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DEVELOPMENT OF A RAPID, DIRECT CHROMATOGRAPHIC ASSAY FOR 0-6 METHYLGUANINE EXCISION IN TISSUES

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DEVELOPMENT OF A RAPID, DIRECT CHROMATOGRAPHIC ASSAY FOR 0-6 METHYLGUANINE EXCISION IN TISSUES

By

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ABSTRACT

DEVELOPMENT OF A RAPID, DIRECT CHROMATOGRAPHIC ASSAY FOR 0-6 METHYLGUANINE EXCISION IN TISSUES

By

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Deficiencies in the repair of 0-6 alkylation lesions in guanine residues of cellular deoxyribonucleic acid (DNA) correlate with enhanced mutagenicity, carcinogenicity and cytotoxicity of agents which produce such lesions. It has been proposed that the cytotoxicity of the cis-platinum (II) anticancer drugs is a result of the formation of an N7-06 platinum chelate in the guanine of cellular DNA. According to this hypothesis, the selective killing of tumor cells sometimes observed with this agent is related to a deficiency in the repair of N7-06 guanyl platinum chelate.

In order to test this theory it was necessary to develop an assay to measure the repair activity for 06 guanyl substituted DNA of various tissues. However, since the N7-06 guanyl-platinum complex has not yet been isolated and adequately characterized, it was judged more suitable to develop an assay by making use of a known adduct, 0-6 methylguanine. A cell-free mouse liver extract served as a source of repair enzymes. The extract was incubated with a methylated DNA substrate. The supernate was then separated

with liquid chromatography. The released 0-6 methylguanine was quantitated by ultraviolet absorbance. Preliminary results indicate that the excision of 0-6 methylguanine is followed by its rapid degradation. These results may explain the failure of other investigators to detect 0-6 methylguanine glycosylase activity.

To my parents,

Ruth and Jerome Wiener

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KEY TO SYMBOLS AND ABBREVIATIONS

Ade	Adenine
AUFS	Absorbance units represented by full scale pen deflection
C_{18}	Waters C_{18} Microbondapak Chromatography column
CH_3CN	Acetonitrile
Cyt	Cytosine
dAMP	2'-Deoxyadenosine-5'-monophosphate
dCMP	2'-Deoxycytidine-5'-monophosphate
dGuo	2'-Deoxyguanosine
DMH	Dimethylhydrazine
DMN	Dimethylnitrosamine
DNA	Deoxyribonucleic acid
DNA-P	Phosphate incorporated into DNA molecular backbone
DNase	Deoxyribonuclease
ENU	N-Ethyl-N-nitrosourea
Gua	Guanine
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
HN2	Nitrogen mustard
M	Molar
mg	Milligram(s)
$MgSO_4$	Magnesium sulfate
min	Minute(s)

ml	Milliliter(s)
mm	Millimeter
mM	Millimolar
MNNG	N-Methyl-N'-nitro-N-nitrosoguanidine
MNU	N-Methyl-N-nitrosourea
N7	7-Methylguanine
ng	Nanogram(s)
$NH_4H_2PO_4$	Ammonium dihydrogen phosphate
NH_4OAc	Ammonium acetate
nm	Nanometer(s)
nM	Nanomolar
06 or 06meGua	0-6 methylguanine
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCX	Whatman strong cation exchange column
SCX column	Whatman SCX-10 Partisil Cation exchange column
Thy	Thymine
Tris	Tris(hydroxymethyl)aminomethane
ul	Microliter(s)
UV	Ultraviolet
v/v	Volume to volume
XP	Xeroderma pigmentosum

CHAPTER ONE

THEORY

A. Introduction

Cis-platinum (II) was shown by Rosenberg, et al., (1965, 1969, 1970) to be cytostatic for Escherichia coli and to be active against Sarcoma 180 and L1210 leukemia in mice. Further studies have confirmed and extended the list of known antineoplastic activities of cis-Pt (II) (Kociba, et al., 1970, Rosenberg and Van Camp, 1970; Leonard, et. al, 1971; Welsch, 1971; Connors, et al., 1971; Cleare and Hoeschele, 1973). After extensive developmental work and numerous clinical trials, the cis-dichlorodiammine platinum (II) derivative (herein referred to as cisplatin, the generic name for this drug) was approved in 1978 by the U. S. Food and Drug Administration for the treatment of testicular and ovarian cancers. Clinical trials have shown some success in the treatment of cancers of the head and neck, bladder, prostate and lung with cisplatin (see Rosencweig, et al., 1977; Rosenberg, 1978).

As is the case with many chemotherapeutic drugs, the mechanism of action of cisplatin and related derivatives is not known. Study of the mechanism of action of these drugs might provide insights helpful in the development of even more effective, chemotherapeutic agents and better cancer treatment protocols as well as providing basic knowledge.

These studies were undertaken to help develop such understanding.

One hypothesis as to the mechanism of action of cis-Pt (II) is that the drug's cytotoxic activity is caused by the binding of cis-Pt (II) to both the N-7 nitrogen atom and the 0-6 oxygen atom of guanine in cellular deoxyribonucleic acid (DNA) (Macquet and Theophanides, 1975; Millard, et al., 1975; Goodgame, et al., 1975; Dehand and Jordanov, 1975; Rosenberg, 1978). Millard, et al., (1975) suggest that cis-Pt (II) binding at the 0-6 site of guanine in tumor cell DNA blocks hydrogen bonding between this oxygen and the cytosine amine group (see Gerchman and Ludlum, 1973) preventing normal Watson-Crick base pairing. It is postulated that this DNA lesion is responsible for the cytotoxic effect of the drug.

Evidence for the mutagenicity of, carcinogenicity of and the deficiency in capacity for repair of alkylation and platination lesions in DNA by certain cancer cells will be reviewed later. Replication of DNA sequences with 0-6 guanyl platination lesions is expected to produce an altered base sequence in daughter strands. However, since the DNA lesions are diluted out by each cell replication in addition to being removed by cellular DNA repair processes, the first cycle of DNA replication is the most important for mutation induction.

It has been demonstrated by a number of workers (discussed later) that those cells with slower rates of repair for the mutagenic 0-6 guanyl alkylation lesion in the guanine of DNA show higher cytotoxicity per unit dose to alkylating

agents which form such lesions than cells with faster repair rates. If the proposed 0-6, N-7 cis-Pt (II) guanyl chelate is repaired by a similar (or the same) mechanism as 0-6 alkylated guanine, then those cells with slower rate of repair for this lesion would be expected to show enhanced cytotoxicity when treated with cis-Pt (II).

Unfortunately, it is not possible to directly test this hypothesis at this time since the 06, N7 cis-Pt (II)-guanine chelate has not yet been isolated and characterized. Therefore, I decided to take a more general approach to the problem, namely, to develop an assay capable of directly monitoring the capacity of tissue to remove chemically substituted bases including 0-6 methylguanine and N-7 methylguanine from methylated DNA. Such an assay could then be utilized to determine whether tumor cells which are sensitive to cis-Pt (II) are slower in the removal of the 0-6 platinated guanyl group from DNA than cells which are not so sensitive to the drug.

The assay was developed through a series of refinements of an initial crude assay procedure. The procedure involved incubation of a methylated DNA substrate with a mouse liver extract. After the reaction was stopped, the supernate was examined quantitatively for methylated bases. High performance liquid chromatography procedures were developed and employed for the separation and detection of minute quantities of 0-6 and N-7 methylguanines removed from the methylated DNA by the tissue extract enzymes. The procedures which were

developed for the assay of the removal of alkylated guanyl groups from DNA are expected to prove equally applicable for study of repair of both alkylation and platination lesions in DNA. However, the chromatographic separation and detection techniques will, in all likelihood, require modification for the assay of the cis-Pt (II)-guanyl adducts from DNA.

Evidence was found for the excision and subsequent degradation of 0-6 methylguanine from methylated DNA by an extract prepared from the livers of normal mice. This assay should prove useful in the comparison of rates of removal of the N7,06-cis-Pt (II)-guanyl adduct from platinum treated DNA by various tissues. However, platinum repair studies will require that: 1) N-7,0-6-cis(II)-guanine chelate is available as standard to identify the adduct excised from DNA in the assay; 2) N-7,0-6-cis-Pt(II)-guanine is stable enough to be quantitatively assayed following removal from DNA; 3) a chromatographic separation for platinated guanines is developed; and 4) the amounts of adduct that are released from the DNA are detectible.

B. Cancer and Cancer Therapy

Neoplasia is a pathological state affecting all cell types in vertebrates. This condition is characterized by uncontrolled cell replication and by metastasis. A wide variety of cellular biochemical anomalies are often found in cancer cells but their role in tumorigenesis is not clear. The known causes of neoplasia are ionizing and ultraviolet

radiation, various chemicals and certain viruses. It is not yet known if these different agents cause cancer by similar mechanisms. In recent years, however, it has been realized that most carcinogenic chemicals and radiation modify DNA and that such DNA lesions appear to be causally involved in carcinogenesis. It is generally believed that DNA lesions cause cell mutations through miscoding during DNA replication or because DNA is not replicated across from such lesions. Since the lesions are distributed randomly in the DNA, the mutations are also random. In a large population of cells treated with a carcinogen, it is assumed that in some of the cells mutations are formed in genes which control cell replication. One or more of such mutations (the number is unknown), and perhaps other events, are postulated to cause a cell to replicate repeatedly since it no longer responds to the normal biological signals which regulate replication. A cell with this property is defined as a tumor cell. The concept of one or more mutagenic lesions resulting in transformation of a normal cell to a tumor cell is often referred to as the somatic mutation theory for the origin of cancer.

An ideal method of cancer therapy will employ agents which selectively damage or kill cancer cells without affecting normal cells. However, this ideal is rarely attainable, since tumor cells taken collectively do not show an enhanced sensitivity to any known agents. Therefore, one then must resort to the use of properties, such as rapid cell proliferation, which cancer cells generally have and which they share

with only a small fraction of somatic cells in order to minimize damage to the latter. For this reason, radiation or chemical agents which are capable of enhanced killing of proliferating cells have often been found to be useful in the treatment of tumors.

C. The Relationship Between Carcinogenesis and the Formation and Persistence of Mutagenic 0-6 Alkylguanine in the DNA of Replicating Cells

1. Mutagenicity and Carcinogenicity of Alkylation Lesions in DNA

The realization that certain chemicals are carcinogenic is relatively recent. The first such report was by Yoshida, who demonstrated in 1932 that oral administration of O-aminoazotoluene regularly induced liver cancer in rats. Similarly, the first report that chemicals are mutagenic was by Thom and Steinberg (1939), who found that nitrous acid caused mutations in Aspergillus. Boveri's (1929) postulate that neoplasms arise from mutations in somatic cells was supported by the report that sarcomas are induced in mice at the site of injection of mustard gas, a known mutagen (Heston, 1953). Recent reviews (Singer, 1975; Lawley,1976 a,b; Pegg, 1977; Singer, 1979) discuss evidence of the mutagenicity and carcinogenicity of alkylating agents.

2. The relationship between the extent of 0-6 guanyl alkylation and mutagenesis and carcinogenesis

Schoental (1958) first suggested that the alkylation of oxygen atoms may be important in carcinogenesis. In support of this, Loveless (1969) found that alkylating agent-induced

mutagenesis of phages correlated only with the extent of 0-6 alkylguanine formation and not with N-7-alkylguanine formation. This led him to hypothesize that 0-6 guanyl alkylation precludes the formation of the normal Watson-Crick guanine-cytosine (G-C) triple hydrogen bond base pairing. Abnormal base pairing was postulated to cause a point mutation during DNA replication. Since Swann and Magee (1968) had found no correlation of carcinogenesis with N-7 methylguanine formation in DNA by alkylating agents, Loveless further suggested that it was the mutation induced by 0-6 guanyl alkylation that was responsible for alkylation-induced carcinogenesis.

Although the N-7 atom of guanine is the most reactive site of action of alkylating agents in DNA, the formation and persistence of this modified base appears to have little correlation with mutagenesis and carcinogenesis (see review in Singer, 1975). No miscoding has been found with N-7 substituted guanines using either RNA templates (Wilhelm and Ludlum, 1966; Ludlum, 1970) or DNA templates (Hendler, et al., 1970). Both 7-alkylguanine and 3-alkylguanine are lost from DNA through depurination from the weakened glycosylic linkages (Margison and O'Connor, 1973). In some cases, enzymatic repair has been observed for these two lesions through glycosylase activity (reviewed in Pegg, 1977; Singer, 1979).

The extent of 0-6 guanyl alkylation produced in DNA by monofunctional alkylating agents has been shown to correlate with their potential carcinogenicity in some in vivo studies

(see Pegg, 1977, pps. 233-234). Only those simple alkylating agents which can produce significant amounts of 0-6 alkylguanine in DNA are carcinogenic (Lawley, 1976b). Some recent experimental findings appear to link the mutagenicity and the carcinogenicity of the 0-6 alkylguanyl lesion. Newbold et al., (1980) compared the mutagenic effects of dimethylsulfonate (DMS), methylmethanesulfonate (MMS) and N-methyl-N-nitrosourea (MNUA) in cultured V79 Chinese hamster cells. Each of the agents' mutagenicity, but not its cytotoxicity, was found to reflect its relative degree of carcinogenicity. The relative degree of mutagenicity of each agent also correlated positively with the relative degree to which it methylated the 0-6 atom of guanine in the cellular DNA. These investigators suggest that 0-6 methylguanine is responsible for most of the mutations induced in mammalian cells by carcinogenic methylating agents. Frei, et al., (1978) observed a quantitative relationship between alkylation at the 0-6 guanyl atom in DNA and tumor yield. The log of the percentage of C57BL mice developing thymoma within 250 days of treatment was charted as a function of the log of i.p. injected dosage of methylnitrosourea (MNU) or ethylnitrosourea (ENU). The response curves for the two drug treatments were parallel. However, dosages of ENU 4.5 times those of MNU dosages were required in order to produce equivalent incidences of thymoma. The investigators reasoned that an event critical to thymoma induction occurs, at a given dose about 4.5 times more frequently for the methylating carcinogen than for the ethylating carcinogen.

