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Base Excision Repair Of Dna In Human Cells: The Apurinic/apyrimidinic Intermediate

Michael Francis Moran

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BASE EXCISION REPAIR OF DNA IN HUMAN CELLS: THE APURINIC/APYRIMIDINIC INTERMEDIATE

by

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ABSTRACT

Base excision repair of DNA and factors that affect DNA incision in human cells were considered in this study.

Treatment of alkylated HeLa cells with 3-aminobenzamide, an inhibitor of poly(ADP-ribose) polymerase, increased the number of DNA strand breaks but did not slow down their rejoining by DNA repair. Therefore, an increase in DNA incision, not a decrease in ligation, results from the inhibition of poly(ADP-ribose) polymerase in alkylated cells. This is consistent with recent findings by others that suggest that repair-patch frequency is increased in alkylated cells when polyADP-ribosylation is inhibited.

Apurinic/apyrimidinic (AP) sites were measured in HeLa cells by digestion of cellular DNA with Escherichia coli endonuclease IV, an AP-specific endonuclease, prior to alkaline elution. The absence of non-specific endonuclease activity allowed endonuclease IV-sensitive AP sites to be detected with the sensitivity of conventional alkaline elution. Cells that were alkylated with dimethylsulfate, but not benzo(a)pyrene diol epoxide, contained AP sites that were repaired along with DNA single-strand breaks during a post-alkylation recovery period. In addition, alkali-labile sites (that become single-strand breaks in the presence of alkali) other than AP sites were resolved in dimethylsulfate-treated cells by comparing the rate of elution of endonuclease IV-digested DNA at pH 12.1 and pH 12.6. Alkali-labile sites were also observed in benzo(a)pyrene diol epoxide-treated cells. However, these alkali-labile sites were not sensitive to endonuclease IV, and hence are not AP sites.

According to the base excision repair model, the sequential action of DNA glycosylases and AP endonucleases is thought to initiate the repair of DNA base damage . This moded was tested in γ-irradiated HeLa cells by endonuclease IV-coupled alkaline elution. AP sites were detected as a transient DNA repair intermediate in γ-irradiated cells. This approach illuminated the operation of base excision repair in human cells by demonstrating the transit of ionizing radiation-induced base lesions, most of which are unknown, through this pathway.

If I have learned anything about science,

it was from my friend Kaney Ebisuzaki.

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NOMENCLATURE

AAAF	N-acetoxy-2-acetylaminofluorene
3AB	3-aminobenzamide
AP	apurinic/apyrimidinic
araC	cytosine-β-D-arabinofuranoside
BP	benzo(a)pyrene
BPDE	anti-trans-7,8-dihydrodiol-9,10-epoxy-benzo(a)pyrene
DMS	dimethylsulfate
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
endo	endodeoxyribonuclease
FaPy	formamidopyrimidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
3mA	3-methyladenine
7mG	7-methylguanine
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
4-NQO	4-nitroquinoline-1-oxide
O^6mG	O^6-methylguanine
O^4mT	O^4-methylthymine
PD	pyrimidine dimer
SDS	sodium dodecylsulfate
TG	thymine glycol
Tris	tris(hydroxymethyl)aminomethane
XP	xeroderma pigmentosum

Chapter 1

GENERAL INTRODUCTION

1.1 DNA damage

Cells can survive constant threats to their genome. Spontaneous DNA damage as well as damage from chemical and physical agents in the environment constantly challenge the integrity of cellular DNA. Consequently, organisms have evolved processes for the tolerance, reversal and removal of damage in their DNA (Friedberg, 1985). Studies of DNA repair mechanisms in bacteria have been greatly aided by the relative ease with which mutants in putative DNA repair genes have been obtained. This luxury has not yet been realized with cultured mammalian cells. The focus of this study is the removal of damage from DNA in mammalian cells.

1.1.1 Spontaneous DNA damage

In addition to DNA damage from external agents, the chemical instability of DNA and the cellular environment itself also contribute to alterations in DNA. Spontaneous hydrolytic reactions such as deamination, depurination, and depyrimidination occur (Singer and Kusmierek, 1982). Deamination of cytosine produces uracil in DNA, and similarly, the deamination of adenine and guanine produces hypoxanthine and xanthine respectively. Uracil and hypoxanthine are potentially mutagenic because they are miscoding lesions whereas xanthine is unable to form base pairs and thus may block DNA synthesis (Lindahl, 1979). The rate of deamination of cytosine is much greater than for the purines. Depurination was calculated to occur with a frequency of several thousand per mammalian cell per day (Lindahl, 1979). This calculation was made by extrapolation from the observed decay of DNA *in vitro* at elevated temperatures (Lindahl and Nyberg, 1972). Similarly, the loss of

pyrimidines is estimated to occur at approximately 1/20 the rate of purine loss (Lindahl and Karlstrom, 1973). These hydrolytic reactions occur at faster rates in single-stranded DNA, and thus may occur preferentially at local single-stranded regions such as DNA replication forks.

Indigenous cellular compounds may also contribute to DNA damage. Free radical species arising from normal aerobic metabolism may react with DNA (Ames, 1983; Cerutti, 1985), and resemble the oxidative damage caused by ionizing (X-ray or γ-ray) radiation. Non-enzymatic alkylation of DNA by cellular S-adenosyl methionine acting as a weak alkylating agent has been demonstrated (Rydberg and Lindahl, 1982; Barrows and Magee, 1982). Alkylnitrosamines are synthesized in mammalian cells from nitrite and secondary and tertiary amines and upon further metabolic activation into a carbonium cation, are capable of covalent interaction with DNA (Taylor and Lijinsky, 1975). The spectrum of DNA damage produced from nitrosamines includes the mutagenic lesions O^6-methylguanine (O^6mG) and O^4-methylthymine (O^4mT) (Singer 1985). Phosphotriesters are also produced, but they are removed very slowly from cellular DNA (Singer, 1985). There are too few data to comment on the biological significance of the phosphotriesters.

1.1.2 Chemical damage

Alkylation of cellular DNA by electrophilic chemicals is mutagenic and carcinogenic and has been extensively reviewed by Singer and associates (Singer, 1979; Singer and Kusmierek, 1982; Singer, 1985). All nitrogens and oxygens in DNA have been shown to be targets of alkylation

and the relative amounts of nitrogen and oxygen damage depends on the given chemical. For example dimethylsulfate (DMS) reacts almost entirely with ring nitrogens of the DNA bases, whereas N-nitroso compounds such as N-methyl-N-nitrosourea (MNU), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and dialkylnitrosamines are more reactive with oxygen in the bases and phosphodiester bonds. Ethylating agents, although less reactive overall, are more reactive with oxygens than are the analogous methylating agents. More complex alkylating agents may require activation into their ultimate reactive form. For example, the unreactive nonpolar compound benzo(a)pyrene (BP) is activated by cytochrome P-450 complexes into a diol epoxide (BPDE) that is thought to be the ultimate carcinogen of BP that reacts directly with DNA (Singer and Kusmierek, 1982). Studies of the effects of BP and other potential carcinogens are complicated by the need for metabolic activation into a reactive form. To circumvent this problem, studies of these alkylation reactions with DNA have been performed using simple alkylating agents that do not require metabolic activation. DMS and MNNG are examples of such agents.

1.1.3 Physical damage

The study of UV radiation damage initiated studies of DNA repair (Kelner, 1949; Dulbecco, 1949). UV light is an efficient DNA damaging agent due to the preferential absorption of 260 nm radiation by DNA. The most intensely studied UV lesion is the cyclobutane pyrimidine dimer, and thymine dimers (PD) in particular. PD result from saturation across the 5-6 double bonds of adjacent thymines (Setlow, 1966) and block the progression of DNA polymerases (Haseltine, 1983). Another intrastrand

DNA cross-link, the (6-4) photoproduct, is alkali-labile and involves cross-linking between the C^6 of a pyrimidine base and the C^5 of its 5' neighbouring pyrimidine (usually a cytosine) (Varghese and Wang, 1968; Lippke et al., 1981; Franklin and Haseltine, 1986). The sequence specificity of nonsense mutations in UV-irradiated E. coli suggested that the (6-4) lesions are mutagenic (Haseltine, 1983; Brash and Haseltine, 1982). Hydrated pyrimidines (5,6-dihydro-6-hydroxy thymidine) and thymine glycol (5,6-dihydroxydihydro thymine) are also minor products of UV- irradiation and the former can spontaneously dehydrate to form reform thymine (Friedberg, 1985).

Unlike the preferential absorption of UV radiation by DNA, ionizing radiation will interact with all components of a cell depending on the mass fraction of a given target (Ward, 1975). DNA damage by ionizing radiation includes the direct ionization of DNA as well as indirect damage from hydroxyl radicals generated by water radiolysis (Ward, 1975). Water makes up 70-90% of a cells mass (Lehninger, 1975), consequently water radiolysis is a major result of ionizing irradiation. Hydroxyl radicals are responsible for the indirect effects of ionizing radiation and account for 60-70% of both the cell killing (Chapman et al., 1973; Roots and Okada, 1975) and the DNA strand breakage (Roots and Okada, 1972) associated with ionizing irradiation, while the direct ionization of DNA may account for the remaining 30-40%. The DNA damage includes mostly damage to the heterocyclic bases as well as single- and double-strand breaks (Cerutti, 1976; Ward, 1986; Wallace, 1983). Hydroxyl radical attack of the 5-6 double bond may be the initial reaction leading to many different pyrimidine derivatives (Scholes

et al., 1960), of which thymine glycol (TG) is the most abundant (Briemer and Lindahl, 1985). Purines are less susceptible to damage by hydroxyl radicals with formamidopyrimidine (FaPy) derivatives being a relatively minor product (Bonicel et al., 1980; Briemer, 1984). Based on model systems, a vast spectrum of ionizing radiation-induced base lesions in DNA is expected (Scholes, 1976; Teoule et al., 1977). At least 21 different thymine radiation products alone were observed, and a similar number of cytosine derivatives probably exists (Teoule et al., 1977). The multitude of γ-ray-induced DNA lesions, many of which may be minor and/or unstable products, has made the assignment of biological end points difficult (Cerutti, 1976). Base damage was estimated to account for more than 85% of the biological inactivation of γ-irradiated PM2 bacteriophage DNA (van der Schans et al., 1973), and Moran and Wallace (1985) found that alkali-labile lesions and the saturated thymine lesions contributed equally to the loss of biological activity of irradiated PM2 DNA. Genetic data from E. coli suggest that radiation-induced base damage leading to base substitutions may be a common mutational event (Glickman et al., 1980).

1.1.4 Different origins of common lesions

It is apparent that many chemical and physical DNA damaging agents produce lesions that are similar to those that arise naturally in cells. Alkylation of purines by simple alkylating agents is similar to the alkylation caused by cellular S-adenosyl methionine, and alkylation by these agents and possibly such carcinogens as BPDE (King et al., 1979), N-acetoxy-2-acetylaminofluorene (AAAF) (Brookes, 1977) and aflatoxin B1 (Wang and Cerutti, 1980) all enhance depurination which also occurs

spontaneously. The deamination of cytosine to generate uracil in DNA is also caused by simple nonalkylating agents such as bisulfite and nitrous acid (Singer and Kusmierek, 1982). The oxidative damage produced by ionizing radiation may be similar to the damage arising from the production of reactive oxygen species by normal aerobic metabolism (Ames, 1983; Cerutti, 1985). Thus, many diverse DNA damaging agents might simply enhance or mimic the reactions that naturally occur in the cellular environment.

1.2 Reversal of DNA damage

Reversal of DNA damage refers to the restoration of the proper DNA sequence without the removal of damaged nucleotides or incorporation of new nucleotides. Enzymatic photoreactivation of cyclobutane pyrimidine dimers occurs in most organisms analyzed, including mammalian cells (Sutherland, 1978). This process is dependent on visible light (Kelner, 1949; Dulbecco, 1949) and is catalyzed by a chromophore-containing enzyme (DNA photolyase) that breaks the cyclobutane ring of the dimer and restores the 5-6 double bonds in the adjacent pyrimidines (Rupert, 1975).

Alkylation of DNA at the O^6 position of guanine by alkylating agents is corrected by the direct transfer of the methyl group to a cysteine residue on an acceptor protein. The persistence of O^6mG in DNA is well correlated with tumor formation (Pegg, 1984; Singer, 1984). The mechanism of repair of O^6mG is similar in both E. coli (Demple et al., 1982), rodent (Pegg et al., 1985) and human cells (Pegg et al., 1982). The methyl group is transfered to an acceptor protein that is

inactivated as a result. In E. coli the ada gene product, a 39,000 molecular weight protein, is the O^6mG methyl transferase that can also repair O^4mT and phosphotriesters (Demple et al., 1985). A proteolytic fragment of the ada protein with a molecular weight of 19,000 is also present in E. coli and has lost the ability to repair phosphotriesters (Margison et al., 1985; McCarthy and Lindahl, 1985). In various mammalian cell extracts both O^6mG and O^4mT were repaired (Becker and Montesano, 1985), but a partially purified O^6mG methyl transferase preparation from rat liver was unable to repair O^4mT suggesting that the two activities are catalyzed by different enzymes (Dolan et al., 1984).

In E. coli the methylated ada protein positively controls the expression of DNA repair genes, including ada itself (also alkA, alkB and aidB), involved in the adaptive response to alkylating agents (Teo et al., 1986). The methyltransferase can be induced several hundred fold in E. coli by prior challenge with a low dose of alkylating agent. Induction of this magnitude in mammalian cells has not been demonstated. Some virally transformed human cell lines and some cell lines derived from human tumors are unable to reactivate MNNG-treated adenovirus (Yarosh et al., 1983). This phenotype is designated as Mer- (methyl repair) and seems to correlate with another phenotype, Mex- (methyl excision), which is characterized by the inability of cells to remove O^6mG from their DNA (Strauss, 1985).

Recently Chetsanga and Grigorian (1985) have isolated an activity from E .coli that is able to catalyze the closure of imidazole ring-opened purines in γ-irradiated DNA. This purine imidazole-ring cyclase requires no cofactors and will not close the FaPy formed from 7-methylguanine in alkylated DNA.

Mammalian cells are reported to contain a DNA binding protein (M_r 120,000) capable of directly inserting the appropriate purines into apurinic sites in duplex DNA (Deutsch and Linn 1979a, 1979b). Apparently the formation of the N-glycosyl bond did not require an energy source such as a nucleotide triphosphate, but it was suggested that the resulting base pairing and base stacking might drive the reaction (Deutsch and Linn, 1979a). It has been suggested that the presumed covalent insertion might actually be very strong noncovalent binding between free guanine and DNA due to the insolubility of free guanine in neutral aqueous solutions (Lindahl, 1982).

1.3 Excision of DNA damage

Excision repair of damage from DNA is generally classified into two model pathways: base excision repair and nucleotide excision repair (Friedberg, 1985). Nucleotide excision repair is initiated by the direct incision of DNA by a damage-specific endonuclease such as the uvrABC complex of E .coli. This enzyme is the product of the uvrA, uvrB and uvrC genes (Howard-Flanders et al., 1966) and is involved in the excision of thymine dimers and also bulky adducts formed by chemicals such as 4-nitroquinoline-1-oxide (4-NQO), BP and AAAF (Murray, 1979). The enzyme is made up of three different polypeptides and requires Mg^{2+} and ATP (Seeberg, 1978; Rupp et al., 1982). This enzyme presumably recognizes general distortions in DNA and incises DNA both 5' and 3' to the damaged site generating a gap of 12 nucleotides after excision of the 12-nucleotide-long oligonucleotide (Sancar and Rupp, 1983). A similar type of broad-specificity activity is thought to be defective in the human disorder xeroderma pigmentosum (XP) (Cleaver, 1968). XP

patients are sensitive to UV radiation, AAAF and other bulky adduct-forming chemicals and suffer a high incidence of skin cancers on areas exposed to sunlight (Setlow, 1978; Friedberg et al., 1979). XP cells in culture were shown to be defective in the incision step of excision repair (Fornace et al., 1976). When purified T4 PD-DNA glycosylase was introduced into UV-irradiated XP cells, DNA repair synthesis was restored, thus demonstrating the defect in the excision of PD in XP cells (Tanaka et al., 1975). The demonstration of the DNA repair defect in XP cells illustrates the critical role of DNA repair in carcinogenesis. At least eight different XP complementation groups have been identified from cell fusion experiments implying that a multi-protein complex might be involved in a uvrABC-like damage-specific endonuclease activity in human cells. The molecular defects in the various XP cell lines have not been identified, but damage-specific DNA binding activities have been identified from various human sources (Feldberg and Grossman, 1976; Tsang and Kuhnlein, 1982). In addition, there are variant complementation groups of XP and afflicted individuals appear phenotypically similar to the classical XP patients, but their derived cultured fibroblasts are proficient in the excision of thymine dimers (Friedberg et al., 1979).

