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THE PURIFICATION AND CHARACTERIZATION OF THE X-RAY ENDONUCLEASE OF ESCHERICHIA COLI

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THE PURIFICATION AND CHARACTERIZATION OF THE X-RAY ENDONUCLEASE OF ESCHERICHIA COLI

by

Harold Lawrence Katcher

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

THE PURIFICATION AND CHARACTERIZATION OF THE X-RAY ENDONUCLEASE OF ESCHERICHIA COLI

by

Harold Lawrence Katcher

Dr. Susan S. Wallace, Advisor

This work concerns the purification and characterization of the X-ray endonuclease of E. coli. The X-ray endonuclease was first detected as an activity present in crude lysates of Escherichia coli that nicked X-irradiated DNA in a dose-dependent manner (Strniste and Wallace, 1975). The X-ray endonuclease was purified by chromatography on DNA-agarose, Sephadex gel filtration, hydroxylapatite chromatography, and phosphocellulose chromatography. Several thousand fold purification was obtained. Parallel assays on modified DNA and oligonucleotide substrates established that the X-ray endonuclease was active on DNA containing apurinic and apyrimidinic sites, thymine glycol and urea residues, and undefined lesions produced by UV and X radiation.

Characterization of the X-ray endonuclease by gel filtration gave a molecular weight of about 25,000 dalton while SDS-polyacrylamide gel electrophoresis of the most purified preparations showed a single band corresponding to a molecular weight of about 13,000 daltons. Glycerol

gradient centrifugation showed two peaks of activity at positions corresponding to 25,000 daltons and 13,000 daltons which indicated that the X-ray endonuclease may be composed of two similar or identical subunits.

Analysis of DNA substrates following X-ray endonuclease treatment showed that the X-ray endonuclease nicked at the 3' side of a base lesion to yield 3'OH and 5'PO termini. Analysis of the acid/alcohol soluble products of the digestion of specifically modified synthetic poly dT:dA by the X-ray endonuclease showed this enzyme to have DNA-glycosylase activities that released both thymine glycol and urea residues from DNA.

Inhibitor studies showed the thymine-glycol endonuclease activity was inhibited by NEM while the AP endonuclease was not. Further studies showed that the thymine glycol-DNA glycosylase activity was NEM sensitive. NEM was also shown to inhibit endonuclease activity on UV-irradiated DNA, X-irradiated DNA, and urea-containing DNA.

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TABLE OF CONTENTS

TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

dA = deoxyadenosine

dT = deoxythymidine

DNA = deoxyribonucleic acid

EDTA = ethylenediaminetetraacetic acid

SDS = sodium dodecyl sulfate

Tris = tris(hydroxymethyl)aminomethane

PREFACE

The ability of bacteria and their viruses to repair radiation damage had been suggested by experimentation occuring as far back as the nineteen thirties (Hollaender, 1935). In the late forties and early fifties, it was shown that bacteriophages inactivated by ultraviolet radiation (UV) could be reactivated by visible light during a post-irradiation period (Kelner, 1949; Dulbecco, 1950). In the early ninteen fifties, various experiments showed that, even in the dark, bacterial recovery from radiation damage was enhanced by storage under conditions unsuitable for growth (liquid holding recovery), and that UV-inactivated bacteriophage could be reactivated by the host cell (host cell reactivation)(Wacker, 1963). It was the isolation of a radiosensitive mutant of Escherichia coli, E. coli B_{s-1} (Hill, 1958), that showed the extent of the active participation of the organism in its recovery from radiation damage. E. coli B_{s-1} was fully two orders of magnitude more sensitive to ultraviolet light than the wild type.

With an easy assay for the cyclobutane-type pyrimidine dimers, the major UV-photoproducts in DNA (Beukers and Berends, 1960; Wang, 1961), the mechanism for this recovery was established. It was determined that in E. coli pyrimidine dimers were produced in DNA in proportion to dose and that these photoproducts were removed from DNA of

wild-type bacteria, but were not removed by the UV-sensitive mutant B_{s-1} and the K12 derivative AB1886 (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964; Riklis, 1965).

The finding that pyrimidine dimers were enzymatically excised from DNA in the form of short (acid soluble) oligonucleotides led to the excision repair hypothesis. In the original excision repair scheme, the following events were postulated:

1. recognition of a lesion by a repair endonuclease which cleaves at or near it;
2. excision of the damage by an exonuclease activity;
3. polymerization opposite the intact strand; and
4. ligation of the nascent strand segment to the unexcised portion of the original strand.

The ability of visible light to effect the disappearance of pyrimidine dimers from DNA also yielded to genetic and enzymological investigation, resulting in a rather complete picture of enzymatic photoreactivation, (Sutherland, 1978). While these two processes, excision repair and photoreactivation, account for the majority of the UV-induced DNA lesions repaired, some lesions are not repaired prior to DNA replication. If a lesion is non-instructional, that is, if it is not a template for a DNA polymerase, the nascent strand opposite such a lesion contains either a gap or a misincorporated nucleotide. In the first situation, recombinational repair might provide

an error-free recombinational mechanism for gap filling, and in the second, an SOS-repair system may provide error-prone repair through the misincorporation of nucleotides opposite such lesions.

Section I. INTRODUCTION

I. Excision Repair

The four steps of the excision repair scheme were considered sufficient to account for excision repair. As the enzymology of the excision repair process was revealed, ambiguities in the ordering of these steps occurred. When it was shown that DNA polymerase I could, *in vitro* both excise pyrimidine dimers and effect resynthesis of the excised strand segment, it could be argued whether *in vivo* synthesis preceded excision or visa versa ("patch and cut" *vs*. "cut and patch"). The finding of DNA glycosylases, enzymes that remove modified bases from DNA without concommitant strand breakage, forced some modifications of the original excision repair hypothesis. Before the discovery of the DNA glycosylases, it was assumed that the incision step of excision repair was catalysed by endonucleases, either damage specific or generalized to classes of damages. Since genetic evidence showed that repair of X-ray and alkylating-agent damage proceded by

different pathways from UV damage repair, it was proposed that two correctional endonucleases exist: a type I correctional endonuclease that would act at monoadduct base damages (such as alkylations and hydrations), and a type II correctional endonuclease that would incise at bulky diadduct damages involving more than one base residue (such as pyrimidine dimers) (Grossman, 1974).

While the uvr system of E. coli K12 was certainly involved in the repair of "bulky" base adducts and diadducts, several endonucleases have been found in E. coli that fit the description of a type I correctional endonuclease. Also, several damage specific endonucleases have been characterized. As far as type II correctional endonucleases are concerned, the uvr system of E. coli, necessary for the removal of bulky damages, requires the products of at least three genes, uvrA, uvrB and uvrC. The mechanism of action is not completely understood. Two apparently simple UV-endonucleases, specific for pyrimidine dimers, have been isolated from T4 infected cells and from Micrococcus luteus . Even these enzymes have a mode of action more complex than originally supposed. The discovery of the DNA gylcosylases has necessitated the addition to the clasical excision repair scheme (nucleotide excision repair) of a base excision repair scheme.

A. Recognition by endonucleases and DNA glycosylases

The first step in the repair of DNA damage requires the recognition of a damage by an endonuclease which acts at or near the damage to produce a strand break (nick). Such a nick can apppear on either side (3' or 5') of a damaged nucleotide, the hydrolysis of the phosphodiester bond yielding either a 3'OH or a 3'PO_4. All known repair endonucleases leave a 3'OH. The following endonucleases will be classified as to the type(s) of damage recognized and the nature of the nick produced.

1. Endonucleases involved in the repair of bulky DNA adducts

a. the uvrA, uvrB, uvrC gene products of E. coli

The endonuclease activity derived from the combined action of the uvrA, uvrB and uvrC gene products of E. coli results in the recognition of and incision at UV-induced cyclobutane-type pyrimidine dimers and other lesions which produce major changes in the secondary structure of the DNA. Substrates for this repair system include pyrimidine dimers that produce intra-strand crosslinks and regions of local denaturation, as well as the products of the interaction of DNA with bifunctional alkylating agents, also productive of crosslinks. In addition, this system

acts in the repair of the inter- and intrastrand crosslinks produced by psoralen in the presence of near UV (Cole et al., 1976), by mitomycin C, and by the bulky adducts formed in DNA by acetylaminofluorene and benzo(a)pyrene (Thielmann, 1976).

While early efforts were hampered by the inability to find differential UV-endonuclease activity in crude extracts of wild-type versus uvrA/uvrB mutants, partial purification of enzyme from wild-type or uvrC mutants yielded an endonuclease absent from uvrA and uvrB cells (Braun and Grossman, 1974). The ability to incise at UV damages in permeabilized cells requires the addition of ATP (Waldstein et al., 1974). The purification of the uvrA, uvrB and uvrC gene products was achieved by the use of a complementation assay. It was demonstrated that none of these complementing extracts had any endonuclease activity alone. When combined and assayed in the presence of ATP and 10-20 mM Mg++, these extracts showed a UV-dependent endonuclease actvity similar in extent to that of the UV-endonuclease of M. luteus (Seeberg et al., 1976). The products of all three genes were required for the ATP-dependent endonuclease (Seeberg, 1978), while a uvrA/uvrB endonuclease did not seem to require ATP (Seeberg, 1976).

The repair of interstrand crosslinks required the gene products of uvrA, uvrB and uvrC as well as the uvrD gene product and polA (DNA-polymerase I) in a two-step process.

The first step of this process is the incision on the 5' side of a crosslink by the uvrABC endonuclease in the presence of ATP to create a 3'OH. This is followed by the uvrD-mediated excision by the 5'-3' exonuclease function of DNA polymerase I (Cole et al., 1976; Yoakum and Cole, 1977). The mechanism of action of the uvrABC system is not known, though the uvrA protein binds single stranded DNA and has no affinity for damaged nucleotides per se. This has been taken as evidence that recognition relies on the changes in secondary structure caused by bulky damages (Seeberg, 1981).

b. the dimer specific endonucleases of Micrococcus luteus and bacteriophage T4.

When extracts of M. luteus are chromatographed on phosphocellulose, five peaks of UV-endonuclease are detected, two of which are specific for pyrimidine dimers (Riazuddin and Grossman, 1978). Both pyrimidine dimer endonucleases produce a nick on the 5' side of a dimer leaving 3'OH and 5'PO_4 termini which may provide sites for DNA-polymerase I attachment. The enzymes produce termini that differ in their sensitivity to bacterial alkaline phosphatase, suggesting that the termini left by these enzymes differ in secondary structure (Nayalama et al., 1971).

The UV-endonuclease encoded by the denV gene of

coliphage T4 is similar to the UV-endonuclease of M. luteus in that it is specific for pyrimidine dimers (Paterson, 1978) and apurinic /apyrimidinic sites, (AP sites), has no requirement for divalent cations, operates in the presence of EDTA, and has a relatively low molecular weight (see Table I). The most interesting similarity between these enzymes is in their mechanism of action. Grossman and coworkers (Grossman et al., 1978) investigated the action of the M. luteus UV-endonuclease on a UV-irradiated fragment of the lac operon, whose entire sequence was known. Analysis of the resulting fragments on a sequencing gel showed that the nick produced seemed to be within the dimer itself. If this fragment was heated to 100oC or treated with alkali prior to sequencing, the nick appeared immediately 5' to the dimer. This was explained by postulating a two-step process based on two separate activities of this enzyme: (1) dimer specific DNA-glycosylase that clips the glycosylic bond between the sugar and the 5' thymine component of the dimer; (2) class I AP endonuclease that nicks at the 3' side of the apyrimidinic site created by the first step. This process leaves a nick with a deoxyribose moiety on its 5' side and a pyrimidine dimer attached by a single glycosylic bond on its 3' side.

Using the fact that UV-irradiation will cleave dimers as well as form them, Radany and Friedberg (1980) demonstrated the UV-mediated release of thymine from

UV-irradiated dimer-containing DNA only after treatment with the denV gene product of T4. This demonstrated that the T4 UV-dimer endonuclease worked by the same mechanism as the M. luteus enzyme. Since the dimer is attached by a single glycosylic bond,in situ cleavage of such a dimer resulted in the liberation of free thymine.

2. Apurinic/apyrimidinic endonucleases

One of the most common DNA lesions occuring in vivo is the apurinic/apyrimidinic site (AP site). This site is normally defined as a nucleotidyl residue in which the N-glycosyl bond between base and deoxyribose is broken, and the deoxyribose is left in the chain in its free aldehyde form. The formation of AP sites can occur through several mechanisms; spontaneous hydrolysis of purines, which occurs at significant rates under physiological conditions (Lindahl and Nyberg, 1972); labilization of purine bases by alkylation; depurination and depyrimidinization by ionizing radiation (Ducolomb et al., 1974); and through the action of DNA glycosylases.

Although there is evidence (to be discussed),that such AP sites may be filled by "base-insertases", it is generally supposed that AP lesions are removed by the excision repair system. All of the E. coli endonucleases supposed to be involved in excision repair (endonucleases III, IV, V, VI and VII) also act at AP sites, except for

the uvrABC endonuclease. A simple tripeptide, lysyltryptophanyl lysine, acts as an AP endonuclease that binds at and nicks AP sites (Behmoarus et al., 1981). AP endonucleases may be classified by the position of the nick they impart relative to the AP site. Class I endonucleases nick at the 3' side of an AP residue while the class II AP endonucleases nick at the 5' side. Both classes produce 3'OH and 5'PO_4 termini. The AP terminus left by the activity of a Class I AP endonuclease acts as a substrate for a Class II AP endonuclease (Mossbaugh and Linn,1981) causing the removal of the deoxyribose residue and providing a binding site for DNA-polymerase I. The following is a description of those E. coli enzymes that are specific for AP sites.

a. endonuclease VI

The history of endonuclease VI has followed a tortuous path. It was first called endonuclease II (Hadi and Goldthwait, 1971). Endonuclease II was characterized as an endonuclease, active on alkylated DNA, which possessed a DNA glycosylase activity as well. Several alkylated bases were reported to be released by thousand-fold purified enzyme, but this result was not reproducible. An endonuclease acting on apurinic DNA was purified by Verly (Verly and Paquete, 1972), following the identical purification procedure used by Goldthwait, and was shown to

be active only on DNA containing AP sites. Verly showed that alkylated purine bases were spontaneously released from DNA leaving apurinic sites. These results explained, in part, the results of Goldthwait (Verly *et al.*, 1973). This enzyme was purified to homogeneity and shown to be the major AP endonuclease of *E. coli* (Verly and Rassart, 1975).

Studies of *E. coli* mutants defective in both exonuclease III and the major AP endonuclease led to the recognition that endonuclease VI and exonuclease III were activities of the same enzyme. Exonuclease III/endonuclease VI is a small protein (28,000 d) that has four known activities:

1. a 3'-5' exonuclease
2. an AP endonuclease
3. an RNase·H activity
4. a DNA phosphatase

In spite of its multiple functions, mutants lacking endonuclease VI/exonuclease III, (*xth*), show normal growth rates and normal UV-sensitivity, but they are slightly sensitive to the alkylating agent, methyl methanesulfonate (MMS)(Ljungquist *et al.*, 1977).

b. endonuclease IV.

Under conditions in which the major AP endonuclease activity of E. coli was absent, *ie.*, in an *xth* mutant, the residual AP endonuclease activity (about 10 percent of the

original activity) was ascribed to an AP endonuclease (Ljungquist et al., 1977). Purified by Ljungquist's group and called endonuclease IV, this enzyme differs from endonuclease VI in several respects, including greater heat stability, lack of a requirement for Mg^{++}, and lack of sensitivity to EDTA (Ljungquist, 1977). Endonuclease IV has neither glycosylase nor exonuclease activity. Its physiological role in E. coli awaits the isolation of mutants.

c. endonuclease VII

AP endonuclease VII differs from those described above in being specific for AP sites on single stranded DNA. The activity has only been described in crude preparations; its role in repair is not known and mutants have not been isolated (Friedberg et al., 1981).

3. Endonucleases that recognize multiple lesions

The enzymes constituting this category appear to recognize altered and unusual bases as well as AP sites. The two enzymes to be discussed here, endonucleases V and III, have been highly purified and characterized.

In addition to these fairly well-characterized enzymes, endonucleases specific for X- and gamma-ray damage in irradiated DNA have been reported in M. luteus (Paterson

and Setlow, 1972) and E. coli (Strniste and Wallace, 1975). The M. luteus preparation remains unpurified (Shon-Bopp et al., 1977). This thesis is concerned with the purification and characterization of the X-ray endonuclease of E. coli.

a. endonuclease V

The small molecule, endonuclease V (Table I), nicks untreated single-stranded DNA. It also nicks untreated native DNA, either supercoiled or relaxed, at 10 percent of the rate of single-stranded DNA. When duplex DNA is depurinated by heat/acid, UV-irradiated, treated with OsO_4 or X-rays, the rate of cleavage increases several fold. The presence of the alkylation products of MMS treatment or the crosslinks from psoralen plus light treatment were not stimulatory (Gates and Linn, 1977). This enzyme is particularly active against duplex DNA containing uracil residues, such as that isolated from the Bacillus subtilis phage, PBS-2. In E. coli, uracil residues are thought to arise through two different mechanisms: spontaneous deamination of cytosine residues (Shapiro and Klein, 1966), or misincorporation of dUMP residues (Tye et al., 1977). Since uracil codes as thymine, the deaminated cytosine residues are mutagenic and their removal and replacement is necessary to maintain genetic intergrity. Analysis of ung (uracil-DNA glycosylase), mutants showed that most uracil residues are removed by uracil-DNA glycosylase (Duncan and

Warner, 1978), but it may be that endonuclease V represents part of an alternative pathway for the removal of uracil residues.

b. endonuclease III

Endonuclease III was first isolated on the basis of its activity toward heavily UV-irradiated DNA (Radman,1976). A similar activity isolated by Linn (Gates and Linn, 1977b) as a by-product of the isolation of endonuclease V and shown to act on duplex DNA damaged by OsO_4, heat/acid, UV and X-rays was assumed to be the same enzyme. But unlike endonuclease V, this enzyme does not nick undamaged DNA either duplex or single stranded, does not require Mg^{++}, and does not nick at uracil residues.

This enzyme, though not homogeneous, is now believed to consist of two activities; a Class I AP endonuclease and a thymine glycol-DNA glycosylase. While it is not clear how these activities relate to active sites, it has been noted that the indole, harmane, inhibits both the AP endonuclease and the DNA glycosylase activities of this enzyme (Warner et al., 1981). This data implies that the two activities share an active site. The associated DNA glycosylase has been shown to remove at least two thymine ring-saturation products, 5,6 dihydro,dihydroxythymine and dihydrothymine. The latter product apparently results from self-irradiation of the tritium-labeled DNA as its occurence in DNA was

independent of the OsO_4 dose. Kinetic studies indicate that the number of thymine glycol residues removed from OsO_4-treated DNA at any time were equal to or slightly greater than the number of nicks produced by this enzyme at that time, thus supporting the hypothesis of a mechanism involving the sequential action of the thymine glycol-DNA glycosylase and the AP endonuclease activities.

4. Recognition by DNA glycosylases

In a detailed investigation of the degradation of uracil-containing DNA by E. coli extracts, it was found that uracil was released from DNA as a free base leaving the DNA unaltered in chain length, but containing apyrimidinic sites (Lindahl, 1974). The enzymes responsible for cleaving the N glycosyl bond joining base to sugar were called DNA glycosylases. In particular, the enzyme responsible for removing uracil residues from DNA was called uracil-DNA glycosylase. At present, several of these enzymes are known in bacteria (Table I).

Uracil-DNA glycosylase was first extensively purified and characterized from E. coli (Lindahl et al., 1977) and from Bacillus subtilis (Cone et al., 1977). Both enzymes have been purified to near homogeneity. Such uracil-DNA glycosylases have been found in many species including man. In spite of its universal occurence, E. coli mutants lacking this enzyme (ung) (Duncan et al., 1978; Riazuddin

and Lindahl, 1978) are normally resistant to the effects of UV, MMS, mitomycin C and naladixic acid, but they are sensitive to bisulfite (Friedberg, 1978) and nitrous acid (DaRoza et al., 1977). Since E. coli deficient in dUTPase (dut) allow the misincorporation of dUMP into DNA, (in place of dTMP), dut ung double mutants should and do accumulate dUMP residues in their DNA (Duncan et al., 1978). The fact that these mutants grow only slightly less well than the wild type indicated that the primary purpose of uracil-DNA glycosylase is to repair the uracil produced by the deamination of cytosine rather than by misincorporation.

Other DNA glycosylases that have been purified and characterized include hypoxanthine-DNA glycosylase, 3-methyladenine-DNA glycosylase and formamidopyrimidine-DNA glycosylase. The first of these enzymes acts on the deamination product of adenine (Karran and Lindahl, 1978), the second on an alkylation product of adenine (Riazuddin and Lindahl, 1978), and the third on formamidopyrimidine, an alkylation product of adenine (Chetsanga, 1981).

All three of these DNA glycosylases are highly specific: the uracil-DNA glycosylase does not recognize deaminated hydroxymethyl-uracil; hypoxanthine-DNA glycosylase does not recognize deaminated guanine residues and 3-methyladenine-DNA glycosylase does not recognize 7-alkylguanine residues (Lindahl, 1981). Other DNA glycosylases in Table II have not yet been purified and

await this step prior to characterization. Of the remaining DNA glycosylases, only the 3-methyladenine-DNA glycosylase has a known mutant lacking in this function, tag (Bachmann et al., 1976). Tag mutants are sensitive to alkylating agents but do not have an increased mutation frequency. As can be seen from Table II, all of the known DNA glycosylases have similar molecular weights (20,000-30,000 daltons) and have no requirement for divalent cations. These enzymes also include those DNA glycosylase activities with associated endonuclease activities.

It should be noted at this point that, except for uracil-DNA glycosylase, the DNA glycosylases without associated endonuclease activity respond to modified purines, while those with endonuclease activities recognize modified pyrimidines. This is significant in light of the fact that there may be enzymes called purine insertases which could complete DNA repair immediately after DNA glycosylase action by merely inserting a purine base in the apurinic site left by the glycosylase. It is generally assumed that the AP sites left by the action of a DNA glycosylase are substrates for one of the several AP endonucleases. In the case of the uracil-DNA glycosylase, the involvement of the major AP endonuclease of E. coli (endonuclease VI) in the repair of uracil residues has been inferred from the fact that, although mutants defective in dUTPase and endonuclease VI (dut xth) grow poorly, and

filamentously and are inviable at temperatures above 30°C, a normal phenotype is exhibited by a (dut ung xth) triple mutant (Lindahl, 1981). While there are several base alkylation products known to be enzymatically released from DNA, DNA glycosylases for these enzymes have not been isolated. It has not been demonstrated that some of the enzymes mentioned and referred to as repair endonucleases actually act as such in vivo. In order to demonstrate this, we must await the isolation of mutants deficient in these enzymes. In the case of the post-incisional processes (steps 2-4) many mutants of the enzymes involved have been clearly shown to be sensitive to DNA damaging treatments.

B. Excision, repolymerization and ligation

The further processing in the repair of lesions which remain covalently bound to DNA depends on the nature of the lesion, the nature and location of the nick relative to the lesion, and the secondary structure of the DNA. There are three types of lesions that remain in the DNA after the incisional steps: altered bases, AP sites, and crosslinks.

1. Removal of damages

In the first demonstrations of excision repair, workers showed that UV-photoproducts were excised from DNA as small oligonucleotides (Boyce and Howard-Flanders, 1964; Carrier

and Setlow, 1964 and Riklis, 1965). While the T4 and the *M. luteus* enzymes are known to leave a deoxyribose moiety on the 5' side of a break, the nature of the break produced by the *E. coli uvrABC* endonuclease *in vivo* is unknown (Kacinski *et al.*, 1981). Evidence indicated that the dimer removal by *E. coli* required the participation of DNA polymerase I. This, together with the fact that dimers are removed in short oligonucleotides, indicated that the nick is located on the 5' side of the dimer (Braun *et al.*, 1976). However, the *uvrABC* endonuclease has been shown to nick on both sides of a pyrimidine dimer *in vitro* (Rupp, personal communication).

2. The involvement of bacterial DNA polymerases.

a. DNA polymerase I, *E. coli*

DNA polymerase I of *E. coli* (Pol I) is a multifunctional enzyme possessing a DNA-directed DNA polymerase, a 3'-5' exonuclease and a 5'-3' exonuclease. It is also capable of carrying out pyrophosphate exchange and pyrophosphorylysis (Kelly *et al.*, 1970). This enzyme has been shown to excise pyrimidine dimers from DNA and effect repair replication *in vitro* (Heijneker *et al.*, 1971).

In order to assess the role of the enzyme and its functions *in vivo*, a series of polymerase I mutants were isolated (*polA*) and these organisms assayed for their sensitivity to UV radiation. While *polA* mutants were more sensitive, no single mutation completely inhibited excision once incision had occured (Hanawalt *et al.*, 1981). It has been found that a *polA* mutant that lacks the polymerase but retains the 5'-3' exonuclease activity is more sensitive to UV radiation then wild type but less so than a *uvrA* mutant (Kanner and Hanawalt, 1970). In such strains, the rate of excision is lower than wild type and the breaks persist longer, but the final extent of excision is nearly that of wild type (Boyle *et al.*, 1970; Cooper and Hunt, 1978). It was originally assumed that the 5'-3' exonuclease was necessary to the polymerization-driven excision process. But experiments with *polAex* mutants that have the DNA polymerase function but lack the 5'-3' exonuclease activity show these mutants to be more UV-sensitive than wild type, but less than *polA1* mutants (Cooper, 1977). At doses lower than 40 J/m^2, dimer excision in *polA* mutants is about as fast as in wild type (Chase and Masker, 1977, 1979). This implies that the polymerization activity of DNA polymerase I is more important to the excision of the photodimers than the 5'-3' exonuclease activity. This surprising fact can be explained by a mechanism involving displacement of the damage-containing strand and degradation of the displaced single-stranded DNA segment by a single-strand-specific

endonuclease or by an exonuclease such as exonuclease VII.

b. DNA polymerase II, *E. coli*

The DNA polymerase II enzyme has a DNA polymerase activity similar to that of DNA polymerase I, but lacks a 5'-3' exonuclease activity (Knippers, 1970). Mutants lacking this enzyme (*polB*) are no more sensitive to UV than wild type (Campbell *et al.*, 1972; Hirota *et al.*, 1972). This enzyme appears responsible for the residual repair of a (*polA polC*^{ts}) double mutant, as the triple mutant, (*polA*, *polB*, *polC*^{ts}) lacked this residium (Strike, 1977).

c. DNA polymerase III, *E. coli*

DNA polymerase III provides the polymerase activity for the *E. coli* "replication complex" (Gefter *et al.*, 1971; Hirota *et al.*, 1971). This enzyme is similar to DNA polymerase I in that it has both 3'-5' and 5'-3' exonuclease activities. Although the 5'-3' exonuclease of DNA polymerase III is not as active as that of DNA polymerase I (Livingstone and Richardson, 1976), this enzyme is capable of excising pyrimidine dimers *in vitro*. *In vivo* it has been shown that in *polA* mutants which rejoin strands slowly the rate of rejoining is drastically reduced even further by an additional *polC* mutation.

It should be noted that the rate of excision in a (polA polC) double mutant is not much lower than wild type (Cooper and Hunt, 1978). More to the point, it was shown that the (polA polC) double mutants are more UV sensitive than a polA mutant alone (Youngs and Smith, 1973). In the absense of DNA polymerase I, it would appear that DNA polymerase III is responsible for base excision and repair replication.

3. Exonucleases

Exonucleases can be naturally divided into those that recognize a 3' terminus and those that recognize a 5' terminus (3'-5' exonucleases versus 5'-3' exonucleases). There are two 5'-3' exonucleases of E. coli, apart from the exonuclease activities of the DNA polymerases; exonuclease V, (the recBC gene product) and exonuclease VII, (the xse gene product). Since, in the classical excision repair hypothesis, the incision occured on the 5' side of the lesion, exonucleases of this type were considered likely repair enzymes. The recBC exonuclease, which functions in recombination, has both 3'-5' and 5'-3' exonuclease activities and acts as an ATP-dependent endonuclease on single-stranded DNA. This enzyme has not been shown to be effective in excision repair (Shaes, 1972). Exonuclease VII, which may act both as a 3'-5' exonuclease and as a 5'-3' exonuclease, has been shown to remove pyrimidine

dimers from UV-irradiated DNA substrate that had been previously nicked by a UV-endonuclease (Chase and Richardson, 1974). Mutants lacking this enzyme, *xseA*, are not UV sensitive (Chase and Richardson, 1977), nor is the rate of UV dimer excision diminished *in vivo* in these mutants. In a *recBC* background, the (*polA*$_{ex}$ *xseA7*) double mutant is only slightly less efficient at dimer removal than *polA*$_{ex}$ single mutants (Chase *et al.*, 1979). It has been determined that the DNA polymerase associated 5'-3' exonucleases are the primary repair exonucleases (Rothman, 1980). The elucidation of the DNA-glycosylase/AP-endonuclease-mediated base-excision process provides a possibility that the AP endonuclease may nick on the 3' side of an AP site, so that a 3' deoxyribose moiety is left on one side of the break. In order to excise this lesion, a 3'-5'exonuclease or a class II AP-endonuclease is required.

4. Repair of AP termini

AP endonucleases may be classified by whether they nick on the 3' side of an AP site (class I) or on the 5' side (class II). Among the class I endonucleases are the AP activities of the *denV* gene product of T4, the UV endonucleases of *M. luteus* and endonuclease III of *E. coli*.

The sites left by the class I AP endonucleases do not

serve as primer termini for DNA polymerase I and must be removed prior to repair replication. These residues were removed *in vitro* by class II AP endonucleases (Mosbaugh and Linn, 1982). Indirect evidence of this *in vivo* is given by the demonstration that mutants lacking endonuclease VI (*xth*), though not markedly sensitive to UV or gamma radiation, are sensitive to alkylation damage (Yajko and Weiss, 1975). Since it is known that AP sites are intermediates in the repair of alkylated DNA, it would seem that the xth gene product is necessary for the repair of AP sites *in vivo* (Weiss *et al.*, 1978). It is not known if endonuclease IV has a role in the repair of 5' AP termini.

5. Ligation

Following repair replication, the joining of the 3'OH of the nascent strand to the 5'PO_4 of the remaining strand segment is the function of DNA ligase. While essential for viability, temperature-sensitive ligase mutants are found to be sensitive to UV if held at non-permissive temperatures for two hours before plating (Pauling and Harm, 1968). Also, in toluenized cells, the presence of a ligase mutation or the ligase inhibitor, nicotinamide mononucleotide (NMN), allows for continued repair replication. Because DNA polymerase I and DNA ligase are known to compete for the same substrate, a nick with a 3'OH, 5'PO_4 terminus, the effect of NMN on repair synthesis

may be explained by assuming that the absense of functional ligase allows the DNA polymerase to reattach at a nick and to reinitiate polymerization. In the absense of ligase the cycles of polymerase attachment to a nick, followed by nick translation and reattachment of polymerase should continue ad infinitum. The concept that DNA polymerase will bind to a nick, "nick translate" processively, and then detach explains why the DNA polymerase I dependent "short patches" are about 20 nucleotides long, since the average processivity of DNA polymerase I in vitro is about 20 nucleotides. Supporting this hypothesis is the result that an altered DNA polymerase I, polA , that has a processivity of three to four nucleotides in vitro, produces patch sizes of about 5 nucleotides in vivo (Matson, 1978).