The formation of 0-6 and N-7 alkylguanines was compared for the thymus as well as the bone marrow of groups of animals a short time after receiving a range of dosages of MNU or ENU. The degree of N-7 methylation was 30 times that of ethylation at equimolar dosage levels. Hence it seemed unlikely that this was the critical event in thymoma induction. However, the 0-6 guanyl methylation-to-ethylation ratio at all dosage levels tested was 5.3. The proximity of this figure to the two alkylating agents' ratio of effectiveness in thymoma induction (4.5) supports the Loveless (1969) hypothesis that 0-6 alkylation is an important factor in DNA miscoding, the induction of mutations and tumor initiation. Frei, et al. (1978) suggest that the slight discrepancy between the ratio of carcinogenic effectiveness (4.5) for methylation and ethylation by the alkylnitrosoureas and their relative extents of alkylation at the 0-6 atom of guanine in DNA (5.3) reflects tumor initiating activity of other alkylated bases which also mispair.

3. Persistence of 0-6 alkylguanine in cellular DNA and mutagenesis and carcinogenesis.

The results of other studies indicate that 0-6 alkylguanine persistence is a critical factor in alkylating agent induced carcinogenesis. Goth and Rajewsky (1974a,b) first demonstrated differential repair capacities of various organs of ten-day old rats for 0-6 ethylguanine lesions in DNA. Higher degrees of persistence of this modified base and increased oncogenesis were found in the brain in contrast to

the liver and kidney. The amounts of 0-6 ethylguanine initially formed in the various organs of fetal, young and adult rats were observed to be approximately the same. But the fetal as well as the young rats consistently had more brain tumors. Although no correlation was found between the degree of 0-6 ethylguanine formation and tumor induction, the rate of elimination of this base was markedly decreased in the fetal and the young rat brain. The decreased ability of the fetal and the young rat brain to remove this mutagenic lesion was suggested to be responsible for the high sensitivity of this tissue to carcinogenesis by ENU. This hypothesis linking 0-6 alkylguanine persistence with carcinogenesis was supported by further studies with MNU in rats (Kleihues and Margison, 1974; Margison and Kleihues, 1975; Kleihues and Margison, 1976). Nicoll, et al. (1977) found less efficient removal of 0-6 methylguanine from kidney, the target organ for carcinogenesis, than from liver following administration of single high dosages of dimethylnitrosamine (DMN) to adult rats. Roberts (1978, Table VIII) reviews papers describing the loss of chemical substituents from the cellular DNA of various organs in experiments in which whole mammals were treated with alkylating agents. In contrast to the results given above, Kleihues, et al. (1980) observed that the adult gerbil is deficient in the removal of 0-6-methylguanine from brain DNA following MNU treatment. However, this organ is not susceptible to oncogenesis with this treatment. Hence, there must be factors other than 0-6 alkylguanine formation

and persistence that are important in alkylating agent-inducing carcinogenesis.

4. Cell replication as a factor in alkylating agent-induced carcinogenesis

Rajewsky, et al. (1976) suggest that the high rate of cell replication, and hence DNA replication, in the developing rat brain may be causally involved in the increased sensitivity of this tissue to carcinogenic agents. Craddock (1973) found that cell replication is necessary in order for methylation lesions to induce carcinogenesis. Although the extent of DNA methylation and the relative degree of 0-6 methylguanine formation remains the same in the DNA of both intact and regenerating livers of DMN-treated rats, carcinogenesis occurs only in the regenerating livers. Further study showed that a single treatment with MNU can induce adenomata in rat liver cells only when they undergo a period of DNA synthesis following treatment. No such increase occurs if the cells are not replicating (Craddock and Frei, 1974). These results suggest that replication of DNA which contains 0-6 methylguanine is a prerequisite for alkylating agent-induced carcinogenesis. This suggestion is supported by Roberts (1978) in his review of studies which compare DNA synthesis and carcinogenesis. Recent studies by Swenberg, et al. (1979) indicate that persistence of the DNA lesions in cells until they have had the opportunity to replicate correlates with carcinogenesis. They administered a single dose of labelled dimethylhydrazine (DMH) subcutaneously to female BD-IX rats. The maximum extent

of alkylation was observed 2 hours after treatment, with the highest concentrations of alkylated bases in the liver, followed by colon, ileum and kidney. The rate of loss of 0-6 methylguanine over three days was slowest for the colon, the principal target organ for carcinogenesis. The investigators suggest that the persistence of 0-6 methylguanine and high rates of cell replication, and hence fixation of mutations, may explain the organ-specific carcinogenicity of DMH.

D. Repair of 0-6 Alkylguanine Lesions in DNA

The mention of persistence or excision of alkylated bases from DNA implies the presence of a repair mechanism. Many reviews deal with the repair of lesions in cellular DNA due to alkylating agents (Friedberg et al., 1977; Strauss, et al., 1977; Pegg, 1977; Roberts, 1978; Singer, 1979). An enzyme activity has been found in rat liver extracts which removes 0-6 methylguanine, but not N-7 methylguanine, activity from methylated DNA (Pegg and Hui, 1978; Pegg, 1978). The activity of this enzyme may be enhanced by long-term, low level exposure of the animals (from which the extract is made) to DMN. However, the enzyme activity is diminished when the animals are treated with high doses of DMN (Montesano, et al., 1979 ; Pegg, 1980; Montesano, et al., 1980). Pegg (1978) reports that no 0-6 methylguanine appears in solution following the reaction of his extract with methylated DNA in vitro. This result will be dealt with in Chapter Two.

E. Inheritable Deficiencies in the Repair of Alkylation Lesions in the DNA of Some Cells with Elevated Tumorigenic Potential and of Some Tumor Cells

Defective DNA repair mechanisms are a characteristic of some tumor cells and may be useful in the selective destruction of these cells. Inherited or induced defects in DNA repair mechanisms may also render otherwise normal cells more sensitive to mutagenic lesions and more likely to subsequently transform to tumor cells. Evidence has been obtained in many laboratories indicating that alkylation repair enzyme activity is deficient in some cells with a high potential for becoming malignant (Pegg, 1977; Goth-Goldstein, 1977; Nicoll, et al., 1975; Margison, et al., 1976; Kleihues and Margison, 1976; Pegg, 1978). Inherited diseases such as Xeroderma pigmentosum, Fanconi's anemia, Bloom's syndrome and ataxia telangiectasia are characterized by high cancer frequencies among patients with these diseases. Xeroderma pigmentosum (XP) is the human pathological state characterized by a hypersensitivity of the skin to ultraviolet (UV) radiation and a tendency toward multiple neoplasm formation on any skin exposed to sunlight. Many XP cell lines have been shown to have a reduced repair capacity for lesions including pyrimidine dimers (Cleaver, 1968), 0-6 alkyguanine (Goth-Goldstein, 1977; Bodell, et al., 1979) and other carcinogen adducts in DNA (Maher, et al., 1976; Setlow and Regan, 1972; Day, et al., 1978; Fraval, et al., 1978, Cleaver, 1973). Scudiero (1980) reported that four of six cell strains derived from patients with the autosomal recessive disorder,

ataxia telangiectasia (AT) had significantly reduced levels of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced repair synthesis compared to controls. Those strains with diminished MNNG-induced repair also had decreased levels of repair following treatment with ionizing radiation (Peterson, 1979). Maurice and Lederrey (1977) observed that lymphocytes from patients with chronic lymphocytic leukemia had a significantly lower nitrogen mustard (HN2)-stimulated tritiated thymidine uptake compared to control lymphocytes. These leukemia leukocytes also were impaired to a greater degree than controls in phytohemagglutinin-stimulated DNA synthesis when pretreated with HN2. The investigators suggest that these findings indicate that some leukemic cells are deficient in a DNA repair mechanism.

Some tumor cells appear deficient in the repair of alkylation damage to DNA. Day, et al. (1980) found that 9 out of 39 human tumor cell strains tested were abnormal in the repair of MNNG-damaged adenovirus 5. These included one (of five) colon tumor strain, one (of four) melanoma strain, one (of two) lung cancer strain, one (only tested) strain derived from epidermoid carcinoma of the neck and four human astrocytoma cell strains, A172, A382, U-87 MG and U-105 MG. Kornblith and Szypko (1978) found variations in susceptibility to killing by bis-1,3-(2-chloroethyl)-1-nitrosourea (BCNU) in low passage human astrocytoma cell cultures prepared from 24 tumors. Day, et al. (1980) suggest that these results indicate that tumors responding well to alkylation chemotherapy

are composed of cells defective in the repair of alkylating agent-induced DNA damage.

The results of Kornblith and Szypko (1978), Day, et al. (1980) as well as those of Salmon, et al. (1978) support the hypothesis that tumors composed of DNA repair-deficient cells may arise in organs composed of repair-proficient cells. Following the hypothesis that most human tumors are monoclonal (Nowell, 1976), Day, et al. (1980) propose that a DNA repair-deficient tumor may arise from a single cell which was somatically produced as a repair-deficient mutant prior to tumorigenesis. If this is so, then DNA repair deficient cells may undergo tumorigenesis more readily than repair proficient cells if treated with an agent which produces DNA lesions for which their repair is deficient.

F. Mutagenicity, Carcinogenicity and Deficiencies in the Repair of Platination Lesions to DNA

Cis-Pt (II) complexes can act as mutagens in bacterial test systems (Beck and Brubaker, 1975; Monti-Bragadin, et al., 1975; Benedict, et al., 1977; Lecointe, et al., 1977) and have significant carcinogenicity in mouse lung and skin and in rat subcutaneous tissue (Leopold, et al., 1979). The results of Andersen's (1979) work with the Salmonella test system of Ames, et al. (1973) indicate that cis-Pt (II) is a base-pair substitution mutant. This type of mutation would be expected from binding at the 0-6 atom of guanyl groups in DNA. Lecointe, et al. (1977) contend that this is the only type of mutation induced by cis-Pt (II)-DNA interactions.

However, Andersen (1979) found evidence for the induction of frameshift mutations in strains TA100 and TA98 of Salmonella typhimurium by cis-Pt (II). Lecointe, et al. (1979) report a correlation between mutagenicity and toxicity to L1210 cells of a series of platinum drugs.

The mutagenic effects of cis-Pt (II) do not seem to be caused by its role in the interstrand crosslinking of DNA or DNA protein crosslinking, which are major binding activities. Zwelling, et al. (1979) found that cis-Pt (II) is mutagenic while trans-Pt (II), which has no anti-tumor activity, is not mutagenic when administered at equitoxic dosage levels to V79 Chinese hamster cells. The two platinum isomers produce comparable degrees of DNA-interstrand crosslinking as indicated by alkaline elution analysis. Furthermore, the nonmutagenic dose of trans-Pt (II) produced a greater degree of DNA-protein crosslinking than the mutagenic dose of cis-Pt (II). Studies of the potentiation of cis-Pt (II)-induced cell killing by hyperthermia in Chinese hamster ovary cells in culture revealed that cell killing is enhanced to a greater degree than DNA interstrand crosslinking by this treatment (Meyn, et al., 1980). This supports the contention that the mechanism of action of cis-Pt (II) involves processes other than DNA interstrand crosslinking.

The failure to repair 0-6,N-7 guanyl platination lesions in DNA prior to cell replication may be the property of some cancer cells which allows cis-Pt (II) to selectively kill them. Fraval, et al. (1978) found that repair-deficient

human fibroblasts in culture are more sensitive to cisplatin than normal human fibroblasts, demonstrating that DNA excision repair contributes to cell survival following platinum treatment. The work of Fraval and Roberts (1979) further supports a direct correlation between the capacity for excision repair of platinum adducts (from DNA in V79 Chinese hamster cells) and an increased ability of the cells to survive. These data support the hypothesis that cis-Pt (II) derives its antitumor activity through its capacity to form adducts in DNA which, if unrepaired, cause cytotoxic effects. Presumably then, most normal cells are expected to survive the drug treatment by repairing platination lesions prior to replication.

CHAPTER TWO

EXPERIMENTAL

A. Objectives

The ultimate objective of this work was to develop techniques to assay the capacity of various tissues to remove 0-6 guanyl platination lesions from DNA to determine the kinetics of that removal. For reasons discussed above, the assay was developed for 0-6 methylguanyl lesion removal from DNA with the intent of later applying a similar technique to assay 0-6 guanyl platination lesion removal. The assay is a straight-forward biochemical reaction. A substrate of methylated DNA is incubated with a fresh extract of the selected organ or tissue. At different times, aliquots are removed from the reaction mixture. These aliquots are inactivated, purified and assayed. The appearance of free 0-6 methylguanine and N-7 methylguanine in the purified reaction mixture is monitored by a liquid chromatographic (LC) separation coupled with UV absorbance quantitation of the chromatographic column effluent. In contrast to this method, most experimental techniques which monitor the repair of alkylation lesions in DNA are much more complex.

The procedures were developed with emphasis on simplicity and convenience of method, as well as significance and reproducibility of results. Furthermore, the methods developed

for the 0-6 methylguanine assay must be applicable to the study of platination lesions. The conventional method of measuring the retention rather than the release of lesions from the DNA by isolating the DNA from various organs of animals treated with the DNA binding agent (in this case cisplatin) followed by a hydrolysis step to depurinate the DNA and subsequent chromatographic separation of the DNA hydrolysate was deemed unfeasible for a number of reasons. Most worthy of note, the hydrolytic depurination procedure was envisaged to dissociate the N7-06 platinum-guanine adduct due to the low pH at which this reaction is carried out. An assay of the total amount of platinum retained in the DNA would not be an accurate measure of repair since cis-Pt (II) forms a variety of binding adducts with DNA. The method of direct monitoring of base adducts released from DNA by the action of tissue extracts fulfills the requirements of simplicity, convenience and adaptability to platinum studies. A major assumption in this approach, however, is that the 0-6 guanyl alkylation lesion (and the N7-06 guanyl platination lesion) is repaired via the excision of the base with the methyl (or cis-Pt (II)) group intact and that this excised adduct remains stable long enough to be detected.