The post-incision reactions are thought to be similar for both base excision repair and nucleotide excision repair. An exonuclease might remove neighbouring nucleotides or deoxyribose, followed by gap-filling by DNA polymerases and finally ligation. The enzymology of these late functions has been extensively described in the context of DNA replication (Kornberg, 1980) and the focus of the ensuing discussion

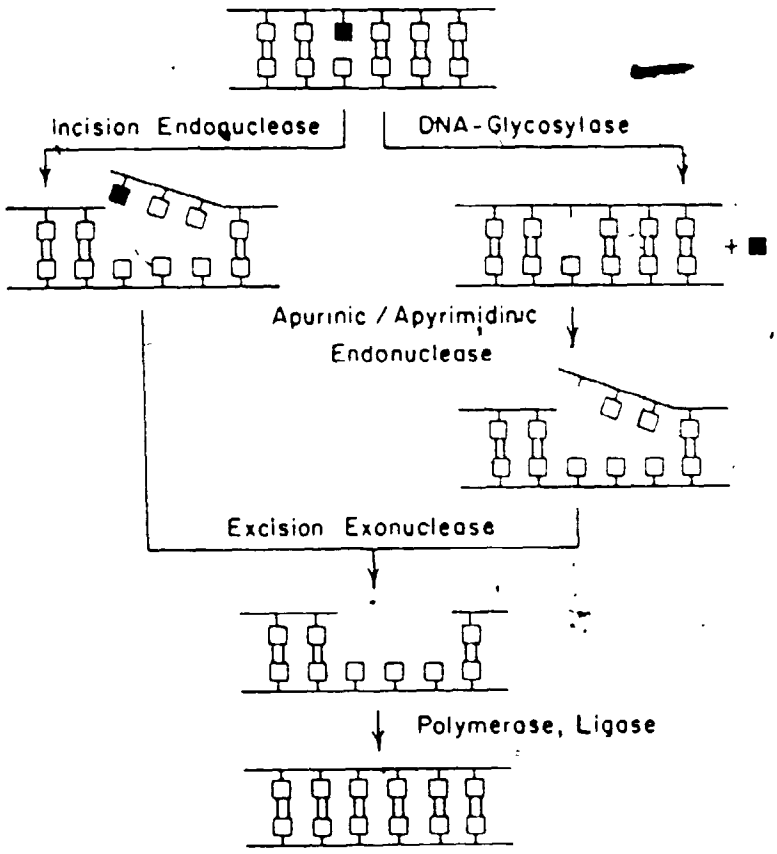
will be on base excision repair in mammalian cells.

1.3.1 Base excision repair and DNA glycosylases

Base excision repair is initiated by breaking the N-glycosyl bond between a base and deoxyribose in DNA either by DNA glycosylase or spontaneously to generate an AP site. The DNA is then nicked at the AP site by an AP-specific endonuclease (fig. 1). Both the DNA glycosylases and AP endonucleases have been found ubiquitously throughout nature (Lindahl, 1979). DNA glycosylases were discovered by Lindahl (1974) who later suggested their potential role in base excision by combined action of DNA glycosylase and AP endonuclease (Lindahl, 1976). The reactions of base excision repair have been demonstrated with purified enzymes in vitro (Evans and Linn, 1984; Mosbaugh and Linn, 1984; Goffin and Verly, 1984), but in the absence of conclusive genetic evidence this pathway remains speculative.

The DNA glycosylases are relatively small (18,000-30,000 molecular weight) single polypeptide proteins without any cofactor requirements. These enzymes catalyze the hydrolysis of the N-glycosyl bond that links a base to deoxyribose in DNA, and there are some DNA glycosylases that have an associated AP endonuclease activity (Duncan, 1981). Enzymes specific for the removal of inappropriate bases arising from misincorporation (in the case of uracil) or deamination of normal bases include uracil-DNA glycosylase that has been found in bacteria (Lindahl, 1974), yeast (Crosby et al., 1981) and mammalian tissue (Borle et al., 1979) and hypoxanthine-DNA glycosylase that has been identified in E. coli (Karran and Lindahl, 1978), HeLa cells (Myrnes et al., 1982) and calf thymus (Karran and Lindahl, 1980). Two different 3-methyladenine

Figure 1. (A) Two model pathways of excision repair. The left sequence depicts nucleotide excision repair initiated by an endonuclease that incises DNA at or near the lesion. Base excision repair is shown on the right: a DNA glycosylase first removes an inappropriate base generating an AP site. The sugar-phosphate backbone of DNA is then broken at the AP site by an AP endonuclease. Subsequent exonuclease activity might remove neighbouring nucleotides followed by DNA synthesis and ligation to close the single-stranded gap. (B) AP endonucleases are classified as class I (3'-APendo) or class II (5'-AP endo) depending on which side of the AP site they incise. (Reproduced from Linn, 1982)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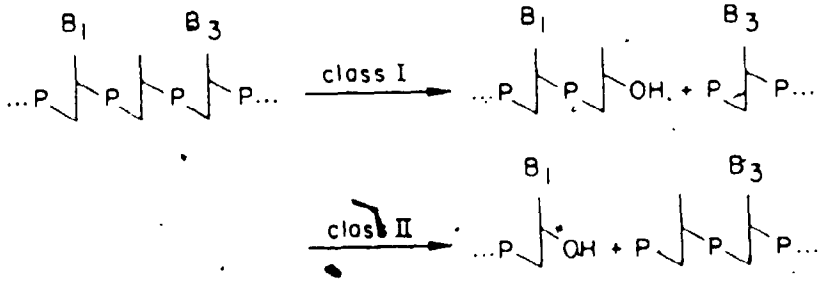
DNA-Glycosylase
Apurinic / Apyrimidinic
Endonuclease
Excision Exonuclease
Polymerase, Ligase


B1
B3
...P
P
P
P...
class I
OH
class II

(3mA)-DNA glycosylases are present in E. coli and are products of the tag and alkA genes. E. coli tag$^-$ and alkA$^-$ strains are sensitive to alkylation (Karran et al., 1980; Evensen and Seeberg, 1982). The tag protein (3mA-DNA glycosylase I) catalyzes the release of 3mA from DNA and is inhibited by free 3mA (Riazuddin and Lindahl, 1978). 3mA-DNA glycosylase I might recognize methyl groups extending into the minor groove of DNA (Lindahl, 1982). The alkA gene product is not inhibited by 3mA and is inducible 20-fold in E. coli as part of the adaptive response to alkylating agents (Nakabeppu and Sekiguchi, 1985). The alkA protein (3mA-DNA glycosylase II) recognizes other N-alkylpurines in addition to 3mA including 7-methylguanine (7mG), 3-methylguanine and 7-methyladenine (Thomas et al., 1982) which suggests that the enzyme might recognize a positively charged purine (Lindahl, 1982). This enzyme has also been reported to release O^2-methylcytosine and O^2-methylthymine from DNA, so its mechanism of substrate recognition may be more complex (McCarthy et al., 1984). A partially purified noninducible 3mA-specific activity from human cells is reminiscent of E. coli 3mA-DNA glycosylase I (Brent, 1979). A partially purified preparation from rat liver contained an activity that released both 3mA and 7mG from DNA. These activities may be from a single enzyme since both showed similar rates of heat inactivation (Cathcart and Goldthwait, 1981). 7mG in alkylated DNA is slowly converted to a methylated FaPy derivative (2,6-diamino-4-hydroxy-5-N-methyl formamidopyrimidine) that has a stabilized N-glycosyl bond (Chetsanga and Lindahl, 1979). Accordingly, FaPy-DNA glycosylases have been detected in bacterial and human cells (Chetsanga and Lindahl, 1979; Chetsanga et al., 1981). This enzyme does not release the FaPy

derivatives formed from adenine and guanine in γ-irradiated DNA (Chetsanga and Grigorian, 1985).

There are DNA glycosylases that also have AP endonuclease activity. Both bacteriophage T4-infected E. coli and Micrococcus luteus contain a PD-DNA glycosylase that has an associated AP endonuclease activity (Friedberg and King, 1969; Yasuda and Sekiguchi, 1970; Shimada et al., 1967). These enzymes hydrolyze the N-glycosyl bond of the 5' base of the dimer and are also able to break the phosphodiester bond between the AP site and the adjacent nucleotide that has the dimer still attached (Haseltine et al., 1980; Radany and Friedberg, 1980). A similar activity has not been identified in eukaryotic cells.

Both E. coli and mammalian cells contain a single enzyme capable of releasing several oxidized thymine derivatives from X- or γ-irradiated DNA. The spectrum of lesions recognized by these enzymes is similar (Helland et al., 1986) and is made up of the major ionizing radiation-induced thymine products including thymine glycols as well as ring-fragmented and ring-contracted derivatives (Breimer and Lindahl, 1985). These enzymes will be refered to as thymine glycol (TG)-DNA glycosylase as suggested by Breimer and Lindahl (1985), to reflect the major lesion in γ-irradiated DNA. TG is also found as a minor product in UV-irradiated DNA, and this activity was first identified using UV-irradiated DNA as a substrate (Bacchetti et al., 1972; Brent, 1973; Radman, 1976). The enzyme has been purified from E. coli by many investigators and has been referred to as X-ray endonuclease (Katcher and Wallace, 1983), endonuclease III (Breimer and Lindahl, 1984) and urea-DNA glycosylase (Breimer and Lindahl, 1980). The mammalian enzyme has also been referred to by various names including endo A (Brent,

1983), urea-DNA glycosylase (Breimer, 1983) and most recently redoxy-endonuclease to reflect the reduction-oxidation reactions that generate the targets of this activity (Helland et al., 1986). TG-DNA glycosylases have an associated AP endonuclease activity that nicks DNA 3' to the AP site generating a 3' deoxyribose and a 5' phosphoryl group (Demple and Linn, 1980). E. coli mutants (nth$^-$) deficient in this enzyme surprisingly are not sensitive to the lethal effects of ionizing radiation (Cunningham and Weiss, 1985). This suggests that there are back-up activities to compensate for the lack of this enzyme, since the thymine lesions are known contribute to the lethality of ionizing radiation (Wallace, 1983). Breimer and Lindahl (1985) have suggested that the fortuitous incorporation of deoxyadenosine-5'-phosphate opposite noncoding thymine lesions might decrease the number of base substitutions that occur in γ-irradiated bacteria (Glickman et al., 1980).

The AP endonuclease activity of the E. coli TG-DNA glycosylase was observed to lag behind its glycosylase activity (Demple and Linn, 1980) and a similar observation was made with the T4 PD-DNA glycosylase (McMillan et al., 1981). This suggests that the two activities of these bifunctional enzymes do not act in a concerted fashion. That is, these enzymes dissociate from DNA after breaking the N-glycosyl bond and must reassociate to incise at the AP site.

The spectrum of possible base adducts in DNA from various DNA damaging agents is far from being completely cataloged. Similarly, there probably exist many more DNA glycosylases that have yet to be discovered. In fact, the general properties of the DNA glycosylases such

as their resistance to inhibition by EDTA and lack of subunit structure and cofactor requirements suggests that the discovery of new DNA glycosylases will proceed and probably lead to the identification of new biologically significant but otherwise chemically elusive DNA lesions.

1.3.2 AP endonucleases

AP endonucleases are commonly found throughout nature (Friedberg et al., 1981). No bacterial or mammalian mutant deficient in a dedicated AP endonuclease (one without other associated activities) has been described. Thus the biological relevance of the AP endonucleases remains open to speculation. A tripeptide has been reported to act as an AP endonuclease which has further called into question the relevance of other associated AP endonuclease activities (Behmoaras et al., 1981). AP endonucleases have been classified as class I (3'-AP endo) or class II (5'-AP endo) depending on which side of the AP sites they incise (Linn, 1982) (fig. 1). In both cases a 3'-OH and a 5'-P are produced. The different activities can be distinguished since the 3'-deoxyribose product of a 3'-AP endo is a poor primer for DNA polymerase I in vitro (Warner et al., 1980). The majority of AP endonuclease activity in bacteria and mammalian cells is of the class II type, and the AP endonuclease activities associated with DNA glycosylases have all been found to be of the class I type (Friedberg, 1985). An exception may be the mammalian TG-DNA glycosylase, for which Helland et al. (1986) have described unpublished results that this enzyme can incise both 3' and 5' to AP sites.

Endo IV of E. coli was purified and characterized by Ljungquist (1977) and is a 5'-AP endo that comprises approximately 10% of the total

class II activity in wild type cells. Endo IV has a molecular weight of 30,000-33,000 and has no cofactor requirements. It is unusually resistant to both salt (50% active in 0.56 M NaCl) and to heat (fully active after heating at 60° C for 5 min). Endo IV is also resistant to EDTA and tRNA and is inhibited by sulfhydryl-reacting agents. This enzyme has not been shown to have any activity other than 5'-AP endonuclease. The AP endonuclease activity of exonuclease III, also known as endo II (Friedberg and Goldthwait, 1969) and endo VI (Verly, 1978), accounts for 90% of the total 5'-AP endonuclease in E. coli (Weiss, 1981). This enzyme has a molecular weight of 28,000, requires Mg^{2+} and is inhibited by EDTA (Gossard and Verly, 1978; Weiss, 1981). Exonuclease III also has intrinsic 3' to 5' exonuclease, 3' phosphatase, and RNase H activities (Lindahl, 1982). E. coli mutants (xth^-) deficient in exonuclease III are not particularly sensitive to simple alkylating agents (Yajko and Weiss, 1975; Kirtikar et al., 1977; Ljungquist et al., 1976). This might be explained by the ability of endo IV or a purine insertase to participate in the repair of AP sites in the absence exo III. E. coli xth^- strains are sensitive to H_2O_2 but not ionizing radiation (Demple et al., 1983). This is surprising since the spectrum of ionizing radiation DNA damage is thought to include the lesions caused by H_2O_2 (Demple and Linn, 1980). E. coli also contain endo VII, a single-strand specific AP endo whose site of incision (3' or 5') has not yet been determined (Bonura et al., 1982). This 60,000 molecular weight protein is larger than most AP endonucleases and no analogous single-strand specific activity has been identified from mammalian sources. Endo V from E. coli is an enzyme active against many damaged DNA

substrates including depurinated DNA (Linn, 1982).

It is interesting that the 3'-AP endonucleases that are associated with DNA glycosylases all create apyrimidinic sites, and that insertase activities have only been described for purines. Thus the repair of purines and pyrimidines might follow different pathways. However, the reality of purine insertases has been questioned (see section 1.6).

Mammalian AP endonucleases were first purified and characterized by Lindahl and associates (Ljungquist and Lindahl, 1974; Ljungquist et al., 1974) and have been purified from various sources including calf liver (Kuebler and Goldthwait, 1977), human placenta (Schaper et al., 1982) and rat liver (Thibodeau and Verly, 1980). In contrast to the bacterial enzymes, the mammalian AP endonucleases require Mg^{2+} and are sensitive to inhibition by EDTA. Linn and coworkers have resolved two types of AP endonucleases from human fibroblasts (Mosbaugh and Linn, 1980) as well as from HeLa cells (Kane and Linn, 1981) and human placenta (Linsley et al., 1977). AP endonuclease I is not retained by phosphocellulose and has a low K_m for AP-containing DNA (4.6 nM). This class I enzyme (3'-AP endo) is unstable during purification and thus it is not well characterized. AP endo I is apparently missing in extracts from XP group D cells (Kuhnlein et al., 1978). AP endonuclease II is a 5'-AP endo with a lower affinity for AP-containing DNA (K_m 44 nM). The AP endo II activity from human placenta elutes as a broad peak from phosphocellulose suggesting that it may be a heterogeneous mixture of enzymes (Linsley et al., 1977). Indeed this 5'-AP endo activity has been localized to different intracellular locations including chromatin, nucleus, and membrane-bound (Thibodeau and Verly, 1980). This distribution has been suggested to reflect the post-translational

processing of the enzyme: the enzyme is initially associated with the endoplasmic reticulum; subsequently transfered to the nucleus; and finally becomes associated with DNA in chromatin (Thibodeau and Verly, 1980). An enzyme from human placenta with both 3' and 5' AP endo activity was described by Grafstrom et al. (1982). It may be that this activity is the same as that associated with the TG-DNA glycosylase from calf thymus described by Helland et al. (1986).

1.4 Factors affecting DNA repair

The association of DNA with protein in the chromatin of mammalian cells poses an additional challenge for DNA repair enzymes. DNA topoisomerase II and poly(ADP-ribose) polymerase (ADPRP) appear to be involved in nucleotide excision repair and base excision repair, respectively. DNA incision is inhibited by novobiocin, an inhibitor of topoisomerase II, in human cells damaged by UV light but not ionizing radiation or simple alkylating agents (Collins and Johnson, 1979; Snyder et al., 1982). Conversely, the repair of damage by ionizing irradiation or alkylation is sensitive to 3-aminobenzamide (3AB), an inhibitor of ADPRP, whereas UV-irradiated cells are unaffected by 3AB (James and Lehmann, 1982; Althaus et al., 1982). At the molecular level, it would appear that a multi-enzyme complex (that is defective in XP cells) that is specific for general distortions in DNA structure recognizes and incises DNA in a topoisomerase II-dependent fashion. Erixon (1986) has recently reviewed these observations and has suggested that topoisomerase II is required to introduce torsional strain into discrete "domains" of DNA in order to allow the recognition and incision of

damaged sites following treatment with UV light or UV-mimetic agents such as 4-NQO and AAAF. According to the model, once the DNA in such a domain sustains a nick, no further incision is tolerated due to the loss of the torsional strain in that domain of DNA. These domains are estimated to be approximately 30 kilobases in length which is close to the size of the DNA loops that are associated with the nuclear matrix (Paulson and Laemmli, 1977). This model explains why the number of strand breaks reaches a plateau (defined by the number of domains in the genome) following DNA damage by UV or UV-mimetic agents that evoke a nucleotide excision repair response (Erixon, 1986). In the case of base excision repair induced by ionizing radiation or simple alkylating agents the level of DNA strand breakage attained is not limited, presumably because the relatively small DNA glycosylases and AP endonucleases are accessible to base lesions in chromatin, and this process is dependent on the involvement of ADPRP. The possible function of ADPRP in DNA repair is discussed more extensively in chapter 2.

The involvement of different eukaryotic DNA polymerases in DNA repair is largely based on the effects of inhibitors of specific enzymes (Collins et al., 1984). Aphidicolin and cytosine arabinofuranoside (araC) are employed as inhibitors of polymerase α, and dideoxythymidine is used to inhibit polymerase β. It appears that no one type of DNA polymerase is exclusively involved in any particular repair response: when one species of polymerase is inhibited, the other appears to be able to compensate (Downes et al., 1983). Th'ng (1984) suggested that the involvement of a particular DNA polymerase will be governed by the size of the single-stranded gap created by exonucleases: polymerase α would fill gaps of 30 nucleotides or more, whereas polymerase β would

fill gaps of 10 nucleotides or less to provide a substrate for DNA ligase. This model is supported by *in vitro* experiments in which HeLa polymerase α decreased the size of gaps (with average lengths of 20-63 nucleotides) down to about 15 nucleotides which were then filled to completion by HeLa polymerase β and sealed by *E. coli* DNA ligase (Moshaugh and Linn, 1984).

