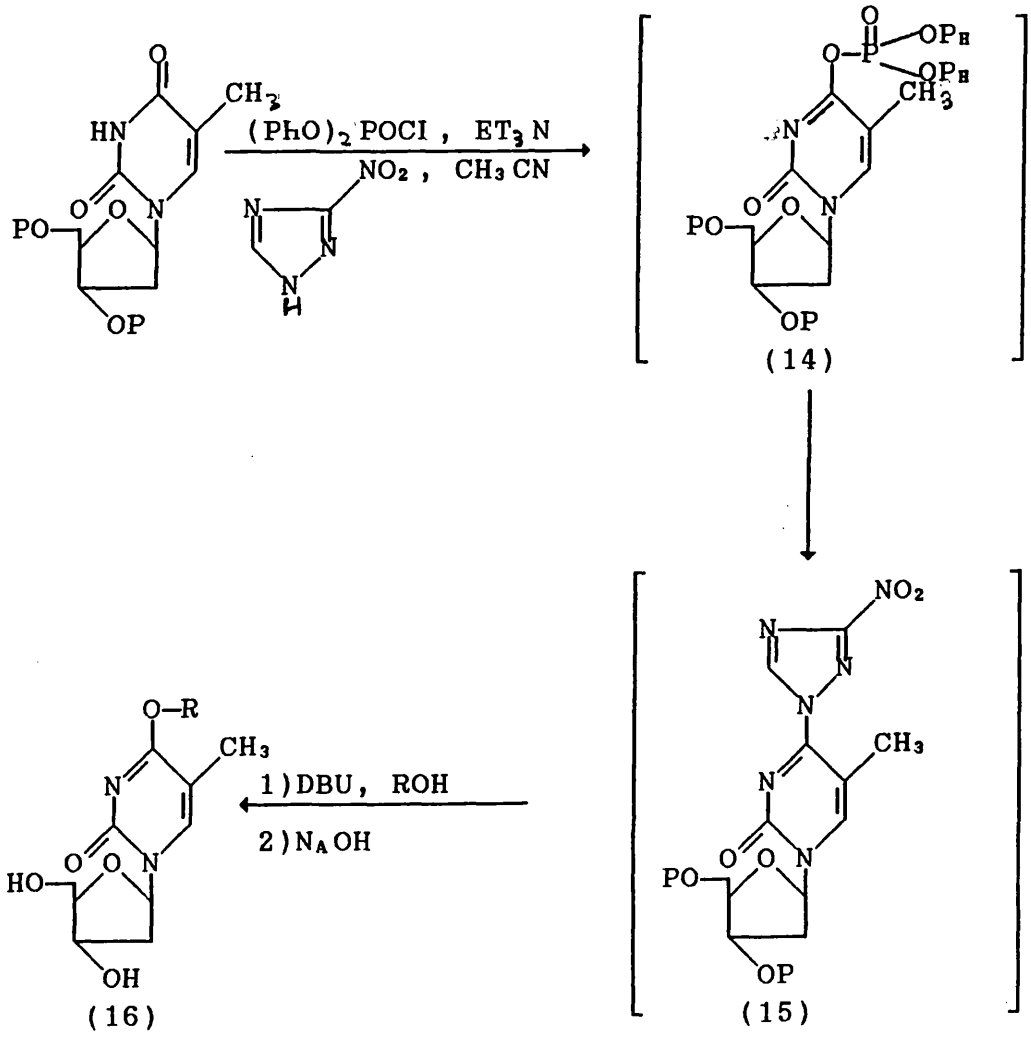


FIGURE 1.14

1.8 DNA ADDUCT ANALYSIS.

The interaction of carcinogens with biological macromolecules is a common phenomena, and is usually associated with adduct formation. For most sub-lethal *in vivo* experiments, only about 1 in 10^5-10^7 nucleotide bases of DNA are modified by interaction with the carcinogen[108]. Thus, very sensitive techniques are required to detect and quantitate the DNA modifications.

The simplest procedure is the use of ^3H or ^{14}C-radiolabelled carcinogens to generate modified DNA bases which are radiolabelled and therefore detectable with high sensitivity[109]. Recently, other methods not requiring the synthesis of radiolabelled carcinogens have been developed. Thus for compounds such as polycyclic aromatic hydrocarbons, either fluorescence or immunofluorescence-spectroscopy is sufficiently sensitive to detect carcinogen-DNA adducts formed *in vivo*[110]. Several other immunochemical methods have been developed, *eg* immuno-slot-blot[111], immuno-electron microscopy[111], and enzyme linked immunosorbent assay (ELISA)[112], the latter being discussed in more detail in Chapter 4.

Immunochemical methods are a sensitive means of detecting DNA adducts where use of radio-labelled substances is not possible, eg in monitoring human exposure to industrial carcinogens. These methods are limited by the availability of carcinogen-DNA adducts required to generate antibodies, and they are only applicable to known carcinogens whose metabolism and interactions with DNA are well characterised. Nonetheless, antibodies have been raised against many DNA-adducts[113-115].

Another recently developed technique uses enzymic ^{32}P-phosphorylation to post-label the DNA-adducts[116]. In this procedure DNA containing bound carcinogen is first hydrolysed to yield 2/-deoxyribonucleoside 3/-phosphates which are then converted into 3/-5/-biphosphate analogues using [Y-^{32}P] ATP and T^{4}-polynucleotide kinase[116]. After chromatographic resolution, the adducts are detected by monitoring the ^{32}P-radiolabel. The method is general and enables a large number of substances to be examined for DNA binding, without prior synthesis of radio-labelled materials. It can be used to monitor human exposure to carcinogens but gives no information on the structure of DNA-adducts.

Other indirect methods can be used to detect DNA damage. For example, double or single-strand breaks in DNA are often the outcome of interaction with carcinogenic agents, and this damage can be observed by monitoring the electrophoretic properties of the DNA[117].

Most modern methods of detecting DNA damage, however, utilize immunochemical procedures for the detection of specific DNA-adducts, and where feasible this evidence is corroborated by post-labelling and other sensitive techniques[111].

1.9 CONCLUSION.

Interaction of simple alkylating agents with DNA has been extensively studied in the literature[9,12]. Interest has now focused on endogenous formation of chemical carcinogens, particular emphasis being paid to the relevance of gastric nitrosation and human cancer[23]. However, although N-nitroso compounds have been detected in gastric aspirates[37], the structure of these compounds is generally unclear.

One possible source of N-nitroso compounds arises from nitrosation of dietary components, particularly proteins, peptides and bile acid conjugates. Initial studies have indicated that the N-nitrosopeptides and N-nitrosobile acid conjugates are mutagenic[29,31], although how they interact with DNA, and the products from such alkylation reactions have not been studied.

THe present work was intended to provide information on how an N-nitrosopeptide (N-(N/-acetyl-L-prolyl)-N-nitrosoglycine) interacts with DNA in vitro, particular emphasis being put on the detection of O^6-alkylG adducts (a potential carcinogenic lesion). The development of an immunochemical assay procedure was employed as a means of monitoring such exposure.

Chapter 2.

SYNTHETIC ROUTES TO O^6-ALKYLATED NUCLEOSIDES. RESULTS AND DISCUSSION.

2.1 PREVIEW.

A major aim of the project was the synthesis of O^6-carboxymethyl-2/-deoxyguanosine (O^6-CMdG) haptens and the generation of polyclonal antibodies for the detection and assay of O^6-CMdG in biological samples. O^6-CMdG was considered to be the most probable antigen resulting from exposure to N-nitrosocompounds produced by endogenous nitrosation reactions. The most likely substrates for endogenous nitrosations in the stomach are dietary amino acids, peptides[18] and bile acid conjugates secreted in the process of fat digestion[25]. These reactions produce diazo and N-nitroso intermediates[26,28] which may act as carboxymethylating agents for DNA. The most significant site for DNA carboxymethylation with regard to cancer induction is the O^6-position of guanine bases. Thus identification and quantitation of O^6-CMdG in biological materials by immunochemical methods may be an exact and quantitative index of exposure to endogenous nitrosation reactions.

2.2.1 SYNTHESIS OF O^6-ALKYL-2/-DEOXYGUANOSINES.

In principle the synthesis of O^6-alkyl-2/-deoxyguanosines can follow either one of two general

methods. The first involves introduction of the O^6-alkyl substituent as an alkoxy moiety with the 2/-deoxyguanosine fragment acting as a electrophile. The second involves introduction of the O^6-alkyl substituent as an alkyl moiety with the 2/-deoxyguanosine fragment acting as a nucleophile.

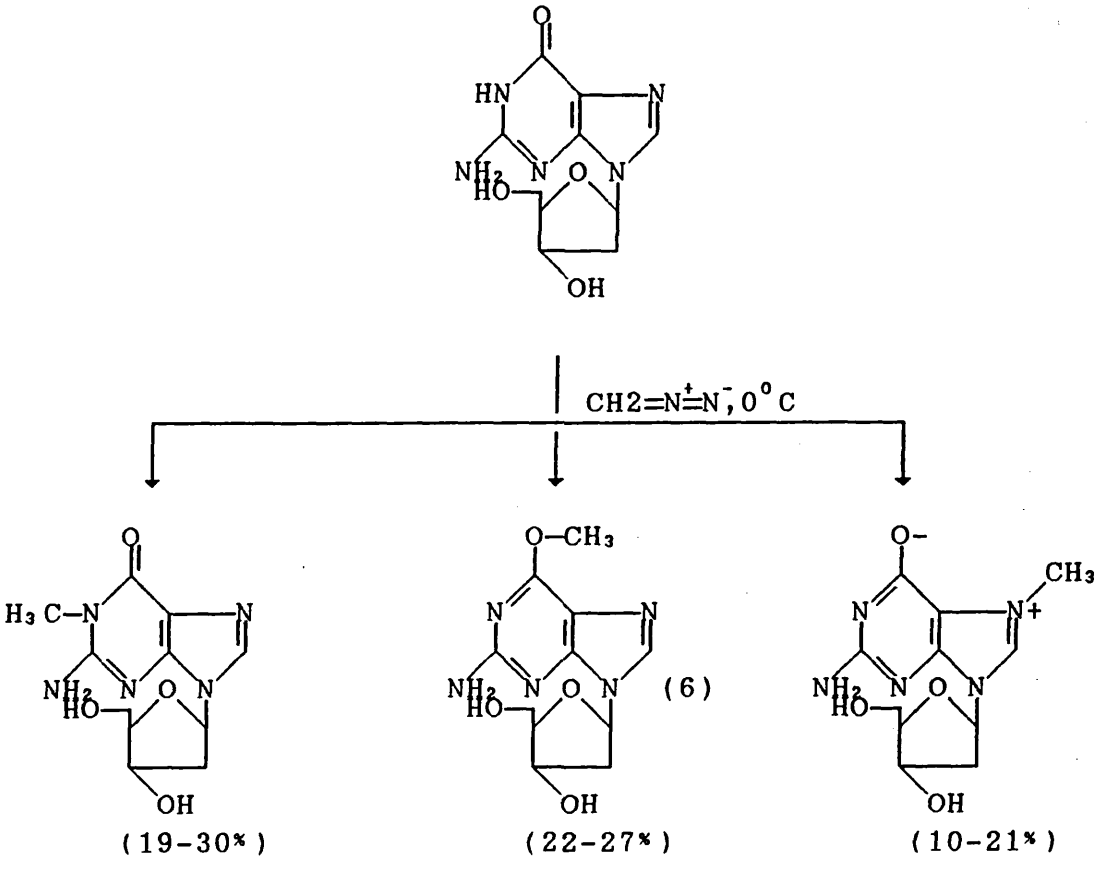


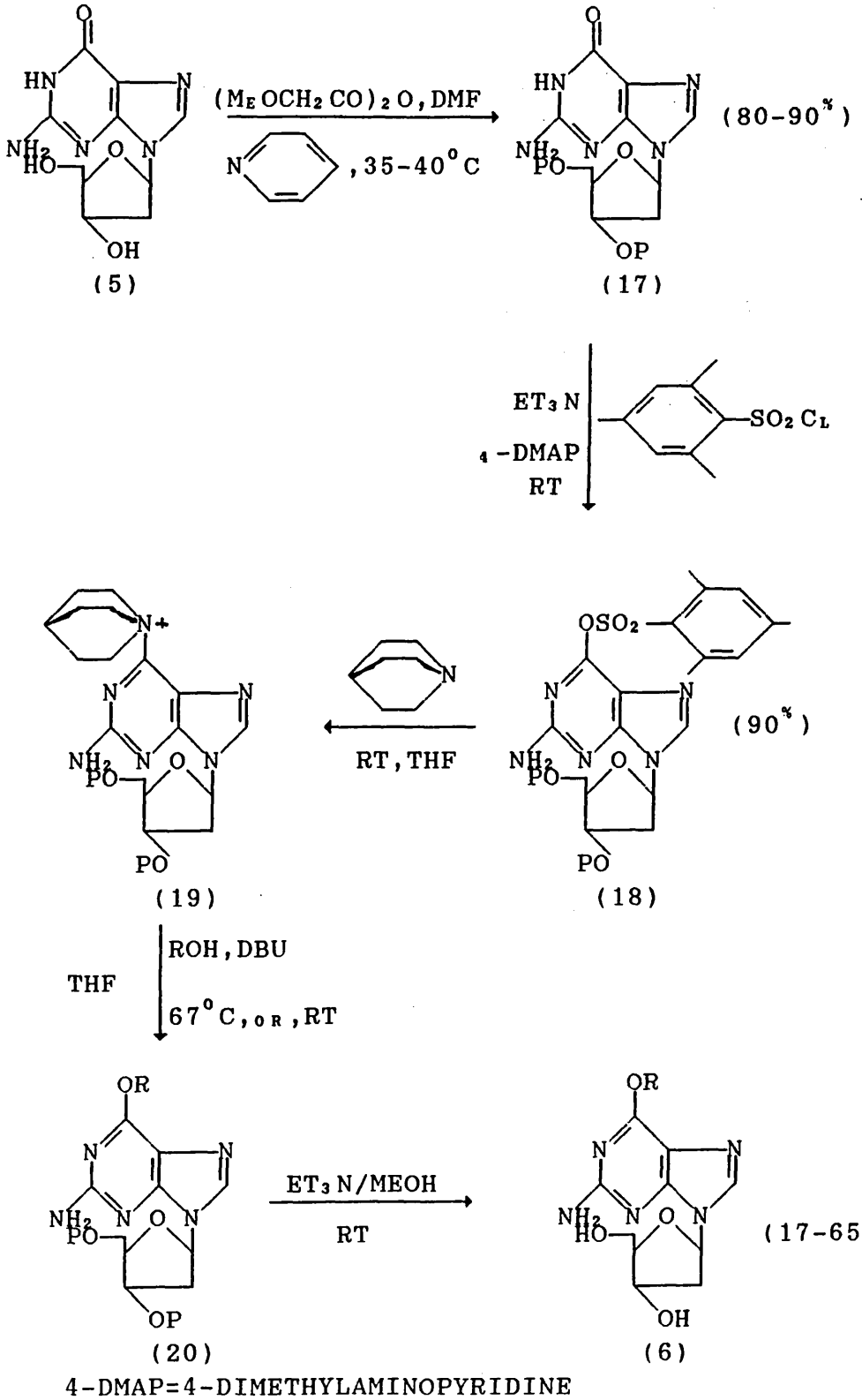
FIGURE 2.1

Direct alkylation of 2/-deoxyguanosine is well known as described in Chapter 1. For example with diazoalkanes several products are obtained with relatively low yields

(22-27%) of the required O^6-alkyl-2'-deoxyguanosines (6) (Figure 2.1)[83,84].The isolation of pure products from the reaction mixture is feasible but difficult.

At an early stage of the project it was decided not to synthesize O^6-CMdG by the alkylation of 2'-deoxyguanosine with diazoacetic acid. The alternative strategy involving unambiguous nucleophilic displacement at the 6-position of 2'-deoxyguanosine was followed using the appropriate glycolic acid reagent.

The procedure for the synthesis of O^6-CMdG was a variant of that described by Gaffney and Jones for other O^6-alkyl-2'-deoxyguanosines[99]. The procedure is outlined in Figure 2.2. It requires conversion of a suitably protected 2'-deoxyguanosine (17) to the O^6-mesityl derivative (18), followed by displacement of the mesityl group with quinuclidine to give a quinuclidine ion intermediate (19). Subsequently the quinuclidine is displaced from (19) by the appropriate alcohol to give the protected O^6-alkyl-2'-deoxyguanosine (20). The final step is removal of the protecting groups from (20) to give the required O^6-alkyl-2'-deoxyguanosine. An indication of the yields for each stage of the synthesis



4-DMAP=4-DIMETHYLAMINOPYRIDINE
DMF=N-N-DIMETHYLFORMAMIDE
P=CH_3OCH_2CO; THF=TETRAHYDROFURAN
DBU=1,8-DIAZABICYCLO[5,4,0]UNDEC-7-ENE

FIGURE 2.2

are also given in Figure 2.2. The yield for the conversion of (18) to (6) varied from 20-70% depending on the structure of the O^6-alkyl substituent. The overall yield for the conversion of (5) to (6) was in the range 17-60% ,again depending on the structure of the O^6-alkyl substituent.

2.2.2 2-DEOXYGUANOSINE PROTECTION.

A major practical difficulty in the synthesis of most nucleoside compounds is their low solubility in organic solvents. Protection of 2/-deoxyribose hydroxyl groups partially overcomes this problem, and reduces undesirable side reactions of the hydroxyl groups throughout the synthesis. These protecting groups have to be stable throughout the synthesis, but removable under mild preferably basic conditions. It was decided to use the 2-methoxyacetyl function to protect the 2/-deoxyribose hydroxyl functions because these fulfil the above criteria[118]. The 2-methoxyacetyl function was preferred to the acetyl protection group because the 2-methoxyacetyl group is more easily removed under basic conditions[97]. The 2-methoxyacetylation was carried out in DMF using pyridine to remove HCl and to catalyse the reaction. The protected 2/-deoxyguanosine (17) was obtained in average yields of 80-90%. Triturating the

residue with $(C_2H_5)_2O$ and then washing with $(C_2H_5)_2O/C_2H_5OH$ produced higher yields ca 90%, than simple recrystalisation from C_2H_5OH (ca 80%).

Conversion of (17) to the O^6-mesityl derivative (18) was carried out without difficulty following the procedure of Gaffney and Jones[99]. Using reaction times of 2h, gave the O^6-mesityl derivative (18) after purification by column chromatography on silica, 60g column $CH_3OH/CHCl_3$ (3:97v/v) eluent, in yields of ca 95%. With longer reaction times than 2h, decreased yields (40-60%) of the O^6-mesityl derivative (18) were obtained, with formation of a fluorescent by-product readily observed by t.l.c. on silica CH_3OH/CH_2Cl_2 (10:90v/v). This by-product was probably the triethylammonium salt from nucleophilic displacement of the mesityl group by triethylamine (see Section 2.2.4).

2.2.3 DIRECT DISPLACEMENT OF THE O^6-MESITYLSULPHONYL GROUP.

Several attempts to displace the mesityl group of (18) directly by alkoxides (Figure 2.3) were unsuccessful. Using either methanol, or methylglycolate under several different reaction conditions, invariably

lead to the precipitation of the protected 2/-deoxyguanosine (17) from the reaction solution, rather than formation of the protected O^6-alkyl-2/-deoxyguanosine (20).

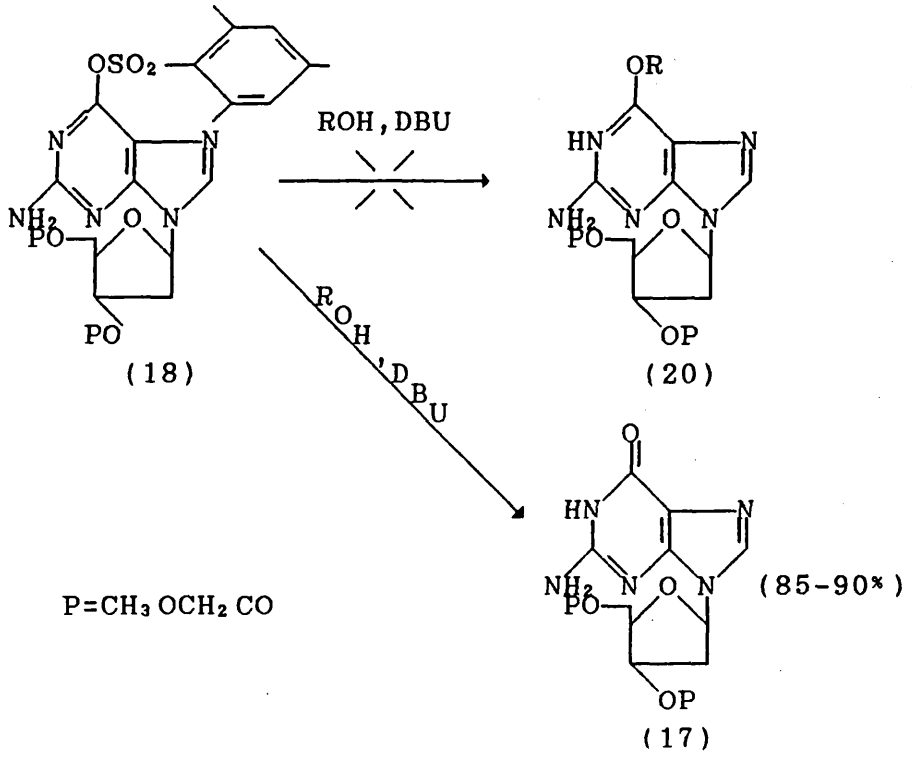


FIGURE 2.3

Gaffney and Jones[99] reported similar results with methanol, although Jones et al[99] were able to successfully displace the 6-O-methane-sulphonyl group by phenoxide ion. No obvious reason is apparent for the different results, but the phenoxide ion is a "softer" nucleophile than the methoxide ion, and steric factors are reduced for the 6-O-met hanesulphonyl substituent.

In any event, it was decided to follow the indirect displacement of the O^6-mesitylsulphonyl group used by Gaffney and Jones[99].

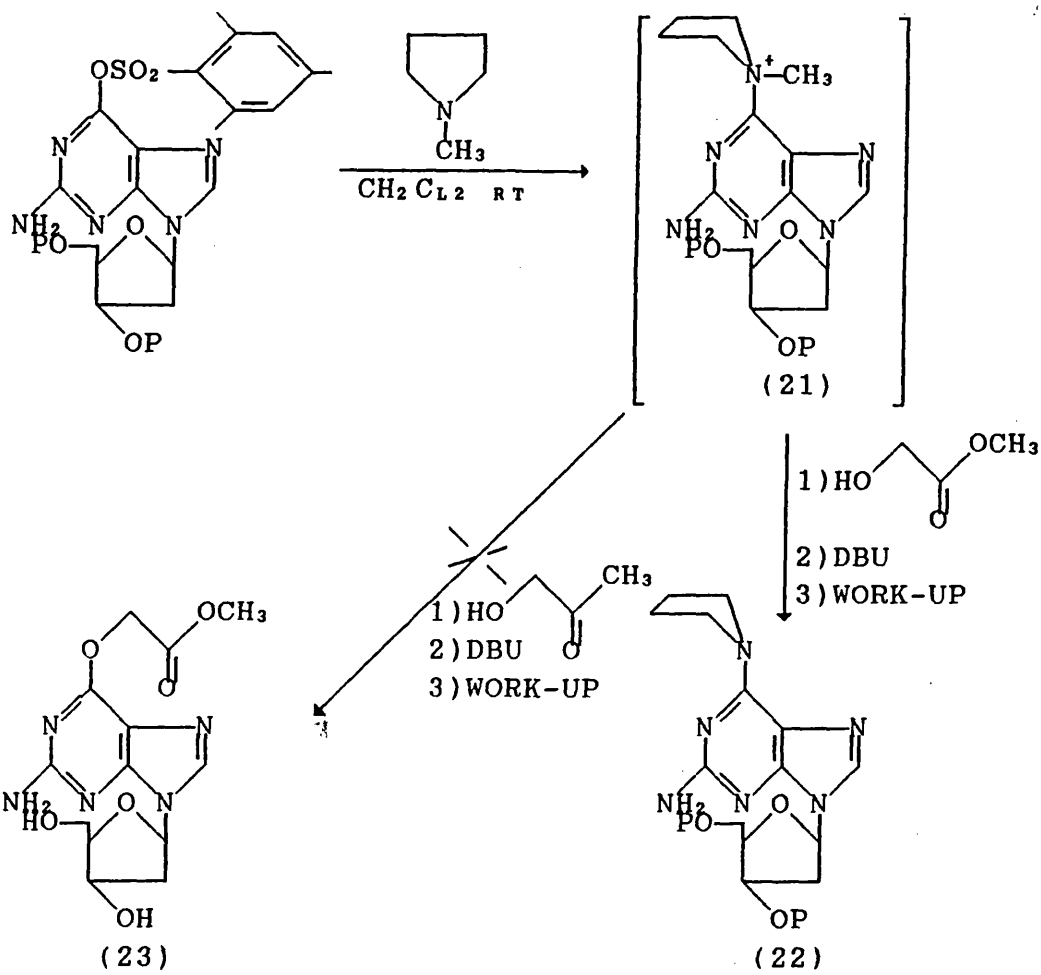
2.2.4 INDIRECT DISPLACEMENT OF THE O^6-MESITYLSULPHONYL GROUP.

Gaffney and Jones advocated the use of trimethylamine to activate the O^6-position of the protected 2/-deoxyguanosine to nucleophilic displacement by alkoxide ions. In early experiments using dry trimethylamine, the only significant product obtained even with excess alcohols was the 6-dimethylamino substituted 2/-deoxyguanosine. It was rationalised that this arose either from demethylation of the trimethylammonium ion intermediate, or by preferential reaction by dimethylamine impurities in the trimethylamine despite purification[120]. It was therefore decided to use other tertiary amines to activate the O^6-mesitylsulphonyl intermediate (18) which were easier to handle than trimethylamine.

N-Methylpyrrolidine was initially chosen as the alternative tertiary amine because it is easier to dry and purify than trimethylamine by conventional

distillation. Exhaustive measures were undertaken to remove all pyrrolidine from the N-methylpyrrolidine. The pyrrolidine impurity reacts faster with the O^6-mesitylsulphonyl intermediate (18) than N-methylpyrrolidine, and the resultant 6-pyrrolidyl compound is stable and unreactive towards alkoxide ions. N-Methylpyrrolidine was therefore purified by fractional distillation from solid KOH, followed by treatment with phenyl isocyanate at room temperature as described in Chapter 5. A second fractional distillation gave a product which on analysis by g.l.c., showed the pyrrolidine content was <0.08%. Reaction with tosylchloride to remove the pyrrolidine impurity was considered as an alternative to treatment with phenyl isocyanate, but discounted because forcing (reflux) conditions and a pyridine catalyst are usually required.

The use of purified N-methylpyrrolidine to activate the 6-position of the protected 2/-deoxyguanosine proved satisfactory for the synthesis of O^6-methyl-2/-deoxyguanosine. Thus following the procedure of Gaffney and Jones[99] reacting (18) with 20 equivalents of N-methylpyrrolidine, followed by reaction with 3 equivalents of DBU and 10 equivalents of CH_3OH gave O^6-methyl-2/-deoxyguanosine in yields of ca 60%. Attempts to synthesize O^6-carboxymethyl methyl ester-2/-deoxyguanosine (23) using methylglycolate in place of CH_3OH, however, were unsuccessful for a wide range of



DBU=1,8-DIAZABICYCLO[5,4,0]UNDEC-7-ENE
P=CH_3OCH_2O

FIGURE 2.4

reaction conditions and different reagent concentrations. The 6-pyrrolidyl-2′-deoxyguanosine (22) was the only product isolated under a variety of reaction conditions in yields of 50-60%.

The preferential formation of (22) cannot be ascribed to the presence of pyrrolidine impurities, which are calculated to give a maximum yield of (22) of only 1.6% under the reaction conditions. Its formation must be associated with the facile the demethylation reaction of the N-methylpyrrolidinium intermediate (21) in preference to its nucleophilic displacement by the methylglycolate anion (Figure 2.4). With hindsight, it is possible that because of steric factors elimination of the methyl group of the N-methylpyrrolidium ion (21) is preferred to nucleophilic displacement by alkoxide ion, especially at the higher reaction temperatures employed for methylglycolate.

To circumvent this problem, quinuclidine was examined as an alternative tertiary amine to activate the O^6-position of 2/-deoxyguanosine. Quinuclidine is sterically less demanding and being a solid, it can be readily purified by either sublimation or recrystalisation. As reported later (Sections 2.2.5-2.2.7) the use of quinuclidine enabled the successful synthesis of O^6-CMdG.

In the course of evaluating the various tertiary amines, the suitability of different solvents and

different glycolate moieties was also investigated. The results of these experiments are conveniently discussed below.

2.2.5 GLYCOLATE MOIETY.

In early experiments the carboxymethylation of (18) was attempted with benzylglycolate, DBU and N-methylpyrrolidine in CH_2Cl_2 heated under reflux. In view of the lack of success the stability of benzylglycolate in CH_2CH_2 in the presence of DBU and N-methylpyrrolidine was investigated. These experiments showed that benzylglycolate was unstable under the reaction conditions. On t.l.c. analysis using silica plate EtOAc/CH_3OH (95:5v/v) eluent, the benzylglycolate decomposed to a more polar product, which was not identified but is probably a glycolate polymer. Similar experiments using methylglycolate in place of benzylglycolate showed by g.l.c. analysis that methylglycolate was stable in the reaction media, with no apparent decrease in methylglycolate concentration in 24h. Consequently, methylglycolate was selected as the most suitable carboxymethyl synthon.

This change had ramifications in the procedure for removal of the 2-methoxyacetyl protecting groups of the

2/-deoxyribose substituent. In particular, the use of heterogeneous reaction conditions and 2.5M NaOH of Gaffney and Jones[99] for the deprotection step was considered too harsh for the methylglycolate substituent. In, concentrated base, hydrolysis of the methylglycolate substituent to glycolate, should proceed readily to give the O^6-CMdG produced as a salt, whose isolation and purification would be difficult. Accordingly, milder, homogeneous reaction conditions, using Et_3N/CH_3OH were employed for the deprotection of the O^6-carboxymethyl methylester-2/-deoxyguanosine compound.

2.2.6 SOLVENT.

Using methylglycolate, displacement of the O^6-mesitylsulphonyl substituent from (18) using CH_2Cl_2 as the reaction solvent, required heating under reflux and long reaction times to obtain (23) in moderate (30%) yields (Table 2.1). Since the reaction involves formation of an ionic alkylammonium ion intermediate (19), the use of more polar solvents should be beneficial. Thus, using THF as the reaction solvent, gave higher yields of (23) under milder reaction conditions and with shorter reaction times. Examination of the results for comparable reactions in CH_2Cl_2 and THF (Table 2.1), shows that reaction in THF at 25°C is ca 12-

Table 2.1. Effect of Solvent on the Yield of O^6-carboxymethyl methyl ester-2′-deoxyguanosine. Initial reagent concentrations were [O^6-mesitylsulphonyl-3′-5′-di-O-methoxyacetyl-2′-deoxyguanosine] (8)=1.27×10^{-1} mole l^{-1}; [Quinuclidine]=6.33×10^{-1} mole l^{-1}; [Methylglycolate]=5.00×10^{-1} mole l^{-1}; [DBU]=1.50×10^{-1} mole l^{-1}.

Solvent	Intermediate Formation 25°C	Reaction Temperature	Reaction Time	Yield
CH_2Cl_2	1.5-2.0h	40°C	24h	29%
THF	3.0-4.0h	25°C	3-4h	70%

fold faster than in CH_2Cl_2 at 40°C. Allowing for the different reaction temperatures this suggests that reaction in THF is ca 20-fold faster than in CH_2Cl_2.