6. Long patch repair

While the excision repair schemes thus far described result in the excision of about 20 nucleotides for each damage (Ben-Ishai and Sharon, 1978; Cooper and Hanawalt, 1971; Setlow and Carrier, 1964), there is another type of excision repair that is not dependent on DNA polymerase I, but requires both the presence of a functional recA gene product and protein synthesis (Cooper and Hanawalt, 1972; Cooper and Hunt, 1978). This process is completely dependent on polB in a polA background and results in patch sizes of several hundred nucleotides (Hanawalt et al.,

1979). Since polB mutants in this background, did not show differential UV sensitivity, it may be concluded that "long patch" repair is not biologically important. It is known that less than 10 percent of damaged sites are repaired by this mode (Campbell et al.,1972). Since the involvement of both the recA gene product and protein synthesis are required, this process may be part of the SOS-repair system.

C. Post-replication repair

If a non-instructional lesion remains in DNA at the time DNA replication is to occur, polymerization would ordinarily proceed up to such a lesion but not past it. DNA replication downstream from this point requires reinitiation with an "Okazaki piece", and a gapped strand is formed (Rupp and Howard-Flanders, 1968). Such "gapped" molecules have been observed indirectly by comparing the molecular weight of genomic DNA obtained using neutral sucrose gradient sedimentation analysis to that weight obtained using alkaline sucrose gradient sedimentation analysis. In UV-irradiated bacteria deficient in excision repair and "post-replication" repair (uvrA recA), the size of the DNA synthesized on the strand opposite the dimer-containing strand was approximately equal to the inter-dimer distance (Sedwick, 1975). The UV survival of E. coli with unexcised dimers involves the participation of

the recA protein. This protein is involved in at least two gap-filling pathways, one error-free and the other error-prone (mutagenic).

1. The recA protein

The recA protein is essential for genetic recombination, recombinational repair and DNA damage-inducible SOS-repair (Lark and Lark, 1978). The recA gene product, which has a molecular weight of 37,000 daltons, acts as a DNA topoisomerase by unwinding a duplex molecule in the presence of single-stranded DNA and either ATP, dATP or ATP(γ)S. This promotes the insertion of a homologous single-stranded DNA molecule into a duplex, causing D loop formation. The enzyme also has a DNA-dependent ATPase activity (Cunningham et al., 1979) and a very specific protease activity that will cleave the repressor of the damage-inducible functions, the lexA gene product, as well as the phage lambda C1 repressor. The lexA protein also represses the synthesis of recA protein. Both the DNA-denaturing and protease activities require Mg , dATP or ATP( )S and a polynucleotide co-factor (Craig and Roberts, 1981; Phizicky and Roberts, 1981). These two activities, protease and DNA topoisomerase, are involved in the two gap-filling pathways. The "recombinase" ability of recA protein catalyzing insertion of single-stranded homologous DNA into duplex molecules is likely to be

involved in the daughter-strand gap-filling "post-replication repair", while the repressor-specific protease is involved in the induction of the error-prone SOS pathway.

2. Daughter-strand repair

Repair of gaps in daughter-strands occurs by the covalent attachment of parental strand material to daughter-strand DNA. Density label experiments showed that daughter-strand DNA was of low molecular weight when the parental organisms were irradiated. When these irradiated organisms were either photo-reactivated or allowed to undergo excision repair prior to replication, daughter strands were of higher molecular weight (Smith and Meun, 1970). Other studies with density-labeled DNA showed that parental DNA, in lengths of about 1000 nucleotides, becomes covalently attached to DNA under conditions which permit daughter-strand gap filling repair (Rupp et al., 1971). This value, 1000 nucleotide inserts, compares favorably with the estimates of gap sizes in daughter DNA, estimates which range in size from 1000 nucleotides (Iyer and Rupp, 1971) to 1,500-40,000 nucleotides (Johnson and McNeill, 1978). Since DNA synthesis may also take place, the inserts need not be as long as the gaps, but if branch migration of single-stranded inserts occurs, they may be even longer than the gap. The genes needed for this type

of recombinational repair include; *lexA* (Ganesan and Seawall, 1975); *recBC* (Youngs and Smith, 1976); *uvrD* (Youngs and Smith, 1976); *recA*, (Smith and Meun, 1970); recF (Rothman, 1975) and the presence of either an active DNA polymerase I or DNA polymerase III (Johnson, 1978). The actual functions of each of these enzymes in the recombinational repair process is still unclear at the molecular level.

3. SOS-repair.

In 1953, it was first reported that UV-irradiated phage were more likely to survive if plated on a lawn of bacteria that had themseves been irradiated with low doses of UV radiation prior to infection (Weigle, 1953). These surviving phage were heavily mutated (Kellenberger and Weigle, 1958). This inducible phenomenon was known as Weigle-reactivation. It was shown to be induced even by the conjugal transfer of UV damaged DNA (Devoret and Radman, 1974). The induced efficiency of phage reactivation began to decline 30 minutes after induction (Defais *et al.*, 1976). This inducible repair, together with evidence that UV-mutagenesis in *E. coli* could be mimicked by a temperature sensitive mutation in the *lexA* gene (*tif*) which caused mutagenesis, phage reactivation and filament formation without UV-irradiation (Castellazzi *et al.*, 1972), led to the SOS-repair hypothesis, that is, that

DNA damage induces an error-prone repair system that not only enhances immediate survival but also may provide genetic flexiblility in dealing with an altered environment by causing a high mutation rate (Radman, 1975). This concept was extended by Witkin to include the other cellular functions induced by DNA damage (Witkin,1976). At present, at least 11 genes in E. coli are known to code for damage-inducible functions (Little and Mount, 1982).

The SOS system, consisting of the genes repressed by the lexA protein, may be in either an "on" or an "off" state. Typically, the system is "off" because the synthesis of the recA protein is inhibited by the presence of its repressor, the lexA protein. When DNA damage occurs, an inducing signal, either a DNA degradation product or even gapped DNA (it is not known which), together with dATP, activates the protease activity of the recA protein, of which a few molecules are available even in the uninduced cell. The recA protease, so induced, proceeds to cleave the lexA repressor (and the C1 repressor of lysogenic lambda phage if present) causing the derepression of recA, lexA (which is auto-regulated) and the other proteins coded for by the so called din (damage inducible) genes.

At this point, recA protein levels rise precipitously so that this protein may account for up to 3 percent of the total cellular protein. In this state, DNA synthesis may proceed past dimers, possibly as a result of the umuC gene

product (Bridges and Lehman, 1982). As repair progresses, the lexA gene product accumulates to a level at which repression of the recA gene occurs again.

There is some evidence that "long patch repair" is an aspect of SOS repair, in that the same conditions, environmental and genetic, are needed for both (Witkin, 1976; Bridges and Mottershead, 1978). It is not known if the 11 din genes are the totality of genes repressed by the lexA protein and knowlege at the molecular level of all but the recA, lexA and uvr genes is lacking.

4. The adaptive response

The adaptive response to alkylating agents is an inducible system of repair that is not controlled by the lexA protein. It was found that bacteria exposed to low levels of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 minutes became refractory to further mutagenesis (Samson and Cairns, 1977). Apparently two classes of lesions are produced by MNNG treatment, one lethal and the other mutagenic (Cairns et al., 1981). The nature of the lethal lesion is unknown but the mutagenic lesion is known to be O^6-methylguanine which in in vitro systems has been shown to code as though it were adenine (Abbott and Saffhill, 1979). Analysis of the DNA of both induced and uninduced bacteria exposed to alkylating agents showed the same levels of two non-mutagenic methyl adducts,

7-methylguanine and 3-methyladenine, but there was considerably less O^6-methylguanine in the DNA of the induced organisms. In addition, it was shown that mutants defective in the adaptive response (ada) did not remove O^6-methylguanine from DNA. Olsson and Lindahl (1980) isolated a protein from an E. coli strain that constituitively expresses the adaptive response and showed it to be a methyl transferase whose substrate is a methyl group on the O position of guanine. These methyl groups are covalently bound to cysteine groups of proteins as S-methyl cysteine (Foote et al., 1980). It is not known whether the methyl transferase is also the methyl acceptor, but the fact that the adaptive response has a limited capacity for the removal of the O^6-methyl groups, with only a few of these groups capable of being removed per cell, is consistent with the fact that the induced demethylating protein accepts the methyl group on its own cysteine residue and then loses activity (Friedberg et al., 1981).

II. IONIZING RADIATION AND DNA

X-ray photons are much more energetic than ultraviolet photons and much less selective in their effects. The interaction of these photons with ordinary molecules causes ionization and the production of free radicals. The proportion of X-ray energy absorbed by any constituent of a mixture is proportional to the mass ratio of that

constituent (Klots, 1968). In the living cell, where water constitutes approximately 75 percent of the cellular mass, water would absorb about 75 percent of the total X-ray energy absorbed while DNA would receive less than one percent. Since the number of species produced by the radiolysis of cellular water and its organic solutes are highly reactive and far outnumber the radicals directly in DNA, they are probably the principal causative agents of DNA damage. Where the G value is the number of molecules of product formed for each 100 eV of energy absorbed, the yields of these various products of ionizing radiation are:

product	G value
OH	2.7
H	0.55
$e^-(aq)$	2.7
H_3O+	2.7
H	0.45
H_2O_2	0.7

(Hutterman et al., 1978).

The values listed above are for neutral water. In general, these values depend on the pH because of secondary processes involving H_3O^+ and OH^- ions. For example, the presence of acids converts aqueous electrons, $e^-(aq)$, to hydrogen atoms ($e^-(aq) + H_3O^+ \rightarrow H + H_2O$), and alkaline conditions lead to the opposite situation,

($H + OH^- \rightarrow e^-(aq) + H_2O$).

In solutions containing oxygen, the perhydroxy radical (superoxide radical) and the perhydroxy radical anion are formed: $H + O_2 \quad HO_2$ (perhydroxy radical), and $e^-(aq) + O_2 \quad O_2^-$ (superoxide radical anion). Singlet oxygen is also produced at a low yield (Singh, 1978). Also, O_2 "fixes" organic radicals formed by removing the species necessary for their regeneration. This "fixing" occurs through the formation of peroxyl radicals: $R'R + O_2^- \rightarrow R'RO_2\cdot$ and the scavenging of $e^-(aq)$ by O_2, $e^-(aq) + O_2 \rightarrow O_2^-$. The organic peroxy radical formed may result in an organic hydroperoxide (ROOH). The biological consequence of the presence of oxygen is a three times greater rate of radiation-induced inactivation of cell proliferation then when irradiation is anoxic (von Sonntag, 1981).

A. Reactions of reactive species generated by the X-irradiation of DNA in aqueous solution

Because of the complexity of DNA and the difficulty of separating the products of radiolysis, many of the initial studies of the radiolysis of DNA were done on model compounds, eg. free sugars, free bases, nucleosides and nucleotides. In general $OH\cdot$, $O\cdot$ and $H\cdot$ react with organic solutes by addition to an unsaturated center or by a hydrogen abstraction reaction. The aqueous electron acts

as a strong nucleophile and will add to organic molecules generating an anion radical which can be protonated (Scholes, 1978). The superoxide radical anion is, however, unreactive with organic chemicals but is involved in electron transfer reactions, hydrogen abstraction and hydrogenation of peroxy radicals (Bors et al., 1974).

The superoxide radical does not affect DNA directly but causes depletion of the cellular pool of sulfhydryl compounds. It also reacts with quinones to produce semi-quinone radicals that can transfer electrons in damaging reactions. Superoxide radicals also regenerate the ferrous ion, leading to an increase in hydroxyl radicals by the Fenton reaction ($Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + {}^{\bullet}OH + OH^-$) (Pryor, 1978; Bors et al., 1979). It should be noted that a superoxide radical generating system of xanthine and xanthine oxidase causes DNA base damage, and that H_2O_2 also appears to be involved in this damage production, because the reaction was inhibited by catalase (Feldberg and Carew, 1981). Also, recent evidence indicates that H_2O_2 causes base damages in DNA that are recognized by endonuclease III (Linn et al., 1982).

B. Final products of radiolysis of DNA

1. Strand breaks

Upon irradiation of DNA in aqueous solution, the

frequency of single-strand breaks increases linearly with dose while that of double-strand breaks increases with the square of the dose. Hagen showed that *in vitro*, double-strand breaks are formed by the coincidence of two single-strand breaks. The maximum distance between the strand breaks that will allow the double-stranded molecule to remain intact is a function of the ionic strength of the medium (Hagen, 1967), such that the number of double-strand breaks produced is inversely proportional to the ionic strength of the solution (Freifelder and Trumbo,1969). *In vivo,* however, double-strand breaks arise linearly with dose, indicating that double-strand breaks originate from a single event (Bohne *et al.*, 1970).

When assaying for the presence of single-strand breaks, the strands of a linear double helix must be separated. This separation is usually done by means of alkali denaturation. However, this measurement includes damages that are labile in alkali, damages that are produced at about one for every two single-strand breaks resulting from the X-irradiation of DNA in aqueous aerated solution (Achey *et al.*, 1971; Bopp and Hagen, 1970). Analysis of the ends produced by the radiolysis of aerated aqueous solutions of DNA reveals that the strand breaks are largely the result of the hydrolysis of the C-3' phosphodiester bond. It has been concluded that radiation-induced strand breaks result from damage to the deoxyribose moiety, causing the cleavage of the C3' phosphodiester bond (von Sonntag *et al.*, 1981).

In vitro, DNA damage occurs mainly through the action of hydroxyl radicals. While 80 percent of hydroxyl radicals react with bases, approximately 20 percent abstract hydrogen atoms from sugars (Scholes et al., 1969). With the abstraction of hydrogen from the 2-deoxyribose residues, at least five different sugar radicals may be formed (that is hydrogens may be abstracted from any of the five carbon atoms present) as the hydroxyl radical is non-selective (Schuchmann and von Sonntag, 1977). If the radical occurs at C-4', that is beta to both the phosphoryl groups at C-3' and C-5', phosphate will be eliminated (Behrens et al., 1978). Three sugars isolated from anoxically-irradiated DNA whether they are in the free form or still bound to DNA, show a reduction at the C-4' position, and the C-4'yl radical is probably their precursor. In the presence of oxygen, some of these products are suppressed, and at least one new sugar damage is formed. In vitro, oxygen causes a doubling of the number of strand breaks, (double- and single-strand breaks, ie., the oxygen enhancement ratio (OER) is 2), while in vivo, double-strand breaks have an OER of 4.9 and single-strand breaks have an OER of 3.6 (Lenartz et al., 1975).

In general two types of frank strand breaks are formed:

1. strand breaks with a phosphate end group on both

the 3' and 5' termini with a small gap in between them, and

2. those with an altered sugar at the 3' end with a 5' phosphate end group

(von Sonntag et al., 1981).

This is in accord with the observation that a radiation-induced strand break is not a substrate for DNA polymerase I (Landbeck and Hagen, 1973). It is interesting to note that 30-40 percent of radiation-induced strand-breaks are amenable to ligation by DNA ligase (Jacobs et al., 1972). The implication of this finding is that the strand breaks of type 2 are ligatable therefore the products of class I AP endonucleases should also be.

2. Alkali-labile lesions

Alkali-labile lesions are the result of sugar damage and/or base release. These sites are expected to have free OH groups at positions C-1', C-2' or C-4', as do AP sites (C-4') or RNA (C-2') (Brown et al., 1953). Two different alkali-labile sites have been characterized in gamma-irradiated DNA. One is a lactone which is a result of base release and converts to an acid in an alkaline environment, and the second results from the loss of carbon C-1' (Dizdaroglu et al., 1977). These alkali-labile sites may also be formed by depurination or depyrimidination following base damage (Lehmann, 1978).

3. Damage to bases

In early studies with the double-stranded replicative form of phage X174, where irradiated DNA without strand breaks can be separated and compared with respect to biological activity to molecules with strand breaks, it was determined that 85 percent of the radiation induced biological inactivation was due to base damage (Taylor and Ginoza, 1967). Further studies with PM2 bacteriophage DNA showed that while only 2 percent of strand breaks were lethal, 87 percent of lethal events were due to base damages (Van Der Schans et al., 1973).

Of the energetic species formed by the radiolysis of water - $\cdot H$, $e^-_{(aq)}$ and $\cdot OH$ -all three will combine with the heterocyclic bases of DNA. In order to determine the nature of the base alterations produced by the X-irradiation of native DNA, the altered bases must be liberated from the polymer. The assay of pyrimidine dimers formed by UV-irradiation was easily accomplished because pyrimidine dimers withstand the harsh conditions of acid hydrolysis necessary to release them from DNA. However, many products of X-irradiation are unstable under these conditions. In order to ascertain what these products are, irradiation of model compounds was performed. Studies of the production of base lesions under aerobic and anaerobic conditions have been undertaken. These studies show that

the most radiosensitive of the bases is thymine, followed by cytosine, adenine and guanine (Myers et al., 1965a; Myers et al., 1965b). The extent of base degradation is highest in the free base, less in the nucleoside, and even less in the nucleotide (Teoule and Cadet, 1978).

In this section the individual base moieties will be discussed with descriptions of the initial products of their radiolysis, their mechanism of formation and their ultimate products (which may differ from the initial products if these are unstable).

a. reactions of water radiolysis products with thymine

When thymine is irradiated in aqueous oxygenated solution, 30 different products are formed that can be separated by two-dimensional thin-layer chromatography (Teoule and Cadet, 1971). The major site of attack in this moiety is the site of unsaturation, the 5,6 double bond. All of the primary species of the radiolysis of water react with this base. The reaction rates of the primary species of water radiolysis with the free bases and nucleosides are in the order; $e^-(aq) > \cdot OH > \cdot H$. In the nucleotides, however, the presence of the negatively charged phosphate lowers the reaction rate of $e^-(aq)$ to less than that of OH.

i. reaction with the hydroxyl radical

The C5-C6 bond is the major site of attack by the hydroxyl radical, and the initial (OH) adducts should have this group on C5 or C6 with unpaired spin on the remaining carbon.

ii. reaction with hydrogen atoms

The yield of these atoms in the radiolysis of water accounts for only about 10 percent of the energetic species produced (G of H = 0.55) and the products formed by reaction with it are relatively minor. In acid solutions, however, where there is a conversion of e^-(aq) to $^\bullet$H, there are considerable quantities of this species. The primary product is the 5-yl radical (Holmes et al., 1967).

iii. reaction with the aqueous electron

The effects of this species can best be observed in deaerated solutions containing an excess of alcohol which scavanges the hydrogen atoms and hydroxyl radicals (Scholes,1968). The efficiency of inactivation of e^-(aq) on bacteriophage is small, about 8 percent (Van Rijn and Lafleur, 1976). The hydrated electron is a nucleophile which adds to organic molecules to produce radical anions which subsequently protonate (Scholes, 1978). It is

believed that reaction with thymine occurs by this mechanism, and it is suggested that the aqueous electron attacks the carbonyl function on C4. Recent *in vivo* evidence suggests that the final products of this reaction, which are not known, rarely result in biological inactivation of bacteriophage DNA (Nabben *et al.*, 1982).

b. products of thymine radiolysis

The most common radiolysis products of thymine residues are the *cis* and *trans* isomers of 5,6 dihydroxy-5,6 dihydrothymine and 5-hydroxymethyl uracil (Ekerts, 1962). These products predominate whether irradiation takes place aerobically or anoxically. These species are believed to result from electron transfer between two hydropyrimidinyl radicals to produce an ion pair. Protonation of this pair leads to dihydrothymine, while solvolysis leads to the various thymine hydrates. Solvolysis of the positive ion derived from 5(6)-hydroxy-5,6-dihydrothyminyl-5 or -6 radical produces the *cis* and *trans* isomers of 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol). In the presense of oxygen, the organic free radical is "fixed" as mentioned earlier and 5(6)hydroperoxy-6(5)hydroxy-5,6-dihydrothymine isomers are thus generated (Cadet and Teoule, 1971). The relative yield of the various hydroperoxides is influenced by pH (Cadet and Teoule, 1974).

c. final products of thymine radiolysis

Thermal decomposition of hydroperoxides generates more stable compounds by three main routes:

1. the hydrolytic degradation of thymine dihydroxy-hydroperoxides yields cis and trans 5,6 dihydroxy-5,6 dihydrothymine, the major relatively stable radiolysis product of thymine produced by gamma -irradiation in solution
2. the loss of water by the degradation of the cis or trans isomers of 6(5)-hyroperoxy-5(6) -hydroxy-5,6-dihydrothymine is a major process (15-20 percent) generating 5-hydroxy -5 methylbarbituric acid; and
3. the hydrolytic opening of the 5,6 bond of thymine hydroxyhydroperoxides produces N'-formyl -N2-pyruvylurea or its cyclic hydantoin form (Teoule and Cadet, 1971, 1978).

The formation of other 5,6 hydroxythymine compounds results from the hydrolysis of their corresponding hydroperoxides. The study of the thymine compounds formed in X-irradiated DNA showed that a variety of base damages resulted. Some of the modified bases remain bound to DNA while others are eliminated. The hydroxyhydroperoxy derivatives have been shown to be attached to DNA. Determination of the extent of the presence of peroxides, diols and "hydrates" can be

accomplished by the "reductive" assay using sodium borohydride. This reducing agent will not react with undamaged DNA but will react with the radiolysis products mentioned above to release small carbon compounds from these modified residues (Hariharan and Cerutti, 1972). An assay for the 5,6-dihydroxy-5,6 dihydrothymine-type lesion in gamma-irradiated DNA has been developed. This assay procedure relies on the fact that acetol is formed by the alkaline hydrolysis of thymine glycols and related compounds. A partial characterization of the modifications of the thymine moieties occuring in DNA gamma-irradiated in aerated aqueous solution was obtained by Cadet et al., (1978), where it was shown that various low molecular weight compounds including formamide, urea and N-substituted urea compounds remain bound to DNA following radiolysis.

d. cytosine

Compared to thymine, much less is known about the radiochemistry of cytosine residues. At neutral, pH both the aqueous electron and the hydroxyl radical react with cytosine to yield the hydropyrimidyl radical and the hydroxypyrimidyl radical (.cytH and .cytOH). Secondary reactions lead to:

1. regeneration of cytosine; and
2. dihydrocytosine derivatives which deaminate to

form uracil (and ammonia) and 5,6 dihydro -uracil.

In the presence of oxygen, cytosine hydroperoxides should be formed, but these are unstable and hard to isolate (Scholes, 1978). The presence of these degradation products in DNA has not been determined.

e. the purines

The major compound produced by the irradiation of adenine in solution under both aerobic and anoxic conditions is 8-hydroxyadenine (Van Hemmen and Bleichrodt, 1971). This implies that the primary event is the attack on the N7-C8 double bond by an hydroxyl radical. Other products are 4,6-diamino-5-formamido-pyrimidine (FaPy), which does not require aqueous electrons for its formation, and 6-amino-8-hydroxy-7,8-dihydropurine which does require conditions that allow electron adducts of adenine to occur (Van Hemmen and Bleichrodt, 1971)

No results are available for guanine as a free base, but imidazole opening of the purine nucleosides and nucleotides has been found by Scholes and Weiss (1952). This imidazole ring opening occcurs via an hydroxyl radical adduct forming at the N7-C8 double bond and should result in the formation of a 4-amino-5-formamidopyrimidine deoxyribose moiety.

4. Radiation-induced crosslinks

Two kinds of ionizing radiation-induced crosslinks have been noted-- DNA-DNA crosslinks and DNA-protein crosslinks. The chemical nature of these lesions are still largely unknown, and the biological relevence of these lesions remains to be determine. Again, studies have had to resort to irradiation under biologically uninteresting conditions.

When dry DNA or highly concentrated solutions of gels of DNA are irradiated at high doses DNA-DNA crosslinking is observed and insoluble gels are formed. DNA-DNA or DNA-protein crosslinks are also formed in phage T1 irradiated at 150 kilorad (Coquerelle and Hagen, 1978). Studies on chromatin irradiated in vitro or in vivo showed that the hydroxyl radical plays a major role in the formation of DNA-protein crosslinks (Mee and Adelstein, 1979). Coquerell and Hagen showed almost twice as many DNA-DNA crosslinks as DNA-protein crosslinks, while Yamamoto noted a considerably higher rate of crosslinking of protein to nucleic acid than crosslinking of nucleic acid to nucleic acid. The rate of cysteine binding to nucleic acids was particularly high (Yamamoto, 1975). It has also been postulated that DNA-DNA crosslinks result from the formation of bonds between the carbonyl products of the radiolysis of the sugar parts of DNA and the base amino groups (Raeva, 1981).

III. REPAIR OF IONIZING RADIATION DAMAGE TO DNA

What little is known about the lesions introduced in DNA by ionizing radiation, particularly base and sugar damages, has largely been determined by studies using model compounds or by irradiating DNA at extremely high doses (Teoule and Cadet, 1978). In the case of base or sugar damage, knowledge of the possible structures of these lesions has been obtained, but the actual occurence of these molecules, as components of DNA is often inferred by indirect methods. One powerful approach to the analysis of the lesions introduced is to use enzymes as probes of DNA structure. This has proved particularly valuable in the analysis of the end groups at radiation-induced strand breaks (von Sonntag *et al.*, 1978). The analysis of the remaining radiation-altered bases in DNA is complicated by their variety, instability and rarity under biologically meaningful conditions of irradiation. Measurements of their presence and removal depend on indirect methods or upon the analysis of those products which are relatively stable. Included in the ionizing radiation damages to be discussed are frank strand breaks, both double- strand and single-strand breaks, alkali-labile lesions, as well as X-ray specific base damages, the radiolysis products of purine and pyrimidine bases. It must be noted here that, though other treatments introduce strand-breaks, *eg.* endonuclease treatment, the strand-breaks produced by

X-irradiation differ in terms of the ends produced. Also, though apurinic and apyrimidinic sites are readily hydrolyzed by beta elimination in alkaline environments, the alkali-labile sites produced by X-irradiation differ in conformation and stability from AP sites. It should be noted that base damages known to be induced by X-rays may also be produced by several other treatments, eg. thymine glycols may be induced by treatment with X-rays, UV-light, OsO4 or permanganate.

A. Single-strand breaks

The principal innovation that led to knowledge of the biological repair of radiation-induced strand-breaks was the technique of McGrath and Williams (1966) that allowed the analysis of unhandled and largely intact bacterial DNA by alkaline sucrose gradient sedimentation. Using gentle lysis on alkaline sucrose gradients, the length of single-stranded DNA was determined to be about one sixth the length of the intact bacterial chromosome. It was noted that immediately after X-irradiation there was a decline in the molecular weight of the single-stranded DNA. With continuing incubation the molecular weight gradually increased until a profile similar to the unirradiated control was reached. It appeared that there were three operationally distinct systems involved in the rejoining process (Towne et al., 1974). Towne defined these as a

Type I strand-break repair which acts immediately, a Type II strand-break repair system which has a half-life of 1-2 minutes, and a Type III strand-break repair which requires 40-60 minutes for completion.

1. Type I repair

The type I repair system rejoins up to 75 percent of the strand-breaks produced by anoxic X-irradiation and about 25 percent of those breaks produced aerobically. The system operates independent of the presence of DNA polymerase I and does not depend on recA. It appeared to be inhibited by heating, cold shock and niacin deprivation. The last two inhibitory conditions indicated the possible involvement of DNA ligase, as this enzyme is cold-sensitive and requires NAD as a co-factor. NAD would be depleted in the absense of niacin (Towne et al., 1972).

The present status of this putative repair system is ambiguous; evidence indicates that if it does exist, it must act in less than 100 milliseconds (Johnson, 1975).

2. Type II repair

Type II repair is DNA polymerase I dependent and recA independent. It operates very quickly, 1-2 minutes at 37 C and 10 minutes at 0 C, and repairs approximately 90 percent of the X-ray-induced single-strand breaks (Towne et al.,

1973). The Type II repair system seems to be the classical excision repair pathway discussed earlier, though it lacks the rate-limiting first step (incision). While DNA polymerase is necessary for type II repair, under conditions where DNA polymerase I is absent, DNA polymerase II or III can replace it (with about 25 percent the efficiency) (Youngs *et al.*, 1974).

3. Type III repair

This system requires complete growth media and the *recA*, *recB*, *lexA*, *uvrD*, *lig* and *polC* gene-products. It is known to produce long patches of repair replication (ca. 1500 nucleotides) (Smith, 1977). The system is *recA* induced (SOS) and is irreversibly inhibited by a 90 minute pre-treatment with chloramphenicol (Youngs *et al.*, 1974) or dinitrophenol both of which selectively block *lexA* dependent repair functions (van der Schueren and Smith, 1974).

B. Double-strand breaks

DNA irradiated in the dry state or *in vivo* shows a linear relationship between dose and double-strand breaks. The mechanism of formation and the composition of the termini of these double-strand breaks is unknown. An early attempt to measure the repair of double-strand breaks in *E.*

coli by Kaplan (1966) indicated that no repair took place. Bonura (1975) used E. coli to examine the production of double-strand breaks in both oxygen and nitrogen atmospheres and could find no evidence of repair. Hutchinson (1973) did however present evidence indicating that a recA-dependent repair of double-strand breaks did take place in E. coli.

In contrast to E. coli, it has been established that radio-resistant bacterium, Micrococcus radiodurans, is able to repair double-strand breaks and to survive conditions which create about 100 double-strand breaks/genome (Kitayama and Matsuyama,1968). Also, work with B. subtilis showed that three double-strand breaks were produced for each lethal hit, which implied that a limited rejoining is possible for this organism (Hutchinson, 1973). The repair of these double-strand breaks was found to correlate well with survival (Hariharan and Hutchinson, 1973).

C. Alkali-labile lesions

The original experiments of McGrath and Williams measured DNA single-strand lengths on alkaline sucrose gradients without taking into account that there are sites produced by ionizing radiation which are converted by alkali into frank strand-breaks. The alkali-labile lesions include sugar damages as well as apurinic and apyrimidinic sites. It should be noted that the damaged sugar residues

believed to result in alkali-labile sites are all apurinic or apyrimidinic and thus technically AP sites (von Sonntag, 1978). It was found that these sites accounted for 20-34 percent of all breaks as measured on alkaline sucrose gradients (Paterson et al.,1973). In the minicell system used by Paterson, true strand breaks were repaired within 10 minutes, while alkali-labile lesions persisted. However, using wild-type E. coli, Towne's group found that after moderate doses all measured strand-breaks, frank strand-breaks and alkali-labile lesions were repaired (Towne et al., 1973). More recently it has been shown that repair of these lesions is partly inhibited in Micrococcus radiophilus (radiodurans) cells treated with chloramphenicol (Kitayama et al., 1981). Again, it must be noted that these X-ray-induced alkali-labile sites are not the AP sites induced by heat/acid treatment or those that arise through the action of DNA glycosylases. The rate constant of the alkali-mediated conversion to frank strand-breaks is much greater for radiation-induced alkali-labile sites than for classical AP sites (Woldhuis and Loman, 1981).