B. Requirements for the Assay

In the development of a new technique, it is essential to designate the specific requirements and specifications to which each of the procedures must adhere. For this analysis,

the experiment may be divided into three phases: the preparation of the tissue extract, the excision reaction of the tissue extract acting on the methylated DNA substrate, and the separation, identification and quantification of the reaction products.

The extraction procedure must be rapid and simple to perform. Care must be taken to exclude tissue components which interfere with excision activity or nonspecifically decompose the methylated DNA substrate (such as lytic enzymes). Substances which adversely affect the separation and identification of the reaction products must also be avoided. Procedures must deter environmental interferences with excision such as temperature, microbial contamination, damaging solvents, lack of appropriate cofactors, etc. These considerations also apply to the reaction conditions. The reaction should be carried out in such a way that kinetics may be easily monitored. Samples should be in a form that facilitates separation and identification. A good separation of 0-6 methylguanine and N-7 methylguanine from all other compounds is required. Rapidity is essential in the preparation for and in the separation itself. The method for quantification of identified reaction products must be sensitive to concentrations of methylated bases on the order of magnitude of nanograms per milliliter.

C. Tissue Extract Preparation

The first step of the experiment is the preparation of a tissue extract with 0-6 guanyl lesion removal activity.

Crude Extract I Preparation

Two ICR Swiss white female mice (Spartan Research Animals, Inc.) aged three to six months were killed by cervical dislocation. The livers were removed, weighed, minced with scalpels and homogenized with a ground glass homogenizer and a loose-fitting pestle. The latter two procedures were carried out over crushed ice. An equal volume of physiological saline solution was added during the homogenization step.

Crude Extract II Preparation

Crude Extract I was purified by dialyzing it against a physiologically isotonic buffer in the dialysis chamber (to be described later) at 2°C. The buffer flow was assisted by a peristaltic pump. This predialysis procedure lasted two to six hours using 400 to 700 ml of buffer.

Purified Tissue Extract I Preparation

Mice (described above) were killed by cervical dislocation with special care not to cause severe internal hemorrhaging in order to prevent the formation of blood clots in the tissue to be extracted. The mouse was operated on aseptically. All work was done with sterile equipment and all dilutions were done with autoclaved solutions. A portable plexiglass hood with a germicidal lamp and a gas microburner was designed by Ms. Khoyi and was constructed under her supervision. The hood was the site of all transfers

of tissue-derived mixtures. After the mouse was killed, its chest cavity was opened, the right atrium was incised and a perfusion of five or ten milliliters (ml) of physiological saline solution was administered through the left ventricle. Heparin was not used in order to avoid possible mitogenic activity. The liver was removed and minced, diluted and homogenized as described for crude extract I. The resulting pinkish-tan suspension was washed several times in physiological saline solution. The cell suspension was washed by centrifuging at 750 revolutions per minute (rpm) for ten minutes in a Sorvall RC2-B centrifuge with either an SS34 or a GSA rotor (g forces of 67 and 90 respectively). Red blood cells were removed from the pellet by pipette prior to resuspending the pellet, which was done by vortexing it in fresh physiological saline solution. Buffy coat was also removed from the sides of the centrifuge tube and the surface of the pellet prior to resuspension. The cells were considered to be adequately washed when blood could not be seen in the pellet. During the final wash, the cells were suspended in twice their volume of 0.28 M Tris-HCl buffer, pH 7.2 (at 37°C according to Sigma Bulletin No. 101, Sigma Chemical Co., St. Louis, Missouri), with 5 millimolar (mM) magnesium sulfate ($MgSO_4$). The suspension was then passed through three layers of cheesecloth. The cell suspension was homogenized in a ground glass homogenizer with a tight fitting pestle in order to release the excision enzymes from the cells. This suspension was then predialyzed as described above and used.

Purified Tissue Extract II Preparation

Instead of predialysis, the purified tissue extract I was centrifuged in the RC2-B centrifuge at 12,000g for ten minutes. The clear yellowish supernate was clarified by pipetting through six layers of cheesecloth. The low density fraction suspended at the top of the supernate in the centrifuge tube was avoided when pipetting.

In order to preserve liver enzyme activities, low temperatures were employed during the extraction procedure. The total workup time, from killing the animal to the final filtering of the extract, was less than an hour provided that the liver had been well perfused.

The above four methods for preparing tissue extracts were compared using the procedures to be described below in Section E. These results indicated that crude extracts were completely unsatisfactory and that only the purified tissue extract II preparation was satisfactory (see below).

D. Purification by Dialysis

Dialysis was chosen as a trial technique for separating excised methylated bases from the other reaction mixture components including methylated DNA substrate, tissue polynucleotides, proteins and other large molecules. Carter and Greenstein (1946) described the dialysis of extracts of both normal and cancerous tissues with DNA. They monitored relative degrees of degradation of the DNA in the presence of various ionic media by spectrophotometric analysis of the dialysates.

A dialysis chamber was designed based on a commercially available device, the TechniLab five milliliter dialysis cell. The cell consists of two matching chambers with capacities of 2.5 milliliters each which may be secured together with nuts and screws while placing the dialysis tubing between the two chambers. The tubing was cut into 2.3 centimeter squares from rolls or sheets. Ports of the compartments were fitted with large gauge stainless steel hypodermic needles to facilitate the transfer of fluids in and out of the chambers. The cell was constructed from acrylic allowing convenient cleaning and sterilization. Also, bubbles were visible and hence easily removed when fluids were added to the chambers. A heating strip, powered through a Variac variable power supply, was wrapped around the chamber to heat it to 37^{o}C for enzyme reactions. Small stirring bars were placed in both compartments of the dialysis chamber. Placing one end of a bar magnet (a large stirring bar) outside one compartment and a non-heating stir plate, with its rotor spinning at a low frequency, outside the other compartment permitted fluid circulation in both compartments. If the apparatus was assembled properly, neither of the internal magnetic stir bars touched the dialysis membrane. The stirring effectively prevented the appearance of concentration gradients at the membrane surfaces.

Squares of dialysis tubing were cut from commercial dialysis tubing (A.H. Thomas Co., Philadelphia). Trace minerals and sulfur were leached from the membranes by

ethanol and bicarbonate/ethylenediaminetetraacetic acid (EDTA) treatments as described by McPhie (1971). Prepared membranes were stored at 4°C in distilled water. The prepared membranes were neither handled manually nor permitted to become dry.

In order to determine whether the dialysis chamber would be applicable to the requirements of the experiment and to gain skill in its workings, a series of preliminary studies were undertaken. It was first necessary to show that large molecules would be excluded by the membrane. Dextran Blue 1000 (Pharmacia, molecular weight 2,000,000 daltons) did not diffuse through the membrane (A.H. Thomas 3787-H45, which excludes molecules with molecular weights greater than 3500) in the dialysis cell.

The next step was to observe the kinetics of diffusion of a molecule approximating the size of 0-6 methylguanine in the dialysis cell. The histochemical stain, methyl green, was initially selected due to its size and apparent ease of quantitation by optical absorption. This particular compound, however, had the unsatisfactory property of fading in the presence of the dialysis membrane.

Another stain, toluidine blue, was selected for the study of diffusion kinetics. On the dialysate side of the membrane a concentration gradient appeared. At this time, the internal stir bar method was implemented. The optical absorption of the dialysate never reached the equilibrium value of one-half of the absorption of the original sample.

Examination of the membrane after the dialysis procedure revealed that a significant amount of the stain was bound to the membrane.

2'-Deoxyguanosine was then studied with regard to its diffusion characteristics in the dialysis cell. There was difficulty quantifying small amounts of this substance directly by absorption on a Cary 17 spectrophotometer. Hence, the Dische color reaction (Clark, 1964) was done on each of the aliquots of deoxyguanosine removed from the dialysate chamber over time. This reaction quantitatively produces a deep blue product with an absorption maximum at 500 nanometers (nm). By monitoring the deoxyguanosine concentration with the Dische reagent, it was possible to compare the diffusion rates through dialysis tubing excluding molecules with molecular weights greater than 3500, 8000 and 12000. The diffusion curves are shown in Figure 1. The results showed that within 120 minutes, the concentration of the dialysate approached 50% that of the original solution. Tubing which excluded molecules of molecular weights of 8000 or more was chosen for use in the studies to be described below. 8000 molecular weight limit tubing was selected for use in further studies. The diffusion of the 2'-deoxyguanosine was also detectible even in the presence of crude liver extract II in the sample chamber of the dialysis cell (not shown). This indicates that the tissue extract does not complex with the dialysis membrane in a manner inhibiting nucleoside diffusion.

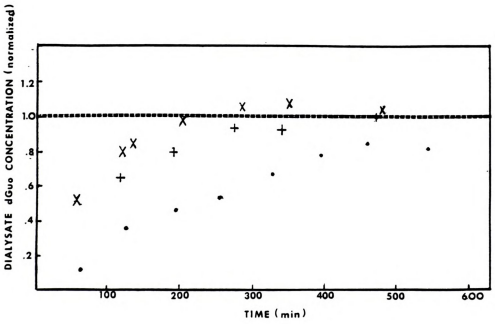


FIGURE 1

Kinetics of diffusion of 2'-deoxyguanosine (dGuo) through dialysis membranes allowing diffusion of molecules with weights greater than 3500 daltons (...), 8000 daltons (+++) and 12000 daltons (XXX) as described in text. Scale of ordinate represents concentration of dGuo in dialysate normalized to one-half the original sample concentration. Hence dashed line represents expected concentrations of dGuo in dialysate at equilibrium.

The dialysis cell was shown to be useful in quantitatively clarifying a nucleoside-containing cell extract for chromatography. Unfortunately, this occurred on a time scale on the order of hours and with a dilution factor. This would later be shown to be unsatisfactory.

E. Chromatography

A method was needed for the separation and quantification of the molecular species in dialysates of a methylated DNA/cell extract mixture. Since nucleotides or nucleosides can be converted to bases by enzymatic hydrolysis, I decided to base the initial chromatographic separation conditions on the methods of Bochert and Webb (1977) and Shaikh, et al. (1978). Without the use of gradient elution (a prohibitively expensive procedure for our laboratory at the time this work was done), simultaneous separation of each of the various methylated bases was not possible (Horvath and Lipsky, 1967).

These chromatographic techniques require solvents of high purity. Distilled water was passed through a Barnstead Ultrapure mixed bed ion exchanger to achieve a resistivity of one million ohm-centimeters. This water was then passed through a Barnstead organic removal cartridge. Then buffers were prepared from one molar stock solutions stored over 1% chloroform. The next purification step, to which all samples were also subjected, was filtration through a prerinsed 0.22 micron Millipore type GS filter. Solvents were filtered under vacuum while samples for chromatography were filtered by pressure with a syringe. Solvents were degassed by

stirring under vacuum. Fresh solvents were prepared daily in nitric acid washed flasks.

Standard chromatography procedures were employed as described in instrument manuals. The Waters model 6000A solvent delivery system with the U6K injector, the Waters model 440 absorbance detector with filters to monitor absorbance at 254 nm and a Houston Omniscribe recorder were used. I used a 250 millimeter (mm) by 4.6 mm (inner diameter) Whatman SCX-10 Partisil cation exchange column (referred to as the 'SCX column') and a Waters 30 cm by 3.9 mm (inner diameter) C_{18} Microbondapak column (referred to as the 'C_{18} column'). Both columns were purchased prepacked by the manufacturer and were handled as prescribed. All separations were done at ambient temperature. As an example of the utility of this technique, a separation of methylated guanines is illustrated in Figure 2. Each compound was dissolved in the solvent (0.1 M ammonium dihydrogen phosphate, pH 3.95). The solution was filtered. An aliquot was injected into the injector with a Hamilton syringe. Chromatography conditions were adapted from the methods of Shaikh, et al. (1978).

A fairly recent innovation for prolonging the lifetime of the microparticle column, the guard column, was employed. This is a precolumn which is packed with large (37-50 micron) silica beads with the same coating as those in the analytical column in use. The use of the guard column was considered to be desirable since it would remove compounds from dialysate

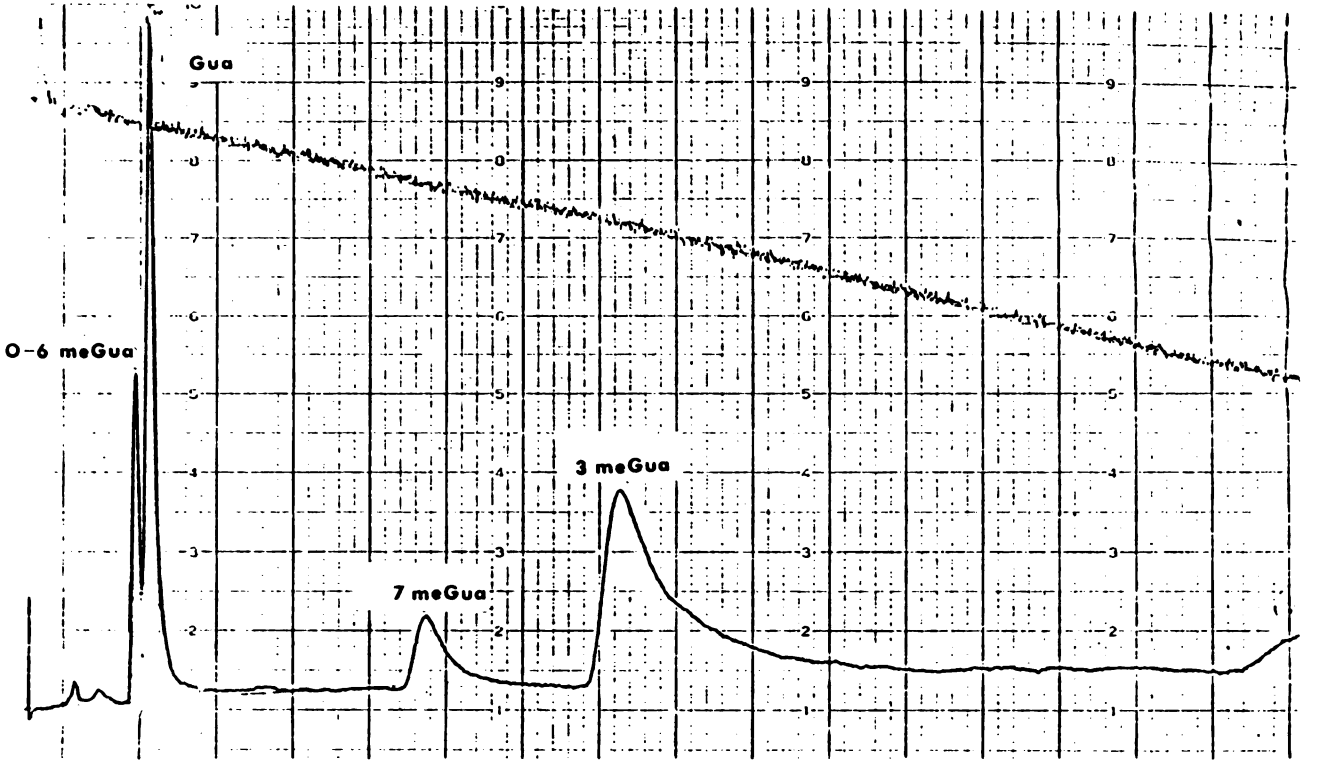


FIGURE 2

Chromatographic separation of methylated guanines in the manner of Shaikh, et al. (1978). Conditions: SCX column, solvent: 0.1 M ammonium dihydrogen phosphate, pH 3.95, flow rate 2 ml/min. Abbreviations: 0-6 meGua- 0-6 methylguanine; Gua-guanine; 7 meGua-7-methylguanine; Gua-guanine; 7 meGua-7-methylguanine; 3 meGua-3-methylguanine. Procedures employed for this and subsequent chromatograms are discussed in Section E.