THF (e=8.9)[121] has a higher dielectric constant than CH_2Cl_2, (e=7.6)[121], so displacement of the tertiary amine from the alkylammonium ion intermediate (19) by methylglycolate is probably the slow step of the reaction. In any event, the use of THF as the reaction media is clearly beneficial.

2.2.7 CONVERSION OF O^6-MESITYLSULPHONYL-3/-5/-DI-O-METHOXY-ACETYL-2/-DEOXYGUANOSINE (18) TO O^6-CARBOXYMETHYL-2/DEOXYGUANOSINE.

These reactions were carried out as a one-pot synthesis without isolation of intermediates. Formation of intermediate (19), however, was monitored by t.l.c. on silica.

Usually quinuclidine (2.5mmol) was added to a solution of the 6-mesitylsulphonyl derivative (18) (0.5mmol) in dry THF, which was then stirred at room temperature under dry nitrogen. The progress of the reaction was monitored by the loss of both O^6-mesitylsulphonyl compound (18) and the formation of a blue fluorescent spot at the baseline, attributed to the quinuclidine ion intermediate (19). These changes were easily followed on silica t.l.c. using EtOAc/CH_3OH (85:15v/v) as eluent. Loss of (18) was usually complete in 3-4h.

The quinuclidine ion intermediate is susceptible to attack by nucleophiles including the alkoxide ion. Hence, the appropriate dry alcohol (5-30mmol) and DBU (1.5-4.0mmol) in dry THF was then added to the above

reaction mixture, which was then stirred at room temperature under dry nitrogen. The progress of this reaction was followed by t.l.c. as before using EtOAc/CH_3OH (85:15v/v) as eluent. DBU was used to generate the alkoxide ion because it is a strong highly hindered base, and significant displacement of

Table 2.2 Reaction conditions employed and resultant yields obtained for the conversion of O^6-mesitylsulphonyl-3′-5′-di-O-methoxyacetyl-2′-deoxyguanosine to O^6-alkyl-2′-deoxyguanosines, via a quinuclidine ion intermediate, using alkoxide ions in THF. Initial reagent concentrations were O^6-mesitylsulphonyl-3′-5′-di-O-methoxyacetyl-2′-deoxyguanosine (8)=1.27×10^{-1} mole l^{-1}; quinuclidine=6.33×10^{-1}.

Compound	[DBU] molel^{-1}	[Alcohol] molel^{-1}	Reaction °C Temperature	Reaction Time h	Yield%
O^6-MedG	0.15	0.5	25	0.5	65
O^6-CMdG	0.15	0.5	25	4.0	65
O^6-Pr^i dG	0.25	2.0	67	24	20
O^6-Bu^t dG	0.4	3.0	67	72	u

u=unobtained.

quinuclidine by DBU alone was not observed. On completion of the reaction , the reaction solution was retained for removal of the 2-methoxyacetyl protecting groups.

The reaction conditions for the second stage of the reaction were varied depending on the bulkiness of the alkoxide ion. Thus nucleophilic substitution of the quinuclidinium ion intermediate (19) by small alkoxide ions (eg methoxide) were usually complete in <4.0h at 25°C,using a slight excess of alcohol (5.0mmol) and DBU (1.5mmol). Substitution by larger alkoxides, such as that from isopropoxide, required higher concentrations of both alcohol (20mmol) and DBU (2.5mmol), longer reaction times (24h) and higher reaction temperatures (up to 67°C). The reaction conditions for all of the O^6-alkyl-2/-deoxyguanosines derivatives synthesized are summarised in Table 2.2. It is evident that substitution by isopropoxide ion requires a 8-fold increase in the reaction time and the yield decreases to 20%. Substitution by t-butoxide proved unsuccessful even under forcing conditions. This confirms that nucleophilic substitution of the quinuclidine ion intermediate (19) by alkoxide ions is subject to steric constraints.

2.2.8 REMOVAL OF THE 2-METHOXYACETYL GROUPS AND PURIFICATION.

The 2-methoxyacetyl protecting groups were readily removed by triethylamine (4 equivalents) at room temperature. Following the removal of the solvent the O^6-alkyl-2/-deoxyguanosine was obtained as a colourless oil. This was purified by column chromatography on silica using EtOAc/CH_3OH (85:15v/v) as eluent. The fraction (rf=0.3-0.4) was isolated from the column, the other components of the reaction generally being present as baseline material in this system. O^6-Alkyl-2/-deoxyguanosines containing simple alkyl functions such as methyl and isopropyl, were further purified by recrystalisation from EtOAc. The O^6-carboxymethyl methylester-2/-deoxyguanosine (23), however, was purified by isocratic reverse phase H.P.L.C. on an ODS column using aqueous CH_3CN (13%) as eluent. This is described in more detail in Chapter 5. The H.P.L.C. trace (Figure 2.5) shows a typical chromatographic separation for (23) using this system. Generally only one impurity was present in the analyte,and that was due to mesitylsulphonic acid.

The H.P.L.C. purification enabled contamination caused by mesitylsulphonic acid to be removed.Pauly *et al*[122] commented on similar difficulties in removing sodium 2,4,6-tri-isopropylbenzene sulphonate for the

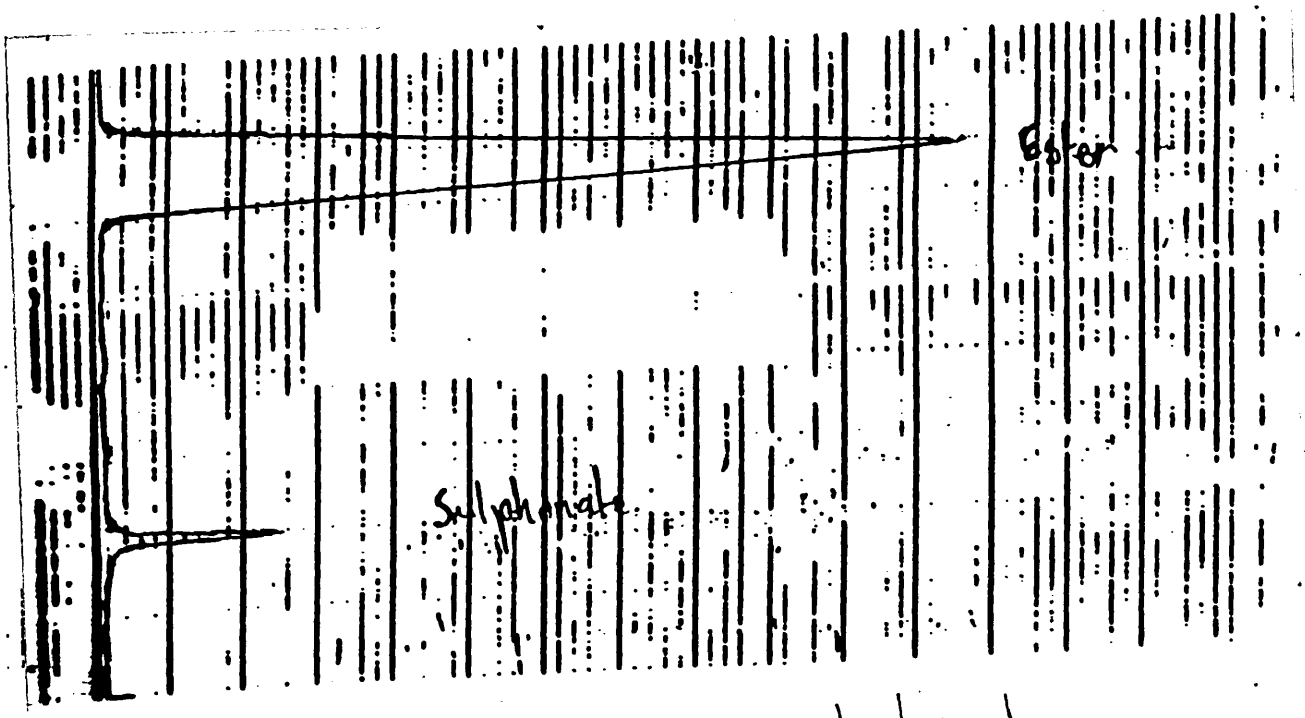


Figure 2.5 Preparative H.P.L.C purification of 06-carboxymethyl methyl ester-2'-deoxyguanosine, using an isocratic elution system (13% CH_3CN/H_2O v/v) at a flow rate of 20 cm^3/min. This system gave a retention time of ca 4 min for the impurity (mesitylene sulphonic acid), and 12 min for the ester. Detection was by UV at 254 nm.

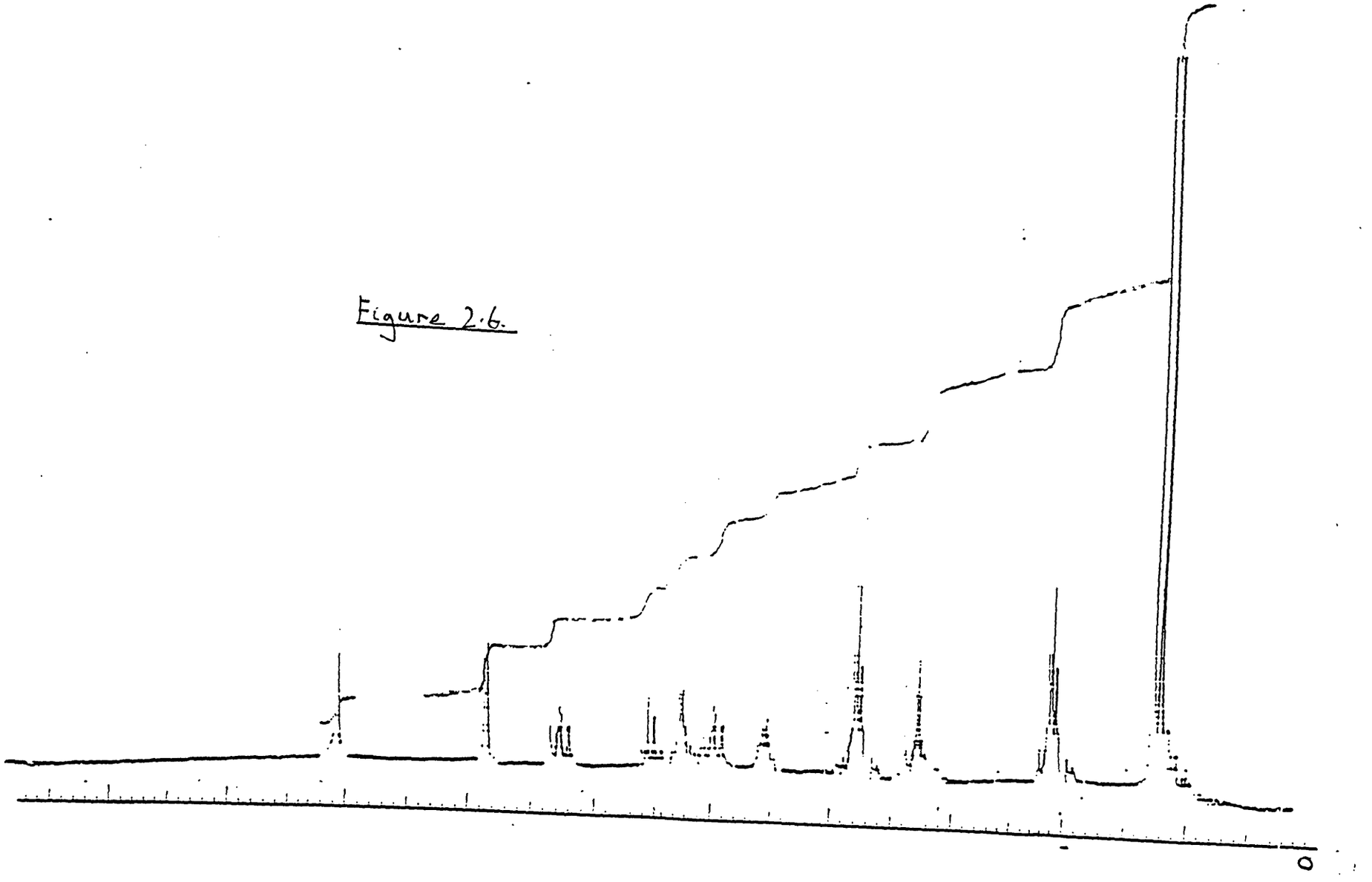
synthesis of N^2-protected O^6-alkyl-2/-deoxyguanosines, and mesitylsulphonyl impurities were present in the mass spectra of several of the compounds synthesized in the present study.

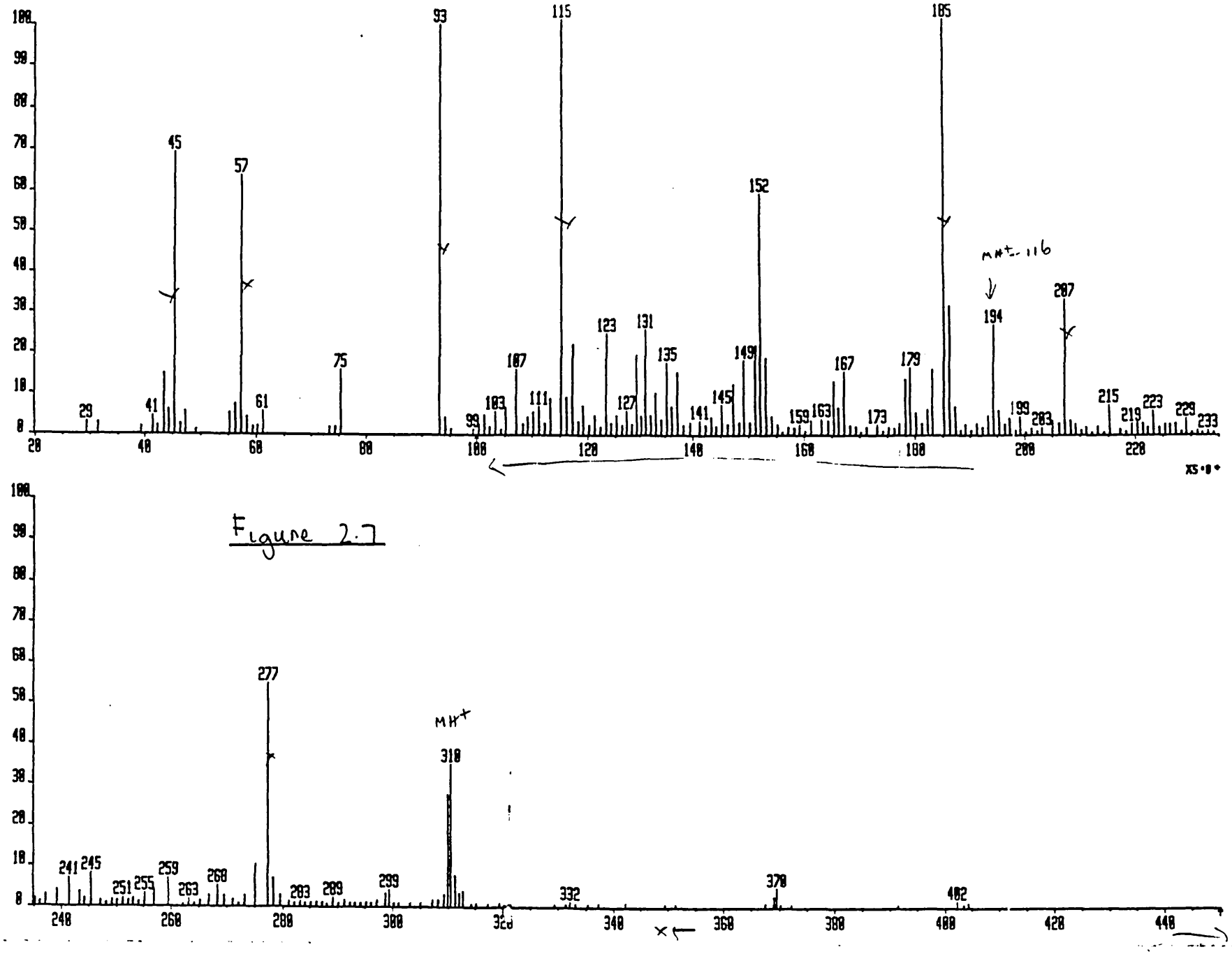
Conversion of (23) to O^6-carboxymethyl-2/-deoxyguanosine (O^6-CMdG) (24), was effected by room temperature hydrolysis using aqueous $Ca(OH)_2$, monitoring the reaction for the loss of (23) (rf=0.3) and the formation of a new baseline spot, by t.l.c. on silica using EtOAc/CH_3OH (85:15v/v) as eluent. The hydrolysis was usually complete in ca 18h. The O^6-CMdG was then isolated as the calcium salt by lyophilization of the reaction solution. The resultant powder was dissolved in ethanol, the residue (unreacted $Ca(OH)_2$) filtered off, and the ethanol removed by evaporation under reduced pressure to give O^6-CMdG in yields of ca 95%. The overall yield for the four step synthetic process was ca 53%.

2.2.9 SPECTROSCOPIC CHARACTERISATION OF O^6-ALKYL-2/-DEOXYGUANOSINES.

The O^6-alkyl-2/-deoxyguanosines were characterised by spectroscopic analysis by uv, ir, nmr and ms. General characteristics are exemplified by those for O^6-Pr^idG.

Figure 2.6.




MH+-116
MH+
Figure 2.7

UV Spectrum. This is very characteristic for all O^6-alkyl-2/-deoxyguanosines. At pH=7, the uv spectrum of O^6-Pr^idG exhibits a peak at λmax=249 (loge=4)due to the purine ring and a second peak at λmax=281 (loge=4) which is characteristic of O^6-substituted compounds.

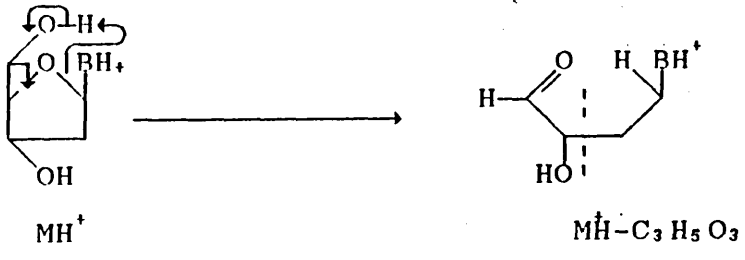
IR Spectrum. These were obtained as KBr dispersions and are relatively complicated, with few peaks characteristic of the O^6-alkyl-2/-deoxyguanosine structure. Thus, O^6-Pr^idG exhibits several peaks between νmax=3100 and 3400cm^{-1} due to the deoxyribose OH groups and the purine $-NH_2$ group, and peaks at νmax=1500cm^{-1} due to the C=C stretch of the purine ring and two peaks at νmax=1250 and 1065cm^{-1} due to the C-O stretch of the O^6-ether linkage.

^{1}H-NMR Spectrum. (d^6-DMSO) These are complicated by the complex pattern of the peaks relating to protons of the deoxyribose group. Nevertheless, the ^{1}H-nmr spectra contains two very characteristic peaks for the 2/-deoxyguanosine moiety, in addition to those for the O^6-alkyl substituents. The most characteristic 2/-deoxyguanosine peak is a singlet at δ=8.07ppm due to the C-8 proton. The other distinctive peak is the C/$_1$-H of the 2/-deoxyribose which appears as a triplet at δ=6.25ppm (Figure 2.6). Peaks at δ=6.87 (2H, brs), 5.30 (1H, m) and 5.00 (1H, t) for O^6-Pr^idG in Figure 2.6 disappear after the addition D_2O, and therefore relate to

the 2-NH_2 of the purine residue, the 3/- 5/-OH groups of the 2/-deoxyribose group. Multiplets at δ=2.25 (2H), 3.35 (2H), 3.80 (1H) and 4.55 (1H) which do not exchange after the addition of D_2O, relate to the $C/_2$, $C/_5$, $C/_4$ and $C/_3$ protons of the sugar. Peaks at δ=1.34 (6H, d, 2xCH_3) and 5.40 (1H, m, CH) in Figure 2.6 relate to the O^6-isopropyl substituent.

Mass Spectra. Both the EI and FAB-positive ion mass spectra contain a fragmentation pattern which is characteristic of all O^6-alkyl-2/-deoxyguanosines. In the case of O^6-Pr^{i}dG, Figure 2.7, the FAB-positive ion exhibits a molecular ion peak at m/z 310 (MH^+) 7%, and other peaks at 221 (MH^+-$C_3H_5O_3$ Figure 2.8) 1%, 194 (MH^+-dR) 12% and 152 (MH^+-dR-O^6-substituent) 5%.

FAB POSITIVE ION FRAGMENTATION OF O^6-ALKYL-2/-DEOXYGUANOSINES.



B=PURINE BASE; DR=2/-DEOXYRIBOSE

FIGURE 2.8

O^6-Carboxymethyl methyl ester-2/-deoxyguanosine undergoes a similar mass spectrum fragmentation pattern

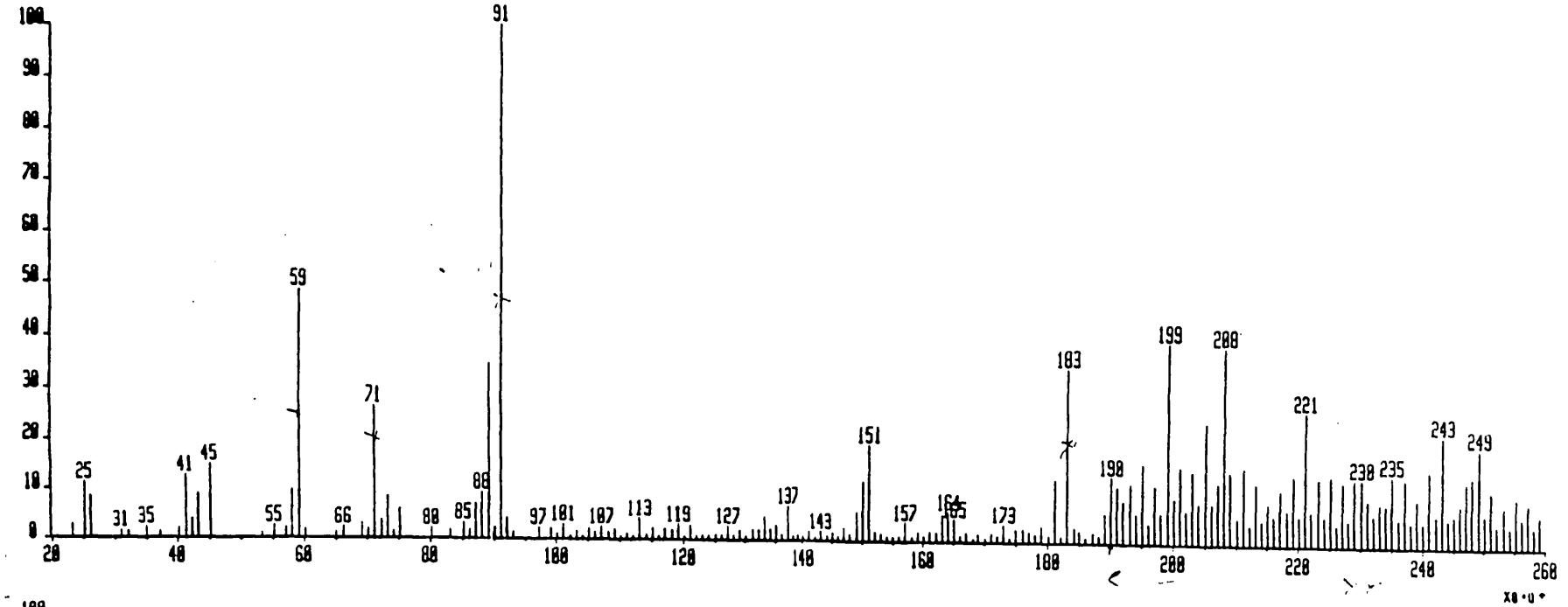
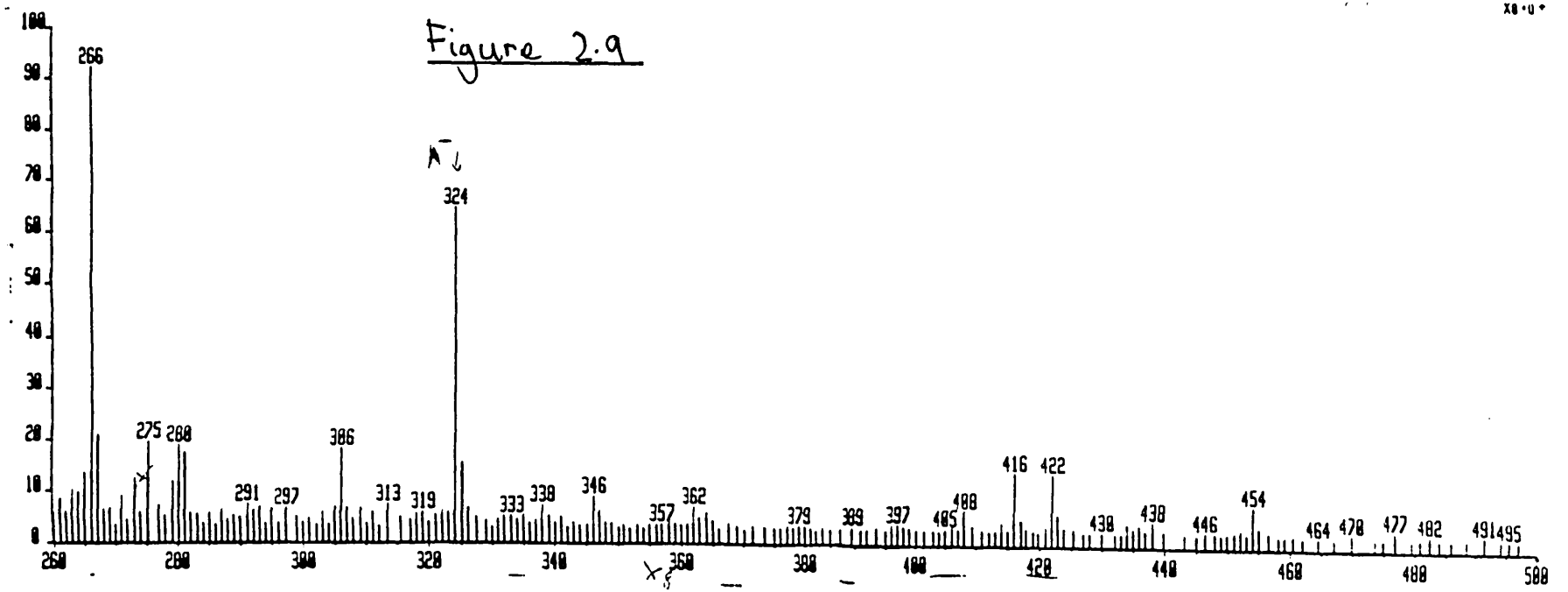

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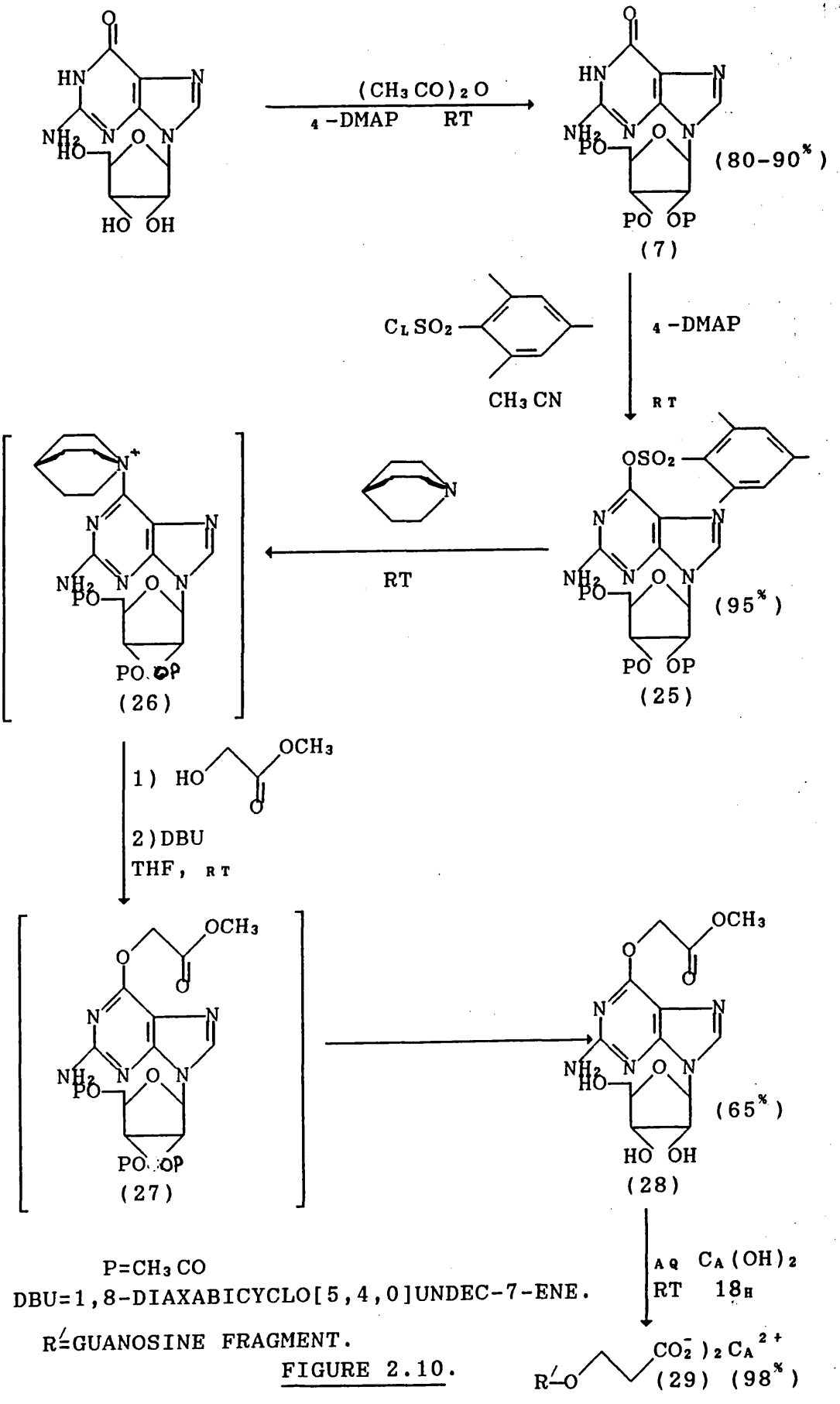
Figure 2.9


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to O^6-Pr^{i}dG. However O^6-CMdG as the calcium salt was analysed by FAB-negative ion, as is shown in Figure 2.9.

2.3.1 SYNTHESIS OF O^6-CARBOXYMETHYLGUANOSINE.

The procedure used for the synthesis of O^6-carboxymethylguanosine (O^6-CMGuo), in general follows the procedure of O^6-alkyl-2′-deoxyguanosines, with the activation of the 6-position of a protected guanosine by a tertiary amine, followed by nucleophilic substitution using an alkoxide ion. The procedure is outlined in Figure 2.10. It requires the conversion of a suitably protected guanosine (7) to the O^6-mesityl derivative (25), followed by displacement of the mesitylsulphonyl group with quinuclidine to give a quinuclidine ion intermediate (26). Subsequently the quinuclidine is displaced from (26) by the methylglycolate to give the protected O^6-carboxymethyl methyl ester guanosine (27). The final step is the removal of the protecting groups from (27) to give the required O^6-alkylguanosine. Conversion of (28) to the free acid involves hydrolysis by aqueous $Ca(OH)_2$. An indication of the yields for each stage of the synthesis are also given in Figure 2.10. The overall yield for the conversion of guanosine to O^6-CMGuo was ca 55%.