Hanawalt and associates have recently provided evidence that DNA repair does not occur uniformly throughout the mammalian genome. Repair of UV-induced pyrimidine dimers occurred more efficiently in amplified dihydrofolate reductase genes than in regions upstream from the gene that were also amplified in Chinese hamster ovary cells (Bohr *et al.*, 1985). The transcriptionally active c-*abl* gene was effeciently repaired in UV-irradiated mouse 3T3 fibroblasts, whereas in the transcriptionally inactive c-*mos* gene the pyrimidine dimers were found to persist (Madhani *et al.*, 1986). The differential repair of these genes was not affected by the growth state of the cells. Thus, the potential for expression of a sequence of DNA may correlate with its likelihood of being repaired.

1.5 Aims of the study

The aim of this study was to investigate factors affecting DNA repair and to define pathways of DNA repair in human cells. The first parameter of DNA repair investigated was the role of ADPRP in repair. This enzyme has a putative role in many cellular processes supported mainly by the effects observed in cells treated with 3-aminobenzamide (3AB), an inhibitor of ADPRP (Ueda and Hayaishi, 1985). My results suggested a role for ADPRP in the early stages of DNA repair, possibly

DNA incision.

The next stage of this investigation was to clarify the early steps in DNA repair required for DNA incision. DMS was employed as a model electrophilic alkylating agent. The approach was to purify bacterial enzymes with defined specificity and use them as probes of lesions in DNA from mammalian cells. Thus the early steps of DNA repair in human cells would be resolved by the identification of intermediates of the process. It was hoped that this approach would yield results that would illuminate the processes involved in the cellular response to more complex but more environmentally prevalent carcinogens such as polyaromatic hydrocarbons and ionizing radiation.

Chapter 2

POLY(ADP-RIBOSE) AND DNA INCISION

2.1 Introduction

Inhibition of poly(ADP-ribose) polymerase (ADPRP) by 3-aminobenzamide (3AB) in cells recovering from alkylation or ionizing radiation damage, has been reported to increase DNA repair replication, DNA strand break frequency, and cell death (Shall, 1983). One explanation of these findings is that DNA ligase II is not activated for DNA repair when ADPRP is inhibited (Creissen and Shall, 1982). In this model the increased DNA strand break frequency, observed during repair in the presence of 3AB, reflects a decreased rate of repair due to reduced ligation. The increased repair replication is interpreted as longer patches being formed at repair sites that fail to be ligated.

Recently, Walker and coworkers (1984) and Cleaver (1985) have found that repair patch size is, in fact, not increased when 3AB is present during DNA repair. Their results suggest that more, not longer, repair patches result from DNA damage when ADPRP is inhibited by 3AB. If increased strand break frequency were due to a failure in ligation, then ligation is necessarily rate limiting under these conditions. In contrast to this constraint, I found that the rate of resealing of DNA strand breaks is unaffected by the presence of 3AB, even though the actual strand break frequency is increased by 3AB. These findings are consistent with the results of Walker *et al.* (1984) and Cleaver (1985), and suggest that DNA incision, and not ligation, is affected by ADP-ribosylation in HeLa cells recovering from alkylation damage.

2.2 Materials and Methods

2.2.1 Cell labelling and alkylation

HeLa S3 cells were grown in Eagle's MEM medium containing 10% fetal calf serum (Gibco), penicillin (50 units/ml) and streptomycin (50 µg/ml) at 37° C in humidified 5% CO_2 in air. Cells were labelled for 40 h with 0.015 µCi/ml [^{14}C]thymidine (58.0 mCi/mmol, New England Nuclear) followed by 5-7 h in fresh medium without label. Treatment of cells with cytosine-β-D-arabinofuranoside (araC) began 30 min prior to DMS addition. DMS (a gift from Dr. I.G.Walker, this university) was first dissolved in methanol before dilution with phosphate-buffered saline (PBS) and addition to cell cultures. The final concentrations with DMS-treated and control cells were 0.003% methanol and 0.3% PBS. Labelled cells in 60 mm dishes were treated with DMS for 2.0 or 2.5 minutes followed by incubation in fresh medium with the indicated chemicals for the recovery period. Cells that were exposed to γ-radiation were kept on ice and exposed at 86 rad/minute under ambient atmosphere with a ^{137}Cs γ-ray emitter (Gammacell 20, Atomic Energy of Canada). 3AB was obtained from Pfaltz and Bauer Inc. and all other chemicals were obtained from Sigma Chemicals unless otherwise indicated.

2.2.2 Alkaline elution

The procedure used was essentially as described by Kohn et al. (1981). Cells were harvested by rinsing with 3.0 ml of ice-cold PBS containing 0.02% EDTA, followed by scraping into 1.0 mL of PBS/EDTA. Cells were diluted with 4.0 ml cold medium and 5.0 ml cold PBS and kept on ice. Approximately 3 X 10^5 cells were loaded onto 2 um polycarbonate

filters (Nucleopore) followed by three washes with 5.0 ml cold PBS. Cells were lysed with 5.0 ml of 2% SDS (BRL), 0.025 M EDTA, pH 9.7. Alkaline elution with 0.02 M EDTA (acid form), tetrapropylammonium hydroxide (1.0 M Aldrich), 0.1% SDS, pH 12.1 was carried out at 0.035-0.038 ml/min. Polycarbonate filters were prepared for scintillation counting by first dissolving in Protosol (New England Nuclear).

2.3 Results

2.3.1 3AB and araC increase DNA strand breakage

Alkaline filter elution was performed with HeLa S3 cells at various times after a brief exposure to a low concentration of DMS. At the concentrations of DMS used in these experiments, there is virtually no cell death (S. Warmels and K. Ebisuzaki, unpublished observations). The slope of the elution curve reflects the presence of alkali-labile sites, predominantly strand breaks, in the DNA and was used to compare strand break frequencies (Kohn et al., 1981). The slope of an elution curve was calculated as the average slope of the individual fractions of the curve. The decrease in the elution slope of cells recovering from DNA damage results from the rejoining of strand breaks by ligation. Therefore, the decrease in the elution slope was followed over time to determine the rate of resealing of DNA breaks during DNA repair.

Fig. 2 shows a typical elution profile from HeLa S3 cells after treatment with DMS. In the presence of a non-toxic concentration (5 mM) of 3AB (S. Warmels and K. Ebisuzaki, unpublished observations), the slope, and therefore the number of strand breaks, has increased. AraC

(30 μM) also increased the strand break frequency, presumably by inhibiting DNA polymerization and thereby preventing subsequent strand rejoining (Mattern et al., 1982). A combination of 3AB and araC further increased the level of breaks in DMS-treated cells. Incubation of cells with 3AB and araC alone did not induce strand breakage (fig. 2).

2.3.2 3AB does not affect DNA strand rejoining

Fig. 3 shows the loss of strand breaks during the recovery period after DMS treatment. In the absence (Fig. 3A) or presence (Fig. 3B) of araC the level of strand breaks present during DNA repair is increased by 3AB. In fig. 4 strand breaks are expressed relative to an initial value of 100% at 10 min after DMS treatment. This allows a comparison of the rate of loss of strand breaks between cells containing different initial levels of breaks. A lower dose of DMS (75 μM for 2.5 min) was used in experiments that included araC due to the potentiation of strand breaks by the drug, especially when used in combination with 3AB (see fig. 2). Fig. 4 shows that without araC both a higher (150 μM for 2.0 min) and lower (50 μM for 2.5 min) dose of DMS resulted in a faster rate of rejoining than when araC was included. Although more breaks exist in cells recovering from damage in the presence of 3AB (figs. 2 and 3), the rate of resealing of these breaks is not affected by inhibition of ADPRP by 3AB (fig. 4). In contrast, araC clearly changes the rate of resealing of breaks, as expected from an inhibition of DNA polymerization. AraC serves as a positive control in fig. 4 by increasing the strand break frequency by limiting ligation. Addition of 3AB to araC-inhibited cells causes a further increase in strand breaks, while the resealing rate is not further decreased. These results suggest

Figure 2. Effect of 3AB and araC on strand break frequency in alklyated HeLa cells. Cells were exposed to 75 μM DMS for 2.5 min and then allowed to recover for 20 min in fresh medium containing: nothing (○); 5 mM 3AB (●); 30 μM araC (□); 30 μM araC and 5 mM 3AB (■). Control cells incubated for 70 min with 30 μM araC and 5 mM 3AB (△). Cells eluted immediately after irradiation with 258 rad of γ-ray (▽).

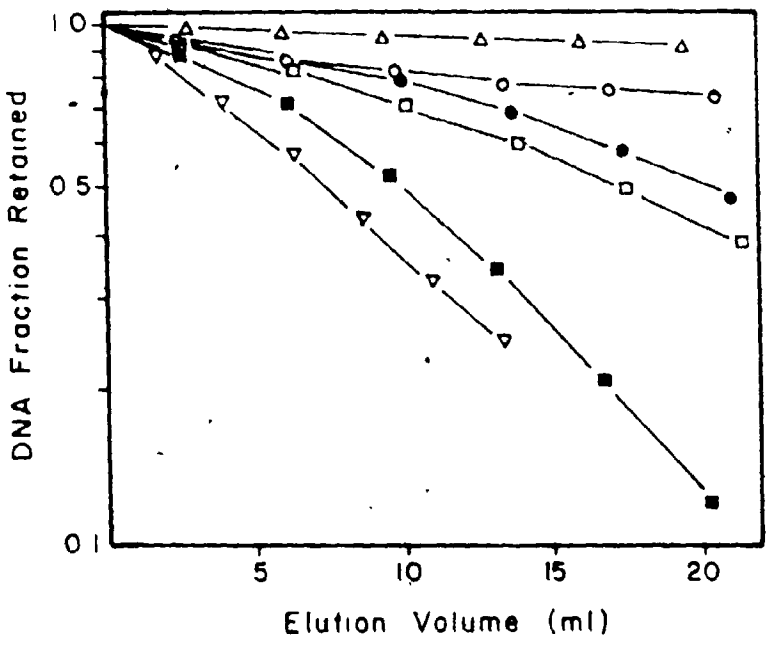

1 0
0 5
0 1
DNA Fraction Retained
5
10
15
20
Elution Volume (ml)

Figure 3. Loss of strand breaks during recovery from DMS damage. Cells exposed to DMS were allowed to recover in the absence (○,△) or presence (●,▲) of 3AB for the indicated time. The DMS dose was 150 μM for 2.0 min in the absence of araC [A (○,●)] or 75 μM for 2.5 min in the presence of araC [B (△,▲)].

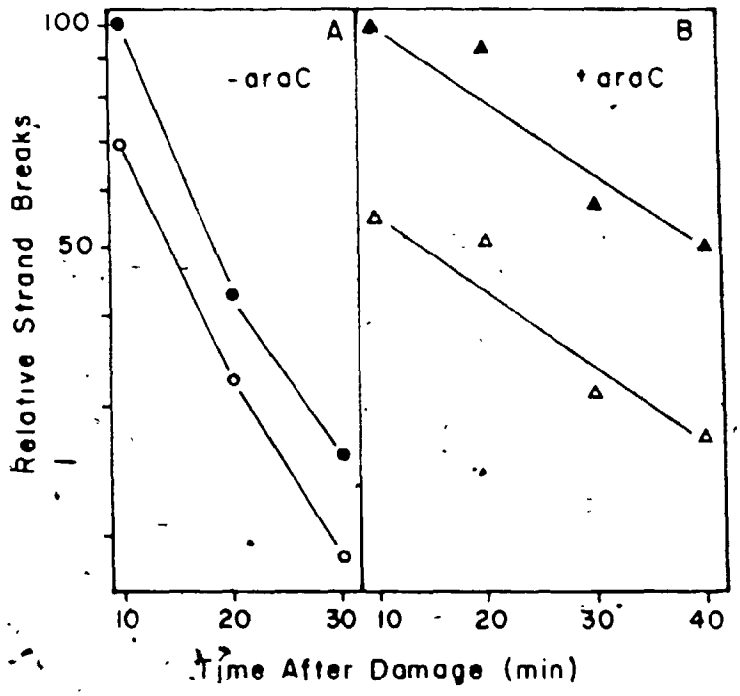
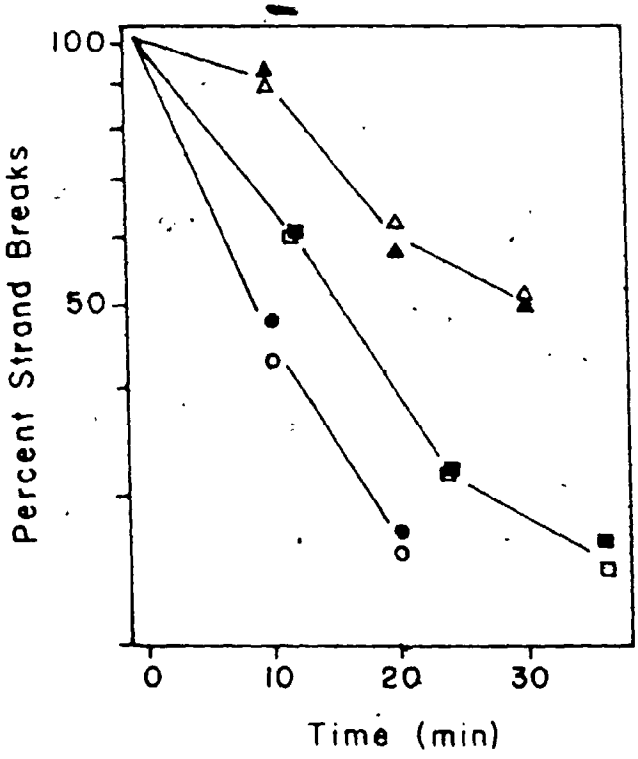

100
50
1
A
-araC
B
+araC
Relative Strand Breaks
10
20
30
10
20
30
40
Time After Damage (min)

Figure 4. Rate of DNA resealing in the absence and presence of 3AB and araC. Strand breaks are expressed relative to an initial value of 100 percent taken at 10 min after DMS exposure. Cells were treated with 75 μM DMS for 2.5 min and allowed to recover with 30 μM araC plus (▲) or minus (△) 5 mM 3AB. Cells incubated without araC after treatment with DMS: 50 μM for 2.5 min (□,■) or 150 μM for 2.0 min (○,●) in the absence (○,△,□) or presence (●,▲,■) of 5 mM 3AB.


100
50
Percent Strand Breaks
0
10
20
30
Time (min)

that the 3AB-induced increase in DNA strand breaks in alkylated HeLa S3 cells is due to an increase in an early repair reaction and not a decrease in ligation.

2.4 Discussion

Poly(ADP-ribose) polymerase is activated by nicked DNA to modify covalently nuclear proteins, including itself, by ADP-ribosylation (Benjamin and Gill, 1980; Adamietz, 1982). Coupled to the degradation of poly(ADP-ribose) by poly(ADP-ribose) glycohydrolase, the level of ADP-ribosylation of proteins may be a mechanism to control DNA-protein interaction (Zahradka and Ebisuzaki, 1982). The production of DNA strand breaks at methylated purines by base excision repair requires the initial formation of apurinic sites by spontaneous acid hydrolysis or enzymatic cleavage of the N-glycosyl bond (Lindahl, 1979). Subsequent AP-endonuclease action would generate a nick in the DNA (Kane and Linn, 1981), capable of activating ADPRP. The observed increase in damage-induced strand breaks when ADPRP is inhibited suggests that ADPRP may act to modulate further strand break formation either by the ADP-ribosylation of an endonuclease/AP-endonuclease or by controlling the accessibility of chromatin to nucleolytic activity.

We question whether an increase in endonuclease or AP-endonuclease activity might be contributing to the increased strand break frequency that has been attributed to decreased ligation. Koide and associates have demonstrated that ADP-ribosylation of Ca^{2+},Mg^{2+}-dependent endonuclease from rat liver (Yoshihara et al., 1975) and bull seminal plasma (Yasuharu et al., 1984) results in enzyme inhibition, probably by

preventing its interaction with DNA. The possibility that the rat liver enzyme is involved in DNA repair has been suggested (Nomura et al., 1981). Cleaver (1985) has recently suggested that the increased repair replication in alkylated cells treated with 3AB might result from increased cutting by Ca^{2+},Mg^{2+}-dependent endonuclease, not necessarily acting at damaged sites. This increased cutting is not observed in undamaged cells treated with 3AB, but might be dependent on altered chromatin structure in damaged cells. The control of DNA incision by ADPRP may explain the observed effects of ADPRP inhibitors on DNA repair. The control of DNA strand break levels may be important for the maintenance of the structural integrity of chromatin during DNA repair.

Chapter 3

ENDONUCLEASE IV-COUPLED ALKALINE ELUTION

3.1 Introduction

Apurinic sites arise spontaneously in DNA by hydrolysis of the N-glycosyl bond at an estimated frequency of 10,000 per day per mammalian cell (Lindahl, 1979). Apurinic sites also result from alkylation of DNA which increases the lability of the N-glycosyl bond of N-alkylpurines (Lawley and Brookes, 1963). In addition, N-alkylpurines may be removed by DNA N-glycosylases that exist in prokaryotic and eukaryotic cells (Lindahl, 1979; Friedberg, 1985; Lindahl, 1982). Loeb (1985) has recently summarized evidence that apurinic sites are mutagenic because of possible nucleotide misincorporation opposite AP sites during DNA replication resulting in transversions. Spontaneous mutations or those induced by DNA damaging agents may result, in part, from the production of apurinic sites in DNA.

To study the dynamics of AP sites during DNA repair a sensitive assay was developed in which E. coli endo IV (Ljungquist, 1977), an AP endonuclease, was coupled with the alkaline elution technique of Kohn et al. (1981). Here I describe an application of this method following treatment of HeLa cells with the alkylating agent DMS and a diol epoxide derivative of the carcinogen benzo(a)pyrene.

