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I am submitting herewith a thesis written by Nadine E. Lee entitled "In Vitro Repair of Ultraviolet-Irradiated Deoxyribonucleic Acid: Characterization of Incision Using the Partially-Purified UvrA, UvrB, and UvrC Gene Products of Escherichia coli." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Bio-medical Sciences.

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IN VITRO REPAIR OF ULTRAVIOLET-IRRADIATED DEOXYRIBONUCLEIC ACID:
CHARACTERIZATION OF INCISION USING THE PARTIALLY-PURIFIED
UvrA, UvrB, AND UvrC GENE PRODUCTS OF ESCHERICHIA COLI

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ABSTRACT

Partially-purified UvrA, UvrB, and UvrC (UvrABC) gene products, prepared by column chromatography of extracts made from sucrose-plasmolyzed Escherichia coli, were used to help characterize in vitro the ability of E. coli to incise ultraviolet-irradiated DNA. The results of these experiments indicated that incision of irradiated PM2 DNA occurred with a 102 (20-40%) efficiency, but that the ability for incision to occur at every dimer was probably not limited by the amount of the gene products, DNA, ATP, Mg^{+} , or KCl added to the reactions nor by the number of dimers in the reactions. Other experiments supported that the efficiency was not enhanced by the addition of the partially-purified UvrD gene product but was enhanced by incubation of the UvrABC gene products prior to the addition of the DNA. The amount of incision was dependent upon the time the reactions were incubated and linearly dependent upon the UV dose applied to the DNA. The data are consistent with incision occurring at a constant proportion of the damage in these reactions and the UvrABC gene products acting by a processive mechanism. The complete in vitro repair of irradiated bacteriophage T7 DNA using enzymes (the partially-purified UvrABC gene products and a crude extract) originating solely from its host was also investigated since this should be instructive relative to repair in E. coli in vitro. Repair was monitored with both physicochemical (restoration of mode molecular weight) and biological (enhanced ability of repaired DNA to form viable phage when

encapsulated in vitro into phage heads) endpoints. The results of these experiments showed no significant difference in biological activity between DNA repaired in vitro or not repaired although incision occurred at as much as 70% of the dimers and although the molecular weight of the DNA was 90% restored in the reactions incubated with the UvrABC proteins and an extract from sucrose-plasmolyzed E. coli. These data support that the completeness of the in vitro repair of bacteriophage T7 DNA in these reactions may be limited in some step other than incision.

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CHAPTER I

INTRODUCTION

A. Necessity for Repair Processes

The preservation of the life and well-being of an individual organism and the continuity of its species depend upon the damage-free maintenance of its deoxyribonucleic acid (DNA). Deleterious alterations can arise in the DNA as a consequence of factors inherent to the internal environment and activity of this hereditary material (e.g., depurination or deamination and the misinsertion or mismatching of bases in replication or recombination). Damage can also occur because of any of a multitude of exogenous insults--including monofunctional alkylating or DNA cross-linking chemicals and ionizing or ultraviolet irradiation. In order for the organism to efficiently cope with this damage, it is imperative that enzymatic repair mechanisms exist (for general reviews, see Hanawalt et al., 1979; Lehmann and Karran, 1981; Hanawalt et al., 1983; Lindahl, 1982).

B. DNA Damage by Ultraviolet Light

The majority of the research directed toward the study of DNA damage (and repair) has concerned the effects of ultraviolet (UV) light on DNA. The principle reason for this is that, although UV-irradiation causes the formation of a variety of photoproducts in the DNA, one is formed predominantly--the cyclobutane pyrimidine

dimer. These dimers can be classified into types dependent upon whether the covalent intrastrand linkage is formed between two adjacent thymines ($\hat{T}\hat{T}$), cytosines ($\hat{C}\hat{C}$), or between one of each ($\hat{C}\hat{T}$ or $\hat{T}\hat{C}$) (Setlow and Carrier, 1966; Carrier and Setlow, 1971; Patrick and Rahn, 1976; Hanawalt et al., 1979; Haseltine et al., 1982). UV-light both forms and directly photoreverses pyrimidine dimers. The relative rates of these two opposing reactions determine the number and distribution of the types of dimers in any particular DNA molecule and are dependent upon the wavelength and fluence of the UV light, the dose of UV that actually reaches the DNA, and the reaction conditions for the irradiation (temperature, pH, etc.) (Setlow and Carrier, 1966; Unrau et al., 1973; Patrick and Rahn, 1976; Haseltine et al., 1980; Haseltine et al., 1982). Under defined conditions for irradiation, both the exposure to and number of stable photoproducts resulting from the UV-irradiation of DNA can be accurately quantitated (Setlow and Carrier, 1966; Ganesan, 1973; Unrau et al., 1973; Wilkins, 1973; Patrick and Rahn, 1976; Haseltine et al., 1980; Haseltine et al., 1982). For E. coli and bacteriophage T7 DNA irradiated with low doses of 254 nm light, about 55 and 0.46 dimers per J/m^2 are formed per genome, respectively (Friedberg and Clayton, 1972; Setlow and Setlow, 1972; Unrau et al., 1973; Patrick and Rahn, 1976; Masker, 1977).

The considerable evidence that has accumulated to implicate pyrimidine dimers as the primary initiators of most of the biological consequences of UV-irradiation includes that this lesion is frequently formed, is quite stable, and (by distorting the DNA helix) has been shown to temporarily stop replication and interrupt transcription

(Setlow et al., 1963; Setlow and Carrier, 1966; Patrick and Rahn, 1976; Haseltine et al., 1982). Other support includes the observation that many organisms produce enzymes for specifically resolving these cyclobutane dimers back to the original bases and that reversal of the damage (directly by UV light or with these photolyases) can result in the restoration of approximately 90% of the biological activity to UV-damaged DNA (Setlow and Setlow, 1972; Sutherland, 1978). The strongest evidence is that some mutants which are very sensitive to killing by UV light are defective in the ability to remove pyrimidine dimers from the DNA and that extremely sensitive (uvrA recA) mutants of Escherichia coli have an average of one lethal lesion per genome at a dose of UV light corresponding to that at which only one pyrimidine dimer per genome is present (Howard-Flanders, 1968; Jagger, 1976).

There has been doubt that pyrimidine dimers are the exclusive cause of the biological effects of UV-light--the suspect lesion being the second most prevalent photoproduct--the pyrimidine (6-4') cytosine lesion (Setlow and Setlow, 1972; Jagger, 1976; Patrick and Rahn, 1976; Witkin, 1976; Hanawalt, 1977; Haseltine et al., 1980). This photoproduct is the precursor to the 6-4'-(pyrimidine-2'-one)-pyrimidine lesion which has been quantitated in acid-hydrolysates to be formed approximately 28% as frequently as the cyclobutane thymine-thymine dimer (or equally frequently as the $\hat{C}T$ or $\hat{T}C$ dimer) in E. coli DNA irradiated with 4000 J/m^2 of 254 nm light (Setlow and Setlow, 1972; Patrick and Rahn, 1976; Haseltine et al., 1980; Haseltine et al.,

1982). Recently Haseltine and his coresearchers showed that a better correlation exists between the formation of the pyrimidine (6-4') cytosine lesion and the amber, ochre, and UGA forward mutational frequencies in the specific hot and warm spots of the lacI sequence of E. coli than between pyrimidine dimers and these mutations (Miller and Coulondre, 1977; Coulondre et al., 1978; Lippke et al., 1981; Brash and Haseltine, 1982; Haseltine et al., 1982).

In addition to providing evidence that many of the biological effects of UV-light are due to pyrimidine dimers, the discovery of a number of mutants very sensitive to ultraviolet light in organisms ranging from viruses to humans has provided the primary impetus for the study of the repair of UV-damaged DNA. This is because the basic assumption for most attempts to understand the biochemical mechanisms of repair is that mutants very sensitive to a particular damaging agent lack at least one of the enzymes involved in the repair of that damage. Also because most of these mutants are also very sensitive to DNA-damaging agents producing other types of bulky lesions, the pyrimidine dimer is considered the prototype lesion for the repair of damage which distorts the DNA helix (Hanawalt, 1977; Hanawalt et al., 1979; Bernstein, 1981). Another reason ultraviolet irradiation has been chosen as a primary agent for the study of DNA damage and repair is that exposure to the ultraviolet wavelengths in sunlight is basically ubiquitous. For humans, the cytotoxic effects of UV-light are experienced as sunburn and the mutagenic and carcinogenic effects as skin lesions and cancers. Humans having the rare genetic disease xeroderma pigmentosum (XP) are very sensitive to UV-light, unable to

normally repair UV-induced damage to the DNA, and also have an extremely high incidence of skin cancers (Cleaver and Bootsma, 1975; Hanawalt, 1977; Arlett and Lehman, 1978).

C. DNA Repair of Damage from Ultraviolet Light

There are several methods by which most cells can cope with the pyrimidine dimers resulting from ultraviolet irradiation--directly reversing, removing, or tolerating them.

1. Photoenzymatic Reactivation

Photoenzymatic reactivation is a rapid rather simple method which directly resolves the pyrimidine dimer back to the original bases. This method requires a single enzyme (photolyase or photo-reactivating enzyme) and visible light in the 310-400 nm range. Photoenzymatic reactivation is specific for cyclobutane pyrimidine dimers and involves breakage of the cyclobutane linkage only (Rupert, 1975; Witkin, 1976; Sutherland, 1978; Lindahl, 1982).

2. Excision Repair

Excision repair is the only method to directly remove the damage from the DNA in the dark. This method involves several enzymatic activities and is usually divided into four steps--incision, excision, repair resynthesis, and ligation (see Figure I-1) (Setlow and Carrier, 1964; Hanawalt et al., 1979).

Incision (Figure I-1a). A damage specific endonuclease locates the lesion in the DNA and usually provides a single strand

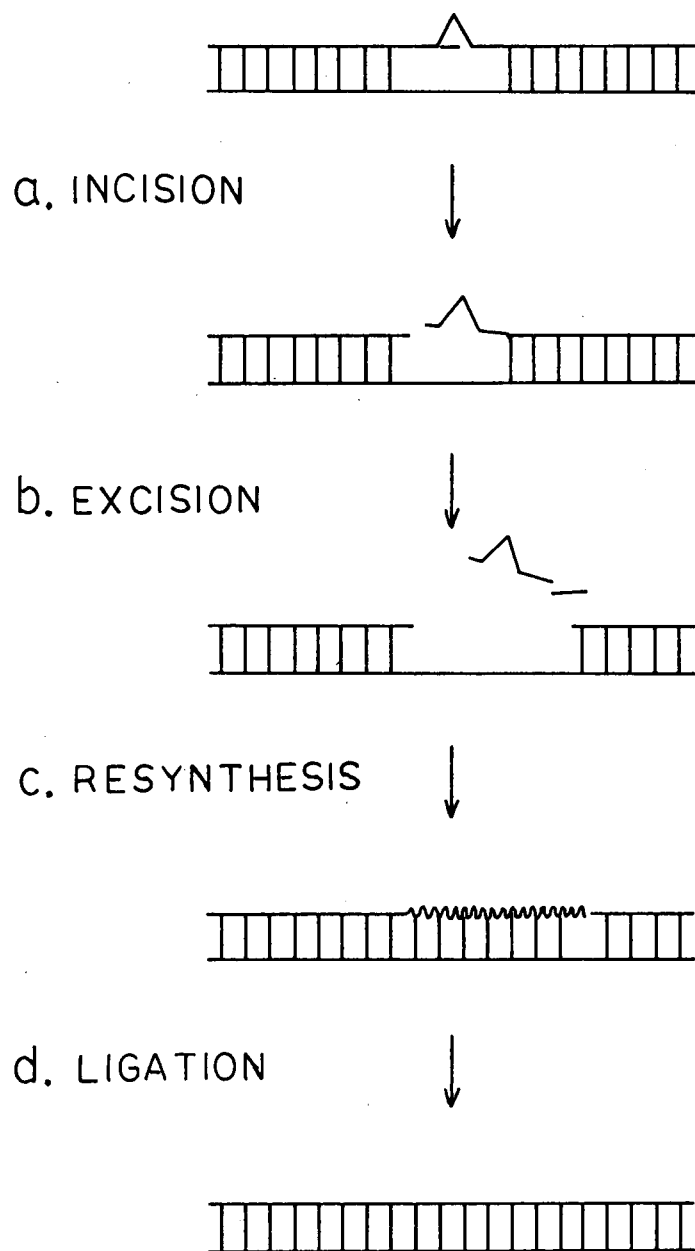


Figure I-1. DNA excision repair of pyrimidine dimers.