P=CH_3CO

DBU=1,8-DIAXABICYCLO[5,4,0]UNDEC-7-ENE.

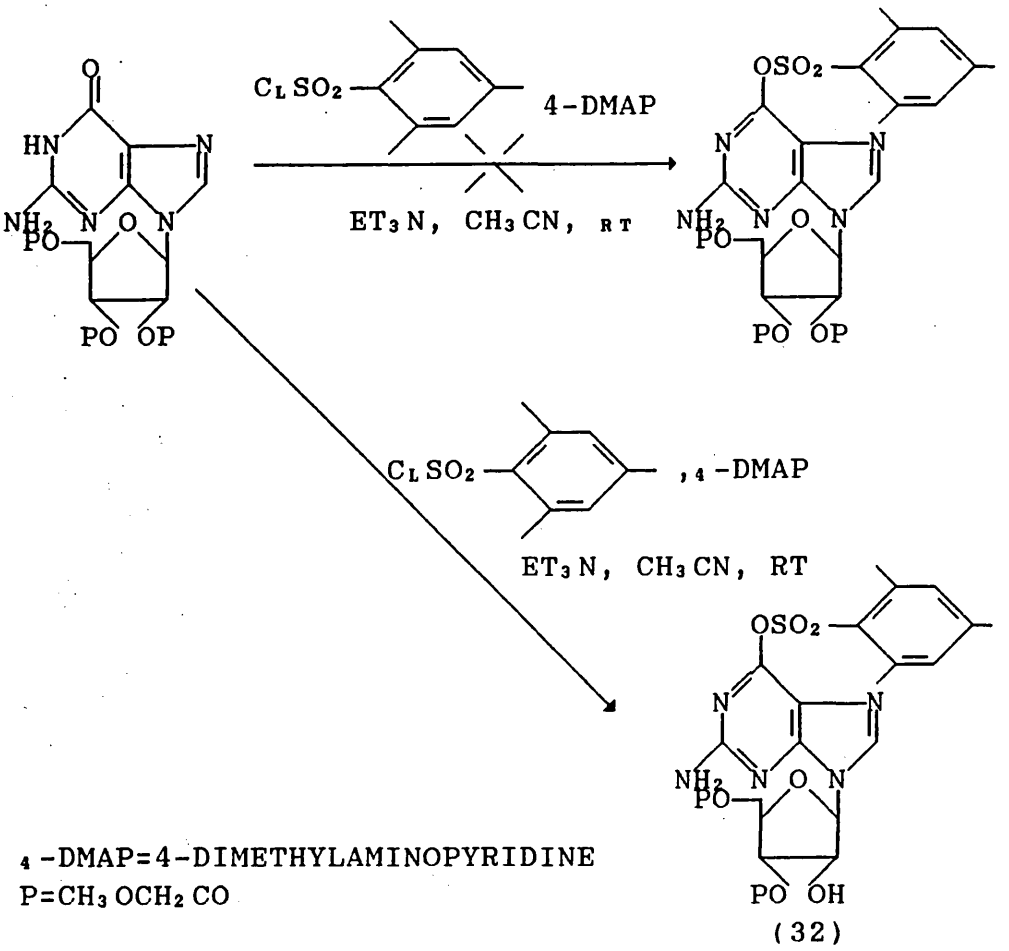
R/=GUANOSINE FRAGMENT.

FIGURE 2.10.

2.3.2 GUANOSINE PROTECTION.

Protection of the ribose hydroxyl functions was essential in order to increase the solubility of guanosine in organic solvents and to reduce undesirable side reactions of the hydroxyl groups throughout the synthesis. Initially, the 2-methoxyacetyl function was chosen to protect the hydroxyl groups. The 2-methoxyacetylation was carried out in DMF using pyridine to remove HCl and to catalyse the reaction. The protected guanosine was obtained in yields of ca 80-90%. Triturating the residue with diethyl ether produced higher yields ca 90% than simple recrystallisation from ethanol ca 80%.

Conversion of (30) to the O^6-mesityl derivative using the Gaffney and Jones procedure[99], did not progress as cleanly as expected. Reaction times of 2h, followed by chromatographic purification of the reaction mixture on silica (60g) using $CHCl_3/CH_3OH$ (97:3v/v) as eluents, failed to give (31) in high yields (Figure 2.11). Instead of isolating (31) in high yields ca 90%, a significant amount of by-product (32) in yields of 50-80% (Figure 2.11) was obtained.



(32)

FIGURE 2.11 BI-PRODUCT FORMATION DURING O^6-MESITYL-GUANOSINE SYNTHESIS.

The by-product was identified by ms and ^{1}H-nmr as missing the 2/-O-methoxyacetyl protection group. The loss of the protecting group probably occurred during chromatographic work up and was caused by acid catalysed hydrolysis of the methoxyacetyl group. Stereochemical effects, the fact that the 2/-OH group of guanosine is axial may explain this observation. Consequently, a more acid stable protecting group was used to protect the ribose hydroxyl functions, and the acetyl group was chosen for this purpose.

Acetylation of guanosine was carried out in acetic anhydride using 4-dimethylaminopyridine as the catalyst and gave 2′-3′-5′-tri-O-acetylguanosine (7) in ca 90-95% yield. Conversion of the protected guanosine (7) to the O^6-mesityl derivative (25) was carried out without difficulty following the procedure of Gaffney and Jones[99]. Using reaction times of 2h gave the O^6-mesityl derivative (26), after purification by column chromatography on silica (60g) $CHCl_3/CH_3OH$ (97:3v/v) as eluent, in yield of ca 90%.

2.3.3 CONVERSION OF O^6-MESITYLSULPHONYL-2′-3′-5′-TRI-O-ACETYLGUANOSINE TO O^6-CARBOXYMETHYLGUANOSINE.

O^6-Carboxymethyl methyl ester guanosine (28) was synthesized from the mesityl derivative (26) in a one pot synthesis without isolation of the intermediates, following a similar procedure to that used in the synthesis of O^6-alkyl-2′-deoxyguanosines. Usually, quinuclidine (2.5mmol) was added to a solution of (26) (0.5mmol) in dry THF (4cm^3), which was stirred at room temperature under dry nitrogen. The progress of the reaction was monitored by t.l.c. on silica using $EtOAc/CH_3OH$ (85:15v/v) as eluent, for the loss of (26), and the formation of a fluorescent spot at the baseline, attributed to the quinuclidine ion intermediate (27). Loss of (26) was usually complete in 3-4h.

Methylglycolate (5mmol) and DBU (1.5mmol) in dry THF ($6cm^3$) were then added to the reaction mixture, which was then stirred at room temperature under dry nitrogen. The progress of the reaction was followed by silica t.l.c. using EtOAc/CH_3OH (85:15v/v) as eluent for the loss of (27). The reaction was usually complete in 4-5h at 25°C, and on completion of the reaction the reaction solution was retained for removal of the protection groups.

2.3.4 REMOVAL OF THE ACETYL PROTECTING GROUPS.

The acetyl protecting groups were removed by triethylamine (6 equivalents) in CH_3OH ($5cm^3$) at room temperature. The resultant oil was purified by column chromatography on silica using EtOAc/CH_3OH (85:15v/v) as eluent. The fraction (rf=0.35) was isolated from the column, and removal of the solvent under vacuum gave (28) as a white crystalline solid. O^6-Carboxymethyl methyl ester guanosine was further purified, however, by isocratic reverse phase H.P.L.C. on an ODS column using aqueous CH_3CN (12%) as eluent. The H.P.L.C. purification enabled contamination due to mesitylsulphonic acid to be removed. Usually, (28) was obtained in yields of ca 65% following isolation and purification.

Conversion of the ester function to an acid was carried out by hydrolysis in aqueous $Ca(OH)_2$. The

resultant O^6-carboxymethyl guanosine was isolated as its calcium salt following lyophilization of the reaction solution.

2.3.5 SPECTROSCOPIC CHARACTERISATION OF O^6-ALKYLGUANOSINES.

O^6-Carboxymethyl methyl ester guanosine and O^6-carboxymethylguanosine were characterised by spectroscopic analysis by uv, ir, nmr and ms. General characteristics are exemplified by those of O^6-carboxymethyl methyl ester guanosine (O^6-CM-Me-ester-Guo).

UV Spectrum. The uv spectrum of this compound was very similar to those of O^6-alkyl-2/-deoxyguanosines. At pH=7, the uv spectrum (Figure 2.12) of O^6-CM-Me-ester-Guo exhibits a peak at λmax=248nm (loge=4) due to the purine ring and a second peak at λ=282nm (loge=4), which is characteristic of O^6-substituted compounds.

IR Spectrum. These were obtained as KBr dispersions and are relatively complicated, with few peaks characteristic of the O^6-alkylated nucleoside. Thus O^6-CM-Me-ester-Guo (Figure 2.13) exhibits several peaks between νmax=3400 and 3100cm^{-1} due to the ribose -OH

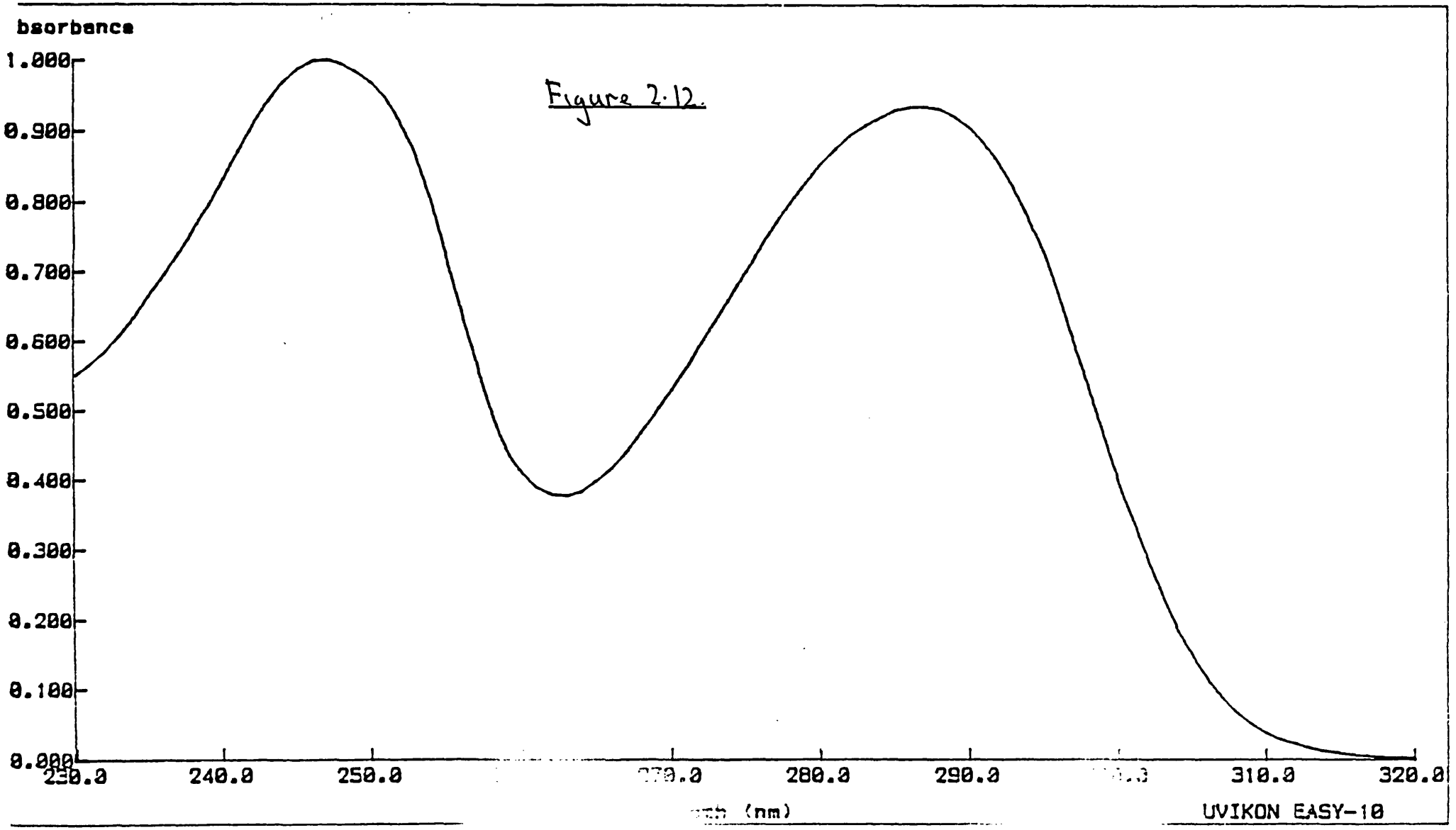

bsorbance
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
230.0
240.0
250.0
280.0
290.0
310.0
320.0
(nm)
UVIKON EASY-10

Figure 2.12.

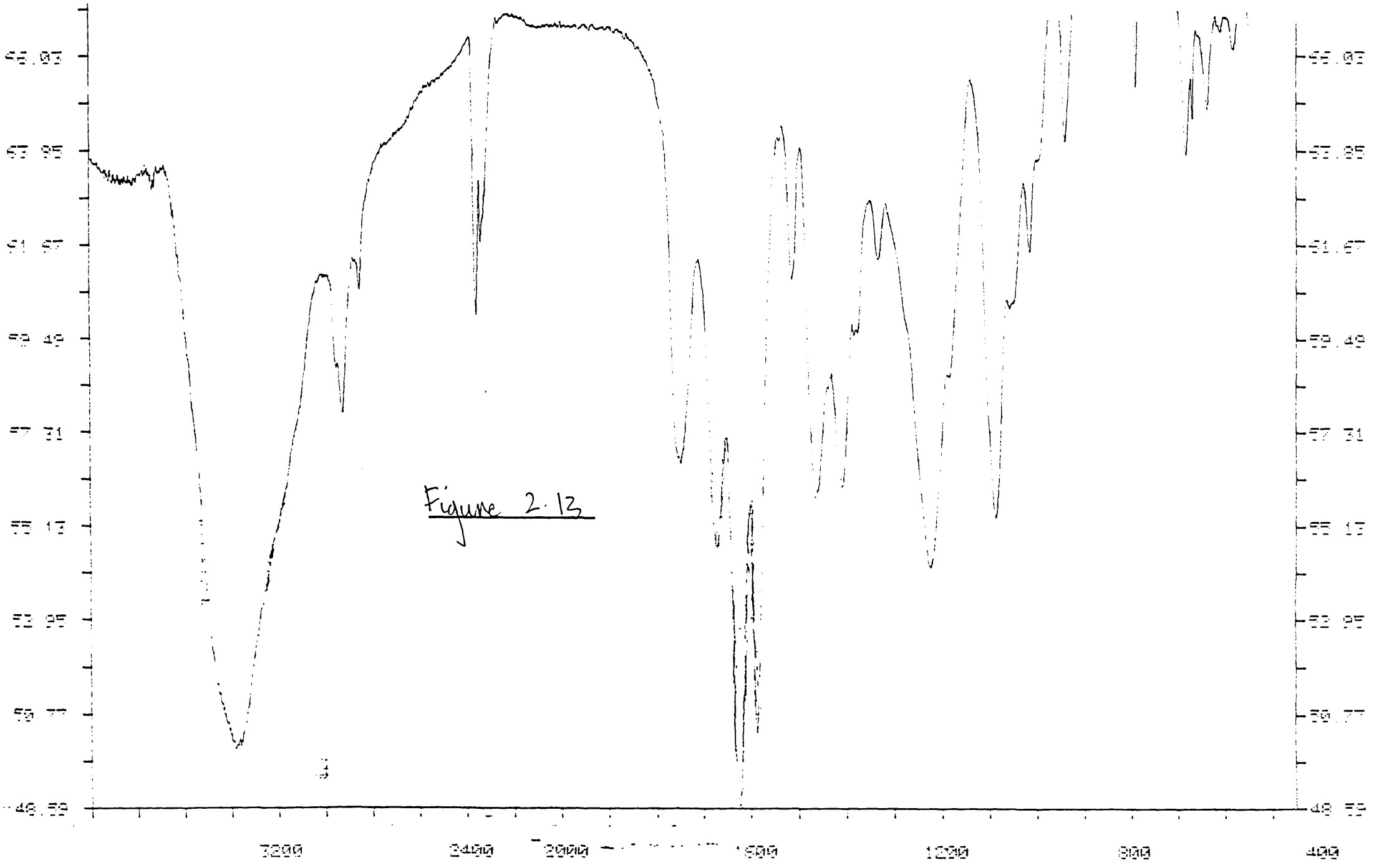


Figure 2.13

groups and the purine $-NH_2$ group. The peak at 1742cm^{-1} was assigned to the C=O stretch of the CO_2Me group and peaks at $\underline{\nu}$max=1580cm^{-1} due to the C=C stretch of the purine ring. Two other peaks at $\underline{\nu}$max=1260 and 1070cm^{-1} were due to the C-O str of the O^6-ether linkage.

^{1}H-nmr Spectrum (d^6-DMSO). These are complicated by the complex pattern of peaks relating to the protons of the ribose group. Nevertheless, the ^{1}H-nmr spectra contains two very characteristic peaks for the guanosine moiety in addition to those for the O^6-alkyl substituents. The most characteristic guanosine peak is a singlet at d=8.11ppm due to the C-8 proton. The other distinctive peak is the $C/_1$-H of the ribose which appears as a doublet at 5.8ppm. Peaks at 5.14 (2H, m), 5.40 (1H, d) and 6.52 (2H, brs) disappear after the addition of D_2O and relate to 2/-3/-5/-OH groups of the ribose and the 2-NH_2 of the purine. Multiplets at δ=3.58 (2H), 3.88 (1H) 4.10 (1H) and 4.46 (1H) relate to the $C/_5$, $C/_4$, $C/_3$ and $C/_2$ protons of the sugar. Peaks at δ=3.61 (3H, s, OCH_3) and δ=4.87 (2h, s, $CH_2CO_2CH_3$) relate to the O^6-alkyl substituent.

Mass Spectrum. In both the EI and FAB positive ion mass spectra, O^6-CM-Me-ester-Guo undergoes a similar fragmentation pattern to that of O^6-Pr^i-2/-dG. In this case the main fragmentation pattern involves the loss the ribose moiety. Thus, the FAB positive ion exhibits a

molecular ion peak at m/z 356 (MH^+) 6% and other peaks at 224 (MH^+-132(ribose)) 8%and 152 (MH^+-ribose-O^6-substituent) 10%.

2.4.1 SYNTHESIS OF N/-(N-ACETYL-L-PROLYL)-N/-NITROSOGLYCINE (APNG).

This compound was required as a suitable reagent for the carboxymethylation of 2/-deoxyguanosine and DNA *in vitro*. It was synthesized by the procedure described by Challis *et al*[28] (Figure 2.14). Salient features of this synthesis are the aprotic nitrosation of the benzyl ester of the dipeptide using liquid N_2O_4 in CH_2Cl_2, in the presence of NaOAc to maintain non-acidic conditions. The benzyl ester is used to solubilize the dipeptide in CH_2Cl_2. Following purification, the benzyl ester group is removed by catalytic hydrogenolysis.

The parent dipeptide, N-acetyl-L-prolylglycine is not commercially available, but is readily synthesized from its constituent amino acids by the usual peptide coupling reactions. Thus, N-acetyl-L-proline (34) was synthesized by reacting L-proline (33) with excess acetic anhydride in the presence of NaOH in yields of *ca* 78%. Glycine benzyl ester was synthesized as the p-TSA

salt from glycine and excess benzyl alcohol, in refluxing benzene in the presence of p-toluene sulphonic acid, in a yield of 90%.

SYNTHESIS OF N'-(N-ACETYL-L-PROLYL)-N'-NITROSOGLYCINE

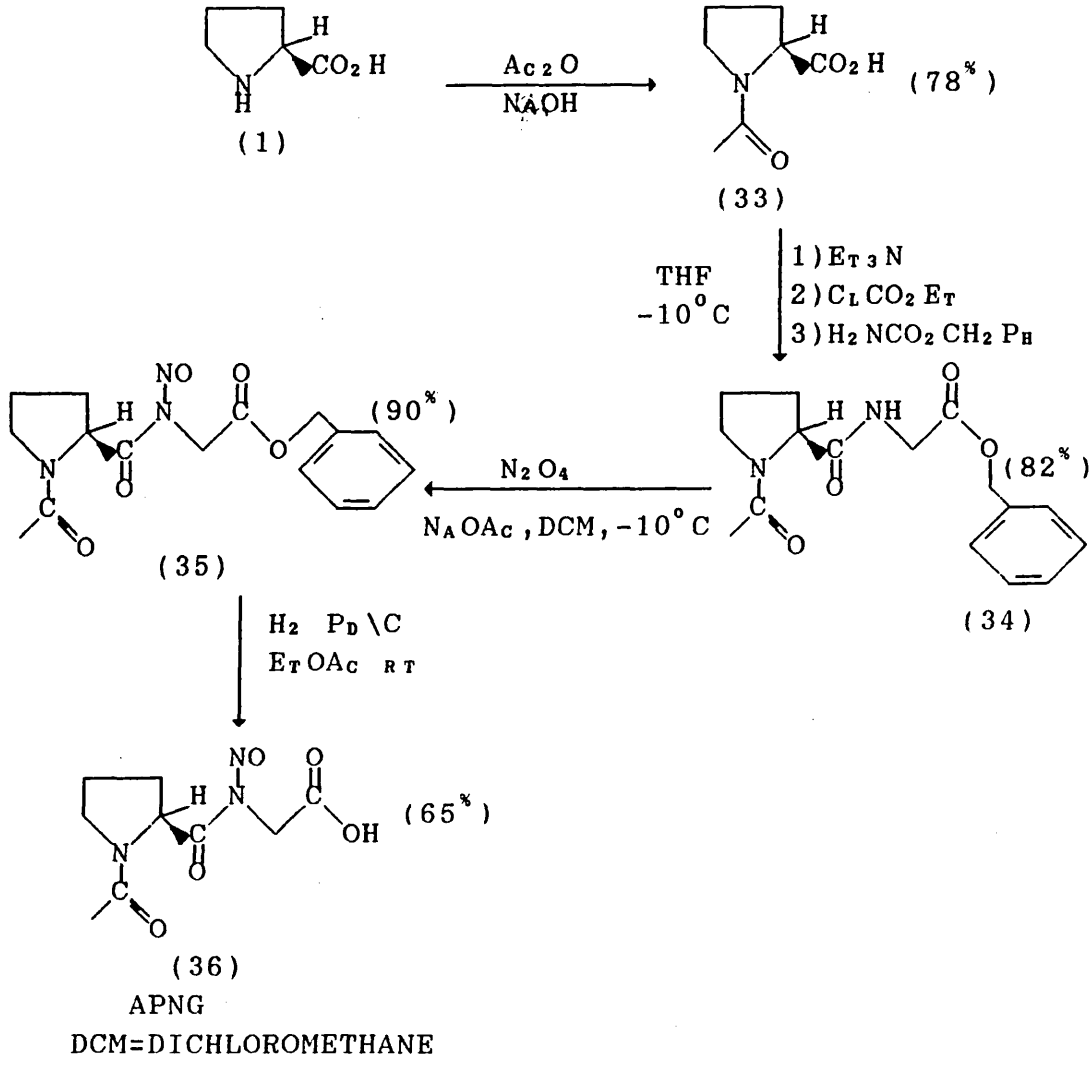


FIGURE 2.14

The coupling of N-acetyl-L-proline (34) with the glycine benzyl ester presented most difficulties in the synthetic sequence. In particular, extreme care was exercised to avoid contamination of the dipeptide (35) with ethylchloroformate, and simplify purification in the later synthetic steps. This was ensured by using only 0.9 equivalents of ethylchloroformate with N-acetyl-L-proline in excess. Neutral glycine benzyl ester is generated in solution by neutralising the conjugate acid of glycine benzyl ester with triethylamine in CH_2Cl_2 at 0°C. The neutral glycine benzyl ester readily polymerizes, and it was used in the coupling reaction immediately. The yield of dipeptide is dependent on temperature, and formation of the mixed anhydride and the subsequent coupling were carried out below -7°C, and allowed to react for 30 min. after the addition of the neutral glycine benzyl ester. At higher reaction temperatures, the yield of the dipeptide is usually reduced. The dipeptide, after purification by column chromatography on silica using EtOAc/CH_3OH (99:1v/v) as eluent, was obtained in a yield of 80%.

Nitrosation of the dipeptide proceeded in accordance with the literature method[28]. The use of an N-protected dipeptide and aprotic non-acidic conditions, favoured amide N-nitrosation and minimized any competing amine N-nitrosation and diazotization reactions. Thus, the N-

nitrosation of the dipeptide was carried out in CH_2Cl_2 using a slight excess of N_2O_4, but a vast excess of NaOAc. The yield of the protected N-nitrosodipeptide (36) is dependent on temperature the nitrosation reaction being carried out below -7°C, and allowed to react for 30 min. after the addition of N_2O_4. Higher reaction temperatures result in reduced yield of (36). Moisture also influences the yield of (36), care must be taken to ensure both the solvent and NaOAc are thoroughly dried. Treatment of the reaction solution with a mildly basic wash and subsequent extraction gave the protected N-nitrosopeptide (36) in a yield of 90-95%.

Considerable difficulty was experienced in the removal of the benzyl ester protecting group to give APNG (37). Following the literature procedure of hydrogenation in C_2H_5OH over Pd/C, invariably resulted in loss of the nitroso group as well as the benzyl group. It was found that hydrogenation in EtOAc/C_2H_5OH (95:5v/v) over Pd/C, followed by recrystallisation from EtOAc gave (37) in yields of 60-70%.

Chapter 3.

ALKYLATION REACTIONS OF 2/-DEOXYGUANOSINE AND DNA WITH APNG *IN VITRO*.

3.1 REACTION OF N-(N/-ACETYL-L-PROLYL)-N-NITROSOGLYCINE (APNG) WITH 2/-DEOXYGUANOSINE AND DNA IN VITRO.

As described in Chapter 1, carcinogenic substances usually exert their toxicity by interaction with cellular DNA. For carcinogenic alkylating agents, this interaction usually results in the alkylation of both phosphate linkages and the component purine and pyrimidine DNA bases[12]. Some of these alkylation reactions (eg of the phosphate linkages and N^7-guanine) are not considered to be part of the carcinogenic process[67,72], but they do induce degradation, (strand breakage and depurination), of the DNA. The products produced by these reactions are, however, a clear indication that carcinogenic substances are interacting with DNA. Other alkylation reactions, (eg to give O^4-alkylthymidine and O^6-alkyl-2/-deoxyguanosine) are considered to be a good indication of carcinogenic activity[70,72,73].

Both N-nitrosopeptides and N-nitrosobile acid conjugates are alkylating agents as described in Chapter 1. Information about the potential cytotoxicity should therefore derive from their interaction with DNA and specifically from the formation of alkylated bases and/or cleavage of DNA strands.

In the present project the alkylating properties of a typical N-nitrosopeptide (APNG) was examined *in vitro*. To keep the study in reasonable proportions, it was decided to examine the reaction of APNG with 2′-deoxyguanosine and with calf-thymus DNA. For both substrates, evidence for an alkylation reaction was sought by monitoring the formation of N^7-carboxymethylguanine (N^7-CMG). N^7-Alkylation is usually the most extensive alkylation of guanine residues in DNA[53,57], and therefore the most sensitive test of alkylation. Evidence for the carcinogenic potential of APNG was sought by monitoring the formation of O^6-carboxymethyl-2′-deoxyguanosine (O^6-CMdG), as O^6-alkylation of DNA is considered to be a good index of carcinogenic activity[72,73].

Usually, the extent of 2′-deoxyguanosine base alkylation by carcinogenic compounds is low, (<5%)[123], and sensitive methods are required to monitor the formation of these products. In the present project it was decided to carry out the assay first by H.P.L.C. and subsequently by immunochemical procedures.

The following text reports and discusses the results for the *in vitro* alkylation of 2′-deoxyguanosine and

calf-thymus DNA by APNG, and describes and evaluates the H.P.L.C. analytical procedures. The immunochemical assays are described, evaluated and discussed in Chapter 4.

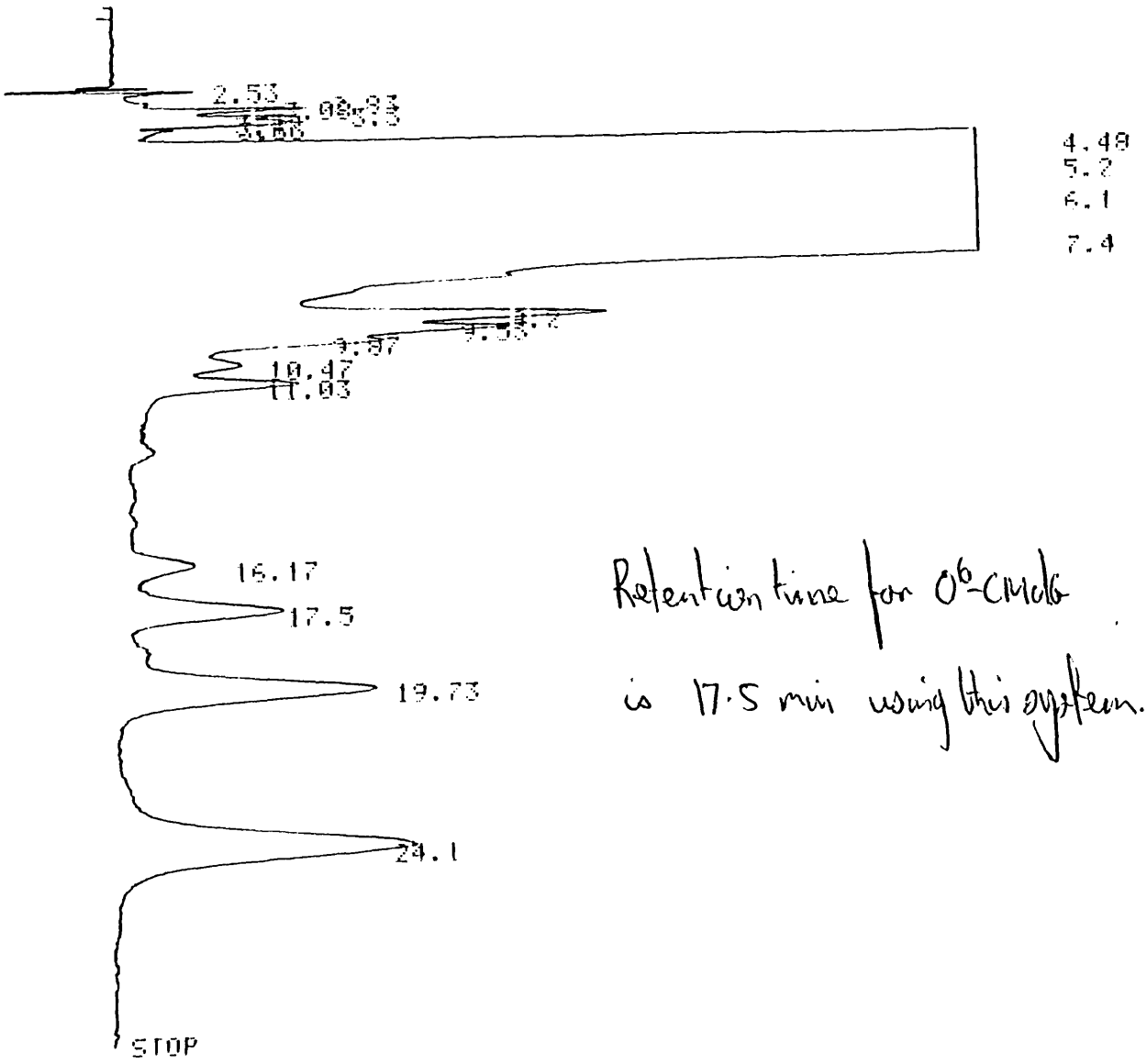
3.2 ANALYTICAL H.P.L.C. PROCEDURES FOR 2/DEOXYGUANOSINE REACTIONS.