3.2 Materials and Methods

3.2.1 Cell growth and treatments

HeLa S3 cells were grown in 100 mm culture dishes with Eagle's MEM medium containing 10% calf serum (Gibco), penicillin (50 units/ml) and streptomycin (50 µg/ml) at 37° C with humidified 5% CO_2 in air. Cells were labelled for 40 h with 0.015 µCi [^{14}C]thymidine per ml (58.0

mCi/mmol) followed by a 3 h chase in fresh medium without label. The labelled cells were then treated with DMS, BPDE or γ-radiation. DMS was dissolved in methanol, diluted in PBS and then added to cell cultures for the indicated time (see figure legends). The DMS-containing medium was then replaced with fresh medium and cells were further incubated as indicated. The final concentrations with both DMS-treated and control cells were 0.003% methanol and 0.3% PBS. anti-trans-7,8-dihydrodiol-9,10-epoxy-benzo(a)pyrene (BPDE) was obtained from the National Cancer Institute's Chemical Carcinogen Repository (Midwest Research Institute). BPDE was dissolved in dimethylsulfoxide (DMSO), absolute ethanol (4:1) and the concentration was confirmed from the absorbance at 345 nm in absolute ethanol using an extinction coefficient of 45,700. Control cells were exposed to an equal amount of ethanol (0.02%) and DMSO (0.08%) without the BPDE. Cells were harvested by rinsing with 2.0 ml of ice-cold PBS-0.02% EDTA, followed by scraping with a rubber policeman into 2.0 ml of PBS-0.02% EDTA and dilution with 4.0 ml of ice-cold medium. Cells were kept at ice-temperature and counted with a Coulter Counter (approximately 1.6×10^6 cells per dish). Cells that were exposed to γ-radiation were first harvested and then irradiated while on ice at 85 rad/min under ambient atmosphere with a ^{137}Cs γ-ray source (Gammacell 20, Atomic Energy of Canada).

3.2.2 AP endonuclease

Endo IV (fraction V) was prepared from E. coli ER22 (endA$^-$) as described by Ljungquist (1981) except that DNA-agarose was used instead of DNA-cellulose. Endo IV activity was determined by

following the conversion of form I DNA to nicked form II molecules that were separated by electrophoresis through 1.0% agarose (Maniatis et al., 1982). Enzyme activity was quantified by cutting out form I and form II bands from ethidium-stained gels and determining the radioactivity by scintillation spectrometry. The reaction mixture (20 ul) contained enzyme diluted into endo IV buffer (0.05 M HEPES-KOH pH 8.2, 1.0 mM EDTA, 0.2 M NaCl, 0.5 mg/ml bovine serum albumin [BSA]) and 1.0 µg [^{14}C]SV40 DNA (approximately 90% form I, 1 X 10^4 cpm/µg). SV40 DNA (Khoury and Lai, 1982) in 0.1 M NaCl, 0.01 M Na-citrate, pH 5.0 was partially depurinated by heating at 70° C for 10 min to introduce an average of 2 AP sites per DNA circle (Lindahl and Nyberg, 1972). Control DNA was not heated at 70° C. The addition of DNA introduced an additional 15 mM NaCl and 1.5 mM Na-citrate to the reaction mixture. Incubation was at 37° C for 15 min.

The final preparation contained 0.04 mg/ml protein (0.20 mg protein/40 g cells) determined by the method of Lowry et al. (1951) as modified by Peterson (1977) and was stored at -70° C in 0.02 M Tris-HCl, pH 8.0, 0.3 M NaCl, 1.0 mM dithiothreitol, 46% glycerol. The enzyme was stable under these conditions (and also at -20° C) for at least several months (data not shown). The specific activity of the enzyme preparation was 2.5 X 10^5 units/mg. One unit of AP endonuclease is defined as the minimum amount of enzyme required to give maximum nicking of 1.0 µg partially depurinated SV40 DNA in the above assay. The enzyme preparation showed no nicking activity with control DNA that was not partially depurinated. A detailed description of the enzyme purification is included in appendix I.

3.2.3 Alkaline elution

The alkaline elution procedure was performed essentially as described by Kohn et al. (1981) with additional steps to accommodate the endo IV incubation. 3×10^5 cells were isolated on polycarbonate filters and washed 3 times with 5.0 ml ice-cold PBS. Cells were then lysed with 5.0 ml of 0.2% sarkosyl (NL-30, CIBA-GEIGY), 2.0 M NaCl, 0.04 M EDTA, pH 10.0. The DNA was rinsed with 5.0 ml of 0.02 M EDTA, pH 9.4, followed by 1.5 ml of endo IV buffer. Flow from the filter holders was then stopped and 1.5 ml of endo IV buffer, containing enzyme as indicated, was added. After incubation at 20-21° C for 1.0 h the reaction mixture was allowed to flow through the filter. The lysis solution and 0.02 M EDTA were at room temperature and the endo IV buffer was at 30° C. The EDTA and subsequent solutions were allowed to flow through the filters by gravity. The filter holders were then connected with tubing to a peristaltic pump and the eluting solution (0.02 M tetrahydryl EDTA, tetrapropylammonium hydroxide, 0.1% SDS, pH 12.1 or pH 12.6) was added. DNA was eluted at 2.0 ml/h for 9 h. Fractions were collected at 1.5 h intervals.

3.3 Results

3.3.1 Specificity of endo IV

DNA from DMS-treated HeLa cells was isolated on polycarbonate filters in preparation for alkaline elution to measure DNA single-strand breaks. To prepare the DNA on the filters for digestion with endo IV, the DNA was first washed with 0.02 M EDTA to remove any remaining lysis solution, followed by rinsing with the endonuclease reaction buffer.

Dithiothreitol was omitted from the endo IV buffer because it caused extensive fragmentation of DNA resulting in a rapid elution of DNA upon alkaline elution. This DNA breakage may be due to radicals or peroxides that arise from an oxygen-dependent reaction in the presence of the reducing agent (Bode, 1967). Enzyme activity was not affected by the absence of dithiothreitol. The difference in the number of strand breaks with and without endo IV treatment is assumed to indicate the number of AP sites.

Endo IV (1000 units) did not nick DNA from either control cells that were not exposed to a damaging agent or cells that had been exposed to γ-radiation (250 rads) while on ice (fig. 5). γ-Irradiated cells were kept at ice-temperature to prevent DNA repair reactions. When DNA from alkylated cells was incubated with endo IV (100 units), additional strand breaks (enzyme-sensitive sites) were detected upon alkaline elution (fig. 6). The increase in DNA elution rate due to the endo IV digestion is attributed to nicking of AP sites by endo IV.

The slope of an elution profile is a linear function of the strand break frequency in the DNA (Kohn et al., 1981). Estimates of single-strand break frequencies are expressed in rad-equivalents and were obtained from a linear calibration curve of γ-ray dose versus elution slope (data not shown). The elution slope was represented by the log fraction of DNA remaining on the filter after 4.5 h of elution. Fig. 7 shows the increase in strand breaks caused by digestion of DNA with endo IV prior to alkaline elution. A maximum increase of approximately 70 rad-equivalents was observed. This corresponds to one AP site per 1.6×10^7 nucleotides, assuming an efficiency of strand break production

of 0.9×10^{-9} per rad per nucleotide (Kohn et al., 1981). Targets of endo IV become limiting as the amount of enzyme is increased beyond approximately 100 units. The saturating dose of endo IV reflects the number of AP sites in the DNA and therefore depends on the degree of alkylation of cells and the number of cells applied to the filter.

3.3.2 AP sites during DNA repair

Fig. 8 shows the appearance and subsequent loss of both strand breaks and AP sites in cells that were exposed to 15.8 μM DMS for 20 min and then allowed to recover in fresh medium for the times shown. DNA repair is demonstrated by the loss of strand breaks during the 2.0-h recovery period. AP sites also decreased during the recovery period in parallel with the loss of strand breaks. An excess amount of enzyme (500 units) was used in each of the experiments shown in fig. 8 to resolve fully the enzyme-sensitive AP sites.

3.3.3 AP and other alkali-labile sites

The effects of the eluting solution pH and the AP endonuclease treatment on the elution profile were studied (fig. 9). AP sites are alkali-labile and are converted to strand breaks in a pH-dependent reaction (Lindahl and Andersson, 1972). An increase in the rate of DNA elution at pH 12.6, compared to the elution rate at pH 12.1, has been used to indicate the presence of alkali-labile sites in DNA from alkylated cells (Kohn et al., 1981). Both elution at pH 12.6 and digestion with endo IV followed by elution at pH 12.1 increased the elution rate when compared with conventional elution at pH 12.1. A saturating dose of endo IV (500 units) was used in these experiments. AP

Figure 5. Absence of AP-sensitive sites in untreated or γ-irradiated HeLa cells. Cells were exposed to 0 (○,●) or 250 rad γ-rays (□,■). Cellular DNA isolated on filters was digested for 1.0 h at 20-21° C with 1000 units endo IV (●,■) or buffer only (○,□) and then eluted at pH 12.1.

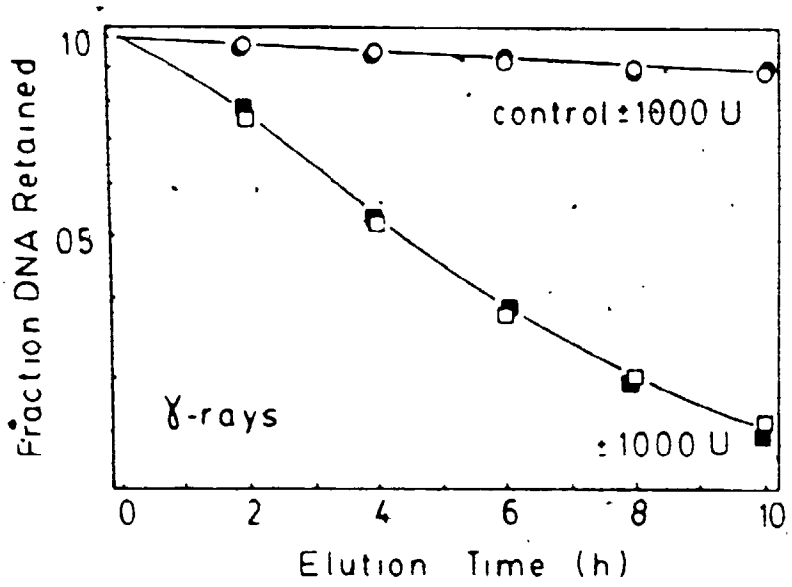

10
05
Fraction DNA Retained
control ± 1000 U
γ-rays
± 1000 U
0
2
4
6
8
10
Elution Time (h)

Figure 6. Endo IV-sensitive sites in DNA from alkylated cells. HeLa cells were alkylated for 3 min with 75 μM DMS and allowed to recover for 10 min. Control cells were not exposed to DMS. Cellular DNA was eluted after digestion with 0 (○,□) or 100 units endo IV (●,■).

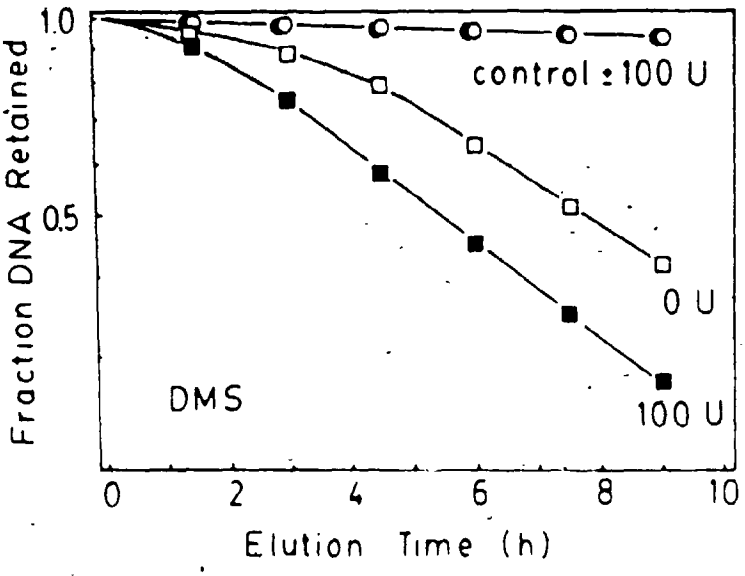

1.0
0.5
Fraction DNA Retained
control ± 100 U
0 U
100 U
DMS
0
2
4
6
8
10
Elution Time (h)

Figure 7. Saturation of endo IV-sensitive sites. HeLa cells were allowed to recover for 10 min after a 3-min exposure to 75 μM DMS. Enzyme digestion with 0–1000 units endo IV as indicated was for 1.0 h. Strand breaks are expressed in rad-equivalents calculated from a γ-ray calibration curve (see Results).

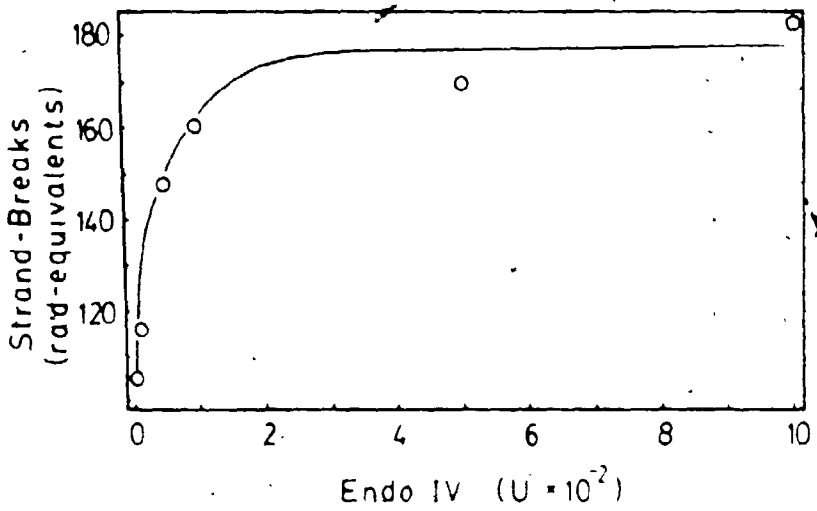

Strand-Breaks
(rad-equivalents)
180
160
140
120
0
2
4
6
8
10
Endo IV (U · 10⁻²)

Figure 8. Repair of AP sites and strand breaks. HeLa cells were allowed to recover for 0-2.0 h after a 20-min exposure to 15.8 μM DMS. Cellular DNA on filters was then digested with 0 (open symbols) or 500 units (closed symbols) endo IV.

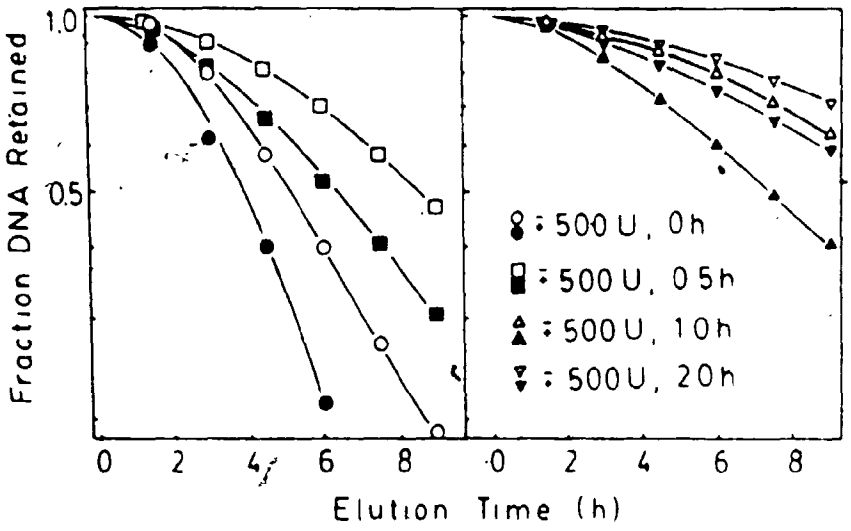
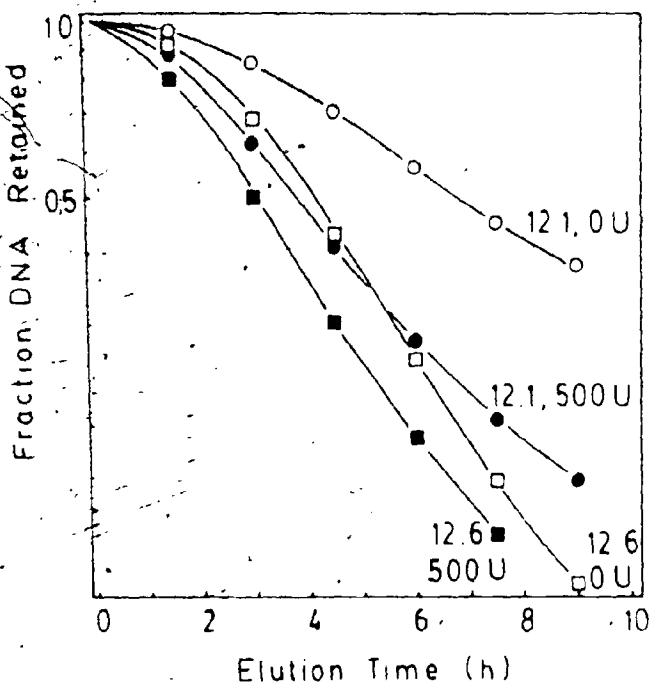

Fraction DNA Retained
1.0
0.5
0
2
4
6
8
0
2
4
6
8
Elution Time (h)
: 500 U, 0h
: 500 U, 0.5h
: 500 U, 1.0h
: 500 U, 2.0h

Figure 9. Endo IV- and pH-sensitivity of AP sites. DNA from alkylated HeLa cells was isolated on filters and digested with 0 (○,□) or 500 units endo IV (●,■) and then eluted at pH 12.1 (○,●) or pH 12.6 (□,■).