While several E. coli endonucleases are known to act at AP sites, (endonucleases III, IV, V, VI and VII), it is not known if all will act at X-ray-induced alkali-labile lesions. As will be seen in Section III (RESULTS), the X-ray endonuclease (probably endonuclease III) does not recognize X-ray-induced alkali-labile lesions though it

quantitatively converts classical AP sites to strand-breaks.

D. Repair of base damage

The difficulty of assessing the amount of base damage in DNA has been mentioned several times. Assessment of the repair of base damage is plagued by the same difficulties. One of the proposed purposes in the purification of the X-ray endonuclease was to find a reliable indicator of ionizing radiation-induced base damage. As mentioned earlier, the indirect methods of Hariharan and Cerutti may be used to quantitate damages of the 5(6) hydroperoxy-6(5)hydroxy-5,6 dihydrothymine-type by the degradative reduction of DNA that had been labeled on the 5 methyl carbon of thymine by sodium borohydride treatment. By quantitation of radio-label on the 3 and 4 carbon fragments released, Hariharan and Cerutti showed that damaged residues are released into the cytoplasm and into the medium following irradiation (Hariharan and Cerutti, 1971).

A similar though simpler method was developed by these authors, using the known alkali-lability of ring-saturated thymine residues of the 5,6 dihydoxy-dihydrothymine-type (thymine glycols). Incubation with alkali (0.2M KOH for 1.5 hours) quantitatively fragments these glycols into free acetol (carrying any label attached to the 5 carbon of the

glycol), while urea and N-conjugated urea residues remain attached to DNA (Roti Roti and Cerutti, 1974). Using this system Hariharan and Cerutti (1974a) found that residues of the thymine glycol-type are removed from X-irradiated DNA or OsO4-treated poly(dA-dT) by extracts of E. coli. Here, 8 to 16 undamaged thymines are removed for each damaged residue. The process seems to involve the DNA polymerase I exonuclease functions. A method related to those above involves the reaction of irradiated DNA with tritium-labeled sodium borohydride. It has been shown that this reaction produces labeled 2,3-dihydroxy-2-methylpropanoic acid which can be resolved by thin layer chromatography on cellulose (Schellenberg et al., 1981).

A different approach to the analysis of radiation-induced base damage developed by Teoule and coworkers relies on the fact that relatively mild hydrolysis in formic acid labilizes the N-glycosylic bond joining saturated thymine residues to DNA. Thus thymine glycols are liberated after 16 hours of incubation in 98 percent formic acid at 90 C, while 5,6-dihydrothymine and 5,hydroxy-5,6 dihydrothymine are released at lower temperatures (Teoule et al., 1977). Although this method may also be used to quantitate 5-hydroxy-5-methyl hydantoin, formyl urea, urea and N-formamide residues attached to DNA, it has not yet been used in repair studies.

The last method to be considered in the quantitation of DNA base damage introduced by ionizing radiation involves the use of enzyme probes specific for these damages. In this case, DNA damage-specific endonucleases are used and the number of additional nicks introduced in irradiated DNA by an endonuclease gives a measure of base damage. Until now only the lesion-specific endonucleases of M. luteus have been used. As mentioned earlier, this organism contains two dimer-specific endonucleases; the other endonucleases have a broad range of specificities (Grossman, 1974). Minicells containing plasmid lambda dv DNA were irradiated anoxically and incubated for varying periods with crude M. luteus extracts to quantitate the number of frank strand breaks and endonuclease-susceptible sites. Under these conditions, approximately equal numbers of each were produced and both types were repaired within 10 minutes (Paterson and Setlow, 1972). Similar techniques have been applied to whole cell lysates to demonstrate this process in bacterial DNA. Such experiments showed, in agreement with Paterson and Setlow, that the endonuclease-susceptible sites were removed from DNA within 10 minutes (Wilkins, 1973). The M. luteus enzyme has been shown to act on both alkali-labile and the alkali-stable X-ray-induced sites. Activity on the alkali-stable but not on alkali-labile sites required Mg^{++} (Hagen et al., 1978).

1. The X-ray endonuclease of *E. coli*.

An endonuclease activity was reported in extracts of *E. coli* that acted on X-ray-induced sites, and named the X-ray endonuclease of *E. coli* (Strniste and Wallace, 1975). The crude enzyme preparations also nicked both OsO_4-treated and depurinated DNA. The remainder of this work concerns the purification and characterization of this enzyme.

Section II. MATERIALS AND METHODS

I. Bacterial and Bacteriophage Strains

A. Eschericia coli

Escherichia coli AB3027, thr-1, leuB6, thi-1, proA2, argE3, his-4, xthA14, polA20, lacY1, galK2, mtl-1, xyl-5, ara-14, rha-6, strA31, tsr-33, lambda-, supE44 obtained from the E. coli Genetic Stock Center, Yale was used for all enzyme preparations. The bacteria were grown in a fermentor in modified Zubay's medium (Zubay et al., 1970) supplemented with Casamino acids and streptomycin sulfate (50 mg/ml) to late log phase, collected by centrifugation and stored at -20oC.

B. Alteromonas espejiana and its bacteriophage

A. espejiana Bal 31 and A. espejiana Bal 31-14 (a thymidine requiring auxotroph) and the alteromonophage PM2 were obtained from H.B. Grey, Jr.

II. Nucleic Acids, Enzymes and Other Reagents

A. Enzymes

DNA polymerase I (E. coli), was purified by the method

of Jovin *et al.*, (1969) through fraction VII. Exonuclease III (*E. coli*), was purchased from Miles Laboratories; phosphodiesterase II (bovine spleen) and alkaline phosphatase (*E. coli*) were purchased from P-L Biochemicals. Uracil DNA glycosylase (*E. coli*) was generously provided by E. Friedberg.

B. Other reagents

Agarose, type A, and streptomycin sulfate, grade b, were purchased from Calbiochem-Behring. Acrylamide and N,N'-bis acrylamide, TEMED and 2-mercaptoethanol were purchased from the Sigma Chemical Co. Dithiothreitol and N',ethylmaleimide were products of the Eastman Kodak Co. Phosphocellulose P11, DEAE-cellulose DE52 and 3MM chromatography paper are were bought from Whatman Ltd. Polygram Cel 300 PEI/UV254 and Polygram Cel 300/UV254MN, were purchased from Brinkman Instruments Inc. Sephadex G75, G100, CM-Sephadex and DEAE-Sephadex were bought from Pharmacia Fine Chemicals. Ag 50WX8 cation exchange resin and AG 1-X8 anion exchange resin, as well as hydroxylapatite (Bio-Gel HTP) and the protein molecular weight standards (Low Molecular Weight Gel Filtration Kit) were purchased from Bio-Rad.

C. Nucleic acids and derivatives

1. Preparation of PM2 DNA

Alteromonas espejiana Ba1 31 was grown at 28°C with aeration to a cell density of 5 x 10^8 bacteria/ml in either Ba1 broth or AMS-4, at which time bacteriophage PM2 was added at a multiplicity of infection (MOI) of 5. Aeration was continued for 4 hours. The culture flask was then incubated at 4°C overnight to complete lysis. The lysate was treated as in the preparation of ^{3}H labeled DNA (see below) to the step where the viral pellet was resuspended. This bacteriophage stock served as the inoculum for preparation of ^{3}H labeled PM2 DNA.

^{3}H labeled PM2 DNA was prepared as described by Strniste and Wallace (1975). Ba1 31-14 (200ml) was grown with aeration to a concentration of 5 x 10^8 bacteria/ml; PM2 bacteriophage were added at an MOI of 5, and 10 minutes after infection, 1 mCi of H-thymidine (82 Ci/mmole) was added with constant aeration. After 30 minutes, an additional 1 mCi of ^{3}H-thymidine was added, and the culture was allowed to incubate with vigorous aeration for 4 hours. The culture flask was then removed from the water-bath and allowed to incubate at 4°C overnight. The lysate was divided amoung six centrifuge tubes and centrifuged in a Sorvall SS-34 rotor at 8,000 rpm (7710 x g) for 10 minutes. The supernatant was centrifuged in six tubes in the SS-34

rotor at 16,500 rpm (32,800 x g) for three hours and the supernatant was discarded. The viral pellets were resuspended overnight with 1.0 ml of 2M NaCl, 0.02 M Tris pH 8.0, 1.0mM EDTA. Those eluates were divided into three 30 ml sucrose gradients (5-20%) containing 2 M NaCl, 0.05 M Tris pH 8.0 and 1 mM EDTA. The gradients were prepared in a Buchler triple outlet mixer (2-5054) using a Buchler 2-5070A Stirrer Assembly and an LKB Multiperpex 2115 peristaltic pump. Bacteriophage, appearing as an irridescent blue band, were removed with a capillary tube connected to a peristaltic pump. The sucrose purified phage preparation was dialyzed overnight against 2ml of 1 M NaCl, 0.02 M Tris pH 8.0, 1 mM EDTA after which Sarkosyl NL-30 (CIBA-Geigy) was added to a final concentration of 0.5 percent. To this was added an equal volume of freshly distilled phenol which had been equilibrated with 0.1 M NaCl, 0.02 M Tris pH 8.0, 1 mM EDTA (pH > 6).The mixture was shaken for 10 minutes, and the first aqueous layer was collected and put aside. The phenol layer was mixed with a small volume (approximately 3 ml) of 0.1 M NaCl, 0.02oM Tris pH 8.0, 1 mM EDTA. The resultant aqueous layer was dialyzed against four 1-liter changes of 0.01 M Tris pH 8.0, 1 mM EDTA, and then quick frozen and stored at -70^{o}C. The DNA preparations contained 8 to 10 ug of DNA at a specific activity of 30,000-50,000 cpm/ug, with more than 90 percent of the DNA in a covalently-closed supercoiled form.

An alternative purification procedure used a direct extaction of the sucrose-purified phage with a 3:1 mixture of chloroform:butanol. The collected bands of sucrose-purified phage were mixed with an equal volume of a 3:1 chloroform:butanol mixture on a Genie Vortex at half speed for 30 seconds and then separated by 2 minutes of spinning on a table-top centrifuge. The upper, aqueous layer was extracted an additional five times and dialyzed against 0.02 M Tris pH 8.0 and stored as above.

To prepare heavily labeled PM2 DNA,(>500,000 cpm/ug DNA) the procedures above were utilized, except that once the bacteria were grown to the proper optical density (0.12 on a Colman Jr. spectrophotometer set at 600 nm) the suspension was spun down in a Sorvall SS-34 rotor for 10 minutes at 6,000 rpm and the bacterial pellet was resuspended in AMS-4 medium prepared without the addition of cold thymidine. Labeling and further preparation was as above.

2. Other nucleic acids and derivatives

(2-^{14}C)poly dT(Na), (2.75 Ci/mMole), was purchased from P-L Biochemicals as was poly dA. ^{3}H-methyl-dTTP(58 Ci/mMole), was purchased from Schwarz-Mann. ^{3}H-methyl dT, (63 Ci/mM), was bought from ICN Pharmaceuticals. E. coli t-RNA was obtained from Boehringer Mannheim. Thymine glycol and thymidine glycol monophosphate, prepared by the

method of Baudisch and Davidson (1925), was a gift of B. Erlanger and cis-thymine glycol was a gift of J. Cadet.

III. Preparation of Substrates

A. X-irradiated DNA

An X-irradiated DNA substrate was prepared by irradiating small volumes (33-100 ul) of ^{3}H PM2 DNA in 0.01 M Tris pH 8.0, 1 mM EDTA and 50 mM KI in air at room temperature with a Picker X-ray source run at 60 kVp and 2.5 mA. The dose rate was 27.6 krad/min as initially determined using bacteriophage T4 survival as the dosimeter. Later determinations of dose were based on the conversion of PM2 Type I to Type II DNA as this correlated to the dose rate as first determined.

B. Apurinic DNA

Apurinic DNA was prepared according to the procedure of Lindahl and Andersson (1972). PM2 DNA was dialyzed overnight against 0.1 M NaCl, 0.01 M sodium citrate pH 5.0, and then heated at 70^{o}C for 10 minutes. After heating, the solution was neutralized by the addition of 5 percent (by volume) of 1 M Tris buffer pH 8.0 and dialyzed against 10 mM Tris pH 8.0, 1 mM EDTA. This produced from one to three alkali labile sites/PM2 DNA molecule.

C. OsO_4-treated DNA

1. Heat denaturation

PM2 DNA (1-2 ml) in 0.01 M Tris pH 8.0, 1 mM EDTA was made 0.02 percent with respect to OsO_4 (Eastman Kodak) and heated for 5 minutes at 70°C. The solutions were then extracted with two volumes of diethyl ether four times and dialyzed against 0.01 M Tris pH 8.0, 1 mM EDTA until no ether smell remained. This produced 1.5 to 3 enzyme-susceptible sites/PM2 DNA molecule.

2. Alkaline denaturation

Following the method of Gates and Linn (1978a), one volume of PM2 DNA in 0.01 M Tris pH 8.0, 1 mM EDTA was partially denatured with four volumes of 0.3 M potassium phosphate buffer pH 12.3, at room temperature, for 30 minutes. Following this, two volumes of 5 M NaCl and three volumes of 1 M potassium phosphate buffer pH 4.0 were added and the DNA dialysed against 0.01 M Tris pH 8.0, 1 mM EDTA. DNA produced by this method was used during several enzyme preparations with results comparable to those obtained when method 1. was used. When it was realized that thymine glycols were unstable in alkaline solution its use as a thymine glycol substrate was discontinued.

D. Apyrimidinic DNA

One volume of a solution of 2.0 M $NaHSO_3$ (1.0 M $Na_2S_2O_3$), 0.5 M Na acetate was adjusted to pH 5.9 with NaOH and added to H-labeled PM2 DNA (of a concentration of 16 ug/ml) and incubated at 37°C for 9 hours. The DNA was dialyzed overnight against 0.05 M Hepes buffer pH 8.0, 1 mM EDTA. This treatment converts a portion of the cytosines to uracil and produces essentially no apurinic sites, as determined by the method of prolonged alkali incubation described below. Uracil bases were removed by incubation at 37°C for 1 hour with a saturating amount of uracil-DNA glycosylase (supplied by E.C. Friedberg). This apyrimidinic DNA was used in reactions which were analysed by both neutral and alkali sucrose gradient sedimentation following prolonged alkaline incubation (see below).

E. OsO_4-treated poly dT

Poly dT (2.75 mCi/mmole) at 1 uCi/ml was brought to 0.4 percent OsO4 and incubated at 55°C for 20 minutes. The solution was cooled to room temperature and the OsO_4 extracted with two volumes of ether four times.

F. Urea-containing poly dT

Approximately half (1 ml) of the poly dT treated with

OsO_4 as above was precipitated by adding Na acetate (from a 3 M stock) to a final concentration of 0.3 M and then adding 3 volumes of punctillious alcohol. These samples were allowed to stand on ice for 30 minutes and then spun for 10 minutes at 10,000 rpm in a SS-34 rotor. The supernatant was discarded and the centrifuge tube washed once with cold ethanol and dried under vacuum. The precipitate was resuspended in 0.2 M KOH (0.5 ml), and left at room temperature for two hours before it was neutralized with 1 M HCl. This modified polynucleotide was then precipitated as above, dried under vacuum and resuspended in 10 mM Tris buffer pH 8.0 containing 10 percent glycerol and 1 mM EDTA and 1 mM mercaptoethanol (Reaction buffer).

G. Ultraviolet-irradiated DNA

A solution of ^{3}H-labeled PM2 DNA in TE buffer at a concentration of 10 ug/ml was irradiated in 0.1 ml aliquots in a watch glass placed about 15 cm from a General Electric germicidal lamp (the UV fluence was 4.4 J/m^2). Irradiation was from 0 to 10 minutes at a rate of about one X-ray endonuclease-susceptible site for each 10 minutes of irradiation.

H. "treated" poly dT:poly dA.

750 ul of (2-^{14}C) poly dT that had been treated with

OsO_4 and subsequently treated with alkali or not, was redissolved in 0.5 ml of reaction buffer together with an equimolar amount of poly dA. The solution was placed in a constant temperature water-bath at 55°C for 1 minute and the water bath was turned off and the solution slow cooled.

IV. Assays for Enzyme Activities

A. Endonuclease assay

Endonuclease assay reaction mixes contained 100-500 ng of PM2 DNA (50,000-100,000 cpm/ug of DNA) in reaction buffer brought to 1 mM mercaptoethanol in a volume of from 45-95 ul. To this was added 5 ul of enzyme (usually at 1 M KCl) and the solution adjusted to 0.1 M KCl. The reactions were incubated for 10 minutes at 37°C and stopped by one of three methods, depending on the nature of further analysis. For agarose gel electrophoresis, 15 ul of stopping solution was added to each 50 ul reaction. The stopping solution consisted of an aqueous solution of 5 percent sodium dodecyl sulfate, 30 percent glycerol, 0.1 M EDTA and 0.05 percent bromophenol blue. If analysis was to determine the total number of strand breaks and alkali-labile sites, the reactions were stopped by the addition of an equal volume of 1 M glycine-NaOH pH 13.1 buffer and allowed to sit at room temperature for 4 hours prior to analysis by alkaline

sucrose gradient centrifugation. Dilution of these reactions with the addition of 50 percent of their volume of water was required to prevent the overlay from sinking into the gradients. Stopping the reactions by the addition of 0.333 reaction volumes of 4 M KCl was used when neutral sucrose gradient sedimentation analysis was to be performed.

B. DNA glycosylase/AP endonuclease assay

This assay relies on a treatment designed to quantitatively convert AP sites to frank strand breaks followed by the determination of strand breaks by alkaline sucrose gradient sedimentation analysis, as described in the section on determination of the number of strand-breaks in PM2 DNA. Reactions were stopped by the addition of an equal volume of 1 M glycine-NaOH buffer pH 13.1 and kept at room temperature for 4 hours. Following this, the solutions were diluted with 50 percent of their volume with water and layered on precooled alkaline sucrose gradients which were then spun at 45,000 rpm in an SW 50.1 rotor for 100 minutes. Control DNA without enzyme treatment was run together with experimental points. The difference in the number of strand-breaks between control and experimental reactions gave the number of either endonuclease-susceptible sites or DNA glycosylase-susceptible sites. Duplicate reactions

analysed in parallel under neutral conditions made it possible to decide whether a DNA glycosylase or endonuclease activity was present. The presense of an AP endonuclease could not be detected by this method.

C. The unit of activity

One unit of endonuclease activity is defined as the conversion of one femtomole of sites into strand-breaks during a 10-minute reaction under the reaction conditions used. One unit of DNA glycosylase activity is defined as the conversion of one femtomole of sites into strand breaks when the DNA glycosylase/AP site assay was used to determine the number of strand breaks.

V. Determination of the Number of Strand Breaks/PM2 DNA Molecule

A. Neutral sucrose gradient centrifugation

Reactions of 50-300 ul containing 100-300 ug of DNA were gently layered onto 5 ml 5-20 percent linear neutral sucrose gradients containing 10 mM Tris pH 8.0 and 1 mM EDTA. The gradients were spun in a SW 50.1 rotor in a Beckman ultracentrifuge at 45,000 rpm for 205 minutes. The gradients were then collected by pumping from the bottoms of the tubes using a peristaltic pump (LKB Multiperpex) run

at a rate of 0.18 ml/12 seconds. Samples were distributed sequentially into 33 minivials. Liquiscint plus water 2.5 ml, (218 ml of water were added to a gallon of Liquiscint) were added to each vial and the series of vials were counted in order. The resulting counts (cpm/vial) were plotted against fraction number typically showed two peaks of radioactivity. The first peak of radioactivity, closest to the bottom of the gradient (29s), represented Type I DNA (covalently closed and supercoiled); the second peak represented Type II DNA (nicked 21-23s). Areas (above a base line) under the two curves were calculated and used to determine the ratio of Type I DNA to total (Type I + Type II) DNA. Since nicking occurs randomly by a Poisson process, the following relationship holds:

$$- \ln (\text{Type I}/(\text{Type I} + \text{Type II DNA})) = B,$$

where B is equal to the average number of breaks/molecule.

B. Alkaline sucrose gradient centrifugation

This procedure is much the same as for neutral sucrose gradient centrifugation, except that the samples were alkali-denatured prior to layering onto 5 to 20 percent sucrose in a solution containing 0.1 M NaOH, 1 M NaCl and 1 mM EDTA. These alkali gradients were spun at 45,000 rpm for 100 minutes, collected, counted and analysed for strand breaks as above.

C. Agarose gel electrophoresis

DNA samples of 150-200 ng contained in 50-65 ul of reaction buffer to which 15 mliters of stopping solution had been added were loaded into the wells of a 2.5 mm thick one-percent agarose gel containing 2 x TEB buffer. These gels were run in a running buffer consisting of 1 x TEB buffer at a constant current of 35-50 mA for each gel run. After three hours, the gels were removed from their supports and placed in TEB buffer containing one ug of ethidium bromide in 200 ml of buffer and allowed to stain for one hour. Following staining, the gels were removed onto Whatman #1 filter papers and illuminated with UV-light (Mineralamp). Two DNA bands were visible corresponding to each lane; the band farthest from the origin was Type I DNA and the band about one cm above it was Type II DNA (Type III, linear DNA migrates faster than Type I under the conditions used). The bands were cut out of the gels, mixed with 0.1 ml of 1 M HCl and autoclaved for 5 minutes. Liquiscint scintillation cocktail was added to each vial (2.5 ml) and the vials counted in a scintillation counter. As above, the ratio of radioactivity in the Type I band to the total radioactivity was calculated and the average number of strand-breaks/PM2 DNA molecule was calculated from this by assuming a Poisson distribution.

VI. Preparation of Chromatography Materials

A. DNA-agarose

DNA-agarose was prepared by the method of Schaller et al. (1972) except that 4 percent agarose was used instead of the 8 percent called for in the original procedure. Calf thymus DNA was dissolved in 0.02 M NaOH to a concentration of 15 mg/ml and heated to 50 C. A 4 percent (w/v) mixture of agarose and water was brought into solution by heating and then allowed to equilibrate in a constant temperature water-bath at 50 C in which the DNA was also kept. When the two solutions had equilibrated they were mixed thoroughly and poured into a large beaker set on ice. The gel was cut into pieces and passed twice through a food strainer. The resulting material was suspended in 1.0 M NaCl, 10 mM Tris pH 7.5, 1mM EDTA. After allowing the fragmented DNA-agarose to settle, the fines were removed from the supernatant by siphoning. This was repeated three times. Prior to use, the DNA-agarose was packed into a column and at least 10 column volumes of 0.25 M KCl in buffer A were run through it at a rate no greater than 2 column volumes/hr. Absorbance of the eluate was read at 260 nm until the UV absorbing material eluting from the column reached a steady state minimum value. The column was washed with 0.2 M KCl in buffer A prior to sample application.

B. Hydroxylapatite

One part of hydroxylapatite, Bio-Gel HTP, was added to six parts of 0.5 M KCl in buffer A with gentle swirling. The gel was allowed to settle for 10 minutes and the fine particles in the cloudy upper layer were decanted. More buffer was added to the settled hydroxylapatite which was resuspended by gentle swirling and the process repeated. The defined adsorbtion matrix was then ready to be poured into a column. The bed volume of the column was determined on the basis that 1 ml of hydroxylapatite binds 0.5 mg of total protein.

C. Sephadex G75 or G100.

Sephadex G75 or G100 was hydrated by boiling for 3 hours (G75) or 5 hours (G100) in about three times the expected settled bed volume of the resin of distilled deionized water. The final settled bed volume was based on water regain; Sephadex G75 yielded 12-15 ml of final volume/gm of dry beads while Sephadex G100 yielded 15-20 ml/g. The hydrated Sephadex was "defined" by placing the suspension into a graduated cylinder containing at least three times the settled bed volume and allowing the beads to settle to an apparent volume 10 percent greater than their fully settled value and syphoning off all the buffer

above the beads as well as the top 10 percent of the partially settled bed. The settled Sephadex was resuspended in 1.0 M KCl in buffer A and allowed to equilibrate to 4°C. All Sephadex columns were poured at the temperature (4°C) at which they were run. A column was poured in a single attempt. Sephadex of known bed volume covered with 50 percent of that volume of 1.0 M KCl in buffer A was swirled to make a thick slurry and poured at once down a glass rod into the column. Column extensions were added to contain the additional volume of the buffer. The Sephadex was allowed to settle for 15 minutes before the flow was started. A Sephadex column was equilibrated with at least two volumes of running buffer before use.

D. Phosphocellulose

Phosphocellulose (Whatman P11) was prepared according the the manufacturer's directions. For every gram dry weight of phosphocellulose, 15 ml of 0.5 M NaOH was used. The ion exchanger was stirred into the NaOH and left for one hour. After this the phosphocellulose was washed by decantation until the pH reached 8. The liquid was poured off and the same volume of 0.5 M HCl was added. After one hour at room temperature, washing was repeated until the pH reached approximately 7. The resulting cation exchanger was resuspended in buffer B, allowed to settle and the cloudy suspension above the settled phosphocellulose was

removed by syphoning and the solution brought to 0.02 percent with sodium azide.

E. CM-cellulose and CM-Sephadex

These matrices were prepared in the same manner as phosphocellulose. DEAE-Sephadex and DEAE-cellulose were prepared in a similar manner, except that the base and acid washing steps were reversed.

VII. Methods Used in the Purification of the X-Ray Endonuclease

A. Growth of *E. coli* AB3027

E. coli AB3027 was grown from a stab culture overnight with vigorous aeration in 30 ml modified Zubay's medium to which streptomycin sulfate (grade B) had been added to a final concentration of 50 ug/ml. MMS sensitivity of the culture was tested by plating 5 ul on a Petri dish with a disk containing 5 ul of MMS. The zones of inhibition were compared to wild-type *E. coli* B and to the *polA* strain, AB1157. The zone of inhibition of AB3027 was 50 percent larger (diameter) than that of the wild type. The overnight was used as an inoculum for a 1-liter overnight culture. After this culture had been analysed as above, it was used to inoculate the three 10-liter vessels of a

fermentor (or three 12-liter Florence flasks filled with 10 liters of modified Zubay's medium in a warm room) and the bacteria grown to late log (OD_{600}=0.4).

B. Breaking open cells

Three volumes of E. coli cells were mixed with one volume of 4 M KCl in buffer A. The cells were then mixed with an equal volume of glass beads (No.16-220, Virtus Co.) and ground for 2 minutes with CO_2 cooling in a Braun Homogenizer MSK (approximately 25 g of cells/2 minute cycle). The lysate was cleared by centrifugation at 12,000 x g for 15 minutes and the pellet discarded. Smaller quantities of cells (less than 10 g) were ground with an equal volume of glass beads in a Sorvall Omnimixer, with 5 cycles of grinding. Each cycle consisted of 3 minutes of grinding at maximum output followed by 2 minutes of cooling (the stainless steel grinding vessel was entirely immersed in an ice-water mixture during the procedure).

C. Removal of nucleic acids

The cleared lysate resulting from the opening of cells (Fraction I) was freed of nucleic acids by precipitation with the polyethelene glycol (PEG 6000). Fraction I was mixed with half its volume of a solution of 30 percent PEG 6000, 1 M KCl, 10 percent glycerol (v/v) 1 mM EDTA, and 1

mM ME and stirred for 30 minutes (at 4°C). The solution was then centrifuged at 4°C at 12,000 rpm in an SS-34 rotor. The pellet (containing the nucleic acids) was discarded and the supernatant was called Fraction II.

D. DNA-agarose chromatography

Fraction II was diluted to 0.25 M KCl with three volumes of cold buffer A and applied to a DNA-agarose column at a rate of 2 column volumes/hour. The DNA-agarose column was constructed so that the ratio of length to diameter was approximately 2:1 and the bed volume was equal (in mls) to half the weight of bacteria used (in gms). Therefore, 200 g of bacteria required a DNA-agarose bed of 100 ml. After the column was loaded, it was washed with three column volumes of 0.3 M KCl in buffer A. The activity was eluted with 2.5 column volumes of 1.0 M KCl in buffer A. Both washing and elution of activity took place at flow rates of 1 column volume/hour. The 1.0 M KCl eluate was called Fraction III. This fraction could be mixed with an equal volume of cold anhydrous glycerol and stored at -20°C for up to one year without loss of activity.

E. Hydroxylapatite chromatography

Fraction III was directly applied to a hydroxylapatite

column constructed such that each ml of bed volume could retain 0.5 mg of the protein contained in Fraction III. If Fraction III had been stored in 50 percent glycerol, it was first mixed with 1 M KCl in buffer A. The loaded column (loaded at about 2 column volumes/hour) was washed with 3 column volumes of 0.1 M buffer B and the activity, Fraction IV, eluted with 2.5 column volumes of 0.3 M buffer B. Fraction IV was concentrated by dialysis against 30 percent PEG 20,000 (w/v) in 1.0 M KCl in buffer A. After about 5 hours of dialysis, the volume of Fraction IV was halved. The volume desired depended on the conditions for the next step in purification.

F. Sephadex chromatography

The volume of Fraction IV loaded onto a column of Sephadex G100 or G75 was equal to between 2 and 10 percent of the column bed volume. Prior to use, the column was equilibrated with at least 2 column volumes of 1 M KCl in buffer A plus 2 mM dithiothreitol instead of 1 mM mercaptoethanol. The columns were run in the ascending mode in the case of the one meter (i.d.= 2.6 cm) advanced Pharmacia column (routinely used), or in the descending mode in the case of simple columns. When Sephadex G100 was used, a 95-cm hydrostatic presure head was maintained with the use of a Marriot flask; if Sephadex G75 was used, the pressure head was increased to about 1.5 m. Columns were

calibrated using Blue Dextran and the Bio-Rad Low Molecular Weight Protein Standards made for this purpose. Active fractions were pooled; the combined active eluate was called Fraction V. This fraction could be stored in 50 percent glycerol at -20°C or kept on wet ice for several weeks without loss of activity.

G. Phosphocellulose chromatography

A 1 ml column made from a tubercullin syringe was used at this step. Fraction V was diluted to 0.25 M KCl with buffer A or buffer C and applied to the column using a Pharamacia peristaltic pump set at about 10 ml/hour. After the sample was loaded, the column was connected to a linear KCl gradient in either buffer A or buffer C (from 0.25 M KCl-0.60 M KCl) and eluted at the same rate as it was applied. The active fraction, Fraction VI, eluted between 0.4 M KCl and 0.5 M KCl. Fraction VI was very unstable and decayed rapidly whether kept on ice or frozen. When mixed with 50 percent glycerol, almost all activity was lost immediately. In this very dilute state (less than 2 ug of protein/ml), the addition of glycerol seemed to destabilize the enzyme.