Many precedents exist for the use of reverse phase H.P.L.C. procedures to analyse biological samples, particularly urine, blood and DNA components[124]. The method seemed ideal for the present work as reverse phase H.P.L.C. offers speed and simplicity coupled with an ability to separate molecules of widely different polarities, (eg thymidine and 2/-deoxyguanosine), by changing the solvent pH and temperature of the column.

Isocratic analysis was considered to be adequate for the assay of 2/-deoxyguanosine (2/-dG) reactions. Hydrolytic work-up procedures followed by gradient elution was considered unnecessary with only one substrate undergoing reaction. Assay of O^6-CMdG was performed on two H.P.L.C. systems, both giving similar results. The use of tetra-n-butylammonium bromide as an ion-pairing reagent at pH=6.43 was considered to be ideal for separating O^6-CMdG from the unreacted 2/-dG. A high

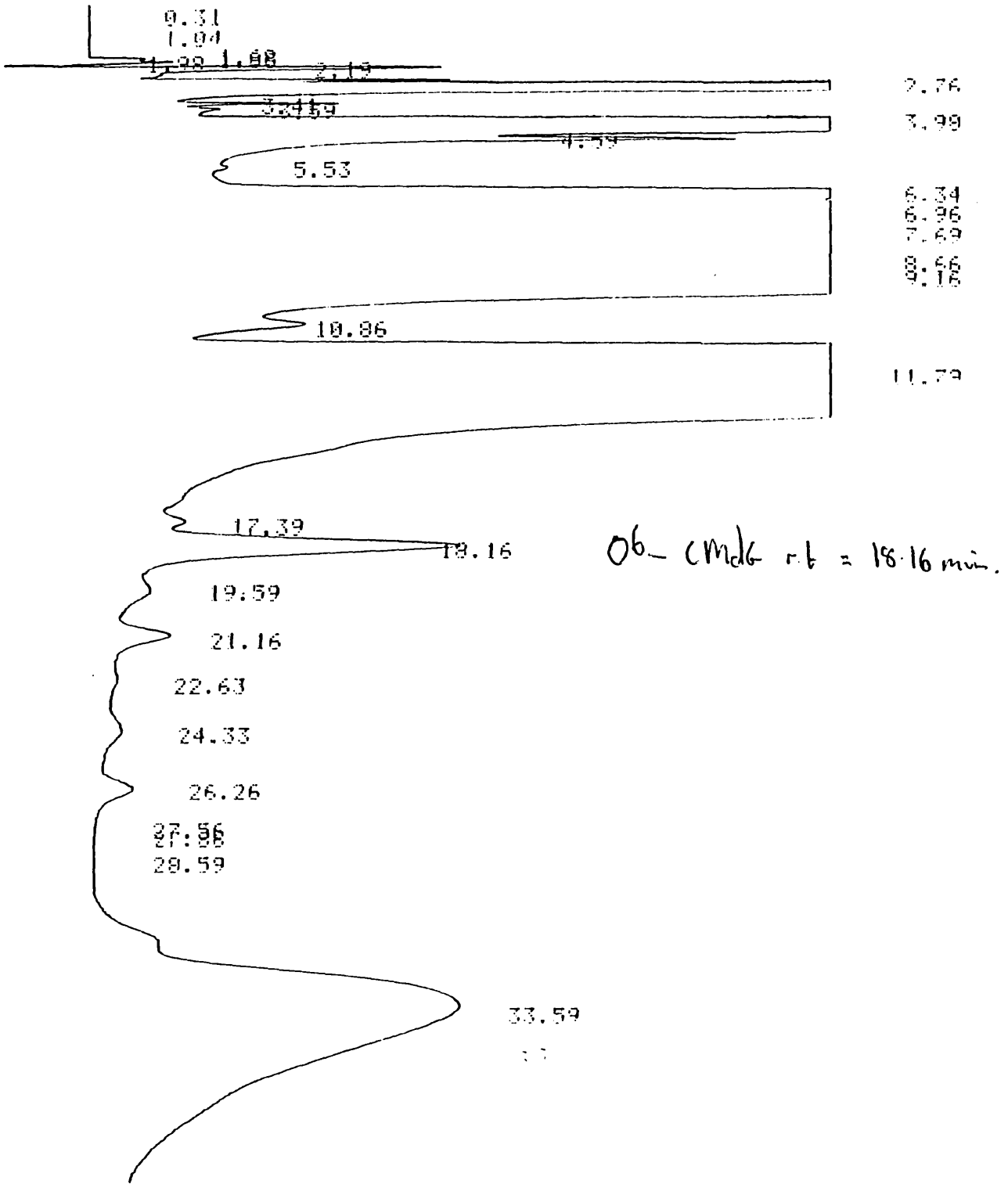
column temperature (45°C) was used to induce nucleoside mobility and enhance separation. Typical retention times for this assay on a Spherisorb 5u C-6 column at 45°C, using 0.02M tetra-n-butylammonium bromide in 0.05M KH_2PO_4 at pH=6.43 (70%), KH_2PO_4 pH=6.43 (28%) and CH_3CN (2%) were ca 17.5min for O^6-CMdG and ca 7.5min for 2′-dG. A typical chromatogram is shown in Figure 3.1. The major difficulty with the ion-pairing assay was the long equilibration time of the stationary column phase with the mobile eluent phase. As long as 1.5-2.0h was often required to ensure that the ion-pairing reagent was evenly dispersed throughout the H.P.L.C. column. Column performance was maintained by thoroughly cleaning the column with H_2O for 1h at the end of each day, followed by 50:50 (v/v) H_2O/CH_3CN overnight.

The second method was used to confirm the analytical results for O^6-CMdG formed from 2′-dG reactions. This employed a more retentive C-8 column at 25°C with 0.05M KH_2PO_4 at pH=4.3. Small amounts (ca 0.8%) of CH_3CN were added to the eluent to increase mobility and improve separation. Typically, with the C-8 column at 25°C, and aqueous 0.05M KH_2PO_4 buffer (pH=4.3):CH_3CN (99.2:0.8v/v), the retention time for O^6-CMdG was ca 18.5min. Again column performance was maintained by cleaning the column with H_2O for 1h, followed by 50:50 (v/v) CH_3CN/H_2O



Retention time for O^6-CMdG is 17.5 min using this system.

Figure 3.1 H.P.L.C analysis for O^6-CMdG in the APNG : dG (1:1 w/w) reaction solution using the ion-pairing eluent system. Detection by uv (262 nm) and A = 0.005. 10 µl injection.



O6-CMdG r.t = 18.16 min.

Figure 32 H.P.L.C analysis for O6-CMdG in the APNG:dG (1:10 w/w) reaction, using the isocratic normal H.P.L.C system with uv detection at 262 nm. A-0.01 50ul injection.

overnight to remove any highly retained material. Examples of typical chromatograms for the O^6-CMdG analysis of 2/-dG reaction solutions are given in Figure 3.1 (ion-pair system) and Figure 3.2 (normal H.P.L.C. system).

Variants of the isocratic ion-pairing H.P.L.C. assay for O^6-CMdG were examined for the analysis of N^7-CMG. However, irrespective of the concentration of ion-pairing reagent, 0.001-0.05M tetra-n-butylammonium bromide, very little retention of N^7-CMG was obtained on several different reverse phase H.P.L.C. columns (C-6 to C-18) at room temperature. A major problem with the ion-pairing reagent at pH=6.43 was the poor peak shape for N^7-CMG. These problems are exemplified in the chromatogram shown in Figure 3.3. The poor chromatography for N^7-CMG may relate to partial protonation of N^7-CMG in the phosphate buffer. A more acidic eluent where N^7-CMG is fully protonated was therefore investigated.

For N^7-CMG with an acidic eluent consisting of aqueous 0.1M heptafluorobutyric acid (HFBA) containing 3% (v/v) CH_3OH and an C-18 silica column at 25°C, a good separation between unreacted 2/-dG and N^7-CMG was obtained. After each analysis the CH_3OH content of the eluent was increased rapidly to clean the column and prevent contamination of subsequent analyses with highly retained material. A re-equilibration period of 15-20min

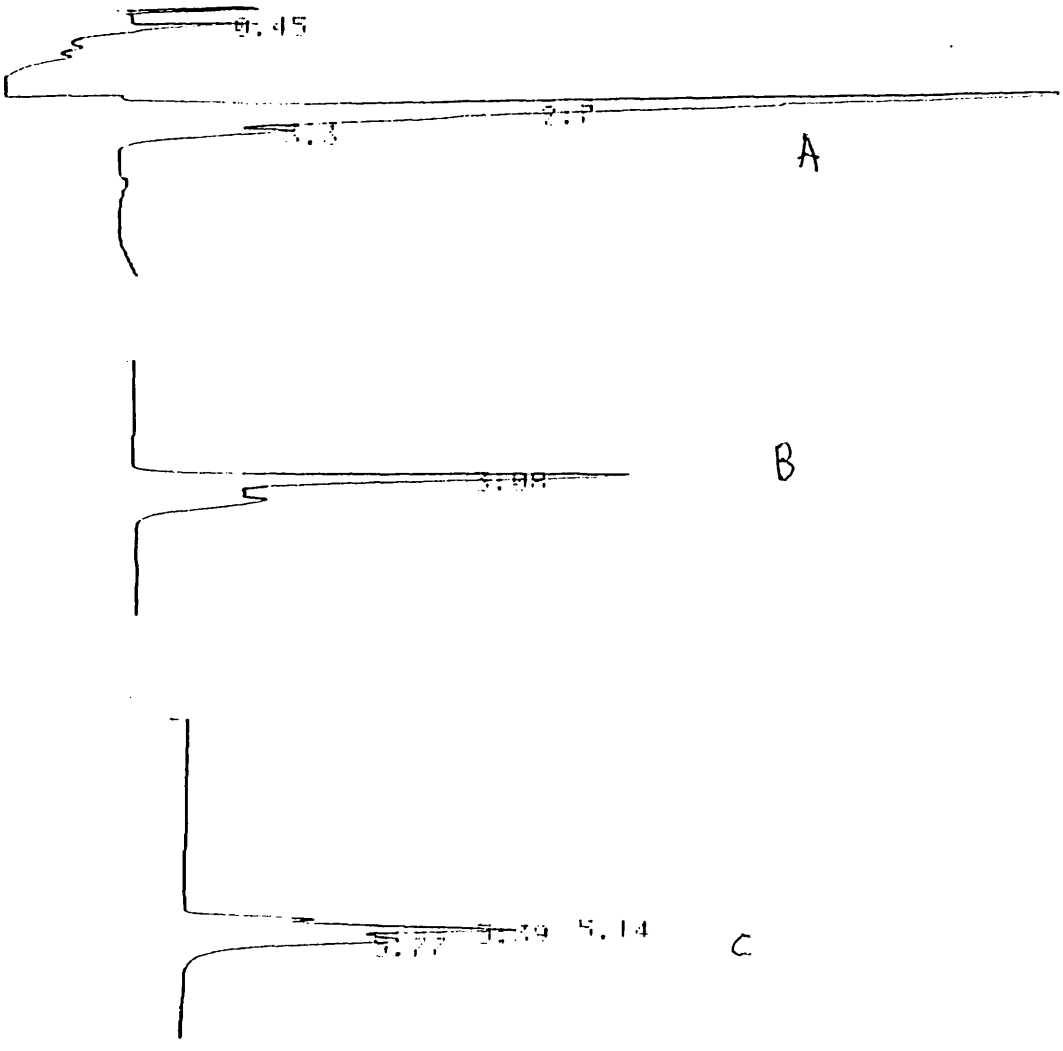


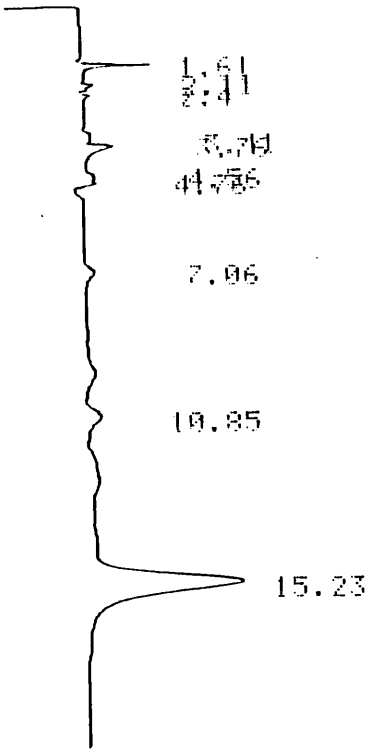
Figure 33 H.P.L.C Analysis of standard N⁷-CH16- on a C-8 column using the ion pairing eluant system:

Figure A represents the standard case of 70% ion pairing agent at pH = 6.43 with 2% CH_3CN at 45°C.

Figure B represents 100% ion pairing agent at 45°C.

Figure C represents 100% ion pairing agent at 23°C.

All the analyses used UV detection at 254 nm and 100 ng injections.



Retention time for N^7-CM6 in this system is 15.23 min.

Figure 3.4. H.P.L.C analysis for N^7-CM6 of the reaction solution (APM6: d6 (1:1 w/w)) using an acidic gradient eluent system with detection by uv at 254nm. A=0.01 10µl injected.

using the original eluent was then required before further analyses. A typical assay using aqueous 0.1M HFBA/CH_3OH with the gradient CH_3OH (3%v/v) for t=0-16min, CH_3OH (3%-80%v/v) from t=16-20min and finally CH_3OH (80%v/v) from t=20-30min on a Spherisorb S_5 ODS/10 column at 25°C gave a retention time of 15.3min for N^7-CMG. A typical chromatogram using this assay is shown in Figure 3.4. A thorough column wash involving H_2O for 1h, followed by 70:30 (v/v) CH_3OH/H_2O overnight was used after each analytical session.

Because of the high sensitivity of the H.P.L.C. assays it was necessary to rigourously purify all of the eluent materials. Without this precaution uv absorbing contaminants generated both large baseline shifts and large artifactual peaks, which made quantitation difficult. This problem was most serious when using gradient elution and changing buffers[125]. Accordingly, H_2O used for the eluents was purified first by glass distillation, and then by passage through a semi-preparative C-18 silica column. The CH_3OH and CH_3CN were H.P.L.C. grade, as were the KH_2PO_4 and HFBA buffer components. All the eluents were stored at 4°C, and used within 2 days of preparation.

Quantitation of the alkylated products from

reactions of 2/-dG were effected by spiking an aliquot of the reaction solution with a known concentration of the authentic product (O^6-CMdG or N^7-CMG). The difference of peak areas between the spiked and unspiked aliquots was used to quantitate the product in the reaction solution. A typical example of the procedure is given in Section 3.3. Generally, where duplicate reactions were performed good agreement was obtained between the duplicates, for both N^7-CMG and O^6-CMdG (see Section 3.1 Table 3.1). When the same reaction was analysed for O^6-CMdG by both the ion-pair and normal H.P.L.C. procedures the results agreed +/_ 2%.

Both normal uv and fluorescence detection was examined to monitor the products from the 2/-dG reactions. Both procedures gave similar limits of detection for O^6-CMdG eg 5ng for uv and 2.5ng for fluorescence detection. As fluorescence detection gave similar sensitivity but required more careful sample and eluent clean-up, O^6-CMdG was monitored by conventional uv at λ=282nm (with a detection limit of ca 5ng), and N^7-CMG at λ=254nm (with a detection limit of ca 10ng).

3.3 REACTION OF APNG WITH 2/-DEOXYGUANOSINE.

As a preliminary to experiments with DNA, the

interaction of 2′-dG with APNG in aqueous buffers at (pH=7.2) at 37°C was investigated in vitro. These experiments were simpler to undertake insofar as the reaction solutions can be analysed without complicated work-up procedures, and only alkylated 2′-deoxyguanosine (or guanine) products can form. The results should reveal, however, the ability of APNG to act as an alkylating agent towards purine bases, and give an indication of the extent of alkylation expected for DNA.

Thus, aqueous 37.5mM 2′-dG in 10mM 2amino(2hydroxymethyl)propane1,3,diol (Tris) (1.0cm^3) at pH=7.2 was reacted with APNG (0.1-1x10^{-3}g 0.4-4x10^{-3}mmol) in DMSO (10-100ul). The mixture was agitated and incubated at 37°C for 48-72h. Loss of APNG absorbance at λ =410nm was used to monitor the extent of reaction. A more detailed account of the experimental method is given in Section 5.4.3.

H.P.L.C. analysis of the reaction solutions were performed without work-up other than dilution with 10mM Tris buffer at pH=7.2 (1.0cm^3), for reactions using the most concentrated reactants. The H.P.L.C. analysis focussed on whether alkylation had occurred at the O^6 or N^7-positions of 2′-dG. Two different isocratic reverse phase H.P.L.C. assays were used to quantitate O^6-

carboxymethylation of 2′-dG. One assay involved 0.05M KH_2PO_4 at pH=4.3 and CH_3CN (99.2:0.8v/v) as eluent on a 5u C-8 silica column at 25°C. The other assay involved 0.02M tetra-n-butylammonium bromide in 0.05M KH_2PO_4 at pH=6.43, 0.05M KH_2PO_4 at pH=6.43 and CH_3CN (70:28:2v/v) as eluent, using a C-6 silica column at 45°C. Both methods are described in more detail in Section 5.7.2. Both gave similar results for the extent of O^6-CMdG formation (see Table 3.1 for details). The quantitation of N^7-CMG was performed at the purine rather than the nucleoside level. These analyses use the 0.1M HFBA/CH_3OH gradient eluent on a Spherisorb C-18 silica column at 25°C as described in Section 5.7.2.

For each reaction solution the O^6-CMdG and N^7-CMG assays were carried out in duplicate and a mean taken. Several of the reactions were carried out in duplicate, and the results for the two experiments agreed to +/_2%. As mentioned previously, the quantitation was performed by spiking the sample solution with a known amount of authentic compound and using the difference in peak areas to estimate the concentration of analyte originally present. The procedure is best illustrated by an actual example for the determination of O^6-CMdG formed in the reaction of APNG:2′-dG (1:1w/w) using the ion-pairing H.P.L.C. assay:

Table 3.1 Effect of [APNG] on yields of O^6-CMdG and N^7-CMG from reaction with 2/-dG at 37°C *in vitro*. Initial [2/-dG]=10mg/cm³ (37.5mM).

10^4 [APNG] M	[APNG] 2/dG (w/w)	Yield O^6-CMdG			Yield N^7-CMG		
		ug	10^3xD mole/moledG	%[c]	ug	10^3xE mole/moledG	%[c]
4.11	0.01	u	u	u	2	0.255[b]	0.03
41.1[dr]	0.1	9[a] 8.5[b]	0.885	0.09	13.5 13.5	1.78[b]	0.18
82.2	0.2	13[a] 13[b]	1.06	0.11	21	2.66[b]	0.27
411[dr]	1	22.5[a] 22[b]	1.79	0.18	29 29	3.69[b]	0.37
2055	5	8[a] 9[b]	0.736	0.07	115	14.6[b]	1.46
4110[dr]	10	1[a] 1[b]	0.082	.008	279 276	35.2	3.52

a=O^6-CMdG analysis using ion-pair H.P.L.C. procedure.

b=O^6-CMdG and N^7-CMG analysis using the normal H.P.L.C. procedure. c=Relative to initial [2/-dG].

dr=Duplicate reaction at a specific APNG:2/-dG ratio.

D=[O^6-CMdG]/[2/-dG]; E=[N^7-CMG]/[2/-dG].

Peak area for 10ul original reaction solution = 42,650

Peak area for 9ul original solution +1ul O^6-CMdG (100ng) = 84,268

Peak area of 9ul original reaction solution = 41,625

Peak area of 1ul O^6-CMdG (100ng) = 42,643

Peak area / ng O^6-CMdG = 426.43

O^6-CMdG / 10ul original reaction solution = 108.6ng

Total reaction solution = $2cm^3$

O^6-CMdG in Total reaction solution = 21.7ug

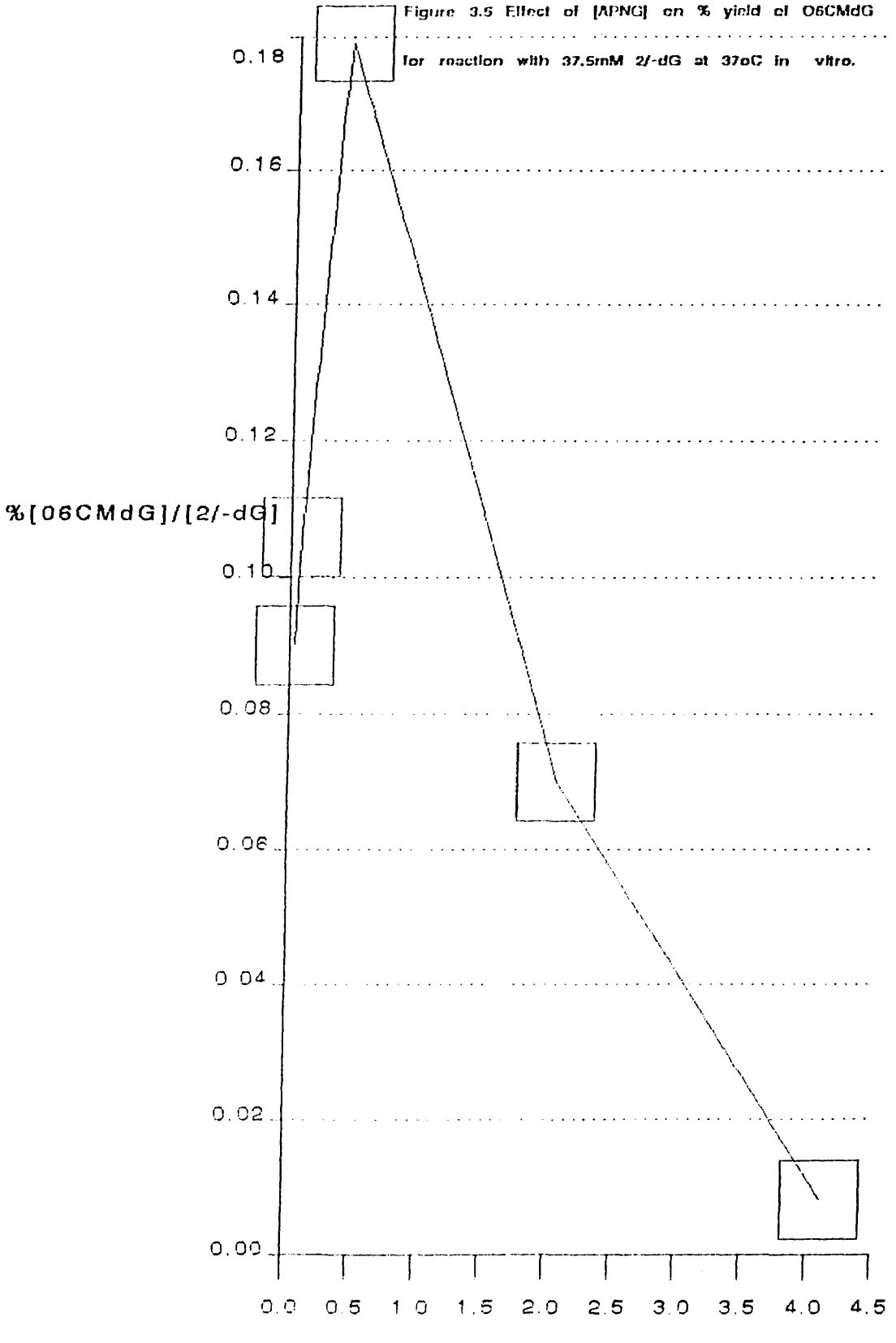
$$\% \text{ Yield moles } O^6\text{-CMdG / mole } 2'\text{-dG} = \frac{6.69\times10^{-8}}{3.75\times10^{-5}} \times 100$$

$$= 0.18\%.$$

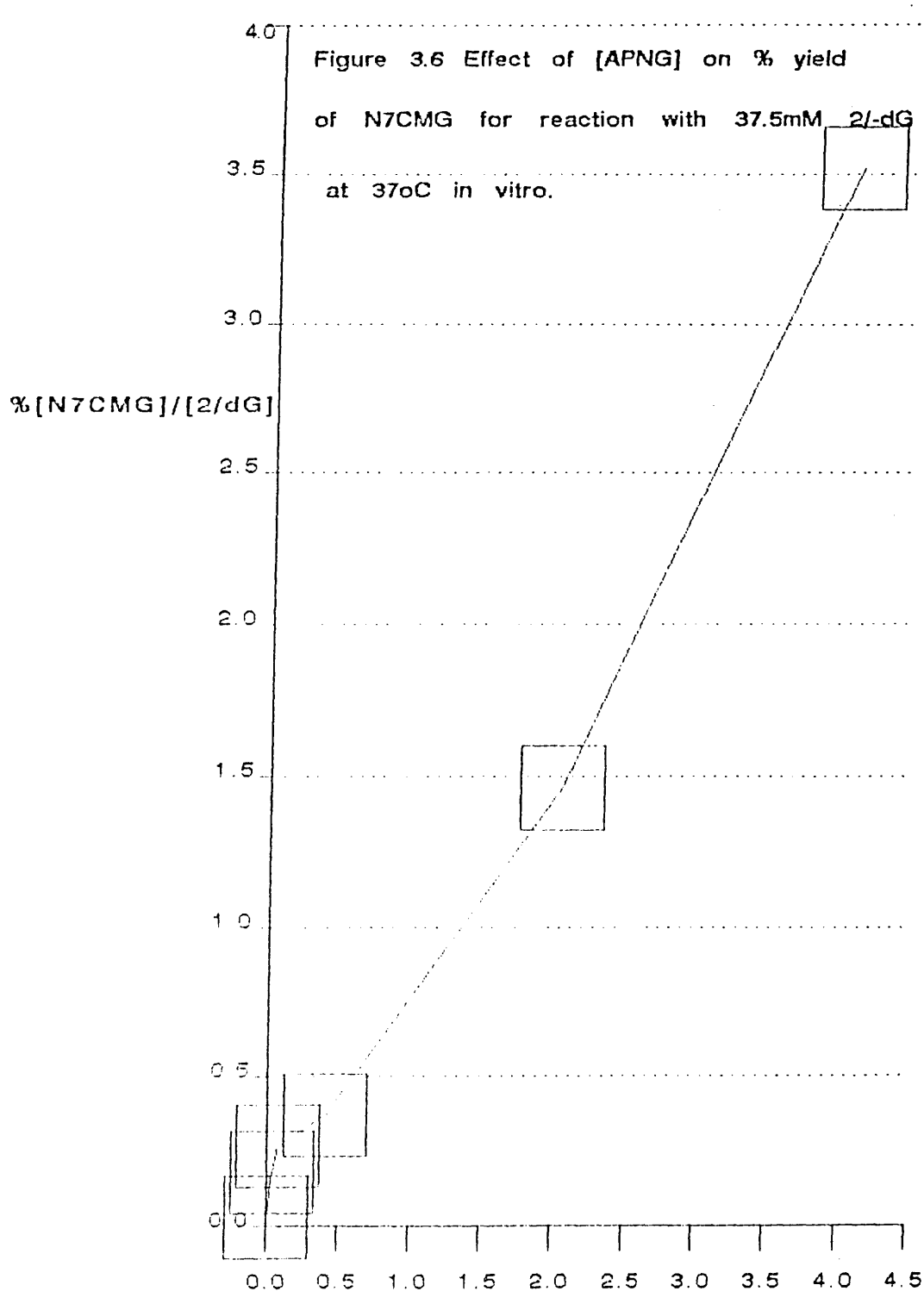
The chromatagrams shown in Figure 3.1 and Figure 3.4 for the reaction of 0.04M APNG with 37.5mM 2/-dG in 10mM Tris buffer (pH=7.2) at 37°C clearly reveal that carboxymethylation proceeds at both the O^6 and N^7-positions of 2/-dG. In Figure 3.1, the peak eluting at ca t= 17.5min corresponds to the O^6-CMdG, whereas the peak corresponding to N^7-CMG elutes at ca t=15min in Figure 3.4.

The effect of APNG concentration on the yields of O^6-CMdG and N^7-CMG from 2/-dG for reaction at 37°C and pH=7.2 *in vitro* are summarised in Table 3.1. It is clear that the formation of N^7-CMG is more extensive than O^6-CMdG. Much higher amounts of N^7-alkylation have been

Figure 3.5 Effect of [APNG] on % yield of O6CMdG for reaction with 37.5mM 2/-dG at 37oC in vitro.



%


Figure 3.6 Effect of [APNG] on % yield
of N7CMG for reaction with 37.5mM 2/-dG
at 37oC in vitro.
%[N7CMG]/[2/dG]
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
10000x[APNG]M
THOUSANDS.

previously noted for the reaction of ethylmethane sulphonate[1,2,3] and diethylsulphate[1,2,3] with guanosine in aqueous media at pH 7.2 and 37°C *in vitro*, and related to the difference in the "hardness" of the O^6 and N^7-positions of guanosine, rather than differences in the sensitivity of detection.

In Figure 3.5, the yield of O^6-CMdG is plotted against the initial concentration of APNG. It can be seen that O^6-CMdG yield passes through a maximum value of 0.2% for an APNG:2/-dG mole ratio of 1:1. With higher molar excess of APNG, the O^6-CMdG yield clearly decreases. The reasons for this behaviour are discussed below.

In Figure 3.6, the yield of N^7-CMG is plotted against the initial concentration of APNG. As noted above the yields of N^7-CMG are always substantially higher than those for O^6-CMdG and it appears to increase linearly with initial APNG concentration. Further, for very low levels of APNG (*eg* APNG:2/-dG ratio of 1:100) only N^7-CMG is detected.

Although the alkylation by APNG proceeds *via* a diazo-intermediate, the levels of 2/-dG alkylation are

lower than for other diazo compounds. For example, Farmer[83] reported yields of 22% O^6-methyl-2/-deoxyguanosine and 21% N^7-methyl-2/-deoxyguanosine when reacting 2/-dG with diazomethane in CH_3OH. The lower yields of carboxymethyl products may relate to extensive N^1-alkylation, dialkylation or the use of aqueous media.

The non-linear relationship between O^6-CMdG and APNG concentration may also relate to extensive formation of dialkylated products, which would not be detected by the present assay. If so, a similar dialkylation of N^7-CMG cannot proceed because N^7-CMG increases linearly with APNG concentration.

3.4 REACTION OF APNG WITH CALF-THYMUS DNA.

These reactions were carried out in a similar manner to those above using calf-thymus DNA in place of 2/-deoxyguanosine. The principal aim was to demonstrate that APNG alkylates the guanine residues in intact DNA under simulated physiological conditions, and to develop and evaluate procedures for the detection and quantitation of the alkylated guanine bases.

Because of its more complex structure, alkylation

may proceed at several sites in intact DNA. These include the phosphate moieties as well as the G,A,T and C bases. Because of the increased number of reactive sites, it is difficult to make a direct comparison between the extent of O^6 and N^7-carboxymethylation of 2/-dG and intact DNA for a given APNG concentration. This problem was partially circumvented by relating the extent of O^6-CMdG and N^7-CMG formation for DNA to its 2/-dG content. This was estimated assuming 19.6% (w/w) DNA refers to 2/-dG bases.

Another important difference for the experiments with intact DNA was the need to release the alkylated 2/-dG bases by hydrolysis prior to H.P.L.C. assay. The examination of suitable [illegible] assay procedures are described and discussed first.