 = pyrimidine dimer,  = new patch of DNA.

break in the phosphodiester backbone at or near the pyrimidine dimer. In the cases of endonuclease V from bacteriophage T4-infected E. coli and of Micrococcus luteus, it has recently been shown that the incision occurs by means of a glycosylase/AP endonuclease. A break is, thus, made in the N-glycosylidic bond between the 5' base of the dimer and the sugar phosphate backbone of the DNA yielding an apurinic/apyrimidinic (AP) site. The phosphodiester backbone is then cleaved between the AP site and the 3' nucleotide of the dimer by an AP endonuclease. For both bacteriophage T4 and M. luteus, the glycosylase and AP endonucleolytic activities are located on the same molecule (Demple and Linn, 1980; Gordon and Haseltine, 1980; Haseltine et al., 1980; Radany and Friedberg, 1980; Warner et al., 1981; Nakabeppu and Sekiguichi, 1981; Lindahl, 1982). In other cases, it is probable that the incision is not a result of glycosyltic and AP endonucleolytic activity but is rather due to direct endonucleolytic cleavage at the site of the dimer--perhaps by a complex of enzymes. In E. coli, the gene products of the uvrA, uvrB, and uvrC genes are necessary for incision (Seeberg et al., 1976; Seeberg and Strike, 1976; Hanawalt et al., 1979; Demple and Linn, 1980; Seeberg and Steinum, 1982). In humans, the suggestion that a complex of enzymes acts in incision is based on the number of complementation groups of xeroderma pigmentosum cells deficient in incision (Cleaver and Bootsma, 1975; Tahaka et al., 1975; Arlett and Lehman, 1978; Hanawalt et al., 1979; Lindahl, 1979; Seeberg, 1981a).

Excision (Figure I-1b). An exonuclease next displaces the damaged strand and probably provides a second break in the phosphodiester backbone. The dimer is released as part of an acid soluble oligonucleotide several basepairs long (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964; Carrier and Setlow, 1966). In vitro, at least three exonucleases of E. coli can remove dimers from UV-irradiated DNA. These include the 5' → 3' exonuclease activities associated with polymerases I and III and exonuclease VII (single-strand specific). Exonuclease V (the RecB,C enzyme) may also be used in excision. There is evidence that concurrent excision and repair resynthesis is more efficient than if these two steps happen successively (Chase et al., 1979; Hanawalt et al., 1979; Lindahl, 1979; Cooper and Hunt, 1981; Hanawalt et al., 1982). Recent results obtained by Rupp and his colleagues (1982) suggest that the UvrA, UvrB, and UvrC gene products (of E. coli) perform concurrent incision and excision by cutting the DNA on both sides of the damage (discussed further in section D).

Resynthesis (Figure I-1c). Any of the three polymerases in E. coli can resynthesize the excised strand by using the intact strand as a template. Polymerase I is currently believed to be the primary one involved in normal (wild-type) repair resynthesis of nicked DNA because it is the only DNA polymerase that can bind at a nick, it is present in the most copies per cell, and polA mutants are UV-sensitive. E. coli polymerases II and III probably require local degradation of the incised region in order to increase the nick to a gap

(Masker et al., 1973; Hanawalt, 1977; Hanawalt et al., 1979). The type of polymerase used probably affects the length of the resynthesis patch. In wildtype E. coli cells, the majority of the patches are 12-30 nucleotides long; the rest (less than 10%) are several hundred basepairs long. Mutants of polymerase I, the uvrD locus, or the recA gene appear to have a greater proportion of long repair patches and to require protein synthesis after irradiation (Cooper and Hanawalt, 1972; Rothman, 1978; Hanawalt et al., 1979; Kuemmerle et al., 1982).

Ligation (Figure I-1d). The continuity of the DNA over the repaired region is restored by DNA polynucleotide ligase (Youngs and Smith, 1977).

3. Postreplicational Repair

The completion of excision repair requires a complementary strand for use as a template for resynthesis. The presence of a dimer at the replication fork is postulated to cause the polymerase to stop at the site of the dimer and resume synthesizing further along the DNA, leaving a gap opposite the dimer. This means that neither strand is then capable of coding for the repair of the other. Postreplicational repair is, therefore, not actually a method to repair DNA but rather a way the cell can cope with the damage if it is not repaired prior to replication. This mechanism, first described by Rupp and Howard-Flanders in 1968, redistributes the damage and provides repairable strands by recombination between the undamaged parental strand and the gapped daughter strand. One of the features

inherent to this model of how the cells cope with the damage is that the dimers can be transferred to daughter strands by the resolution of the Holliday structure (strand isomerization), thus diluting the amount of damage that the daughter cells must eventually repair (Rupp and Howard-Flanders, 1968; Ganesan, 1974; Hanawalt, 1977; Hanawalt et al., 1979; Dodson and Hadden, 1980).

4. SOS (Inducible) Repair

Weigle (1953) exposed E. coli cells to a low dose of ultra-violet light and then infected them with UV-irradiated bacteriophage lambda. He found that there was an increased survival of the phage as well as an increased mutation frequency in the repaired phage--phenomena which became known as Weigle or UV reactivation. Since that time, other damage which causes the unscheduled stopping of DNA replication has been shown to cause an increased mutational frequency (and survival) after DNA damage in bacteria or phage. It has also been shown that the reactivation requires protein synthesis and that other altered cellular processes (including induction of prophages, cellular filamentation, and increased DNA degradation) result as well as the increased mutagenesis (Defais, 1976; Witkin, 1976; Gottesman, 1981; Little and Mount, 1982). Radman (1975) called this cluster of responses of E. coli to damage the "SOS response" and hypothesized that they were linked to a repair pathway which was error-prone (hence, the other names--SOS repair, error-prone repair, and inducible repair). The control of the SOS repair system is complex and depends upon at least two proteins--the RecA protein (a protease to

cleave the repressors) and the LexA protein (which regulates the synthesis of the RecA protein). A model has been proposed to account for the SOS response and depends upon the increased synthesis and activation of the RecA protein to a proteolytically active form resulting in derepression of the operons controlling the SOS response (Radman, 1976; Witkin, 1976; Little and Mount, 1982). The increased mutational frequency is proposed to be a consequence of the decreased fidelity of DNA synthesis which can result from an alteration in the DNA polymerase activity (because of a relaxed 3' → 5' editing function, a reversal (insertion of nucleotides) of the exonuclease activity of the polymerase, or of an altered error-prone form of DNA polymerase) (Villani et al., 1978; Schroeder, 1979; Lehmann and Karran, 1981; Little and Mount, 1982). Kenyon and Walker have identified a series of loci which appear to be under the recA⁺, lexA⁺ regulatory control and are damage-inducible (selectively derepressed after DNA damage). They have found the uvrA, uvrB, and umuC genes to be among these (Kenyon and Walker, 1980; Fogliano and Schendel, 1981; Kenyon and Walker, 1981; van den Berg et al., 1981).

D. The uvrA, uvrB and uvrC Gene Products

1. Discovery and Characterization of the Mutants

By 1966, three loci controlling UV sensitivity--named the uvrA, uvrB, and uvrC genes--had been located in widely dispersed positions in the chromosome of Escherichia coli (map positions at 91, 17, and 42 minutes, respectively). Mutants for any of these genes are very sensitive to ultraviolet irradiation, unable to perform host

cell reactivation of irradiated bacteriophages, and unable to excise pyrimidine dimers (Boyce and Hoard-Flanders, 1964; Setlow and Carrier, 1964; van de Putte et al., 1965; Howard-Flanders et al., 1966, Bachman et al., 1976; Hanawalt et al., 1979).

The uvrA and uvrB mutants are unable to incise damaged DNA (Rupp and Howard-Flanders, 1968; Shimada et al., 1968; Seeberg and Johansen, 1973). For mutations in either of these loci, the UV dose at which 37% of the cells survive ($1/e$ = the dose at which an average of one lethal lesion per E. coli cell is present assuming a Poisson distribution of the damage) is about 0.8 J/m^2 --corresponding to approximately 50 dimers per genome (254 nm light). At this level of survival, wild-type bacteria and recA mutants can cope with 3000 and 20 dimers per genome, respectively. The fact that recA uvrA mutants can survive with only an average of one dimer per genome is strong evidence that the only two pathways of dark-phase repair are the uvrA⁺-dependent excision repair and the recA⁺-dependent postreplicational repair (Howard-Flanders, 1968; Jagger, 1976; Hanawalt, 1977).

The role of the uvrC is very poorly understood. Mutants in the uvrC locus are excision defective; however, the actual deficiency in cells in vivo or quasi in vitro systems has been attributed to two temporally distinguishable phenomena. Some reports claim that the uvrC mutants are defective also in the incision step such that incision is absent or abnormal in these mutants (Shimada et al., 1968; Taketo et al., 1972; Seeberg and Strike, 1976; Seeberg et al., 1980). Others claims that the defect is in a post-incision, pre-excision step and that the UvrC gene product acts to coordinate a multienzymatic

complex of incision and excision activities (Seeberg and Johansen, 1973), to modify the termini formed by incision for subsequent steps (Kato, 1972), or to prevent abortive ligation (Seeberg and Rupp, 1975; Sharma and Moses, 1979). In addition to the fact that some investigators report incision in uvrC mutants in vivo and others do not, the issue is further confused by a discrepancy between the results obtained in vivo and in vitro by the same investigators. Seeberg and his coresearchers have shown that in vivo uvrC mutants do incise UV-irradiated DNA, but that the incision is not normal--being slower and having a UV-dose dependence for the number of dimers incised that terminates at a much lower dose than for wild-type cells (20 J/m^2 vs 60 J/m^2) (Seeberg and Johansen, 1973; Seeberg et al., 1980). With crude extracts of E. coli in vitro, however, they have shown that incision will not occur without the UvrC gene product (Seeberg, 1978; Seeberg et al., 1980; Seeberg, 1981a). Two theories have been proposed to explain these differences. The first is that the uvrC mutants produce an altered form of the UvrC gene product which is induced along with other SOS responses and which will permit abnormal incision to occur (Seeberg et al., 1980). In general, the results of the experiments investigating the role of the uvrC gene product listed above do not conflict with their theory. The second is that the uvrC gene is essential; all mutants must, therefore, have a residual activity which may be detectable only under certain conditions (Sancar et al., 1981c). Nevertheless, the results obtained, particularly by Seeberg, Rupp, and Strike (1980), support that the UvrC gene product does participate in the incision step of

repair and acts to do more than just to prevent abortive ligation.

A mutant in the uvrD gene differs from one in the uvrA, uvrB, or uvrC genes in that uvrD mutants are less sensitive to ultraviolet light and can perform normal incision in irradiated DNA. In addition to being unable to excise dimers, the pleotropic phenotypes of mutants in this cistron (including uvrD, uvrE, recL, and mutU) encompass deficiencies in postreplication repair, genetic recombination, and host cell reactivation and an increased spontaneous mutational frequency (Howard-Flanders, 1968; Shimada et al., 1968; Taketo et al., 1972; Kuemmerle and Masker, 1980; Kushner et al., 1981; Kuemmerle et al., 1982; Oeda et al., 1982).

2. Other Damage

Besides ultraviolet light, the uvrA, uvrB, and uvrC mutants are very sensitive to damage by bulky lesions (such as those formed by 4-nitroquinoline-1-oxide and 7-bromo-methylbenz(a)anthracene) and interstrand cross-links (such as those formed by psoralen plus light, mitomycin C, nitrogen mustard, nitrofurans, and other bifunctional alkylating agents) (Hanawalt and Haynes, 1965; Ikenaga et al., 1975; Cole et al., 1976; Yoakum and Cole, 1977). The ability of the uvrA⁺, uvrB⁺, uvrC⁺-dependent excision repair pathway to repair so many types of damage probably means that the damage itself is not recognized, but rather that a distortion in the DNA helix is identified by one or more of these gene products and distinguished as damage (Hanawalt and Haynes, 1965; Howard-Flanders et al., 1966; Lindahl, 1979).

3. Isolation of the Gene Products

Until recently it was believed that the enzymes responsible for incision at pyrimidine dimers in other organisms would have very similar properties to those coded for by M. luteus or bacteriophage T4 (Gordon and Haseltine, 1980; Seeberg, 1981a). This belief received considerable support from a paper by Braun and Grossman (1974) in which they described the partial purification of an approximately 14,000 dalton endonuclease from E. coli that appeared to incise specifically at pyrimidine dimers, was absent in uvrA and uvrB mutants (but present in normal amounts in uvrC mutants), and required no cofactors. They called this enzyme correndonuclease II or the uvrAB endonuclease. Subsequent work primarily by Seeberg has shown that the uvrA, uvrB, and uvrC (UvrABC)-dependent endonuclease is instead a much more complex enzymatic activity than this correndonuclease II, is composed of large molecular weight components, requires ATP and Mg^{++} , and requires the functional gene product of the uvrC as well as the uvrA and uvrB genes. It is now known that the enzymes from M. luteus and bacteriophage T4 which incise UV-irradiated DNA actually are glycosylase/AP endonucleases, are specific for pyrimidine dimers, and are coded for by single genes. These properties contrast with what appears to be true in organisms such as yeast and humans and could indicate that the UvrABC endonuclease of E. coli may be more representative of repair in yeast and humans (Seeberg et al., 1976; Seeberg and Strike, 1976; Seeberg, 1978; Seeberg, 1981a; Lindahl, 1982).