1.0
0.5
Fraction DNA Retained
12.1, 0 U
12.1, 500 U
12.6
500 U
12.6
0 U
0
2
4
6
8
10
Elution Time (h)

sites and other alkali-labile sites become strand breaks during the elution period at pH 12.6 resulting in an increase in the elution-curve slope with time. In contrast, the endo IV digestion is completed before the elution begins, thus giving a more linear elution profile. These complicating factors make it difficult to compare the two elution profiles because of their different shapes. Elution of endo IV-treated DNA was faster at pH 12.6 than at pH 12.1. This may be due to strand break production at alkali-labile sites other than AP sites during the elution at pH 12.6 and/or alkali-catalyzed hydrolysis of DNA at this higher pH. It is assumed that all AP sites are accessible to endo IV during the pre-elution digestion. However, it is possible that AP sites that were inaccessible to endo IV may contribute to the increased rate of elution observed by endo IV-digested DNA at pH 12.6.

3.3.4 Benzo(a)pyrene diol epoxide

When cells were alkylated with BPDE alkali-labile sites in cellular DNA were produced. In contrast, the elution of DNA from untreated control cells was unaffected by the pH of the eluting solution (fig. 10). To investigate whether the alkali-sensitive sites were AP sites, BPDE-treated cells were analyzed by endo IV-coupled alkaline elution. Fig. 11 shows that 2.5 h after the addition of BPDE the cells did contain alkali-labile sites, but there were no AP sites.

3.4 Discussion

Endo IV from *E. coli* was used to detect AP sites in HeLa cell DNA. Nonspecific endonuclease activity was not associated with the endo IV

Figure 10. pH-sensitive sites in BPDE-treated cells. Cells were treated with 0 (○,●), 1.0 uM (□,■) or 5.0 uM (△,▲) BPDE for 1.0 h and then analyzed by alkaline elution at pH 12.1 (○,□,△) or pH 12.6 (●,■,▲).

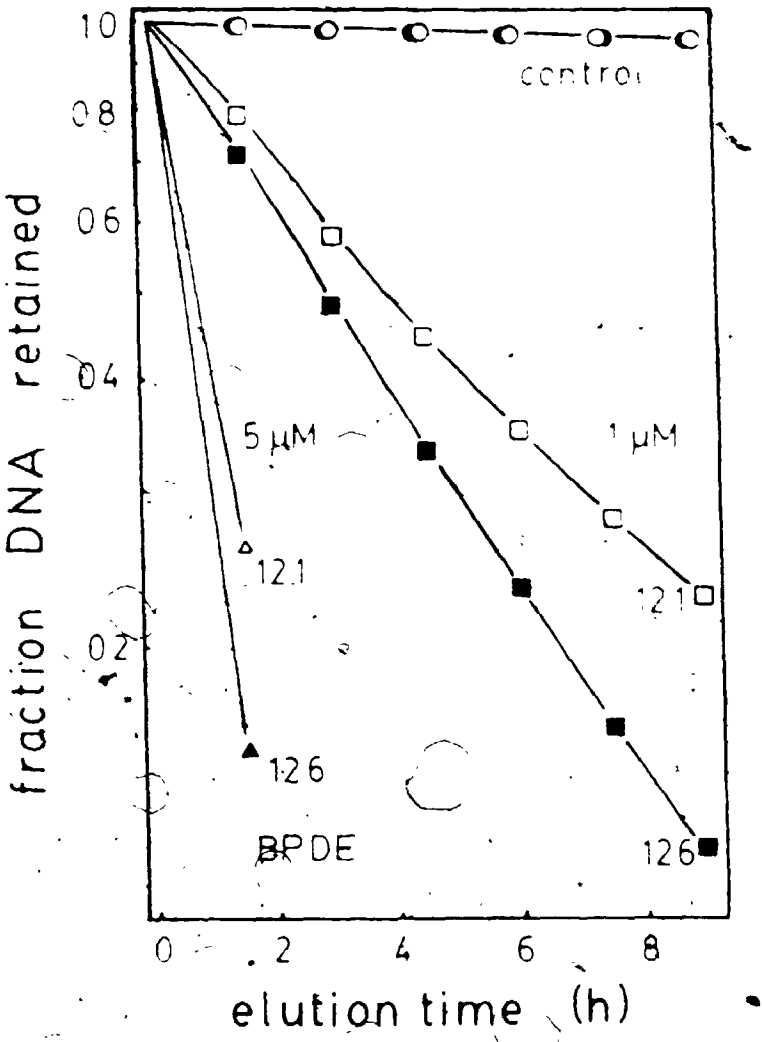
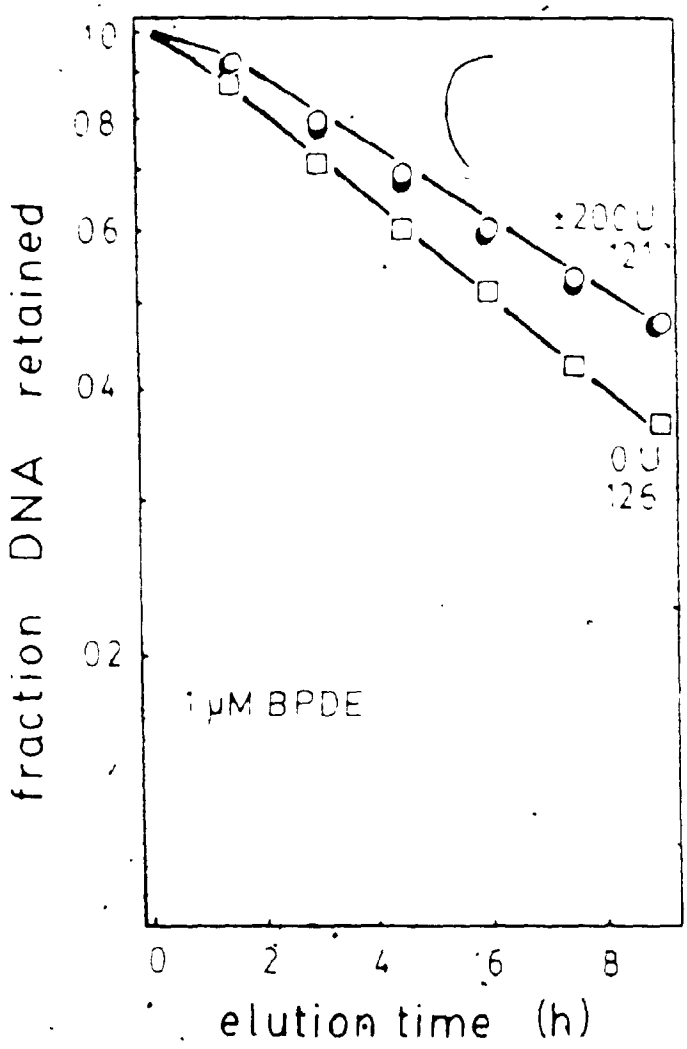

10
08
06
04
02
fraction DNA retained
control
5 μM
1 μM
12.1
12.1
12.6
BPDE
12.6
0
2
4
6
8
elution time (h)

Figure 11. Lack of AP sites in BPDE-treated cells. Cells were treated with 1.0 uM BPDE for 2.5 h and then analyzed by endo IV-coupled alkaline elution. Cellular DNA was digested with 0 (○,□) or 200 units (●) of endo IV and then eluted at pH 12.1 (○,●) or pH 12.6 (□).


1.0
0.8
0.6
0.4
0.2
fraction DNA retained
+200 U
121
0 U
125
1 μM BPDE
0
2
4
6
8
elution time (h)

treatment. This allowed AP sites to be detected with a sensitivity similar to the sensitivity of conventional alkaline elution, which is on the order of one lesion per 10^7 nucleotides (Kohn et al., 1981). Analysis of enzyme-sensitive sites has been used previously in the study of DNA repair. Both the bacteriophage T4 denV gene product (Williams and Cleaver, 1979) and an extract from Micrococcus luteus (Paterson et al., 1973; Fornace, 1982) have been used to detect thymine dimers in DNA, and AP sites in purified viral DNA have been measured with AP endonuclease (Brent et al., 1978; Wallace, 1983).

AP sites were demonstrated to be an intermediate in DNA repair in alkylated HeLa cells. Since AP sites are relatively stable under physiological conditions, with an estimated half-life of at least 100 h (Lindahl and Andersson, 1972), the observed appearance and subsequent loss of AP sites during DNA repair (see Results and fig. 8) suggest the involvement of enzymatic repair processes. The existence of mammalian N-methylpurine-specific DNA glycosylases and AP endonucleases suggest that N-methylpurines, the major alkylation products in DMS-treated DNA (Singer and Kusmierek, 1982) are removed from DNA by base excision repair, initiated by the sequential action of these enzymes (Lindahl, 1982). Purine insertion (Deutsch and Linn, 1979a) at apurinic sites is also a possible explanation.

Simple alkylating agents like DMS are often employed as model electrophilic compounds to gain an understanding of the chemical interactions of more complex carcinogens that require metabolic activation. BPDE is the metabolite of benzo(a)pyrene that reacts covalently with DNA and is a potent mutagen and carcinogen. Many different BPDE adducts are formed in DNA, but their relative

contributions to the biological effects of this alkyating agent are unknown. There is evidence that many chemical carcinogens including simple alkylating agents (Lawley and Brookes, 1963; Drinkwater et al., 1980) and chemicals that form bulky adducts on DNA such as BPDE (Drinkwater et al., 1980; Gamper et al., 1980; Sage and Haseltine, 1984) and β-propiolactone (Schaaper et al., 1982) cause the formation of AP sites in DNA. There was a positive qualitative correlation between mutagenicity and the production of AP sites by these chemicals (Drinkwater et al., 1980). A high incidence of transversions was found among the spectrum of mutations caused by BPDE, aflatoxin B1 and AAAF in bacteria (Foster et al., 1983; Eisenstadt et al., 1982), similar to the mutations induced by AP sites (Schaaper et al., 1983). Depurinated SV40 DNA was mutagenic in transfected mammalian cells (Gentil et al., 1984), and DNA polymerases in vitro can insert purine nucleotides opposite presumed AP sites producing transversions (Kunkel et al., 1983). This led to the suggestion that a common mechanism of chemical-induced mutation may involve the intermediate formation of AP sites in DNA (Schaaper et al., 1982). The detection of AP sites and their relative persistence during DNA repair, as noted in the experiments with DMS, lend support to this suggestion at least for the simple alkylating agents. However, the finding that the BPDE-induced alkali-labile sites are not AP sites clearly refutes the generality of this view.

The ability to measure AP sites in mammalian cells may facilitate further studies of agents that induce AP sites, and the associated DNA repair reactions.

Chapter 4

BASE EXCISION REPAIR IN γ-IRRADIATED HUMAN CELLS

4.1 Introduction

Ionizing radiation is a DNA damaging agent and a common environmental mutagen. In addition to DNA strand breaks, a vast spectrum of base lesions exists in γ-irradiated DNA (Hutchinson, 1985; Ward, 1975; Cerutti, 1976). Several DNA glycosylases capable of releasing inappropriate or altered bases from DNA have been described (Lindahl, 1979, 1982). Only two such enzymes, the thymine glycol-DNA glycosylase that is specific for several 5-6- saturated thymine derivatives, and the formamidopyrimidine-DNA glycosylase, that releases imidazole ring-opened purines, have been implicated in the repair of ionizing radiation damage (Breimer and Lindahl, 1985). Since the spectrum of ionizing radiation-induced base lesions probably far exceed the range of lesions recognized by these two enzymes, the mechanism of repair of a large fraction of base damage remains to be resolved. Although genetic evidence, particularly from mammalian cells, is lacking, the sequential action of DNA glycosylases and the equally ubiquitous AP endonucleases (Linn, 1982) is thought to initiate the base excision repair of damaged bases (Lindahl, 1976). As an alternative to a genetic approach, I sought to test the base excision repair model in γ-irradiated cells by monitoring for the appearance of AP sites, a predicted intermediate in this pathway. E. coli endo IV, an AP-specific enzyme (Ljungquist, 1977), was used to resolve AP sites as a transient DNA repair intermediate in HeLa cells recovering from γ-irradiation.

4.2 Materials and Methods

HeLa S3 cells were grown, labelled with [^{14}C]thymidine and

harvested as described previously. Cells in ice-cold medium (6×10^5/ml) were maintained in an ice-water bath and irradiated under ambient atmosphere at a dose-rate of 85 rad/min with a ^{137}Cs γ-ray source. A post-irradiation recovery period, if included, was initiated by incubating irradiated cells at 37° C for the indicated time. After the recovery period, cells were diluted with 9 volumes of ice-cold PBS and loaded onto polycarbonate filters. The endo IV-coupled alkaline elution procedure was as described previously.

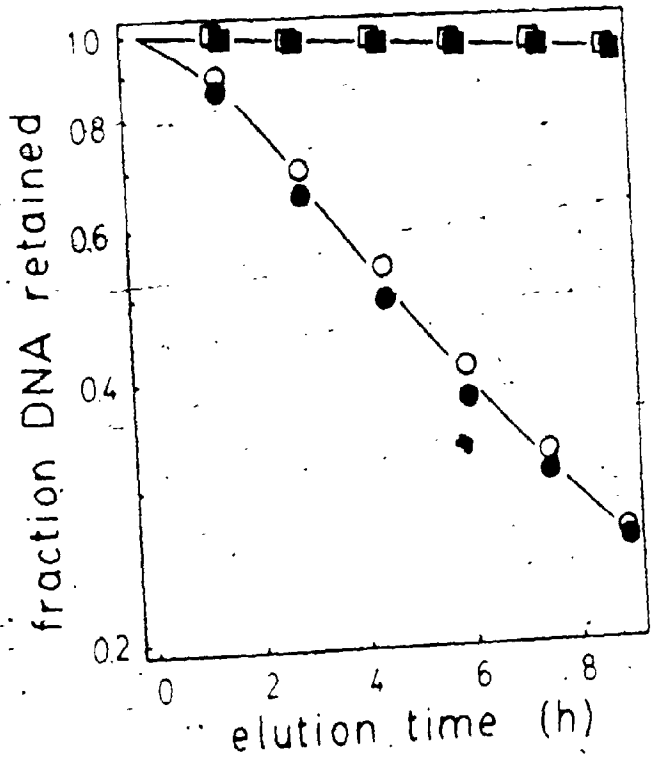
4.3 Results

4.3.1 AP sites after γ-irradiation

We have used a highly sensitive enzyme-coupled alkaline filter elution method to monitor the appearance of AP sites after γ-irradiation of HeLa cells. Cells were collected and then lysed on polycarbonate filters. The cellular DNA on the filters was washed free of the lysis solution, digested with endo IV to generate nicks at AP sites and then eluted at pH 12.1. The rate of elution of DNA is a linear function of the DNA single-strand break frequency (Kohn et al., 1981). The increase in elution rate of endo IV-digested DNA, compared with undigested DNA, is attributed to the nicking of DNA at AP sites by the enzyme.

The enzyme did not nick DNA from either control cells that were not damaged or γ-irradiated cells that were kept at ice temperature to prevent initiation of DNA repair (fig. 12). In contrast, cellular DNA from DMS-treated cells did contain AP sites that were incised by

Figure 12. Alkaline filter elution of DNA from undamaged and γ-irradiated HeLa cells. Cellular DNA on filters was digested with 0 (open symbols) or 100 units of endo IV (closed symbols) and then eluted at pH 12.1. The DNA from cells irradiated with 375 rad γ-rays (circles) contained single-strand breaks causing the increased rate of elution of DNA compared with the unirradiated control (squares).


fraction DNA retained
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
elution time (h)

endo IV (chapter 3, fig. 6). When γ-irradiated cells were allowed a post-irradiation recovery period in growth medium at 37° C strand breaks were rapidly repaired and AP sites were rapidly generated and subsequently removed (fig. 13).

The endonuclease was present in saturating amounts in all experiments. Fig. 14 shows that 100 units of endo IV was sufficient to saturate the endonuclease-sensitive sites at 2 min post-irradiation, when the maximum number of AP sites was observed (see fig. 13). Single-strand break frequencies are expressed in rad equivalents (the dose of γ-ray that generates an equivalent rate of DNA elution) obtained from the gamma ray calibration curve (inset, fig. 15). Using the calibration curve, the number of strand breaks and AP sites from the experiment depicted in fig. 13 were determined and are shown in fig. 15. The number of lesions per cell was obtained by assuming an efficiency of DNA strand break production by γ-radiation of 0.9×10^{-9} per rad per nucleotide (Kohn et al., 1981), and a DNA content of 14.4 pg per HeLa cell (Shapiro, 1976). The number of AP sites was calculated from the increase in DNA strand breaks due to the endo IV treatment. Cells initially had approximately 8700 single-strand breaks per cell and this number decreased by half in approximately 4 min, which is similar to the 3-4 min half-time observed with HeLa cells reported by Ward (1981). AP sites reached a maximum of approximately 1200 per cell after 2 min of repair, and decreased with a half-time of less than 4 min. The observed decrease in AP sites was not accompanied by a corresponding increase the number of strand breaks suggesting that the post-incision repair reactions are not rate limiting.

Figure 13. Endo IV-sensitive AP sites appear in the DNA of γ-irradiated cells upon incubation at 37° C. After irradiation with 375 rad, cells were incubated at 37° C for the indicated time to allow DNA repair. AP sites were revealed in cells by digestion of cellular DNA with endo IV prior to elution.