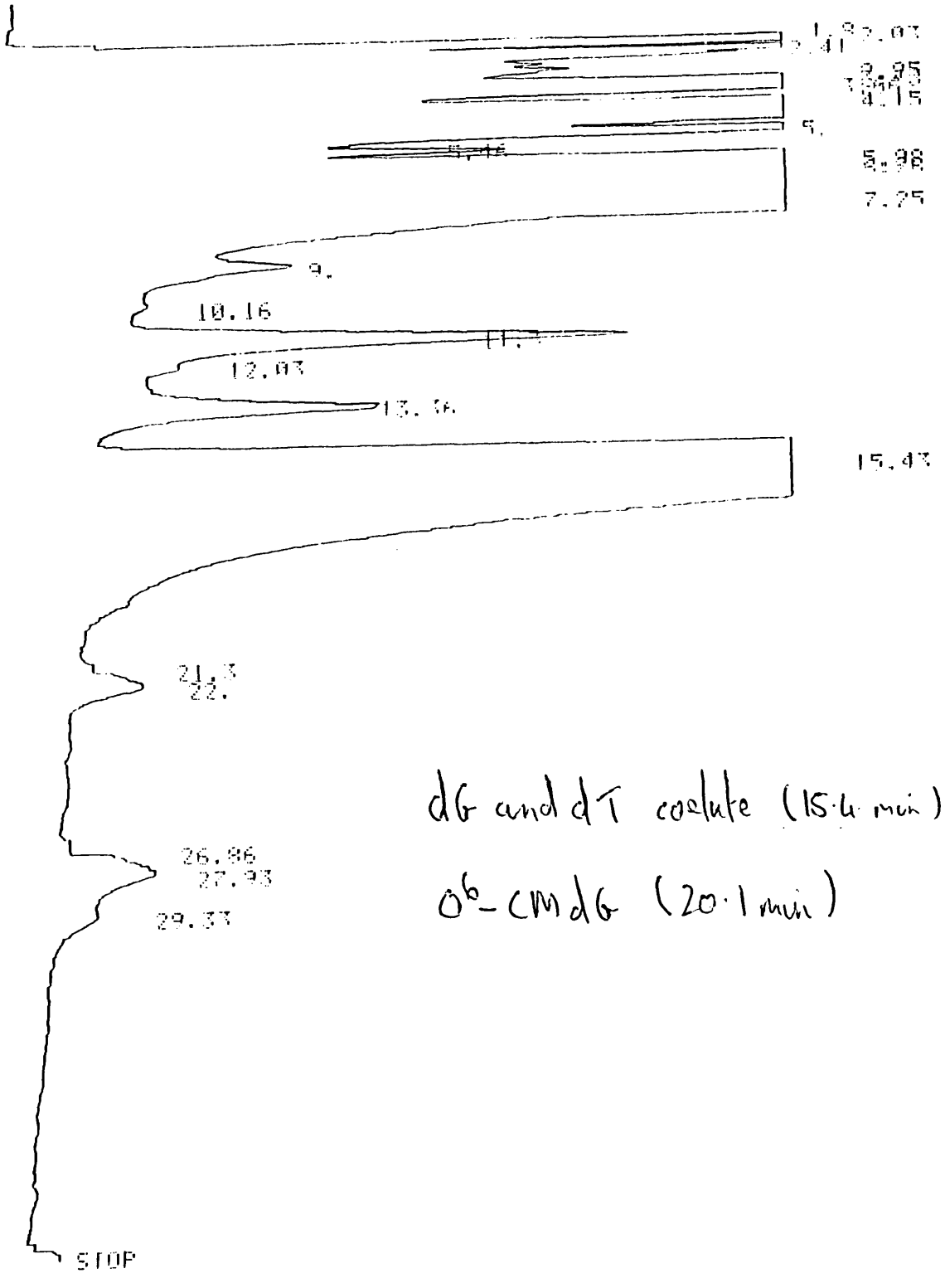
3.5 ANALYTICAL H.P.L.C. PROCEDURES FOR DNA ALKYLATION REACTIONS.

Reverse phase H.P.L.C. analysis of intact DNA treated with APNG for the presence of carboxymethylated-2/-deoxyguanosine adducts, is more difficult than the corresponding analysis of the 2/-dG reactions. The extra difficulties arise through the greater number of

potential adducts that can form from intact DNA, and separating these from the unmodified components of DNA at either nucleotide, nucleoside or purine/pyrimidine base level.

Initial work concentrated on the development of assays for O^6-CMdG but this proved very difficult and impracticable because of several interfering co-eluting compounds from DNA. Both isocratic and gradient procedures are available in the literature for the quantitation of DNA alkylation products at the nucleoside level. Attempts to adapt the two isocratic assays for O^6-CMdG for the 2/-dG reaction solutions, described in Section 3.2, were unsuccessful. Isocratic analysis of the DNA reactions was extremely difficult. Several problems were encountered, including inadequate separation of unreacted nucleoside components, inadequate buffering capacity of the eluent and a lack of sensitivity arising from small amount of DNA reaction solution (50ul equivalent to 7ug of DNA) that could be injected onto the column. Similar problems with buffering by the eluent were encountered with the H.P.L.C.-ELISA assay Section 4.4. Typical examples of the forementioned problems are given in Figure 3.7 (inadequate buffering capacity).

Because of the difficulties encountered in analysing DNA at the nucleoside level, it was decided to hydrolyse



dG and dT coelute (15.4 min)

O^6-CMdG (20.1 min)

Figure 3.7 Enzyme hydrolysed DNA

Analysis was performed on a C-18 silica column using isocratic CH_3CN/H_2O (1.5 : 98.5 v/v) at room temperature by UV (261nm).

the DNA further to its purine and pyrimidine components, and to analyse these products, enabling quantitation of both N^7 and O^6-carboxymethylguanine (O^6-CMG) in a single assay. An acidic gradient H.P.L.C. gave adequate separation of the DNA components. Thus, 100ug of hydrolysed DNA could be analysed using aqueous HFBA (0.1%w/w)/CH_3OH as eluent with a Hypersil C-18 silica column at 25°C at a flow of 1.0cm^3/min. The gradient used was 5% CH_3OH for 5min, rising to 20% CH_3OH and then 80% CH_3OH up to 15 and 25min, respectively, followed by 80% CH_3OH for a further 5min and decreasing to 5% CH_3OH over the final 5min. This gradient gave retention times for N^7-CMG and O^6-CMG of ca 7.9min and 17.5min respectively.

Analysis of the DNA at the purine level allowed the use of more sensitive fluorescence rather than conventional uv detection methods. The increased sensitivity of fluorescence detection of alkylated purines arises from the suppression of the emission spectra of the unmodified purine bases[125] eg guanine, relative to their carboxymethylated analogues. A more detailed account of the fluorescence detection is given in Section 5.7.2.

Quantitation of O^6-CMG and N^7-CMG in hydrolysed DNA

samples was effected by spiking the samples with a known amount of authentic compound and measuring the difference in peak area between spiked and unspiked samples. Duplicate analyses were performed for each reaction solution and a mean value obtained. The reproducibility of the total procedure was checked by carrying out duplicate reactions. Usually, duplicate samples of the same reaction solution agreed to +\-2% and duplicate reactions to +/-1%, as shown in Table 3.2.

3.6 HYDROLYTIC DECOMPOSITION OF DNA.

Unlike the 2/-dG reactions direct analysis of the APNG treated DNA reaction solutions proved impossible. Purification and hydrolysis of the DNA was required prior to analysis. The purification was effected by precipitation with 2 volumes of absolute C_2H_5OH to 1 volume of the reaction solution at 0°C, followed by centrifugation at 30,000 r.p.m. The supernatant fluid was discarded and the resultant pellet was dried under vacuum overnight and retained (see Section 5.5.3). Hydrolysis of the DNA pellet to its constituent nucleosides in two stages was instigated first. The enzyme Nuclease P1 (Sigma) at pH=5.3 and 37°C was used to cleave the DNA to its constituent nucleotide

monophosphates. Then, either the enzyme bacterial alkaline phosphatase (BAP) (BDH) or calf-intestine alkaline phosphatase (Boehringer) at pH=8.9 and 37°C was used to convert the nucleotide monophosphates to nucleosides. This procedure is a variant of the ordinary enzyme procedure described fully in Section 5.6.2.1.

One difficulty encountered with the enzymatic hydrolysis of DNA is the limited amounts of DNA that could be hydrolysed in one reaction. Most enzyme hydrolyses limit the amount of DNA to 50-100ug. Above this weight enzyme saturation occurs with incomplete hydrolysis. Originally BAP was used for the conversion of nucleotides to nucleosides. BAP, however is used as a colloidal suspension, and after hydrolysis it was necessary to filter the solutions before analysis could proceed. The use of more active calf-intestine alkaline phosphatase (Boehringer) removed the need for this sample filtration.

Non-enzymatic methods for hydrolysis of the DNA pellet were also examined. Acid hydrolysis is a simple and rapid technique compared to enzymatic hydrolysis but the DNA is reduced to its constituent purine and pyrimidine bases. Large amounts of DNA (1.0-5.0mg) could be hydrolysed by acid in small volumes, (100-500ul),

leading to higher concentrations of DNA for analysis by reverse phase H.P.L.C. Typically acid hydrolysis was performed by heating DNA (1.0mg) in aqueous HFBA 20% (v/v) (100ul) at 70°C for 30min. Subsequently H.P.L.C. analysis was carried out on 10ul aliquots (100ug of DNA) of the above solution. Further details of the experimental methods are given in Sections 5.5-5.7. Acid hydrolysis gives the constituent purine and pyrimidine bases. This is advantageous because a single assay procedure can be used to quantitate both the O^6 and N^7-carboxymethylguanine residues of DNA.

Quantitation of N^7-CMG and O^6-CMG was best performed using fluorescence detection for the H.P.L.C. assay. Determination of the optimum conditions for these analyses were determined using a fluorimeter. For a fixed excitation wavelength, (initially chosen at λ max=282nm), the emission was monitored to reveal maximum emission at λ=354nm. The quantitation O^6-CMG and N^7-CMG by fluorescence using excitation at λ=286nm and monitoring the emission over λ=300-400nm, gave detection limits of 2.0ng and 5.0ng respectively.

Thus analysis of acid hydrolysed DNA (100ug 10ul) was performed on a reverse phase C-18 silica column at 25°C using a HFBA/CH_3OH gradient system at 1.0cm^3/min. A typical chromatogram obtained for acid hydrolysed DNA

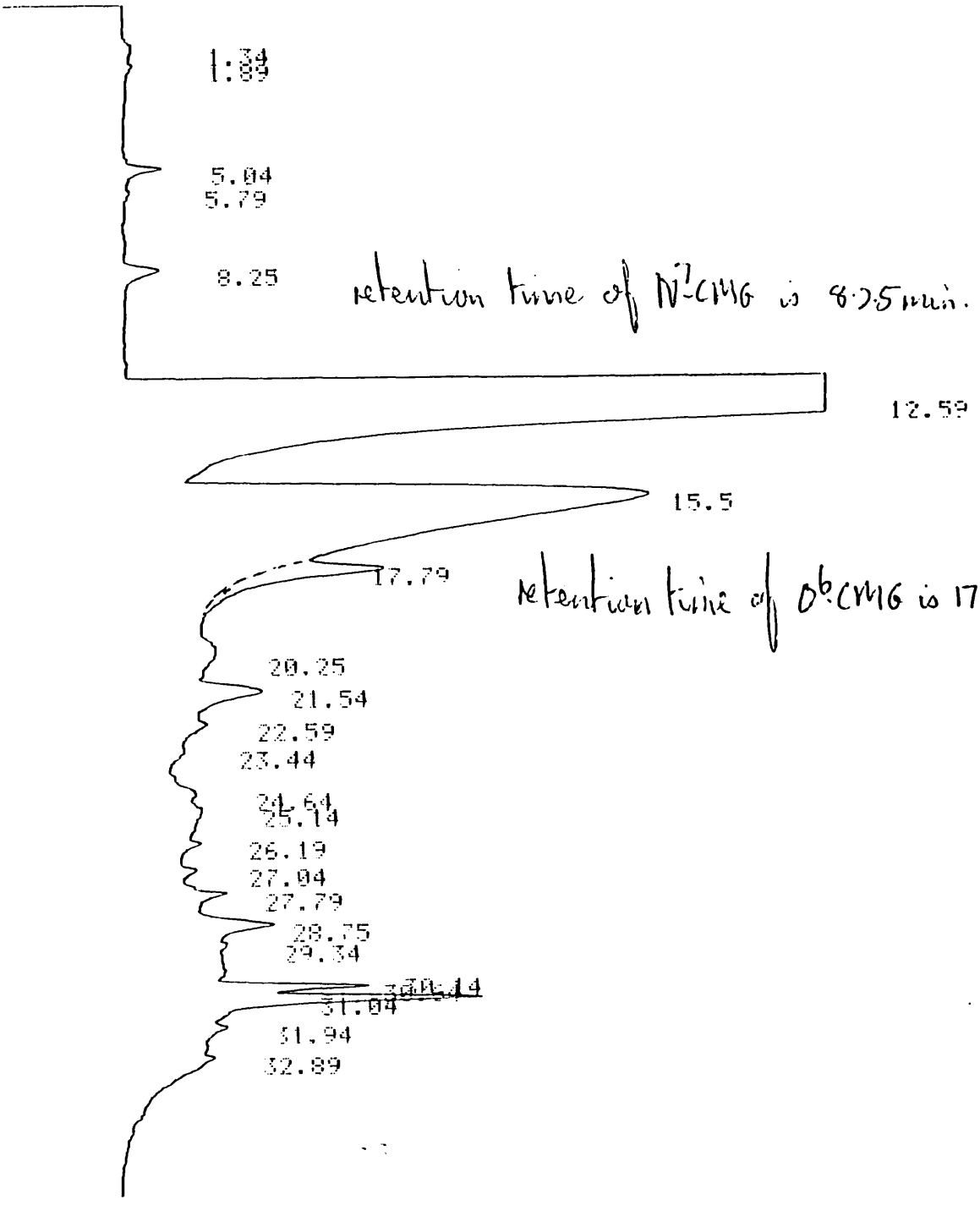


Figure 3.8 H.P.L.C analysis of acid hydrolyzed DNA using a HFBA/CH_3OH gradient system on a C-18 silica column. 10 ml (100 ng) of DNA was injected and detection was by fluorescence at ex = 286 nm. DNA: APNG (1:1 w/w) analyzed

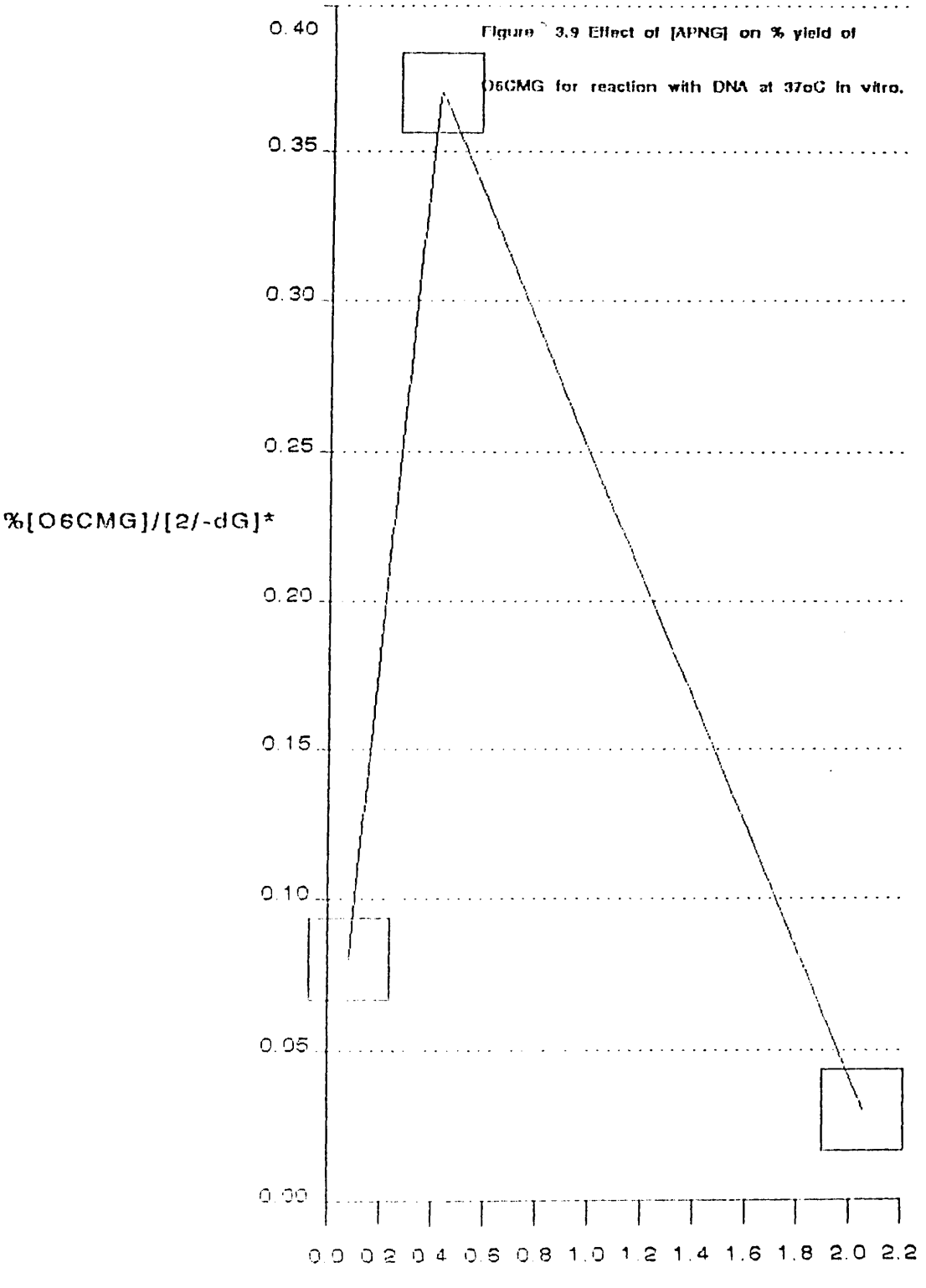
is given in Figure 3.8. Generally, adequate separation was obtained between N^7-CMG, O^6-CMG and the component purines and pyrimidines. However, quantitation of the extent of 2/-dG residues in DNA was performed on an alternative C-18 silica H.P.L.C. system (see Chapter 4 for details).

3.7 DNA ALKYLATION REACTIONS.

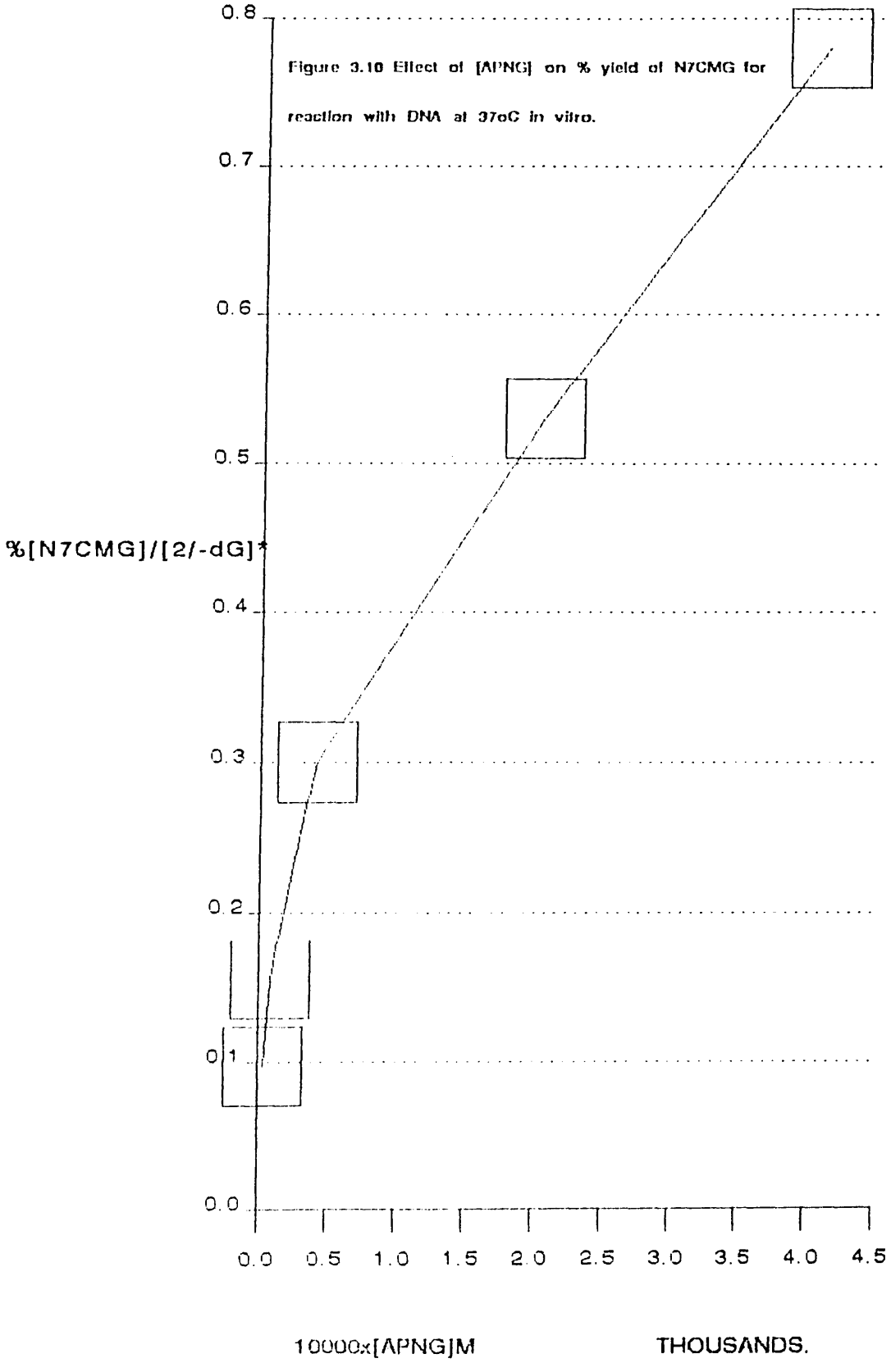
These were carried out on aqueous calf-thymus DNA in 10mM Tris buffer at pH=7.2 at 37°C. Thus, APNG (41.1-4110x10^{-7}moles) in DMSO (10-100ul) was added to the DNA solution (1.0cm^3 10mg/cm^3) at pH=7.2. The mixture was thoroughly shaken then incubated at 37°C for 48-72h, monitoring the extent of reaction by loss of the APNG absorbance at λ=410nm. The mixture was then cooled to 0°C, the DNA precipitated by the addition of absolute C_2H_5OH (2cm^3) followed by $(C_2H_5)_2O$ (2cm^3), then dried under vacuum. The DNA pellet was then hydrolysed in acid at 70°C. A more detailed description of the methods are given in Section 3.5. Subsequent H.P.L.C. analysis of the hydrolysed solution indicated that alkylation had occurred at both the N^7 and O^6-positions of the guanine residues. The results are summarised in Table 3.2 and Figures 3.9 and 3.10 for treatment of DNA with different amounts of APNG.

Variations in the yield of O^6-CMG with [APNG] are

Figure 3.9 Effect of [APNG] on % yield of O6CMG for reaction with DNA at 37oC in vitro.



[2/-dG]* calculated assuming 19.61ug 2/-dG per 100ug DNA.



[2/-dG]* calculated assuming 19.61ug 2/-dG per 100ug DNA.

plotted in Figure 3.9 which shows that the yield passes through a maximum for [APNG]/[DNA]=1 (w/w). Variation in the yield of N^7-CMG with [APNG] in Figure 3.10 shows that the yield increases steadily with [APNG], but clearly it falls of at the highest [APNG]. For the lowest concentrations of APNG (41.1×10^{-4}M) evidence for N^7-alkylation only was obtained.

Table 3.2 Variation of the yields of O^6-CMG and N^7-CMG with [APNG] for the reaction with DNA *in vitro* at 37°C. Initial [DNA]=10mg/cm³.

10^4[APNG]	[APNG]/[DNA]	Yield O^6-CMG			Yield N^7-CMG		
		ng	10^4xA mole moledG	%[c]	ng	10^4xB mole moledG	%[c]
41.1	0.1	u	u	u	14.8	9.65	.097
82.2	0.2	12.5	8.14	0.08	24.2	15	.156
411	1	57.3 58.1	37.3 37.8	0.37	46.3 47.0	30.6	.30
2055	5	4.7	3.05	0.03	82.2	53.5	.53
4110	10	u	u	u	119.7 120.4	77.9 78.5	.78

A=[O^6-CMG]/[2′-dG][c]; B=[N^7-CMG]/[2′-dG][c]

u=Beyond limits of detection.

c=The amount of 2′-dG was estimated by assuming DNA containing 19.6% (w/w) of 2′-dG.

It is not clear why reduced levels of O^6-CMG and N^7-CMG are obtained for the highest [APNG]. Although appreciable dialkylation of the guanine residues may occur , this cannot be the only explanation. It is possible that incomplete precipation of the reacted DNA is an additional factor. For reactions with APNG:DNA < (1:1w/w), the DNA pellet obtained on precipitation with C_2H_5OH was solid, indicating a well-defined structure. For reactions with APNG:DNA > (1:1w/w) precipitation of the DNA was difficult because the pellet was a sticky solid, indicating structural breakdown of the DNA. High levels of base alkylation often results in low molecular weight DNA fragments which are soluble in C_2H_5OH. Structural breakdown of DNA results from phosphate ester alkylation and generation of phosphotriesters. Phosphotriesters are labile, resulting in strand cleavage and DNA fragmentation.

3.8 CONCLUSIONS.

The results show that APNG reacts with both 2′-dG and 2′-dG residues of calf-thymus DNA *in vitro* under simulated physiological conditions (37°C, pH=7.2). Several products are formed, two of which are O^6-CMdG (O^6-CMG) and N^7-CMG. Both these compounds can be

detected (after suitable work-up of the reaction solutions) by reverse phase H.P.L.C. with a limit of detection of ca 2.0ng for O^6-CMdG (O^6-CMG) and ca 5.0ng for N^7-CMG.

The extent of O^6-CMdG and N^7-CMG formation is dependent on the concentration of APNG. Carboxymethylation is site dependent, however, and the yields of N^7-CMG are greater than O^6-CMdG under all conditions for both 2/-dG and DNA. The N^7-CMG yields increase steadily with increasing [APNG], whereas those for O^6-CMdG pass through a maximum value. For concentrations of APNG $<82.2 \times 10^{-4}$M N^7-CMG is found but O^6-CMdG is probably below the limit of detection.

These in vitro experiments suggest that APNG will also alkylate the residues of DNA in vivo. The formation of O^6-CMdG is of particular interest because O^6-alkyl-2/-deoxyguanosine adduct formation in vivo is commonly associated with carcinogenic substances. Thus, APNG and related N-nitrosopeptides and N-nitrosobile acid conjugates may well be carcinogenic.

Extension of the above studies using low (sub-lethal) doses of APNG in vivo, will result in low levels of DNA-base alkylation and much smaller amounts of DNA

available for analysis. Thus, highly sensitive methods will be required to detect O^6-CMG and N^7-CMG formation *in vivo*. Typically, *in vivo* modification of 1 in 10^5-10^7 DNA bases is expected which will give sub-picomole levels of O^6-CMdG and N^7-CMG formation. It is unlikely that the H.P.L.C. assays described above will be sufficiently sensitive for these measurements. The development, application and use of a more sensitive ELISA immunochemical method to detect and quantitate levels of O^6-CMdG in DNA samples, is therefore described and discussed in Chapter 4.

Chapter 4.

IMMUNOCHEMICAL ANALYSIS OF DNA.

4.1 INTRODUCTION.

The development of immunological assays for the detection of normal and modified nucleic acids has advanced rapidly in recent years. These assays, which complement other biochemical and physical methods, are especially useful because of their simplicity and sensitivity.

A wide variety of environmental mutagens and carcinogens, chemotherapeutic agents, as well as uv light, cause structural alterations to cellular macromolecules. Structural alteration of genomic deoxyribonucleic acid, DNA, is considered to be most important for the induction of cancer[126]. The detection of carcinogen-modified DNA components requires highly sensitive methods, because usually only small amounts of DNA are modified. A major advantage of immuno-assay is that the DNA fragment or chemical adduct of interest does not have to be labelled with radio-isotopes. This is especially important for DNA from cells which are slowly proliferating, and therefore difficult to label with high levels of radio-activity, and where the radio-active label is attached to the compound, which binds to DNA. For the latter, the utility of the radio-chromatographic assay is limited by the specific activity of radio-

labelled compounds, and the difficulty or expense of synthesizing radio-labelled compounds[127].

The formation of O^6-alkyl-2/-deoxyguanosine adducts in DNA through exposure to alkylating agents has been closely linked with carcinogenesis[48,59,64], and is related to subsequent mispairing of the modified DNA bases during replication. Many of these O^6-alkyl-DNA adducts have been analysed by immunological methods[128,129,130]. For the present study involving the ability of N-(N/-acetyl-L-prolyl)-N-nitrosoglycine and N-nitrosoglycocholic acid to alkylate DNA, an immunological method using an Enzyme Linked Immunosorbent Assay (ELISA) procedure was investigated for the detection of O^6-carboxymethyl-2/-deoxyguanosine (O^6-CMdG). The development and evaluation of the ELISA procedure carried out in conjuction with Dr. Shuker at I.A.R.C. is outlined below.

4.2.1 ELISA PROCEDURE.

Competitive ELISA (cELISA) is one of the most commonly used immunoassay procedures. Its basic principle is that a controlled amount of antibody will partition itself between free antigen in the sample under analysis and immobilized antigen on a solid surface. Competition for the controlled amount of antibody by the

immobilized hapten and the free hapten in solution follows the usual law of mass action, and is therefore influenced only by the relative amounts of antigens and the equilibrium constant for the antibody-hapten interaction[131].

The first step in the ELISA procedure is to immobilize a suitable form of the antigen on a plastic surface and to wash away any unbound material. Simple adsorption onto a specially prepared polystyrene surface is sufficient for the immobilization. In the second step a solution of unbound antigen is added either from a standard or from the sample to be analysed and the antibody then added to the free and immobilised antigen mixture. High concentrations of unbound antigen in solution reduce antibody binding to antigen immobilized on the plate. At the end of a suitable incubation period, the solution phase is discarded leaving behind a proportion of the antibody bound to the immobilized hapten. The proportion remaining is of course inversely proportional to the amount of unbound antigen present in the test solution. The final step in the assay is the detection of antibody bound to the immobilized antigen. For cELISA this is measured by some form of enzyme activity associated with the antibody. The enzyme can be either directly conjugated to the antigen specific antibody using the second step, or introduced

subsequently via an enzyme conjugated second antibody that specifically binds to the original anti-hapten antibody. Many enzymes are suitable[131], for example peroxidase, alkaline phosphatase and many chromogenic substrates to measure the enzyme activity are commercially available.

The advantage of cELISA over other immunoassay procedures is its ability to amplify the response from small amounts of antigen by means of prolonged reaction times for determining the enzyme activity. Thus cELISA's with very low detection limits (<1fmole of DNA adduct) have been described by Harris et al[132], and by Van der Laken et al[133].

4.2.2 NON-EQUILIBRIUM cELISA ASSAY[112,131].

This is a variant of the standard cELISA procedure. Binding between the free hapten and the antibody is promoted in preference to the immobilized antigen-antibody binding by carrying out the assay at a reduced temperature (eg 4°C) and with short reaction times. The method gives increased sensitivity but is less reproducible without careful attention to experimental detail.