It is advantageous to the investigation of the biochemical mechanisms by which a particular reaction occurs to be able to isolate the enzymes and study the reactions in vitro. The difficulty in studying the reaction of a multienzyme complex in vitro is that the components of this complex are very likely to be separated in the purification procedures. In order to isolate the individual subunits, therefore, one must know exactly how each component benefits the entire system or know some distinguishing property about it. This is seldom feasible. Seeberg used an assay system for in vitro complementation in order to isolate and begin to characterize the products of the uvrA, uvrB, and uvrC genes. One of the primary factors contributing to his success was the use of sucrose plasmolysis--a very gentle way to prepare extracts containing labile proteins (other methods to prepare extracts of E. coli do not preserve the endonucleolytic activity) (Wickner and Hurwitz, 1972; Seeberg et al., 1976; Masker, 1977). In 1976, Seeberg, Nissen-Meyer, and Strike showed that extracts from uvrA, uvrB, or uvrC mutants were individually not able to incise UV-damaged DNA in vitro but the combination of any two of these extracts would complement the deficiencies so that incision occurred. Furthermore, they showed that the identification of the presence of one of these Uvr⁺ gene products in the fractions from column chromatography can be facilitated by assaying for the restoration of the endonucleolytic activity by complementation of an extract from a mutant defective in that gene (Seeberg et al., 1976; Seeberg, 1978a).

Using this in vitro complementation technique, Seeberg and his coworkers have partially purified the UvrA, UvrB, and UvrC gene products by ion-exchange chromatography. They have shown that the UvrA gene product has an affinity for cellulose phosphate and that the UvrB and UvrC gene products have an affinity for diethylaminoethyl cellulose (DEAE). The UvrB and UvrC gene products co-chromatograph--at least at early stages (DEAE cellulose) of their purification--and may exist as a complex (Seeberg, 1978a, 1981a,b). It has also been shown that none of the gene products incise DNA alone, but the combination of all three results in incision of irradiated DNA by a mechanism which appears distinct from that of a glycosylase (Seeberg, 1978a; Demple and Linn, 1980). That the target lesion is the pyrimidine dimer was determined by the observation that photo-enzymatic reversal resulted in the loss of sites in the irradiated DNA which are sensitive to the UvrABC gene products. The isolated gene products also have a broad substrate specificity as reported for the UvrABC endonuclease in vivo (Seeberg, 1981a,b).

Despite the advances in the study of the activity and in the partial purification of the UvrABC gene products which have been possible using the in vitro complementation system, the low copy number of the gene products in the cell and their lability in vitro have greatly complicated the attempts to complete purification of all the individual gene products (Lindahl, 1982). All three genes have been cloned now onto multicopy plasmids or bacteriophage vectors. Using a second ingenious method--the maxicell technique of Sancar, Rupp and coworkers (which does not require a functional assay)--rapid

chromatographic procedures to purify the UvrABC gene products to radiochemical homogeneity have been developed (Sancar et al., 1979; Kacinski et al., 1981; Sancar et al., 1981a,b,c; Yoakum and Grossman, 1981). Rupp and his coresearchers (1982) have recently purified all three gene products in biologically active forms. Using sequencing gels they have investigated the location of the incision by the UvrABC endonuclease. They have preliminarily reported that the UvrABC gene products incise DNA approximately seven nucleotides 5' to the damage and approximately three nucleotides 3' to the damage. This indicates that the UvrABC gene products excise twelve nucleotides (and the damage) as part of their unique "incisive" activity.

4. Properties of the Purified Proteins

The best characterized of the three components of the UvrABC endonuclease is the UvrA protein--a 114,000 dalton, monomeric protein that is normally present in E. coli at 20 (Sancar et al., 1981a) to 100 (Seeberg and Steinum, 1982) copies per cell. The UvrA gene product is an ATPase and a DNA binding-protein which binds weakly to non-irradiated double-stranded DNA and strongly to single-stranded or irradiated double-stranded DNA (Seeberg, 1978b; Seeberg, 1981a; Seeberg and Steinum, 1982). ADP abolishes binding to the DNA. GTP or ATP [γ S] (adenosine 5'-[γ -thio] triphosphate--a nonhydrolyzable analog of ATP) prevents both specific-binding to single-stranded or irradiated double-stranded DNA and incision (Seeberg and Strike, 1976; Kacinski et al., 1981; Seeberg, 1981a; Seeberg and Steinum, 1982). Seeberg and Steinum have proposed that the ATPase activity

of this gene product may be the cause of the requirements for Mg^{++} and the hydrolysis of ATP (to ADP + P_i) in incision by the UvrABC endonuclease. They have further postulated that the UvrA gene product is the damage recognition component of the endonuclease--recognizing regions in the DNA in which the base pairing has been impaired rather than the damage itself. These hypotheses suggest that the hydrolysis of ATP may allow changes in the strength of DNA binding. This would allow the UvrA proteins to move to different regions in the DNA in order to locate damage (Kacinski et al., 1981; Seeberg, 1981a; Seeberg and Steinum, 1982). The ATPase of the UvrA protein is, however, not DNA-dependent, as evidenced by the fact that in the absence of DNA, the UvrA protein still utilizes approximately 17 molecules of ATP per minute at 37°C (Seeberg and Steinum, 1982). A significant advance in the ability to isolate the UvrA protein was recently accomplished by the linkage of the transcription of the uvrA gene to the lambda phage promoter P_L on plasmid pKC30. This permits amplification of the UvrA gene product to approximately 7% of the total cellular protein (Yoakum et al., 1982).

The UvrB gene product is an 84,000 dalton protein which is present per cell at about seven times the amount of the UvrA protein (140 copies per cell; Sancar et al., 1981b). It has no DNA binding or ATPase activity by itself, but binds to single-stranded DNA in the presence of the UvrA gene product (Kacinski et al., 1981; Seeberg, 1981). Only the carboxyl end of this protein appears to be necessary for the repair activity (Pannekoek et al., 1980). It has been postulated that this protein may be involved in other DNA metabolic

activities besides excision repair because the polA uvrB double mutant is inviable (polA uvrA shows a decreased viability), and because the UvrB gene product is present in a larger copy number than the UvrA or UvrC gene products (Shizuya and Dykhuizen, 1972; Morimyo and Shimagu, 1976; Sancar et al., 1981b; Lindahl, 1982).

The UvrC gene product is a 70,000 dalton protein present in as few as 10 copies per cell. It has been partially-purified by the maxicell method but is extremely labile in vitro. It binds to single-stranded DNA and, therefore, evidence suggests the UvrC protein can be purified quickly by denatured DNA cellulose chromatography (Kacinski et al., 1981; Sancar et al., 1981c; Yoakum and Grossman, 1981).

The radiolabelled gene product from a cloned UvrD gene has been partially-purified (using gel electrophoresis to monitor the purification) by Oeda et al. (1982). They found this protein to be a 75,000 dalton DNA-dependent ATPase and postulated that it might be identical with DNA helicase II (also DNA-dependent ATPase I).

5. Expression

The amount of the UvrA, UvrB, and UvrC gene products expressed constitutively appears to be sufficient for a cell to cope with the levels of damage it might normally experience. It was, therefore, surprising when it was discovered that the uvrA gene was under the regulatory control of the recA, lexA system and induced to four times normal level a part of the SOS response (Witkin, 1976; Kenyon and Walker, 1980; Kacinski et al., 1981; Kenyon and Walker, 1981; Lindahl, 1982; Sancar et al., 1982a; Seeberg and Steinum, 1982). The uvrA,

recA, and lexA genes have similar operators which are believed to be regulated by the LexA protein (Sancar et al., 1982a). The regulation of the uvrB gene is more complex because the uvrB gene contains LexA-dependent and LexA-independent promoters (Sancar et al., 1982b) and because only some types of damage induce the uvrB gene (UV and nalidixic acid, but not mitomycin C) (Fogliano and Schendel, 1981; Kenyon and Walker, 1981). In the absence of recA⁺, lexA⁺ controls, the transcription of the uvrB gene is postulated to be controlled by the uvrC gene (van den Berg et al., 1981).

E. DNA Repair in Vitro

The ultimate goal for purifying the UvrA, UvrB, and UvrC gene products is to better understand the mechanisms of repair. The work done previously with the partially-purified gene products concerned only the incision step of repair (Seeberg, 1978; Seeberg et al., 1980; Seeberg, 1981a,b; Seeberg and Steinum, 1982). Complete repair dependent upon the UvrABC endonuclease has been studied in a quasi in vitro situation with cells permeabilized with toluene (Masker et al., 1973; Waldstein et al., 1974; Sharon et al., 1975; Masker, 1976; Strike, 1977; Yoakum and Cole, 1977; Sharma and Moses, 1979; Seeberg et al., 1980) and by sucrose plasmolysis (Seeberg and Strike, 1976; Seeberg et al., 1980). This method is well suited for the investigation of the requirements in repair for various cofactors and small molecular weight proteins. By this technique the requirements for ATP and Mg⁺⁺ in the incision step, for all four dNTP's in the repair resynthesis step, and for NAD⁺ in the ligation step were first

discovered (Waldstein et al., 1974; Sharon et al., 1975; Masker, 1976; Seeberg and Strike, 1976; Strike, 1977; Yoakum and Cole, 1977). The quasi in vitro studies are, however, less defined than can be possible with true in vitro analyses of repair, are not permeable to enzymes, have not been able to resolve the role of the UvrC gene product, and (for unknown reasons) are permissive for incision to occur at a maximum of 30% of the dimers formed in the DNA upon irradiation (Waldstein et al., 1974; Sharon et al., 1975; Deutsch et al., 1976; Seeberg and Strike, 1976; Seeberg et al., 1980). This low efficiency made the investigation of incision in slightly irradiated or low molecular weight DNA difficult (even using the very sensitive technique of alkaline sucrose gradient centrifugation). To circumvent this difficulty, the purified, highly efficient glycosylase/AP endonucleases from M. luteus or bacteriophage T4-infected E. coli cells have been used to incise the irradiated DNA (Friedberg et al., 1974; Masker, 1977; Kuemmerle and Masker, 1977). Masker (1977) exposed UV-irradiated bacteriophage T7 DNA to the glycosylase/AP endonuclease of M. luteus to incise the damaged sites and then to a gently lysed extract from E. coli which supplied the components for the other steps of repair. The completion of the DNA repair process in vitro by this method was demonstrated by several biophysical criterion--the restoration of the molecular weight of the DNA, the removal of glycosylase/AP endonuclease sensitive sites from the DNA, and the performance of UV-dependent repair synthesis. Kuemmerle and Masker (1977) also showed the completion of DNA repair in vitro as determined by a more stringent, biological criteria. They showed

that if the bacteriophage T7 DNA was repaired as above (using the M. luteus glycosylase/AP endonuclease and a crude extract of E. coli) and the DNA was then encapsulated into phage heads in vitro, then there was a significantly greater number of resulting viable phage than for unrepaired DNA--indicating that about 80% of the damage had been removed from the irradiated phage DNA in these reactions.

F. Specific Aims of This Research

The major aim of this research was to investigate the repair of DNA in vitro using partially-purified enzymes (and crude extracts) originating solely from E. coli since this would be more representative of the in vivo repair situation. The use of only endogenous proteins for complete in vitro repair has been achieved for M. luteus (Hamilton et al., 1974), but in E. coli the severely limited amounts of incision performed by crude extracts from this organism have prevented this (Seeberg et al., 1976; Masker, 1977). We postulated that the use of partially-purified UvrA, UvrB, and UvrC gene products would alleviate this obstacle. Because the sensitivity of the experimental method required that the incision step be performed efficiently and because the reactions using the partially-purified gene products differed from those using the M. luteus glycosylase/AP endonuclease in that the reactions for incision and the subsequent steps of repair had to be performed concurrently (W. E. Masker, unpublished observation), the first requirement for achieving complete DNA repair as proposed was to optimize incision by the partially-purified UvrABC gene products without hindering the activities which are necessary

for complete repair in the reaction. This characterization of incision also was intended to provide insights into the mechanisms of action of the UvrABC endonuclease. Three reasons prompted the use of the small, covalently closed-circular DNA of bacteriophage PM2 for the investigation of incision by the UvrABC gene products. First, centrifugation of closed-circular DNAs through alkaline sucrose gradients under the conditions used in these experiments completely separated unnicked (Form I = closed circular) from nicked (Form II = relaxed circular and Form III = linear) DNA. The fact that the radioactivity was located in only two widely separated peaks made the calculation of the number of breaks per molecule by this method not only simpler to do (see Chapter II, section J), but also considerably less sensitive to slight perturbations (such as minor disturbances in the gradient resulting from high glycerol concentrations; centrifugation of gradients to be compared in centrifuges which differed slightly in acceleration, timing, and braking; etc.) than was possible when calculating the molecular weight changes by incision of a large DNA like that of E. coli. Second, the use of the PM2 DNA provided a more sensitive means of detecting and dealing with the presence of contaminating non-UV-specific endonucleases. Third, the purification of PM2 DNA was rather routinely performed in this laboratory. By modifying the reaction components and conditions for the incubation of the partially-purified UvrABC gene products with this PM2 DNA and using the technique of alkaline sucrose gradient centrifugation to determine the amount of incision resulting, it was possible to investigate the mechanism of action of the UvrABC gene

products and to begin an investigation of the later steps in the excision repair process. The results of the investigation of the incision of UV-irradiated PM2 DNA are presented in Chapter III.

After determining any minor modifications which might be necessary for the optimization of the reaction conditions for the incision of bacteriophage T7 DNA instead of PM2 DNA, complete in vitro repair of UV-irradiated bacteriophage T7 DNA using enzymes and extracts only from E. coli was attempted.