samples which would bind irreversibly to the column, reducing its efficiency. Crude extracts appear to contain many such substances. When installed in the Waters guard column, the Whatman cation exchange guard packing had the effect of spreading sample peak widths in an unsatisfactory manner when chromatographic procedures were done as described above. The only packing that yielded no peak spreading problem with the SCX column was one composed of 37-50 micron diameter glass beads with no coating (Whatman).

The performance of the C_{18} column was unaltered by the guard column with C_{18} packing when separating bases with the procedures described above. The guard column problem with the SCX column was confirmed by Herron and Shank at the University of California at Irvine in a personal communication. In their work, they also found that in order to achieve reproducible results with the Whatman SCX column, it was necessary to change the buffer eluant as the column aged. Based upon these considerations as well as the economy of a longer lifetime, the column was selected for the following work.

A chromatographic separation was developed for 0-6 and N-7 methylguanines for the C_{18} column with the corresponding guard column. The procedure was based upon that of Christiensen and Whitsett (1979) for the separation of methylated caffeine derivatives. An eluant of 0.010 M ammonium dihydrogen phosphate, pH 4.0 with 2.5% acetonitrile (v/v), was employed with a flow rate of 1.5 ml per minute. In later experiments,

acetate was substituted for phosphate due to its volatility. This property is useful if samples are to be collected, dehydrated, desalted, resuspended and rechromatographed. These eluants were prepared from stock solutions of ammonium hydroxide, phosphoric acid and acetic acid respectively. Glass-distilled, spectral grade acetonitrile was used (Burdick and Jackson, Muskegon, Michigan).

F. Standards

Methylated nucleic acid bases were purchased from Sigma Chemical Co., St. Louis, Missouri. A series of standard solutions of the nucleic acid bases and their methyl derivatives were prepared. Approximately 0.1 milligram of each substance was tared and dissolved in 100 ml of the ammonium dihydrogen phosphate/magnesium sulfate buffer used for incubating the repair assay reactants. In order to dissolve the samples, stirring was required for periods of time ranging from an hour to several days. Heating the vessels to 37°C accelerated this solvation of the standards. Stock solutions of all compounds but 0-6 and N-7 methylguanines were stored at -20°C. Standard solutions were prepared fresh daily from frozen stocks. Standard solutions of 0-6 and N-7 methylguanines were prepared on the day of the experiment.

Preparation of 0-6 Methylguanine

First the substrate of 6-chloroguanine hydrochloride was prepared by reacting one gram of 6-chloroguanine (Sigma) with 65 ml of 17% (v/v) hydrochloric acid for 80 minutes at 25°C.

6-Chloroguanine hydrochloride was recovered as a fine yellowish-white powder. In a procedure adapted from Balsiger and Montgomery (1960), 2.9 millimole of the 6-chloroguanine hydrochloride was refluxed with 24 millimoles of sodium methoxide (MC/B) in methanol (20 ml) for eight hours. After cooling, 20 mmole of acetic acid was added. Volatile materials were removed by vacuum. The product was recrystallized in water. The white powder derived from this procedure showed two peaks in chromatographic analysis with the SCX column. The earlier, more water soluble peak coeluted with 6-chloroguanine standard. Recrystallization of the product enhanced the later peak, while a trace of the early peak remained. The late peak coeluted with a genuine sample of 0-6 methylguanine donated by Dr. D.B. Ludlum.

Preparation of Methylated DNA

Calf thymus DNA (grade I, Sigma) was methylated in vitro in the manner of Craddock (1969). In this procedure, 10 ml solutions of DNA (10 mg) and MNNG (30 mg) in 0.02 M sodium phosphate buffer, pH 7.5, were combined and incubated in the dark, with shaking, at 38^{o}C. for 30 minutes. The solution was cooled. DNA was precipitated with 20 ml of 1% cetyltrimethylammonium bromide. It was washed successively with water, 2% (v/v) sodium acetate in 70% (v/v) ethanol overnight in the cold, ethanol, ethanol-ether and ether. The product was dried under nitrogen.

Hydrolysis of this methylated DNA and some of the untreated DNA was performed by a modification of the

technique of Lawley and Thatcher (1970). The methylated and control DNA samples (7.0 and 9.0 micromoles respectively) as well as standards of 0-6 and N-7 methylguanine and quanine were each dissolved overnight in 0.2 ml of water. Twenty microliters of one molar hydrochloric acid were added to the 0.1 ml dissolved samples. The solutions were maintained at 67°C while stirring. The methylated DNA was too viscous to stir. White precipitates were found in the DNA solutions following this procedure. (See Figure 3). Areas under peaks were weighed and compared to derive the amounts of methylated guanines in the DNA. 2.05×10^{-5} Moles of 0-6 methylguanine were calculated (from comparison of Figures 3C and 3D) per mole of DNA-phosphate (DNA-P). All calculations use a molecular weight of 300 for each more of DNA-P. The weight of the 7-methylguanine peak in Figure 3B correlated with a value of 3.8×10^{-4} moles of 7-methylguanine per mole of DNA-P. Dividing the latter value into the former produces an 0-6/N-7 methylation ratio of 0.054. A single preparation of methylated DNA (without cysteine) was made for all experiments in which its use is mentioned.

Procedure for methylation of DNA in the presence of cysteine

DNA was methylated as described in Craddock's (1969) procedure employing cysteine. This procedure is the same as described above except that 1 ml of a 20 mg/ml solution of cysteine (Sigma) in the appropriate buffer was added to the DNA solution immediately before the addition of MNNG. In order to characterize their respective degrees of

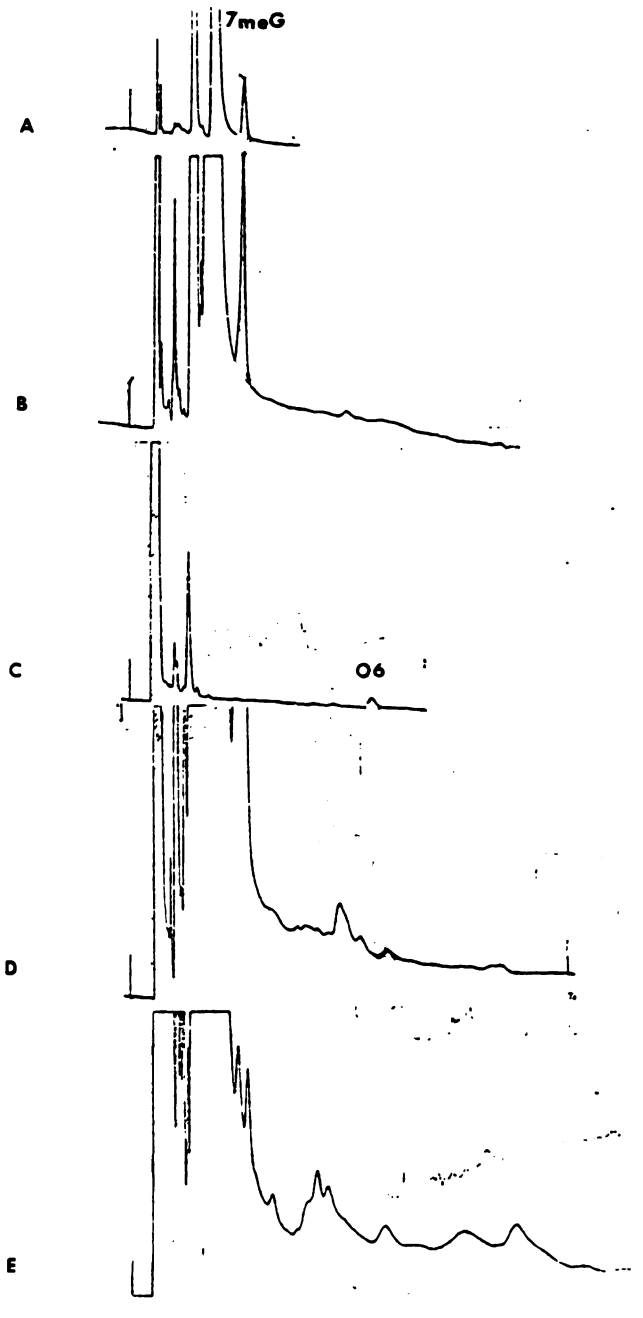


FIGURE 3

Chromatographic separation of samples from hydrolysis (by technique of Lawley and Thatcher, 1970) of DNA methylated without cysteine. A) 2.5 X 10^{-11} Moles of 7-methylguanine standard. B) 11 ul sample of hydrolysate of 7.0 X 10^{-6} moles of methylated DNA in 0.22 ml reaction mixture. C) 5.5 X 10^{-11} Moles of 0-6 methylguanine standard. D) 82 ul of methylated DNA hydrolysate (same as B). E) 70 ul sample of hydrolysate of 9.0 X 10^{-6} moles of normal DNA in 0.22 ml reaction mixture. Chromatography conditions: C_{18} column, eluant: 10 mM ammonium acetate (NH_4OAc), pH 4.22, with 2.5% acetonitrile (CH_3CN), flow rate 1.5 ml/min. Elution volumes of 0-6 methylguanine in C and D are 26.04 and 26.07 ml respectively, while in E the closest peak elutes at 25.62 ml.

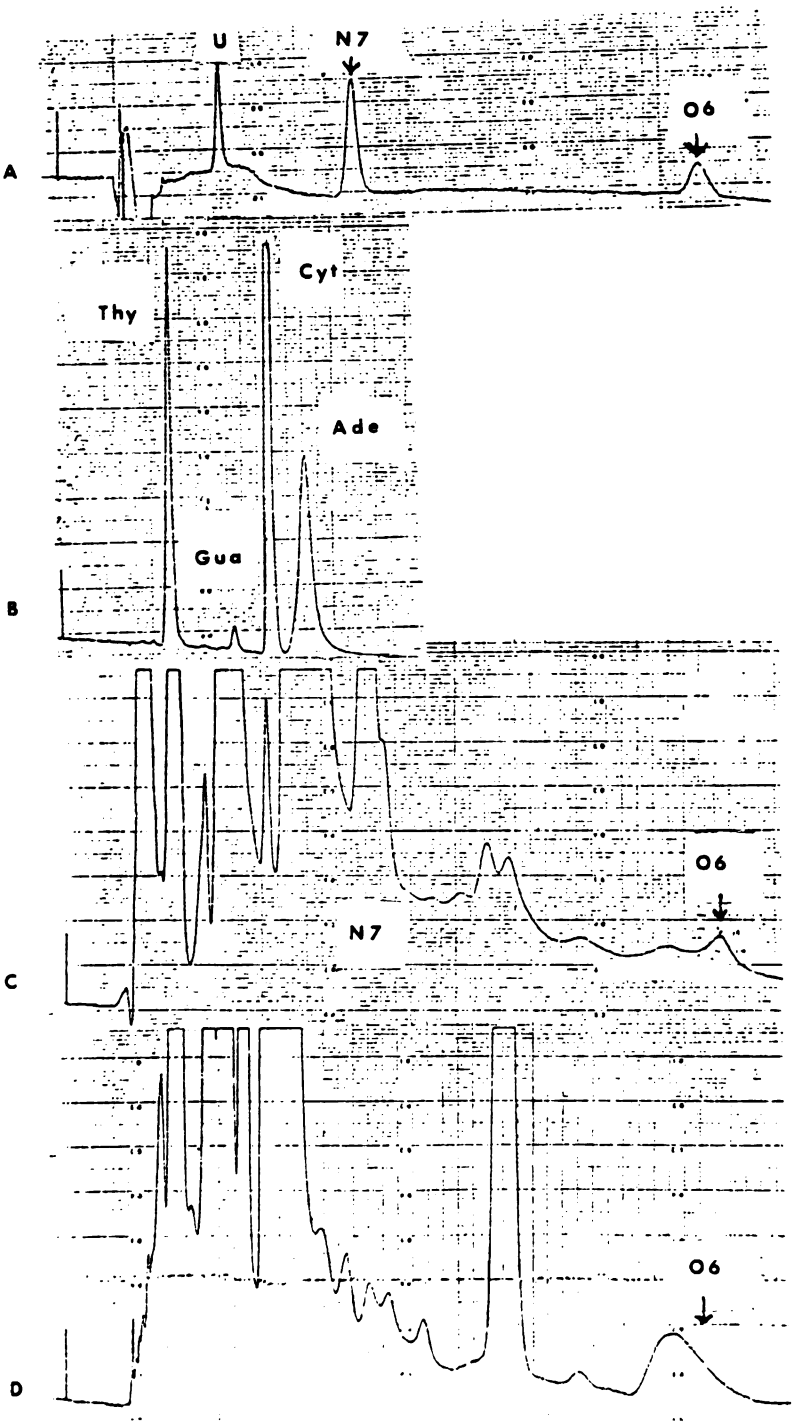
methylation, the methylated and control DNA's were hydrolyzed according to the procedure Lawley and Thatcher (1970) by incubating them over 16 hours in 0.1 M hydrochloric acid at 37°C. A black precipitate formed on the bottoms of the reaction vessels after they were dried over nitrogen. When one ml of 10 mM NH_4OAc at pH 3.85 with 2.5% CH_3CN was added to the tubes to resuspend the samples, the black precipitate remained insoluble. These solutions were chromatographed as illustrated in Figure 4. Yields were again calculated by weighing the peaks. The yield of 0-6 methylguanine increased 200-fold over the yield from the reaction without cysteine to 5.5×10^{-3} moles per mole of methylated DNA-P. This figure contains an adjustment for the 87.4% yield of 0-6 methylguanine in this hydrolysis procedure (Bochert and Webb, 1978). Craddock (1969) found the yield of N-7 methylguanine in this reaction to be 1.4×10^{-2} moles per mole DNA-P (assuming 25% of bases are guanine). This indicates that this product has an 0-6/N-7 methylation ratio of 0.45. A single preparation of DNA methylated in the presence of cysteine and this was used for all experiments in which its use is mentioned.