H. Other chromatographies

In some procedures DEAE-cellulose chromatography was

used prior to phosphocellulose. This procedure was discontinued due to the extreme loss of activity encountered. The X-ray endonuclease eluted from this matrix at an ionic strength of less than 0.2 M KCl. CM-cellulose was also tried, but discontinued due to poor yields and purification; the enzyme eluted from this matrix at about 0.1 M KCl.

VIII. Analysis of Protein Molecular Weight and Purity

A. Glycerol gradients

For determination of the sedimentation coefficient of the endonuclease activities, a 50 ul sample of Fraction V, stored in 50 percent glycerol, was diluted with 150 ul of 1 M KCl, 10 mM Tris pH 8.0, 1 mM ME, 1omM EDTA. Samples of 175 ul were layered onto 5 ml 20-40 percent glycerol gradients containing 0.02 M potassium phosphate buffer pH 8.0, 1omM ME, 1omM EDTA. The gradients were centrifuged in a Beckman SW 50.1 rotor at 46,000 rpm for 48 hours at 2^{o}C. Fractions were collected using the same techniques as for sucrose gradient centrifugation, except that a single gradient was collected at a time, and the collection was done in the cold room at 4^{o}C. Five ul of each fraction were assayed on both AP DNA and OsO_4-treated DNA. The distance between the top of the gradient tube and the peak of activity was determined and the molecular weight of the

protein was approximated by calculating its position in the gradient relative to the positions of marker proteins run in different gradient tubes at the same time. Bovine serum albumin, chymotrypsinogen and ribonuclease A were used as standards. Molecular weight calculations were made acccording to the relationship derived by Martin and Ames (1961), according to which the distance traveled by the unknown protein, D(x) and distance travelled from the meniscus by the standard protein, D(s), are related to the molecular weight of the unknown protein, MW(x) and the molecular weight of standard protein by the equation:

$$D(x)/D(s) = (MW(x)/MW(s)^{3/2}.$$

This equation assumes that both proteins are spherical.

B. SDS-Polyacrylamide gel electrophoresis

For SDS-polyacrylamide gel electrophoresis a modification of the method of Laemlli (1970) was used. Protein solutions were dialysed against dry Sephadex G200 until dryness. The dialysis tubes were washed with three 25 ul aliquots of TGS buffer and this solution was brought to 10 percent glycerol, 5 percent mercaptoethanol, 2 percent SDS and 0.0025 percent bromophenol blue.

A discontinous electrophoresis system was used; the upper stacking gel was 3 percent acrylamide (from a 30 percent stock of 30:1 acrylamide:bis acrylamide), 0.125 M Tris-HCl pH 6.8 and 0.1 percent SDS, and the lower

separating gel was 12.5 percent acrylamide (30:1 as before) containing 0.375 M Tris-HCl pH 8.8 and 0.1 percent SDS. These solutions (20 ml of stacking and 40 ml of separating gel solutions for each gel cast) were deaerated for 2 minutes prior to the addition of SDS. To polymerize these gels, 200 ul of fresh 10 percent ammonium persulfate and 20 ul of TEMED were added to the 40 ml of the separating gel solution and 100 ul of fresh ammonium persulfate and 10 ul of TEMED were added to the 20 ml of the stacking gel.

TGS buffer was used in both upper and lower chambers. To electrophorese, a constant current of 50 mA was maintained until the bromophenol blue dye marker reached the bottom of the gel (about 7 hours). The gel was then fixed and stained by submersion overnight in a solution of 0.1 percent Commassie Brilliante Blue, 50 percent methanol and 7.5 percent acetic acid. This was followed by 2 hours of destaining in 50 percent methanol, 7.5 percent acetic acid followed by several changes of a solution of 5 percent methanol, 7.5 percent acetic acid until protein bands were easily visible. Molecular weight standards (Bio-Rad molecular weight standards for SDS-PAGE) were run in separate lanes in each run. The molecular weight was determined by plotting the distance of marker proteins from the start of the separating gel against the logarithm of the molecular weight (semi-log), and comparing the distance traveled by the unknown proteins to the straight line obtained by this procedure.

C. Sephadex gel filtration chromatography

Sephadex gel filtration chromatography was performed as in the preparative step discussed above, but the Pharmacia column (1 m x 2.6 cm id.) was calibrated with three different proteins (BSA, ribonuclease A and ovalbumin), as well as Blue Dextran. Also, for the purpose of molecular weight determination, the volume of the sample was 2 percent of the total Sephadex bed volume. The Kav of each protein analysed was calculated from the formula:

$$Kav = (Ve-Vo)/(Vt-VO);$$

where Ve is the elution volume, VO the void volume, and Vt is the total bed volume of the column. When the Kav of the standards was plotted against their molecular weights, a straight line was determined that was used to calculate the molecular weight of the X-ray endonuclease.

IX. Determination of the Nature of the Termini Left by the Endonuclease Activity of the X-ray Endonuclease

A. The DNA polymerase I assay

1. Preparation of the DNA polymerase I substrates

Aliquots of unlabeled PM2 DNA were treated with either OsO_4 or acid/heat to produce 2-3 enzyme susceptible

sites/molecule and dialysed against 10 mM Tris pH 8.0, 1 mM EDTA overnight. Each reaction used 15 ul of DNA at 25 ul/ml, and the reactions were brought to 90 ul with 25 mM Tris pH 7.5. Aliquots of the three substrates- untreated, heat/acid-treated and OsO_4-treated PM2 DNA- were each examined in the following four ways:

1. a 10 ul aliquot of 1 M KCl was added and the reactions were incubated at 37°C for 20 minutes and then incubated at 70°C for 3 minutes followed by the addition of 3 ul of 0.04 M $MgCl_2$.
2. Several fold saturating X-ray endonuclease was added and the reactions incubated for 20 minutes at 37°C; after the reactions were incubated for 3 minutes at 70°C, 3 ul of 0.4 M $MgCl_2$ was added.
3. Exactly 10 ul of 1 M KCl was added and the reactions incubated 10 minutes at 37°C. After incubation, 3 ul of 0.4 M $MgCl_2$ and two units of exonuclease III were added, and the reactions incubated at 37°C for an additional 10 minutes. Following this the reactions were placed in a 70°C waterbath for 3 minutes.
4. Reactions were incubated for 10 minutes in a several fold excess of X-ray endonuclease, and 3 ul of 0.4 M KCl added to each reaction. Then two units of exonuclease III were added

and incubation at 37°C continued for 10 minutes. The reactions were then placed in a 70°C waterbath for 3 minutes then cooled in an ice bath.

2. DNA polymerase I reactions

To each of the above reactions was added: 20 nmoles each of dATP, dGTP, dCTP (ca. 10 ul of each) and 0.8 nMoles of dTTP, together with 2.5 mCi (5 ul) of ^{3}H-(methyl) dTTP at a specific activity of 58 Ci/mmole, and 25 ul of 1 M KCl. One unit of DNA polymerase I (1 ul) was then added to each reaction. While still on ice, 25 ul of reaction mix was removed from each vessel and spotted onto a Whatman GF/A filter presoaked with ice-cold 0.2M sodium pyrophosphate. These filters were placed on a filtering appartatus to which a vacuum was applied and they were washed with a 10 percent TCA solution for 30 seconds. After an additional 10-second wash with ice-cold 95 percent ethanol, the filters were spread on a sheet of aluminum foil and allowed to dry at room temperature. The reactions were then placed in a 37°C waterbath and aliquots were drawn every 30 minutes and treated as above. The dried filters were placed in minivials and 3.5 ml of Liquiscint scintillation cocktail was added to each minivial.

B. Calf spleen phosphodiesterase sensitivity

1. Treatment with bacterial alkaline phosphatase (BALP)

OsO_4-treated PM2 DNA (2 ug at 50,000 cpm/ug) was treated with X-ray endonuclease as in steps A and B of the preparation of substrates for the polymerase assay. The solution was divided equally into two aliquots and placed in a 65°C waterbath. After 2 minutes of incubation, one unit of bacterial alkaline phosphatase was placed into one of the DNA-containing tubes and both tubes were allowed to incubate at 65°C for 30 minutes. The solutions placed in a 37°C water bath and allowed 5 minutes to come to equilibrium.

2. Treatment with calf spleen phosphodiesterase

Each tube of DNA in the procedure above was divided into six equal aliquots and into three of these was placed 0.1 milliunits of calf spleen phosphodiesterase and the reaction were incubated for 30 minutes. Following this 1 ug of tRNA was added to each reaction and enough 100 percent TCA to bring the final solution to 10 percent. The reactions were placed on ice. After 30 minutes the reactions were spun down for 2 minutes in an Eppendorf centrifuge, the supernatant was pipeted off an placed directly into scintillation cocktail and the precipitate

was washed once with cold 10 percent TCA, resuspended in 10 mM Tris buffer pH 8.0, and placed in scintillation fluid. All samples were counted, and triplicated samples were found to differ by less than 5 percent.

X. Analysis of the Products of Digestion of Polynucleotides with X-Ray Endonuclease

A. Analysis of the molecular weights of the oligonucleotide products

For the analysis of the (2-^{14}C) poly dT:dA, a variation of the method of Maniatis et al., (1975) was followed. Slab gels (2.5 mm thick x 9 cm long) containing 5 percent acrylamide, (acrylamide:N,N'-bis acrylamide, 30:1) were polymerized in TEB buffer containing 7 M urea and run in a vertical gel electrophoresis apparatus, the reservoirs of which were filled with 20 mM sodium phosphate pH 7.5. The samples containing oligonucleotide were ethanol precipitated, dried under vacuum, redissolved in 98 percent deionized formamide, and heated in a boiling waterbath for 2 minutes. The denatured oligonucleotide solutions were then brought to 0.025 percent bromophenol blue and loaded into the precast slots in the gel (12 tooth comb). The gel was run at a constant voltage of 10 v/cm (90 volts) until the dye marker reached the bottom of the gel. Under these conditions the dye marker should migrate with

oligonucleotides less than or equal to 20 nucleotides in length. After electrophoresis, gels were sliced so that each lane was cut into 1-cm segments, left in scintillation cocktail overnight and then counted.

B. Analysis of the acid/alcohol soluble products of the digestion of OsO_4-treated or the OsO_4/alkali-treated (2-^{14}C) poly dT

1. Paper chromatography

One-meter squares of Whatman 3MM paper were marked into lanes 1.5 inches wide. Samples in 5 ul aliquots were spotted onto the centers of each lane 4 inches from the top of the paper sheets, until the sample source was depleted. The supernatant of the alcohol precipitation was brought to dryness on an Evapomix and redissolved in the minimum necessary amount of punctillious alcohol. The supernatants of TCA precipitation of polynucleotide-containing reactions were lyophilized to dryness and redissolved in as little water as possible. Thymine, thymidine, TMP, cis thymine glycol, cis-trans thymine glycol, thymine glycol nucleoside monophosphate and urea were used as markers and run in parallel in other lanes. All or an appropriate selection of these were run simultaineously with unknowns.

After sample spots were dry, the loaded sheets were placed in glass chromatography chambers pre-equilibrated

with solvent and chromatography was initiated by the addition of solvent to a trough at the top of the tank. The solvent used consisted of the upper phase of a mixture of four parts of ethyl acetate, which was mixed with one part of 1-propyl alcohol and two parts of water. Chromatography was stopped after 9 hours and the chromatograms were dried at room temperature.

2. Thin layer chromatography (TLC)

a. cellulose TLC

Using a #2 pencil, each plastic-backed plate was separated into lanes 1.5 inches wide (6/plate), and a light pencil line was drawn perpendicular to the lanes 2 cm from the bottom of the plate. A TLC chromatography tank was pre-equilibrated with solvent (propanol:water, 3:1) placed as a 1 cm layer at the bottom of the tank and covering it. Samples of the acid or alcohol soluble supernatants and appropriate markers were spotted onto the center of each lane 2 cm from the bottom in 2 ul aliquots (a hair dryer was used to dry the spots after applications). The plates were then carefully placed in the chromatography tank, the cover replaced and chromatography continued until the solvent front was 1-2 cm from the top of the plate.

b. polyethyleneimine (PEI)-cellulose thin layer chromatography

TLC using this matrix was performed as above, except that water was used as the solvent. Polyphosphates remained at the origin.

3. Visualization of markers

For the visualization of thymine, thymidine and dTMP markers the chromatograms were viewed under UV light. Urea was visualized by spraying the chromatogram with Warner's reagent, a solution of 10 percent p-dimethyl-aminobenzaldehyde in concentrated HCl, which was mixed with 4 volumes of acetone immedeately before use (Ekert, 1962). Thymine glycol and derivatives, thymidine glycol and the monophosphate were visualized by a modification of the technique of Fink et al., (1956). The chromatograms were first sprayed with 1 M methanolic HCl, dried and then sprayed with Warner's reagent. The thymine glycol derivatives were characterized by a yellow spot which over time became pink, blue and yellow.

XI. Determination of Reaction Optima and Inhibitors

A. Salt

A 4 M solution of KCl in reaction buffer or a 5 M solution of NaCl in reaction buffer was diluted appropriately into the reaction mixes prior to the addition of 5 ul of the X-ray endonuclease (Fraction VI), diluted so that approximately 30-40 percent of X-ray endonuclease susceptible sites were converted during a half-hour incubation in reaction buffer at 0.1M KCl. The molarity of KCl added with the diluted enzyme was taken into consideration in calculating the final salt concentration.

B. pH

Solutions of 200 mM Tris base were brought to the desired pH by the addition of HCl and diluted to 100 mM; the pH was measured on a Radiometer pH meter. These solutions were brought to 1 mM DTT and placed in the cold room. Aliquots of PM2 DNA in 10 mM Tris pH 8.0, 1 mM EDTA (1 ml) were placed in narrow (0.25 inch) dialysis tubing and the tubes allowed to dialyze against the 100 mM Tris solutions overnight. After dialysis (which previous studies had shown to be at equilibrium after 5 hours under these conditions), the dialysis tubes were removed and the dialysis solutions heated to 37 C, at which time the pH was

read. The DNA was assayed immediately using saturating amounts of the X-ray endonuclease. For the determination of the inhibition by N,ethylmaleimide (NEM), a 0.2 M solution of this substance was made up in reaction buffer and appropriately diluted into the reactions prior to the addition of enzyme.

C. Heat inactivation

A 2-ml aliquot of buffer A containing 1.0 M KCl was placed in a Wassermann tube and heated in a constant-temperature water bath for 5 minutes before addition of a 100-ul aliquot of a preparation of the Sephadex eluate, Fraction V. The solution was stirred by filling and emptying the pipet tip. A 100 ul aliquot was taken and placed in a tube embedded in ice. This process was repeated every 5 minutes to a total of 25 minutes. These timed samples were distributed in 5 ul aliquots to reaction tubes containing 200 ng of either AP or X-irradiated PM2 DNA. Each point was run in triplicate for each substrate. Following a 10-minute incubation, the reactions were stopped and AP-DNA reactions were analysed by neutral sucrose gradient sedimentation. The X-irradiated DNA reactions were analysed by alkaline sucrose gradient sedimentation.

Section III RESULTS

I. Purification

A. Opening cells

Four methods of breaking open cells were tried; grinding with glass beads in Braun Homogenizer, grinding with glass beads in a Sorvall omnimixer, sonication using a Branson Sonifier, and a lysozyme-EDTA method. Of these methods the first two gave comparable results with the resulting specific activity of the X-ray endonuclease being between 1,000 and 10,000 units/mg bacterial protein. Treatment by use of the Sonifier or lysozyme-EDTA gave yields of enzyme an order of magnitude lower while yielding as much protein/cell.

B. Removal of nucleic acids

The precipitation of the nucleic acids with a 10 percent solution of PEG 6,000 has several definite advantages over other methods tried. While precipitation of nucleic acids by streptomycin sulfate was efficient at removing DNA from bacterial lysates that had been cleared of ribosomes by ultracentrifugation (100 minutes at 27,000 rpm), there was considerable loss of endonuclease activity. PEG 6,000 precipitation had two advantages. First, a

separate ultracentrifugation step was not needed; Fraction II was used directly after a low speed spin in an SS 34 rotor (10 minutes at 10,000 rpm) and still gave an A_{260}/A_{280} ratio indicative of the absense of nucleic acids. The second advantage was that this method resulted in some purification with no loss of activity.

C. DNA-agarose chromatography

DNA agarose prepared by the method of Schaller et al., (1972) is an affinity matrix for proteins for single-stranded-DNA-binding proteins which Schaller states account for about 6 percent of all E. coli proteins. With this procedure about 13 percent of the soluble protein in Fraction II bound. This method has disadvantages in the purification of the X-ray endonuclease. There was an apparent loss of 75 percent of the activity loaded onto the column. The column was constantly eluting DNA at a rate of 0.1 percent of the total DNA eluting with each column volume of put-through. The X-ray endonuclease eluted in a broad peak of from 0.6-1.0 M KCl when gradient elution was used. There was, however, one advantage that outweighed the disadvantages: a 10-30 fold purification at this step. Table II shows the number of X-ray endonuclease-susceptible sites/PM2 DNA molecule/kilorad in PM2 DNA X-irradiated in the presense of 50 mM KI. As can be seen during the DNA agarose chromatography there was an apparent separation of

the X-ray endonuclease and another activity, endonuclease or DNA glycosylase which acts on X-irradiated DNA. As controls of unirradiated DNA were run in parallel with the crude fractions, the possibility of non-specific endonuclease activity was ruled out. Also, as the number of enzyme-induced strand breaks was the same under both the endonuclease assay and the DNA glycolsylase assay we could be sure that the sites nicked by these crude preparations were alkali-stable. Fractions I and II appeared to recognize about 40 percent more sites than the fractions following DNA-agarose chromatography, implying that another X-ray specific endonuclease or DNA glycosylase was present in the crude preparations.

D. Hydroxylapatite chromatography

The hydroxylapatite column was used to concentrate, remove glycerol and achieve some degree of purification with no loss of activity (see Table II). One important function of this chromatographic procedure was to remove uracil-DNA glycosylase which was known to elute from hydroxylapatite at an ionic strength of less than 0.1 M phosphate (Lindahl, 1978). Since it is known that uracil residues derived from cytosine are radiolysis products of DNA, this enzyme should add to the total number of enzyme-susceptible sites/kilorad of X-rays. Studies using gradient elution (from 0.1 M buffer B-0.5 M buffer B)

showed the X-ray endonuclease activity to elute in a broad peak from 0.2 M to 0.3 M buffer B together with the majority of proteins present in Fraction III. One distinct advantage of this hydroxylapatite as a column matrix was that samples could be loaded onto this column with ionic strengths of KCl equal to 1 molar. As with DNA-agarose, stepwise elution proved to be the most efficient mode, as no purification in excess of that acheived in step-wise elution was obtained by gradient elution, and the latter procedure resulted in much higher volumes. The column was washed with 2.5 column volumes of 0.1 M buffer B and was eluted with a similar volume of 0.3 M buffer B. The active eluate was immediately concentrated against 30 percent PEG 20,000, in 1 M KCl in buffer A until it reached a volume between 2 percent and 10 percent of the bed volume of the Sephadex column (G75 or G100) on which it was chromatographed as quickly as possible. There was some evidence that the preparation was unstable at this stage, but this was not adequately tested.

E. Sephadex (molecular exclusion) chromatography

Fraction IV, concentrated to less than 10 percent of the bed volume of the Sephadex column, was applied to a column of either Sephadex G75 or G100 using 1 M KCl in buffer A as the running buffer. Under preparative conditions, the X-ray endonuclease activity eluted with a

Kav corresponding to a protein of molecular weight between 20,000 and 40,000 daltons. There was better than a 10-fold purification at this step (see Table 4) with little or no loss of activity. If, at this step, a running buffer with a KCl concentration of less than 0.1 M was used, the activity was found exclusively in the void volume (of either a G100 or a G75 matrix) with a 90-95 percent loss of activity. Dialysis of this low ionic strength eluate against high ionic strength buffers did not restore activity.

F. Phosphocellulose chromatography

Due to the high binding capacity of phosphocellulose and the low protein concentration of the Sephadex eluate (an average of 50 ul/ml), a small column was constructed using a disposable tubercullin syringe. Early efforts used gradient elution with a KCl gradient in buffer C (based on 10 mM potassium phosphate buffer pH 8.0) while latter efforts used a KCl gradient in buffer A (based on 10 mM Tris pH 8.0). The switch in buffers was originally based on developing an assay involving S1 nuclease, which is inhibited by the presence of phosphate, and by the desire to keep the same buffer system throughout the purification. In either case the X-ray endonuclease activity eluted between 0.4 and 0.5 M KCl when a gradient of 0.3 M-0.6 M KCl was contained in 10 column volumes. The eluate from

this column was unstable and activity was rapidly lost if added to glycerol (final concentration 50 percent) at -20°C or if concentrated against 30 percent PEG 20,000 in 1 M KCl in buffer A.

G. Notes on the purification

The X-ray endonuclease was stable up to and including the penultimate step of the purification scheme as given in Table I (Sephadex chromatography), provided some precautions were taken. In a typical preparation, the purification was taken to Fraction V as rapidly as possible and stored at that stage in 50 percent glycerol at -20°C while aliquots were removed for phosphocellulose chromatography as needed.

The important caveats in the purification procedures are as follows: The ionic strength must be over the equivalent of 0.2 M KCl at all times. Figure I illustrates that with dialysis against decreasing KCl concentrations in buffer A there was an increasing loss of enzyme activity. It should be noted in this regard that enzyme appeared to aggregate at a low ionic strength (the results of the Sephadex chromatography at low ionic strength). Failure to include glycerol in any of the buffers resulted in considerable loss in activity. Finally, failure to include mercaptoethanol or dithiothreitol in chromatography or storage buffers resulted in the specific loss of the

ability to nick OsO_4-treated or X-irradiated DNA substrates without altering the AP endonuclease activity. Table 4 shows the purification of the X-ray endonuclease of E. coli in terms of yields of activity in terms of its activity on X-irradiated DNA.

II. Substrate Specificity

A. X-irradiated DNA

1. Alkali-labile sites

The X-ray endonuclease was purified on the basis of endonuclease activity on sites in DNA that are the result of X-irradiation. The original report of Strniste and Wallace (1975) showed that in crude preparations both alkali-labile and alkali-stable lesions were nicked. Additional purification based on endonuclease/DNA glycosylase activity towards DNA X-irradiated in the presense of 50 mM KI led to a loss of the ability to nick at X-ray-induced alkali-labile sites. KI is an hydroxyl radical trap and was used to increase the ratio of enzyme-susceptible sites to frank strand breaks that are one consequence of X-irradiation without KI.

Figure 2 shows the X-ray dose response of saturating quantities of X-ray endonuclease using both the endonuclease assay and the DNA glycosylase assay (+

alkali). As can be readily calculated from the data presented in Figure 2 or in Table 3, the DNA glycosylase assay showed the same number of enzyme-susceptible sites as the endonuclease assay at each dose. This indicates that the alkali-labile sites in X-irradiated DNA are not nicked by the X-ray endonuclease. In order to substantiate this unexpected result, an experiment were performed in which aliqouts of X-irradiated DNA were treated with: saturating amounts of a yeast (Saccharomyces cerevisae) class II AP endonuclease, active solely against the alkali-labile sites in X-irradiated DNA (P. Armel personal communication); saturating amounts of the X-ray endonuclease; or sequentially with saturating amounts of the yeast AP endonuclease, which was subsequently inactivated by heating at 70°C for three minutes and the DNA was then treated with saturating amounts of the X-ray endonuclease (Table 5). Identical results obtained when this experiment was performed using the major AP endonuclease of E. coli, exonuclease III/endonuclease VI in place of the yeast endonuclease. These results confirmed that the majority of alkali-labile sites produced in DNA by X rays in the presense of 50 mM KI are not susceptible to the X-ray endonuclease.

2. Alkali-stable X-ray endonuclease susceptible sites

As can be seen from Table 3, purification of the X-ray endonuclease beyond fraction II resulted in a definite number of enzyme-susceptible sites produced for each kilorad of X rays. This number, 0.035+/-.005 X-ray endonuclease-susceptible sites/PM2 molecule/kilorad of X rays in the presense of 50 mM KI, remains a constant for all preparations of the X-ray endonuclease past Fraction II and for all DNA preparations. Since it was known that X-irradiation introduces base lesions of the thymine glycol type, an experiment was performed to determine the relationship between X-ray endonuclease-susceptible sites and thymine glycol residues produced by X-irradiation. Heavily labeled ^{3}H PM2 DNA (500,000 cpm/ug DNA) was X-irradiated and simultaneously assayed for both X-ray endonuclease-susceptible sites and thymine glycol residues by use of the alkaline-degradative procedure of Hariharan (1980). The results (Figure 3) show that no more than 40 percent of the enzyme-susceptible sites are provided by thymine glycol-type lesions. The nature of the other enzyme susceptible sites will be discussed later.

B. Apurinic sites

The apurinic sites generated by heat/acid treatment

(Lindahl and Andersson, 1972) are substrates for the X-ray endonuclease. As shown in Figure 4, the X-ray endonuclease incised at about 85 percent of the total sites convertible to strand breaks as determined by the DNA glycosylase/AP endonuclease assay. Whether AP DNA was treated with X-ray endonuclease or was not treated prior to the DNA glycosylase/endonuclease assay, the number of alkali induced strand breaks remained the same. This indicated that the only sites recognized by the X-ray endonuclease in heat/acid-treated DNA are the alkali-labile, apurinic sites. It is supposed that continued incubation with X-ray endonuclease would be necessary to convert all AP sites to strand breaks, as the velocity of the X-ray endonuclease at this low substrate concentration approaches zero asymptotically.

C. Apyrimidinic DNA

Since pyrimidines are more sensitive to ionizing radiation than purines (see Introduction) it was possible that the alkali-labile sites produced in DNA by X-irradiation were primarily apyrimidinic. In order to test whether the X-ray endonuclease recognized apyrimidinic sites as well as apurinic sites, it was necessary to make DNA with a pure population of apyrimidinic sites. This was accomplished by a two step process:

1. ^{3}H-PM2 DNA was incubated for nine hours at 37^{o}C

in 2M $NaHSO_3$ to cause the deamination of cytosine residues producing uracil residues (Lindahl, 1978);

2. uracil residues resulting from the first step were removed with E. coli uracil-DNA glycosylase (kindly supplied by Dr. Errol C. Friedberg).

It can be seen from Table 6 that, while uracil-containing DNA was not a substrate for the X-ray endonuclease, apyrimidinic DNA was. Incubation of the apyrimidinic DNA with alkali showed the presense of 0.2 apyrimidinic sites, which was equal to the number obtained with X-ray endonuclease digestion.

D. Osmium tetroxide (OsO_4)-treatment

Treatment of DNA with OsO_4 at elevated temperatures or at high pH produces thymine glycol residues almost exclusively (Beer et al., 1966; Burton and Riley, 1966). The same treatment also produces alkali-stable, X-ray endonuclease-susceptible sites. Highly labeled, freshly prepared PM2 DNA, treated for varying times with OsO_4, was assayed for X-ray endonuclease-susceptible sites by incubating with X-ray endonuclease and analyzing by agarose gel electrophoresis. The same PM2 DNA was also assayed for thymine glycol-type residues by the procedure of Hariharan (1980). A linear relationship was observed between the number of thymine glycols present and the number of X-ray

endonuclease-susceptible sites (Figure 5) with respect to OsO_4 dose (assuming that the dose was proportional to time of incubation at high temperature in the presence of OsO_4, see Figure 6). Although the data (Figure 5) indicate that there are approximately two thymine glycols present for each enzyme-susceptible site, the chemical analysis for the production of thymine glycols at these low levels is uncertain enough that a one-to-one relationship cannot be ruled out. It should be born in mind that the calculation of the number of thymine glycols present in a DNA preparation by the method of Hariharan depends on the use of an empirical multiplicative constant derived under experimental conditions different from those of these.

E. Ultraviolet-irradiated DNA

PM2 DNA irradiated with ultraviolet radiation at 254 nm shows a dose-dependent production of enzyme-susceptible sites (Figure 7). These sites occur at a rate of about one-seventieth (1/70) of the rate at which cyclobutane-type pyrimidine dimers are formed. Figure 7 shows the result of neutral sucrose gradient sedimentation analysis of UV-irradiated PM2 DNA with and without incubation with the X-ray endonuclease. The same DNA analyzed by use of the DNA glycosylase assay showed complete breakdown of the Type I DNA.

F. OsO_4-treated poly dT

An aliquot of (2-^{14}C)-labeled poly dT with a chain length of 400-600 nucleotides (P-L Biochemicals), was treated with OsO_4 to produce several thymine glycol residues per chain. X-ray endonuclease was reacted with either the OsO_4-treated poly dT or with the OsO_4-treated poly dT that had previously been hybridized to poly dA. Untreated poly dT and poly dT:dA with and without X-ray endonuclease treatment were run simutaneously as controls. The results of the analysis of the polynucleotides in terms of chain lengths (Maniatis, 1976) showed (Figure 8) that untreated poly dT, untreated poly dT:dA, and OsO_4-treated poly dT were substrates for the X-ray endonuclease, although there does seem to be some degradation of the OsO_4-treated poly dT. However, OsO_4-treated poly dT:dA is a good substrate for this enzyme indicating that modified thymine residues are substrate sites for the X-ray endonuclease. Other studies, to be discussed, show that these sites are alkali-stable.

G. OsO_4/Alkali-treated (2-^{14}C) poly dT:dA

Aliquots of the OsO_4-treated (2-^{14}C) poly dT were alcohol precipitated and resuspended in 0.2 M KOH. These reactions were incubated at room temperature for 2 hours and neutralized with HCl. The polynucleotide was then

alcohol precipitated, dried in vacuum and hybridized to an equimolar quantity of poly dA. Aliquots of this substrate were then treated and analyzed as above. In Figure 8 it can be seen that OsO_4-treated, alkali-treated poly dT annealed to poly dA was a good substrate for the X-ray endonuclease. In this case the enzyme appeared to recognize thymine glycol fragmentation products. Since the alkali incubation did not significantly reduce the molecular weight of the polynucleotides analysed, it may be concluded that the sites imparted by OsO_4-treatment were alkali-stable.

H. OsO_4/alkali-treated PM2 DNA

OsO_4-treated PM2 DNA was incubated in 0.1 M NaOH for 12 hours, neutralized and examined for X-ray endonuclease-susceptible sites. As can be seen from Table 7, this substrate was stable to alkali-incubation and had as many enzyme-susceptible sites as the same DNA without alkali treatment.