Bacteriophage T7, a genetically and biochemically well characterized coliphage, represented an excellent system for use in the proposed investigation because it permitted the monitoring of the completion of repair by both biophysical and biological endpoints (see Figure I-2) (Studier, 1969; Studier, 1972; Hausmann, 1977; Kuemmerle and Masker, 1977; Masker, 1977). Bacteriophage T7 has a linear duplex chromosome with a molecular weight of 26×10^6 daltons. The advantages of the use of this DNA for the first endpoint chosen--the restoration of the mode molecular weight (as determined by alkaline sucrose gradient centrifugation)--are that the DNA is conveniently small and that it has been well-characterized biophysically (Studier, 1965; Masker, 1977; McDonnell et al., 1977). The most advantageous feature of bacteriophage T7 for these studies is, however, that the DNA can be encapsulated in vitro into phage heads using cell-free extracts of bacteriophage T7-infected E. coli cells--thus permitting a very sensitive biological endpoint for the monitoring of DNA repair (Kerr and Sadowski, 1974; Kuemmerle and Masker, 1977; Earnshaw and Casjens, 1980; Masker et al., 1981). Masker and

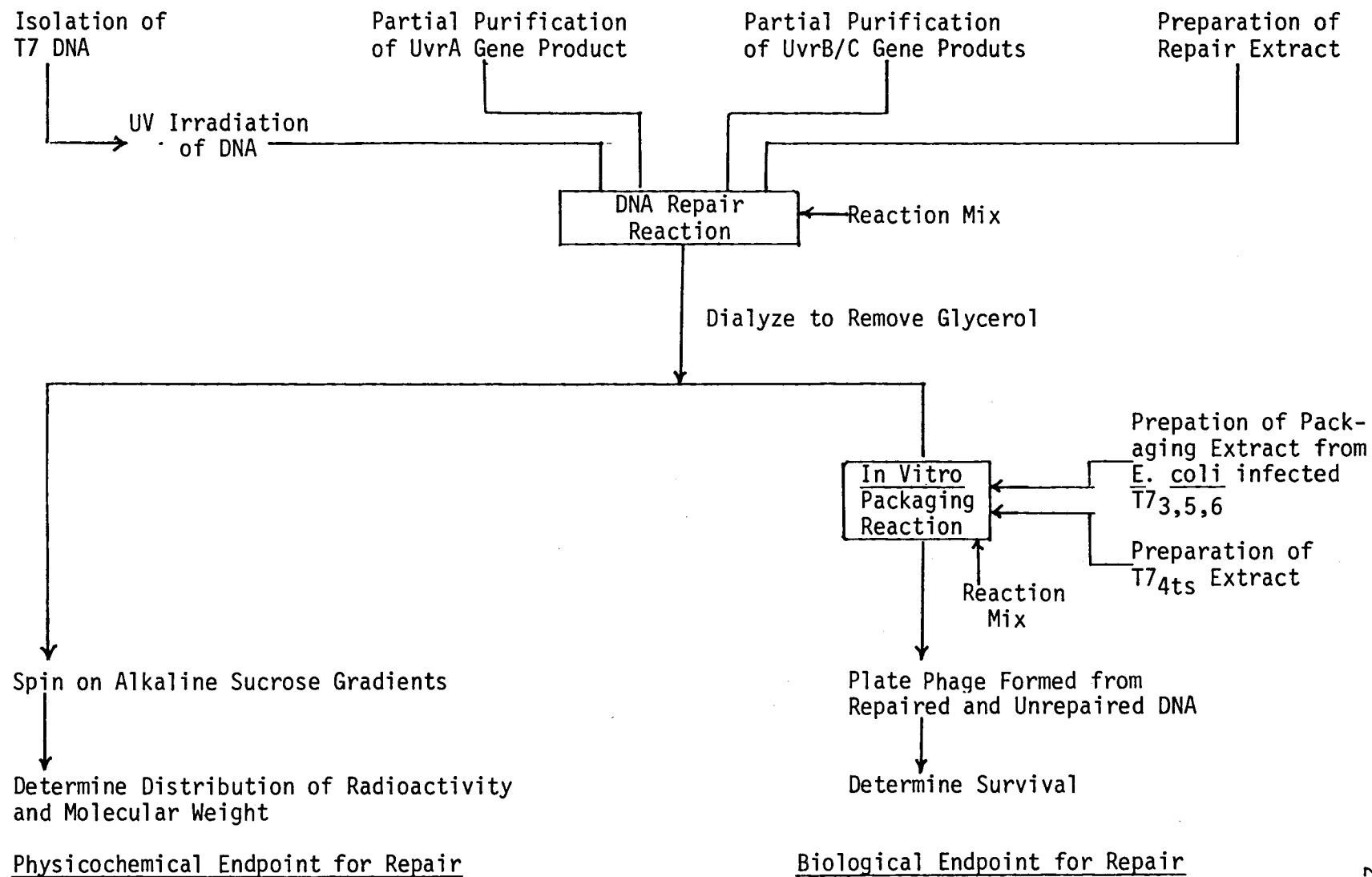


Figure I-2. Protocol for monitoring the in vitro repair of bacteriophage T7 DNA.

his coresearchers have considerably refined the original in vitro packaging system of Kerr and Sadowski (1974) and have achieved packaging efficiencies as high as 3-5% viable phage produced per phage genome equivalent. These packaging reactions are completely dependent upon exogeneous DNA and no detectable recombination occurs in them; therefore, the phage produced are not the result of recombinogenic "rescue" of the damaged exogeneous DNA. In addition, for bacteriophage T7 DNA damaged by ultraviolet light or alkylating agents, the number of viable phage particles produced after in vitro packaging closely parallels the survival of similarly damaged whole phage (Kuemmerle and Masker, 1977; Dodson and Masker, 1981; Masker et al., 1981; Dodson et al., 1982; Masker and Serwer, 1982). Thus, after incubating UV-irradiated T7 DNA with the partially-purified UvrA, UvrB, and UvrC gene products and a crude extract of E. coli prepared by sucrose plasmolysis, the extent of in vitro repair could be monitored by biophysical and biological endpoints which can also indicate how representative this in vitro repair is of repair in vivo. The results of the experiments investigating in vitro repair are presented in Chapter IV.

CHAPTER II

MATERIALS AND METHODS

A. Bacterial Strains and Bacteriophage

The strains of Escherichia coli K-12 used in this study are shown in Table II-1. The host bacteria for the PM2 phage is Pseudomonas BAL-31 (Espejo and Canelo, 1968).

Wild-type bacteriophage T7 (T7_{wt} and T7 amber mutants were from the collection of F. W. Studier. Amber mutations utilized in this study include am29, am28, and am147 which occur in gene 3 (endonuclease), gene 5 (a subunit of DNA polymerase), and in gene 6 (exonuclease), respectively. In the text, the amber mutations are designated by subscripts referring only to the gene in which they occur. For example, T7 bacteriophage containing all three amber mutations listed above is written T7_{3,5,6}. Bacteriophage T7 with a temperature-sensitive mutation (ts101) in gene 4 (primase and DNA-dependent nucleoside triphosphatase), also from F. W. Studier, is designated T7_{4ts}. The T7 phage was grown as described by Studier (1969).

B. Chemicals and Enzymes

Deoxyribonucleoside triphosphates and [methyl-³H]thymidine were purchased from Schwarz/Mann Bioresearch. Lysozyme, nicotinamide adenine dinucleotide (NAD⁺) and dithiothreitol (DTT, Cleland's reagent), all grade A, were obtained from Calbiochem-Behring. Crystalline

TABLE II-1
BACTERIAL STRAINS

Strain	Geneotype	Source
W3110	<u>thyA</u> , <u>sup</u> ⁰	P. Hanawalt
JC6720	F ⁻ , <u>endA</u> , <u>recB21</u> , <u>recC22</u> , <u>gal</u> , <u>thi</u>	N. Willetts
TN207	F ⁻ , <u>uvrA6</u> , <u>thyA</u> , <u>sup</u> ⁰ (deriv. of W3110)	W. Masker
AB2499	F ⁻ , <u>uvrB5</u> , <u>leu</u> , <u>arg</u> , <u>his</u> , <u>pro</u> , <u>thr</u> , <u>thy</u> , <u>thi</u> , <u>lae</u> , <u>ara</u> , <u>gal</u> , <u>mtl</u> , <u>xyl</u> , <u>str</u> ^R , <u>T6</u> ^R , <u>λS</u>	A. Ganesan
KLC647	<u>recA1</u> , <u>uvrA6</u> , <u>ΔxonA</u>	J. Chase
KLC658	= KLC647 carrying plasmid 1996 plasmid = <u>uvrA</u> ⁺ , <u>tet</u> ^R , <u>amp</u> ^R	J. Chase

bovine serum albumin (BSA), Pentex grade, was purchased from Miles Laboratories. Spermidine, morpholinopropanesulfonic acid (MOPS), β -nicotinamide mononucleotide (NMN), and tris(hydroxymethyl)amino-methane (Tris, Trizma base) were obtained from Sigma. Fisher was the source for disodium ethylenediamine tetraacetate (EDTA) and [ethylene-bis-(oxyethylenetriolo)]tetraacetic acid (EGTA). The 2-mercaptoethanol was purchased from Eastman Kodak. Permablend I (scintillation grade--a 91% POP, 9% POPOP scintillant) was obtained from Packard. Pharmacia was the source for Dextran 10. Cellulose phosphate (P11, phosphocellulose) and diethylaminoethyl cellulose (DE52, DEAE) were purchased from Whatman and prepared according to the manufacturer's instructions.

The glycosylase/AP endonuclease isolated from Micrococcus luteus that specifically incises at pyrimidine dimers was a kind gift from W. L. Carrier (prepared by a modification of the procedure described in Carrier and Setlow, 1970). The enzyme was stored at -20°C in 10 mM Tris-HCl, 1 mM 2-mercaptoethanol, 10% (vol/vol) ethylene glycol, and 1 mg/ml BSA.

The UvrD gene product isolated from E. coli strain AB2499 was the kind gift of Nancy B. Kuemmerle. The UvrD gene product used in these experiments came from the most active fractions after Sepharose column chromatography and was stored at -20°C in 50% glycerol (N. B. Kuemmerle and W. E. Masker, personal communication).

C. Media

The M9 media consists of 42 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 8.6 mM NaCl, 18.7 mM NH_4Cl , 0.1 mM CaCl_2 , 1 mM MgSO_4 , and 0.4% (wt/vol) glucose (Miller, 1972). The media was routinely "completed" with 4 $\mu\text{g/ml}$ thymine, 0.2% (wt/vol) casamino acids, and 1 $\mu\text{g/ml}$ thiamine.

L-broth consists of 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter (Miller, 1972). This was routinely supplemented with 10 $\mu\text{g/ml}$ thymine.

AMS media (= BAL-broth) consists of 9 mM KCl, 48 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.44 M NaCl, 13.5 mM CaCl_2 , and 8 g/liter nutrient broth (Espejo and Canelo, 1968).

T-broth consists of 140 mM NaCl and 10 g/liter tryptone (Miller, 1972). T-soft and AMS-soft agar, used in plating, were formed by the addition of 6.5 grams of agar per liter of T-broth and AMS media, respectively. Similarly, the addition of 15 grams agar per liter formed mixtures suitable for pouring T-plates and AMS plates. Calcium was added to the AMS solutions immediately prior to use.

Low-salt T7 diluent consists of 20 mM Tris-HCl, pH = 7.5, 10 mM MgSO_4 , 100 mM NaCl, and 10 $\mu\text{g/ml}$ gelatin. PM2 diluent consists of 1 M NaCl and 0.01 M Tris-HCl, pH = 8.0.

D. DNA

PM2 bacteriophage was grown by a modification of the method of Espejo and Canelo (1968) as described. Pseudomonas BAL-31 was grown in 500 ml of AMS media at 26°C with rapid shaking. When the

bacterial culture reached a turbidity of 0.1 (measured with a Lumetron Colorimeter), approximately 5×10^{11} pfu of PM2 bacteriophage were added. Ten minutes later, approximately 1 mCi of ^3H -thymidine was added to the culture and infection was continued until lysis was complete. After the NaCl concentration was increased to 1.0 M, cellular debris was removed by centrifugation at 5000 rpm for 20 minutes in a Sorvall GSA rotor and filtration of the supernatant through glass wool. The lysate was chilled and titered using AMS-plates and cool (45 $^{\circ}$) AMS-soft agar for the plating. The phage were cleaned by three high-speed (29,500 rpm for 90 minutes in a Spinco type 30 rotor at 4 $^{\circ}$ C) alternated with two low-speed (8000 rpm for 10-15 minutes in a Sorvall SS34 rotor at 4 $^{\circ}$ C) centrifugations. The phage was further isolated by equilibrium centrifugation in CsCl. This involved layering 0.5 ml (approximately 3×10^{12} pfu) concentrated PM2 phage in PM2 diluent onto the top of each of two rotor tubes containing 0.45 g CsCl per ml PM2 diluent and spinning at 37,000 rpm for 24 hours at 8 $^{\circ}$ C in a Spinco SW50 swinging bucket rotor. The phage band was collected and dialyzed against two changes of PM2 diluent to remove the CsCl and briefly against one change of 20 mM Tris-HCl, pH = 8.0 (all approximately 2000 X volume). The DNA was extracted with phenol three times and the phenol was removed by dialysis against 10 mM Tris, pH = 7.5, 0.1 mM EDTA (7 to 8 changes at 2000 X volume). The DNA concentration was spectrophotometrically determined. The integrity (% Form I DNA) was determined by centrifugation in alkaline sucrose gradients as described in Chapter II, section J.