G. Model Assays

The three techniques including the use of the dialysis incubation cell, the chromatographic separation and the tissue extraction were each developed and well-practiced. The combination of the three and further

FIGURE 4

Chromatographic separation of hydrolysate (see text) of DNA methylated in the presence of cysteine. A) Standards: 1.7 X 10^{-10} Moles of N-7 methylguanine. U signifies peak from previous injection. B) Standards of adenine (Ade), cytosine (Cyt), guanine (Gua) and thymine (Thy). C) 20 ul of hydrolysate of 3.3 millimoles of methylated DNA resuspended in one ml of solvent. D) 20 ul of hydrolysate of 3.3 millimoles of normal DNA resuspended in one ml of solvent (control). Chromatography conditions: C_{18} column, eluant: 10 mM NH_4OAc, pH 3.85, with 2.5% CH_3CN, flow rate 1 ml/min.


A
U
N7
O6
B
Thy
Cyt
Ade
Gua
C
O6
N7
D
O6

refinement was the next step in this project.

A model enzymatic reaction was attempted with an enzyme of known potency to determine whether the system was capable of monitoring such a reaction. The reaction mixture consisted of 0.5 mg/ml of deoxyribonuclease I (DNase, Sigma) and single stranded DNA (prepared by K. Strong) in 0.1 M HEPES, pH 7.5 with 5 mM $MgSO_4$ at 37°C. Standards of 2'-deoxyadenosine monophosphate (dAMP), 2'-deoxycytosine monophosphate (dCMP), and 2'-deoxyguanosine monophosphate (dGMP) were made up in the same buffer as the reaction medium. Thymidine monophosphate (TMP) of satisfactory purity was not available. The chromatographic separation was performed on the C_{18} column with a solvent of 0.1 M HEPES, pH 6.8, flowing at 1 ml/min.

A chromatogram of dialysate of DNase I plus crude liver cell extract is shown in Figure 5. The multiplicity of unidentified peaks illustrates the difficulties of working with crude organ extracts. After a few such attempts and procedural modifications (including the use of the heating strip as described above), it was found that the assembled apparatus could indeed be used to monitor this enzyme reaction. In Figure 6 and 7, the resulting chromatograms are illustrated. Note the relative multiplicity of peaks in the DNase digest of methylated DNA (Figure 7) compared to the digest of normal DNA (Figure 6). The appearance of such small peaks in both digests which were disproportionate to the amount of DNA substrate employed,

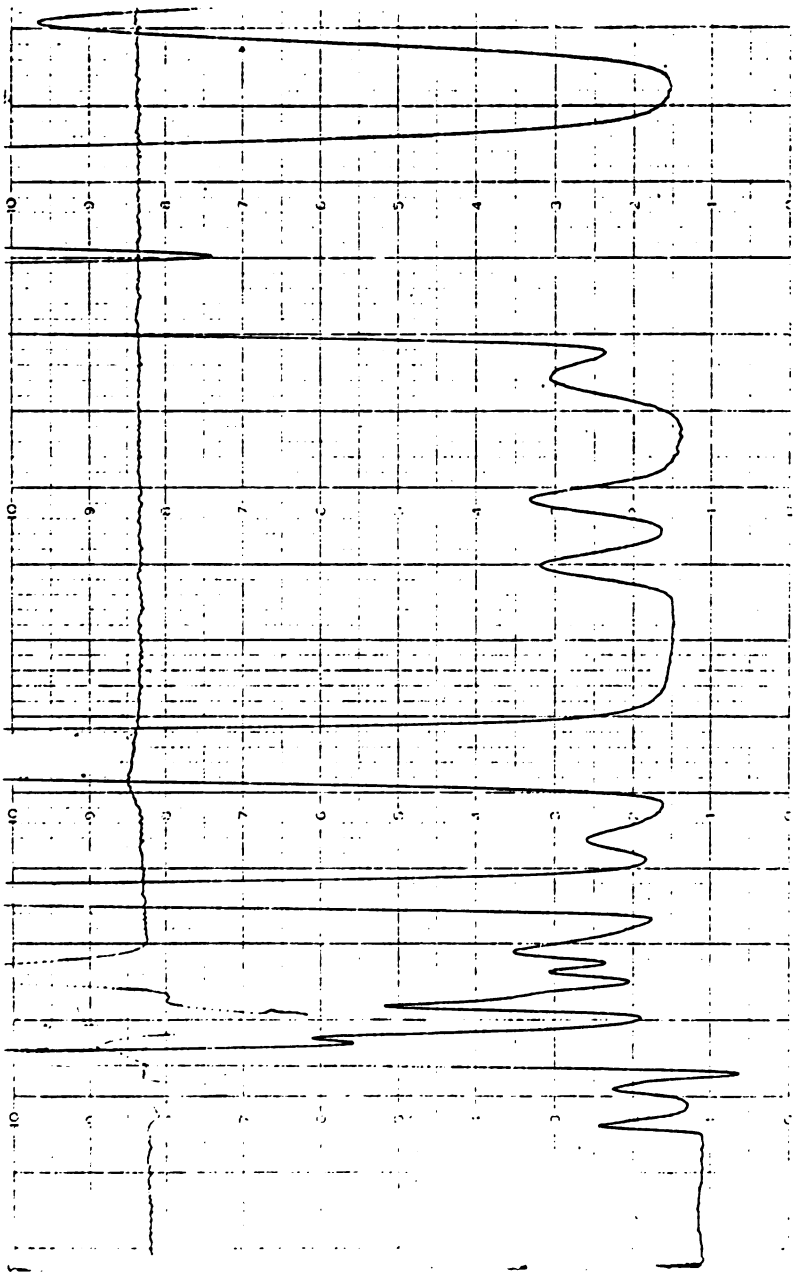


FIGURE 5

Chromatographic separation of 10 ul of 65 minute dialysate of 1 mg/ml DNase I plus crude liver cell extract II incubated in 0.1 M HEPES with 5 mM $MgSO_4$, pH 7.5. Chromatography conditions: C_{18} column, solvent: 0.1 M HEPES, pH 6.8, flow rate 1 ml/min. Chromatography terms and the significance of this chromatogram are discussed in the text.

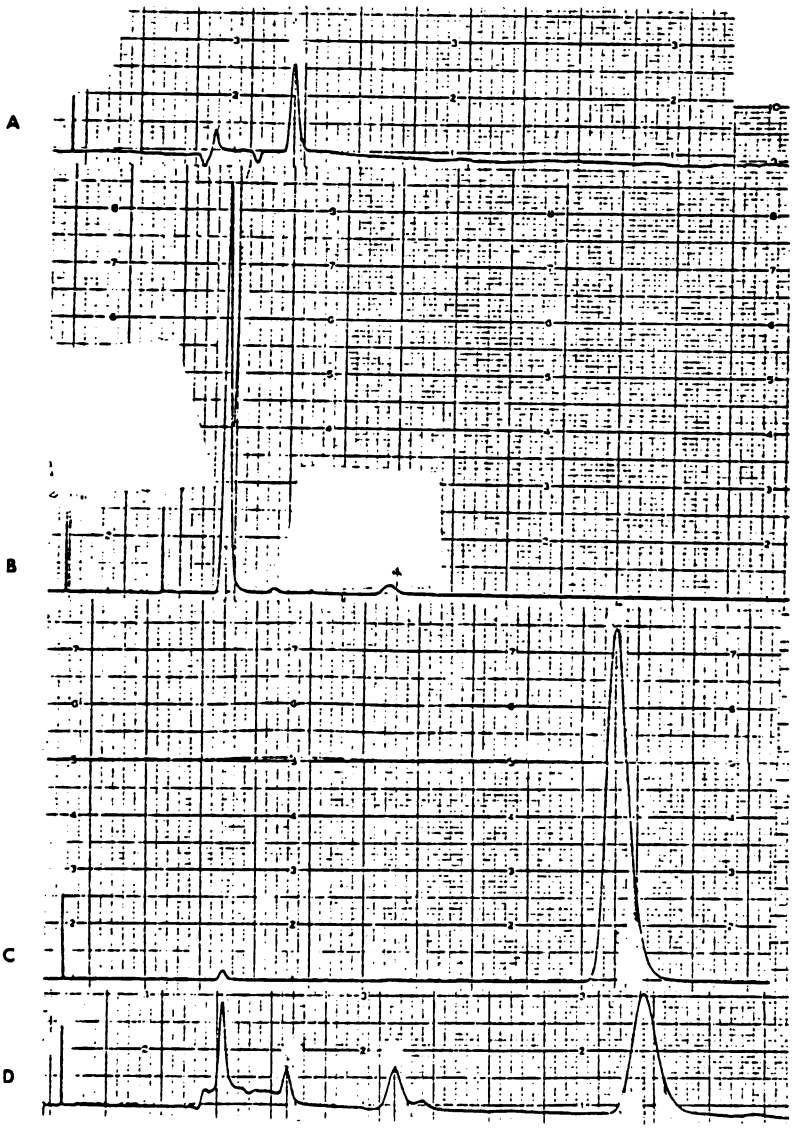


FIGURE 6

Chromatographic separation of dialysates of 5 X 10^{-4} M DNA-P plus 0.55 mg/ml of DNase I incubated in 0.1 M HEPES, pH 7.5 with 2.5 mM $MgSO_4$ at 38.5°C. A) Control-incubation meida only. B) 6.3 X 10^{-6}grams dCMP standard. 1.0 absorbance units represented by full scale pen deflection (AUFS). C) 1.1 X 10^{-5} grams dAMP standard. 1.0 AUFS. D) 10 ul of 83 minute DNA/DNase I dialysate. 0.01 AUFS. Chromatography conditions: C_{18} column, solvent: 0.1 M HEPES, pH 6.8, flow rate 1 ml/min.

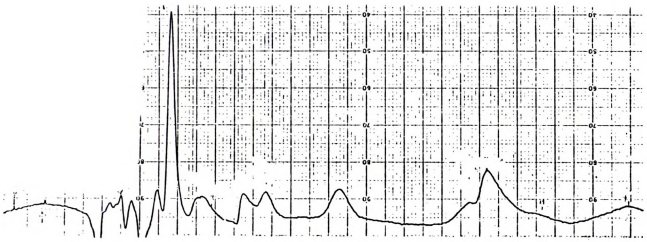


FIGURE 7

Chromatographic separation of 100 ul sample of 230 minute dialysate of digest of 0.190 mg/ml of methylated DNA plus 1.0 mg/ml DNase I in 0.1 M HEPES, pH 7.5 with 5 mM $MgSO_4$. Chromatographic conditions: C_{18} column, flow rate 1 ml/min.

even with the detector at the highest sensitivity setting, accompanied by a multiplicity of late peaks (not shown) was a baffling result initially. The problem was not in the dialysis step since a mixture of deoxyribonucleotide standdards dialysed quantitatively and without difficulty. (See Figure 8.) A closer examination of the literature concerning DNase helped to solve this puzzle. The products of exhaustive digestion of DNA by DNase I are: deoxyribonucleotides, 5%; deoxyribodinucleotides, 60% and deoxyribotrinucleotides, 25% (Vanecko and Laskowski, 1961). The dialysis cell allowed the effective diffusion of the smaller enzymic reaction products while restricting larger deoxyribonucleotide fragments as well as the enzyme from dialysing.

In order to effect a higher yield of mononucleotides in this model system for the enzymatic degradation of DNA, an additional enzyme, snake venom phosphodiesterase ((DNase II), was employed. This enzyme is an endonuclease while DNase I is an exonuclease. The DNase I reaction was performed at pH 7.5. Then a DNase II solution with appropriate buffer to change the reaction solution to a pH of 8.8 was added. This procedure was attempted twice with only a limited degree of success. Attempting to change the pH of the mixture in the dialysis chamber may have been the cause of the poor results.

The effectiveness of the method was proven by monitoring the DNase I/DNA reaction successfully. With proficiency in

FIGURE 8

Chromatographic separation of 10 ul of 95 minute dialysate of deoxyribonucleotide standards in 0.1 M HEPES, pH 6.7. Chromatography conditions: C_{18} column, eluant 0.1 M $NH_4H_2PO_4$, pH 3.95 flow rate 1 ml/min.

the operation of each of the instruments and each of the techniques well practiced, the combination of techniques through the incubation of methylated DNA with a tissue extract was attempted. The liver was chosen as the first trial tissue because it has high 0-6 methylguanine repair activity in mice (Buecheler and Kleihues, 1977) and hence should be the most easily assayable organ for this activity. Furthermore, the liver is a relatively large organ necessitating the killing of a minimal number of mice for the experiments. The liver is also convenient since it is easily minced and homogenized due to its relatively low amount of connective tissue compared to many other organs.