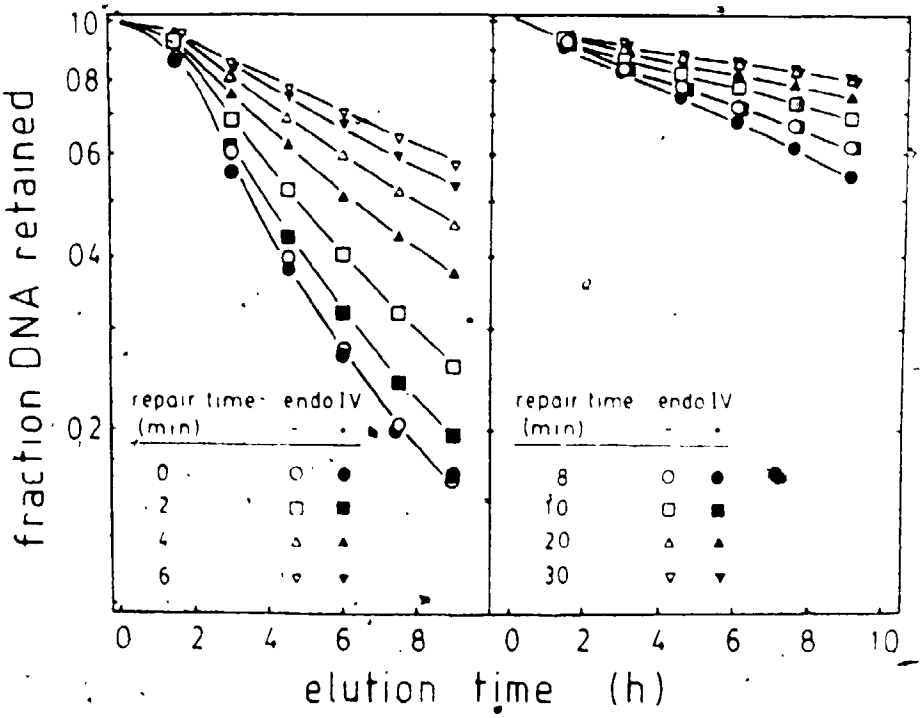

fraction DNA retained
1.0
0.8
0.6
0.4
0.2
repair time (min)
endo IV
0
2
4
6
8
10
20
30
0 2 4 6 8
0 2 4 6 8 10
elution time (h)

Figure 14. Defining a saturating dose of endo IV. Cells were allowed a 2 min recovery period at 37° C following γ-irradiation (375 rad) to allow for the accumulation of AP sites. Cellular DNA on filters was then digested with 0 to 200 units of endo IV and eluted at pH 12.1. The additional strand breaks arise by nicking of DNA at AP sites by endo IV.

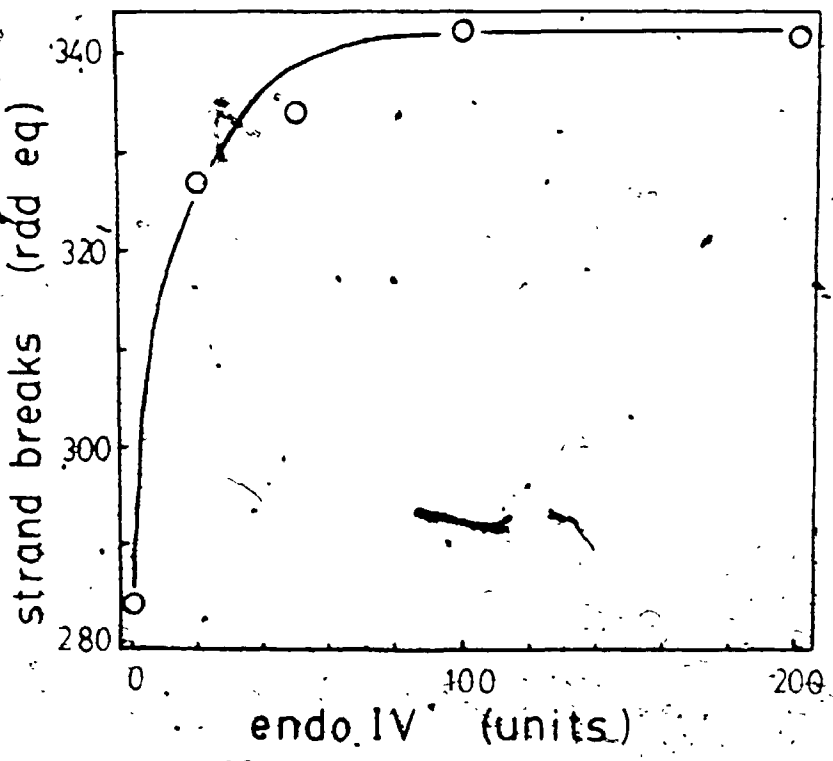
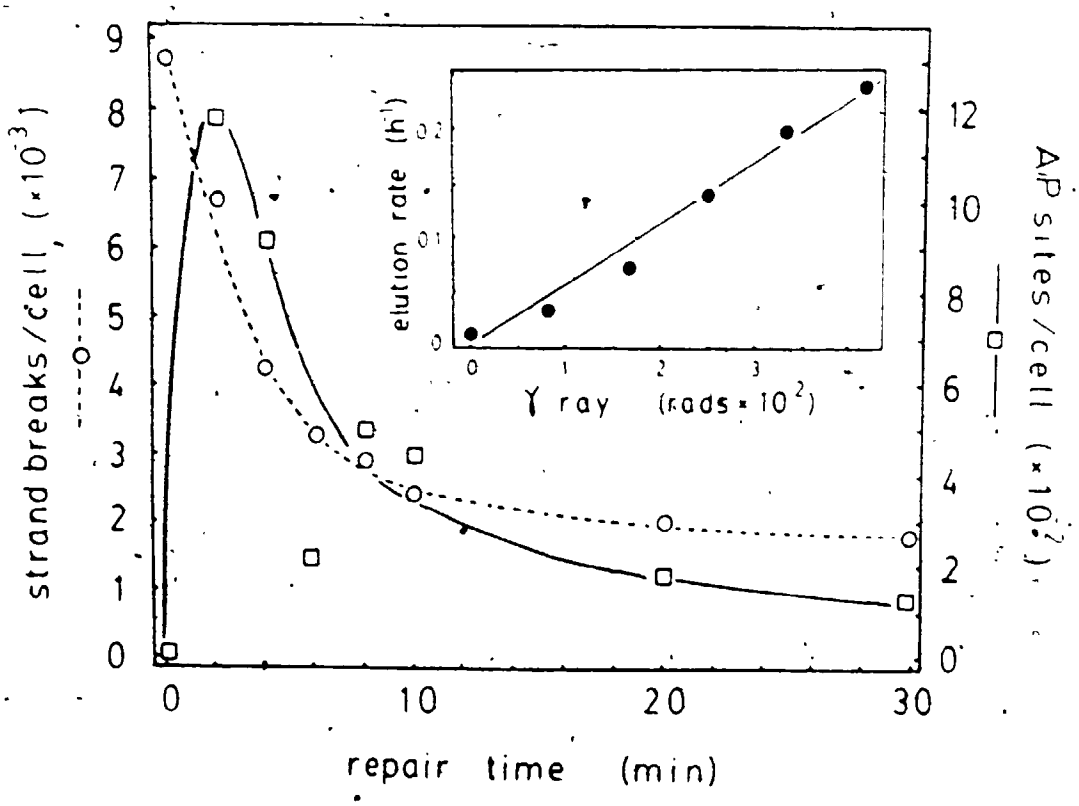

strand breaks (rad eq)
340
320
300
280
0
100
200
endo IV (units)

Figure 15. The repair of DNA strand breaks and AP sites following γ-irradiation. Irradiated cells (375 rad) were allowed the indicated post-irradiation repair period and analyzed for strand breaks and AP sites by endo IV-coupled alkaline elution. The number of strand breaks and AP sites per cell were derived as explained in section 4.3. The calibration curve (inset) was obtained by plotting the elution rate (the slope of an elution profile over the 9h elution period) versus the dose of γ-radiation from cells that were irradiated as indicated while at 0° C and then immediately subjected to alkaline elution. The line was fitted by linear regression analysis.


strand breaks/cell (×10⁻³)
AP sites/cell (×10²)
repair time (min)
elution rate (h⁻¹)
γ ray (rads × 10²)
0
10
20
30
0
1
2
3
0.2
0.1
0
12
10
8
6
4
2
9
8
7
6
5
4
3
2
1

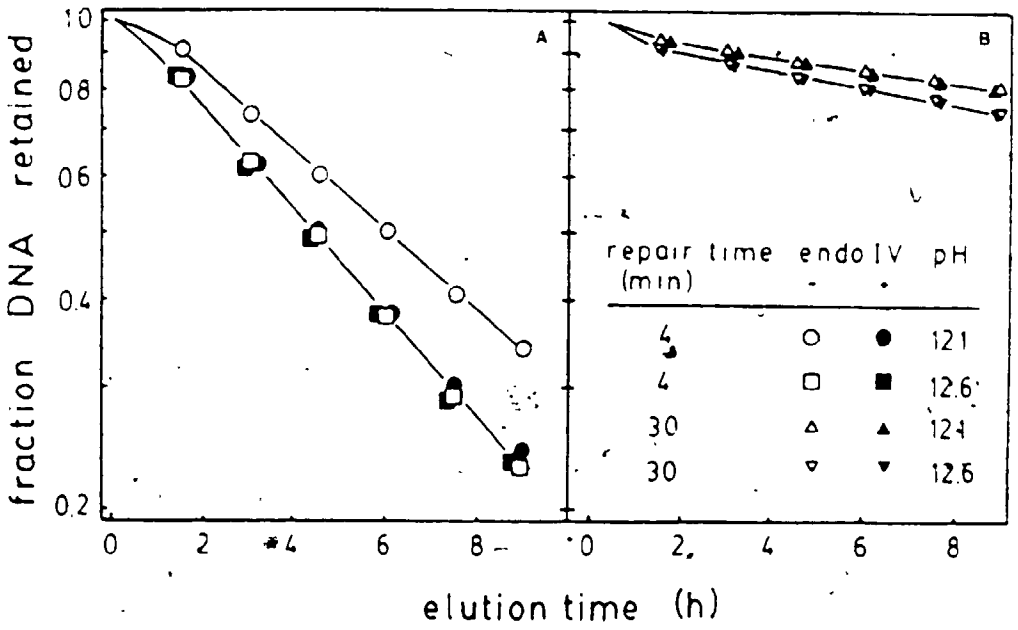
4.3.2 Alkali-labile sites after γ-irradiation

The effect of pH on the elution of DNA from γ-irradiated cells was investigated. In fig. 16A it is seen that at 4 min post-irradiation the DNA elution rate at pH 12.1 after endo IV digestion is equivalent to the elution of digested or undigested DNA at pH 12.6. This implies that AP sites make up the total of alkali-labile sites in the DNA. There was no further increase in the elution rate at pH 12.6 when DNA was first digested with endo IV. Thus alkali-labile sites other than AP sites are not present after this dose (375 rad) of γ-radiation. This result also suggests that the non-AP alkali-labile sites observed with DMS-treated cells (chapter 3, fig. 9) are not due to a population of AP sites that are inaccessible to endo IV. At 30 min post-irradiation the AP sites have been repaired and endo IV digestion of DNA did not increase the elution rate (fig. 16B). However, the presence of non-AP alkali-labile sites is indicated by the slightly faster rate of DNA elution at pH 12.6 compared to pH 12.1, without any effect of prior exposure to the AP endonuclease. This may indicate either the accumulation of some non-AP alkali-labile sites during the 30 min recovery period, or that these sites were not resolvable against the background of endo IV-sensitive sites observed at 4 min after irradiaion.

4.4 Discussion

Pathways of DNA repair could be determined by the identification of the intermediates in the repair process. The transient nature of the AP sites observed in this study, reaching a maximum by 2 min, suggests that they are an early intermediate in a DNA repair pathway.

Figure 16. pH- and endo IV-sensitive sites in γ-irradiated cells. Cells were irradiated (375 rad) and allowed a 4 min (A) or 30 min (B) recovery period to allow DNA repair. Cellular DNA was digested with 0 (open symbols) or 100 units (closed symbols) of endo IV and then eluted at pH 12.1 or pH 12.6 as indicated.


fraction DNA retained
1.0
0.8
0.6
0.4
0.2
A
B
repair time (min)
endo IV
pH
4
12.1
4
12.6
30
12.1
30
12.6
0
2
4
6
8
elution time (h)

and that their removal may be rate limiting. The post-incision reactions of nucleotide excision, gap-filling, and ligation may be common for the repair of the strand breaks that were present initially and for base excision repair (Friedberg, 1985). The direct insertion of purines into apurinic sites would also effect the loss of AP sites (Deutsch and Linn, 1979a). The relatively low dose of γ-radiation (375 rad) commonly used in this study allows approximately 30% survival of the colony-forming ability of the cells (data not shown) suggesting that the observed DNA strand break repair and AP site turnover reflect a physiologically meaningful response.

Although there are a multitude of unknown damaged bases formed in DNA after γ-irradiation (Hutchinson, 1985; Cerutti, 1976), the assay of AP sites provides an assessment of the transit of base lesions through the base excision repair pathway. AP sites are presumably generated by DNA glycosylases that release damaged bases from DNA, and removed by DNA incision by AP endonucleases (Lindahl, 1976; Warner et al., 1980). The relative activities of these enzymes would therefore control the pool size of AP intermediates that arise during the repair of a much larger population of base lesions. Ward (1985) has recently calculated that ionizing radiation-induced base damage in cellular DNA is more than twice as frequent as the sugar damage that causes DNA strand breaks. We conclude that the AP sites detected in this study are intermediates produced during base excision repair and thus provide evidence that this pathway constitutes an important component of the cellular response to DNA damage in γ-irradiated human cells.

Chapter 5

EPILOGUE

The goal of this study was to define cellular responses to DNA damage in human cells. The first parameter of DNA repair investigated was the role of polyADP-ribosylation. I began with the argument that the increase in DNA strand breaks that accompanies inhibition of poly(ADP-ribose) polymerase that had been attributed to a failure in ligation could also be due to an increase in DNA incision. My approach was to use the alkaline elution technique to measure DNA strand break frequencies following damage of cells. By measuring the strand break frequencies in cells at various times after the initial damage, a kinetic profile of DNA resealing was obtained. My results indicated that the inhibition of ADPRP by 3AB results in an increase in DNA strand breaks, but there was no change in the resealing kinetics. Parallel experiments employed cytosine arabinoside to inhibit DNA synthesis during repair. These experiments implied that ADPRP is involved in DNA repair at a step prior to DNA polymerization. PolyADP-ribosylation is required following injury by agents such as simple alkylating agents and ionizing radiation that cause predominantly discrete base lesions in DNA. The above findings suggest that polyADP-ribosylation affects an early step in DNA repair, possibly an endonuclease reaction or a function that is required for DNA incision and a base excision repair pathway is implicated.

The next phase of this study was to define the events of DNA repair following treatment of cells with the simple alkylating agent, dimethylsulfate. A highly sensitive method of estimating AP sites in cellular DNA was developed by coupling endonuclease IV with the alkaline elution procedure. Cells that were alkylated with DMS contained AP sites that were repaired along with DNA strand breaks

during a post-damage recovery period. These results demonstrated that DNA alkylation products are repaired, at least in part, via an AP intermediate suggesting a repair pathway initiated by DNA glycosylases followed by DNA incision by AP endonuclease.

The simple alkylating agents are often employed as model compounds of the more complex mutagenic and carcinogenic electrophilic alkylating agents (Singer, 1985). It was reasoned that since the spectrum of base substitutions induced by both AP sites and certain chemical carcinogens is the same, then the chemical adducts probably induce depurination and the resulting AP sites contribute to the mutagenicity of these chemicals (Loeb, 1985). This model is supported by the fact that these chemicals also produce alkali-labile sites in DNA in vitro. Since AP sites are themselves alkali-labile, the chemical-induced alkali-labile sites have been assumed to be AP sites (Sage and Haseltine, 1984). However, I found that the BPDE-induced alkali-labile sites were not AP sites. This does not support the model of the depurination-induced mutagenicity of BPDE, but it does not exclude it for other untested agents.

Base excision repair was demonstrated in γ-irradiated cells by monitoring for AP sites, a predicted intermediate in this pathway. Endo IV-coupled alkaline elution was used to resolve AP sites as a transient DNA repair intermediate in cells recovering from γ-irradiation. This approach has the advantage that the identity of base lesions traversing this pathway need not be known.

An unexpected finding during these studies was the detection of a class of lesions defined as non-AP alkali-labile sites. Like AP

sites they become strand breaks upon prolonged exposure to alkali, but they are distinct because they are not substrates of endo IV. Fig. 26 summarizes the appearance of this type of lesion. The identities of the non-AP alkali-labile sites are not known, but the ability to measure these lesions apart from AP sites (by digesting cellular DNA with endo IV prior to alkaline elution) should facilitate their further characterization.

To further my studies on base excision repair and the role of ADPRP in this process, another enzyme was purified to extend the enzyme-coupled alkaline elution approach. 3mA-DNA glycosylase II was purified from E. coli (see appendix II). This enzyme releases N-alkylpurines such as 3mA and 7mG from alkylated DNA. Pre-digestion of cellular DNA from alkylated cells with this enzyme followed by endo IV-coupled alkaline elution would allow the kinetics of base removal to be studied in alkylated cells. The effect of ADPRP function on this aspect of DNA repair could then be analyzed. Specifically, are the 3AB-induced DNA strand breaks accompanied by an increased release of alkylated bases from DNA? Preliminary results indicate that 3mA-DNA glycosylase II is able to operate in conjunction with the alkaline elution procedure.