Unlabelled and ^3H -labelled T7 DNA was prepared from wild-type T7 bacteriophage grown on E. coli strain W3110 (sup^0) using the method of Richardson (Richardson et al., 1964; Richardson, 1966).

All DNA concentrations are expressed as nucleotide phosphorus equivalents.

E. Conditions for Ultraviolet Irradiation of the DNA

The 254 nm light was provided by a pair of germicidal lamps with an incident dose of 0.50 or 1.48 J/m^2 per second (determined by an International Light Germicidal/Erythema Radiometer IL 570). The DNA was irradiated in cold deep-well microscope slides with constant gentle stirring. Unless otherwise noted, the DNA was irradiated in 0.175 ml quantities at a concentration of 15 μM . Subsequent manipulations with all irradiated DNAs were performed under yellow lights.

F. Partial Purification of the UvrA Gene Product

In general, the partially purified UvrA gene product was obtained using the method of Seeberg (see Figure II-1) from KLC658, a strain carrying the uvrA⁺ gene on plasmid 1996 and, therefore, overproducing the UvrA gene product (Seeberg, 1978). The UvrA protein was also isolated for use in some of the T7 experiments from strain JC6720 because of this strain's recB,C genotype, but the UvrA⁺ activity per ml was less in this case. The bacteria were grown in three 600 ml cultures in completed M9 at 37°C with rapid shaking. When the OD_{590} reached 1.0 (cell density = $5 \times 10^8/\text{ml}$), the cells were harvested by centrifugation in ice-cold rotor bottles at 6000

STEP

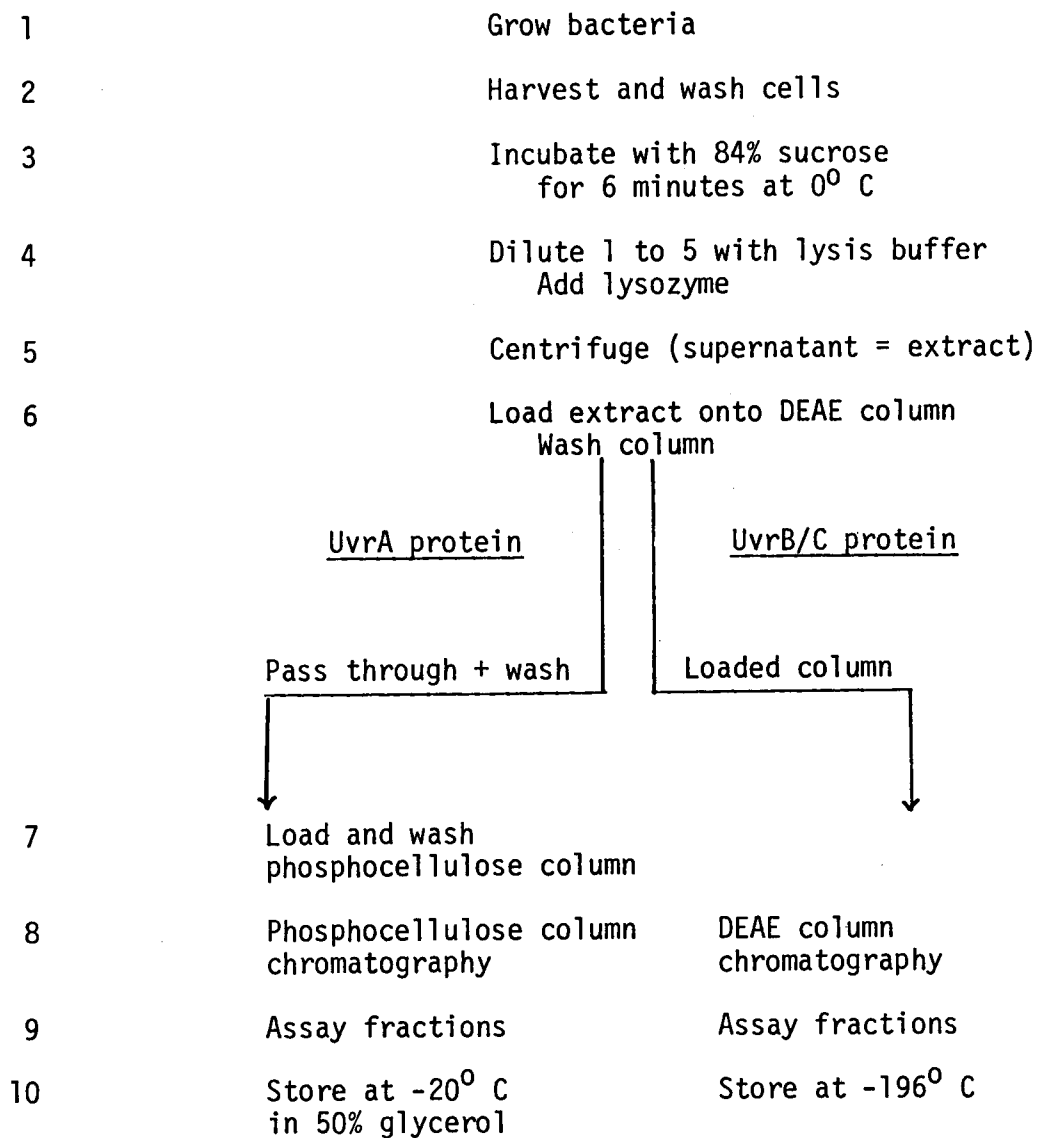


Figure II-1. Protocol for partial purification of the UvrA and UvrB/C gene products.

rpm for 10 minutes in a Sorvall GSA rotor. The cell pellets were washed once by resuspension in 108 ml (12 ml/bottle) ice cold 40 mM Tris HCl, pH = 7.7, transferred to four ice-cold pastic tubes, and centrifugation at 7500 rpm for 10 minutes in a Sorvall SS34 rotor. One drop of lysis buffer (5.6 mM MOPS, pH = 7.55, 11 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT)) was added to each tube and the cell pellets were resuspended thoroughly with an ice-cold stir rod. Three milliliters of ice cold buffer P (2.4 M (84% wt/vol) sucrose, 40 mM Tris, pH = 8.0, 10 mM EDTA) was allowed to drip into each tube as the cells were rapidly stirred. The thoroughly resuspended cells were allowed to incubate 6 minutes at 0°C; then 12 ml of ice-cold lysis buffer was rapidly added to each of the four tubes and the diluted mixture was stirred three strokes. A volume of 0.055 ml freshly prepared 30 µg/ml lysozyme (in water) was added to each tube and the mixture was stirred very gently once. The reaction was allowed to incubate undisturbed at 0°C for 45 minutes. It was then spun at 15,000 rpm (27,000 X g) for 20 minutes in a Sorvall SS34 rotor. The supernatant was carefully decanted into an ice-cold flask and stored on ice from zero to six hours before beginning the chromatography. This extract was loaded onto a 0.9 x 8 cm cold DEAE-cellulose column which had been equilibrated with Buffer A + 0.1 M KCl (Buffer A consists of 50 mM MOPS, pH = 7.55, 9 mM 2-mercaptoethanol, 1 mM EDTA; Buffer B is the same as Buffer A except it is 25% (vol/vol) glycerol). The pass-through was collected in a flask at ice temperatures and was pooled with a 20 ml wash of the column with Buffer B + 0.1 M KCl. This pool was loaded into a cold 0.9 x 8 cm phosphocellulose column

which was equilibrated with Buffer A + 0.1 M KCl. The column was washed with 20 ml of Buffer B + 0.1 M KCl. In order to elute the UvrA gene product from the column, a potassium chloride gradient was run using 20 ml each 0.1 M and 0.4 M KCl in Buffer B. As each 2 ml fraction was collected, the majority of the fraction was gently stirred into ice-cold glycerol (so that the final concentration of each fraction was 50% (vol/vol) glycerol) and stored at -20° C. The fractions were assayed by the method below (Chapter II, section J) to determine the peak incision activity using 0.012 ml fraction from the column and 0.012 ml UvrB/C protein preparation in a 0.126 ml reaction with 150-180 picomoles of ^3H -labelled PM2 DNA, irradiated with 15-20 J/m^2 . The peak fractions were 0.45 to 0.6 mg/ml protein. Figure II-2 shows the results of the complementation assays and the protein determinations from the fractions of a typical column. In the text, the volumes of the protein preparations added to a reaction, rather than the amount of protein, are designated in order to emphasize that the latter is a measure of the total protein in the preparations and may not be always proportional to the amount of the individual Uvr⁺ proteins in these preparations. The partially-purified UvrA gene product will maintain its activity for at least a month if it is stored at -20° C.

G. Partial Purification of UvrB/C Gene Products

The partial purification of the UvrB and UvrC gene products was accomplished by the method of Seeberg (1978) as diagrammed in Figure II-1. For this E. coli strain JC6720 (recB,C) was used in order to

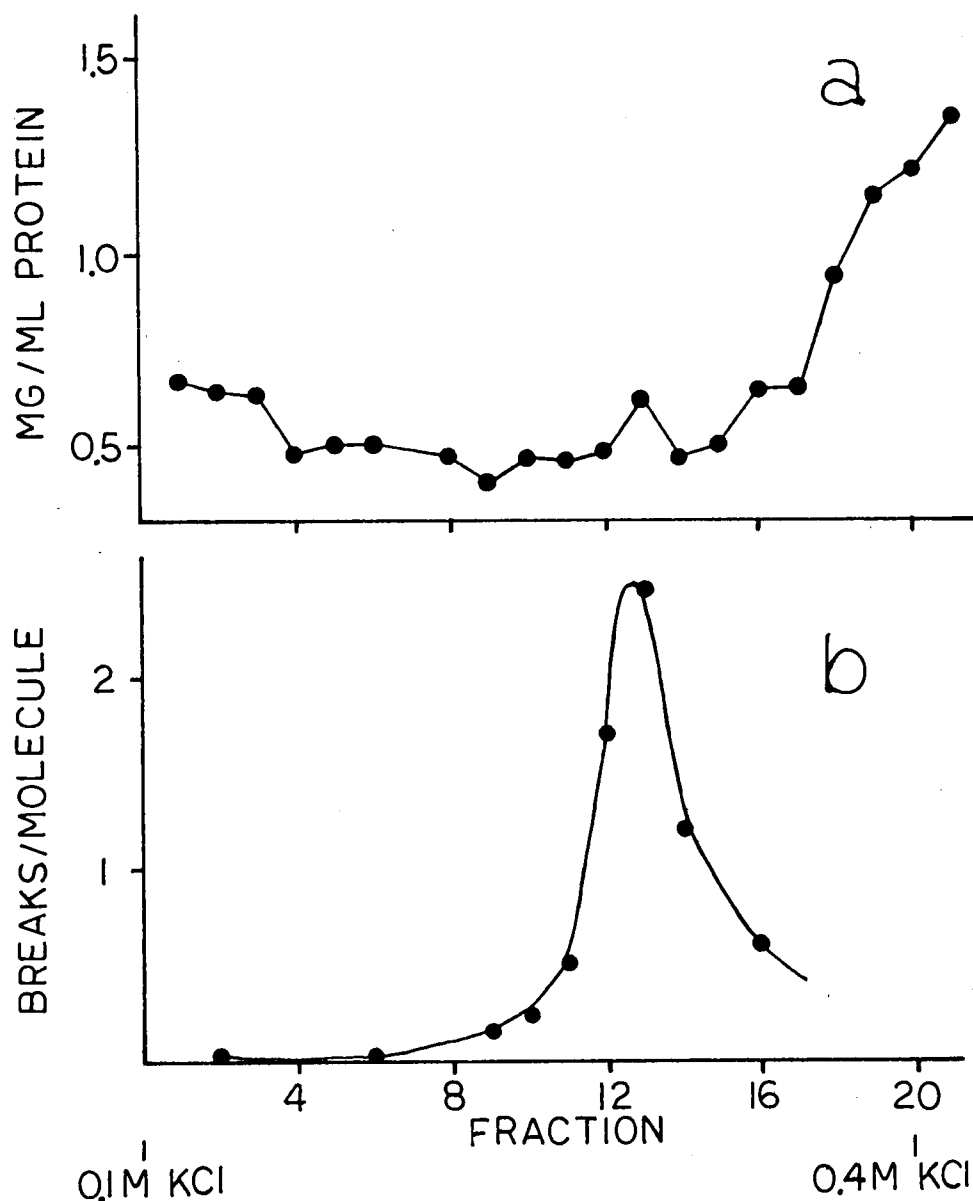


Figure II-2. *In vitro* complementation activity and amount of protein in the fractions of an UvrA protein preparation.