Dialysates of both crude extracts provided unsatisfactory chromatograms. The dialysates produced large peaks obliterating any other UV absorption peaks which may have appeared from methylated bases in the chromatographic separation. (See Figure 5.) This was not due to a failure of the dialysis chamber since the dialysates remained transparent despite the presence of the brown, particulate-laden fluid on the other side of the membrane. The extraction procedure was hence modified to include perfusion of the animal and washing of blood from the liver cell suspension. Blood may cause interferences due to high amounts of proteolytic enzymes (which are expected to destroy excision enzyme activity) and high amounts of adenine derivatives (Miech and Tung, 1970) which are expected to yield peaks obliterating chromatographic spectra. The purified liver cell extract II had a considerable

reduction in background peaks (not shown).

A preliminary test was performed to examine the sensitivity of the apparatus to minute quantities of methylated bases. Purified liver cell extract I was predialysed. Then a mixture of methylated base standards including 6.7 nanomoles of 0-6 methylguanine was added to the extract in the sample chamber of the dialysis cell. Within 30 minutes, the presence of 6.7×10^{-9} moles of 0-6 methylguanine in the sample chamber was detected in a 100 ul sample of the dialysate by UV absorbance following chromatographic separation (see Figure 9). This sensitivity could have been improved by taking a larger aliquot and by allowing a longer diffusion time. Assuming a 10^{-4} conversion of guanine to 0-6 methylguanine in the methylated DNA (see section F) 2.0×10^{-5} moles of methylated DNA (about the capacity of the 2.5 ml sample chamber) should yield about 2.0×10^{-9} moles of 0-6 methylguanine following 100% excision by the liver extract. Hence, the results of the above experiment suggest that low level 0-6 methylguanine excision from DNA may not be detectible with this system.

These considerations were explored in the following experiments where methylated DNA and purified liver cell extract I were dialysed to find barely detectible peaks which coeluted with 0-6 methylguanine standards. The purified liver extract I was predialysed. Methylated DNA (2.3 micromoles) was added to the heated incubation chamber. Dialysates were injected into the U6K injector. No sign of 0-6 methylguanine

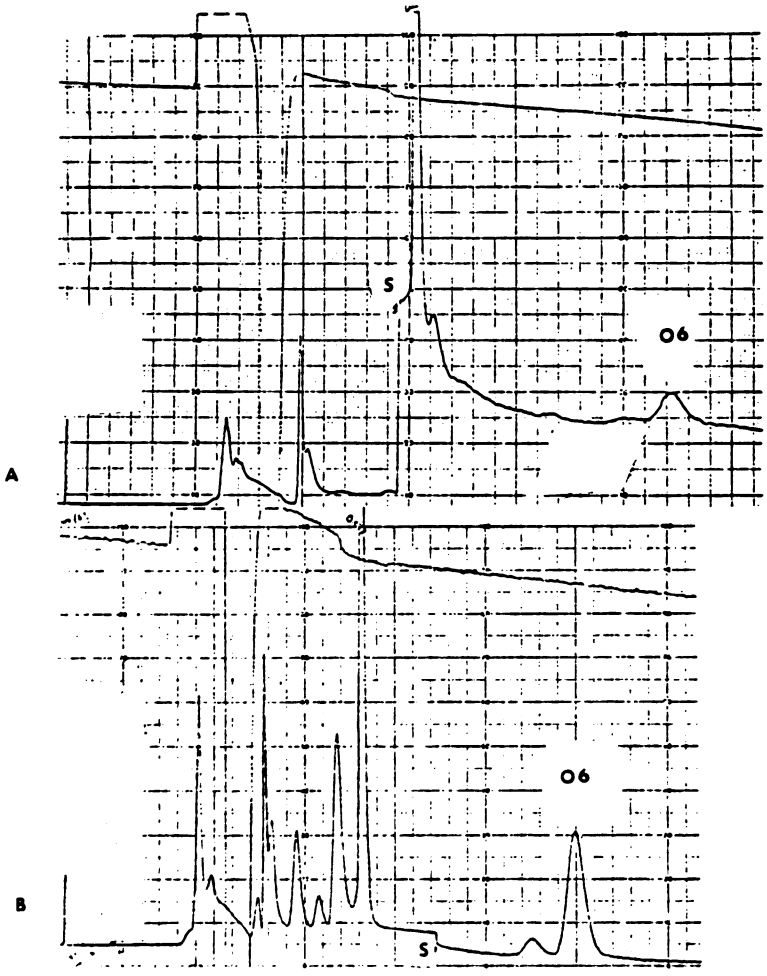


FIGURE 9

Chromatographic separation of A) 100 ul of 30 minute dialysate of mixture of standard nucleic acid bases and methylated purines incubated with purified liver cell extract I. 6.7 X 10^{-9} Moles of 0-6 methylguanine were present in the mixture. B) mixture of standards without treament. Chromatography conditions: C_{18} column, eluant 0.010 M $NH_4H_2PO_4$, pH 4.0, flow rate 2 ml/min. The chromatograms have been reduced by different degrees. S denotes change in detector sensitivity.

appeared in early samples. After 97 minutes, the entire dialysate (1.5 ml in volume) was injected into the U6K injector. A small blip, representing a fraction of 0.001 absorbance unit, eluted at a position corresponding to 0-6 methylguanine. This encouraging result was shadowed by the fact that this peak was but a shoulder on a much larger peak defying quantitation. Furthermore, the injection of such a large volume to find such a small peak causes poor sensitivity in the assay. Tissues less capable of this repair could not be studied with this assay since they would cause release of 0-6 methylguanine well below the limit of detection.

The next step toward better sensitivity was to increase the amount of methylated DNA substrate. Up to five milligrams of methylated DNA was dissolved in one milliliter of buffer and triturated five times through a 22 gauge needle by syringe. The trituration was performed in order to reduce the viscosity of the solution while accommodating a greater amount of substrate.

Other experimental design improvements were made to facilitate enzymic activity. At this time, the incubation and predialysis medium as well as standard solutions were changed to 0.28 M Tris-HCl buffer,(pH 7.22 at 37^{o}C)with 5 mM magnesium sulfate. In spite of these improvements, again only a small blip appeared at the 0-6 methylguanine elution point as a shoulder on another peak. A large peak appeared at the 7-methylguanine elution point.

H. Reassessement and Revision of Technique

In a given dialysis experiment using 1.7×10^{-5} moles of methylated DNA as a substrate, the highest amount of 0-6 methylguanine that could be released is 3.4×10^{-10} moles. At diffusion equilibrium, this would be dissolved in the 5 ml volume constituting the sample and dialysate compartments of the dialysis cell. The detection limit for quantitative measurements with the Waters absorbance detector with the 254 nm filter is 1.2×10^{-11} moles for 0-6 methylguanine and 1.2×10^{-12}moles for 7-methylguanine. Assuming total 0-6 methylguanine excision from a 1.7×10^{-5} mole methylated DNA substrate, the smallest aliquot which would be taken from the dialysate compartment which contain a detectible amount of the excised base is 440 ul. It is necessary to have 0-6 methylguanine released in quantities well above the detection limits in the repair proficient cell populations in order to quantitatively assay various tissues with deficient repair capacities.

Three possible approaches toward improving sensitivity were devised. Increasing the amount of methylated DNA substrate and the amount of methylation within the DNA is one such possible approach. However, there is a limit to how concentrated a DNA solution should be used. The methylated DNA must be well dissolved (i.e., the solution is completely clear with no visible bubbles or gel bodies) before beginning the incubation. The highest concentration which may be used in a DNA solution without causing excess viscosity is

suggested to be approximately 5 mg/ml with trituration (Dr. J.J. McCormick, personal communication). A higher degree of methylation was deemed feasible on a moderate level by using Craddock's (1969) technique of adding cysteine to the methylation reaction solution (described above.) This procedure was carried out. Increasing the amount of methylating agent in the reaction mixture is not a feasible alternative since the procedure already involves a vast excess of MNNG.

The detector sensitivity could also be improved. Herron and Shank (preprint of an article accepted for Analytical Biochemistry) quantitatively detect 0-6 methylguanine to lower limits of 9×10^{-13} moles and 7-methylguanine to 4.2×10^{-11} moles with a Farrand fluorescence detector. With this type of detector, a complete excision of the 0-6 methylguanine from a solution containing 5 mg of DNA methylated in the presence of cysteine (described and characterized above) could be detected in 32 microliters (ul) of dialysate at equilibrium.

The Farrand fluorescence detector appears to be the detector best suited for this type of experiment. The construction achieves such sensitivity through a combination of a 150 watt xenon lamp source, a excitation monochromator, a 10 ul quartz flow cell, emission bandpass filters and photomultiplier detection. Mercury lamps which have a weak emission in the range of 295 nm are totally unsuitable since 0-6 methylguanine is excited at 295 nm (See Figure 10).

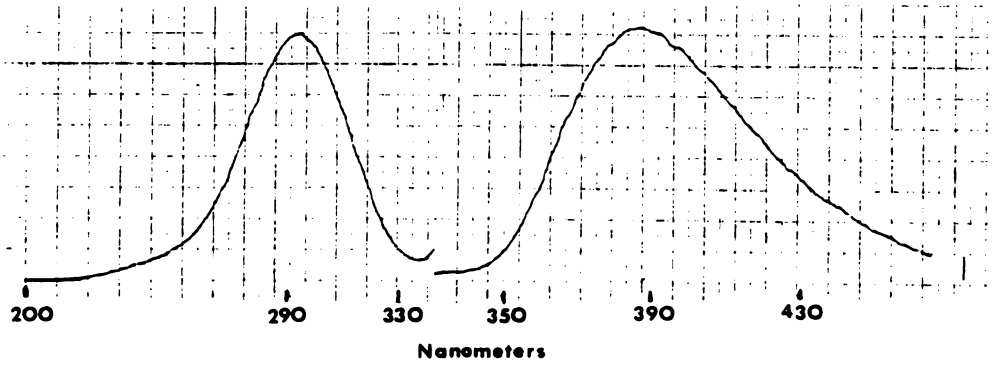


FIGURE 10

Fluorescent absorption and emission spectra of 0-6 methylguanine in 10 mM NH_4OAc at pH 4 with 2.5% CH_3CN. Taken on an Aminco-Bowman spectrophotofluorometer.

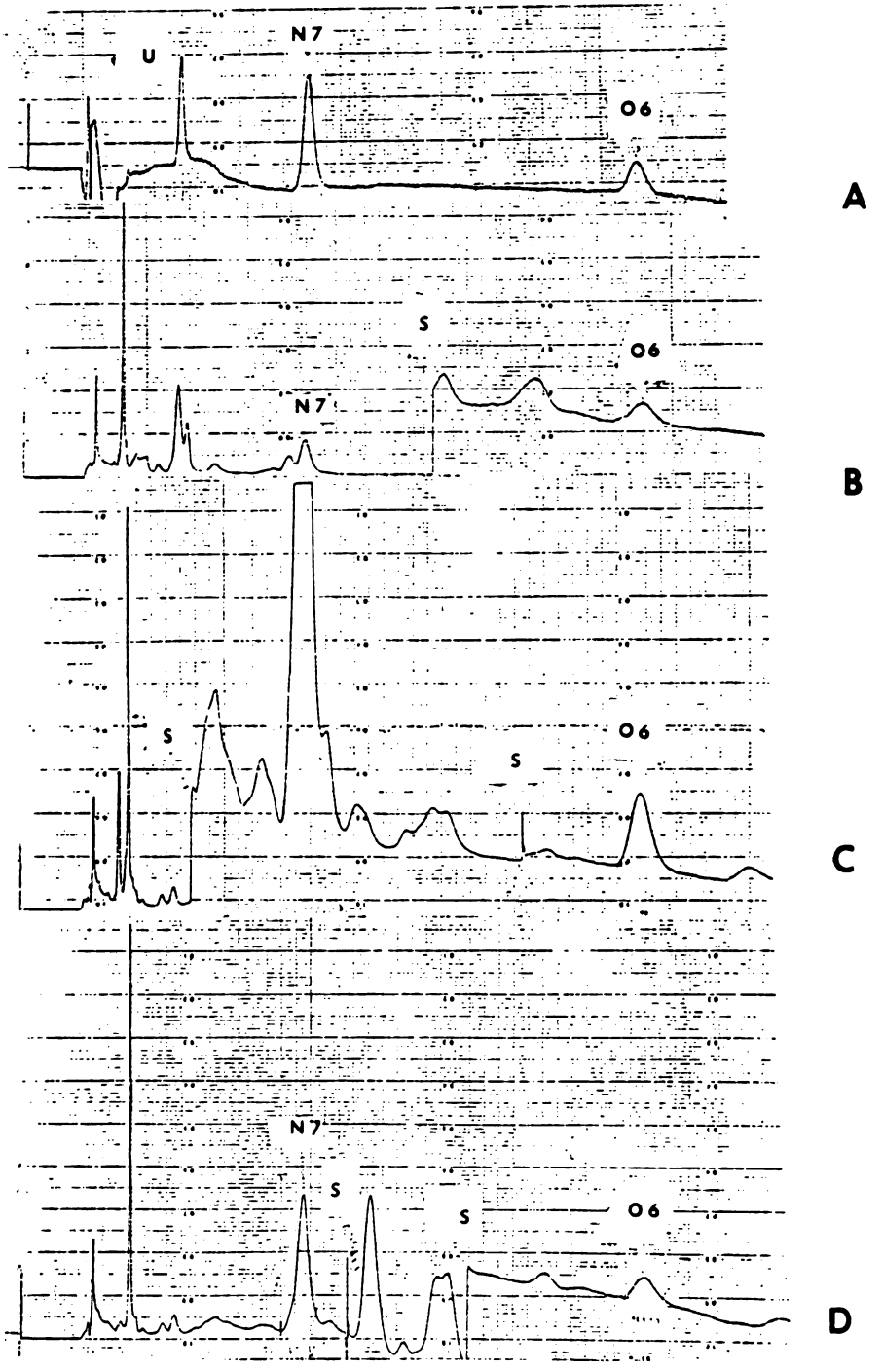
A third approach to increasing sensitivity is the elimination of all steps in the analysis which may dilute the 0-6 methylguanine product yield, including dialysis. This signifies a radical revision of the experimental design. The tissue extracts must be prepared free of blood and cells and clear of all suspended precipitates as outlined in the purified cell extract II procedure section.