4.3 CROSS REACTIVITY OF THE RABBIT ANTISERUM, CMGuo1.

The basis of an immunochemical assay is the generation of an antiserum by treatment of the host animal with the adduct of interest, (or structurally related compound), and following isolation from blood by centrifugation, after a suitable incubation period, the serum is used to determine adduct levels in samples of interest. The rabbit antiserum, O^6-CMGuo1, was prepared in this way, by immunizing rabbits with the protein adduct O^6-carboxymethylguanosine-methylated bovine serum albumin (O^6-CMGuo-m-BSA), and subsequently isolating the serum from blood. A detailed description of the synthesis of O^6-CMGuo m-BSA and rabbit immunization procedure are given in Section 5.9.

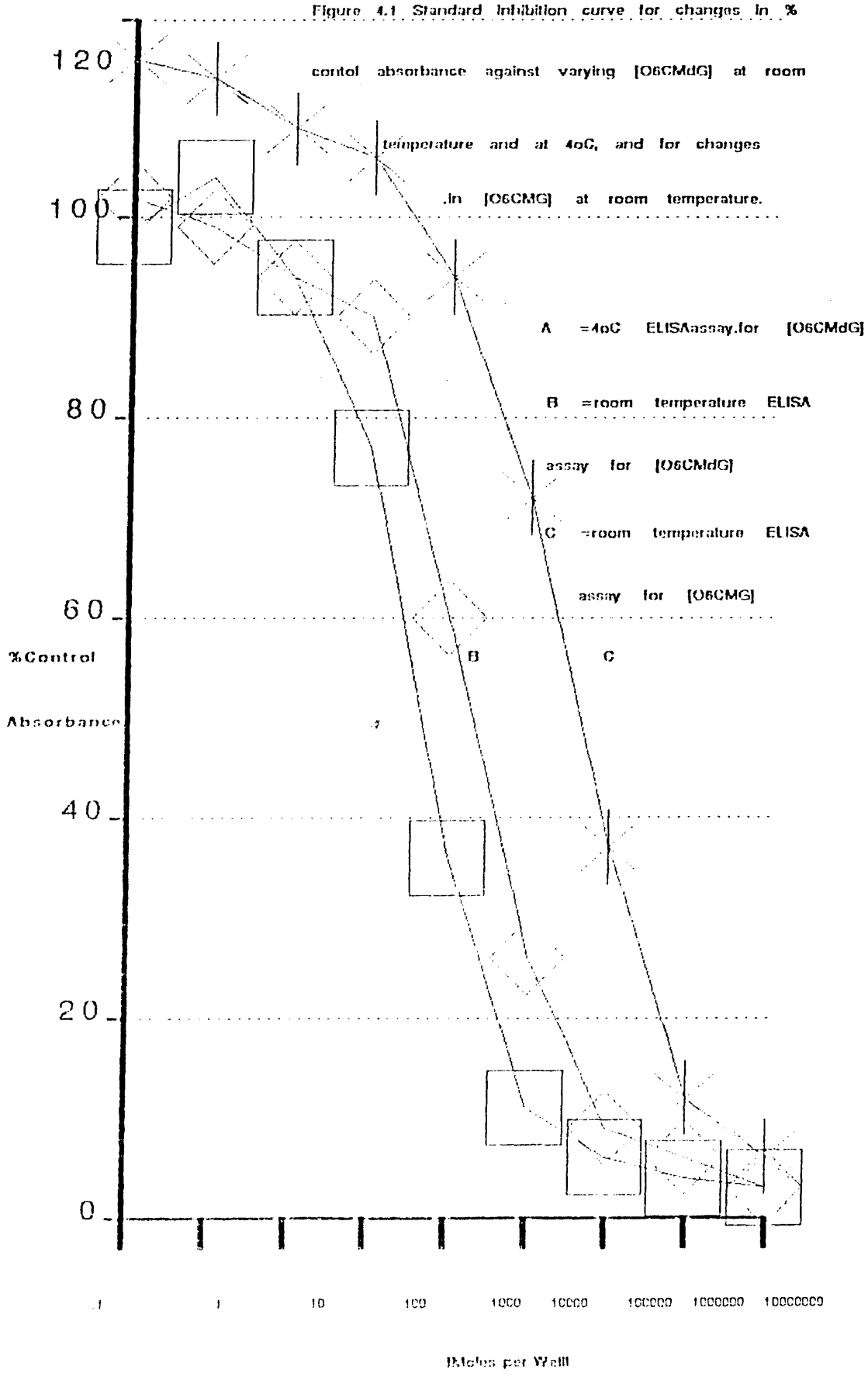
The first requirements of any immunoassay is that the antibody (monoclonal or polyclonal) "recognizes" the analyte at low levels and with a high degree of selectivity; ie the antibody should have minimal "cross-reactivity" with structurally related compounds.

The cross-reactivity of CMGuo1 with modified and unmodified nuleosides was determined by the room

temperature ELISA procedure. This procedure is described in detail for O^6-CMdG in Section 5.6.1.2. Thus, varying concentrations of the compounds of interest (0.1-10^7fmoles in PBS (25ul)), (eg nucleosides, O^6-CMdG and O^6-CMG), are placed in wells of the pre-coated ELISA plate (O^6-CMGuo-ovalb (5ng/well)) and the antiserum added. After a 90min incubation period a second antiserum is added, horse-radish peroxidase-linked goat anti-rabbit immunoglobulin G and the plate re-incubated for 90min. Subsequently, a colour marker is added, and the optical density of each well of the plate determined. The cross-reactivity of the reagent is determined as an inhibition curve, by plotting the % absorbance of each well relative to that of a control against reagent concentration. A typical example is shown in Figure 4.1.

The inhibition curve, Figure 4.1, at room temperature, shows the variation of control absorbance (as %) versus antigen (O^6-CMdG) concentration. The sigmoidal curve obtained (Figure 4.1) is typical of antigen-antibody binding curves. The 50% inhibition obtained with 2pmoles of O^6-CMdG per well is comparable with those found for other room temperature ELISA procedures; for example, Shuker[134] found 50% inhibition at the 10 pmole level for the immunoassay of N^7-methylguanine by a similar polyclonal antiserum.

Figure 4.1 Standard Inhibition curve for changes in % control absorbance against varying [O6CMdG] at room temperature and at 4oC, and for changes in [O6CMG] at room temperature.



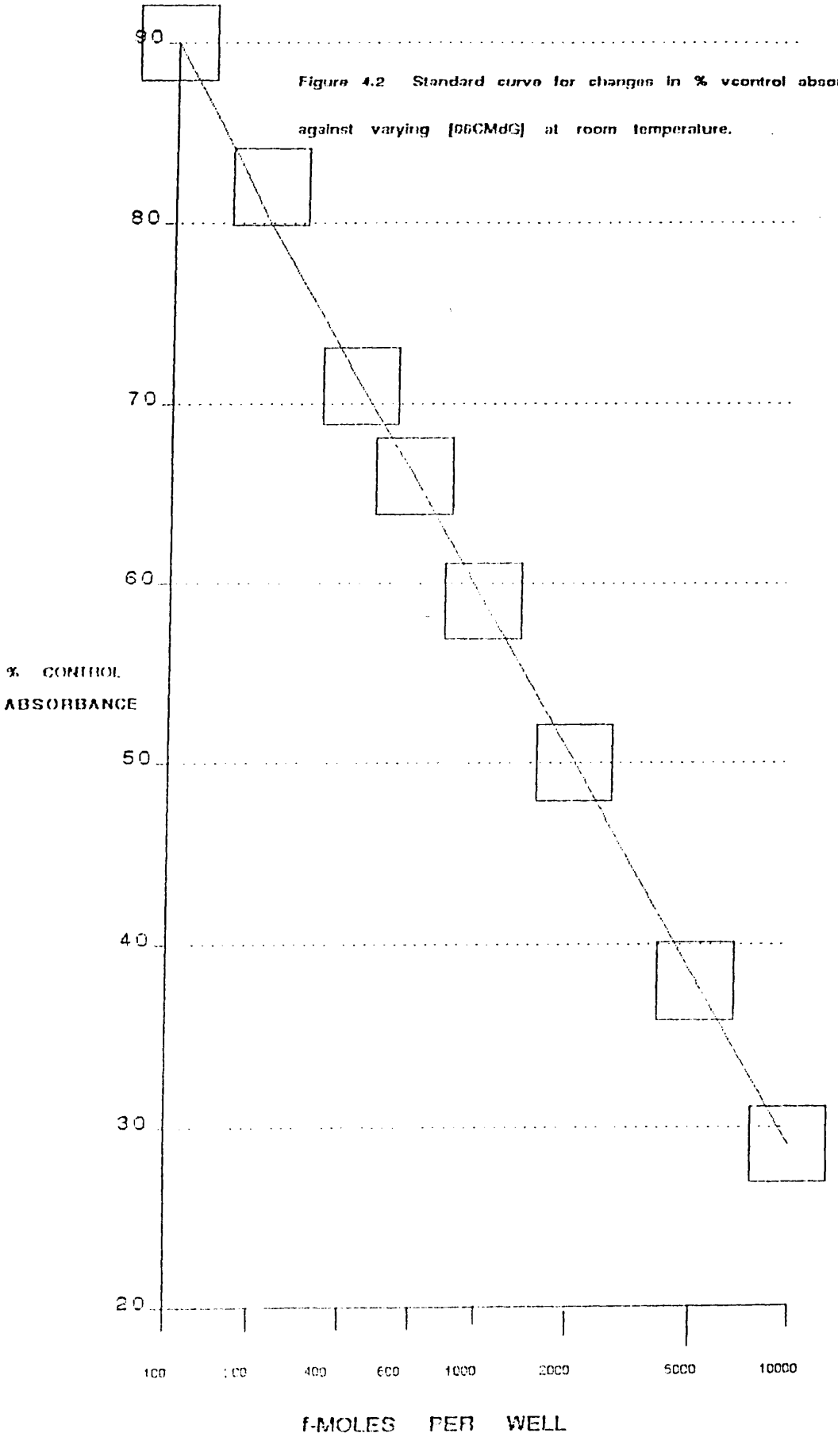


Figure 4.2 Standard curve for changes in % vcontrol absorbance against varying [66CMdG] at room temperature.

Cross-Reactivity of modified and unmodified with the rabbit anti-serum CMGuo1 for the ELISA assay at room temperature.

Substrate	[Substrate] to achieve 50% Inhibition fMoles	[Substrate] to achieve 80% Inhibition fMoles
O^6-CMdG	$2x10^3$	$2x10^4$
O^6-CMG	$6x10^4$	$5x10^5$
O^6-MedG	$>10^7$	$>10^7$
O^6-EtdG	$>10^7$	$>10^7$
2/-dG	$>10^7$	$>10^7$
N^7-CMG	$>10^7$	$>10^7$
N^3-CMA	$>10^7$	$>10^7$
2/-dA	$>10^7$	$>10^7$
2/-dC	$>10^7$	$>10^7$
2/-dT	$>10^7$	$>10^7$

O^6-MedG = O^6-methyl-2/-deoxyguanosine.

O^6-EtdG = O^6-ethyl-2/-deoxyguanosine.

N^3-CMA = N^3-carboxymethyladenine.

Table 4.1.

The effect of assay temperature is also reported in Figure 4.1. At 4°C the inhibition curve shows a 50% inhibition for 700 fmoles of O^6-CMdG (ie a 3-fold increase relative to the room temperature assay). This increase was much smaller than anticipated, and compares unfavourably with the 50-100 fold increase achieved for N^7-methylguanine antibodies, by Shuker[135]. The small effect of temperature may relate to the type of antigen binding with the O^6-CMdG-antibody. For uncharged adducts, eg O^6-methyl-2′-deoxyguanosine, the antigen-antibody binding is thought to result from Van der Waals interactions. Thus, any reduction in temperature should decrease the vibrational and rotational energies associated with the antigen-antibody interaction, and therefore increase the Van der Waals binding and hence the sensitivity of the assay.

In the case of an antigen bearing a polar group such as O^6-CMdG, antigen-antibody binding may be dominated by ionic interactions, which are less susceptible to temperature dependent changes in the rotational and vibrational energies of the antibody and the antigen. Thus, carrying out the immunoassay at 4°C instead of room temperature would produce a small change in sensitivity.

The cross-reactivity of the antiserum CMGuo1 with other nucleosides including 2′-deoxyguanosine, 2′-

deoxyadenosine and 3-carboxymethyladenine was examined at room temperature. These results are summarised in Table 4.1 in the form of concentrations required to achieve 50% and 80% inhibition. Low cross-reactivity is evident for the deoxynucleosides dA, dT, dC and dG. No cross-reactivity was seen for simple alkyl substituents on the O^6-position of dG, eg O^6-methyldG and O^6-ethyldG. This shows that antibody binding is dependent on the structure of the O^6-alkyl substituent. Further no cross-reactivity was observed with either N^7-carboxymethylguanine (N^7-CMG) or 3-carboxymethyladenine, indicating that the antibody only recognizes the carboxymethyl component present at the O^6-position of the 2'-deoxyguanosine nucleoside. Comparision of the inhibition curves for O^6-CMdG and O^6-carboxymethylguanine (O^6-CMG) shown in Figure 4.1, shows that the antibody will bind but is 30 times less sensitive to the modified base than to the modified deoxyguanosine nucleoside. This implies that the presence of an N^9-deoxyribose residue is important for the antigen-antibody binding and recognition.

The above results show that the antiserum is highly specific for O^6-CMdG, with insignificant cross-reactivity for other modified or unmodified nucleosides. The sensitivity of the antiserum is lower than desired, but comparable with other polyclonal antisera under similar experimental conditions.

4.4 H.P.L.C. ELISA METHOD.

The validity of the ELISA assay for O^6-CMdG was tested by comparison with the H.P.L.C./fluorescence procedure for O^6-CMdG in DNA. In practice the two assays were combined by first carrying out the enzymatic hydrolysis of DNA to the nucleoside level, analysing the samples by H.P.L.C. with fluorescence detection, collecting the fraction containing O^6-CMdG and analysing this by the ELISA method. As a further comparative test the DNA was analysed for O^6-CMG after an appropriate acid hydrolysis.

The enzymatic hydrolysis of the reacted DNA was performed by two procedures, using either Ordinary Enzyme or New Enzyme (see Section 5.6.2 for details). The hydrolysate DNA was then analysed on an Altrex Ultrasphere C-18 silica column using an aqueous HFBA (0.1%w/w) /CH_3OH gradient system with a flow rate of 1.0cm^3/min at 25°C. A more detailed description of the method can be found in Section 5.7.2.2. The O^6-CMdG was quantitated with a dual uv/fluorescence detection system. Typical examples of chromatograms obtained are shown in Figure 4.3 and Figure 4.4. For the acid hydrolysis of DNA, analysis was performed at the purine level on a Hypersil ODS2 C-18 silica column using an aqueous HFBA (0.1%w/w)/CH_3OH gradient system with a flow rate of 1.0cm^3/min at 25°C.

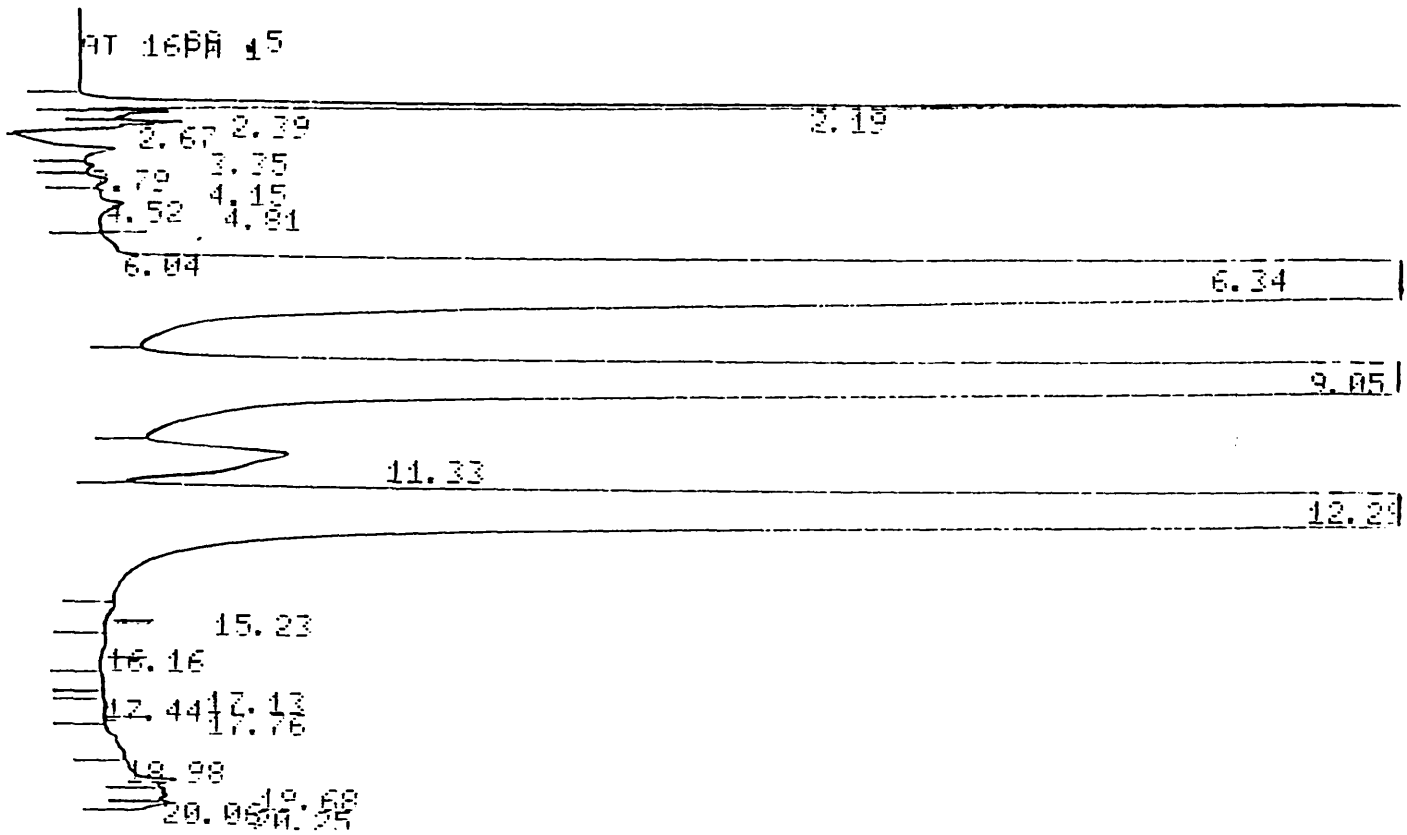


Figure 4.3. H.P.L.C analysis by uv at 260 nm of the enzyme hydrolyzed DNA (new enzyme method used) on a C-18 silica column using a HFBA/CH_3OH gradient-eluent system. 200 ml of the enzyme hydrolyzed DNA was injected. 10 DNA: 1 APNG w/w was used.

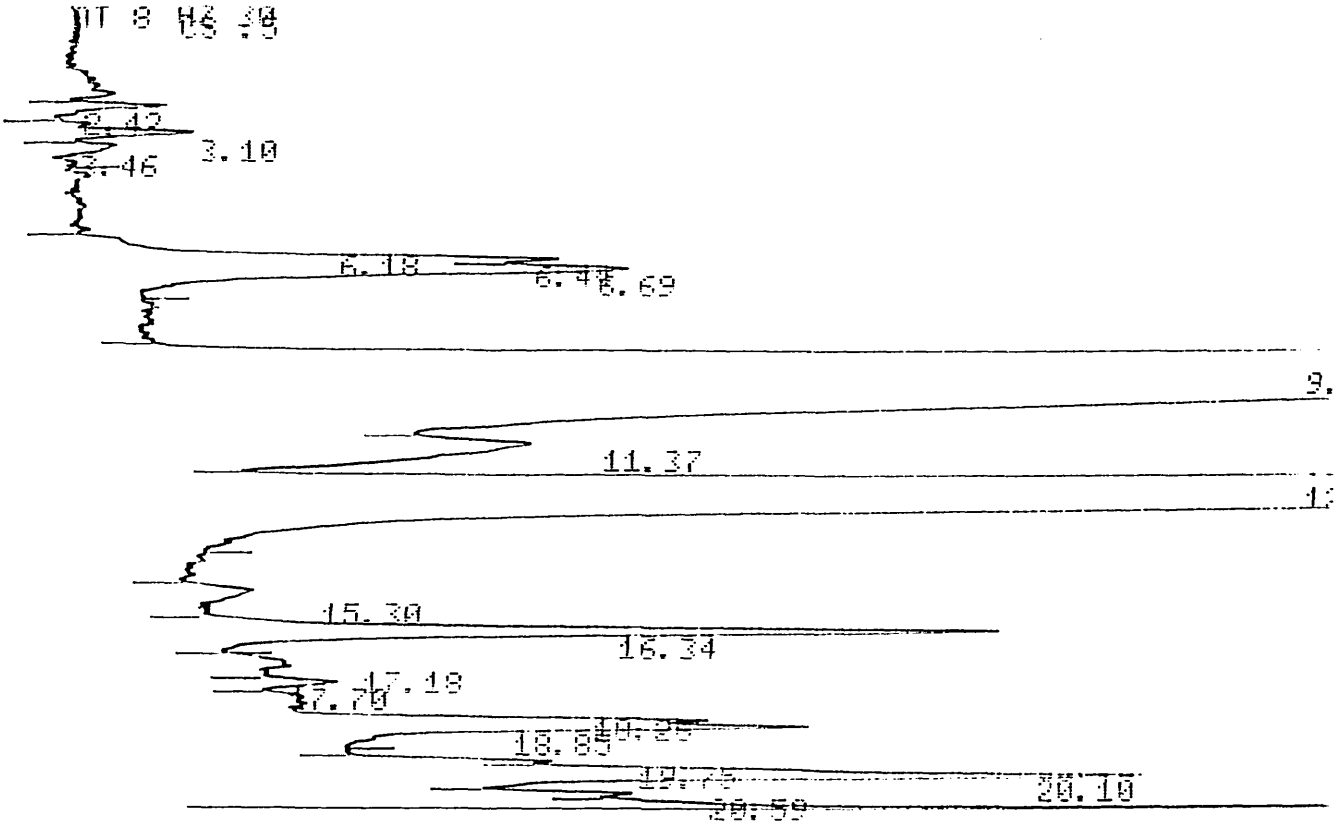


Figure 4.4 H.P.L.C analysis of the enzyme hydrolysed DNA (new enzyme method used) on a C-18 silica column using a HFBA/CH3OH gradient eluent system. 200µl of the enzyme hydrolysed DNA solution was injected and the 10DNA:1APNG (w/w) treated DNA was used. Detection was performed by fluorescence (Ex 266 nm) and under these conditions the retention time for O^6-CMdG was 16.34 min.

Table 4.2 Comparison of levels of O^6-CMdG obtained for different APNG treated DNA samples, by different analytical methods used in conjunction with the room temperature ELISA assay. Quantitation of the amount of O^6-CMdG adduct formation (ng) is performed on 100ug of DNA.

Analytical method for O^6-CMdG quantitation	[APNG]:[DNA] (1:1w/w) ng	[APNG]:[DNA] (1:5w/w) ng	[APNG]:[DNA] (1:10w/w) ng
Direct analysis intact DNA by the room temperature ELISA assay.	26	1.09	0.286
Ord Enz hydrolysis + H.P.L.C. + room temperature ELISA assay.	4.7	2.6	a
New Enz hydrolysis + H.P.L.C. + room temperature ELISA assay	2.0	2.6	a
Acid hydrolysis + fluorescence detection.	89 57[b] (O^6-CMG)	18 13[b] (O^6-CMG)	a

a=See Table 3.2; b=Below limits of detection.

Quantitation was by fluorescence, and a detailed description of methods employed and results obtained

during the alkylation and subsequent analysis of DNA treated with APNG *in vitro* at 37°C, are given in Chapter 3.

The results are summarised and compared in Table 4.2, where it is apparent that analysis by H.P.L.C./ELISA gave lower levels of O^6-CMdG compared with the levels of O^6-CMG determined by H.P.L.C./fluorescence after acid hydrolysis. Previous analyses of authentic O^6-CMdG by H.P.L.C./fluorescence indicated no problems from the decomposition of the standard solutions by the acidic eluent (0.1% aqueous heptafluorobutyric acid (HFBA)). Only 1 peak for O^6-CMdG and no peaks corresponding to O^6-CMG or G were detected by H.P.L.C./fluoresence analysis. Analysis by H.P.L.C./fluorescence (286nm excitation, >340nm emission) gave a limit of detection of 500pg (1.5pmole) for O^6-CMdG, compared to 100fmoles by H.P.L.C./ELISA analysis, indicating that ELISA analysis for O^6-CMdG gave *ca* a 15-fold increase in sensitivity over the H.P.L.C./fluorescence method.

Examination of the results in Table 4.3 shows that the analysis of 5ng of authentic O^6-CMdG by ELISA after passage through the H.P.L.C. column and/or after storage in the H.P.L.C. eluent for 180min, gave values of 2.4ng and 2.6ng O^6-CMdG, respectively. Clearly, decomposition of O^6-CMdG occurs in the eluent buffer, mainly after

collection of the fraction containing O^6-CMdG. The stability of O^6-CMdG in dilute acid was therefore examined independently. At room temperature (ca 20°C), in 0.1% aqueous HFBA, O^6-CMdG decomposed with a $t_{1/2}$=180min, (as determined by analysis of a solution by H.P.L.C. fluorescence assay at timed intervals). By comparison, the decomposition of O^6-MedG is much slower, ($t_{1/2}$=240h at 38°C in 0.5M HCl)[136]. The rapid hydrolysis of O^6-CMdG may relate to protonation of the N^7-position by the neighbouring O^6-carboxymethyl group, leading to rapid depurination (Figure 4.5).

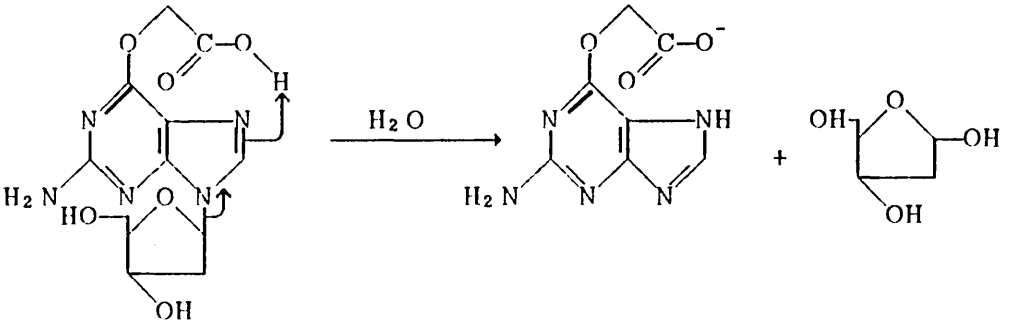


FIGURE 4.5

Whatever, the increased instability of O^6-CMdG in aqueous acid is an unfortunate complication which limits the range of conditions for both the H.P.L.C. assay and the ELISA assay. Decomposition may be minimized by rapid neutralisation of the H.P.L.C. eluent with NH_3. This possibility has yet to be tested.

Table 4.3 Comparison between the effects of H.P.L.C. analysis and storage in the buffer eluents, on the quantitation of the levels of O^6-CMdG by the room temperature ELISA assay. 5ng of authentic O^6-CMdG was used as substrate.

Substrate	Substrate after H.P.L.C. analysis and storage in buffer eluent (3h) ng.	Substrate after storage in buffer eluent (3h) only ng.
O^6-CMdG	2.4	2.6

Rapid acid-catalysed decomposition does not fully account for the underestimation of O^6-CMdG present in DNA, after enzyme hydrolysis and ELISA assay (Table 4.2). The effect of the enzyme hydrolysis procedure was determined by examining two, different enzyme procedures. These were "Ord Enz", (which requires denaturing of the DNA to obtain single strands), and "New Enz", (which is effective on double stranded DNA). Both procedures gave similar low results for the O^6-CMdG content. The highest reduction applied to reactions using equal weights of APNG and DNA (ie 1:1 w/w ratio), which should contain the highest amounts of carboxymethylated DNA bases. This implies that the presence of other carboxymethylated DNA adducts, possibly phosphotriesters, prevent the hydrolytic enzymes from functioning properly. One

possible solution to the underestimation is therefore to analyse DNA directly by the ELISA method (see later).

4.5 DIRECT DNA ANALYSIS.

In view of the problems encountered with decomposition of O^6-CMdG during the H.P.L.C. purification and enzyme hydrolysis procedures, the feasibility of analysing the intact DNA after carboxymethylation was examined. Cross-reactivity data (Table 4.1) indicated that neither unmodified DNA, nor DNA adducts other than O^6-CMdG, would interfere.

Preliminary results shown in Table 4.2, suggest that for the 5:1 (w/w) DNA:APNG reaction similar results are obtained for the analysis of intact DNA and O^6-CMdG following enzymatic hydrolysis. For the 1:1 (w/w) DNA:APNG the two analysis are more divergent (eg 26ng for the intact DNA versus 4.7ng of O^6-CMdG following hydrolysis). This is tentative evidence that enzyme hydrolysis may be being hindered, especially for highly modified DNA. Nonetheless, intact DNA analysis also gives lower levels of O^6-CMdG relative to those for O^6-CMG following acid hydrolysis. The preliminary results suggest that the O^6-CMdG adduct is recognized in a DNA matrix and that intact DNA analysis is therefore

possible, but further work is required to make intact DNA analysis quantitative.

A single experiment was also carried out to see if O^6-CMdG could be detected in rat liver DNA following treatment with APNG. Two rats (male Sprague-Dawley 180-190g) were treated with APNG (10mg) in P.B.S. ($0.5cm^3$) by oral gavage. Two other rats were treated only to serve as control animals. After 6h, the rats were sacrificed by decapitation and the livers were removed and frozen in liquid nitrogen. DNA was extracted from portions (2g) of the liver by a standard phenol extraction procedure[137]. O^6-CMdG could not be detected in the samples either by H.P.L.C./fluorescence following hydrolysis or by ELISA on the intact DNA.

4.6 CONCLUSION.

The results above show that the polyclonal antiserum derived from rabbits by treatment with O^6-carboxymethylguanosine-m-BSA-ovalbumin is specific for O^6-CMdG, with a high degree of selectivity. Its sensitivity at 4°C is lower than other polyclonal antisera possibly due to strong ionic interactions in the antigen-antibody complex.

Increased immunochemical sensitivity could be obtained by the development of a monoclonal antibody (Mab) possessing a high affinity constant for binding with O^6-CMdG. For example Ademkiewicz et al[111] have shown that a specific Mab for O^6-ethyl-2/-deoxyguanosine derived from mice, has an affinity constant greater than $10^{10}M^{-1}$, and shows 50% inhibition at 40fmoles of O^6-ethyl-2/-deoxyguanosine.

It is important to understand that the sensitivity associated with both polyclonal antisera and Mabs is dependent on the carrier protein coupled to the hapten. Muller and Rajewsky[112] found that the sensitivity for the O^6-ethyldG hapten varied widely with both the type of carrier protein and the type of immunization procedure. Thus for the O^6-CMdG hapten a change in either the carrier protein or the immunization method may increase the sensitivity of the antiserum. These facts have yet to be investigated. As each antiserum possess a unique mixture of antibodies, it is conceivable that a Mab, (derived for example from a mouse polyclonal antiserum) will give increased sensitivity towards O^6-CMdG at 4°C, depending on the exact nature of the binding site. Thus further work to increase sensitivity should probably concentrate on the development of a Mab.

The present results revealed a large discrepancy for the quantitation of O^6-CMdG obtained by enzyme hydrolysis, acid hydrolysis and direct DNA analysis. It may be beneficial to concentrate further developments on the direct DNA analysis, to circumvent problems arising from incomplete hydrolysis of the DNA.