An extract from *E. coli* strain KLC658 was prepared by sucrose plasmolysis and passed through a DEAE-cellulose column. The pass-through was loaded onto a phosphocellulose column and chromatography was performed using a linear gradient of KCl (0.1 to 0.4 M). Details are as described in the text. (a) The amount of protein in each fraction was determined by the method of Lowry et al., 1951. (b) The ability of each fraction to complement the partially-purified UvrB/C proteins in order to incise irradiated PM2 DNA was determined by the alkaline sucrose gradient method described in Chapter II, section J.

prevent contamination of the UvrB/C gene products by exonuclease V (Masker, unpublished observation). The procedure for the formation of the extract was identical with that used for the UvrA gene product (steps 1-7 in Figure II-1). The extract was loaded immediately onto a 0.9 x 8 cm DEAE-cellulose column which had been equilibrated with Buffer A + 0.1 M KCl. The column was then washed with 20 ml Buffer B + 0.1 M KCl. The UvrB and UvrC gene products (which probably elute as a complex (Seeberg and Strike, 1976)) are eluted from the column with a potassium chloride gradient formed from 20 ml each 0.1 M and 0.6 M KCl in Buffer B. Twenty 2-ml fractions were collected and stored on ice. In early preparations, the fractions were assayed by the "incision" assay below (Chapter II, section J) and the two fractions with peak activities were pooled, divided into portions of convenient size, and frozen in liquid nitrogen. Because the UvrB/C proteins are labile and the assay takes about 4 hours to complete, in later preparations portions of probable peak fractions (around 0.3 M KCl) were frozen as soon as each fraction was eluted from the column. Assay of the unfrozen fractions was performed immediately thereafter to determine which to retain. The UvrB/C peak fractions were 1.4 to 2.4 mg/ml protein. The partially-purified UvrB/C protein appears to be very stable when stored in liquid nitrogen. Figure II-3 shows the results of complementation assays and the protein determinations from the fractions of a typical column.

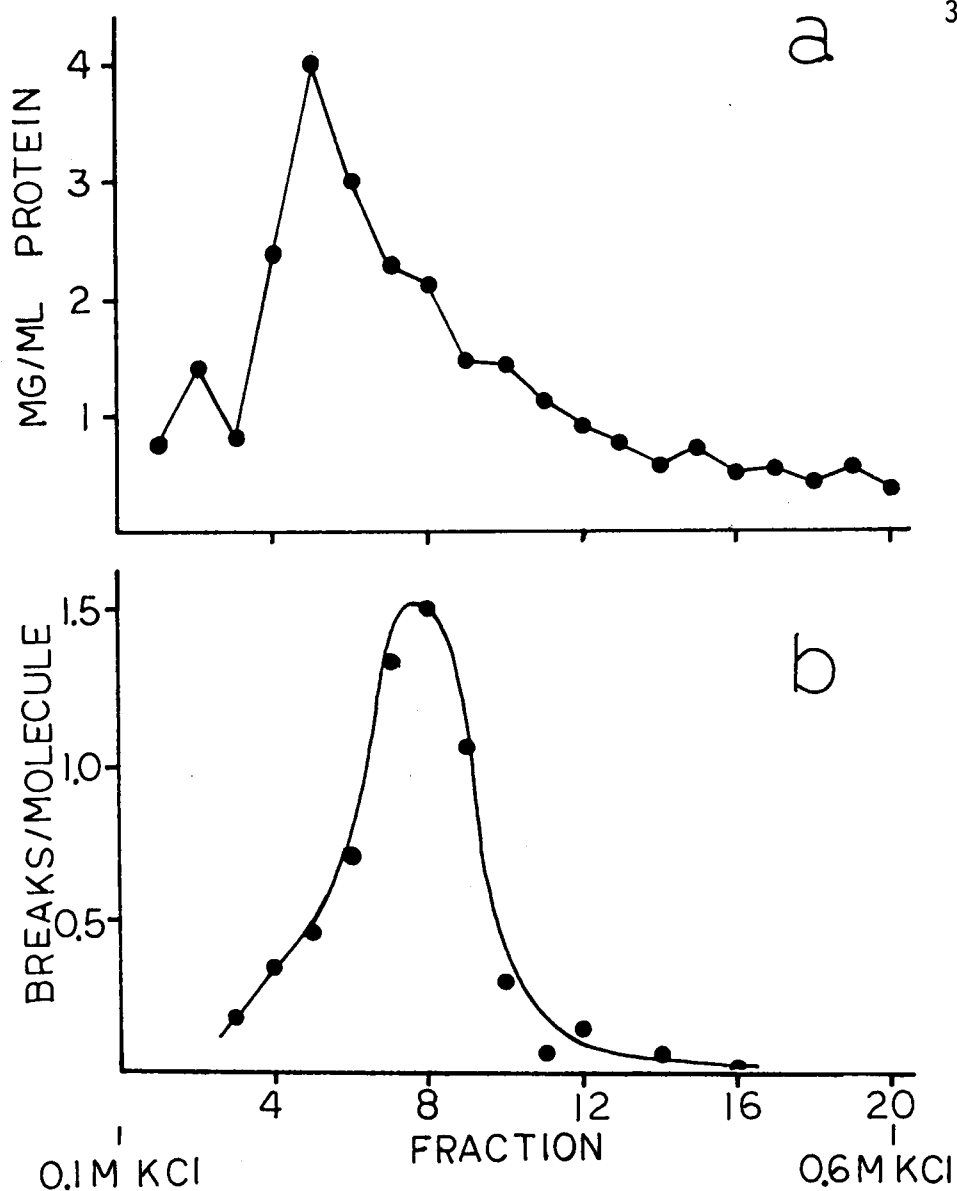


Figure II-3. In vitro complementation activity and amount of protein in the fractions of an UvrB/C protein preparation.

An extract from *E. coli* strain JC6720 (*recB,C*) was prepared by sucrose plasmolysis. Column chromatography to separate the proteins in this extract which bind to DEAE-cellulose was performed using a linear gradient of KCl (0.1 to 0.6 M). Details are as described in the text. (a) The amount of protein in each fraction was determined by the method of Lowry et al., 1951. (b) The ability of each fraction to complement the partially-purified UvrA protein in order to incise irradiated PM2 DNA was determined by the alkaline sucrose gradient method described in Chapter II, section J.

H. Preparation of Extracts by Sucrose Plasmolysis

Purification of the UvrA, UvrB, and UvrC proteins was based on a complementation assay using a "donor fraction" and "receptor extract." (A receptor extract is an extract which contains all the components of a reaction except the component which is being assayed; therefore, when this component is "received," the reaction is complete.) The preparation of the extracts was essentially the same as a small scale preparation of the extract for the isolation of the partially-purified UvrA or UvrB/C gene products. A volume of 100 ml of bacteria was grown in completed M9 at 37° C with rapid shaking. When the cell density reached 5×10^8 cells/ml ($OD_{590} = 1.0$), 40 ml of the culture was harvested by centrifugation in two ice-cold tubes at 5000 rpm for 10 minutes in a Sorvall SS34 rotor (0° C). Because there were so few cells, they were not washed. The pellet was re-suspended thoroughly by rapid stirring as 0.025 ml Buffer B was allowed to drip into each tube. After 5 minutes incubation at 0° C, 1 ml of ice-cold lysis buffer containing 125 µg/ml lysozyme was rapidly added to each tube. The reactions were allowed to incubate on ice undisturbed for 45 minutes and then the two tubes were spun in a SS34 rotor at 12,500 rpm for 15 minutes. The supernatant was carefully removed with a Pasteur pipette. This extract was stored on ice and used as quickly as possible.

I. Preparation of Phage-Infected Cell Extracts for Packaging

Extracts prepared from phage-infected cells contain all the components necessary for the encapsulation of exogenously provided T7 DNA into phage heads to form viable phage (Kerr and Sadowski, 1974; Kuemmerle and Masker, 1977). The T7_{3,5,6} phage is used for the infection because the three amber mutations in the phage are in genes necessary for DNA synthesis and recombination--thereby, limiting endogenous DNA to that of the infecting phage and preventing recombinogenic "rescue" of the UV-damaged exogenous DNA. The phage formed from the endogenous DNA will not form plaques on suppressor-free indicator bacteria; therefore, the number of phage surviving depends entirely on the addition of exogenous DNA in a manner linear over a range of 1 to 1000 picomoles of DNA per 0.030 ml reaction (Kuemmerle and Masker, 1977). The phage-infected cell extracts were prepared by the method described in the paper by Kuemmerle and Masker (1977) with the addition of a final centrifugation step. Three milliliters of an overnight culture of E. coli suppression-free bacteria, strain W3110, were diluted into 600 ml L-broth supplemented with 10 µg/ml thymine and grown at 30° C with rapid shaking until the cell density reached 5×10^8 cells per ml ($OD_{590} = 1.0$). The cells were infected with T7_{3,5,6} at a multiplicity of infection of three. Infection was continued for exactly 18.5 minutes at 30° C with rapid shaking and then was stopped quickly by cooling of the flask in an ice-water bath. The ice-cold culture was poured into ice-cold rotor

bottles and centrifuged at 3300 rpm for 5 minutes in a Sorvall GSA rotor. The pellets were thoroughly resuspended in a total added volume of 7.2 ml ice-cold low salt T7-diluent, pooled, and frozen in polycarbonate 40 rotor tubes under liquid nitrogen. Phage-infected cells could be stored for months by this method.

Immediately before use, the phage-infected cell preparation was thawed rapidly at 30° C, one-fortieth volume of freshly prepared lysozyme solution (1 mg/ml lysozyme in 15 mM Tris·HCl, pH = 7.5, 7.5 mM MgSO₄, 0.25 M EDTA) was added, and the mixture was incubated at 30° C for 2 minutes. After chilling on ice, this lysate was centrifuged at 16,000 rpm for 20 minutes in a Spinco 40 rotor and used immediately.

Extracts prepared from T7_{4ts} infected cells and isolated procapsids were the kind gifts of Marilyn Maupin and were prepared as described recently (Dodson et al., 1982, Masker and Serwer, 1982) and stored in liquid nitrogen until immediately prior to use. The addition of a T7_{4ts} extract (and the procapsids) improved the packaging efficiencies of the in vitro packaging reactions considerably (to 0.3 to 3%, in general), probably because of the addition of the gene 6 product, but did not result in measurable levels of in vitro recombination (Masker et al., 1978; W. E. Masker, unpublished observation). W. E. Masker generously provided some of the protein layer of an extract of bacteriophage T7-infected E. coli cells which had been partially-fractionated by sedimentation through neutral sucrose. This preparation was stored in liquid nitrogen and used in some of the in vitro packaging reactions in order to eliminate the endogenous DNA and phage present in the crude packaging extracts.

J. Reaction Conditions for the Incision of PM2 DNA

The reaction conditions used to measure incision of UV-irradiated DNA by the UvrABC gene products were varied in an attempt to improve the efficiency of the incision reaction. A typical reaction mixture of 0.126 ml contained 40 mM MOPS, pH = 7.55, 100 mM KCl, 15.5 mM MgSO₄, 1 mM EDTA, 1 mM DTT, 2.0 mM ATP, and 3.65 mM NMN (final concentrations). To this 180 picomoles of UV-irradiated, ³H-labelled PM2 DNA, 0.018 ml of the peak fractions of a UvrA protein preparation (approximately 0.55 mg/ml total protein), and 0.018 ml of the peak fractions of a UvrB/C protein preparation (approximately 1.8 mg/ml total protein) were added. The UvrB/C fractions were thawed on ice just prior to use. The reaction was incubated for 45 minutes at 37⁰ C and stopped by the addition of ice cold EDTA to a final concentration of 45 mM and by chilling the tubes on ice. A volume of 0.110 ml of this reaction was layered onto a preformed alkaline sucrose gradient (5 to 20% linear gradient of sucrose in 0.5 M NaCl, 0.01 M EDTA, 0.2 M NaOH). The gradients were immediately centrifuged at 49,000 rpm for 50 minutes at 20⁰ C in a Spinco SW56 rotor. Twenty fractions were collected from the bottom of the gradient onto Whatman number 17 chromatography paper strips as described by Carrier and Setlow (1971). DNA was precipitated by immersing the strips in 5% trichloroacetic acid for 15 minutes at room temperature, followed by two washes (12 minutes each) in ethanol. When the strips were completely dry, they were cut into shell vials. Radioactivity was determined (5 minutes for each fraction) in a Packard Tri-Carb

scintillation spectrometer using scintillation fluid consisting of 3 g Packard Permablend per liter of toluene. The sums of the radioactivity in the peak regions associated with Form I DNA (supercoiled, covalently closed circular, faster sedimenting DNA) and Forms II and III DNA (relaxed, nicked circular and linear DNA) were corrected for background radiation and used to calculate the percent Form I (unnicked) DNA as below.

$$\% \text{ Form I} = \frac{\text{Form I}}{\text{Form I} + \text{Form (II + III)}} \times 100.$$

The distribution of dimers over a large number of DNA molecules follows a Poisson distribution (Friedberg and Clayton, 1972). The average number of breaks per molecule can be calculated using the equation below by assuming that the proportion of unnicked (Form I) DNA molecules also follows a Poisson distribution (i.e., that incision at the dimers is random over the molecules).

$$\text{Breaks/molecule} = - \ln \left(\frac{\text{Form I}}{\text{Form I} + \text{Form (II + III)}} \right)$$

The number of UV damage-specific breaks per molecule caused by the UvrABC gene products was determined by subtracting the number of breaks per molecule calculated with reactions in which unirradiated DNA was incubated with the UvrABC gene products from the number calculated with the reactions using UV-irradiated DNA.