A revised procedure was formulated which took advantage of these considerations. An aliquot of the purified liver cell extract II is added to incubation vessels containing solutions of normal DNA, methylated DNA, a mixture of methylated DNA and methylated guanine standards, and a control with solvent only at 37°C. Samples are removed from the tubes over time and precipitated with a 4% incremental volume of 95% ethanol. The samples are then Millipore filtered and chromatographed.

This procedure allows rapid kinetics to be observed. (The dialysis chamber required 45 minutes to reach a fraction of equilibrium concentration in the dialysate). Some results are shown in Figure 11. The peaks for the substituted guanines are substantial. An 0-6 methylguanine peak is produced from methylated DNA incubated with purified liver cell extract II (Figures 11C and 11D) which is not found in the normal DNA/extract incubation mixture (not shown) nor in the extract alone. The elution of these peaks at the same volume as similarly treated standards provides evidence that 0-6 and N-7 methylguanines are excised. The appearance of

FIGURE 11

Chromatographic separation of reaction mixtures containing methylated (in the presence of cysteine) DNA and purified liver cell extract II as described in text. A) Standards: 1.73×10^{-10} moles of 0-6 methylguanine (0-6) and 1.09×10^{-10} moles of 7-methylguanine (N7). B) 195 ul sample from 1.65 ml meaction mixture containing 3.7×10^{-6} moles methylated DNA-P and 1.09×10^{-9} moles of 7-methylguanine standard and 1.7×10^{-9} moles of 0-6 methylguanine standard. This was incubated for 25 minutes at 37°C. Amount of 0-6 methylguanine represented by peak is less than amount of standard which was originally present indicating that some degradation of this substance has occurred. C) 100 ul sample from 1.65 ml reaction mixture containing 1.62×10^{-5} moles of methylated DNA after 16 minute incubating. D) 99 ul of methylated DNA incubated for 40 minutes (same mixture as C). 0-6 methylguanine peak decreases from C to D. U denotes peak from previous injection. S denotes shift in detector sensitivity.


U
N7
O6
A
S
O6
N7
B
S
O6
S
C
N7
S
S
O6
D

a nearby peak in the DNA/extract incubation mixture (not shown) leaves the exact identity of the 0-6 methylguanine peak in question. Although critical peaks were collected, dried, redissolved in eluant and rechromatographed with the SCX column, the concentrations were too low to be detected by these techniques.

In these experiments another interesting phenomenon was observed. The peak corresponding to 0-6 methylguanine in both the methylated DNA digest and the methylated DNA with methylguanine standards digest appeared to reach a maximum within one-half hour of incubation. As seen by comparing Figures 11C and 11D, the area of the peak appeared to decrease in samples incubated for longer periods of time. This occurred to such a degree that 0-6 methylguanine levels in the standard-containing digest fell below the original micromolar concentration of standard which was added to the digest (See Figure 11B). (Note that N-7 methylguanine concentrations remained constant. This does not appear clearly due to inconsistency in absorbance meter scale amplification changes). This suggests that a glycosylytic base excision is followed by decomposition within the tissues. This may explain Pegg's (1978b) conclusion that 0-6 methylguanine is not removed intact following the reaction of rat liver extracts with methylated DNA <u>in vitro</u>.

CHAPTER THREE

SUMMARY

A model system has been developed to compare and assay the capacity of different organs and tissues for the removal of methylation lesions from DNA. Early results indicate that there is a hydrolytic excision and subsequent degradation of 0-6 methylguanine from methylated DNA when incubated with cell-free mouse liver extracts. While others have argued against this mechanism, their techniques may not have detected such a transient intermediate. This two-step process may be related to the two types of 0-6 methylguanine repair observed by Jeggo, et al. (1977), Schendel and Robins (1978) and Robins and Cairns (1979) in E. coli.

CHAPTER FOUR

RECOMMENDATIONS

The experiments indicating 0-6 methylguanine excision should be repeated with better quantitation. A protein assay and an assay for lytic enzymes in the extract would be desirable. Placing 0-6 methylguanine standard into the control DNA reaction mixture will help to determine that the nearby peaks found in this reaction mixture are distinct from the genuine 0-6 methylguanine peak. Larger peaks should be collected from the methylated DNA incubation mixture for chromatography on other columns in order to confirm that the substance coeluting with 0-6 methylguanine will do so under a variety of conditions. More detailed studies of time dependence and degree of methylation dependence of 0-6 methylguanine excision are in order. Other tissues than liver, including the repair-deficient brain, as well as tumors, should be observed for their 0-6 methylguanine excision propperties. Extracts from animals with chronic exposure to methylating carcinogens should be observed in order to determine if excision capacity can be induced.

The parallel experiment with cis-platination repair should be done. In this experiment, in vitro, cis-platinated DNA incubated with a tissue extract is separated and quantitated with chromatographic methods. Standards of N-7/0-6

chelate with platinum will be needed for the study in order to identify the excised base-metal adduct. Again tissues will be compared for repair capacity with platinum-susceptible tumor cells. Cultured cells may also be examined for platination repair capacity. Of particular interest are repair deficient cell lines such as Xeroderma pigmentosum as well as the L1210 platinum-susceptible and resistant cell lines. Other incubation conditions may be varied to determine their importance in the excision reaction. Such variables include specific ion concentrations, and ionic strength of the incubation medium. Incubation temperature may prove to be a particularly important variable. It has been reported that small temperature differences can determine DNA repair and survival in radiation damaged E. coli (R. Shenkar, Ph.D. Thesis, Michigan State University, 1979).

BIBLIOGRAPHY

BIBLIOGRAPHY

Ames, B.N., Lee, F.D., Durston, W.E., "An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens", Proc. Nat. Acad. Sci. (USA), 70:782-786 (1973).

Andersen, K.S., "Platinum (II) Complexes Generate Frame Shift Mutations in Test Strains of Salmonella Typhimurium", Mutat. Res., 67:209-214 (1979).

Balsiger, R. W., Montgomery, J.S., "Synthesis of Potential Anticancer Agents. XXV. "Preparation of 6-Alkoxy-2-amino Purines", Journal of Organic Chemistry, 25:1573-1575 (1960).

Beck, D.J., Brubaker, R.R., "Mutagenic Properties of cis-Platinum (II) Diamminedichloride in Escherichia coli", Mutat. Res., 27:181-189 (1975).

Benedict, W.F., Baker, M.S. Haroun, L., Choi, E., Ames, B.N., "Mutagenicity of Cancer Chemotherapeutic Agents in the Salmonella/Microsome Test", Cancer Res., 37:2209-2213 (1977).

Bochert, T.G., Webb, J., "The Use of Liquid Ion-Exchange Chromatography for the Determination of Alkylated Nucleic Acid Bases in Maternal and Fetal Tissues", Methods in Prenatal Toxicology, Prenatal Workshop Paper, pp. 456-464, Neubert, D., et al., eds. (1977).

Bodell, W.J., Singer, B., Thomas, G.H., Cleaver, J.E., "Evidence for Removal at Different Rates of O-Ethyl-pyrimidines and Ethylphosphotriesters in Two Human Fibroblast Cell Lines', Nucleic Acids Research, 6:2819-2829 (1979).

Boveri, T., Origin of Malignant Tumors (translated by M. Boveri), Williams and Wilkins: Baltimore (1929).

Buecheler, J., Kleihues, P., "Excision of O^6-Methylguanine from DNA of various Mouse Tissues Following a Single Injection of N-Methyl-N-Nitrosourea", Chem.-Biol. Interactions, 16:325-333 (1977).

Carter, C.E., Greenstein, J.P., "Studies on the Enzymatic Degradation of Nucleic Acids", Journal of the National Cancer Institute, 7:29-45 (1946).

Christiensen, H.D., Whitsett, T.L., "Measurements of Xanthines and their Metabolites by Means of HPLC", Biological/ Biomedical Applications of Liquid Chromatography, pp. 507-537, Hawk, G.L., ed., Dekker: N.Y. (1979).

Clark, J.M., Jr. ed., Experimental Biochemistry, Chapter 28, "Partial Characterization of DNA", San Francisco, W.H. Freeman, (1964).

Cleare, J.J., Hoeschele, J.O., "Studies on the Antitumor Activity of Group VIII Transition Metal Complexes. Part 1. Platinum (II) Complexes", Bioinorganic Chem. 2:187-210 (1973).

Cleaver, J.E., "Defective Repair Replication of DNA in Xeroderma Pigmentosum", Nature (Lond.), 218:652-656 (1968).

Cleaver, J.E., "DNA Repair with Purines and Pyrimidines in Radiation and Carcinogen-Damaged Normal and XP Human Cells", Cancer Res., 33:362-369 (1973).

Connors, T.A., Jones, M., Ross, W.C.J., Braddock, P.D., Khokhar, A.R., Tobe, M.L., "New Platinum Complexes with Antitumor Activity", Chem-Biol. Interactions,5:415-424 (1972).

Craddock, V.M., "The Reaction of N-Methyl-N'-Nitro-N-Nitroso-guanidine With Deoxyribonucleic Acid", Biochemical Journal, 106:921-922 (1968).

Craddock, V.M., "Study of the Mechanism and Lack of Deamination of Deoxyribonucleic Acid by N-methyl-N'-nitro-N-nitroso guanidine", Biochemical Journal, 111:615-620 (1969).

Craddock, V.M., "The Pattern of Methylated Purines Formed in DNA of Intact and Regenerating Liver of Rats Treated with the Carcinogen Dimethynitrosamine", Biochimica et Biophysica Acta, 312:202-210 (1973).

Craddock, V.M., Frei, J.V., "Induction of Liver Cells Adeno-mata in the Rat by a Single Treatment with MNU Given at Various Times After Partial Hepatectomy", Br. J. Cancer, 30:503-511 (1974).

Day, R.S., Scudiero, D., Dimattina, M., "Excision Repair by Human Fibroblasts of DNA Damaged by r-7, t-8-Dihydroxy-t-9, 10-Oxy-7,8,9,10-Tetrahydrobenzo (a) Pyrene", Mut. Res., 50:383-394 (1978).

Day, R.S., III, Ziolkowski, C.H.J., Scudiero, D.A., Meyer, S.A., Mattern, M.R., "Human Tumor Cell Strains Defective in the Repair of Alkylation Damage", Carcinogenesis, 1:21-32 (1980).

Dehand, J., Jordanov, J., "Interaction of cis-Diaminotoluene-platinum (II) with Nucleosides: Evidence for Guanosine O(6)·N(7) Chelation by Platinum", Journal of the Chemical Society, London, Chem. Communications, 15:598-599 (1976).

Fraval, H.N.A., Rawlings, C.J., Roberts, J.J., "Increased Sensitivity of UV-Repair Deficient Human Cells to DNA Bound Platinum Products", Mut. Res.,51:121-132 (1978).

Fraval, H.N.A., Roberts, J.J., "Excision Repair of cis-Diamminedichloro-platinum (II)-Induced Damage to DNA of Chinese Hamster Cells", Cancer Res., 39:1793-1797 (1979).

Frei, J.V., Swenson, D.H., Warren, W., Lawley, P.D., "Alkylation of Deoxyribonucleic Acid in vitro in Various Organs of C57BL Mice by the Carcinogens N-Methyl-N-Nitrosourea, N-Ethyl-N-nitrosourea and Ethylmethanesulphonate in Relation to Induction of Thymic Lymphoma", Biochemical Journal, 174:1031-1044 (1978).

Friedberg, E.C., Cook, K.H., Duncan, J., et al., "DNA Repair Enzymes in Mammalian Cells", Photochem. Photobiol. Rev. 2:263-322 (1977).

Gerchman, L.L., Ludlum, D.B., "The Properties of O^6-Methylguanine in Templates for RNA Polymerase", Biochimica et Biophysica Acta, 308:310-316 (1973).

Goodgame, D.M.L., Jeeves, I., Phillips, F.L., Skapski, A.C., "Possible Mode of Action of Anti-Tumor Platinum Drugs-X-Ray Evidence for cis Binding by Platinum of Two Inosine-5'-Monophosphate Molecules via the N(7) Positions", Biochim. Biophys. Acta., 378:153-157 (1975).

Goth, R., Rajewsky, M.F., "Persistence of 06-Ethylguanine in Rat Brain DNA: Correlation with Nervous System-Specific Carcinogenesis by Ethylnitrosourea", Proc. Nat. Acad. Sci. (USA), 71(3):639-643 (1974a).

Goth, R., Rajewsky, M.F., "Molecular and Cellular Mechanisms Associated with Pulse-Carcinogenesis in the Rat Nervous System by Ethylnitrosourea: Ethylation of Nucleic Acids and Elimination Rates of Ethylated Bases from the DNA of Different Tissues", Z. Krebsforsch,82:37-64 (1974b).

Goth-Goldstein, R., "Repair of DNA Damaged by Alkylating Carcinogens is Defective in Xeroderma Pigmentosum-derived Fibroblasts", Nature, 267:81-82 (1977).

Hendler, S., Furer, E., Srinivasan, P.R., "Synthesis and Chemical Properties of Monomers and Polymers Containing 7-Methylguanine and Investigation of Their Substrate of Template Properties for Bacterial DNA or RNA Polymerases", Biochemistry, 9:4141-4152 (1970).

Heston, W.E., "Occurrence of Tumors in Mice Injected Subcutaneously with Sulfur Mustard and Nitrogen Mustard and Nitrogen Mustard", J. Nat. Cancer Inst., 14:131-140 (1953).