The results summarized above give rise to questions that might be addressed in the future. The lack of AP sites in BPDE-treated cells suggests that other putative AP-producing agents such as AAAF and aflatoxin B1 should be investigated. These experiments would test the hypothesis that AP sites make an important contribution to the mutagenicity of these carcinogenic agents. The endo IV-coupled alkaline elution method might also be employed to test the reported AP

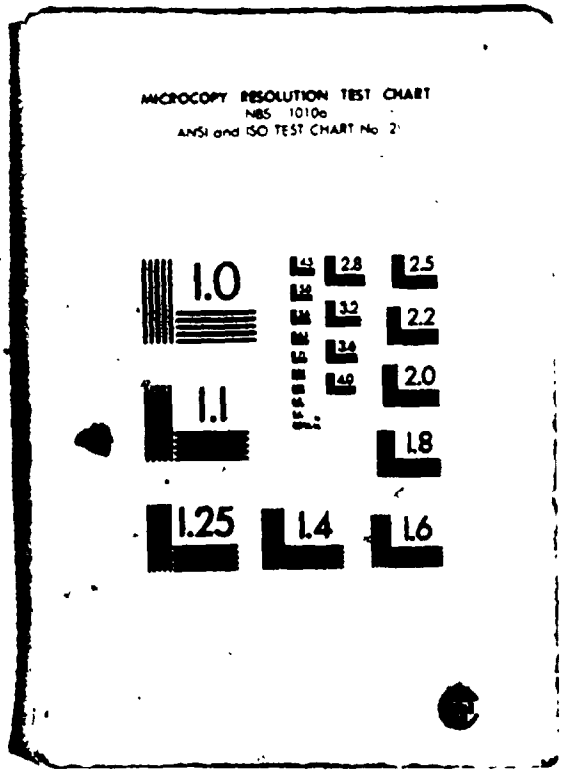
endonuclease deficiency in group D XP cells (Kuhnlein et al., 1978). If these cells are lacking an AP endonuclease involved in the repair of UV-induced damage, then they should contain a population of endo IV-sensitive sites following UV-irradiation. The enzyme-coupled alkaline elution approach could be further extended by purifying additional DNA glycosylases to analyze the repair of more DNA base lesions. The thymine glycol-DNA glycosylase (the nth gene product) of E. coli seems ideally suited for such an application. This enzyme is resistant to inhibition by EDTA and Katcher and Wallace (1983) have observed that even fractions from the early stages of the enzyme purification are devoid of contaminating nuclease activity. This enzyme would facilitate the study of the repair of the major thymine base lesions in γ-irradiated human cells.

In conclusion, the results described in this study clarify the role of polyADP-ribosylation in DNA repair and demonstrate that base excision repair is an important component of the cellular response to DNA damage in human cells.

Figure 17. DNA lesions detectable by endo IV-coupled alkaline elution. The presence (+) or absence (-) of three types of lesion is summarized.

DNA Lesion	Damaging Agent		
	DMS	γ-RAY[a]	BPDE
Strand Breaks	+	+	+
AP sites	+	+	-
non-AP alkali-labile sites	+	-	+
			-

a. A small number of non-AP alkali-labile sites were revealed after most of the strand breaks and AP sites were repaired.


MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No 2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8

Appendix I

ENDONUCLEASE IV

I.1 Introduction

Endonuclease IV was first identified in E. coli xth mutants that lack exonuclease III, the major AP endonuclease of E. coli. The enzyme catalyzes the introduction of single-strand breaks at apurinic and apyrimidinic sites in double-stranded DNA (Ljungquist, 1977). This class II AP endonuclease produces a 3'-OH and a 5'-deoxyribose phosphate at AP sites. Endo IV was prepared essentially as described by Ljungquist (1980) except a nicked-circle assay was used to monitor enzyme activity and DNA-agarose was used instead of DNA-cellulose.

I.2 Purification of endo IV

E.coli ER22 (endA$^-$) was grown in M9 medium supplemented with 0.2% casamino acids and 0.1% yeast extract (Difco) and harvested in the logarithmic growth phase. The cell paste was frozen and stored at -70° C. All buffers contained 1.0 mM dithiothreitol and centrifugations were at 20,000 x g for 20 min. All steps were performed at 0-4° C unless otherwise stated. 40 g of wet cells were thawed at room temperature for 5 min and mixed with 40 ml buffer A (0.05 M Tris-HCl, pH 8.0). Cells were crushed three times at 15,000 psi with a French pressure cell and the cell debris was removed by centrifugation. To the cloudy supernatant (93 ml) was added an equal volume of 1.6% (w/v) streptomycin sulfate (P-L Biochemicals) in buffer A. The suspension was slowly stirred for 30 min, allowed to stand for another 30 min and then centrifuged.

To the cloudy supernatant (fraction I, 182 ml) was added 0.265 g/ml solid ammonium sulfate (ultrapure grade, Schwarz/Mann) over a period of 30 min with gentle stirring. After another 30 min without

stirring the precipitate was removed by centrifugation. An additional 0.127 g/ml ammonium sulfate was added to the supernatant (200 ml) as described above, and the resulting precipitate was collected by centrifugation. The pellet was dissolved in 24 ml of buffer B (0.05 M potassium phosphate, 0.2 M NaCl, pH 7.4) and the solution (34 ml) was then distributed into dialysis bags. After 10 h dialysis against 1 l buffer B the volume of the solution increased to 40 ml (fraction II).

Fraction II was applied in two lots to a column (2.6 x 100 cm) of Sephadex G-75 that had been equilibrated with buffer B. The sample was eluted from the column with 1 l of buffer B at a flow-rate maintained by gravity (140 cm-height of hydrostatic pressure). Fractions were assayed for AP endonuclease activity as described in chapter 3 and were monitored for absorbance at 280 nm. AP endonuclease activity eluted from the column after the bulk of protein and active fractions were pooled. Next, the pooled fractions were heated at 65° C for 5 min and then rapidly chilled in an ice-water bath for 30 min. The resulting precipitate was removed by centrifugation and discarded. The supernatant was dialyzed against 3 l of buffer C (0.02 M Tris-HCl, pH 8.0, 5% glycerol) for 12 h (fracion III, 60 ml).

DNA-agarose was prepared and chromatography was performed as described by Schaller et al. (1972). Dialyzed fraction III was applied to a column (0.9 x 30 cm) of DNA- agarose equilibrated with buffer C. The column was then washed with 60 ml of buffer C that eluted most of the applied protein. Endo IV was recovered from the column with a batch-elution of 0.3 M NaCl in buffer C. Fractions (4.3 ml) were collected and active fractions were pooled (fraction IV, 37 ml).

Fraction IV was passed through a column of DEAE-cellulose (DE52, Whatmann) to remove any traces of nucleic acid remaining from the DNA-agarose step. The sample was applied to the column (2 ml of DEAE-cellulose in a 5 ml syringe plugged with glass wool) and recovered in the flow-through. The eluate (40 ml) was concentrated by ultrafiltration with a PM10 filter (Amicon), adjusted to 46% glycerol, distributed into 0.5 ml aliquots and stored at -20 or -70° C (fraction V, 5.0 ml). The enzyme activity was stable for at least several months under these conditions.

I.3 Results and discussion

Fig. 17 shows an agarose gel (ethidium stained) that was used to resolve form I and form II DNA molecules that were digested with samples eluted from the DNA-agarose column. The AP endonuclease activity was recovered upon the addition of 0.3 M NaCl to the column. The conversion of partially depurinated form I DNA into nicked form II molecules by column fractions 32-42 reflects the release of endo IV activity from the DNA-agarose. A comparison of lanes 4 and 12 in fig. 17 shows that the endonuclease activity was completely dependent on DNA containing AP sites since fraction 32 was only able to incise DNA that had been partially depurinated by heating at 70° C. A detailed description of the AP endonuclease assay is included in chapter 3 (section 3.2.2). Fig. 19 shows the activity of the endo IV (fraction V) and was used to define the unit of enzyme activity.

Fig. 20 summarizes the results of the enzyme purification. The final preparation (fraction V) was purified approximately 3600-fold with a recovery of approximately 50%. Ljungquist (1981) achieved a

similar purification with a recovery of 20%. The apparent increase in total enzyme activity between fractions III and V might be due to the instability of the AP endonuclease activity in these fractions. The samples used to determine enzyme activity in these fractions had been stored at 0° C for several days and their inherent endo IV activity may have decreased during that period. The protein profiles at the various stages of the purification are shown in fig. 21. Lane 5 (fraction V) of this 10% SDS-polyacrylamide gel appears to contain 2-3 proteins (depending on the staining procedure) with a major band migrating at a position expected for endo IV (M_r 30,000) as expected from the observations of Ljungquist (1980).

Figure 18. Elution of endo IV from DNA-agarose. (lanes 1-12) Samples (1.7 ul) of column fractions (as indicated) were incubated with 0.14 ug of SV40 form I DNA that did (+) or did not (-) contain AP sites. (lanes 13-14) Buffer only was incubated with the indicated DNA. Following electrophoresis, the 1.0% agarose gel was stained with ethidium bromide and illuminated with a UV source. AP-specific endonuclease activity was recovered when 0.3 M NaCl was added to the column. This activity did not incise DNA that did not contain AP sites (lane 12).

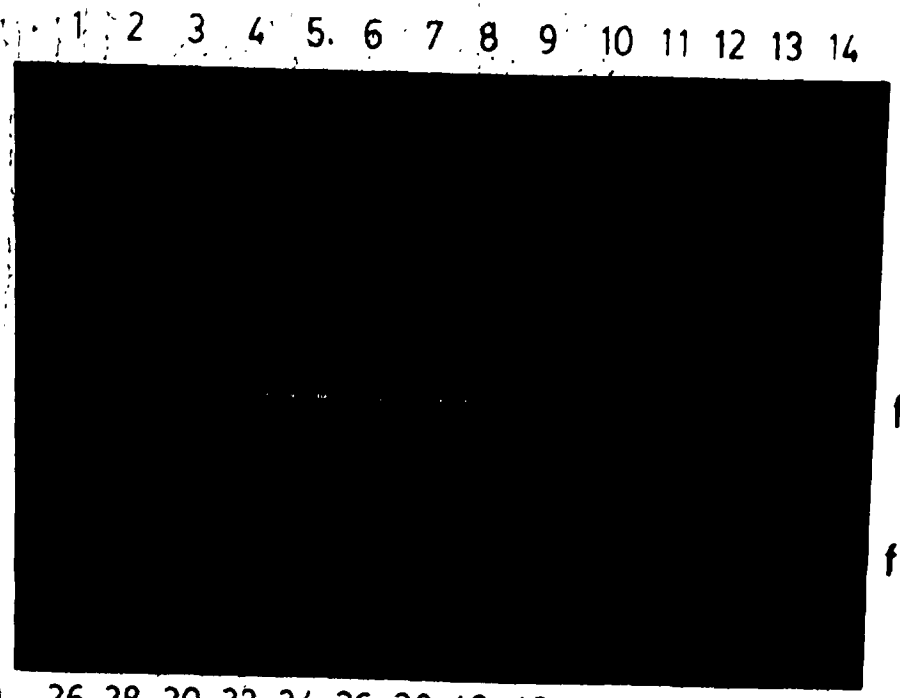

1 2 3 4 5. 6 7 8 9 10 11 12 13 14
form II
form I
fraction 26 28 30 32 34 36 38 40 42 44 46 32 - -
± AP sites + + + + + + + + + + + - - +
0.3 M NaCl

Figure 19. Quantification of endo IV activity. [^{14}C]-labelled SV40 DNA (1.0 ug) was resolved by agarose gel electrophoresis following digestion with the indicated amount of endo IV (fraction V). Form I and II DNA bands were cut out and their radioactivity was measured. The minimum amount of enzyme required to fully nick the substrate in the standard assay is defined as one unit of AP endonuclease activity and is indicated by the arrow.

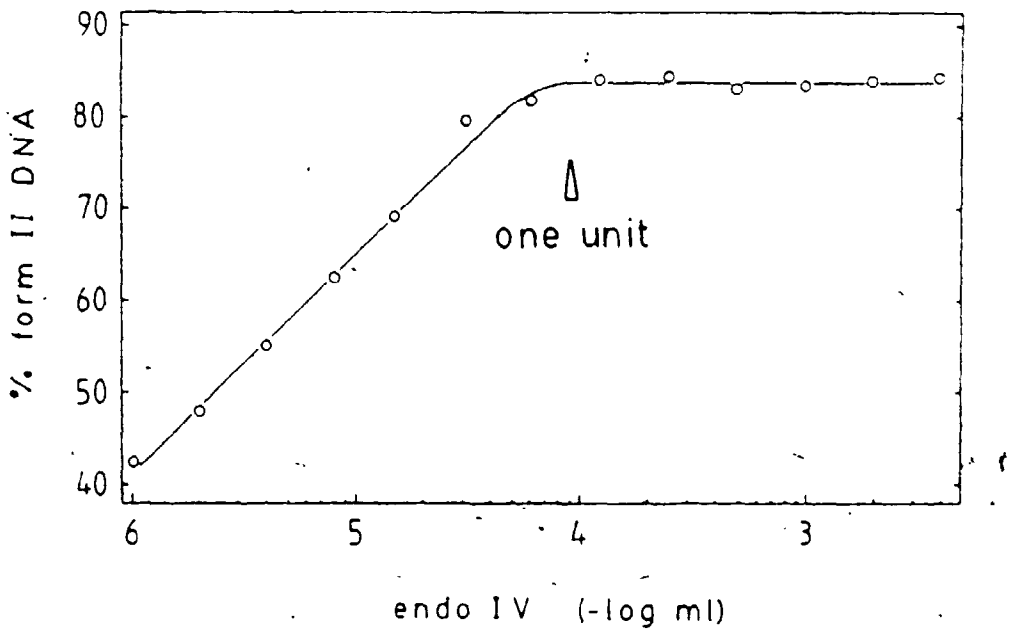
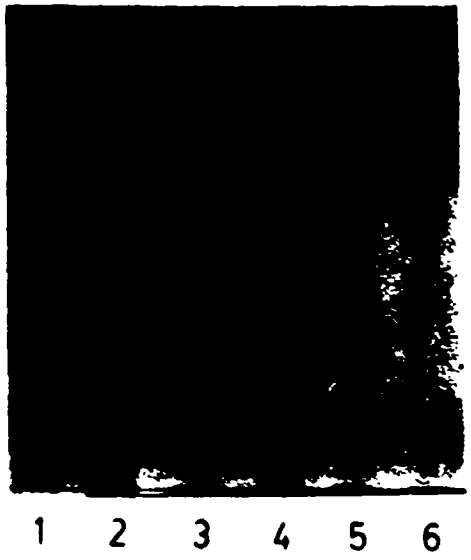

90
80
70
60
50
40
% form II DNA
one unit
6
5
4
3
endo I V (-log ml)

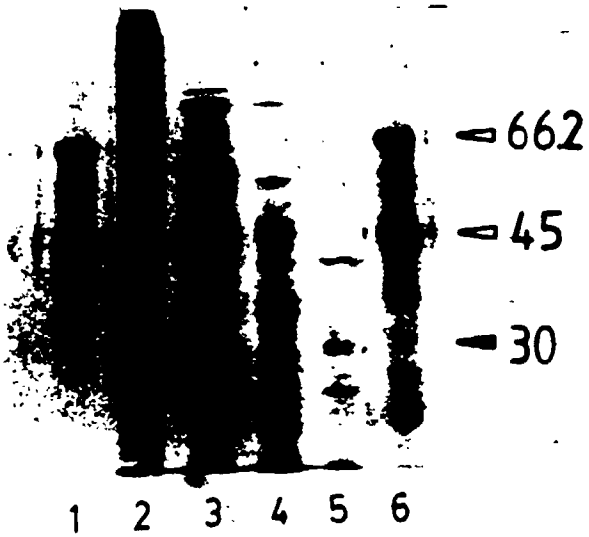
Figure 20. Purification of endo IV. AP endonuclease activity was measured with the standard assay and protein concentration was determined according to Lowry et al. (1951) as modified by Peterson (1977)..

FRACTION	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	PURIFICATION
	mg	units	units/mg	-fold
II Ammonium sulfate	1420	98700	69.5	1
III Sephadex G-75	204	38000	185	2.7
IV DNA-agarose	0.96	40500	42200	607
V DEAE cellulose	0.20	50000	250000	3600

Figure 21. Protein profile during endo IV purification. Samples were subjected to electrophoresis through a 10% polyacrylamide gel with a 4% stacking gel in the presence of SDS as described by Laemmli (1970). The same gel was stained first with 0.25% Coomassie Brilliant Blue (upper gel) and then, following destaining, with silver stain. Lanes 1 and 6 contain the molecular weight markers (5 µg of each) bovine serum albumin (M_r 66,200) and ovalbumin (M_r 45,000) as indicated by the open arrows. The closed arrow indicates the position expected for a 30,000 molecular weight protein. Lane 2, 45 ug fraction II (ammonium sulfate); lane 3, 34 µg fraction III (Sephadex G-75); lane 4, 15 µg of fraction III after the 65° C heat step; lane 5, 0.8 µg fraction V (concentrated DEAE-cellulose eluate).



$M_r \times 10^{-3}$



Appendix II

3-METHYLADENINE-DNA GLYCOSYLASE II

II.1 Introduction

3mA-DNA glycosylase II is the product of the *alkA* gene in *E. coli*. The gene was cloned and its product was purified and characterized by Nakabeppu *et al.* (1984). The *alkA* protein catalyzes the release of N7 and N3-methylpurines from DNA by breaking the appropriate N-glycosyl bonds. The enzyme also releases the analogous ethylated bases as well as some O-alkylpyrimidines from DNA (McCarthy *et al.*, 1984). The *alkA* protein was purified for use with the endo IV-coupled alkaline elution procedure to extend our studies of the early steps of base excision repair of DNA in human cells.

II.2 Purification of 3mA-DNA glycosylase II

The enzyme activity was monitored by measuring the release of [^{3}H]methyl-labelled bases from alkylated DNA into an ethanol-soluble fraction as described by Nakabeppu *et al.* (1984). The reaction mixture (50 µl) contained 1.8 µg of [^{3}H]MNU-treated calf thymus DNA (8060 cpm/µg) prepared according to Cathcart and Goldthwait (1981), 0.07 M Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 mg/ml BSA and enzyme. After 20 min at 37° C, the reaction was stopped by the addition of an equal volume of 1 mg/ml of BSA, 1 mg/ml denatured calf thymus DNA, 0.2 M NaCl followed by the addition of 200 ul of ethanol. After 15 min at -70° C the mixture was centrifuged (15,000 x g for 10 min) and 250 µl of the supernatant was taken as the ethanol-soluble fraction. The radioactivity in this fraction was determined with a liquid scintillation counter. One unit of 3mA-DNA glycosylase activity is defined as the activity required to release 1 pmol of methylated base per min from the DNA.

The alkA protein was purified from E. coli DH1 harbouring a plasmid (pYN1000, graciously provided by Dr. M. Sekiguchi, Kyushu University, Japan) that contains the inducible wild-type alkA gene. Cells were grown, induced with MMS and harvested as described by Nakabeppu et al. (1984) and the subsequent purification steps were adapted from their procedure. Centrifugations were at 20,000 x g for 20 min and all steps were performed at 0-4° C unless indicated otherwise.