I. Other substrates

Since the lesions produced by the OsO_4 treatment of DNA, thymine glycols, and the thymine fragmentation products resulting from the alkali treatment of thymine glycols both appeared to be substrates for the X-ray endonuclease, it was expected that other treatments known

to produce similar products in DNA should give rise the enzyme-susceptible sites. Treatment of DNA with $KMnO_4$ produces thymine hydrates and thymine ring-fragmentation products as well as the ring-saturation and ring-fragmentation products of cytosine and guanine residues (Iida and Hayatsu, 1970). PM2 DNA incubated at several concentrations of $KMnO_4$ and assayed for nicks following incubation with X-ray endonuclease showed total conversion to Type II DNA, while non-enzyme treated controls were essentially Type I. The substrate sites produced in DNA by this treatment could not be ascribed to base lesions- instead of apurinic/apyrimidinic sites -because the DNA was totally degraded in the presense of alkali (Table 6). Studies of the activity of the X-ray endonuclease towards this substrate in the presense of 10 mM NEM showed inhibition comparparable to the inhibition exhibited against OsO_4-treated or X-irradiated DNA, rather than the virtual lack of inhibition shown against AP DNA. This may be taken as evidence that base lesions rather than AP sites were nicked. It should also be noted that, here again, species of alkali-labile sites were produced that were not a substrate for the X-ray endonuclease.

Since NaBH is known to fragment thymine glycol residues while not reacting with undamaged DNA, (Hariharan and Cerutti, 1971) PM2 DNA containing OsO_4 induced X-ray endonuclease-susceptible sites was incubated with NaBH4 for 12 or 24 hours. The number of enzyme-susceptible sites did

not decrease, as they would if thymine glycol fragments were not substrate sites for the X-ray endonuclease. Nor would they remaine the same, as they would if they were such substrate sites. Instead the number of X-ray endonuclease-susceptible sites increased. Untreated PM2 DNA similarly incubated showed a similar production of enzyme-susceptible sites (Table 7). The nature of these sites is unknown except that they are stable to alkali incubation (they were quantified using the DNA glycosylase assay). Since it has been shown (Lindahl, 1971) that AP sites are alkali-stable following NaBH reduction, alkali-stability of these endonuclease-susceptible sites is not sufficient to show that base lesions rather than AP sites are nicked. Support for the contention that it is base lesions which are acted on in this substrate comes from two sources:

1. DNA was treated with $NaBH_4$ at 4°C at a high pH (ca. pH 10);
2. NEM caused inhibition of the X-ray endonuclease against this substrate is similar to the inhibition against OsO_4-treated DNA.

Since it is not known if NEM inhibits X-ray endonuclease activity against $NaBH_4$-reduced AP sites, it may not be concluded that the $NaBH_4$-induced-X-ray endonuclease susceptible sites are base damages.

J. Co-elution of the DNA-endonuclease/DNA-glycosylase activities of the X-ray endonuclease towards several substrates

During each preparation of purified X-ray endonuclease, the fractions resulting from the various chromatographic procedures were simultaneously analyzed with several different substrates. Figure 9 shows the elution profiles of activity from a phosphocellulose column directed against OsO_4-treated DNA using the DNA glycosylase assay and against DNA containing apurinic sites using agarose gel electrophoretic analysis of that substrate at neutral pH. Such studies were done at each stage of purification with both UV-irradiated and X-irradiated DNA as well as the substrates mentioned above. Both the endonuclease assay and the DNA glycosylase assay were used where appropriate. At no point during purification listed did a separation of activities occur. Additional studies using DEAE-cellulose and CM-cellulosic ion exchangers failed to separate any of these activities from each other.

III. Molecular Properties.

A. Molecular weight

Three means were used to determine the molecular weight of the X-ray endonuclease: gel filtration chromatography

with Sephadex G75 and G100, glycerol gradient sedimentation analysis, and SDS-polyacrylamide gel electrophoresis.

Sephadex chromatography on either G75 or G100 calibrated columns in the presense of 1 M KCl consistantly yielded a figure of 25,000 daltons +/- 10 percent (Figure 11).

Glycerol gradient sedimentation analysis of the Sephadex purified Fraction V, assayed on both AP DNA and OsO_4-treated DNA, showed two major peaks of activity with both substrates (see Figure 10). The larger peak corresponded to an average s value of 2.6-2.7 when compared to the markers run at the same time. This was equivalent to a molecular weight of 26,000 daltons +/- 20 percent using the Studier relationship. Assuming a spherical protein, it can be determined that;

$$R(s)^{3/2}M(s) = M(u),$$

where Rs = distance traveled by the unknown protein/the distance traveled by the standard, and M(s) and M(u) are the molecular weights of the standard and unknown proteins respectively. The smaller peak which present when AP endonuclease or OsO_4 endonuclease activity was assayed corresponded to a molecular weight of approximately 12,000 daltons +/- 20 percent (again see Figure 10). As can be seen, a third small peak of activity was also present with OsO_4-treated DNA.

The third method of assessing molecular weight depended on the use of a pure preparation of enzyme. The purity of

the preparation was, however, difficult to estimate due to the substantial loss of activity during the last step of the purification scheme (phosphocellulose chromatography) and the extremely low protein concentration (less than 2 ug of protein/ml). In some cases, the requirement for 5 ug of protein for an accurate concentration determination (using the Bio-Rad protein assay in its "micro" form) would take an entire preparation derived from 40-50 grams of cells. An entire phosphocellulose active fraction of 40 grams of cells was concentrated against dry Sephadex G200 to dryness and analysed by the Laemlli procedure (Materials and Methods), using the Pharmacia Molecular Weight standards for SDS-PAGE. It can be seen (Figure 11), that Fraction VI showed a single darkly staining band, corresponding to a molecular weight of 12,500 daltons. Confirming these results were the SDS-gels run on the 1000 fold purified Fraction V. This fraction, which should have included only proteins of a molecular weight near 25,000 daltons (based on the Kav's of the eluted proteins), showed only three bands, corresponding to molecular weight of about 10,000 daltons, 13,000 daltons and 15,000 daltons. This result indicated that all of the proteins eluting at this step of purification showed subunit structure. It should be noted that the enzyme, represented only a small fraction of the total protein in Fraction V, and was mostly lost by Fraction VI, so that the evidence presented for subunit structure should not be taken as definitive.

IV. Optima and Inhibitor Studies

A. Salt and pH optima

Studies showing the effect of KCl and NaCl concentration on enzyme activity were undertaken. There were some differences in the response of the X-ray endonuclease towards OsO_4-treated and AP DNA with respect to ionic strength. As can be seen from Figure 12, the activity against OsO_4 sites showed optimum activity at 0.10 M KCl with 50 percent inhibition at 0.05 M and 0.15 M KCl while the salt optimum against AP sites was between 0.05 and 0.10 M KCl with 50 percent inhibition at 0.02 M and 0.12 M KCl. Similar results were obtained with NaCl.

Studies of the pH dependence of the activity of the most purified fractions also showed differences in the enzyme's response to AP- and OsO_4-treated DNA. Figure 13 shows the activity against AP DNA to have a sharp pH optimum at pH 7.5 with 50 percent inhibition at 0.3 pH units on either side of this, while the endonuclease activity against OsO_4-treated DNA had a broader optimum at pH 7.0 with 50 percent inhibition at pH 6.2 and pH 8.1.

B. Heat lability

Studies assessing the effect of heat on the kinetics of

the loss of activity of the enzyme on two substrates, AP DNA and X-irradiated DNA, showed the activity against X-irradiated DNA to be more labile (Figure 14). While 50 percent of the activity against AP DNA remained after 10 minutes of heating at 45°C, the same reduction in activity occurred in only 5 minutes when X-irradiated DNA was the substrate. As this experiment was performed with Fraction V, which was clearly not homogeneous, the significance of these results is not clear.

C. Differential sensitivity to N'ethylmaleimide

The differential response to this sulfhydral group blocking reagent of the AP- and the OsO_4-endonuclease activities of the most purified preparations is quite marked (Figure 15). While the activity against OsO_4 -treated PM2 DNA was reduced by 70 percent at a N'ethylmaleimide concentration of 10 mM, there was little if any inhibition of the AP-endonuclease activity. Using the most purified preparations of the X-ray endonuclease, the NEM sensitivity of the enzyme to other substrates was assayed. It was found that 10 mM NEM reduced enzyme activity against UV-irradiated DNA (Figure 16 c), X-irradiated DNA (Figure 16, a and b), thymine fragment-containing DNA (Table 6), and $NaBH_4$-treated DNA to approximately the same extent as OsO_4-treated DNA.

D. Other inhibitors

PM2 DNA type I is itself a weak inhibitor of the X-ray endonuclease against OsO_4 damages. It is not known if PM2 DNA in its relaxed form, type II, is equally inhibitory. Single-stranded DNA is a good inhibitor, concentrations of calf-thymus DNA (on the order of 50 ug/ml) which showed no inhibition of the X-ray endonuclease activity; completely inhibited this activity after heat denaturation and quick cooling.

V. Nature of the Nick

A. Polymerase I assay

PM2 DNA, either untreated, treated with OsO_4 to produce about 3 enzyme-susceptible sites/molecule, or with heat/acid to produce about 1 AP site/molecule was incubated with the X-ray endonuclease followed by incubation with DNA polymerase I (*E. coli*) as described in MATERIAL AND METHODS. Figure 17 shows that the endonucleolytic nicks introduced by the X-ray endonuclease were not effective substrates for DNA polymerase I unless exonuclease III/endonuclease VI (*E. coli*) acted prior to the polymerase. This experiment indicates that the X-ray endonuclease nicked on the 3' side of the damage, leaving a deoxyribose moiety with a 3'OH. This moiety can be

effectively cleaved on the 5' side by exonuclease III/endonuclease VI, thus removing the deoxyribose residue and providing a polymerase I primer terminus site (Warner et al.. 1980). An alternate possibility however, is that the X-ray endonuclease cleaves at the 5' side of the damage leaving a 3'PO_4 that can be subsequently removed by the 3' phosphatate activity of exonuclease III/endonuclease VI (Richardson and Kornberg, 1964), generating a polymerase binding site.

B. Calf spleen phosphodiesterase (phosphodiesterase II) digestion

Phosphodiesterase II is a 5'-3' exonuclease that is inhibited from acting by a 5' PO_4 group. OsO_4-treated DNA was incubated with saturating amounts of X-ray endonuclease and divided into aliquots which were either left untreated or treated with BALP. These aliquots of OsO_4-treated, X-ray endonuclease-treated PM2 DNA with and without BALP treatment were incubated with phospodiesterase II and the extent of solubilization of the H-labeled DNA measured. The results shown in Table 8, indicate that the PO_4 group was left at the 5' position, meaning that the OH group should be in the 3' position and should be an effective primer terminus site for DNA polymerase I if the base at this position was intact.

VI. DNA Glycosylase Activity

Both thin layer chromatography (TLC) and paper chromatographic analysis (Figures 18 and 19) showed the acid soluble products of exhaustively digested, heavily labeled OsO_4-treated ^{3}H PM2 DNA (1-10 percent of the thymine residues converted to thymine glycol) to be thymine glycol as revealed by co-migration of the labeled products with cis-thymine glycol and a mixture of cis- and trans-thymine glycol. The glycosylase activity, in addition to cleaving thymine glycols, also removed urea fragments as shown in Figures 19 and 20.

Here, OsO_4-treated (2-^{14}C)poly dT annealed to poly dA, was treated with alkali to fragment the thymine glycols and then incubated with the X-ray endonuclease. Both paper chromatographic and TLC systems,showed that a labeled fragment was released that co-migrated with urea. The TCA supernatant of the digestion of heavily labeled, highly X-irradiated PM2 DNA by the X-ray endonuclease was analyzed by TLC on polyethyleneimine (PEI)-cellulose. The analsis showed an enzyme-dependent release of material that did not migrate with either thymine, thymidine or TMP (Figure 22). This release was inhibited by the presense of 10 mM NEM. In an experiment where thymine glycol containing-DNA was incubated with X-ray endonuclease in the presence or absence of 10 mM NEM, it can be clearly seen (Figure 22) that NEM inhibits the thymine glycol-DNA glycosylase:

Section IV DISCUSSION

I. Purification

The purification system developed to purify the X-ray endonuclease was fast and until the last step, efficient. The result of the purification was probably a homogeneous protein (further analysis by SDS-PAGE will be necessary to prove this). The order of the steps used in this purification was unusual in that the highly specific affinity chromatography step, DNA-agarose chromatography, was the first rather than the last step. I chose to do this in order to accomplish three aims: 1. reduce the volumes of buffer and chromatographic matrix needed in subsequent chromatographic steps; 2. separate the single-stranded-DNA-binding proteins from potential proteases present in crude fractions; and 3. remove the single-stranded DNA that continually elutes from the DNA-agarose column. The phosphocellulose step of the purification scheme is powerful in that X-ray endonuclease bounds tightly to this matrix and good separation was effected. Nevertheless, the large loss of activity was certainly a detriment and may be to some extent avoidable.

A. The assay

There are several different methods of quantitatively

separating Type I PM2 DNA from Type II aside from the techniques given in MATERIALS AND METHODS. These methods include separation by means of a two phase aqueous system consisting of PEG 6,000 and Dextran T500, by nitrocellulose filter assay and by hydroxylapatite chromatography. While all these methods gave reasonable estimates of the number of breaks/molecule caused by known doses of X-radiation, they did not work in the presence of the X-ray endonuclease. The hydroxylapatite chromatography was slow, and both the PEG-Dextran and the filter assay showed a significant increase in the number of strand-breaks/PM2 DNA molecule when the X-ray endonuclease was present, even when the DNA was untreated. Under these conditions sucrose gradient sedementation analysis showed no X-ray endonuclease activity against untreated DNA. The development of the agarose gel electrophoretic system to assay endonuclease activity proved extremely valuable. While other agarose gel electrophoresis systems had to be run at low currents to effect a clean separation, and consequently took on the order of 20 hours, the use of a discontinuous buffer system (2 x TEB buffer in the gel and 1 x TEB running buffer; see MATERIALS AND METHODS), allowed currents of 35-50 mA/gel to effect an adequate separation in 2.5 hours. Using this method, as many as forty reactions could be run at a time with strictly quantitative results and excellent reproduceability. Thus, a theoretical maximum of 120 reactions could be analysed in

an 8 hour day, which was almost ten fold the number that could be assayed using the neutral sucrose gradient sedimentation method.

The DNA glycosylase/endonuclease assay was developed in order to ascertain whether there was a separation of endonuclease from DNA-glycosylase activities during the course of purification. This method will show strand breaks at every apurinic/apyrimidinic site produced either chemically or enzymatically. It will also show endonuclease produced nicks as strand breaks. Parallel studies using either neutral sucrose gradients or agarose gel electrophoresis were used to distinquish between these cases. Efforts to apply the techniques of agarose gel electrophoresis in alkaline solution met with little success, they required long running times and resulted in poor separations. Attempts, to improve this method, perhaps by the introduction of a discontinuous buffer system, would be worth trying.

B. DNA-agarose chromatography

DNA-agarose chromatography, an example of affinity chromatography, proved a very powerful separatory step but had the disadvantages of loss of activity and "bleeding" DNA. As was mentioned previously, some of the apparent loss of activity may be due to the separation of another X-irradiated-DNA-specific endonuclease or DNA glycosylase

from the X-ray endonuclease. Alternatively, the loss of activity may arise through the binding of short pieces of single-standed DNA to the X-ray endonuclease where it could act as a competitive inhibitor blocking the enzyme's active center. These difficulties could be circumvented by covalently linking the single-stranded DNA to an agarose (or Sephadex) support either through cyanogen bromide activation of the polysaccharide or through the use of single-stranded circular DNA, which has been shown to be trapped by polymerizing agarose. A well-washed column constructed in either of these ways should have no DNA in the eluate and could possibly be used in a salt gradient elution mode to improve both the yield and purity of the enzyme preparation.

C. Phosphocellulose chromatography

During some preparations Tris buffer was used to elute the X-ray endonuclease (for reasons given in the RESULTS section) and samples were applied too quickly. Tris is known to complex with the phosphate groups of the phosphocellulose and so reduce the number of sites to which enzyme will bind as well as provide a heterogeneous matrix (Maniatis et al., 1982). As was described in MATERIALS AND METHODS, the enzyme-containing samples were applied to the phosphocellulose column at a rate of approximately 10 column volumes/hour.

D. Other chromatographic procedures

Should the final product of this purification scheme prove other than completely homogeneous (it is at least several thousand-fold purified), other separation procedures may be sought. Those chromatography procedures that require the application or elution of the X-ray endonuclease at low salt concentrations (DEAE- or CM-cellulose) should be avoided if possible as the enzyme loses activity at low salt concentrations. The use of another affinity matrix such as heparin-Sepharose, Affi-gel Blue, or double-stranded DNA-cellulose in very small volumes as a final step (perhaps in place of the phosphocellulose step) may serve to give a pure and stable preparation. Also, it would be worthwhile to try to stabilize the final preparation by the addition of protein (BSA or gelatin that is nuclease-free).

II. Comparison to Other E. coli Repair Enzymes

Because of its broad specificity, the X-ray endonuclease is easily differentiated from the AP-specific endonucleases IV (Ljungquist, 1977) and VII (Friedberg et al., 1981), as well as from the AP endonucleases associated with exonuclease III/endonuclease VI (Verly and Rassart, 1975). The ability to distinquish the X-ray endonuclease

from endonuclease VI was ensured by using both an xth mutant (Weiss, 1976) for the starting material and an assay procedure in which the activity of AP-specific endonucleases would not be observed, ie., the DNA-glycosylase assay in which alkali-labile lesions, including AP sites, are converted to strand breaks independently of the presence of AP-endonucleases. It should also be noted that, in distinction to endonuclease VI, the X-ray endonuclease does not incise the alkali-labile sites produced by X-rays in vitro in the presence of KI. The fact that the X-ray endonuclease does not incise untreated PM2 DNA distinquishes it from endonuclease I (Lehman et al., 1962).

Endonucleases V and III have a similarity to the X-ray endonuclease. Endonuclease V, in its ability to incise UV-irradiated DNA, OsO_4-treated DNA, and X-irradiated DNA, is like the X-ray endonuclease; however, it prefers a single-stranded substrate, nicks untreated single-stranded DNA, requires Mg++, has a pH optimum of 9.5, and acts particularly well on uracil-containing DNA (Gates and Linn, 1977a). Since the X-ray endonuclease has none of these attributes, endonuclease V is eliminated as a possibility.

The X-ray endonuclease is similar to endonuclease III in both its molecular and catalytic properties. Endonuclease III, originally purified by its activity with heavily UV-irradiated DNA, has nearly the same salt and pH optima, sedimentation coefficient, and molecular weight (as

determined by gel filtration) as the X-ray endonuclease. It is also sensitive to inhibition by single-stranded DNA and 1 M NaCl (Radman, 1976). An enzyme found as a by-product of the purification of endonuclease V, purified using a heavily UV-irradiated DNA substrate, was assumed to be endonuclease III (Gates and Linn, 1977b) and was shown to nick AP DNA, X-irradiated DNA and OsO_4-treated DNA. However, it was not determined whether the sites nicked in the X-irradiated DNA were stable to alkali. Endonuclease III was recently shown to possess an associated DNA glycosylase specific for 5,6 hydrated thymine moieties (thymine glycol-type residues) (Demple and Linn, 1980). Although it was not acknowledged, this enzyme may also have acted at urea residues in OsO_4-treated DNA since the method used to produce the 5,6 hydrated thymine residues in double-stranded DNA (OsO_4 oxidation of alkali-denatured PM2 DNA) employed a pH at which the glycols produced were unstable. The half-life of thymine glycols being less than 20 minutes at pH 12.0 (Iida and Hayatsu, 1970). Thus, a significant number of glycols should have been fragmented during the procedure used in the studies of Gates and Linn.

Of the seven DNA glycosylases reported to occur in E. coli, only the uracil-DNA glycosylase may be definitively ruled out as being an activity of the X-ray endonuclease, as the latter does not nick uracil-containing DNA (Table 6). Both the 5,6 hydrated thymine-DNA glycosylase activity of endonuclease III and the urea-DNA glycosylase activity,

reported in a 10-fold purified preparation of E. coli (Breimer and Lindahl, 1981), appear to be components of the X-ray endonuclease. The urea-DNA glycosylase, like the X-ray endonuclease, has a molecular weight on the order of 25,000 daltons, does not require Mg^{++}, and has a pH optimum (for the release of urea) of between 7.4 and 7.8. These properties are consistent with those of endonuclease III and the X-ray endonuclease. If the uracil-DNA glycosylase like the X-ray endonuclease, represent the same protein, then the separation of the urea-DNA glycosylase from both the hypoxanthine-DNA glycosylase and the formamidopyrimidine-DNA glycosylase during its purification (Breimer and Lindahl, 1980) implies that these activities are not components of the X-ray endonuclease. Similarly, the inability of highly purified preparations of 3-methyladenine-DNA glycosylase to release free urea from the substrate used to demonstrate the urea-DNA glycosylase activity indicates that this activity is distinct from the X-ray endonuclease. The relationship between the X-ray endonuclease and the 7-methylguanine-DNA glycosylase remains unknown.

In summary, it would appear that the X-ray endonuclease is a multifunctional enzyme consisting of a bifunctional (at least) DNA glycosylase and a class I AP endonuclease whose aspects include those activities ascribed to endonuclease III and the urea-DNA glycosylase.

III. Substrate Specificity

The damages produced by X-irradiating DNA in dilute aqueous solution may be broadly classified as; frank strand breaks, alkali-stable or alkali-labile base and/or sugar damages. Alkali-lability depends on whether incubation in alkali converts damages into strand scissions. When PM2 DNA irradiated in a dilute aqueous solution of KI is a substrate, the X-ray endonuclease recognizes mainly the alkali-stable base damages - presumably ring saturation and fragmentation products of the bases still attached to DNA - but does not nick the majority of alkali-labile damages. This is demonstrated in part by the observation that the number of X-ray endonuclease-susceptible sites remains the same whether or not this number is obtained using the endonuclease assay or the DNA glycosylase assay (Figure 2). Supporting this conclusion are the results of the experiments involving the sequential actions of the AP endonucleases (yeast and endonuclease VI) and the X-ray endonuclease, where the total number of nicks/PM2 DNA molecule resulting from the combined action of these enzymes is equal to the sum of the contributions of the AP endonuclease and the X-ray endonuclease acting alone. These results appear paradoxical, especially since the X-ray endonuclease acts both at the apurinic sites generated by heat/acid treatment and the at apyrimidinic sites generated by the action of the uracil-DNA glycosylase

on uracil-containing DNA (Figure 4 and Table 6). Alkali-labile sites in X-irradiated DNA are not equivalent to the "clean" AP sites resulting from the procedures discussed above, in which the depurinated or depyrimidinated sugar remains intact in its aldehyde or hemiacetyl form. Instead, the X-ray-induced alkali-labile sites consist of damaged sugar moieties lacking base residues (von Sonntag and Schulte-Frohlinde, 1978). These more complex alkali-labile sites appear not to be substrates for the X-ray endonuclease although they are substrates for other AP endonucleases (Armel and Wallace, 1978). Other evidence (Lafleur et al., 1981) that these sites are not pure AP sites comes from experiments that show that the rate constant for the alkali-mediated conversion of X-ray-induced sites to strand breaks is considerably larger than for the "clean" AP sites. The alkali-lable lesions produced by X-irradiation are less stable than "clean" AP sites produced by heat/acid treatment (Katcher and Wallace, 1978).

The X-ray endonuclease was also shown to nick the site of thymine glycol residues specifically produced in DNA by OsO_4 treatment, the site of the minor photoproducts resulting from UV-irradiation and the ring fragmentation products (apparently including urea residues) resulting from KMnO' oxidation and from the action of alkali-induced degradation of thymine glycol-type residues (Figures 6, 7, 8 and Table 6). Analysis of a complex substrate such as

that produced by the X-irradiation of DNA reveals many more X-ray endonuclease-susceptible sites than can be accounted for by products of the 5,6 dihydroxy-dihydrothymine-type (Figure 3) as measured by the alkali-degradative method of Hariharan (1980). As the lesions known to be recognized by the X-ray endonuclease, apurinic sites, apyrimidinic sites, and ring saturated thymine residues all belong to the class of lesions that cause minor distortions of the DNA helix (Cerutti, 1975b), it would be interesting to see if other members of this class - ring saturation, ring contraction and ring fragmentation products of the pyrimidines - are also substrates for this enzyme.

Another set of results that appears at first paradoxical are those analyzing the activity of the X-ray endonuclease against UV-irradiated DNA: When the number of X-ray endonuclease susceptible sites was compared to the UV dose, an approximate linear relationship was observed with about one endonuclease site formed for a dose which should result in the formation of about 70 thymine dimers. When duplicates of these reactions were analyzed using the DNA glycosylase/endonuclease assay, there was complete conversion of the irradiated DNA to its nicked form even at doses that result in the formation of less than 0.5 X-ray endonuclease sites/PM2 DNA molecule. Since the endonuclease assay system used cannot resolve type I DNA in the presence of call or more nicks/PM2 DNA molecule, it must be assumed these alkali-labile sites are an order or

magnitude more prevalent than the endonuclease susceptible sites. Furthermore, it was shown that the high degree of inhibition provided by the presence of 10 mM NEM makes the activity with this substrate similar to the endonuclease/glycosylase activities against OsO_4-treated and X-irradiated DNA, and not to the class I AP endonuclease activity possessed by the X-ray endonuclease. This apparent difficulty can be resolved by assuming that the alkali-labile sites produced by UV-irradiation are not AP sites. Recent evidence in support of this hypothesis comes from the work of Rupp (1983), indicating the presence of a newly noted UV-induced DNA base lesion which is alkali-labile at a frequency of approximately an order of magnitude less than pyrimidine dimers.

Alkali-stable X-ray endonuclease sites also appear to be produced by incubation of PM2 DNA at $4^{o}C$ with a solution of sodium borohydride disolved in carbonate buffer, pH 9.6. DNA subjected to this treatment, whether untreated or treated with OsO_4, appears to acquire about 1.5 X-ray endonuclease-susceptible sites for every 24 hours of incubation. It unlikely that these sites are AP sites that were rendered alkali-stable by the borohydride reduction of its free aldehyde group (Goldthwaite and Hadi, 1972) for two reasons:

1. AP sites should not form at the low temperature and high pH of the incubation conditions ; and
2. The X-ray endonuclease is inhibited from nicking

in response to these lesions by the presense of 10 mM NEM.

It is not known whether the X-ray endonuclease nicks at "reduced" AP sites, or whether this reaction is inhibited by NEM. Whether the X-ray endonuclease will react to reduced AP sites should be determined, because the ability to nick at such sites is a parameter in the classification of AP endonucleases (Mosbaugh and Linn, 1980). Although the alternative possibility that sodium borohydride could reduce the double bond of a pyrimidine to produce a ring saturation product such as dihydrothymine, already been shown to be a substrate for endonuclease III, has been contradicted (John Ward, personal communication). Analysis of the supernatants resulting from alcohol or acid precipitation of X-ray endonuclease-treated, sodium borohydride-treated DNA by high pressure liquid chromatography (HPLC) or by gas chromatography, followed by mass spectroscopy, should tell us much about substrate specificity and the mechanism of action of this enzyme.

IV. The Mechanism of Action of the X-ray Endonuclease

While the AP endonuclease activity of the X-ray endonuclease is virtually uninhibited by the presense of NEM (Figure 15), the endonuclease activity on both thymine glycol- and urea-containing DNA are substantially inhibited by NEM (Table 6), as are the thymine glycol-DNA glycosylase

and the thymine "fragment" (urea?)-DNA glycosylase (Figure 19 and Table 6).

These data, taken together with the data derived from the glycerol gradient sedimentation studies (Figure 10) and the analysis by SDS-polyacrylamide gel electrophoresis (Figure 11) suggest that the X-ray endonuclease is a dimer of subunits with the same or nearly the same molecular weight and with two independent or partially independent active centers. One of these active centers appears to be a Class I AP endonuclease (that is it cleaves on the 3' side of an AP site). The other active center(s) appears to be a base-damage-specific DNA glycosylase. The endonuclease activity on base damaged DNA would therefore be the result of the concerted action of both active centers.

If the thymine-glycol-DNA glycosylase activity and the urea-DNA glycosylase activity share the same active center, this multisubstrate DNA glycosylase might also recognize other base damages that share characteristics of both the the thymine glycol and urea residues. These might include those lesions that cause minor distortions of the DNA helix, or lack aromaticity, or are improperly base-paired, or cannot base-stack properly. Alternatively, the two DNA glycosylase activities might involve separate or overlapping active sites with unique specificities. Since both DNA glycosylase activities are NEM-inhibitible, these alternatives remain unresolved. Analysis of those products

of the X-irradiation of DNA whose release is mediated by the X-ray endonuclease should support one hypothesis or the other.

A third possibility is that the AP endonuclease activity and the damage-specific DNA glycosylase activity belong to two totally independent enzymes that happen to fortuitously co-purify through a great number of chromatographic and other separatory procedures and have the same sedimentation characteristics. This is extremely unlikely If each of the eight separatory steps gave a 2-fold purification, the probability is about .004 that two unrelated enzymes would remain together at the last step. In general the probability the two protein should co-purify is equal to the inverse of the purification factor.

The AP endonuclease activity of the X-ray endonuclease is a class I AP endonuclease and presumably acts after the DNA glycosylase removes the base lesion. On an AP substrate, the endonuclease activity gives rise to a nick which is not a primer terminus for DNA polymerase I (Figure 17) and should not be a substrate for the DNA ligase of E. coli. In conjunction with either a class II AP endonuclease or a 3'-5' exonuclease it should produce a gap that must be filled before ligation. It is still possible, however, that the X-ray endonuclease is a multifunctional endonuclease as well as a DNA glycosylase and that the endonuclease acts prior to the action of the DNA glycosylase. Analysis of the X-ray endonuclease digestion

products as well as quantitative and kinetic studies are needed to distinquish among these possibilities.

V. An Endonuclease Involved in Ionizing Radiation Repair?

The damages intorduced into DNA by X rays result from the production of high energy species such as free radicals, hydrated electrons and peroxides. These species are also produced by a variety of other reactions that take place during aerobic metabolism. Interaction of DNA with the high energy photons of UV-light produce base lesions similar to those produced by ionizing radiation (Hariharan and Cerutti, 1977). Metabolic processes produce hydroxyl radicals, hydrogen peroxide and superoxide radicals (Fridovich, 1978) which would produce DNA damages similar to damages produced by ionizing radiation if these species occur in the vicinity of DNA. Radiation arriving from space in addition to radiation from the decay of terrestrial atoms give sufficient reason for an organism whose genetic integrity must be maintained to have enzymes to repair the resultant lesions. To elaborate a repair enzyme such as the X-ray endonuclease, which is able to perform the first step(s) of excision-repair on a variety of such damages, would clearly be an economical solution.