Shuker and Margison[138] have shown that O^6-CMdG is probably not repaired by the methyl transferase enzyme, which increases the probability that formation of O^6-CMdG in DNA is a pre-mutagenic lesion. Analysis of urine for the presence of O^6-CMdG, would give further evidence towards its method of repair. Urine analysis would involve the ELISA procedure, possibly involving a clean-up procedure using an immuno-affinity column. A positive result would tend to indicate that O^6-CMdG is lost from DNA via an excision repair process.

The present work has shown that it is possible to make specific polyclonal antibodies against O^6-CMdG. A more sensitive ELISA procedure, based on a Mab, could be used, not only to quantitate O^6-CMdG in DNA directly, but also to quantitate excised O^6-CMdG in urine.

Chapter 5.

EXPERIMENTAL.

5.1 SYNTHESIS.

5.1.1 INSTRUMENTATION.

Melting points were measured on a Gallenkamp hot-stage and are uncorrected. Infra-red spectra were recorded on a Perkin-Elmer 298 or 1420 grating spectrophotometer , and calibrated against polystyrene. ^{1}H-Nmr spectra were recorded on either a Joel FX90Q spectrometer or a Joel FX400Q spectrometer in the solvents indicated, using tetramethylsilane (TMS) as the internal standard. Uv and visible spectra were recorded on either a Pye-Unicam SP8-5000 or a Cecil CE599 spectrophotometer in the solvents indicated, calibrated with a Holmium filter. Mass spectra were provided by Dr. J.A.Challis using VG-7070 and VG-20-250 instruments. PH measurements were made on a PT 1-6 Universal pH meter, calibrated against standard buffer solutions. Micro analysis were provided by Medac Ltd. University of Brunel.

5.1.2 REAGENTS AND CHEMICALS.

The nucleic acid bases were supplied by Cruachem Ltd., Aired, Glasgow, (2/-deoxyguanosine, thymidine),

Sigma Chemical Co. Ltd. (2/-deoxyadenosine) and Aldrich Chemical Co. Ltd. (2/-deoxycytosine). The calf thymus DNA was supplied by B.D.H. Ltd. Nuclease P1 and Bacterial Alkaline Phosphatase were supplied by Sigma Chemical Co. Ltd. DNAase1 and Snake Venom Phosphodiesterase were supplied by Boehringer Ltd. All of these compounds were used without further purification. Methylated Bovine Serum Albumin (m-BSA), Ovalbumin and Horseradish Peroxidase-linked Goat Anti-rabbit Immunoglobulin G were all supplied by Boehringer Ltd. The Enzyme Linked Immunosorbent Assay (ELISA) plates were supplied by Dynatech, Plochingen, F.R.G. The proteins, anti-rabbit antiserum and the ELISA plates were all used without prior purification. L-Proline was purchased from the Sigma Chemical Co. Ltd. and used without prior purification. Methylglycolate was purchased from Kodak Ltd. and purified by distillation before use. Quinuclidine was purchased from Aldrich Chemical Co. Ltd., and purified by sublimation prior to use. Solvents and other reagents eg alcohols, were purified by standard techniques prior to use[139].

5.2 SYNTHESIS OF NUCLEOSIDES.

5.2.1 N-METHYLPYRROLIDINE.

N-Methylpyrrolidine (Aldrich 60cm^3) was heated under

reflux over KOH (3.0g) for 12h, then fractionally distilled (bp 80-81°C[140]). After adding phenyl isocyanate (3.0g 25mmol) to the N-methylpyrrolidine (40cm³ 370mmol), the solution was stirred at room temperature for 3h; filtration followed by redistillation, gave N-methylpyrrolidine (bp 80-81°C) containing pyrrolidine (<0.08%) by g.l.c. analysis (see later).

5.2.2 METHOXYACETIC ANHYDRIDE.

Methoxyacetic acid (20g 0.2mol) and acetic anhydride (20g 0.2mol) were heated under reflux for 24h. The reaction mixture was then fractionally distilled through a glass helices column to give methoxyacetic anhydride. Yield 12.3g (67%); bp 73-75°C at 1mm Hg (lit[141] 115-120°C at 15mm Hg); ν_{max} (neat) 2960 (C-H str), 2850 (C-H str), 1850 (C=O str), 1770 (C=O str), 1230 (C-O str) and 1055cm^{-1} (C-O str); δ_H ($CDCl_3$) 5.89 (4H, s, 2xCH_2), 6.65 (6H, s, 2xCH_3).

5.2.3. 3′-5′-DI-O-METHOXYACETYL-2′-DEOXYGUANOSINE (17).

2′-Deoxyguanosine (1.0g 3.60mmol) was suspended in anhydrous DMF (Analar Gold label) (16cm³) containing pyridine (4cm³). To the stirred suspension at room temperature, methoxyacetic anhydride (2.45g 15mmol) was first added, and then the mixture was heated at 40°C for 2h. The solution then became homogeneous, and it was allowed to cool to room temperature. Methanol (0.5cm³) was then added and the solution stirred for a further 30min at room temperature. The solvents were evaporated under reduced pressure and the residue was filtered, then washed with cold ethanol. Recrystallisation from ethanol gave (17) as white crystals. Yield 1.18g (81%); mp 176-177°C (lit[112] 176-178°C); Vmax (nujol) 3450 (N-H str), 3350 (N-H str), 1750 (C=O ester str), 1700 (C=O amide str), 1230cm⁻¹ (C-O str); δ_H (d^6-DMSO) 2.50 (1H, m, 2′-H), 2.93 (1H, m, 2′-H), 3.30 (6H, s, 2xCH_3), 3.92 (4H, 2s, 2xCH_2), 1.10 (2H, s, 5′-H_2), 4.30 (1H, m, 4′-H), 5.38 (1H, m, 3′-H), 6.17 (1H, t, 1′-H), 6.53 (2H, brs, NH_2), 7.95 (1H, s, 8-H) and 10.70 (1H, brs, NH); m/z (FAB positive ion) 412 (MH^+) 20%, 152 (MH^+-260) 100%.

5.2.4 O^6-(2,4,6-TRIMETHYLBENZENE SULPHONYL)-3′-5′-DI-O-METHOXYACETYL-2′-DEOXYGUANOSINE (18).

3′-5′-Di-O-methoxyacetyl-2′-deoxyguanosine (1.0g 2.4mmol), 2-mesitylsulphonyl chloride (1.2g 5.5mmol) and 4-dimethylaminopyridine (150mg 1.2mmol) were suspended in CH_3CN (10cm³). Triethylamine (1.15g 11.4mmol) was added dropwise, and the suspension stirred room temperature. After 2h, this solution was concentrated under reduced pressure, the residue redissolved in $CHCl_3/CH_3OH$ (90:10v/v) (10cm³) and then column chromatograph on silica (60g) using $CHCl_3$ (70cm³), then $CHCl_3/CH_3OH$ (97:3v/v) as eluents, to give the product (18) as a brown glass. This glass was dissolved in the minimum volume of CH_2Cl_2 and precipitated with petroleum ether (40-60°C) to give (18) as pale yellow crystals. Yield 1.35g (95%); mp 140-112°C (lit[142] 142-143°C); ν max (nujol) 3450 (N-H str), 3350 (N-H str), 1750 (C=O ester str), 1350 (SO_2-O str), 1230 (C-O str) and 1160cm^{-1} (SO_2-O str); δH ($CDCl_3$) 2.31 (3H, s, p-CH_3), 2.50 (1H, m, 2′-H), 2.75 (6H, s, 2xo-CH_3), 2.93 (1H, m, 2′-H), 3.35 (6H, 2s, 2xCH_3), 3.92 (4H, 2s, 2xCH_2), 4.10 (2H, s, 5′-H_2), 4.40 (1H, m, 4′-H), 4.95 (2H, brs, NH_2), 5.50 (1H, m, 3′-H), 6.30 (1H, t, 1′-H), 6.95 (2H, brs, arom) and 7.90 (1H, s, 8-H); m/z (EI) 593 (M⁺) 12%, 333 (M⁺-260) 30%.

5.2.5 2/-3/-5/-TRI-O-METHOXYACETYLGUANOSINE (30).

Guanosine (1.0g 3.54mmol) was suspended in dry DMF (16cm^3) containing pyridine (6cm^3) and the suspension stirred at room temperature for 10 min. Methoxyacetic anhydride (3.92g 24mmol) was added to the suspension which was then heated at 40°C for 3h. After this time, the mixture became homogeneous, and it was allowed to cool to room temperature. CH_3OH (1.0cm^3) was added, and the solution stirred for a further 45 min at room temperature. The solvents were then evaporated under reduced pressure, the residue filtered, washed with ice cold C_2H_5OH, and then recrystallized from C_2H_5OH to give (30) as white crystals. Yield 1.50g (85%); mp 219-220°C (lit.[12] 219-220°C); ν_{max} (nujol) 3450 (N-H str), 3350 (N-H str), 1750 (C=O ester str), 1700 (C=O amide str) and 1230 (C-O str); δ_H (d^6 DMSO) 3.30 (9H, 3s, 3xCH_3), 3.95 (2H, s, CH_2), 1.05 (4H, 2s, 2xCH_2), 4.40 (2H, brs, 5/-H_2), 5.60 (2H, m, 2/-H 3/-H), 5.90 (1H, m, 2/-H), 6.10 (1H, d, 1/-H), 6.55 (2H, brs, NH_2), 7.90 (1H, s, 8-H) and 10.70 (1H, brs, NH); m/z (FAB positive ion) 500 (MH^+) 18%, 152 (NH^+-348) 100 %.

5.2.6 2/-3/-5/-TRI-O-ACETYLGUANOSINE (7).

Guanosine (2.0g 7.1mmol) was suspended in acetic

anhydride (25cm³) containing pyridine (4cm³) and 4-dimethylaminopyridine (100mg 1mmol). The suspension was heated at 45°C for 2h to affect homogeneity and it was then allowed to cool to room temperature. CH_3OH (1.0cm³) was added, and the solution stirred for a further 30 min at room temperature. The solvent was removed under reduced pressure to give a solid residue which was washed with diethyl ether (20cm³), CH_2Cl_2 (20cm³), then recrystallized from C_2H_5OH to give (7) as white crystals. Yield 2.96g (98%); mp 224-225°C (lit[143] 225-226°C); Vmax (nujol) 3450 (N-H str), 3350 (N-H str), 1730 (C=O ester str), 1700 (C=O amide str) and 1230 (C-O str); δH (d^6 DMSO) 2.03 (3H, s, CH_3CO), 2.07 (3H, s, CH_3CO), 2.08 (3H, s, CH_3CO), 4.35 (2H, brs, 5/-H_2), 5.50 (2H, m, 2/-H 3/-H), 5.80 (1H, m, 2/-H), 6.05 (1H, d, 1/-H), 6.55 (2H, brs, NH_2), 7.96 (1H, s, 8-H) and 10.75 (1H, brs, NH); m/z (FAB positive ion) 410 (MH^+) 22%, 152 (MH^+-258) 100%.

5.2.7 O^6-(2,4,6-TRIMETHYLBENZENESULPHONYL)-2/-3/-5/-TRI-O-ACETYLGUANOSINE (25).

2/-3/-5/-Tri-O-acetylguanosine (1.0g 2.4mmol), 2-mesitylsulphonyl chloride (1.05g 4.8mmol) and 4-dimethylaminopyridine (130mg 1.06mmol) were suspended in

CH_3CN ($12cm^3$). Triethylamine (1.10g 9.6mmol) was added dropwise and the suspension stirred at room temperature. After 2h, the residue was evaporated to dryness under reduced pressure, and the residue redissolved in $CHCl_3/CH_3OH$ (90:10v/v) ($2cm^3$) and was chromatographed on silica (60g) using $CHCl_3$ ($70cm^3$) then $CHCl_3/CH_3OH$ (97:3v/v) as eluents, to give the product (25) as a brown solid. The glassy solid was dissolved in the minimum volume of CH_2Cl_2 and precipitated with petroleum ether (40-60°C) to give (25) as yellow crystals. Yield 1.31g (91%) mp 140-141°C (lit[114] 141-142°C); ν_{max} (nujol) 3450 (N-H str), 3350 (N-H str), 1730 (C=O ester str), 1350 (SO_2-O str), 1230 (C-O str) and 1160 cm^{-1} (SO_2-O str); δ_H ($CDCl_3$) 2.03 (3H, s, CH_3CO), 2.07 (3H, s, CH_3CO), 2.08 (3H, s, CH_3CO), 2.30 (3H, s, p-CH_3), 2.70 (6H, s, o-CH_3), 4.35 (2H, brs, 5′-H_2), 5.60 (2H, m, 2′-H 3′-H), 5.85 (1H, m, 2′-H), 6.00 (1H, brd, 1′-H), 6.50 (2H, brs, NH_2), 6.9 (2H, s, arom) and 7.8 (1H, s, 8-H); m/z (FAB positive ion) 592 (MH^+) 18%, 334 (MH^+-258) 50%.

5.2.8 O^6-(2,4,6-TRIMETHYLBENZENESULPHONYL)-2′-3′-5′-TRI-O-METHOXYACETYLGUANOSINE (31).

2′-3′-5-Tri-O-methoxyacetylguanosine (1.0g 2.0mmol), 2-mesitylsulphonyl chloride (1.0g 4mmol) and 4-

dimethylaminopyridine (130mg 1.06mmol) were suspended in dry CH_3CN (11cm³). Triethylamine (1.0g 9.0mmol) was added dropwise and the suspension stirred at room temperature. After 2h, the mixture was evaporated to dryness under reduced pressure and the residue redissolved in $CHCl_3/CH_3OH$ (90:10v/v) (2cm³) and was chromatographed on silica (70g) using $CHCl_3$ (90cm³) then $CHCl_3/CH_3OH$ as eluents. This gave (31) as a brown glassy solid after removal of the solvents under reduced pressure. The glassy solid was dissolved in the minimum volume of CH_2Cl_2 and precipated with petroleum ether (40-60°C) to give (31) as pale yellow crystals. Yield 0.24g; mp 139-140°C; Vmax (nujol) 3450 (N-H str), 3350 (N-H str), 1742 (C=O ester str), 1350 (SO_2-O str), 1230 (C-O str) and 1166cm⁻¹ (SO_2-O str); δH ($CDCl_3$) 2.30 (3H, s, p-CH_3), 2.70 (6H, s, 2xo-CH_3), 3.30 (9H, s, 3xCH_3), 4.10 (6H, 3s, 3xCH_2), 4.40 (2H, brs, 5′-H_2), 5.50 (2H, m, 3′-H 4′-H), 5.85 (1H, m, 2′-H), 6.0 (1H, brd, 1′-H), 6.50 (2H, brs, NH_2), 7.0 (2H, s, arom), 7.9 (1H, s, 8-H); m/z (FAB positive ion) 682 (MH^+) 12%, 334 (MH^+-348) 18%, 152 (MH^+-348-182) 100%.

During the separation of the reaction material, a significant amount of by-product (32) was isolated from the column as a viscous oil. Yield (50-80%); Vmax (nujol) 3450-3100 (N-H and O-H str), 1742 (C=O ester str), 1350 (SO_2-O str), 1230 (C-O str) and 1100cm⁻¹ (SO_2-O str); dH ($CDCl_3$) 2.30 (3H, s, p-CH_3), 2.70 (6H, s, 2xo-

CH_3), 3.30 (6H, s, 3xCH_3), 4.10 (4H, s, 2xCH_2), 4.40 (3H, brs, 5/-H_2 2/-H), 5.45 (3H, m, 3/-H 4/-H 2/-OH), 5.95 (1H, brd, 1/-H), 6.50 (2H, brs, NH_2), 7.0 (2H, s, arom) and 7.9 (1H, s, 8-H); m/z (FAB positive ion) 610 (MH^+) 10%, 334 (MH^+-276) 22%, 152 (MH^+-276-182) 100 %.

5.2.9 O^6-METHYL-2/-DEOXYGUANOSINE.

O^6-(2,4,6-trimethylbenzenesulphonyl)-3/-5/-Di-O-methoxyacetyl-2/-deoxyguanosine (18) (0.3g 0.5mmol) was dissolved in dry CH_2Cl_2 (5cm^3) and purified N-methylpyrrolidine (0.8cm^3 10mmol) was added. The solution was then stirred at room temperature under dry nitrogen. The progress of the reaction was monitored by the loss of (18) and formation of a blue fluorescent baseline spot, (believed to be the N-methylpyrrolidinium ion intermediate), using silica t.l.c. developed with EtOAc/CH_3OH (85:15v/v).

After 2h, when no further changes were evident by t.l.c., dry CH_3OH (0.32g 10mmol) in CH_2Cl_2 (3cm^3) and 1,8-diazabicyclo[5,4,0] undec-7-ene (DBU) in CH_2Cl_2 (3cm^3) was added to the reaction solution, which was then heated under reflux for 3h. After cooling to room temperature aqueous 2.0M NaOH (1cm^3) was added to the

reaction mixture. After stirring for 10min, the mixture was neutralised with NH_4Cl, and the products extracted with EtOAc/CH_3OH (99:1v/v) (2x40cm^3). The combined organic layers were dried (Na_2SO_4) and evaporated under reduced pressure to an oily residue. The residue was column chromatographed on silica using EtOAc/CH_3OH (85:15v/v) as eluent, to give O^6-methyl-2′-deoxyguanosine (6) as a white solid. Recrystallisation from EtOAc gave (6) as a white crystalline solid. Yield 0.08g (60%); mp 121-122°C (lit[83] 122-123°C); λmax (pH=7) 249, 280nm (e=8390 8302); νmax (KBr) 3300 (N-H O-H, br, str), 2850 (C-H str), 1650 (C=N str), 1580 (C=C str) and 1250cm^{-1} (C-O str); δH (d^6-DMSO) 2.30 (2H, m, 2′-H), 3.55 (2H, m, 5′-H_2), 3.80 (1H, m, 4′-H), 3.95 (3H, s, CH_3), 4.35 (1H, m, 3′-H), 5.0 (1H, t, 5′-OH), 5.30 (1H, d, 3′-OH), 6.22 (1H, t, 1′-H), 6.45 (2H, brs, NH_2) and 8.08 (1H, s, 8-H); m/z (FAB positive ion) 282 (MH^+)12%, 166 (MH^+-116) 35%.

5.2.10 N^6-PYRROLIDYL-2′-DEOXYGUANOSINE.

A significant by-product in the attempted formation of O^6-carboxymethyl methyl ester-2′-deoxyguanosine, using purified N-methylpyrrolidine as the activating amine, was the isolation of N^6-pyrrolidyl-2′-deoxyguanosine. O^6-(2,4,6-trimethylbenzenesulphonyl)-3′-5′-di-O-methoxyacetyl-2′-deoxyguanosine (0.3g 0.5mmol) was dissolved in dry CH_2Cl_2 (5cm^3) and purified N-

methylpyrrolidine added (0.8cm³ 10mmol), and the solution stirred at room temperature under dry nitrogen for 2h, monitoring the loss of (18) by t.l.c. on silica developed in EtOAc/CH_3OH (85:15v/v).

Dry methylglycolate (0.45g 5mmol) in CH_2Cl_2 (3cm³) and DBU (0.24g 1.5mmol) in CH_2Cl_2 (3cm³) was added to the reaction solution, and the stirring solution heated under reflux for 4h. After cooling to room temperature, the solution was concentrated under reduced pressure. The residue was then dissolved in CH_3OH (5cm³) and treated with 0.4M methanolic Et_3N (5cm³), and the solution stirred at room temperature for 10min. Removal of the solvents by evaporation under reduced pressure, gave an oil which was extracted with EtOAc/CH_3OH (99:1v/v) (2x40cm³), and the resultant organic layers combined and dried over Na_2SO_4. The residue was chromatographed on silica (40g) using EtOAc/CH_3OH (85:15v/v) as eluents to give (22) as a cream coloured powder. Recrystallisation from EtOAc gave (22) as a crystalline solid. Yield 0.09g (54%); mp 105-106°C; νmax (KBr) 3400-3100 (N-H O-H, br, str), 2800 (C-H str), 1650 (C=N str) and 1580cm^{-1} (C=C str); δH (d^6-DMSO) 1.70 (4H, m, 2xCH_2), 2.10 (4H, m, 2xCH_2), 2.30 (2H, m, 2′-H), 3.55 (2H, m, 5′-H_2), 3.80 (1H, m, 4′-H), 5.0 (1H, t, 5′-OH), 5.30 (1H, d, 3′-OH), 6.30 (1H, t, 1′-H), 6.45 (2H, brs, NH_2) and 8.08 (1H, s, 8-H); m/z (FAB positive ion) 321 (MH^+) 24%, 205 (MH^+-116) 45%.

5.2.11 GENERAL PROCEDURE FOR THE SYNTHESIS OF O^6-SUBSTITUTED-2/-DEOXYGUANOSINES.

O^6-(2,4,6-Trimethylbenzenesulphonyl)-3/-5/-di-O-methoxyacetyl-2/-deoxyguanosine (18) (0.3g 0.5mmol) was dissolved in dry THF (4cm^3) and quinuclidine (0.32g 2.5mmol) was added and the solution stirred at room temperature under dry nitrogen. Progress of the reaction was monitored by loss of (18) and the formation of a blue fluorescent spot at the baseline, (believed to be the quinuclidinium ion intermediate (19)), using silica t.l.c. developed with EtOAc/CH_3OH (85:15v/v). The formation of (19) was usually complete in ca 3h at room temperature (Figure 2.2).

The appropriate dry alcohol (10-30mmol) and DBU (1.5-2.5) were added to the reaction mixture, which was either stirred at room temperature, or heated under reflux depending on the structure of the alcohol, until the blue fluorescent spot, visible at the baseline on silica t.l.c. developed with EtOAc/CH_3OH (85:15v/v), had disappeared. This required from 30min to 24h, with longer reaction times being required for more sterically hindered alcohols. After cooling to room temperature, the solvent was removed under reduced pressure, and the

resultant residue dissolved in CH_3OH ($5cm^3$) and treated with 0.4M methanolic Et_3N ($6cm^3$) and then stirred at room temperature for 10min. Evaporation of the organic solvents under reduced pressure gave a residual oil, which was then column chromatographed on silica (40g) using EtOAc/CH_3OH as eluent. The appropriate product containing fractions identified by t.l.c. on silica were combined, dried using Na_2SO_4, filtered and evaporated under reduced pressure to give white solids. Purification either by preparative H.P.L.C., or by recrystalisation from EtOAc gave the O^6-substituted-2/-deoxyguanosines as white crystalline solids. The reagent concentrations for each O^6-substituted compound, reaction conditions and physical properties are summarised in detail below.

5.2.11.1 O^6-METHYL-2/-DEOXYGUANOSINE (O^6-MedG) (6).

Dry CH_3OH (0.16g 5mmol) in THF ($3cm^3$) and DBU (0.24 1.5mmol) in THF ($3cm^3$) were added to (19) and the solution stirred at room temperature for 30min. After deprotection using methanolic Et_3N and purification described above, O^6-MedG was recrystallized from EtOAc and isolated as a white crystalline solid. Yield 0.09g (65%); mp 122-123°C (lit.[93] 121-123°C); λmax (pH=7) 249, 280nm (e=8550 8410); νmax (KBr) 3300 (N-H O-H, br, str), 2850 (C-H str), 1650 (C=N str) and 1580cm^{-1} (C=C str); δH (d^6-DMSO) 2.30 (2H, m,2/-H), 3.55 (2H, m, 5/-H), 3.80

(1H, m, 4/-H), 3.95 (3H, s, CH_3), 4.35 (1H, m, 3/-H), 5.0 (1H, t, 5/-OH), 5.30 (1H, d, 3/-OH), 6.22 (1H, t, 1/-H), 6.45 (2H, brs, NH_2) and 8.08 (1H, s, 8-H); m/z (FAB positive ion) 282 (MH^+) 10%, 166 (MH^+-116) 32%.

5.2.11.2 O^6-ISOPROPYL-2/-DEOXYGUANOSINE (O^6-Pr^idG) (6a).

Dry isopropanol (1.20g 20mmol) in THF (3cm^3) and DBU (0.4g 2.5mmol) in THF (3cm^3) were added to (19) and the solution heated under reflux for ca 24h. After deprotection by methanolic Et_3N and purification as described earlier, O^6-Pr^idG was recrystalised from EtOAc and isolated as a white crystalline solid. Yield 0.03g (20%); mp 104-106°C (lit[145] 105-107°C); λmax (pH=7) 249, 281nm (e=8760, 9160); νmax (KBr) 3300 (N-H O-H, br, str), 2850 (C-H str), 1650 (C=N str), 1580 (C=C str) and 1250cm^{-1} (C-O str); δH (d^6-DMSO) 1.34 (6H, d, 2xCH_3), 2.25 (2H, m, 2/-H_2), 3.55 (2H, m, 5/-H_2), 3.80 (1H, m, 4/-H), 5.00 (1H, t, 5/-OH), 5.30 (1H, m, 3/-OH), 5.40 (1H, m, CH), 6.25 (1H, m, 1/-H), 6.87 (2H, brs, NH_2) and 8.07 (1H, s, 8-H); m/z (FAB positive ion) 310 (MH^+) 7%, 194 (MH^+-116) 12%.

5.2.11.3 O^6-TERT-BUTYL-2/-DEOXYGUANOSINE (O^6-Bu^tdG).

Dry t-butanol (2.25g 30mmol) in THF (3cm^3) and DBU

(0.64g 4.0mmol) were added to (19) and the solution heated under reflux for 72h. No observable decomposition of the blue fluorescent quaternary quinuclidinium ion intermediate was apparent during this time. CH_3OH (0.32g 10mmol) in THF (3cm^3) was added to the cooled reaction and the solution stirred at room temperature for 30min. After deprotection with methanolic Et_3N, and purification by column chromatography and recrystalisation, O^6-MedG was isolated as a white crystalline solid. Yield 0.08g (60%); mp 122-123°C (lit[83] 121-123°C).

5.2.11.4 O^6-CARBOXYMETHYL METHYL ESTER-2/-DEOXYGUANOSINE.

Dry methylglycolate (0.44g, 5mmol) in THF (3cm^3) and DBU (0.24g, 1.5mmol) in THF (3cm^3) were added to (19) and the solution stirred at room temperature for 3-4h. After deprotection using methanolic Et_3N and purification by column chromatography on silica (30g) using CH_3OH/EtOAc (15:85v/v) as eluent, (23) was further purified by semi-preparative H.P.L.C. on a Phase Sep S10 ODS2 C-18 silica column, using CH_3CN/H_2O (13/87v/v) isocratic eluent system. A detailed description of the conditions used for this procedure are given in Section 5.7.1. After removal of the eluent by lyophilization, (23) was

obtained as white crystaline solid. Yield 0.11g (68%); mp 83-84°C. λmax (pH=7) 248, 281 (10040, 9890); νmax (KBr) 3300 (N-H, O-H str), 2800 (C-H str), 1752 (C=O str), 1650 (C=N str), 1580 (C=C str), 1250 (C-O str) and 1065cm^{-1} (C-O str). dH (d^6-DMSO) 2.30 (2H, m, 2′-H), 3.55 (2H, m, 5′-H_2), 3.61 (3H, s, OCH_3), 3.80 (1H, m, 4′-H), 4.35 (1H, m, 3′-H), 4.81 (2H, s, $CH_2CO_2CH_3$), 5.0 (1H, t, 5′-OH), 5.30 (1H, d, 3′-OH), 6.22 (1H, t, 1′-H), 6.45 (2H, brs, NH_2) and 8.10 (1H, s, 8-H); m/z (FAB positive ion) 340 (MH^+) 10%, 224 (MH^+-116) 20% and 152 (MH^+-alkyl-116) 32%.

5.2.11.5 O^6-CARBOXYMETHYL METHYL ESTER-GUANOSINE (28).

O^6-(2,4,6-Trimethylbenzenesulphonyl)-2′-3′-5′-Tri-O-acetylguanosine (25) (0.3g, 0.5mmol) was dissolved in dry THF (4cm^3) and quinuclidine was added, and the solution was stirred at room temperature under dry N_2. Progress of the reaction was monitored by loss of (25) and the fomation of a blue fluorescent spot at the baseline, (believed to be the quinuclidine salt (26)), using silica t.l.c. developed with EtOAc/CH_3OH (85:15v/v). The formation of the quinuclidine ion intermediate (26) was

usually complete in ca 3-4h at room temperature (Figure 2.10).

Dry methylglycolate (0.44g, 5mmol) in THF (3cm^3) and DBU (0.24g, 1.5mmol) in THF (3cm^3) were added to (26) and the solution stirred at room temperature until the blue fluorescent spot, visible at the baseline on silica t.l.c. using EtOAc/CH_3OH (85:15v/v) had disappeared. After 4h, the solution was cooled to room temperature, the solvent removed under reduced pressure, and the resultant residue dissolved in CH_3OH (5cm^3) and treated with 0.4M methanolic triethylamine (9cm^3) and then stirred at room temperature for 12min. Evaporation of the solvent under reduced pressure gave a residual oil, which was column chromatographed on silica (40g) using EtOAc/CH_3OH (85/15v/v) as eluent. The appropriate product containing fractions identified by t.l.c. on silica were combined, dried using Na_2SO_4, filtered and evaporated under reduced pressure to give (28) as a white solid. (28) was further purified by semi-preparative H.P.L.C. on a Phase Sep C-18 silica column using CH_3CN/H_2O (12:88v/v) as eluent at 20cm^3/min at room temperature. A more detailed description of the H.P.L.C. conditions employed for the purification are given in Section 5.7. After removal of the solvent by lyophilization, (23) was isolated as a white crystaline solid. Yield 0.11g (62%); mp 123-124°C. λmax (pH=7) 248, 281 (10300, 9730); νmax (KBr) 3300 (N-H, O-H str),

2860 (C-H str), 1752 (C=O str), 1580 (C=C str), 1250 (C-O str) and 1065cm^{-1} (C-O str); dH (d^6-DMSO) 3.58 (2H, m, 5/-H), 3.61 (3H, s, OCH_3), 3.88 (1H, m, 4/-H), 4.10 (1H, m, 3/-H), 4.46 (1H, m, 2/-H), 4.87 (2H, s, $CH_2CO_2CH_3$), 5.14 (2H, m, 5/-OH, 2/-OH), 5.40 (1H, d, 3/-OH), 5.80 (1H, d, 1/-H), 6.52 (2H, brs, NH_2) 8.11 (1H, s, 8-H); m/z (FAB positive ion), 356 (MH^+)12%, 224 (MH^+-132)30%.

5.2.11.6 O^6-CARBOXYMETHYL-2/-DEOXYGUANOSINE (24).

O^6-Carboxymethyl methyl ester-2/-deoxyguanosine (23) (0.042g, 0.12mmol) was dissolved in aqueous 4mM $Ca(OH)_2$ (30cm^3) and the solution stirred at room temperature. Loss of (23) was monitored by t.l.c. on silica using EtOAc/CH_3OH (85:15v/v) as eluent. After 18h, lyophilization of the reaction solution gave (24) as a white fluffy solid. The solid was dissolved in C_2H_5OH, and the insoluble residue removed. The solvent was removed under reduced pressure, to give (24) as a white solid. Yield (0.040g) (98%); mp >250°C. Found C 39.60, H 4.44, N 19.30 and O 30.75. (Calc C 41.73, H 4.05, N 20.28 and O 27.82). λmax (pH=7) 248, 282 (9980, 9760); νmax (KBr) 3300 (N-H, O-H br str), 2860 (C-H str), 1650 (C=N str), 1620 (C=O str), 1580 (C=C str), 1250 (C-O str) and 1065cm^{-1} (C-O str); dH (d^6-DMSO) 2.30 (2H, m, 2/-H), 3.55 (2H, m, 5/-H), 3.80 (1H, m, 4/-H), 4.35 (1H, m, 3/-

H), 4.64 (2H, s, CH_2), 5.0 (1H, t, 5/-OH), 5.30 (1H, d, 3/-OH), 6.22 (1H, t, 1/-H), 6.45 (2H, brs, NH_2) and 8.09 (1H, s, 8-H); m/z (FAB negative ion) 324 (M^-) 8%, 151 (M--alkyl-116) 20%.