The wet cell paste (10 g) was diluted with buffer A (0.02 M Tris-HCl, pH 8.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 5% glycerol) containing 0.3 M NaCl and crushed three times at 15,000 psi with a French pressure cell. After removing the cell debris by centrifugation, the supernatant (fraction I, 35 ml) was mixed with an equal volume of DEAE-cellulose that had been equilibrated with buffer A containing 0.3 M NaCl. After a low-speed centrifugation (1500 x g for 10 min) the supernatant was recovered. The DEAE-cellulose pellet was back-extracted once and centrifuged again. The combined supernatants were dialyzed against buffer A (fraction II, 70 ml). Fraction II was applied to a column (0.9 x 30 cm) of DNA-agarose previously equilibrated with buffer A. After washing the column with 60 ml of buffer A, the column was developed with a 200 ml linear gradient of NaCl (0-0.5 M) in buffer A. Samples (300 µl) were taken from the column fractions (2.0 ml), precipitated with 750 µl of acetone and then analyzed for protein by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue. Fractions 50-100 (0.25-0,5 M NaCl) revealed a major band corresponding to the molecular weight of the alkA protein (M_r 30,000) (fig. 23). The

pooled fractions were concentrated to 5.0 ml by ultrafiltration with a PM10 filter, diluted with water to approximately 30 ml, and re-concentrated. The resulting precipitate was removed by centrifugation and the supernatant was adjusted to 40% glycerol and stored at -20 ° C for several weeks (fraction III, 6 ml).

Fraction III was thawed, dialyzed against buffer A and applied to a column (0.9 x 20 cm) of phosphocellulose (P11, Whatmann). Under these conditions (pH 8.5) few proteins other than the alkA protein bind to the phosphocellulose. After washing the column with 60 ml of buffer A, the column was developed with a 200 ml linear gradient of NaCl (0-0.3 M) in buffer A. The glycosylase activity eluted as a single peak with 0.18 M NaCl. Fractions 58-66 (2 ml each) were pooled and then concentrated by counterdialysis against buffer A containing 30% polyethylene glycol (PEG 6000), adjusted to contain 20% glycerol, distributed into 1.0 ml aliquots and stored at -20° C (fraction IV, 6.9 ml).

II.3 Results and discussion

The purification of 3mA-DNA glycosylase II is summarized in fig. 22. The enzyme was purified approximately 60-fold with a recovery of 12%. This compares favourably with the procedure of Nakabeppu et al. (1984) who obtained a 42-fold purification with a recovery of 22% using a protocol that did not involve diluting the sample with water ("Ebisuzaki extraction"); placed the cation-exchange chromatography before the DNA-agarose step, and included a gel filtration column. Fig. 23 shows the elution of the alkA protein from the DNA-agarose

column. The Ehisuzaki extraction, not surprisingly, resulted in the precipitation of many proteins from the sample, while most of the alkA protein remained in solution (fig. 24). The alkA protein eluted as a single peak from the phosphocellulose column with 0.18 M salt (fig. 25). The enzyme activity varied linearly with the amount of fraction IV added, showing two components (fig. 26). This biphasic response probably reflects the rapid release of 3mA, which comprises approximately 10% of the alkylated bases in the substrate, followed by the slower release of 7mG (75% of alkylated bases) from the alkylated DNA (Thomas et al., 1982).

Figure 22. Purification of 3mA-DNA glycosylase II. DNA glycosylase activity was determined as described in section II.2 and protein concentrations were determined by the method of Lowry et al. (1951) as modified by Peterson (1977).

FRACTION	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	PURIFICATION
	mg	units	units/mg	-fold
II DEAE-cellulose	420	9100	21.7	1
III DNA-agarose	2.6	2424	925	43
IV Phospho-cellulose	0.83	1079	1300	60

Figure 23. DNA-agarose chromatography. Samples from column fractions were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis. Lanes 2 and 3, 20 µl each of column flow-through and wash, respectively; lanes 4-19, fractions collected during the linear gradient of increasing salt concentration as indicated. The fractions that eluted between 0.25 and 0.5 M NaCl (lanes 12-19) contained a major M_r 30,000 protein (indicated by the closed arrow) as expected for 3mA-DNA glycosylase II. Lanes 1 and 20, molecular weight markers were bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), and soybean trypsin inhibitor (M_r 21,500).

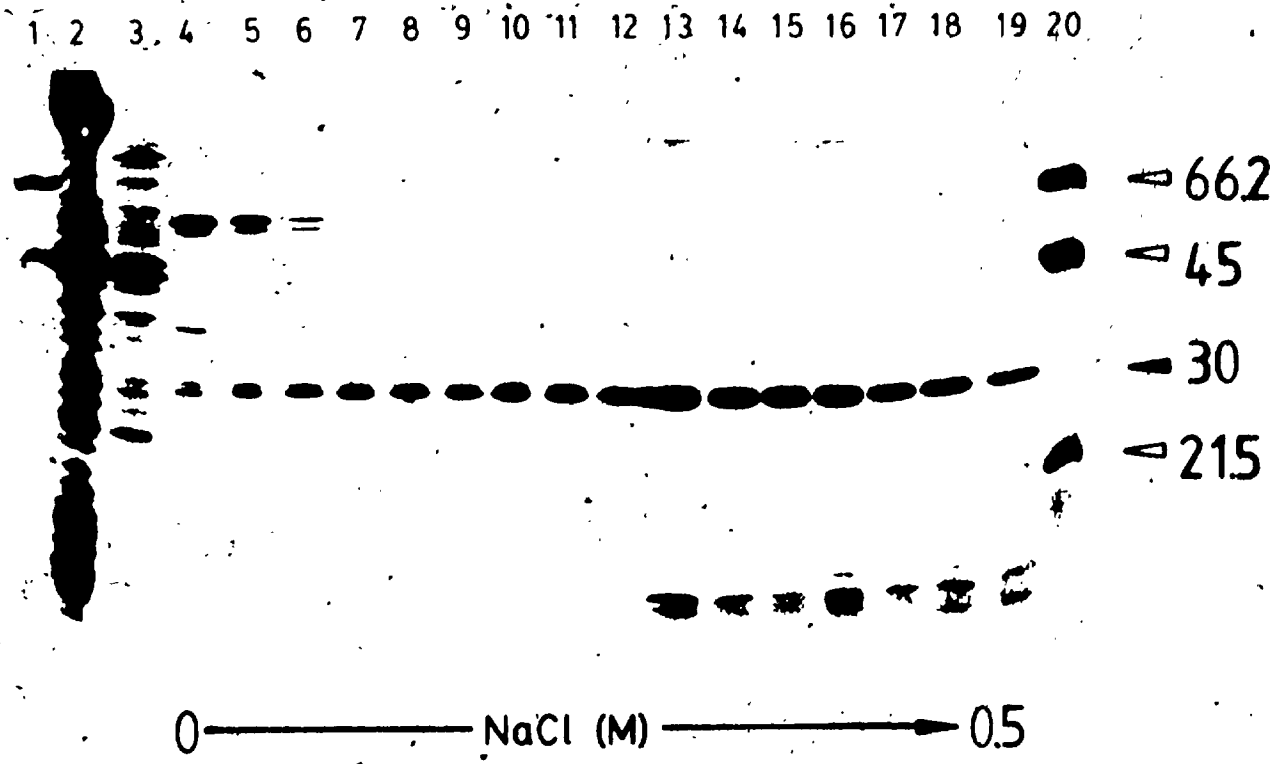

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
662
45
30
215
0
NaCl (M)
0.5

Figure 24. Water extraction of 3mA-DNA glycosylase II. Samples were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis with molecular weight markers as described above. Lanes 2 and 3, 20 µl each of the DNA-agarose flow-through and wash, respectively; lane 4, 20 µl of the resuspended precipitate (5 ml in buffer A) that resulted from the dilution with water of the pooled DNA-agarose fractions; lane 5, 3.6 µg of fraction III (pooled DNA-agarose fractions after dilution, clarification, and concentration).

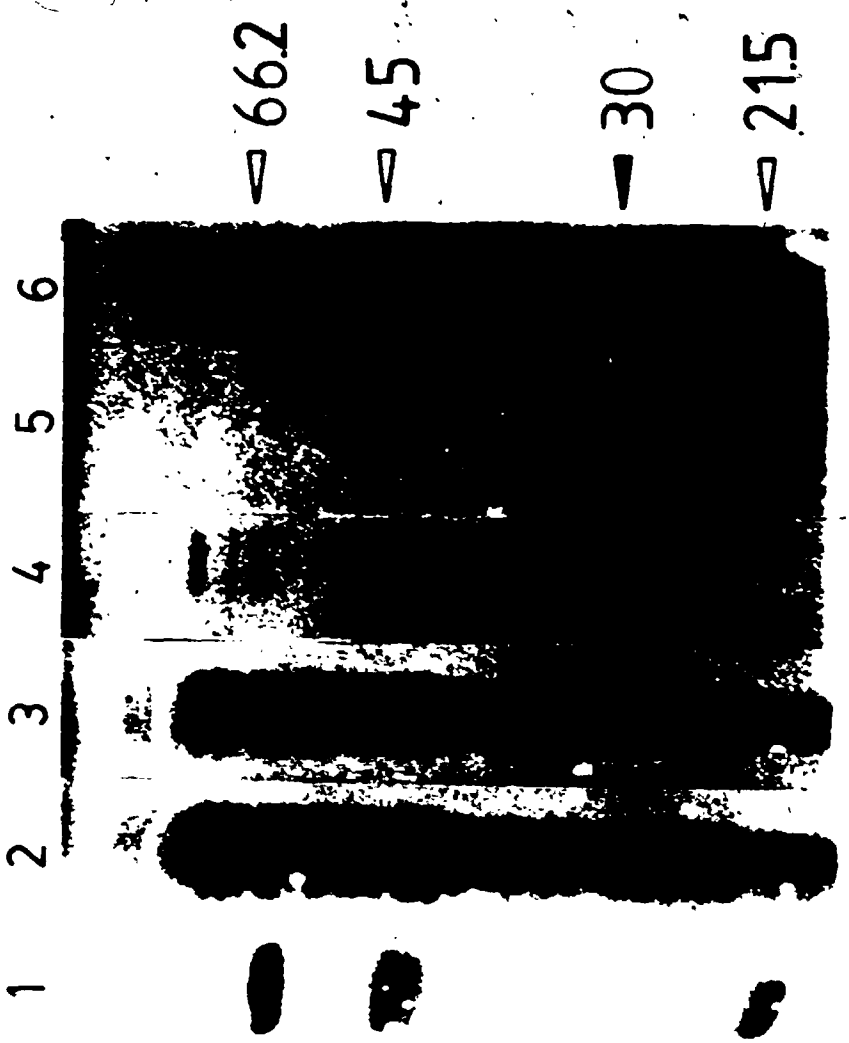

662
45
30
215
6
5
4
3
2
1

Figure 25. Phosphocellulose chromatography of 3mA-DNA glycosylase II. The enzyme was retained by the cation exchange resin even at pH 8.5. The column was developed with a linear salt gradient (×) and enzyme activity (○) was recovered in fractions 58-66.

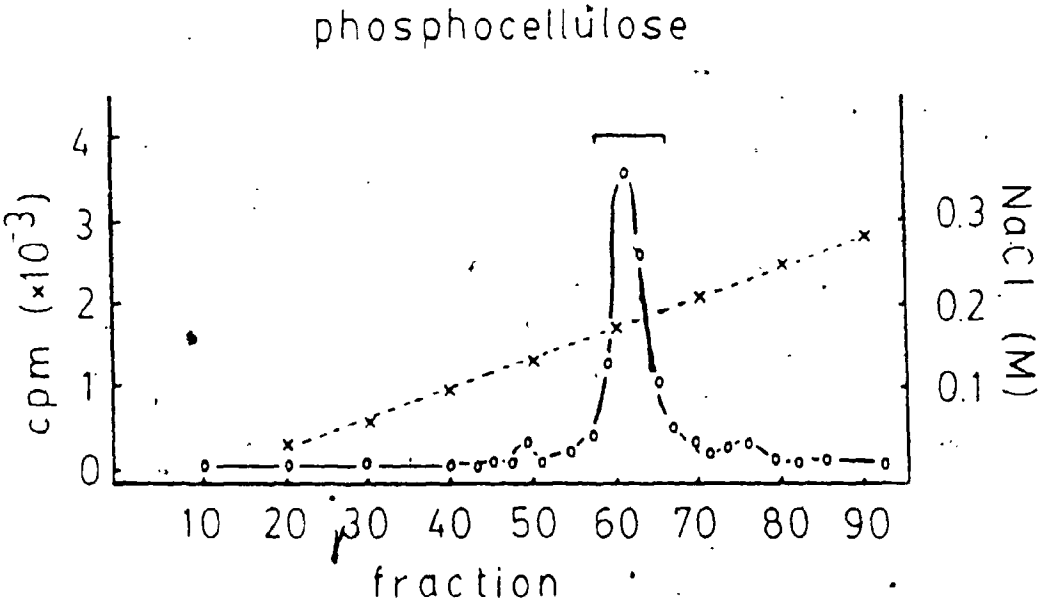
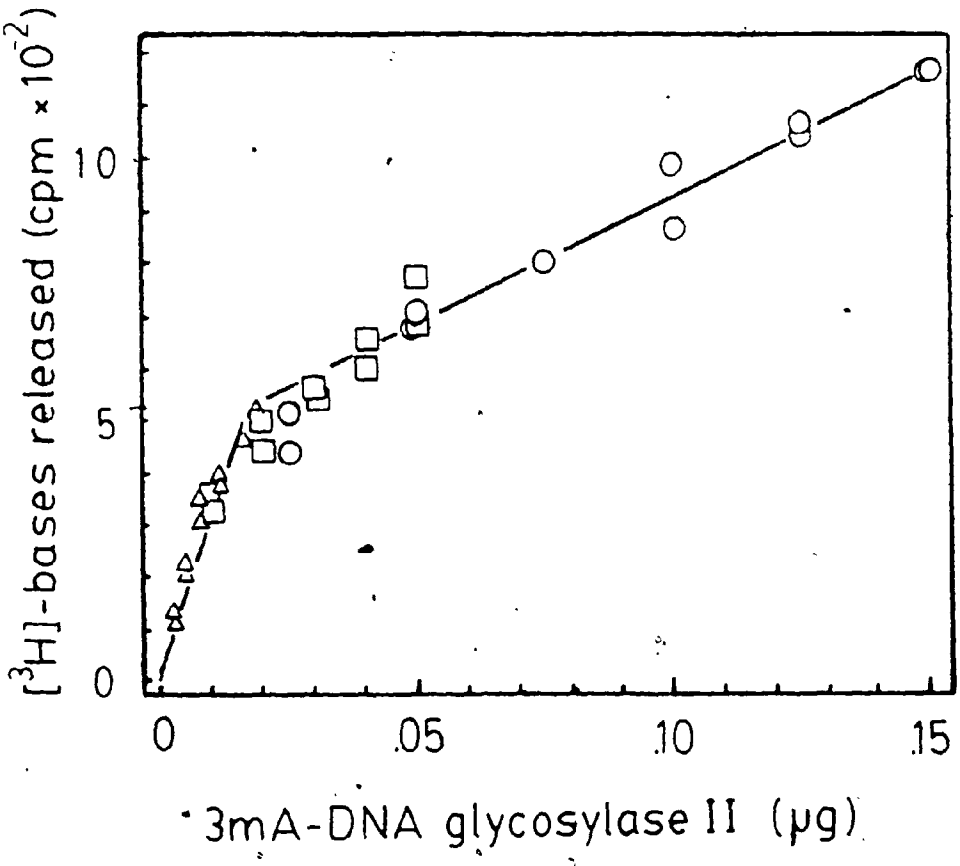

phosphocellulose
cpm (×10⁻³)
0
1
2
3
4
NaCl (M)
0.1
0.2
0.3
10 20 30 40 50 60 70 80 90
fraction

Figure 26. Activity of 3mA-DNA glycosylase II. Fraction IV of the enzyme preparation catalyzed the release of bases from [^{3}H]MNU-treated DNA in a biphasic manner. Enzyme activity was determined as described in section II.2. The different symbols represent separate experiments.


[3H]-bases released (cpm × 10-2)
0
5
10
0
.05
.10
.15
3mA-DNA glycosylase II (μg)

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I.1 Introduction

Endonuclease IV was first identified in E. coli xth mutants that lack exonuclease III, the major AP endonuclease of E. coli. The enzyme catalyzes the introduction of single-strand breaks at apurinic and apyrimidinic sites in double-stranded DNA (Ljungquist, 1977). This class II AP endonuclease produces a 3'-OH and a 5'-deoxyribose phosphate at AP sites. Endo IV was prepared essentially as described by Ljungquist (1980) except a nicked-circle assay was used to monitor enzyme activity and DNA-agarose was used instead of DNA-cellulose.

I.2 Purification of endo IV

E.coli ER22 (endA$^-$) was grown in M9 medium supplemented with 0.2% casamino acids and 0.1% yeast extract (Difco) and harvested in the logarithmic growth phase. The cell paste was frozen and stored at -70° C. All buffers contained 1.0 mM dithiothreitol and centrifugations were at 20,000 x g for 20 min. All steps were performed at 0-4° C unless otherwise stated. 40 g of wet cells were thawed at room temperature for 5 min and mixed with 40 ml buffer A (0.05 M Tris-HCl, pH 8.0). Cells were crushed three times at 15,000 psi with a French pressure cell and the cell debris was removed by centrifugation. To the cloudy supernatant (93 ml) was added an equal volume of 1.6% (w/v) streptomycin sulfate (P-L Biochemicals) in buffer A. The suspension was slowly stirred for 30 min, allowed to stand for another 30 min and then centrifuged.

To the cloudy supernatant (fraction I, 182 ml) was added 0.265 g/ml solid ammonium sulfate (ultrapure grade, Schwarz/Mann) over a period of 30 min with gentle stirring. After another 30 min without