VI. Directions for Further Research

The X-ray endonuclease is presumed to be a repair endonuclease that recognizes a limited class of DNA damages, some of which are known to be lethal and mutagenic. In order to fully understand the repair of ionizing radiation damage and the extent of damage that may occur in the course of normal metabolic activity, both the substrate specity of the various activities of the X-ray endonuclease and the phenotype of mutants lacking X-ray endonuclease activity (or activities) must be determined. The determination of the phenotype can be accomplished definitively by the study of mutants lacking the DNA-damage specific DNA glycosylase.

Mass screening isolates for mutants lacking DNA glycosylase activity against OsO_4-treated DNA has already begun. The ability of the X-ray endonuclease to function in the absence of magnesium and in the presence of 5mM EDTA enables crude bacterial lysates produced by freeze-thaw cycles to be used with because there is little or no non-specific endonuclease activity. Another way to explore the *in vitro* function of the X-ray endonuclease is to isolate the gene(s) coding it and introduce it into wild-type hosts on vectors that allow expression. Survival data on such hosts versus wild-type may reveal the effects of increased activity of the endonuclease.

While the value of the X-ray endonuclease to *E. coli*

remains in doubt, its value to researchers interested in quantitating the nature, extent and repair X-ray damages or damages resulting from other treatments known to be lethal or mutagenic. It remains, however, to completely characterize this enzyme in terms of substrate specificity. At least two different base lesions, thymine glycol and urea, appear to be substrates of the DNA glycosylase activity of the X-ray endonuclease but studies of the complex substrate produced by X rays on DNA indicate that there may be a larger set of lesions recognized by this activity. Two approaches might be used to determine which base lesions are substrates: synthesis of polynucleotides containing defined base lesions, and examination of the acid/alcohol souluble products liberated by the X-ray endonuclease from X-irradiated or UV-irradiated substrates. The second approach could be implemented by means of powerful analytic techniques such as gas chromotography or high pressure liquid chromatography (HPLC) followed by mass spectrographic analysis. This combination of a purification technique followed by mass spectrography should show exactly what is released from DNA.

Once the X-ray endonuclease has been completely characterized in terms of its substrate specifity and mode of action, it will be a powerful tool for the quantitation of DNA damage in higher organisms. Coupled with modern molecular biological techniques, discrete segments of the eukaryotic genome may be examined for the occurence of

X-ray endonuclease-susceptible sites at different times following irradiation or mutagen treatment. Studies of the kinetics of repair in various organisms or cell lines with respect to age or genotype are obvious applications. Further along the road, one might speculate that this enzyme in combination with others, may be introduced into human cells with reduced functioning or even into healthy cells to help cope with environmental stresses that normal cells are unable to deal with effectively.

Section V REFERENCES

Achey, P.M., Billen, D. and Beltrana, H.P. (1971) Single-strand breaks in gamma-irradiated X 174 DNA induced by exposure to alkali. Int. J. Radiat. Biol. 20, 501-504

Bachmann, B.J., Low, K.B., Taylor,A.L. (1976) Recalibrated linkage map of Escherichia coli K-12. Bacteriol. Rev. 40, 116-167

Behmoaras, T., Toulme, J.J. and Helene, C. (1981) Specific recognition of apurinic sites in DNA by a tryptophan-containing peptide. Proc. Natl. Acad. Sci. USA 78, 926-930.

Beukers, R. and Berends, W. (1960) Isolation and idenification of the irradiation product of thymine. Biochim. Biophys. Acta. 41. 550

Bohne, C., Coquerelle, T., Hagen, U (1970) Radiation sensitivity of bacteriophage DNA. II. Breaks and cross-links after irradiation in vivo. Int. J. Radiat. Biol. 17, 205-215

Bonura, T., Towne C.D., Smith, K.C. and Kaplan, H.S. (1970)

The influence of oxygen on the yeild of DNA double-strand breaks in X-irradiated E. coli K12. Radiat. Res. 63, 567-577

Bopp, A. and Hagen, U. (1970) End Group Determination in Gamma-irradiated DNA. Biochim. Biophys. Acta 209, 320-326

Bors, W., Michel, C. and Saran, M (1979) Superoxide anions do not react with hydroperoxides. FEBS Letters 107, 403-406

Bors, W., Saran, M., Lengfelder, E., Spottl, R. and Michel, C. (1974) The relevance of the superoxide radical anion in biological systems. Curr. Top. Radiat. Res. Q. 9, 247

Boyle, J.M., Paterson, M.C. and Setlow, R.B. (1964) Excision-repair properties of and Escherichia coli mutant deficient in DNA polymerase. Nature 226, 708-710

Boyce, R.P. and Howard-Flanders, P. (1964) The release of UV-induced thymine dimers from DNA in E. coli K12. Proc. Natl. Acad. Sci. USA. 51, 293-300

Braun, A.G. and Grossman, L. (1974) An endonuclease from E. coli that acts preferentially on UV-irradiated DNA and is absent from the uvrA and uvrB mutants. Proc. Natl. Acad. Sci. USA 71, 1838-1842

Braun, A.G., Radman, M. and Grossman, L. (1976) Enzymatic repair of DNA: sites of hydrolysis by the Escherichia coli endonuclease specific for pyrimidine dimers (Correndonuclease II). Biochem. 15. 4116-4120

Bridges, B. and Lehman, A. (1982) Inducible responses to DNA damage. Nature 298, 118-119

Bridges, B.A. and Mothershead, R.P. (1978) Mutagenic DNA repair in Escherichia coli. VIII. Involvement of DNA polymerase III in constituitive and inducible mutagenic repair after ultraviolet and gamma irradiation. Mol. gen. Genet. 162, 35-41

Brown, D.M., Fasman, G.D. Magrath, D.I., Todd A.R. Cochran, W. and Woolfson, M.M. (1953). Structure of adenylic acids a and b. Nature (Lond) 172, 1184

Cadet, J. and Teoule, R. (1971) Peroxydes formes par action du rayonnement gamma sur la thymine en solution aqueouse aeree. Biochim. Biophys. Acta. 238, 8-26

Cadet, J. and Teoule, R. (1974) Radiation chemistry of nucleic acids, characterization of thymine hydroxyhydro-peroxides. Biochem. Biophys. Res. Commun. 59, 1047-1052

Castellazzi, M., George, J., Buttin, G. (1972) Prophage induction and cell division in E. coli linked (recA, zab) and unlinked (lex) suppressors of tif-1 mediated induction and filamentation. Mol. gen. Genet. 119, 153-174

Cairns, J., Robbins, P., Sedgewick, B., Talmud, P. (1981) The inducible repair of alkylated DNA. Prog. Nucleic Acids Res. Mol. Biol. 26, 237-244

Chase, J.W. and Masker, W.E. (1977) Deoxyribonucleic acid repair in Escherichia coli mutants deficient in the 5'-3' exonuclease activity of deoxyribonucleic acid polymerase I and exonuclease VII. J. Bacteriol. 130, 667-675

Chase, J.W., Masker, W.E. and Murphy, J.B. (1979) Pyrimidine dimer excision in Escherichia coli strains deficient in exonucleases V and VII and in the 5'-3' exonuclease of DNA polymerase I. J. Bacteriol. 137, 234-242

Chetsanga, C.J., Lozon, M. Makaroff, C. and Savage, L. (1981) Purification and characterization of Escherichia coli formamidopyrimidine-DNA glycosylase that excises damaged 7-methyltuanine from deoxyribosenucleic acid. Biochem. 20, 5201-5206

Cole, R.S., Levitan, D. and Sinden, R.S. (1976) Removal of

psoralen interstrand cross-links from DNA of Escherichia coli, mechanisms and genetic control. J. Mol. Biol. 103, 39-59.

Cone, R., Duncan, J. and Hamilton, L. and Friedberg, E.C. (1977) Partial purification and characterization of a uracil DNA N-glycosylase from Bacillus subtilis. Biochem. 16, 3194-3201.

Cooper, P. (1977) Excision-repair mutants of Escherichia coli deficient in DNA polymerase I and/or its associated 5'-3' exonuclease. Mol. gen. Genet. 150, 1-12.

Cooper, P.K. and Hunt, J.G. (1978) Alternate pathways for excision and resynthesis in Escherichia coli: DNA polymerase III role? in DNA Repair Mechanisms (P.C. Hanawalt, E.C. Friedberg and C.F. Fox eds.) Academic Press, New York pp. 255-260

Da Roza, R., Friedberg, E.C., Duncan, B.K. and Warner, H.R. (1977) Repair of nitrous acid damage to DNA in Escherichia coli. Biochem. 16, 4934-4939

Defais, M., Caillet-Fauquet, P., Fox, M.S. and Radman, M. (1976) Induction kinetics of mutagenic DNA repair activity in E. coli following ultraviolet irradiation. Mol. gen. Genet. 148, 125-130.

Demple, B. and Linn, S. (1980) DNA N-glycosylases and UV repair. Nature (Lond) 287, 203-208.

Devoret,R., George, J. and Radman, M. (1974) Indirect ultraviolet-reactivation of phage lambda. Proc. Natl. Acad. Sci. USA 71, 144-147.

Duncan, B.K., Rockstroh, P.A. and Warner, H.R. (1978) Escherichia coli K-12 mutants deficient in uracil-DNA glycosylase. J. Bacteriol. 134, 1039-1045

Ekert, B (1962) Effects of gamma-rays on thymine in deaerated solutions. Nature(Lond) 194, 278-279.

Foote, R.S., Mitra, S. and Pal, B.C. (1980) Demethylation of O -methylguanine in a synthetic DNA polymer by and inducible activity in Escherichia coli. Biochem. Biophys. Res. Commun. 97, 654-659

Friedberg, E.C., Anderson, T., Bonura, T., Cone, R. and Simmons, R. (1978) Base excision repair of DNA. in DNA Repair Mechanisms (P.C. Hanawalt, E.C. Friedberg and C.F. Fox eds.) Academic Press New York

Friedberg, E.C., Bonura, T., Love, S.D., McMillan, Radany, R.A. and Schultz, E. (1980) The repair of DNA damage:

Recent developments and insights. J. Supramol. Struct. Cell Biochem. 16, 91-103

Freifelder, D. and Trumbo, B. (1969) Matching of single-strand breaks to form double-strand breaks in DNA. Biopolymers 7, 681-693

Ganesan, A.K. and Seawall, E. (1975) The effect of lexA and recF mutations on post-replication repair and DNA synthesis in Escherichia coli K-12. Mol. gen. Genet. 141, 189-205.

Gates, F.T.III and Linn, S. (1977a) Endonuclease V of Escherichia coli. J. Biol. Chem. 252, 1647-1653.

Gates, F.T.III and Linn, S. (1977b) Endonuclease from Escherichia coli that acts specifically upon duplex DNA damaged by ultraviolet light, osmium tetroxide, acid or X-rays. J. Biol. Chem. 252, 2802-2807.

Grossman, L., Riazzudin, S. Haseltine, W.A. and Landau, C. (1978) Nucleotide excision repair of damaged DNA. Cold Spring Harbor Symp. Quant. Biol. 43, 947-955

Hadi, S.M. and Goldthwait, D.A. (1971) Endonuclease II of Escherichia coli. degradation of partially depurinated deoxyribonucleic acid. Biochem. 10, 4986-4994

Hagen, U. (1967) Bestimmung von Einzel- und Doppelbruchen in Bestrahlter Desoxyribonukleinsaure durch die Moleckularge-wichtsverteilung. Biochim. Biophys. Acta. 134, 45-58

Hanawalt, P.C., Cooper, P.K. and Smith C.A. (1981) Repair replication schemes in bacteria and human cells. Prog. Nucleic Acids Res. Mol. Biol. 26, 181-196.

Hariharan, P.V. and Cerutti, P.A. (1971) Repair of gamma-ray induced thymine damage in M. luteus. Nat. New Biol. 229, 247-249.

Hariharan, P.V. and Hutchinson, F. (1973) Neutral sucrose gradient sedimentation of very large DNA from Bacillus subtilis II. Double-strand breaks formed by gamma ray irradiation of the cells. J. Mol. Biol. 75, 479-494

Heijneker, H.L., Pannekoek, H., Oasterbaan, R.A., Pouwels, P.H., Bron, S., Arwert, F. and Venaema, G. (1971) In Vitro excision-repair of ultraviolet-irradiated transforming DNA from Bacillus subtilis. Proc. Natl. Acad. Sci. USA 68, 2967-2971

Holmes, D.E., Ingalls, R.B. and Myers, L.S. (1967) ESR spectra of free radicals induced in nucleic acid, purine

and pyrimidine bases and selected analogues by exposure to hydrogen atoms. Int. J. Radiat. Biol. 13, 225-234

Hollaender, A. (1935) foreword in Molecular Mechanisms for the Repair of DNA (P.C. Hanawalt and R.B. Setlow eds.) Plenum Press, New York and London (1975)

Hutchinson, F. (1973) The lesions produced by ultraviolet light on DNA containing 5-bromo-uracil Rev. Biophys. 6 201-246

Iyer, V.N. and Rupp, W.D. (1971) Usefulness of benzoylated naphthoylated DEAE-cellulose to distinquish and fractionate double-strand DNA bearing different extents of single-stranded regions. Biochim. Biophys. Acta. 228, 117-126

Jacobs, A., Bopp, A. and Hagen, U. (1972) In vitro repair of single-strand breaks in gamma-irradiated DNA by polynucleotide ligase. Int. J. Radiat. Biol. 22, 431-435

Johnson, I. (1975) The Radiobiology of strand breakage. in Molecular Mechanisms for the Repair of DNA, Volume B. (P.C. Hanawalt and R.B. Setlow eds.) Plenum Press, New York and London pp. 459-469

Johnson, R.C. (1978) Reduction of postreplication DNA

repair in two Escherichia coli mutants with temperature-sensitive polymerase III activity: implications for the postreplication repair pathway. J. Bacteriol. 136, 125-130

Johnson, R.C. and McNeill, W.F. (1978) Electron microscopy of UV-induced post replication repair of daughter strand gaps. in DNA Repair Mechanisms (P.C. Hanawalt, E.C. Friedberg and C.F. Fox eds.) Academic Press New York pp. 95-99.

Kanner, L. and Hanawalt, P. (1970) Repair deficiency in a bacterial mutant defective in DNA polymerase. Biochem. Biophys. Res. Commun. 39, 149-155.

Kaplan, H.S. (1966) DNA strand scission and loss of viability after X-irradiation of normal and sensitized bacterial cells. Proc. Natl. Acad. Sci. USA 55, 1442-1446

Karran, P. and Lindahl, T. (1978) Enzymatic excision of free hypoxanthine from polydeoxynucleotides and DNA containing deoxyinosine monophosphate residues. J. Biol. Chem. 253, 5877-5879

Kellenberger, G. and Weigle, J. (1958) Etude au moyen des rayon ultraviolet de l'interaction entre bacteriophage

tempere et bacterie hote. Biochim. Biophys. Acta. 30, 112-124.

Kelly, R.B., Cozzarelli, N.R., Deutscher, M.P., Lehman, I.R. and Kornberg, A. (1970) Enzymatic synthesis of deoxyribonucleic acid XXXII. Replication of duplex deoxyribonucleic acid by polymerease at a single-strand break. J. Biol. Chem. 245, 39-45

Kitayama, S. and Matsuyama, A. (1968) Possibility of the repair of double-strand scissions in Micrococcus radiodurans DNA caused by gamma rays. Biochem. Biophys. Res. Commun. 33, 418-422.

Kitayama, S., Harsojo, S and Matsuyama, A. (1981) Sensitization of Micrococcus radiophilus to gamma-rays by post-irradiation incubation with chloramphenicol or at non-permissive temperatures. J. Radiat. Res. 31(3/4), 257-262

Landbeck, L. and Hagen, U. (1973) Action of DNA polymerase I on gamma-irradiated DNA. Biochim. Biophys. Acta. 331, 318-327

Lehman, I.R. (1978) Repair processes for radiation-induced DNA damage in Effects of Ionizing Radiation on DNA. (J. Hutterman, W. Kohnlein, R. Teoule

eds.) Springer-Verlage (Berlin, Heidelberg, New York) pp. 312-334.

Lehman, I.R. and Chien, J.R. (1973) Persistence of deoxyribonucleic acid polymerase I and Its 5'-3' exonuclease activity in polA mutants of Escherichia coli K12. J. Biol. Chem. 248. 7717-7723.

Lennartz, M., Coquerelle, J. and Hagen, U. (1975) Modification of end-groups in DNA strand breaks of irradiated thymocytes during early repair. Int. J. Radiat. Biol. 26, 181-185.

Lindahl, T. An N-glycosidase from Escherichia coli that releases free uracil from DNA containing deaminated cytosine residues. Proc. Natl. Acad. Sci. (USA) 71, 3649-3653

Lindahl, T. (1976) New class of enzymes acting on damaged DNA. Nature (Lond) 259, 64-66.

Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B. and Sperens, B. DNA N-Glycosidases J. Biol. Chem. 252, 3286-3294.

Little, J.W. and Mount, D.W. (1982) The S.O.S. regulatory system of Escherichia coli. Cell 29, 11-22.

Ljungquist, S. (1977) A new endonuclease from Escherichia coli acting at apurinic sites in DNA. J. Biol. Chem. 252, 2808-2814.

Ljungquist, S. Lindahl, T and Howard-Flanders, P. (1976) Methyl methane sulfonate-sensitive mutant of Escherichia coli deficient in an endonuclease specific for apurinic sites in deoxribonucleic acid. J. Bacteriol. 126, 646-653.

Mayers, L.S., Ward, J.F., Tsukamoto, W.T., Holmes, D.E. (1965) Site of attack in the radiolysis of pyrimidine compounds. Nature (Lond) 208, 1086-1087

Mayers, L.S., Ward, J.F., Tsukamoto, W.T. Holmes, D.E. and Julia, J.P.. (1965) Radiolysis of thymine in aqueous solution; change in site of attack with change in pH. Science 148, 1234-1235.

Mc Grath, R.A. and Williams, R.W. (1966) Reconstruction in vivo of irradiated Escherichia coli deoxyribonucleic acid; the rejoining of broken pieces. Nature (Lond) 212, 534-535

Mitzel-Landbeck, L., Schutz, G. and Hagen, U. (1976) In Vitro repair of radiation induced strand breaks in DNA. Biochim. Biophys. Acta. 432, 145-153.

Mosbaugh, D.W. and Linn, S. (1980) Further

characterization of human fibroblast apurinic/apyrimidinic DNA Endonucleases. J. Biol. Chem. 255, 11,743-11,752

Nabben, F.J., Karman, J.P. and Lohman H. (1982) Inactivation of biologically active DNA by hydrated electrons. Int. J. Radiat. Biol. 42, 23-30

Olsson, M. and Lindahl, T. (1980) Repair of alkylated DNA in Escherichia coli. J. Biol. Chem. 255, 10,569-10,571

Paterson, M.C., Boyle, J.M. and Setlow, R.B. (1971) Ultraviolet- and x-ray-induced responses of a deoxyribonucleic acid polymerase-deficient mutant of Escherichia coli. J. Bacteriol. 107, 61-67

Paterson, M.C. and Setlow, R.B. (1972) Endonucleolytic activity from Micrococcus luteus that acts on gamma-ray-induced damage in plasmid DNA of Escherichia coli minicells. Proc. Natl. Acad. Sci. USA. 69, 2927-2931

Paterson, M.C., Roozen, K.J. and Setlow, R.B. (1973) Gamma-ray induced chain breaks and alkali-labile bonds in lambda dv DNA of Escherichia coli minicells irradiated under aerobic and anoxic conditions. Int. J. Radiat. Biol. 23, 495-508

Pryor, W.A. (1978) The formation of free radicals and the

consequences of their reactions *in vivo*. Photochem. Photobiol. *28*, 787-801.

Radman, M. (1975) SOS repair hypothesis: Phenomonology of an inducible DNA repair which is accompanied by mutagenisis. *in* "Molecular Mechanisms for the Repair of DNA" (P.C. Hanawalt and R.B. Setlow eds.) Plenum Press, (New York and London) p. 355

Radman, M. (1976) An endonuclease from *Escherichia coli* that introduces single polynucleotide chain scissions in ultraviolet-irradiated DNA. J. Biol. Chem *251*, 1438-1445

Riazzuddin, S. and Grossman, L (1977) Micrococcus luteus correndonuclease I. Resolution and purification of two exonucleases specific for DNA containing pyrimidine dimers. J. Biol. Chem *252*, 6280-6286

Riazzuddin, S. and Lindahl, T. (1978) Properties of 3-methyladenine-DNA glycosylase from Escherichia coli. Biochem. *17*, 2110-2118

Richardson, C.C., Lehman, I.R. and Kornberg, A. (1964) A Deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*. J. Biol. Chem *239*, 251-258

Riklis, E. (1965) Mechanisms of repair of ultraviolet

irradiated DNA *in vivo* and *in vitro*. Can. J. Biochem. *43*, 1207-1219

Rothman, R.H., Kato, T. and Clark, A.J. (1975) The beginning of an investigation of the role of *recF* in the pathways of metabolism of ultraviolet-irradiated DNA in *Escherichia coli*. *in* "Molecular Mechanisms for the Repair of DNA" (P.C. Hanawalt and R.B. Setlow eds.) Plenum Press, New York and London pp. 283-291

Rupp, W.D., Wilde, C.F. III, Reno, D.L. and Howard-Flanders, P. (1971) Exchanges between DNA strands in ultraviolet-irradiated *Escherichia coli*. J. Mol. Biol. *61*, 25-44

Samson, L. and Cairns, J. (1977) A new pathway for DNA repair in *Escherichia coli*. Nature (Lond) *267*, 281

Scholes, G. (1968) Radiolysis of nucleic acids and their components in aqueous solution. *in* "Radiation Chemistry of Aqueous Solutions" (G. Stein ed.) Interscience (London) pp. 259-285

Scholes, G. (1978) Primary events in the radiolysis of aqueous solutions of nucleic acids and related substances. *in* "Effects of Ionizing Radiation on DNA" (J. Hutterman, W.

Kohnlein, R. Teoule eds.) Springer-Verlage (Berlin, Heidelberg, New York) pp. 153-170

Scholes, G., Willson, R.L. and Ebert, M. (1969) Pulse radiolysis of aqueous solutions of deoxyribonucleotides and DNA: Reactions with hydroxy-radicals. Chem. Commun. 17-18

Scholes, G. and Weiss, J. (1952) Chemical action of x-rays on nucleic acids and related substances in aqueous solution. Exp. Cell Res. Suppl. 2, 219.

Sedgewick, S.G. (1975) Genetic and kinetic evidence for different types of postreplication repair in Escherichia coli B. J. Bact. 123, 154-161

Seeberg, E. (1978) Reconstitution of and Escherichia coli repair endonuclease activity from uvrA and uvrB+/uvrC+ gene products. Proc. Natl. Acad. Sci. USA 75, 2569-2574

Seeberg, E., Nissen-Mayer, J. and Strike, P. (1976) Incision of ultraviolet irradiated DNA by extracts of E. coli requires three different gene products. Nature (Lond) 263, 524-525

Shapiro, R. and Klein, R.S. (1966) The deamination of cytidine and cytosine by acidic buffer solutions, mutagenic implications. Biochem. 5, 2358-2362

Singh, A. (1978) Introduction: Interconversion of singlet oxygen and related species. Photochem. Photobiol. 28, 429-433

Singh, A and Singh, H. (1982) Time-scale and nature of radiation-biological damage: approaches to radiation protection and post-irradiation therapy. Progress in Biophysics and Mol. Biol. 39, 110-142

Sonntag, von C., Hagen, U. Schon-Bopp, A., Schulte-Frohlinde, D. radiation-induced strand breaks in DNA: Chemical and enzymatic analysis of end groups and mechanistic aspects. Advances in Radiat. Biol. 9, 109-142

Smith, K.C. (1977) Multiple pathways of DNA repair and their possible roles in mutagenesis. National Cancer Institute Monograph #50.

Smith, K.C. and Meun, D.H.C. (1970) Repair of radiation-induced damage in Escherichia coli: I. Effect of rec mutations on post-replication repair of damage due to ultraviolet radiation. J. Mol. Biol. 51, 459-472

Strniste, G.F. and Wallace, S.S. (1975) Endonucleolytic incision of x-irradiated deoxyribonucleic acid by extracts

of Escherichia coli. Proc. Natl. Acad. Sci. USA 72, 1997-2001

Sutherland, B.M. (1978) Enzymatic photoreactivation of DNA. in "DNA Repair Mechanisms" (P.C. Hanawalt, E.C. Friedberg and C.F. Fox eds.) Academic Press New York pp. 113-122

Tait, R.C., Harris, A.L. and Smith, D.W. (1974) DNA repair in Escherichia coli mutants deficient in DNA polymerases I, II, and/or III. Proc. Natl. Acad. Sci. USA 71, 675-679

Taylor, W.D. and Ginoza, W. (1967) Correlation of gamma-ray inactivation and strand scission in the replicative form of X174 bacteriophage DNA. Proc. Natl. Acad. Sci. USA 58, 1753-1758

Teoule, R. and Cadet, J. (1971) Radiolysis of thymine in aerated aqueous solution. J. Am. Chem. Soc., Chem. Commun. 1269-1270

Teoule, R. and Cadet, J. (1978) Radiation-induced degradation of the base components in DNA and related substances- final products. in "Effects of Ionizing Radiation on DNA." (J. Hutterman, W. Kohnlein, R. Teoule

eds.) Springer-Verlage (Berlin, Heidelberg, New York) pp. 171-202

Thielmann, H.W. (1976) Carcinogen-induced DNA repair in nucleotide-permiable Escherichia coli cells. Analysis of DNA repair induced by the carcinogen N-acetoxy-N2-actylaminoflourene and 7-bromomethylbenz(∝)anthracene. Eur. J. of Biochem. 61, 501-513

Towne, C.D., Smith, K.C. and Kaplan, H.S. (1973) The repair of DNA single-strand breaks in Escherichia coli K12 x-irradiated in the presense or absense of oxygen; the influence of repair on cell survival. Radiat. Res. 55, 334-345

Towne, C.D., Smith, K.C. and Kaplan, H.S. (1974) Repair of x-ray damage to bacterial DNA. in Curr. Top. Radiat. Res., (M. Ebert and H. Howard eds.) pp. 351-398

Tye, B.K., Nyman, P.O., Lehman, I.R., Hochhauser, S. and Weiss, B. (1977) Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA. Proc. Natl. Acad. Sci. USA 74, 154-157

Van Der Schans, G.P., Bleichrodt, J.F. and Blok, J. (1973) Contributions of various types of damage to inactivation of

biologically active double-stranded circular DNA by gamma-irradiation. Int. J. Radiat. Biol. 23, 133-150

Van Der Schueren, E. and Smith, K.C. (1974) Inhibition of the exrA gene-dependent branch of the DNA excision repair system in Escherichia coli K12 by 2,4, dinitrophenol. Photochem. Photobiol. 19, 95-102

Van Rijn, C.J.S. and Lafleur M.V.M. (1976) Competition between biologically-active DNA and Protective agents for radicals formed in water. Int. J. Radiat. Biol. 29, 571

Verly, W.G. and Paquette, Y. (1972) An endonuclease for depurinated DNA in Escherichia coli B. Can. J. Biochem. 50, 217-224

Verly, W.G., Paquette, Y. and Thibodeau, L. (1973) Nuclease for DNA apurinic sites may be involved in the maintenance of DNA in normal cells. Nat. New Biol. 244, 67-68

Verly, W.G. and Rassart, E. (1975) Purification of Escherichia coli endonuclease specific for apurinic sites in DNA. J. Biol. Chem. 250, 8214-8219

Wacker, A. (1963) Molecular mechanisms of radiation effects. Prog. Nucleic Acid Res. 1, 369-399

Waldstein, E.A, Sharon, R. and Ben-Ishai, R. (1974) Role of ATP in excision repair of ultraviolet radiation damage in Escherichia coli. Proc. Natl. Acad. Sci. USA 71, 2651-2654

Warner, H.R., Perrson, M.L. Benson, R.J. Mosbaugh, D.W. and Linn, S. (1982) Selective inhibition by harmane of the apurinic/apyrimidinic endonuclease activity of phage T4 induced UV endonuclease. Nucleic Acids Res. 9, 6083-6092

Weiss, B. (1976) Endonuclease II of Escherichia coli is exonuclease III. J. Biol. Chem. 251, 1896-1901

Weigle, J.J. (1953) Induction of mutations in a bacterial virus. Proc. Natl. Acad. Sci. USA 39, 628-636

Witkin, E.M. (1976) Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol. Rev. 40, 869-907

Yajko, D.M. and Weiss, B. (1975) Mutations simultaneously affecting endonuclease II and exonuclease III. Proc. Natl. Acad. Sci. USA 72, 688-692

Yamamoto, O. (1975) Radiation-induced binding of some protein and nucleic acid constituents with macromolecular

components in cell systems. Radiat. Res. 61, 261-273

Yoakum, G.H. and Cole, R.S. (1977) Roles of ATP in removal of psorolen cross-links from DNA in Escherichia coli permiabilized by treatment with toluene. J. Biol. Chem. 252, 7023-7030

Youngs, D.A., Van Der Schueren, E. and Smith, K.C. (1974) Separate branches of the uvr gene-dependent excision repair process in ultraviolet-irradiated Escherichia coli K12 cells: their dependence of growth media and the polA, recA, recB and exrA genes. J. Bacteriol. 117, 717-725

Table 1 DNA Glycosylases

NAME	SOURCES	SUBSTRATES	MOLECULAR WT (daltons)	pH OPTIMA	NEM SENSITIVE ?	Mg^{++} ?
3-methyladenine-DNA glycosylase	*E. coli* *B. subtilis* *M. luteus*	3-methyladenine and 3-ethyladenine in ds-DNA	20,000	7.2-7.8	?	-
3-methladenine-DNA glycosylase II	*E. coli*	3-methyladenine, 3-methylguanine, 7-methyladenine, 7-methylguanine.	27,000	-	--	-
hypoxanthine-DNA glycosylase	*E. coli*	hypoxanthine in ds DNA	30,000	-	--	NO
uracil-DNA glycosylase	*E. coli* *B. subtilis* calf, human	ring-opened 7-methyl-guanine in ds DNA	24,500	8.0	-	-
formamidopyrimidine-DNA glycosylase	*E. coli*	ring-opened 7-methyl-guanine in ds DNA	30,000	-	-	-
urea-DNA glycosylase	*E. coli*	urea residues in ds DNA	20,000	7.0	-	NO
thymine glycol-DNA glycosylase (endonuclease III	*E. coli*	thymine glycol and dihydrothymine in ds DNA	25,000	7.5	-	NO

Table 2. PUTATIVE ESCHERICHIA COLI REPAIR ENDONUCLEASE

NAME	SUBSTRATES	OTHER ACTIVITIES	MOLECULAR WEIGHT(d)	pH OPTIMUM	NEM SENSITIVE	CO-FACTORS
Endonuclease III	AP DNA, UV-, and X-irradiated ds DNA, thymine glycol residues in ds DNA	thymine-glycol-DNA glycosylase	25,000	7.5	-	NO
Endonuclease IV	AP ds DNA	NO	30,000-33,000	8.0-8.5	YES	NO
Endonuclease V	AP DNA, ssDNA, uracil containing DNA and UV-, and X-irradiated DNA	NO	20,000	9.5	-	absolute Mg^{++} requirement
Endonuclease VI (exonuclease III)	AP ds DNA	RNase, exonuclease, phosphatase	28,000	8.0-8.5	--	Mg^{++} needed for exonuclease activity
Endonuclease VII	AP ss DNA	-	56,000 ?	7.0	YES	NO

Table 3. Enzyme-susceptible sites in X-irradiated DNA

Fraction	(Strand breaks/PM2 DNA molecule) Endonuclease assay	DNA glycosylase assay
I	0.057	0.053
II	0.053	0.057
III	0.037	0.035
IV	0.042	NT
V	0.041	NT
VI	0.037	0.042

Table 4. Purification of the X-ray endonuclease of Escherichia coli.