Horvath, C.G., Lipsky, S.R., "Peak Capacity in Chromatography", Analytical Chemistry, 39:1893 (1967).

Jeggo, P., Defair, M., Samson, L., Schendel, P., "An Adaptive Response of E. coli to Low Levels of Alkylating Agent; Comparison with Previously Characterized DNA Repair Pathways", Molecular and General Genetics,157:109 (1977).

Kleihues, P., Bamborschke, S., Doerjer, G., "Persistence of Alkylated DNA Bases in the Mongolian Gerbil (Meriones Unguiculatus) Following a Single Dose of MNU", Carcinogenesis, 1:111-113 (1980).

Kleihues, P., Margison, G.P., "Carcinogenicity of N-Methyl-N-Nitrosourea: Possible Role of Excision Repair of 06-Methylguanine from DNA", Journal of the National Cancer Institute, 53(6):1839-1841 (1974).

Kleihues, P., Margison, G.P., "Exhaustion and Recovery of Repair Excision of 06-Methylguanine from Rat Liver DNA," Nature, (London), 259:153-155 (1976).

Kociba, R.J., Sleight, S.D., Rosenberg, B., "Inhibition of Dunning Ascitic Leukemia and Walter 256 Carcinosarcoma with cis-Diamminedichloroplatinum (NSC-119875)", Cancer Chemother. Rep., 54:325-328 (1970).

Kornblith, P.L., Szypko, P.E., "Variation in the Response of Human Brain Tumors to BCNU in vitro", J. Neurosurg., 48:580-586 (1978).

Lawley, P.D., "Methylation of DNA by Carcinogens: Some Applications of Chemical Analytical Methods", Screening Tests in Chemical Carcinogenesis, Montesano, R., Burtsch, H., Tomatis, L., eds., International Agency for Research on Cancer, Lyon, pp. 181-210 (1976a).

Lawley, P.D., "Carcinogenesis by Alkylating Agents", Chapter 4, Chemical Carcinogens, pps. 83-244, Searle, C.E., ed., ACS Monograph 173, Washington, American Chemical Society (1976b).

Lawley, P.D., Thatcher, C.J., "Methylation of DNA in Cultured Mammalian Cells by MNNG", Biochemical Journal, 116:693-707 (1970).

Lecointe, P., Macquet, J.P., Butour, J.L., "Correlation Between the Toxicity of Platinum Drugs to L-1210 Leukemia Cells and Their Mutagenic Properties", Biochem. Biophys. Res. Commun., 90(1): 209-213 (1979).

Lecointe, P., Macquet, J.P., Butour, J.L., Paoletti, C., "Relative Efficiencies of a Series of Square-Planar Platinum (II) Compounds on Salmonella Mutagenesis", Mutat. Res., 48:139-144 (1977).

Leonard, B.J., Eccleston, E., Jones, D., Todd, P., Walpole, A., "Antileukemic and Nephrotoxic Properties of Platinum Compounds", Nature, 234:43-34 (1971).

Leopold, W.R., Miller, E.C., Miller, J.A., "Carcinogenicity of Antitumor cis-Platinum (II) Coordination Complexes in the Mouse and Rat", Cancer Res., 39:913-918 (1979).

Loveless, A., "Possible Relevance of 0-6 Alkylation of Deoxyguanosine to the Mutagenicity and Carcinogenicity of Nitrosamines and Nitrosamides", Nature, 223:206-207 (1969).

Ludlum, D.B., "Methylated Polydeoxyribocytidylic Acid Templates for RNA Polymerase", Biochim. Biophys. Acta, 247:412-418 (1971).

Macquet, J.P., Theophanides, T., "DNA-Platinum Interactions in vitro with cis- and trans-$Pt(NH_3)_2Cl_2$", Bioinorganic Chemistry, 5:59-66 (1975).

Maher, V.M., Curren, R.D., Ouellette, L.M., McCormick, J.J., "Effects of DNA Repair on the Frequency of Mutations of Induced in Human Cells by Ultraviolet Irradiation and Chemical Carcinogens", in Fundamentals of Cancer Prevention, eds., Magee, P.N. et al., University Park Press: Baltimore, pp. 363-382 (1976).

Margison, G.P., Kleihues, P., "Chemical Carcinogenesis in the Nervous System - Preferential Accumulation of 06-Methylguanine in Rat Brain DNA During Repetitive Administration of MNU", Biochem. J., 148:521-525 (1975).

Margison, G.P., Margison, J.M., Montesano, R., "Methylated Purines in the DNA of Various Syrian Golden Hamster Tissues after Administration of a Hepatocarcinogenic Dose of DMN", Biochemical Journal, 157(3): 627-634 (1976).

Margison, G.P., O'Connor, P.J., "Biological Implications of the Instability of the N-Glycosidic Bond of 3-Methyldeoxyadenosine in DNA", Biochimica Biophysica Acta, 331: 349 (1973).

Maurice, P.A., Lederrey, C., "Increased Sensitivity of Chronic Lymphocytic Leukemia Lymphocytes to Alkylating Agents Due to a Deficient DNA Rapair Mechanism", European J. Cancer, 13: 1033-1039 (1977).

McPhie, P., "Dialysis", Chapter 4, Methods in Enzymology, Volume 22, Jakoby, W.B., ed., New York: Academic Press, pp. 23-32 (1971).

Meyn, R.E., Corry, P.M., Fletcher, S.E., Demetriades, M., "Thermal Enhancement of DNA Damage in Mammalian Cells Treated with cis-Diamminedichloroplatinum (II)", Cancer Res., 40:1136-1139.

Miech, R.P., Tung, M.C., "Adenine Nucleotides in Blood", Biochemical Medicine, 4:435-439 (1970).

Millard, M.M., Macquet, J.P., Theophanides, T., "X-Ray Photoelectron Spectroscopy of DNA-Pt Complexes. Evidence of 06(gua)-N7(gua) Chelation of DNA with cis-dichlorodiammine Platinum (II)", Biochimica Biophysica Acta, 402(2), 166-170, (1975).

Montesano, R., Bresil, H., Margison, G.P., "Increased Excision of 06-Methylguanine from Rat Liver DNA After Chronic Administration of Dimethylnitrosamine", Cancer Res., 39:1798-1802 (1979).

Montesano, R., Bresil, H., Planche-Martel, G., Margison, G.P., Pegg, A.E., "Effect of Chronic Treatment of Rats with Dimethylnitrosamine on the Removal of O^6-methylguanine from DNA", Cancer Research, 40:452-458 (1980).

Monti-Bragadin, C., Tamaro, M., Banfi, E., "Mutagenic Activity of Platinum and Ruthenium Complexes", Chem.-Biol. Interact., 469-472 (1975).

Newbold, R.F., Warren, W., Medcalf, A.S.C., Amos, J., "Mutagenicity of Carcinogenic Methylating Agents is Associated with a Specific DNA modification", Nature, 283:596-599 (1980).

Nicoll, N.W. Swann, P.F., Pegg, A.E., "Effect of Dimethylnitrosamine on Persistence of Methylated Guanines in Rat Liver and Kidney DNA", Nature, (London), 254:261-262 (1975).

Nicoll, J.W., Swann, P.F., Pegg, A.E., "The Accumulation of O^6-Methylguanine in the Liver and Kidney DNA of Rats Treated with Dimethylnitrosamine for a Short or a Long Period", Chemico-Biological Interactions, 16:301-308 (1977).

Nowell, P.C., "The Clonal Evolution of Tumor Cell Populations", Science, 194:23-28 (1976).

O'Connor, P.J., Capps, M.J., Craig, A.W., "Comparative Studies of the Hepatocarcinogen DMN in vivo: Reaction Sites in Rat Liver DNA and the Significance of Their Relative Stabilities", Br. J. Cancer, 27:153-166 (1973).

Paterson, M.C., Anderson, A.K., Smith, B.P., Smith, P.J., "Enhanced Radiosensitivity of Cultured Fibroblasts from Ataxia Telangiectasia Heterozygotes by Defective Colony-Forming Ability and Reduced DNA Replication After Hypoxic -irradiation", Cancer Res., 39:3725-3734 (1979).

Pegg, A.E., "Formation and Metabolism of Alkylated Nucleosides: Possible Role in Carcinogenesis by Nitroso Compounds and Alkylating Agents" in Klein, G., Weinhouse, S.,eds., Advances in Cancer Research, Volume 25, pps. 195-269 (1977).

Pegg, A.E., "Enzymatic Removal of O^6-Methylguanine from DNA by Mammalian Cell Extracts", Biochemical and Biophysical Research Communications, 84(1):166-173 (1978).

Pegg, A.E., "Formation and Subsequent Repair of Alkylation Lesions in Tissues of Rodents Treated with Nitrosamines", Arch. Toxicol., in press (1980).

Pegg, A.E., Hui, G., "Formation and Subsequent Removal of O^6-Methylguanine from Deoxyribonucleic Acid in Rat Liver and Kidney after Small Doses of Dimethylnitrosamine", Biochemical Journal, 173: 739-748 (1978).

Rajewsky, M.F., Augenlicht, L.H., Biessmann, H., Goth, R., Hulser, D.F., Laerun, O.D., Lomakina, L.Y., "Nervous System-Specific Carcinogenesis by Ethylnitrosourea in the Rat: Molecular and Cellular Aspects", Origins of Human Cancer, Hiatts, H., Watson, J., Winsten, J., eds., Cold Spring Harbor, pp. 709-726 (1977).

Roberts, J.J., "The Repair of DNA Modified by Cytotoxic, Mutagenic, and Carcinogenic Chemicals", Advanc. Radiat. Biol., 9:211-435 (1978).

Robins, P., Cairns, J., "Quantitation of the Adaptive Response to Alkylating Agents", Nature, 280:74-76 (1979).

Rosenberg, B., "Platinum Complex-DNA Interactions and Anti-cancer Activity", Biochimie, 60(9):859-867 (1978).

Rosenberg, B., Van Camp, L., "The Successful Regression of Large Solid Sarcoma 180 Tumors by Platinum Compounds", Cancer Res., 30:1799-1802 (1970).

Rosenberg, B., Van Camp, L., Krigas, T., "Inhibition of Cell Division in Escherichia coli by Electrolysis Products from a Platinum Electode", Nature, 205:698-699 (1965).

Rosenberg, B., Van Camp, L., Trosko, J.E., Mansour, V., "Platinum Compounds-A New Class of Potent Antitumour Agents", Nature, 222:385-386 (1969).

Rosencweig, M., Von Hoff, D.D., Penta, J.S., Muggia, F.M., "Clinical Status of cis-Diamminedichloroplatinum (II)", J. Clin Hematol. Oncol., 7:672-678 (1977).

Salmon, S.E., Hamburger, A.W., Soehnlen, B., Durie, B.G.M., Alberts, D.S., Moon, T.E., "Quantitation of Differential Sensitivity of Human-Tumor Stem Cells to Anticancer Drugs", New Eng. J. Med., 298:1321-1327 (1978).

Schoental, R., "Carcinogenic Activity and O-Aklylation", Nature, 182:719-720 (1958).

Scudiero, D.A., "Decreased DNA Repair Synthesis and Defective Colony-Forming Ability of Ataxia Telangiectasia Fibroblast Cell Strains Treated with MNNG", Cancer Research, 40:984-990 (1980).

Setlow, R.B., Regan, J.D., "Defective Repair of AAF Lesions in the DNA of XP Cells", Biochem. Biophys. Res. Comm., 46:1019-1024 (1972).

Shaikh, B., Huang, S-K.S., Potzer, N., Zielinsky, W.L., Jr., "Separation of Methylated Purines by High Pressure Liquid Chromatography", Journal of Liquid Chromatography, 1(1), 75-88 (1978).

Singer, B., "The Chemical Effects of Nucleic Acid Alkylation and Their Reaction to Mutagenesis and Carcinogenesis", Prog. Nucleic Acid Res. Mol. Biol., 15:219-284 (1975).

Singer, B., "N-Nitroso Alkylating Agents: Formation and Persistence of Alkyl Derivatives in Mammalian Nucleic Acids as Contributing Factors in Carcinogenesis", Journal of the National Cancer Institute, 62(6):1329-1339 (1979).

Strauss, B.S., Karren, P., Higgins, N.P., "Alkylation Damage in Mammalian Cells", J. Toxicol. Environ. Health, 2:1395-1414 (1977).

Swann, P.F., and Magee, P.N., "Nitrosamine Induced Carcinogenesis", Biochemical Journal, 110:39-47 (1968).

Swenberg, J.A., Cooper, H.K.,Buecheler, J., Kleihues, P., "1,2-Dimethylhydrazine-Induced Methylation of DNA Bases in Various Rat Organs and the Effect of Pretreatment with Disulfiram", Cancer Res.,39:465-467 (1979).

Thom, C., Steinberg, T.S., "The Chemical Induction of Genetic Changes in Fungi", Proc. Nat. Acad. Sci (USA), 25:329-335 (1930).

Vanecko, S., Laskowski, M. Sr., "Studies of the Specificity of Deoxyribonuclease I. III. Hydrolysis of Chains Carrying a Monoesterified Phosphate on Carbon 5", Journal of Biological Chemistry, 237:3312-3316 (1961).

Welsch, C.W., "Growth Inhibition of Rat Mammary Carcinoma Induced by cis-Platinum Diamminedichloride", J. Nat. Cancer Inst., 47:1071-1074 (1971).

Wilhelm, R.C., Ludlum, D.B., "Coding Properties of 7-Methylguanine", Science, 153(3742):1403-1405 (1966).

Yoshida, T., "Histopathologische Untersuchungen mit Ainiduvazotoluol (o-Toluol-azo-o-toluidin)", Transactions of the Japanese Pathology Society, 22:934-946 (1932).

Zwelling, L.A., Bradley, M.O., Sharkey, N.A., Anderson, T., Kohn, K.W., "Mutagenicity, Cytotoxicity, and DNA Crosslinking in V-79 Chinese Hamster Cells Treated with cis-Platinum (II)Diamminedichloride and trans-Platinum (II) Diamminedichloride", Mutat. Res., 67:271-280 (1979).