K. Reaction Conditions for the in Vitro Repair of Bacteriophage T7 DNA

A typical reaction mixture for the in vitro repair of bacteriophage T7 contained (final concentrations) 28 mM Tris·HCl, pH = 7.7,

16.7 mM MgSO_4 , 3.1 mM 2-mercaptoethanol, 2.1 mM ATP, 0.26 mM NAD^+ , and 0.08 mM each deoxyribonucleotide phosphate (dNTP) (plus 3.1 mM 2-mercaptoethanol, 32.7 mM MOPS, pH = 7.55, 0.65 mM EDTA, and 110 mM KCl added to the reaction as components of the protein preparations or crude extract). To this was added 1.63 nanomoles of ^3H -labelled T7 DNA (14.9 cpm/picomole) which had been irradiated with 0 to 24 J/m^2 of 254 nm light, 0.144 ml of the peak fraction of a UvrA protein preparation (0.5 mg/ml protein) isolated from *E. coli* strain JC6720 (recB,C), 0.080 ml of the peak fraction pool of a UvrB/C protein preparation (1.8 mg/ml protein) which had been thawed on ice just prior to use, and 0.160 ml of an extract of *E. coli* strain JC6720 prepared by sucrose plasmolysis (see Chapter II, section H). The reaction was incubated for 40 minutes at 35° C and stopped by adding ice-cold EDTA to a final concentration of 45 mM and by chilling the tubes on ice. In control reactions (no repair expected), mixtures of Buffer B made equivalent in glycerol and KCl to those of the UvrA and UvrB/C preparations and lysis buffer (without lysozyme) were added instead of the UvrABC proteins and the repair extract, respectively. In the reactions to determine the amount of incision, a final concentration of 3.65 mM NMN (to prevent ligation) was substituted for the NAD^+ and deoxyribonucleotide phosphates. In order to package this DNA, the glycerol (which accounted for 18% of the volume) had to be removed. This was done by an 8-hour dialysis against ice-cold 10 mM Tris·HCl, pH = 7.5, 0.1 mM EDTA and a 4-hour dialysis against ice-cold 25 mM Tris·HCl, pH = 7.5 (both at 4000 times volume). Because the glycerol disturbs the sucrose gradients, the DNA could be

centrifuged without dialysis only if it was diluted at least 2:1 with 25 mM Tris·HCl. Alkaline sucrose gradient centrifugation, fraction collection, and scintillation spectrometry were performed as described for PM2 DNA except the gradients were centrifuged for 120 minutes, 30 fractions were collected, and the radioactivity in each fraction was determined after 10 minute "counts" in a Packard Scintillation counter.

The weight average molecular weight (M_w) is defined by the equation below using the adaptations to Charlesby's (1954) original equation as discussed by Youngs and Smith (1976):

$$M_w = k \sum f_i d_i^{2.63}$$

In this, f_i refers to the proportion of the recovered radioactivity associated with the i^{th} fraction and d_i refers to the distance of the i^{th} fraction sediments from the meniscus. The constant k was determined using bacteriophage T7 DNA (which had been neither reacted nor dialyzed) as a standard, and assuming a molecular weight in alkali of 13×10^6 daltons (Studier, 1972). The distance sedimented for this standard DNA was defined by the leading edge of the peak fractions. Finally fractions near the top and bottom containing little radioactivity were excluded from the calculation.

L. Reaction Conditions for in Vitro Packaging of Bacteriophage T7 DNA

After dialysis DNA (0.0065 ml) from the in vitro repair reaction was diluted into a reaction mix consisting of (final volumes) 0.020 ml packaging extract (from T7_{3,5,6} infected E. coli as

described above), 0.00021 ml extract from bacteriophage T7_{4ts} infected *E. coli*, 4.3 mg/ml BSA, 4.0 mM 2-mercaptoethanol, 4.3 mM Tris·HCl, pH = 7.5, 7.1 mM MgCl₂, and 2.8 mM spermidine such that the final volume was 0.030 ml. (In some experiments, 0.015 ml of the protein layer of an extract partially-fractionated by sedimentation through neutral sucrose and 0.005 ml of isolated procapsids (diluted 1:1 in a solution containing 1 mg/μl dextran 10 in low salt T7 diluent) were added instead of the packaging extract in order to improve the efficiency of packaging (see Chapter II, section I)). These reactions were incubated at 30° C for one hour and stopped by the addition of 1 ml of ice-cold low salt T7 diluent and by chilling the tubes on ice. To 0.1 ml of this mixture appropriately diluted, was added 0.3 ml strain TN207 (*uvrA*⁻, *sup*⁰) bacteria, and 2.5 ml T-soft agar and this mixture was plated on T-agar plates. The plates were incubated at 44° C for 2-3 hours and the number of resulting plaques counted.

The suppressor-free indicator bacteria and the incubation at the non-permissive temperature was expected to virtually eliminate all contaminating phage formed from packaging endogeneous DNA. This was experimentally monitored by determining the number of plaques formed in a packaging reaction with no exogenous DNA. To correct for dilution (or loss) of the DNA during dialysis, 0.200 ml samples of dialyzed DNA were filtered through G/C glass fiber filters using 4 ml ice-cold M PP_i, 1 N HCl to which approximately 0.1 mg salmon sperm DNA had been added. Filters were washed twice with ice-cold ethanol, dried, and the radioactivity determined in a liquid

scintillation counter. The plaque counts were corrected for DNA recovery by a dilution correction factor (equal to the number of counts per minute expected on the filter divided by the actual number counted). Dilution ranged from 30 to 60% of the original volume.

Each of the packaging reactions were done in quadruplicate and plated in duplicate. Each data point graphed represents the mean of the data obtained from these eight determinations. The standard deviations were, in general, about 20% or less of the mean.

M. Protein Determinations

The amount of protein in each fraction from the UvrA and UvrB/C protein preparations was ascertained by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

CHAPTER III

THE INCISION OF ULTRAVIOLET-IRRADIATED PM2 DNA BY THE UvrA, UvrB, AND UvrC GENE PRODUCTS

This section of the thesis presents some basic characterization of the ability of the partially-purified UvrA, UvrB, and UvrC (UvrABC) gene products to incise DNA in a UV-specific manner. It also shows the experimentation done to optimize the reaction conditions for incision at pyrimidine dimers in PM2 DNA and to investigate the mechanism of action by which the UvrABC gene products incise irradiated DNA in vitro.

A. Quantitation of the Efficiency of Incision by Comparison with the M. luteus Glycosylase/AP Endonuclease

The data from the work of Seeberg and his colleagues showed that the number of incisions performed by the UvrABC gene products (whether in plasmolyzed cells in a pseudo in vivo situation or as cell-free extracts or partial-purified protein preparations in an in vitro complementation assay) was fewer than the number of dimers formed in the DNA by the doses of ultraviolet light used--although the efficiency of incision (breaks per dimer) varied greatly (Seeberg and Strike, 1976; Seeberg et al., 1978; Seeberg, 1978; Seeberg, 1981). In order to investigate whether the partially-purified UvrABC gene products would act in vitro (and under the reaction conditions used in this study) with a low efficiency of dimer

incision and to actually quantitate this efficiency, a comparison was made between the amount of incision performed when the partially-purified UvrABC gene products acted alone to incise UV-irradiated DNA and when the Micrococcus luteus glycosylase/AP endonuclease (a well-characterized UV-specific glycosylase/AP endonuclease (Riazuddin and Grossman, 1977a,b; Gordon and Haseltine, 1981)) was added subsequently to the same reaction in order to complete incision at all dimers.

Quantities of 105 picomoles of ^3H -labelled PM2 DNA (50.1 cm/picomole, irradiated with 0, 5, 10, or 15 J/m^2), 0.020 ml of partially-purified UvrA protein preparation (0.6 mg/ml protein), and 0.030 ml of UvrB/C protein preparation (1.4 mg/ml protein) were added to reaction mixes consisting of 34 mM MOPS, pH = 7.5, 119 mM KCl, 13 mM MgSO_4 , 1 mM EDTA, 1 mM DTT, 1 mM ATP, and 3.8 mM NMN (final concentrations) to yield a total volume of 0.252 ml. After a 20 minute incubation at 37° C, the reactions were stopped by the addition of ice-cold EDTA to a final concentration of 20 mM and by chilling the tubes on ice. The reactions at each dose of ultraviolet irradiation were divided into two equal portions. To one portion, additional ice-cold EDTA was added to result in a final concentration of 50 mM. To the other portion was added 0.010 ml of the M. luteus glycosylase/AP endonuclease (an amount known to be in excess of that required to cause one incision at every pyrimidine dimer (Setlow et al., 1975; Masker, 1977)). The reactions to which the M. luteus glycosylase/AP endonuclease had been added were incubated 15 minutes at 30° C and they were stopped by the addition of ice-cold EDTA to a final

concentration of 50 mM and by again chilling the tubes on ice. The number of breaks per molecule formed in the DNA in each reaction was calculated (as presented in Chapter II, section J and Figure III-1) by centrifugation through alkaline sucrose gradients and determination of the distribution of radioactivity between Form I and total DNA. The results are shown in Figure III-2.

The presence of the UvrABC gene products did not inhibit the action of the M. luteus glycosylase/AP endonuclease (data not shown). In addition, because the reaction to which the M. luteus glycosylase/AP endonuclease was added to incise DNA irradiated with a dose of 15 J/m² left less than 2% Form I DNA, this point was omitted from the graph.

The data in Figure III-2 shows linear amounts of incision both when the UvrABC gene products incise the DNA alone and when the M. luteus glycosylase/AP endonuclease also incised the DNA. The slopes of these lines are 0.066 and 0.303 breaks per molecule per J/m² ultra-violet irradiation of the DNA, respectively. A comparison of these slopes shows that the partially-purified UvrABC gene products act in vitro with an efficiency approximately 22% of that of the M. luteus glycosylase/AP endonuclease.

The number of dimers formed per DNA molecule varies linearly with low (biological) doses of ultraviolet light applied to the DNA (Setlow and Carrier, 1966; Unrau et al., 1973; Masker, 1977). This can be seen directly in the reactions with the M. luteus glycosylase/AP endonuclease because this enzyme is known to be UV-specific and to act quantitatively such that one single-strand break is introduced

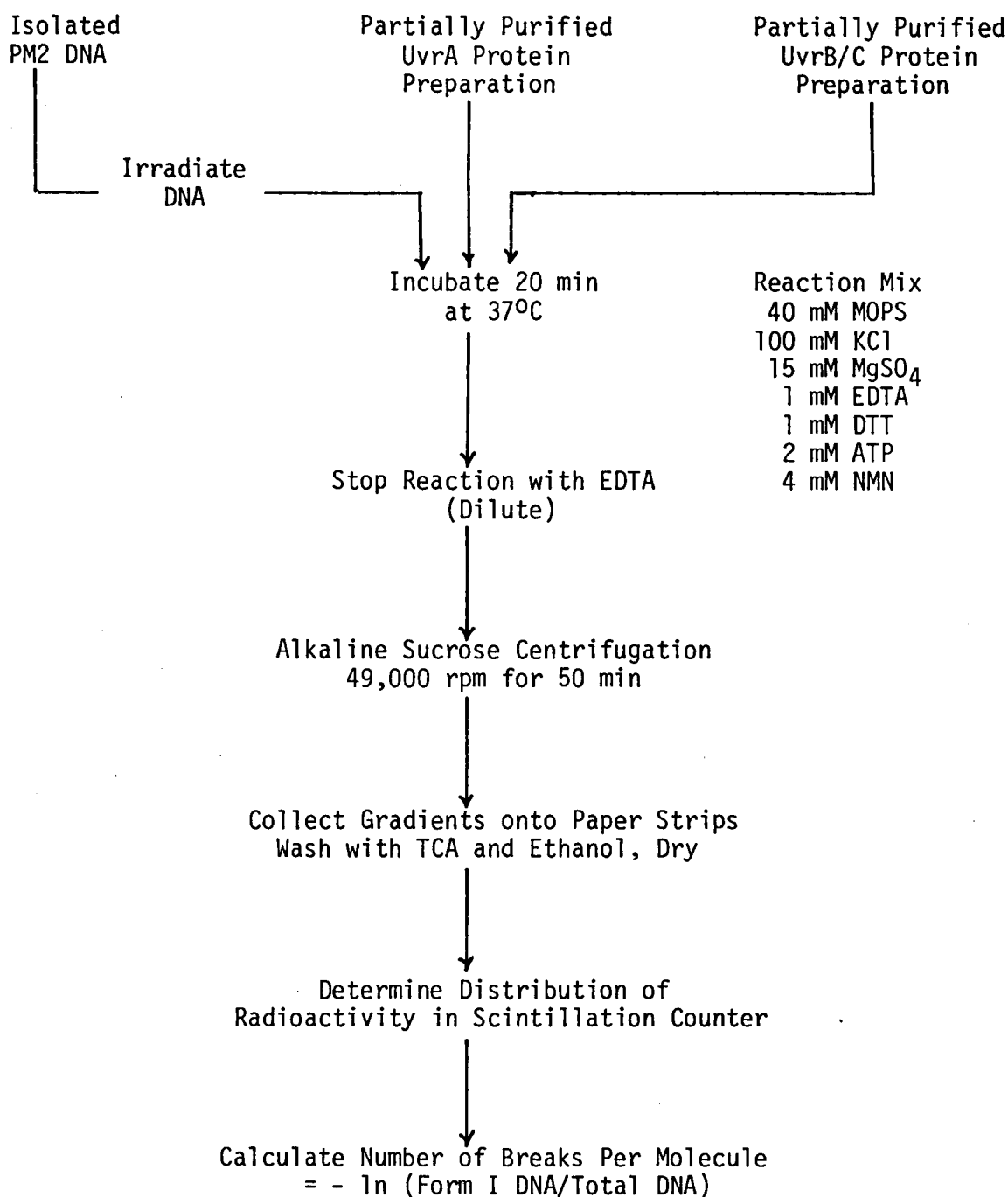


Figure III-1. Protocol for Determination of the Amount of Incision of UV-Irradiated PM2 DNA.