Fraction Number	Description	Protein Conc. (mg/ml)	Specific Activity unit/mg protein	Yield
I	crude extract	19	7,000	100%
II	PEG 6000 supt.	8	13,000	100%
III	DNA-agarose 1 M KCl eluate	0.3	320,000	25%
IV	hydroxylapatite	0.9	780,000	23%
V	Sephadex G75	0.06	4×10^6	19%
VI	phosphocellulose	0.01	single band on SDS gels	0.3%

Table 5. Substrate specificity of the X-ray endonuclease on X-irradiated PM2 DNA.

X ray dose	Enzymes	breaks/mol.	enzyme-induced breaks/mol.
0 krad	none	0.23	-
20 krad	none	0.55	-
20 krad	yeast AP endonuclease E	0.95	0.40
20 krad	X-ray endonuclease	1.33	0.80
20 krad	yeast AP endo. + X-ray endo.	1.98	1.40

Table 6. X-ray endonuclease substrate specificity

	Enzyme-induced sites				
Type of DNA treatment	Alkali-labile sites ?	Alkali-stable sites ?	NEM inhibition ?	DNA glycosylase ?	Probable lesion
Acid/heat	yes	no	no	-	apurinic sites
Bisulfite	no	yes	NT [1]	-	uracil residues
Bisulfite, ura-DNA glycosylase	yes	no	NT [1]	-	apyrimidinic sites
OsO_4-treatment	no	yes	yes	yes	thymine glycol residues
OsO_4/alkali treatment	no	yes	yes	yes	urea- residues

Table 6. X-ray endonuclease substrate specificity (cont.)

Type of DNA treatment	Alkali-labile sites ?	Alkali-stable sites ?	NEM inhibition ?	DNA glycosylase ?	Probable lesion
X-irradiation	no	yes	yes	yes	thymine ring-saturation and degradation products
$KMnO_4$	?[2]	yes	NT	NT	base fragment residues
UV-irradiation	?[2]	yes	yes	NT	pyrimidine photo-hydrates

1. NT= not tested

2. Alkali sensitivity of sites could no be tested as alkali cause complete conversion to type II.

Table 7. Reaction of X-ray endonuclease with degradation products of thymine glycol residues.

Substrate	breaks/PM2 DNA molecule		
	no enzyme	+ enzyme	+ enzyme, + NEM
OsO_4-treated DNA	0.18	1.08	0.59
OsO_4-treated DNA	0.15	0.97	0.63
untreated DNA 12 hours $NaBH_4$	0.59	2.84	?
2' OsO_4, 0 hrs $NaBH_4$	0.18	1.08	0.59
2' OsO_4, 12 hrs $NaBH_4$	0.26	3.29	2.20
2' OsO_4, 25 hrs $NaBH_4$	0.12	4.48	1.64
4' OsO_4, 0 hrs $NaBH_4$	0.21	1.40	0.44
4' OsO_4, 12 hrs $NaBH_4$	0.34	3.10	1.42

Table 8. Determination of the presence of a 5' PO_4 at an X-ray endonuclease-induced nick.

Reaction conditions	% Acid soluble radioactivity
OsO_4-treated DNA + X-ray endonuclease	13
OsO_4-treated DNA + X-ray endonuclease + BALP	11
OsO_4-treated DNA + X-ray endonuclease + spleen phosphodiesterase	13
OsO_4-treated DNA + X-ray endonuclease + BALP + spleen phosphodiesterase	67

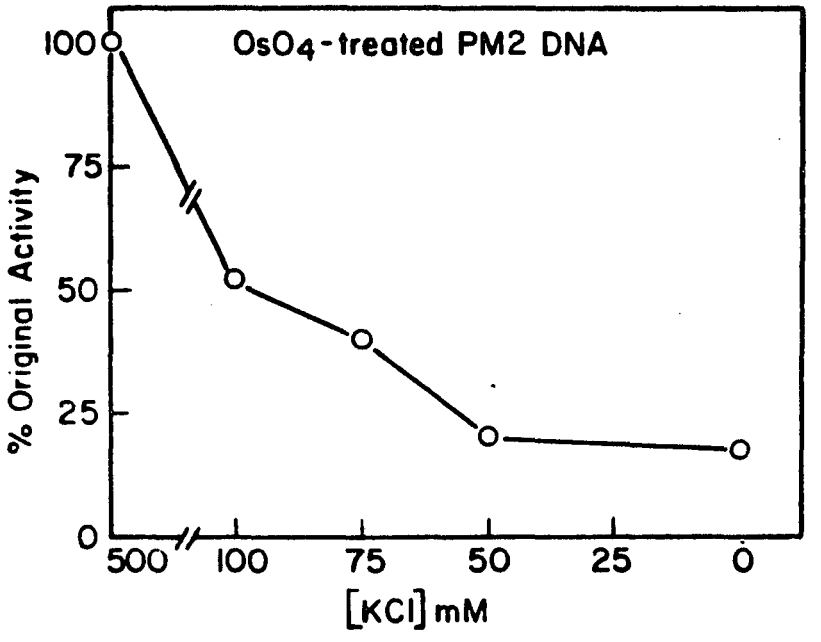

Figure 1. Dialysis of X-ray endonuclease against buffers of low ionic strength.

A highly active preparation of the X-ray endonuclease (Fraction V) was dialysed overnight against solutions of buffer A with the concentrations of KCl indicated. The dialysed samples were diluted 1:10 in buffer A at 1 M KCl and further dilutions were performed in this buffer to assess the endonuclease activity remaining. Concommitant protein concentration measurements (Bio-Rad protein assay "micro" procedure) showed no discernable difference between samples.

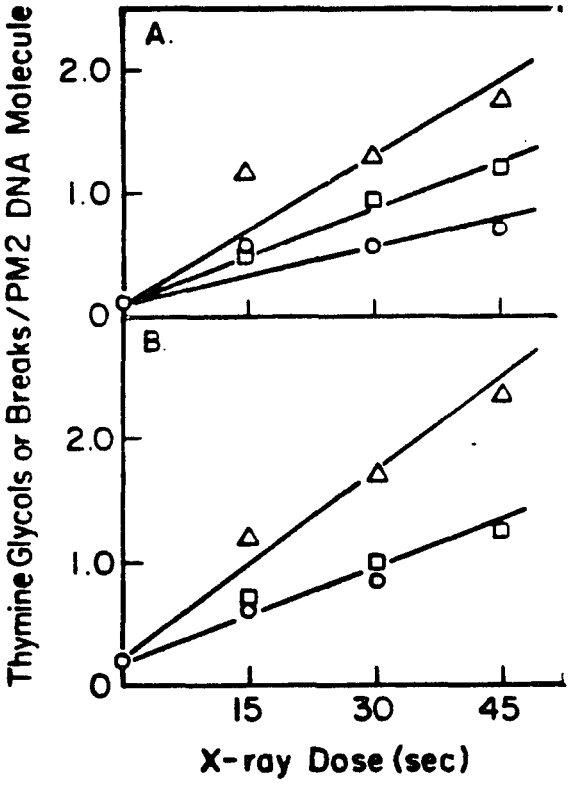

Figure 2. The relationship between X-ray endonuclease sites, strand breaks and alkali-labile lesions in X-irradiated DNA.
A. ^{3}H PM2 was X-irradiated in the presence of 50 mM KI at the indicated times and strand breaks measured immediately by neutral sucrose gradient centrifugation (Δ) or after incubation with saturating amounts of X-ray endonuclease (Fraction VI) (o). B. ^{3}H PM2 DNA treated as above was incubated with an equal volume of 1 M glycine-NaOH buffer (pH 13.1) for 4 hours prior to analysis by alkaline subrose gradient centrifugation. As above, (Δ) represents no enzyme treatment, (o) X-ray endonuclease digestion and (□) X-ray endonuclease digestion in 10 mM NEM.

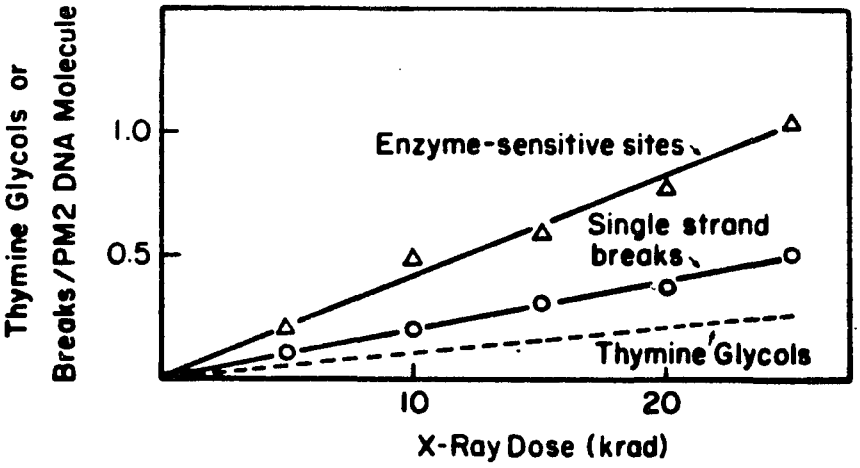

Figure 3. The relationship between X-ray-induced, endonuclease-susceptible sites and X ray-induced thymine glycols.
^{3}H PM2 DNA (500,000 cpm/ug DNA) was X-irradiated in 10 mM Tris pH 7.5, 50 mM KI and 200 ng aliquots were assayed for X-ray endonuclease-susceptible sites by agarose gel electrophoresis. Simultaneously, 4 ug aliquots of the same DNA preparations were assayed for thymine glycol content by the alkali-degradative procedure of Hariharan (1980).

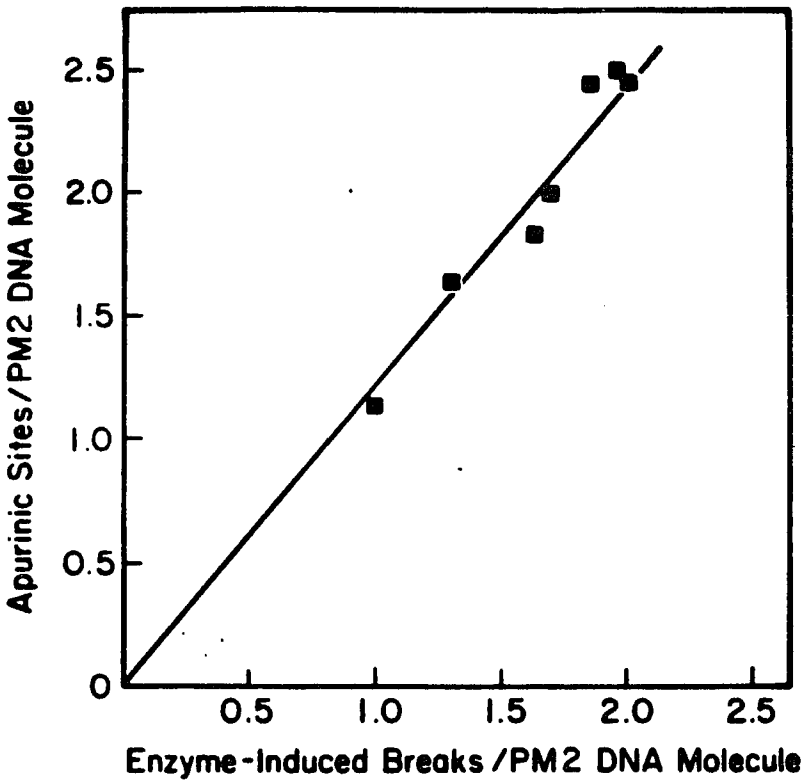

Figure 4. Relationship between X-ray endonuclease-susceptible sites and AP sites in heat/acid treated DNA.

^{3}H PM2 DNA (10 ug/ml) in 0.1 M NaCl, 0.01 M sodium citrate, pH 5.0 was heated for varying periods at 70°C and subsequently neutralized with 1 M Tris, pH 7.5. The depurinated DNA was either incubated with saturating amounts of X-ray endonuclease or incubated for 4 hours at room temperature with equal volumes of 1 M glycine-KOH buffer pH 13.1 and analyzed by neutral sucrose gradient centrifugation analysis or by alkaline sucrose gradient centrifugation analysis respectively as described as described in MATERIALS AND METHODS.

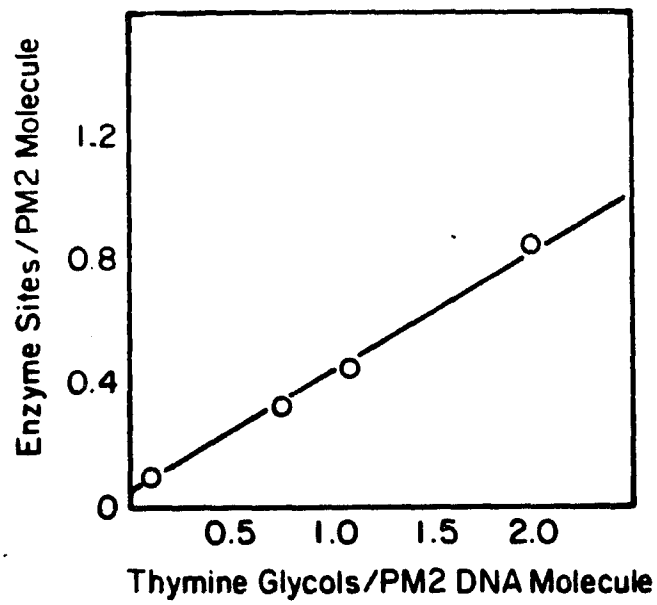

Figure 5. The relationship between thymine glycols and X-ray endonuclease-susceptible sites in OsO_4-treated DNA. ^3H PM2 DNA (500,000 cpm/ug), was treated PM2 DNA with 0.02% OsO_4 at 70°C and simultaneously assayed for thymine glycols by the procedure of Hariharan (1980) and for X-ray endonuclease-susceptible sites using gel electrophoresis.

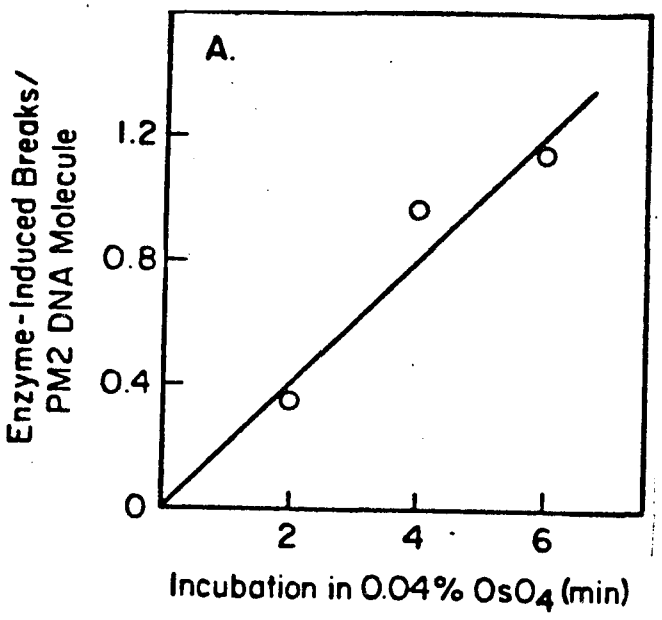

Figure 6. The relationship between incubation in OsO_4 and the appearance of X-ray endonuclease-susceptible sites.

^3H PM2 DNA at 55°C was brought to 0.04% OsO_4 from a 4% stock solution. Aliquots were removed immediately and at the times indicated and digested with saturating amounts of X-ray endonuclease. The DNA was analyzed by the DNA glycosylase/endonuclease assay. Neither strand breaks nor alkali-labile sites were generated during the incubation.

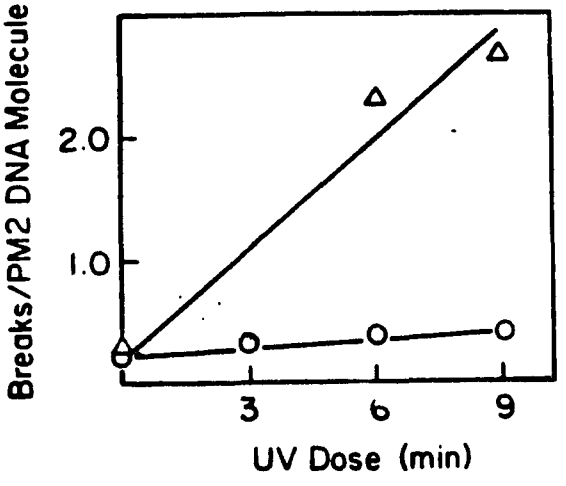

Figure 7. The relationship between UV dose and the induction of X-ray endonuclease-susceptible sites.
^{3}H PM2 DNA was irradiated at a fluence of 4.4 J/m^2 for the durations indicated and either analysed by neutral sucrose gradient centrifugation analysis directly or following extensive X-ray endonuclease digestion.

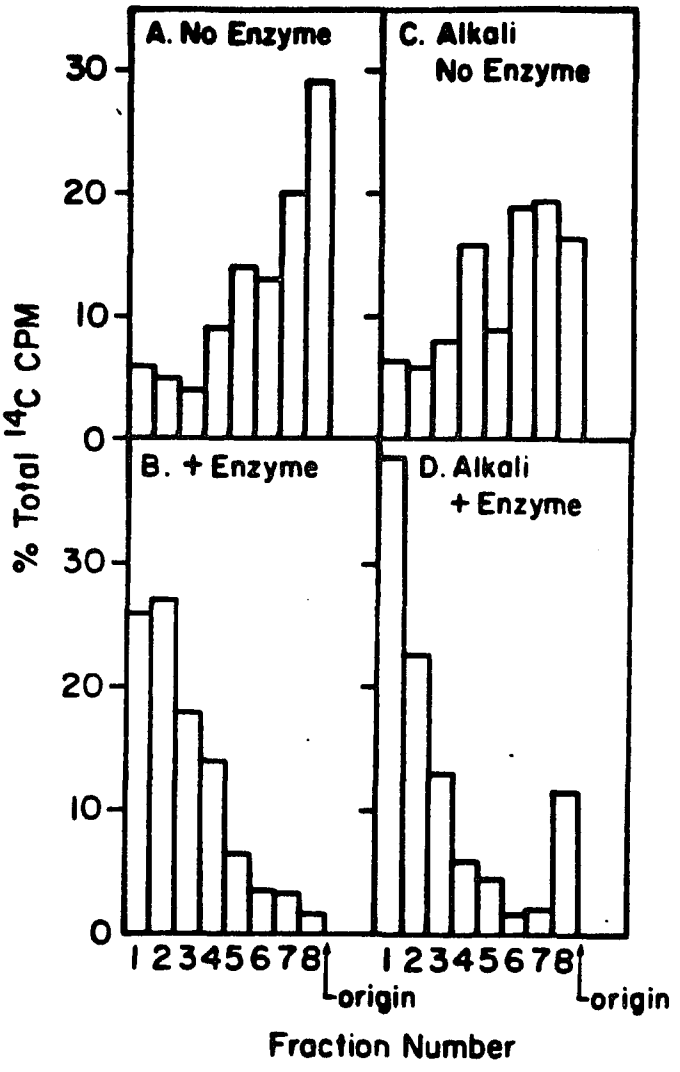

Figure 8. Polyacrylamide gel electrophoresis of poly-dA:dT containing either thymine glycol residues or thymine fragment (urea) residues.

(A) and (B). Poly (2-^{14}C) dT was incubated with 0.4% OsO_4, extracted with diethyl ether, precipitated and annealed with poly dA. The duplex was either directly precipitated and redissolved in 98% formamide (A) or first digested with saturating X-ray endonuclease (B). The denatured polynucleotides were then analyzed by electrophoresis on 5% polyacrylamide gels containing 7 M urea. (C) and (D). OsO_4-treated poly (2-^{14}C)dT (as above) was incubated in 0.2 M KOH prior to tre4atment as above. (C). No X-ray endonuclease treatment. (D). Following digestion with saturating X-ray endonuclease.

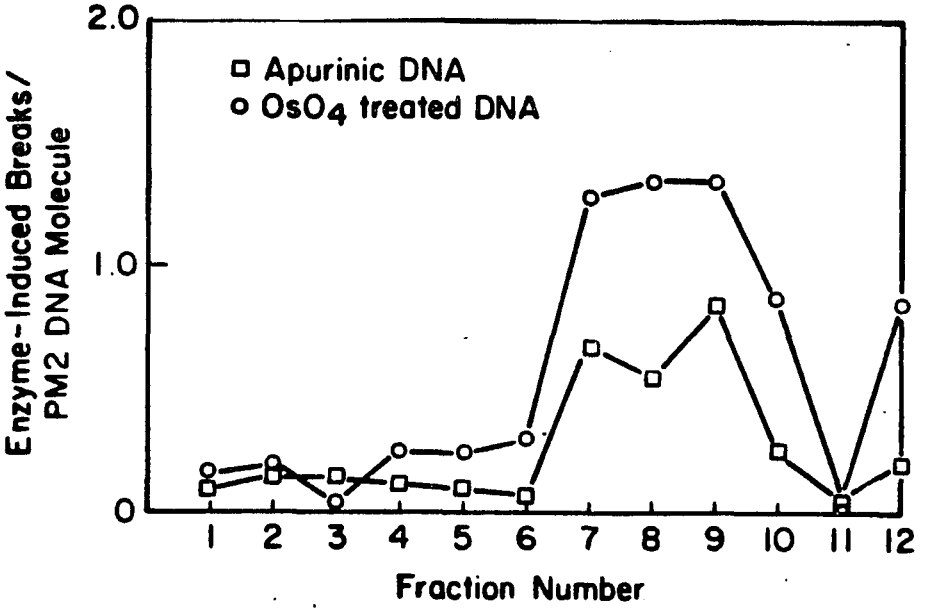

Figure 9. Co-elution from phosphocellulose of endonuclease activities against AP and OsO_4-treated DNA.

X-ray endonuclease (Fraction V) was applied to 0.5 x 3 cm column and eluted with a 10 column volume gradient of 0.25-0.60 M KCl in buffer A. Fractions (1 ml) were simultaneously assayed for DNA glycosylase/endonuclease activity on OsO_4-treated DNA and endonuclease activity on AP DNA, as described in MATERIALS AND METHODS. Both activities co-elute between 0.4-0.5 M KCl.

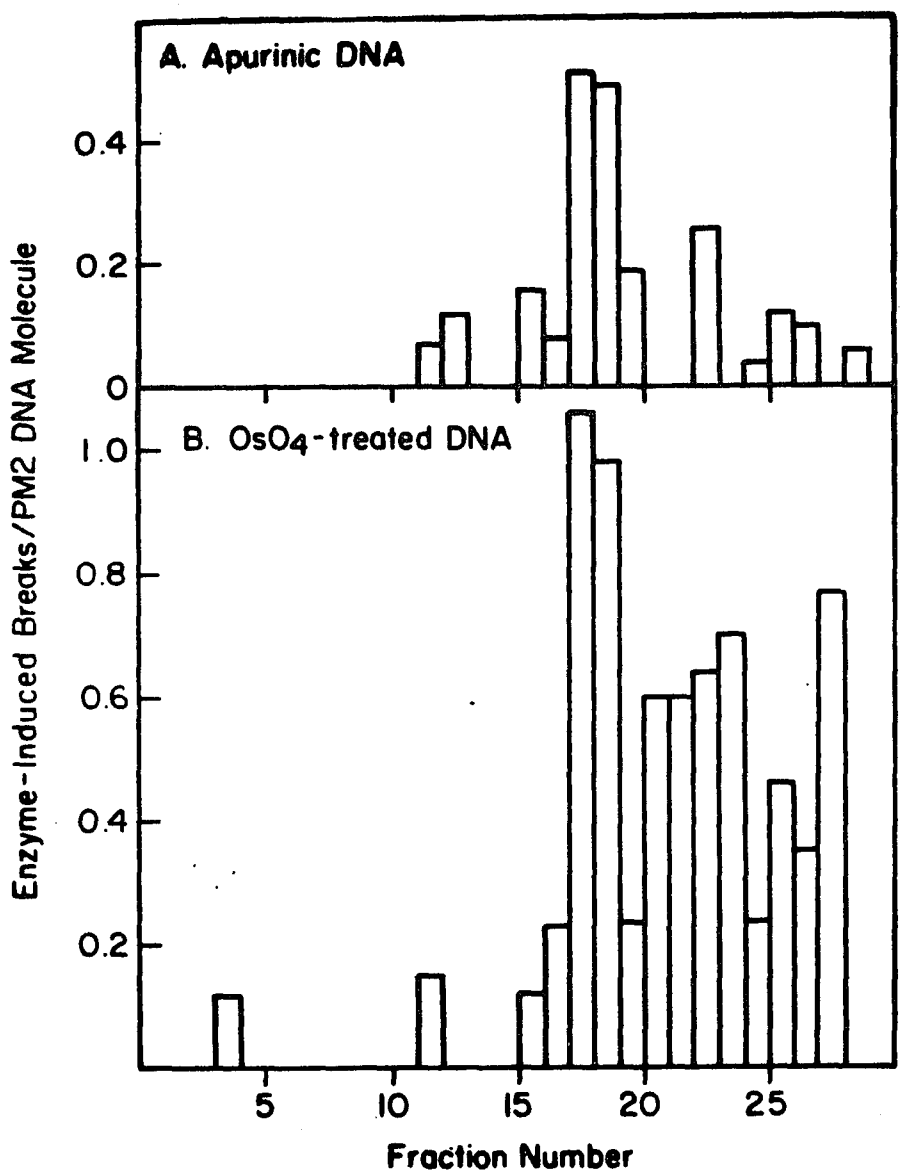

Figure 10. Glycerol gradient analysis of X-ray endonuclease.

Aliquots of Fraction V were layered onto 5 ml 20-40% glycerol gradients in 0.02 M potassium phosphate buffer containing 1 mM mercaptoethanol, 1 mM EDTA at 2°C and spun at 46,000 rpm for 48 hours at in an SW 50.1 rotor. Standard proteins were run in separate gradients. X-ray endonuclease containing gradients were collected in 33 vials in the cold and simultaneously assayed for activity against OsO_4-treated and AP DNA. Protein standards, bovine serum albulmin, chymotrypsinogen, and ribonuclease A were assayed for protein concentration by the Bio-Rad "micro" assay.

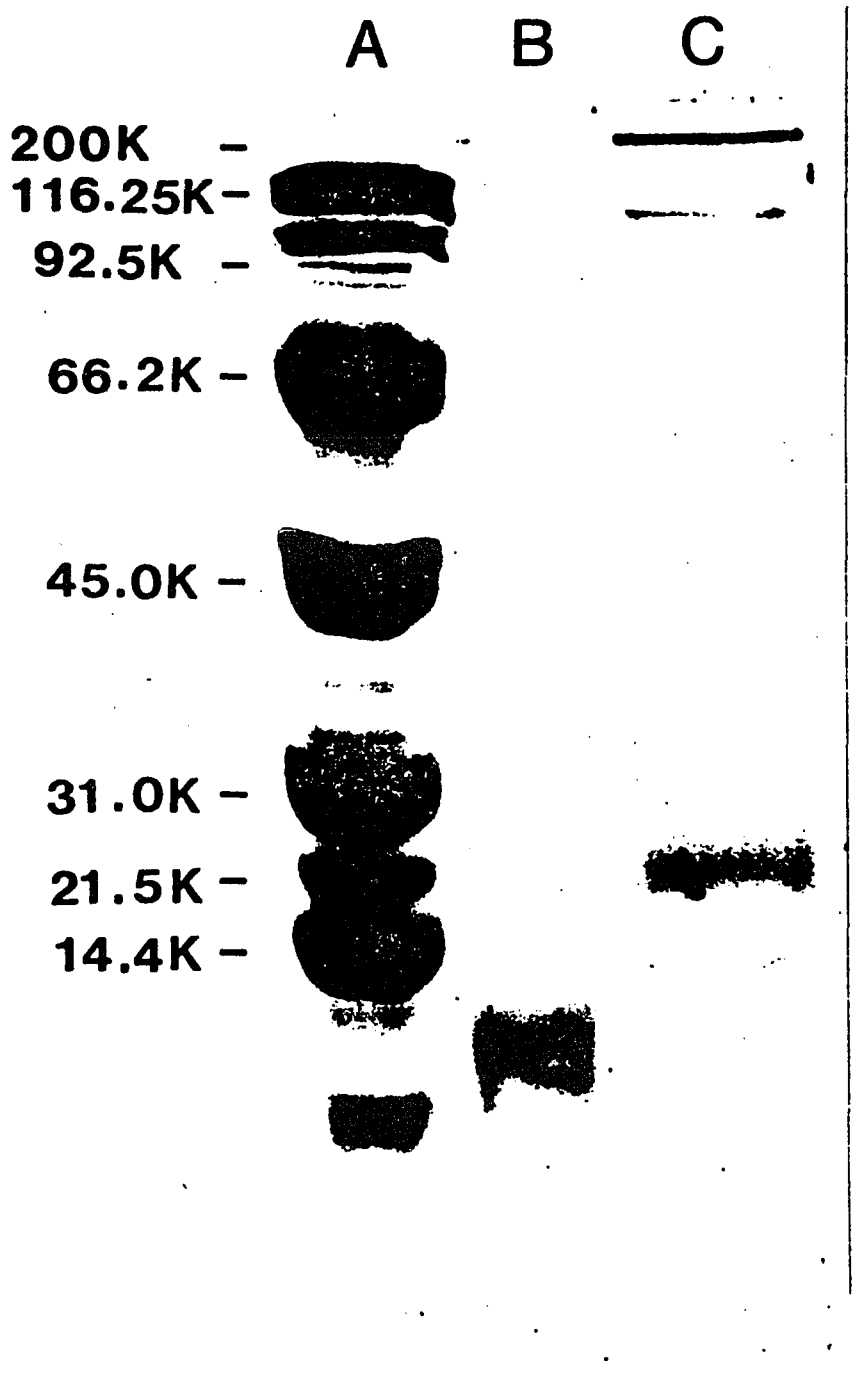

Figure 11. SDS-polyacrylamide gel electrophoresis of X-ray endonuclease.