5.2.11.7 O^6-CARBOXYMETHYLGUANOSINE (29).

O^6-Carboxymethyl methyl ester guanosine (28) (0.042g 0.11mmol) was dissolved in 4mM aqueous $Ca(OH)_2$ solution (30cm^3) and the solution stirred at room temperature. Loss of (28) was monitored by t.l.c. on silica using EtOAc/CH_3OH (85:15v/v) as eluent. After 18h, lyophilization of the reaction solution gave (29) as a white fluffy solid. The solid was dissolved in C_2H_5OH, and the insoluble residue removed. The solvent was removed under reduced pressure to give (29) as a white solid. Yield 0.04g (97%) mp >250°C. Found C 38.90, H 4.35, N 18.90 and O 34.45. (Calc C 40.29, H 3.97, N 19.83 and O 31.72). λmax (pH 7) 248, 282nm, (10020, 9880); νmax (KBr) 3300 (O-H, N-H str), 2880 (C-H str), 1650 (C=N str), 1620 (C=O str), 1580 (C=C str), 1250 (C-O str) and 1065cm^{-1} (C-O str); dH (d^6-DMSO) 400MHz, 3.58 (2H, m, 5/-H_2), 3.88 (1H, m, 4/-H), 4.10 (1H, m, 3/-H), 4.46 (1H, m, 2/-H), 4.62 (2H, s, CH_2), 5.14 (2H, m, 5/-

OH, 2-OH), 5.40 (1H, m, 3/-OH), 5.82 (1H, d, 1/-H), 6.52 (2h, brs, NH_2), 8.09 (1H, s, 8-H); m/z (FAB negative ion) 340 (M^-) 7%, 151 (M^--alkyl-116) 20%.

5.3 SYNTHESIS OF N-NITROSODIPEPTIDES.

5.3.1 N-ACETYL-L-PROLINE (34).

L-Proline (10g 87.5mmol) was dissolved in 2M NaOH ($40cm^3$), the resultant solution cooled on ice and stirred. $(CH_3CO)_2O$ was then added dropwise to the stirring solution over a period of 20min. The temperature of the reaction solution was maintained at 0-5°C during the addition and for 30min afterwards, the solution was then stirred at room temperature for a further 18h. 2M H_2SO_4 (ca $25cm^3$) was then added to the solution, pH=3.0, and the resultant mixture extracted with $CHCl_3$ ($3x200cm^3$). The organic layer was dried over Na_2SO_4, and the solvent removed under reduced pressure. The resultant powder was recrystalised from EtOAc, to give N-acetyl-L-proline as a white crystaline solid. Yield 9.9g (72%); mp 118-119°C (lit[145] 118-119°C); Vmax (nujol), 3346 (O-H str), 1742 (C=O acid str) and 1625cm^{-1}

(C=O amide str); dH ($CDCl_3$) 2.00 (4H, m, 3-H_2 4-H_2), 2.10 (3H, s, CH_3), 3.45 (2H, m, 5-H_2), 4.40 (1H, m, 2-H) and 9.00 (1H, s, O-H).

5.3.2 N-ACETYL-L-PROLYLGLYCINE BENZYL ESTER (35).

N-Acetyl-L-proline (0.78g 5mmol) was dissolved in THF (15cm^3) containing Et_3N (0.51g 5mmol). The resulting mixture was cooled to -10°C in an ice salt bath. Ethylchloroformate (463mg 4.5mmol) in THF (5cm^3) was added dropwise to the solution over a period of 5min. The reaction mixture (a white slurry) was stirred at -10°C for 30min. Glycine benzyl ester (1.7g 5mmol) in THF (10cm^3) was then added to the solution which was maintained at a temperature of -7°C to -10°C for 30min. The mixture was then allowed to warm to an ambient temperature. The solvent was removed under reduced pressure, the residue dissolved in CH_2Cl_2 (30cm^3), and the resultant solution was then washed with 0.01M aqueous HCl (2x20cm^3), saturated aqueous $NaHCO_3$ (2x20cm^3), H_2O (2x20cm^3) and saturated aqueous NaCl (2x20cm^3). After separation, the organic fraction was dried over Na_2SO_4. Filtration and solvent evaporation gave (35) as a pale yellow oil. Further purification by column chromatography on silica (100g) using EtOAc/CH_3OH

(99:1v/v) as eluent gave (35) as a colourless oil. Yield 1.18g (81%) (lit[146] 78%); Vmax (thin film) 3305 (N-H str), 1750 (C=O ester str), 1690 (C=O amide str) and 1620cm^{-1} (C=O amide str); dH ($CDCl_3$) 2.00 (4H, m, 3-H_2 4-H_2), 2.10 (3H, s, CH_3), 3.45 (2H, m, 5-H_2), 4.05 (2H, s, $CH_2CO_2CH_2Ph$), 4.60 (1H, m, 2-H), 5.10 (2H, s, CH_2Ph), 7.30 (5H, brs, arom) and 7.60 (1H, brs, NH).

5.3.3 N-(N/-ACETYL-L-PROLYL)-N-NITROSOGLYCINE BENZYL ESTER (36).

N-Acetyl-L-prolylglycine benzyl ester (1.52g 5mmol) was dissolved in CH_2Cl_2 (30cm^3) at -10°C containing anhydrous NaOAc (1.64g 20mmol). Liquid N_2O_4 (340ul 5.5mmol) was added slowly to the mixture over a period of 1min and stirring continued at -10°C for 30min. H_2O (50cm^3) was added, the organic phase separated, then washed with 5% aqueous $NaHCO_3$ (4x20cm^3), H_2O (2x20cm^3) and saturated NaCl (20cm^3) and dried using Na_2SO_4. Filtration and removal of the solvent under reduced pressure gave (36) as a yellow/orange oil. Yield 1.55g (94%) (lit[90%][146]; Vmax (film) 1750 (C=O ester str), 1740 (C=O nitrosamide str), 1660 (C=O amide str) and 1510cm^{-1} (N=O str); dH 2.0 (4H, m, 3-H_2 4-H_2), 2.10 (3H, s, CH_3),

s, CH_3), 3.6 (2H, m, 5-H_2), 4.65 (2H, s, $\underline{CH_2}CO_2CH_2Ph$), 5.10 (2H, s, $\underline{CH_2}Ph$), 5.7 (1H, m, 2-H) and 7.30 (5H, brs, arom).

5.3.4 N-(N/-ACETYL-L-PROLYL)-N-NITROSOGLYCINE (37).

N-(N/-Acetyl-L-prolyl)-N-nitrosoglycine benzyl ester (600mg 1.8mmol) was dissolved in EtOAc/C_2H_5OH (95:5v/v) (20cm^3) and Pd/C 10% (60mg 0.1 equivalents) added. The solution was stirred at room temperature under a hydrogen atmosphere at atmospheric pressure and the hydrogen uptake monitored. On completion of the uptake of hydrogen (43.2cm^3), the catalyst was removed by filtration and the solvent under reduced pressure. The resulting yellow solid was recrystalised from EtOAc (45-50°C) to give (37) (APNG) as a crystaline yellow solid. Yield 302mg (68%); mp 110-111°C (lit[146] 109-110°C); Vmax (nujol) 3200-2700 (O-H acid str), 1740 (C=O nitrosamide str), 1720 (C=O acid str), 1640 (C=O amide str) and 1510cm^{-1} (N=O str); dH ($CDCl_3$) 2.0 (4H, m, 3-H_2 4-H_2), 2.10 (3H, s, CH_3), 3.6 (2H, m, 5-H_2), 4.60 (2H, brs, $\underline{CH_2}CO_2H$), 5.7 (1H, m, 2-H) and 9.0 (1H, brs , OH). m/z (FAB positive ion) 244 (MH^+) 7%, 215 (MH^+-NO) 18%, 140 (MH^+-N(NO)CH_2CO_2H) 54%, 112 (MH^+-CON(NO)CH_2CO_2H) 65%, 70 (MH^+-Ac-CON(NO)CH_2CO_2H) 90%.

5.4 ALKYLATION OF 2/-DEOXYGUANOSINE. GENERAL PROCEDURE.

5.4.1 PREPARATION OF STOCK 2/-DEOXYGUANOSINE SOLUTION.

2/-Deoxyguanosine (200mg 0.75mmol) was suspended in aqueous 10mM 2-amino(2-hydroxymethyl)propane1,3,diol (Tris) (20cm^3) buffer at pH=7.2 and heated at 50°C to obtain a homogeneous solution. The solution with gentle agitation was then incubated at 37°C for 24h. The concentration of 2/-deoxyguanosine in this solution was ca 37.5mM.

5.4.2 ALKYLATION AND ANALYSIS.

To the 37.5mM aqueous 2/-deoxyguanosine solution in Tris buffer (1.0cm^3) was added N-(N/-acetyl-L-prolyl)-N-nitrosoglycine (APNG) (0.1-10^{-3}g 0.4-0.004mmol) in DMSO (10-100ul). The reaction mixture was monitored by disappearance of the APNG absorbance at λmax=410nm, and generally incubation of the reaction solution at 37°C was

continued for a further 24h after the absorbance was undetectable to ensure complete reaction. The reaction solution was then analysed for O^6-carboxymethyl-2/-deoxyguanosine (O^6-CMdG) and N^7-carboxymethylguanine (N^7-CMG) by H.P.L.C. without additional work-up. Details of the conditions used for the H.P.L.C. analysis are given in Section 5.7. Details of a typical reaction are given below.

5.4.3 REACTION OF APNG WITH 2/-DG (1:1w/w).

To the stock dG solution (1.0cm^3) was added APNG ($1x10^{-2}$g 0.04mmol) in DMSO (10ul). The resultant solution was thoroughly mixed and incubated at 37°C for 72h. Aliquots of the reaction solution (10ul) were then analysed for O^6-CMdG and N^7-CMG by H.P.L.C.

5.5 ALKYLATION OF DNA. GENERAL PROCEDURE.

5.5.1 PREPARATION OF THE STOCK DNA SOLUTION.

Calf thymus DNA (150mg) was suspended in 10mM Tris buffer (15cm^3) at pH=7.2, and after thorough mixing

incubated at 37°C for 48h with gentle agitation. The resulting solution was homogeneous.

5.5.2 ALKYLATION AND ANALYSIS.

To the stock DNA solution (1.0cm^3) wasadded APNG (0.1-1x10^{-3}g 0.4-4x10^{-3}mmol) in DMSO (10-100ul). The solution was incubated at 37°C for 48-72h, monitoring the loss of the N-nitrosodipeptide as before by monitoring the absorbance at λmax=410nm. The DNA was then precipated from the solution as a pellet as described below. The resultant pellet was then hydrolysed to either the nucleoside or purine level, and analysed for the presence of carboxymethylated components. The acid hydrolysis procedure is described in detail in Section 5.5.4. and the enzyme hydrolysis procedures are described in Section 5.6.5. H.P.L.C. procedures for the analysis of nucleosides and purines are given in Section 5.7.

5.5.3 DNA PRECIPATION[125].

To the reaction solution (1.0cm^3) was added ice cold C_2H_5OH (2cm^3) and the mixture was mixed thoroughly (vortexed), cooled at 0°C for 30min revortexed, and then

centrifuged at 30,000r.p.m. for 5min. The supernatant was discarded and the resultant pellet was washed with C_2H_5OH (1.0cm^3), centrifuged, and the supernatant C_2H_5OH layer discarded. Finally, the DNA pellet was washed with $(C_2H_5)_2O$ and dried under vacuum.

5.5.4 GENERAL PROCEDURE FOR THE ACID HYDROLYSIS OF DNA.

The dry DNA pellet (1.0-5.0mg) was suspended in 20% aqueous HFBA (100-500ul) and heated at 70°C for 30min to produce a homogeneous solution. The solution was cooled and retained for H.P.L.C. analysis of constituent and modified nucleic acid bases (see Section 5.7.2).

5.6 IMMUNOCHEMICAL PROCEDURES.

5.6.1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) METHODS.

5.6.1.1 PLATE PREPARATION.

A stock solution was prepared by dissolving O^6-carboxymethylguanosine-ovalbumin (O^6-CMGuo-ovalb) (see Section 5.8 for synthesis) 1.8mg in phosphate buffered saline solution (PBS) (5.92cm^3) pH=7.2, containing laurylsulphate (0.1%w/w) and sodium azide (0.62%w/w). PBS (5dm^3) contains NaCl (40g 0.68mol), KCl (1.0g 13.4mmol), KH_2PO_4 (1.0g 7.35mmol) and $Na_2HPO_4.2H_2O$ (5.75g 32.3mmol). The resulting stock solution contained 250ug/cm^3.

An aliquot of the stock solution (20ul) was dissolved in PBS (40cm^3), to give a solution containing O^6-CMGuo-ovalb 5ng/40ul. To each well of the 96 well polystyrene microtitre plate, an aliquot (40ul) of the O^6-CMGuo-ovalb (5ng/40ul) was added, and the plates dried by evaporation at 37°C. The resultant plates contained O^6-CMGuo-ovalb (5ng) per well.

5.6.1.2 ROOM TEMPERATURE ELISA PROCEDURE.

The above plate coated with O^6-CMGuo-ovalb was

washed with PBS Tween ($6x50cm^3$) and then thoroughly dried. PBS Tween ($5dm^3$) contains PBS and Tween ($2.5cm^3$). Standard solutions of O^6-CMdG were prepared in PBS so that the concentration of O^6-CMdG varied between 0.1fmol to 10^7fmol/25ul. An aliquot of O^6-CMdG in PBS (25ul) was pipetted on to the plate a row at a time for each of the standard solutions. Usually two rows on the ELISA plate were used for controls. To each of these rows was added PBS (25ul per well) and to the reference row was added PBS (50ul per well). Polyclonal rabbit antiserum (CMGuo1) (1 in $2x10^5$ dilution in PBS of the neat antiserum) (25ul), prepared from rabbits immunized with O^6-carboxymethylguanosine-methylated Bovine Serum Ovalbumin (O^6-CMGuo-mBSA) (see Section 5.8), was added to each well in the plate except the reference row. The plate was then incubated at room temperature for 90min. The supernatant liquid was then decanted from the ELISA plate, and the plate washed in PBS Tween ($6x50cm^3$). Horse-radish peroxidase-linked goat anti-rabbit immunoglobulin G (Boehringer) (1 in 10^3 dilution in PBS) (50ul) was added to every well on the plate. The plate was re-incubated at room temperature for 90min.

After 90min, the supernatant was discarded, and the plate washed with PBS Tween ($6x50cm^3$) and H_2O ($5ocm^3$). Aqueous citrate buffer pH5.3 (50ul), from a stock

solution of 10cm^3 containing 3/3/5/5/-tetramethylbenzidine (1mg) (1mg/100ul $(CH_3)_2SO$) and H_2O_2 (2ul, 30%w/w), was added to each well of the plate, and the plate incubated for 15min at room temperature to allow colour developement to occur. 1M HCl (50ul) was added to each well, and the optical density at λ=450nm for each well of the plate was measured by an automatic plate reader. Generally, the control wells of the plate gave an optical density of ca 0.7 after calibration with the blank rows (those containing no CMGuo1),and the optical densities of the sample rows after calibration, were usually calculated relative to the control absorbance. Typical results for room temperature ELISA assay are given in Section 4.3.

Generally, for O^6-CMdG concentrations in the range 80 to 10^4 fmoles per well, there was an inverse linear relationship in the observed optical densities at λ=450nm for each of the sample containing wells relative to the control values. Thus, 80fmoles per well corresponded to 85% of the control optical density, and 10^4 fmoles per well ca 25% of the control value. O^6-CMdG (80fmole per well) was the limit of detection for O^6-CMdG under room temperature ELISA conditions using the CMGuo1 antiserum.

5.6.1.3 NON-EQUILIBRIUM ELISA ASSAY PROCEDURE AT 4°C.

The procedure was generally similar to that used for the room temperature ELISA assay (see Section 5.6.1.2), with the initial steps being performed at 4°C instead of at room temperature. Thus, the first washes of the ELISA plate with PBS Tween (6x50cm^3) prior to the addition of any reagents to the plate, was performed at 4°C. The reagents, (O^6-CMdG (0.1-10^7 fmole/25ul PBS) and the CMGuo1 rabbit antiserum) were cooled to 4°C, and then added to the plate at 4°C. Incubation for 15min only, was then performed at 4°C, the supernatant discarded and the plate re-washed with PBS Tween (6x50cm^3) at 4°C. Subsequent transformations were carried out at room temperature as for the room temperature ELISA procedure. Thus, horse-radish peroxidase-linked goat anti-rabbit immunoglobulin G, (1 in 10^3 dilution in PBS) (50ul) was added to each well on the plate and the plate incubated at room temperature for 90min. After 90min, the supernatant was discarded and washed with PBS Tween (6x50cm^3) and H_2O (50cm^3). Aqueous citrate buffer pH 5.3 (50ul), from a solution containing 3/3/5/5/-tetramethyl benzidine (1mg/100ul $(CH_3)_2SO$) and H_2O_2 (2ul, 30%w/w), was added to each well of the plate and incubated at room temperature for 45min to allow colour to develop. 1M HCl (50ul) was added to each well and the optical density at λ=450nm for

each well of the plate monitored for each well. The reagent concentrations and volumes used for this assay were identical to those in the room temperature ELISA assay, and typical results obtained using this procedure are given in Section 4.3.

5.6.2 DNA ENZYME HYDROLYSIS FOR THE ELISA ASSAY.

5.6.2.1 ORDINARY ENZYME PROCEDURE (ORD ENZ)[125].

Calf-thymus DNA, BDH, (50ug) in 10mM Tris buffer (100ul) at pH 7.2 was denatured by heating at 100°C for 5min. The solution was immediately cooled on ice. Aqueous 30mM NaOAc at pH 5.3 (200ul), 20mM $ZnSO_4$ pH 5.3 (10ul), a solution of Nuclease P1 Boehringer, (1mg of enzyme in 30mM NaOAc ($1cm^3$) at pH 5.3) (20ul) and calf intestine alkaline phosphatase, Boehringer (7500 units per mg diluted to 1.87 units per ul in 10mM Tris at pH 7.2) (2ul), were then added to the DNA solution. The resultant mixture was incubated at 37°C for 2h, after which 0.5M Tris buffer (40ul) was added, and the mixture incubated at 37°C for a further 2h. The sample was then stored at 4°C prior to H.P.L.C. analysis (ses Section 5.7).

5.6.2.2 NEW ENZYME METHOD (NEW ENZ).

To a solution of calf-thymus DNA, BDH, (100ug) in 10mM Tris buffer (200ul) at pH 7.2, was added a mixture of 200mM $MgCl_2$ (10ul), DNAase1, (5ug/ul) (1ul), and the solution was incubated at 37°C for 1h. Snake venom phosphodiesterase, Boehringer, (2ug/ul) (1ul), and calf intestine alkaline phosphatase, Boehringer, (12units/ul) (1ul), were then added to the mixture and the solution re-incubated at 37°C for 2h. The sample was then stored at 4°C prior to H.P.L.C. analysis (see Section 5.7).

5.6.2.3 HYDROLYSIS OF DNA FOR COMPARATIVE FLUORESCENCE, H.P.L.C. AND ELISA ANALYSIS.

Samples of DNA (50-100ug) which had been reacted with an N-nitrosopeptide (APNG), were dissolved in 10mM Tris buffer (100-200ul) and then hydrolysed by treatment with enzymes effective for either single-stranded or double-stranded DNA (Ord Enz or New Enz), as described in Sections 5.6.2.1-5.2.6.2. Usually, aliquots of the hydrolysed DNA mixture (20-50ul, 25ug DNA maximum), were analysed for the separate nucleoside components by reverse phase analytical H.P.L.C., on an Altrex

Ultasphere C-18 silica column using an aqueous 0.1M HFBA(w/w)/CH_3OH gradient system and fluorescence detection (see Section 5.7). The relevant nucleoside fraction,(retention time 15-18min, $3cm^3$), that containing the O^6-CMdG, (retention time ca 16.3min), was collected and the solvent aqueous 0.1M HFBA(w/w)/CH_3OH removed under vacuum on a Speed Vac. The residue was dissolved in PBS (200ul) and analysed by the room temperature ELISA assay, as described in Section 5.6.1.2.

Typically, (4x25ul) aliquots of the sample solution were analysed by ELISA for the presence of O^6-CMdG. Comparison between the relative optical density of (to the control) of the unknown sample, and the relative optical densities of known O^6-CMdG concentrations, as described in Section 4.3 and Figure 4.1, enabled the levels of O^6-CMdG in the unknown to be quantitated.

5.7 H.P.L.C. METHODS.

5.7.1 PURIFICATION OF THE SYNTHESIS PRODUCTS.

5.7.1.1 O^6-CARBOXYMETHYL METHYL ESTER-2/-DEOXYGUANOSINE.

This compound was purified by semi-preparative H.P.L.C. procedure using a Phase Sep S10 ODS2 column (25cmx20mm I.D) at 25°C, with an isocratic eluent system CH_3CN/H_2O (13:87v/v) at a flow rate of 20cm³/min. Detection was by uv at λ=254nm.Typically, for 500ul injections, 1.0-2.0mg, the retention time was 12min. The major impurity had a retention time of 4min in this system.

5.7.1.2 O^6-CARBOXYMETHYL METHYL ESTER GUANOSINE.

This compound was purified by semi-preparative H.P.L.C. procedure using a Phase Sep S10 ODS2 column (25cmx20mm I.D.) at 25°C, with an isocratic eluent system CH_3CN/H_2O (12:88v/v) at a flow of 20cm³/min. Detection was by uv at λ=254nm. Typically, for 500ul injections, (1.0-2.0mg), the retention time was 12min. The major impurity 2-mesityl sulphonate had a retention time of 3.2min in this system.

5.7.2 ANALYSIS OF ALKYLATED DNA BASES.

5.7.2.1 NUCLEOSIDES.

5.7.2.2 O^6CARBOXYMETHYL-2/-DEOXYGUANOSINE (O^6-CMdG).

Analytical analysis of this compound in solutions of 2/-deoxyguanosine treated with APNG, was performed on a Spherisorb 5u Hexyl C-6 silica column (25cmx5mm I.D.) at 45°C using an isocratic eluent system. The eluents consisted of 0.02M tetra-n-butylammonium bromide in 0.05M KH_2PO_4 at pH 6.43 (70%), 0.05M KH_2PO_4 at pH 6.43 (28%) and CH_3CN (2%), the flow rate being 1.2cm³/min. Typical retention times for samples (20ul) were 18min. The limit of detection for O^6-CMdG by uv analysis λ=281nm was ca 5.0ng. A typical chromatogram is given in Section 3.4.

An alternative analytical method for O^6-CMdG quantitation was performed on a Apex 2 5u Octyl C-8 silica column (25cmx4.6mm I.D.) at 25°C using an isocratic eluent system. The eluents consisted of 0.05M KH_2PO_4 at pH 4.3 and CH_3CN (99.2:0.8v/v), at a flow rate of 1.2cm³/min. A typical retention time for O^6-CMdG using this system was 17.5min. An example of a standard chromatogram using this system is given in Section 3.4.

The analytical system used in conjunction with the ELISA assay (Section 5.6.1) to quantitate the amount of O^6-CMdG present in APNG treated DNA, was performed on a Altrex Ultrasphere 5u ODS C-18 silica column (12.5cmx4.6mm I.D.) at 25°C, using a 0.1M HFBA/CH_3OH gradient eluent system at a flow rate of 1.0cm^3/min. The gradient used was 3% CH_3OH for 5min, rising to 80% CH_3OH after 20min, followed by 80% CH_3OH for a further 5min, and decreasing to 3% CH_3OH over a further 5min. The gradient followed the Walters Curve No 7. A dual uv and fluorescence detection system was employed to quantitate the levels of O^6-CMdG. A typical retention time using this system for O^6-CMdG was 16.3min and a limit of detection 500pg using an Applied Bioanalytical fluorescence detector.

5.7.2.3 GUANINE BASES.

5.7.2.4 N^7-CARBOXYMETHYLGUANINE (N^7-CMG).

Analytical analysis of this compound in solutions of 2'-deoxyguanosine treated with APNG, was performed on a Spherisorb 5u S5 ODS2 C-18 silica column (20cmx4.6mmI.D.) at 25°C, using a 0.1M aqueous HFBA/CH_3OH gradient system at a flow rate of 1.0cm^3/min. The gradient used 3% CH_3OH for 15min, rising to 80% over the next 5min, and

remaining at 80% CH_3OH for a further 10min. Finally, the gradient was decreased to 3% CH_3OH over a further period of 5min. A typical retention times for N^7-CMG using this system was ca 14min. Quantitation by uv λ=254nm gave a limit of detection of ca 10ng for N^7-CMG.

An alternative analytical procedure for the quantitation of N^7-CMG in acid hydrolysed DNA pre-treated with APNG, was performed on a Hypersil 5u ODS C-18 silica column (25cmx4.6mm I.D.) at 25°C, using a 0.1% (w/w) aqueous HFBA/CH_3OH gradient system at a flow rate of 1.0cm^3/min. The gradient used was 5% CH_3OH for 5min, rising to 20% and then 80% CH_3OH up to 15 and 25min, respectively, followed by 80% CH_3OH for a further 5min and decreasing to 5min and decreasing to 5% CH_3OH over the final 5min. This gradient system gave a retention time of ca 7.5min for N^7-CMG. Quantitation by fluorescence detection using Spectrovision SP 200, with excitation at λ=286nm and monitoring the emission over λ =300-400nm gave a detection limit of 5.0ng.

5.7.2.5 O^6-CARBOXYMETHYLGUANINE.

Analytical analysis of this compound in solutions of acid hydrolysed DNA, which had been treated with APNG,

was performed on a Hypersil 5u ODS C-18 silica column (25cmx4.6mm I.D.) at 25°C, using a 0.1% (w/w) HFBA/CH_3OH gradient system at a flow rate of 1.0cm^3/min. The gradient, identical to that used for N^7-CMG analysis Section 5.7.2.4, used was 5% CH_3OH for 5min, rising to 20% CH_3OH and then 80% CH_3OH up to 15 and 25min, respectively, followed by 80% CH_3OH for a further 5min and decreasing to 5% CH_3OH over the final 5min. The gradient gave a retention time of 17min for O^6-CMG. Quantitation by fluorescence detection using a Spectrovision SP 200 fluorescence detector, with excitation at λ=286nm and monitoring the emission over Δ =300-400nm gave a limit of detection of 2.0ng for O^6-CMG, using typical sample sizes of 100ug DNA (10ul of analyte).

5.8 G.C. ANALYSIS.

5.8.1 ANALYSIS OF N-METHYLPYRROLIDINE FOR PYRROLIDINE.

Analytical analysis for the presence of pyrrolidine in N-methylpyrrolidine (Aldrich), after pre-treatment with phenyl isocynate, was performed on a BP01 capillary

column. The oven temperature was 180°C and the detector temperature was 250°C. The flow rate for the caarrier gases, were He=0.5Kg/cm^3, Air=1.1Kg/cm^3 and H_2=0.7Kg/cm^3. Detection of pyrrolidine was performed using a Flame Ionisation Detector (FID). A typical retention time for pyrrolidine using this system was 7.5min and generally 1ul samples were analysed at a sensitivity of 16 and a limit of detection of 50ng.

5.8.1 METHYLGLYCOLATE ANALYSIS.

Analysis of the possibility of decomposition of methylglycolate in the presence of DBU and N-methylpyrrolidine under refluxing conditions, was performed on a SP1000 column at an oven temperature of 130°C using a FID detector. The flow rate for the carrier gases were Air=20psi, N_2=30psi and H_2=20psi. Sample analysis (10ul) of the reaction solution indicated that there was no apparent decrease in methylglycolate concentration (retention time ca 6min) over a period of 72h.

5.9 ADDENDUM.

5.9.1 PREPARATION OF THE O^6-CMGuo CONJUGATED WITH m-BSA.

The following experiments were performed by Dr. D.E.G.Shuker at the I.A.R.C. in Lyon France. O^6-Carboxymethylguanosine (O^6-CMGuo) (Ca^{++} salt, 10mg) in H_2O (0.5cm^3) was treated with $NaIO_4$ (21.4mg, 100umoles) in H_2O (0.5ul) and stirred at room temperature (20°C) for 15min. Ethylene glycol (5ul) was added to stop the reaction, and the resultant mixture was added to methylated bovine serum albumin (m-BSA) (10mg) in H_2O adjusted to pH 9.5 with 0.2M Na_2CO_3, and kept at room temperature for 45min. The Schiffs base was stabilized by addition of $NaCNBH_4$ (0.5ul of 30mg/cm^3 freshly made solution) and left at 4°C for 3h, with one drop of octanol added to inhibit foaming. The solution was dialysed overnight against phosphate buffered saline solution (PBS) and then separated on a Sephadex G-50 column, and the relevant fractions collected and concentrated overnight at 4°C in a ProDimem apparatus and then lyophilized.

5.9.2 IMMUNIZATION OF THE RABBITS AND ISOLATION OF THE RABBIT ANTISERUM CMGuo1.

O^6-CMGuo conjugated with m-BSA (2.5mg) was dissolved in PBS (2.5cm^3) and to this solution was added Freuds complete adjuvant (2.5cm^3). 1cm^3 of the resultant mixture was injected in 100ul aliquots subcutaneously at multiple sites on the shaved backs of two rabbits. After 2weeks the first test bleed of the rabbits took place. The rabbits were bled from the lateral ear vein into 50cm^3 centrifuge tubes and the resultant fluid left overnight at 37°C and then after standing at 37°Cfor 1h, centrifuged at 1000g for 10min. After 3weeks the rabbits were given an other injection of O^6-CMGuo m-BSA adduct, (1mg in PBS/Freuds adjuvant(1:1w/w) (2cm^3), 1cm^3 per rabbit in 500ul aliquots into the hind quarter. Two months later, the rabbits were given a further booster injection of O^6-CMGuo m-BSA adduct, (1mg in PBS/Freuds adjuvant(1:1w/w) (2cm^3)), 1cm^3 per rabbit in 500ul aliquots into the hind quarter. Two weeks later the rabbits were bled from the lateral ear vein, and the resultant fluid stored at 4°C overnight. The serum was then stored at 37°C for 1h, and centrifuged at 1000g for 10min. The supernatant (CMGuo1) was decanted off, and stored in 1cm^3 aliquots at -30°C.

5.9.3 PREPARATION OF O^6-CMGuo CONJUGATED WITH OVALBUMIN.

O^6-CMGuo (Ca^{++} salt, 10mg) in H_2O (0.5cm^3) was treated with $NaIO_4$ (21.4mg, 100ul) and stirred at room temperature for 15min. Ethylene glycol (5ul) was added to stop the reaction and this solution was added to ovalbumin (10mg) in H_2O (1cm^3) adjusted to pH 9.5 with 0.2M Na_2CO_3 and kept at room temperatrure for 45min. The Schiffs base was stabilised by addition of $NaCNBH_4$ (0.5cm^3 of 30mg/cm^3 freshly made in H_2O) and left at 4°C for 3h, with one drop of octanol to prevent foaming. The resultant mixture was dialysed overnight against PBS and then eluted through a Sephadex G-50 column. The relevant fractions were collected and concentrated in a ProDimem evaporator overnight and the samples lyophilized.

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