Samples were prepared and run according to the procedure of Laemmli (1970) as described in MATERIALS AND METHODS. Lane A, molecular weight standard proteins (Bio-Rad Laboratories, low molecular weight standards for SDS gel electrophoresis); Lane B, phosphocellulose Fraction (VI); Lane C, DNA-agarose, Fraction III.

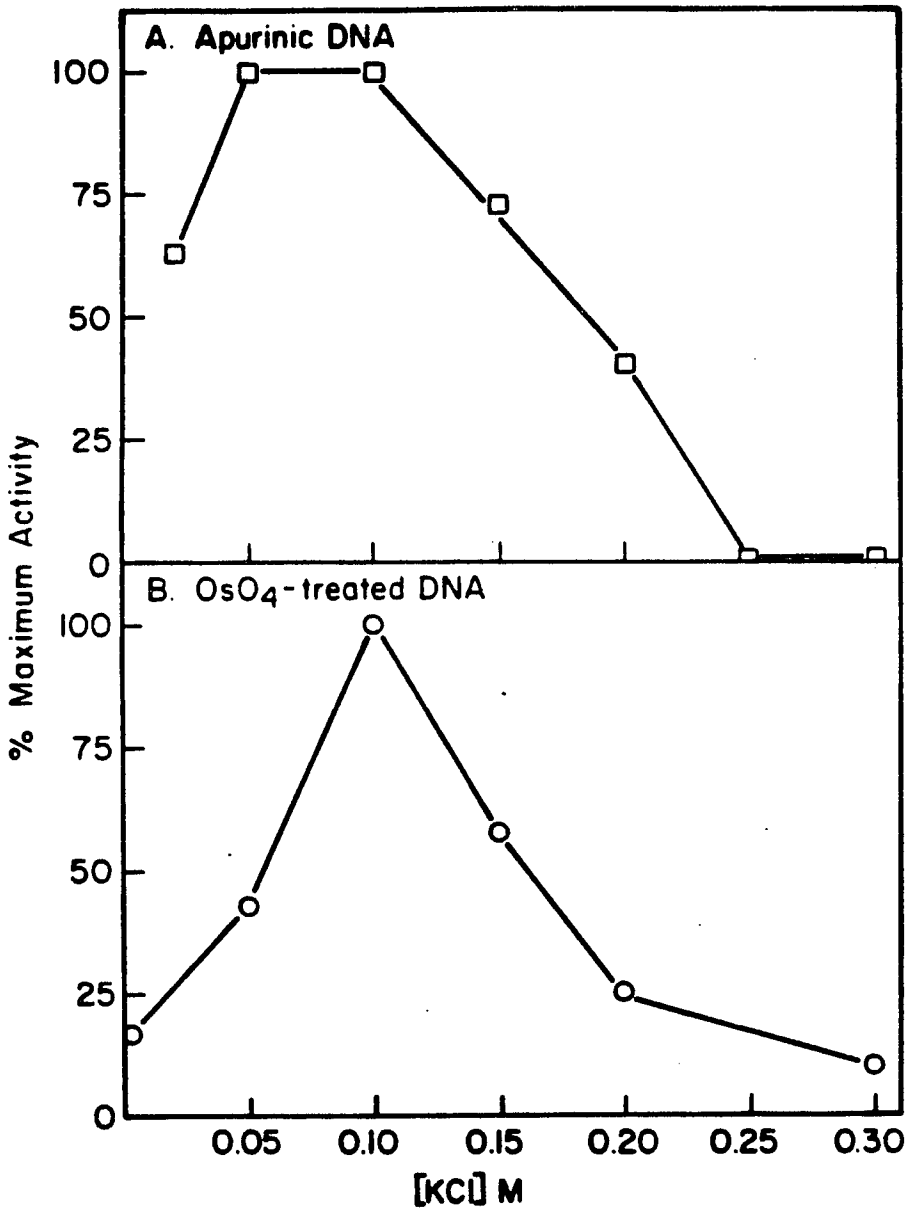

Figure 12. The effects of KCl on the activities of the X-ray endonuclease.

^{3}H PM2 DNA treated either to produce AP sites (A) or OsO_4 damages (B) was incubated with subsaturating amounts of the X-ray endonuclease (Fraction VI) at the KCl concentrations indicated. Strand breaks were quantitated by either agarose gel electrophoresis or neutral sucrose gradient centrifugation.

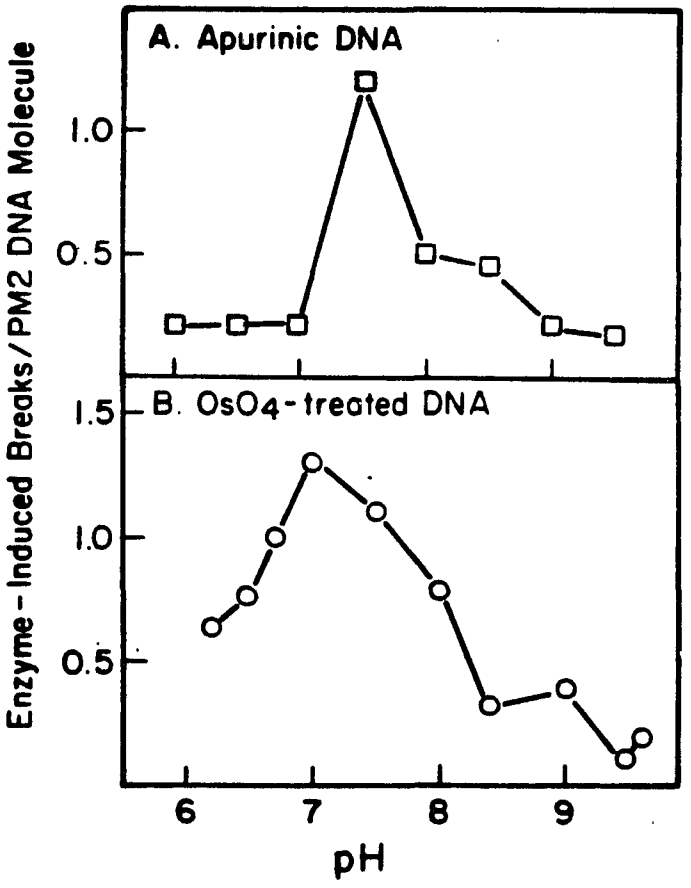

Figure 13. The effect of pH on the activities of the X-ray endonuclease.

^{3}H PM2 DNA containing either AP sites (A) or OsO_4 damages (B), was incubated with subsaturating amounts of the X-ray endonuclease (Fraction VI) at the indicated pH. and assayed for strand breaks by either agarose gel electrophoresis or neutral sucrose gradient centrifugation.

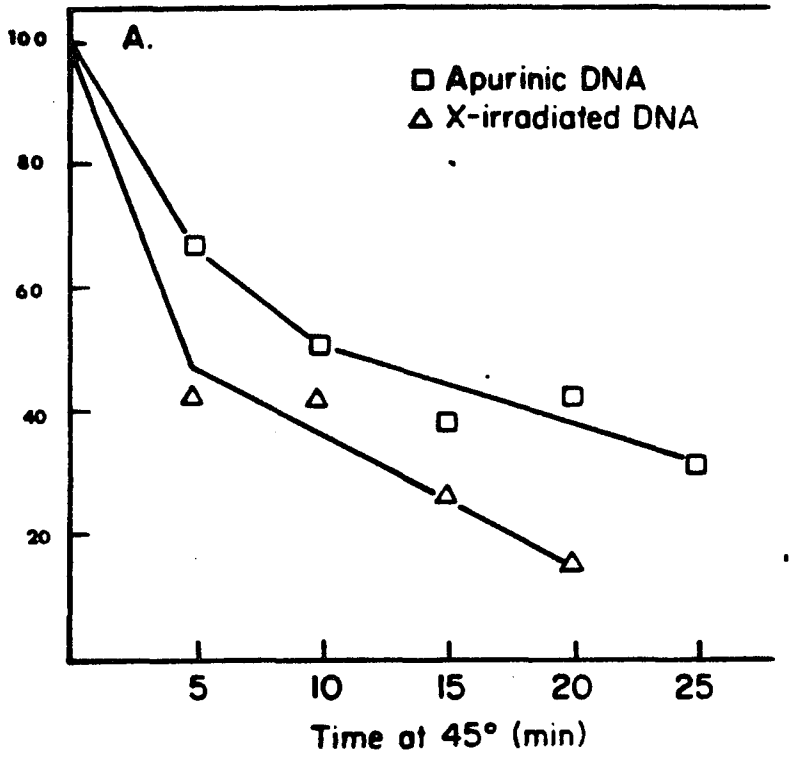

Figure 14. Heat inactivation of the activities of the X-ray endonuclease.

Samples (100 ul) of the X-ray endonuclease (Fraction V) were heated at 45°C. Aliquotes were removed immediately and at 5 minute intervals, diluted and simultaneously assayed for activity on AP DNA ( ) or X-irradiated DNA ( ) by neutral sucrose gradient centrifugation.

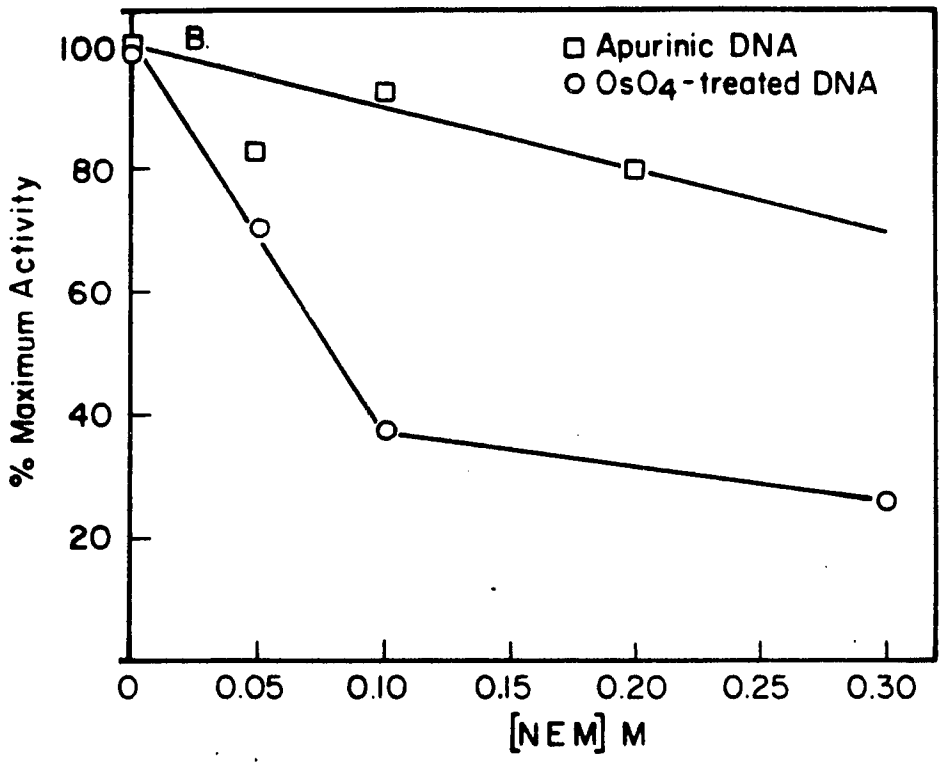

Figure 15. Differential effects of NEM on the activities of the X-ray endonuclease.

^3H PM2 DNA containing either AP sites, or OsO_4-induced damages was treated with subsaturating amounts of X-ray endonuclease (Fraction VI) at concentrations of NEM indicated and assayed by neutral sucrose gradient centrifugation analysis.

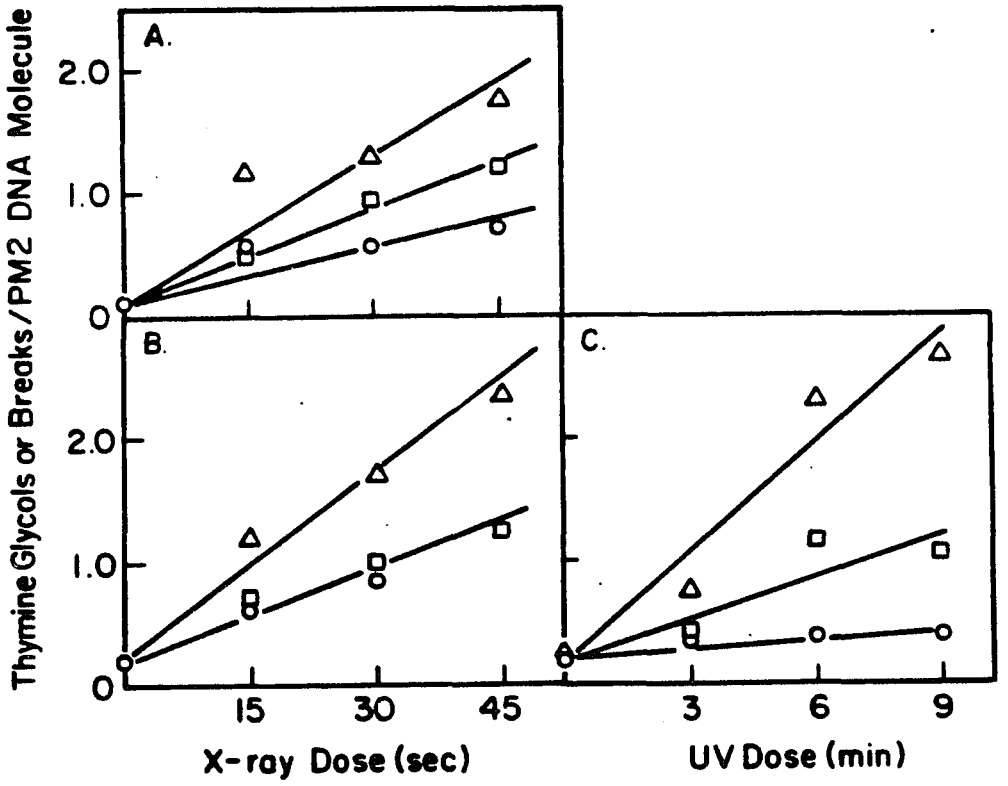

Figure 16. Effect of NEM on the X-ray endonuclease activities towards X-irradiated and UV-irradiated DNA substrates.

^{3}H PM2 DNA, X-irradiated in Tris buffer 0.05 M KI or UV-irradiated as described in MATERIALS AND METHODS was either analysed directly by agarose gel electrophoresis analysis (A and C) or by the endonuclease/DNA glycosylase assay (B), (Δ); or was either incubated with saturating amounts of X-ray endonuclease without NEM (□), or incubated with saturating amounts of X-ray endonuclease in the presence of 10 mM NEM (○) prior to analysis.

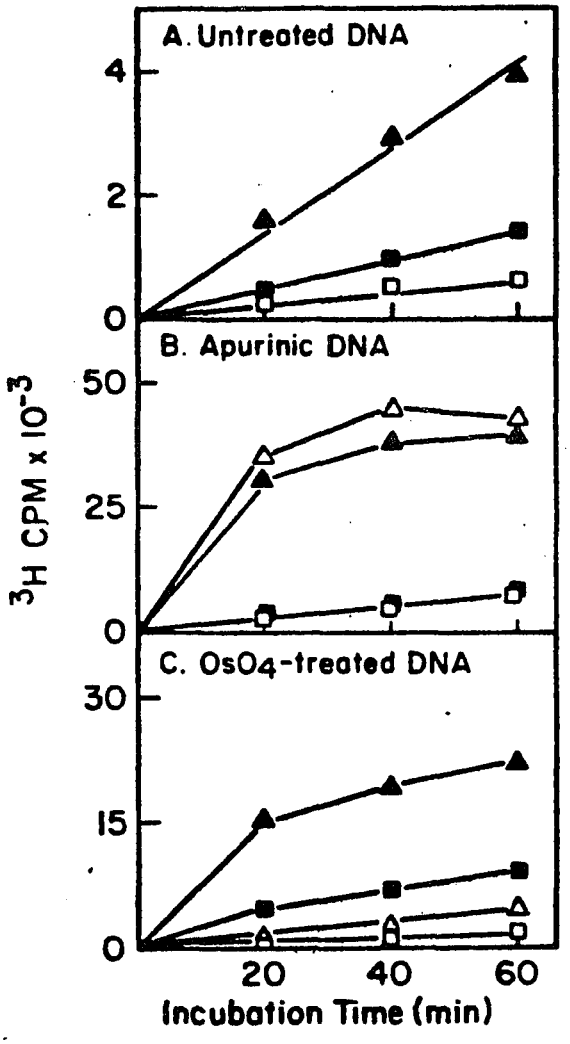

Figure 17. X-ray endonuclease-induced nicks as substrates for E. coli DNA polymerase I.

Unlabeled PM2 DNA, either untreated (A), containing apurinic sites (B), or treated with OsO_4 (C), was incubated without enzyme (□), with exonuclease III/endonuclease VI (△), with X-ray endonuclease (Fraction VI) (■), or with X-ray endonuclease followed by exonuclease III/endonuclease VI (▲). These reactions were then heated to inactivate the endonucleases and added to a reaction mixture containing DNA polymerase I, Mg^{++} and all four dNTPs (^{3}H-dTTP) as described in MATERIALS AND METHODS. Aliquots were removed initially and at 20 minute intervals and the acid insoluble radioactivity determined.

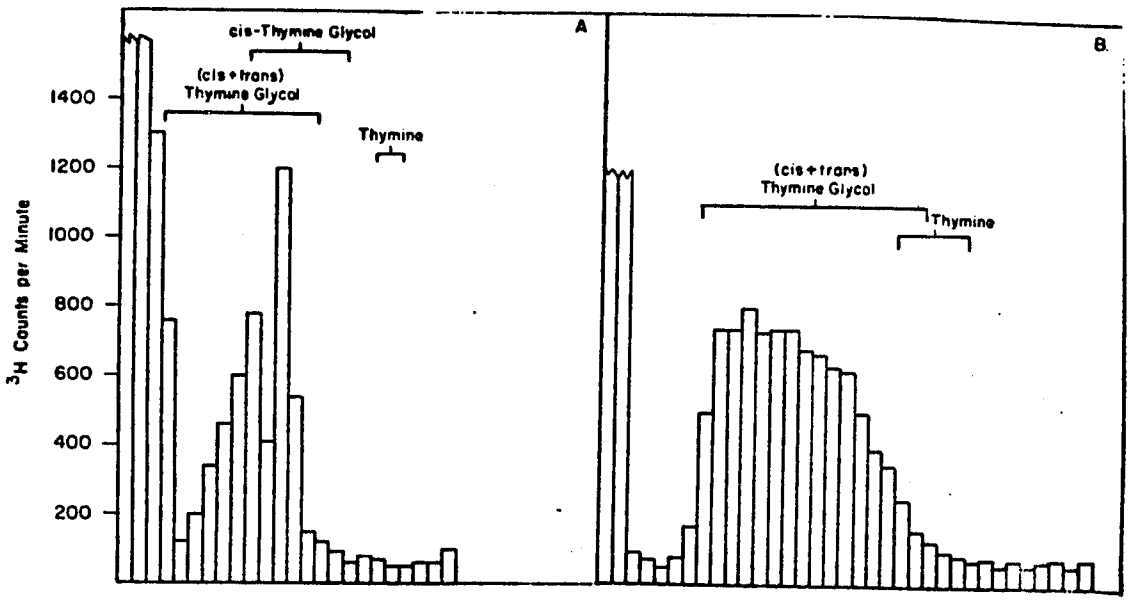

Figure 18. Analysis of products released after incubation of OsO_4-treated DNA with X-ray endonuclease.

^{3}H PM2 DNA (>500,000 cpm/ug DNA) was extensively treated with OsO_4 (see MATERIALS AND METHODS) and then incubated overnight with saturating quantities of enzyme. The trichloroacetic acid soluble fraction was taken to dryness and samples were spotted onto both:
(A) Polygram Cel 300/UV_{254}MN thin layer plates and developed in propanol/water (3:1). The developed plates were dried and cut into 1 cm squares and counted.
(B) Whatmann 3MM chromatography paper and developed in the descending mode with the upper phase of a mixture of ethyl acetate, n,propyl alcohol, H_2O (4:2:1). The developed chromatograms were cut into 1 inch squares and eluted with water. TMP, thymine glycol monophosphate and polynucleotides remained at the origin in both systems.

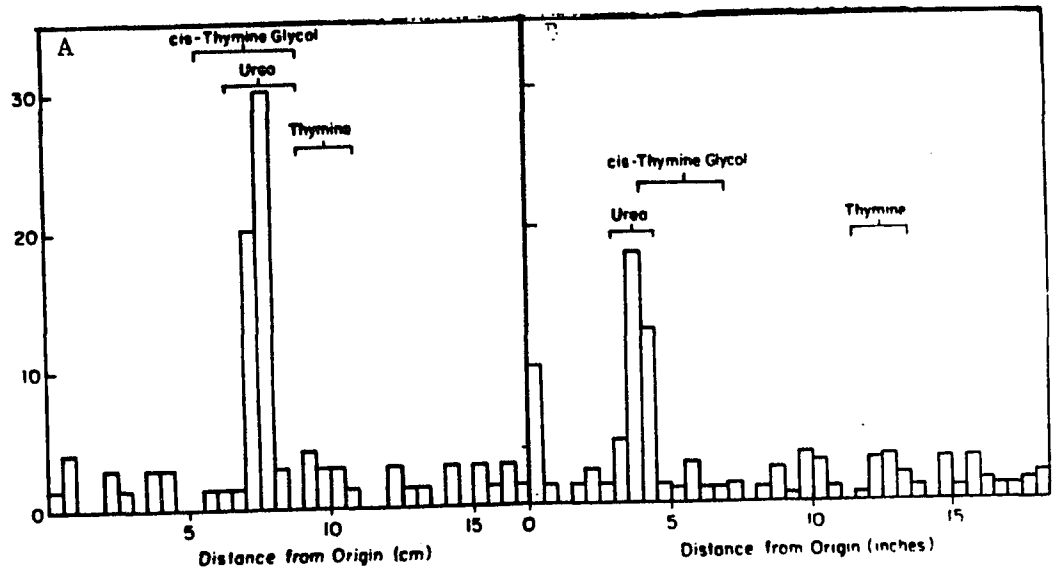

Figure 19. Analysis of products released after X-ray endonuclease digestion of OsO_4 treated-(2-^{14}C) poly dT:dA followed by alkaline hydrolysis.

OsO_4-treated-(2-^{14}C) poly dT:dA was incubated overnight at 37°C with X-ray endonuclease or without. The reactions were cooled in an ice bath and alcohol precipitated as in MATERIALS AND METHODS. The supernatants were spotted onto either: (A) Polygram Cel 300/UV_{254}MN thin layer plates, developed in propanol:water (3:1) and cut into 1 cm squares and counted; or, (B) Whatmann 3MM chromatography paper and developed with the upper phase of a mixture of ethyl acetate, n-propyl alcohol and water (4:1:2), cut into squares and counted. Authentic markers were run in parallel.

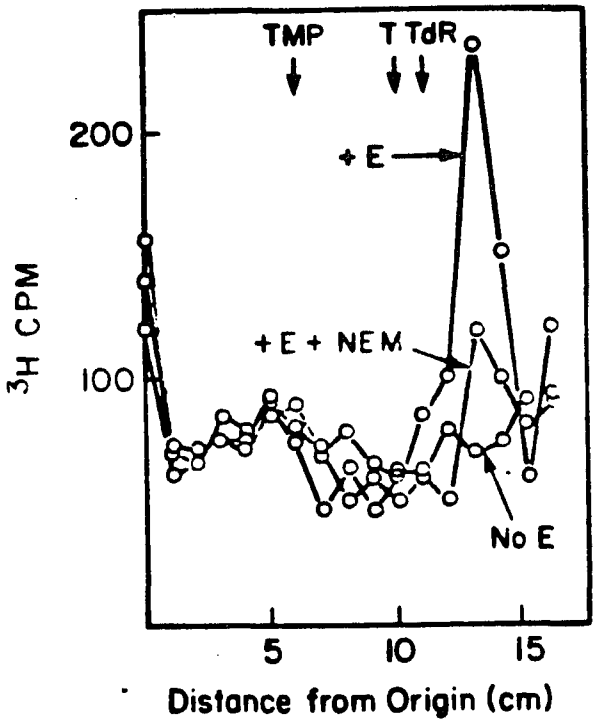

Figure 20. PEI-cellulose chromatography of acid-soluble products released by X-ray endonuclease digestion of X-irradiated DNA.

Heavily ^{3}H labeled PM2 DNA (>500,000 cpm/ug) was incubated overnight without enzyme, with saturating quantities of X-ray endonuclease or with saturating endonuclease in the presence of 10 mM NEM. The reactions were spotted onto PEI-cellulose thin layer chromatography plates and developed with water. Authentic standards were run in parallel lanes. After the water migrated ca. 9 cm the plates were dried, cut into 1 cm squares, each square was eluted with 1 ml of water overnight, mixed Liquiscint scintillation cocktail and counted.

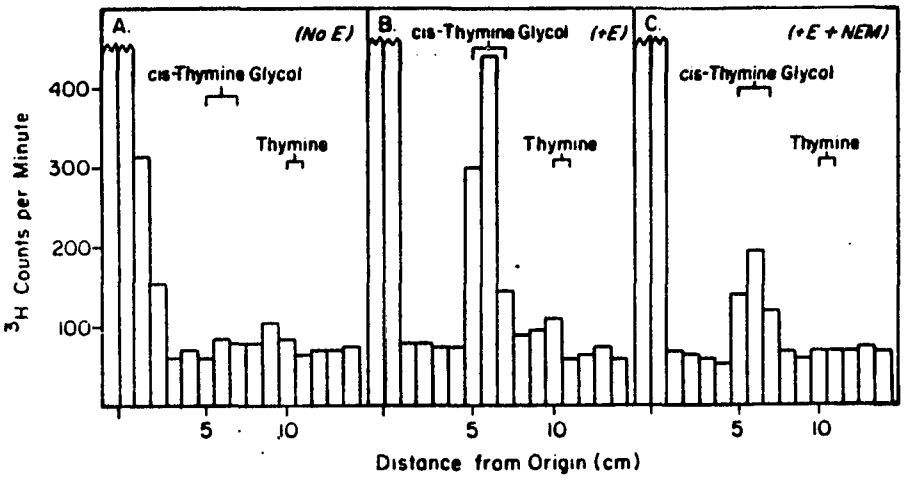

Figure 21. The effect of NEM on the thymine glycol-DNA glycosylase activity of the X-ray endonuclease.

Highly ^{3}H labeled (500,000 cpm/ug) heavily OsO_4-treated DNA (see MATERIALS AND METHODS) was incubated without enzyme (A), with saturating amounts of X-ray endonuclease (B), or with saturating X-ray endonuclease in the presence of 10 mM NEM (C). The reactions were spotted onto Polygram Cel 300/UV_{254}MN cellulose thin layer plates and developed with propanol:water (3:1). The developed plates were cut into 1 cm squares and counted.

Appendix BUFFERS AND MEDIA

I. Media for the growth of *E. coli*

A. Liquid Media

1. Modified Zubay's Broth

for each 1. add:		after autoclaving add.	
KH_2PO_4	5.6 g	25% glucose	40 ml
K_2HPO_4	28.9 g	10% casamino acids*	50 ml
yeast extract	10 g	thymine to	0.1%
0.1% thiamine	1.0 ml		

* modification for the growth of AB3027.

2. Hershey Broth

for each 1 add:		after autoclaving add:	
Nutrient broth	8 g	10% casamino acids	50 ml
Bacto-peptone	5 g	0.1% thiamine	0.2 ml
glucose	3 g		
NaCl	8 g		
Na citrate	2 g		
H_2O to	1 l		

B. Solid Media

1. Top Layer Agar.

for each liter add:		after autoclaving add:	
Bacto-tryptone	10 g	0.1% thiamine	0.2 ml
Bacto-agar	6 g		
NaCl	8 g		
Na citrate	2 g		

2. Bottom Layer Agar.

for each liter add:		after autoclaving add:	
Bacto-tryptone	13 g	0.1% thiamine	0.2 ml
Bacto-agar	12 g		
glucose	1.2 g		
NaCl	8 g		
Na citrate	2 g		

II. Media for the growth of Alteramonas espejiana

1. Bal Broth

for one liter add:		after autoclaving add:	
Nutrient broth	8 g	7.8% $MgSO_4.7H_2O$	100 ml
NaCl	26 g	15% $CaCl_2.2H_2O$	10 ml
KCl	0.7 g		

2. AMS-4

for each liter add:		after autoclaving add:	
NaCl	26 g	15% $CaCl_2.2H_2O$	10 ml
Tris base	12 g	0.24% KH_2PO_4	10 ml
Na_2SO_4	6.92 g	2.5% glucose	50 ml
NH_4Cl	1.1 g	0.1% thymidine	25 ml
$MgCl_2.6H_2O$	0.94 g		
KCl	0.7 g		
H_2O	850 ml		
HCl	to pH 7.3		

B. Solid Media

1. Bal Top Layer Agar

for each liter add:		after autoclaving add:	
Bacto-tryptone	8 g	7.8% $MgSO_4.7H_2O$	100 ml
Bacto-agar	5 g	15% $CaCl_2.2H_2O$	10 ml
KCl	0.7 g		
NaCl	26 g		
H_2O	890 ml		

2. Bal Bottom Layer Agar

for each liter add:		after autoclaving add:	
Nutrient Broth	9 g	7.8% $MgSO_4.7H_2O$	100 ml
Bacto Agar	10 g	15% $CaCl_2.2H_2O$	10 ml
KCl	0.7 g		
NaCl	26 g		
H_2O	890 ml		

III. Buffers used in chromatography

1. Buffer A - for each liter add:

1 M Tris buffer, pH 8.0	10 ml
200 mM EDTA	5 ml
glycerol (anhydrous)	100 ml
2-mercaptoethanol	0.07 ml
KCl	to desired concentration
H_2O	to 1 l

2. Buffer B - for each liter add:

1 M KPO_4 buffer pH 8.0	10 ml
200 mM EDTA	0.5 ml
KCl	to desired concentration
2-mercaptoethanol	0.07 ml

3. Buffer C - for each 100 mls add:

1 M KPO_4 buffer pH 6.5	1.0 ml
glycerol (anhydrous)	10 ml
200 mM EDTA	0.5 ml
2-mercaptoethanol	7 ul
KCl	to desired concentration
H_2O	to 100 ml

IV. Buffers used in electrophoresis

1. 20 x TEB buffer

Tris base	108 g
boric acid	55.65 g
EDTA (disodium)	4.65 g

2. Phosphate running buffer

200 mM NaH_2PO_4	16 ml
200 mM Na_2HPO_4	84 ml
H_2O	900 ml

3. TGS Buffer

Tris base	2.1 g
glycine	10.08 g
10% SDS	693 ml