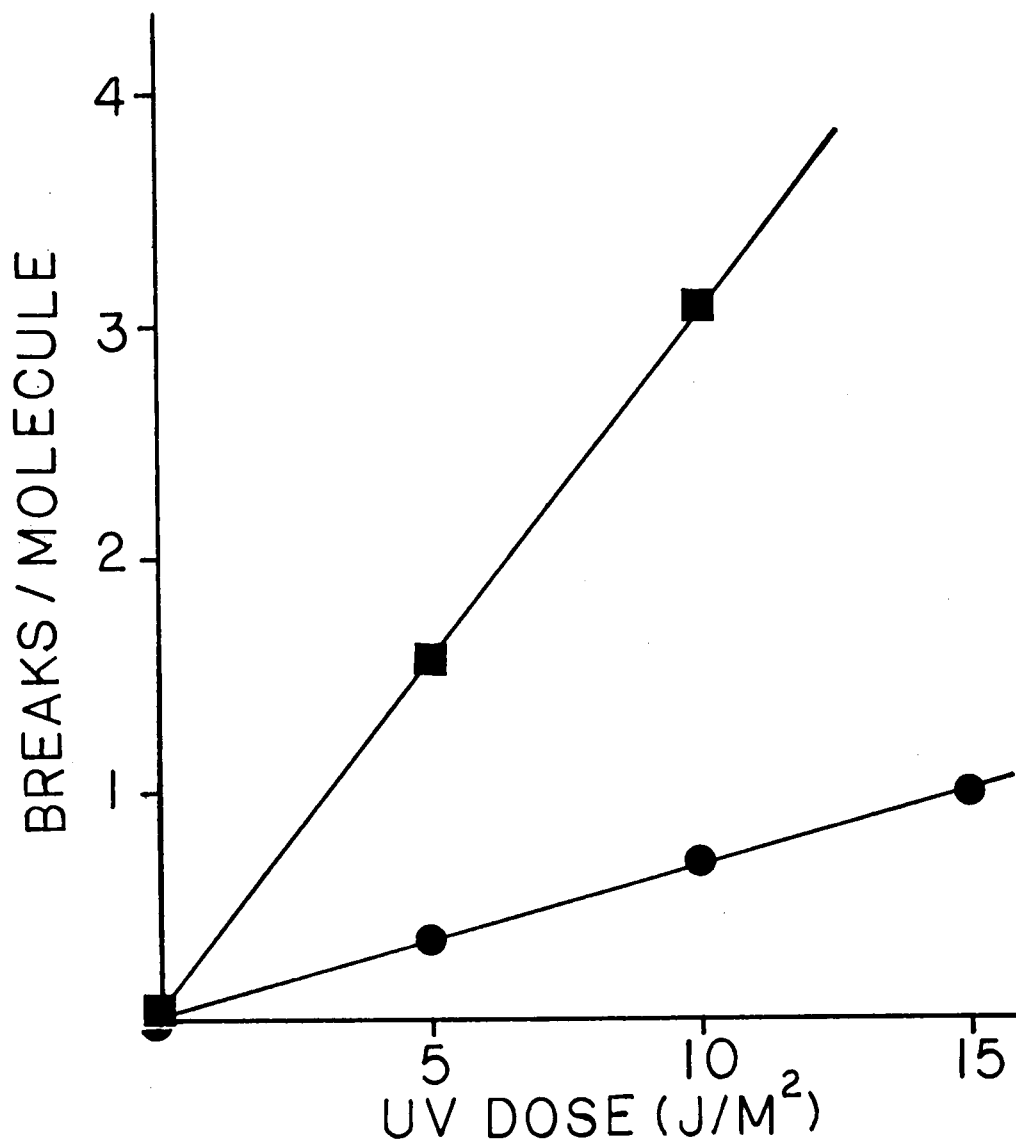


Figure III-2. Quantitation of the efficiency of incision of the UvrA, UvrB, and UvrC gene products by comparison with the *M. luteus* glycosylase/AP endonuclease.

³H-labelled PM2 DNA was irradiated at various UV doses and incised by the partially-purified UvrA, UvrB, and UvrC gene products (●). One-half of each reaction was further incubated with an amount of *Micrococcus luteus* glycosylase/AP endonuclease sufficient for incision to occur at all dimers (■). After both sets of reactions were stopped, they were centrifuged through alkaline sucrose gradients and the radioactivity in the fractions collected was determined in order to ascertain the percentage of Form I (unnicked) DNA and corresponding number of breaks per molecule. Reaction conditions and radioactivity were as described in the text. The data was corrected for non-UV-specific breakage.

for each dimer (Carrier and Setlow, 1970; Setlow, Carrier, and Stewart, 1975; Haseltine et al., 1980). The results of this experiment show that irradiation of PM2 DNA with 1 J/m^2 of ultraviolet light under the conditions used in these experiments caused the formation of an average of 0.3 dimers per PM2 genome.

Another point to be noted about the results of this experiment is that the number of breaks per molecule made by the partially-purified UvrABC gene products in vitro is a constant proportion of the total number of dimers formed in the DNA regardless of UV dose.

B. UV Dose Dependence

The functional dependence between the amount of incision performed by the partially-purified UvrABC gene products and the UV dose applied to the DNA was measured. To do this the number of sample points and the UV dose range over which the investigation was performed were increased from the previous experiment because non-linearity in the relationship is more likely in the extreme regions of UV dose (where there are either very many or very few dimers per molecule). A quantity of 150 picomoles of ^3H -labelled PM2 DNA (22.5 cpm/picomole) which had been irradiated with between 0 and 30 J/m^2 was added to a reaction mixture consisting of 32.7 mM MOPS, pH = 7.5, 113 mM KCl, 15.6 mM MgSO_4 , 1 mM EDTA, 0.7 mM DTT, 1.3 mM ATP, and 3.8 mM NMN (final concentrations). Immediately thereafter, 0.012 ml each of the partially-purified UvrA and UvrB/C protein preparations (preparations are 0.5 and 2.2 mg/ml protein respectively) were added to the reactions. These 0.126 ml reactions were

incubated at 37° C for 20 minutes and stopped by the addition of ice-cold EDTA to a final concentration of 45 mM and by chilling the tubes on ice. The number of breaks per molecule was calculated after centrifugation of the reaction through alkaline sucrose gradients and determination of the distribution of radioactivity in the fractions collected (Chapter II, section J and Figure III-1). The results are shown in Figure III-3. This shows a linear dependence (correlation = 0.977) between breaks per molecule and UV dose (or dimers per molecule). The slope is 0.036 breaks per molecule per J/m², reflecting an efficiency of incision of only 12%. The same results with an efficiency of 25% were obtained in a similar experiment when the UvrA protein preparation was freshly prepared (data not shown).

No organism is capable of maintaining its normal DNA metabolic activities in the presence of DNA damage (for reviews, see Witkin, 1976; Hanawalt et al., 1979; Lehmann and Karran, 1981). Therefore, the inability of these in vitro reactions to quantitatively remove dimers must indicate either that the in vitro reaction conditions used in these experiments are not sufficiently mimicking the in vitro situation or that, in dark phase repair in vivo, the relative role of the excision repair process associated with the UvrABC gene products is poorly understood relative to other means of immediately coping with the damage. Possible reasons for the former (summarized in Table III-1) include the following:

The quantities and activities of the components of the reactions used to assay incision of the PM2 DNA could influence the efficiency of incision. One way this is possible is that the amounts

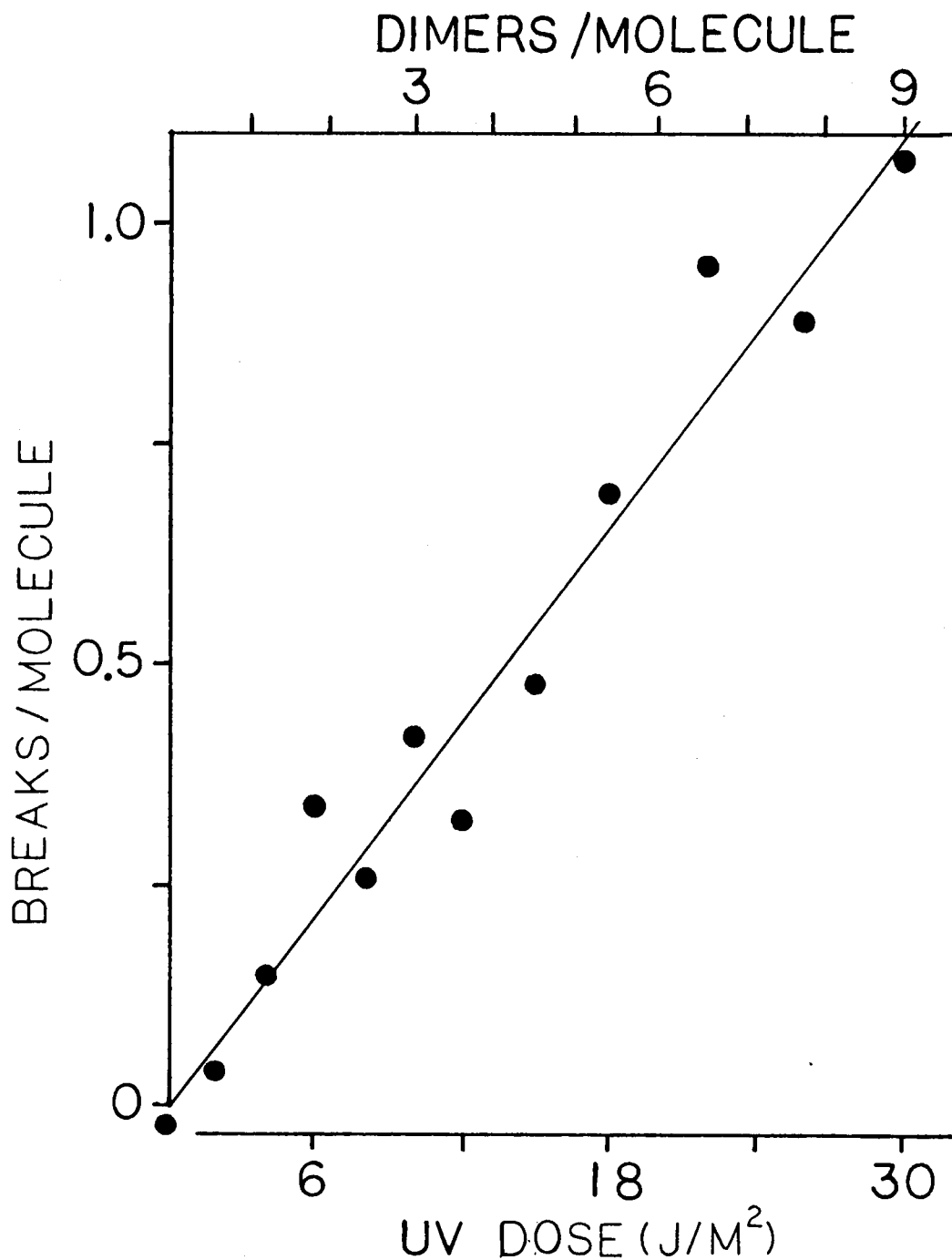


Figure III-3. Effect of UV dose applied to the DNA on the amount of incision.

³H-labelled PM2 DNA was irradiated with various UV doses and incised by the partially-purified UvrA, UvrB, and UvrC gene products. These reactions were treated as described in Figure III-2 and in the text. The data was corrected for non-UV-specific breakage of the DNA.

TABLE III-1

POSSIBLE REASONS FOR THE LOW EFFICIENCY OF INCISION OBTAINED IN VITRO
WITH THE PARTIALLY-PURIFIED UvrA AND UvrB/C GENE PRODUCTS

Possible Reason	Possible Cause
1. (a) Limited amount or (b) Lack of some components	(a) UvrA, UvrB, or UvrC gene product (b) UvrD gene product
2. Limited amount or lack of some cofactor(s)	ATP, Mg ⁺⁺
3. Physical conditions of the reaction not optimal	pH, KCl concentration, total protein concentration, osmolarity, temperature
4. Factors dependent on the DNA (a) Binding site (b) Other factors which influence DNA protein interactions	(a) Number of binding sites, distribution (b) Sequence, pyrimidine tracts
5. Inhibitory factors (a) Large amounts of UvrA, UvrB, or UvrC gene products inhibitory (b) Presence of inhibitory factors in the protein preparations	(a) Competition between DNA-associated or free forms (b) Glycerol, other proteins
6. Incision at only a subset of the dimers (a) Type (b) Location	(a) $\hat{T}T$, $\hat{T}C$, $\hat{C}T$, $\hat{C}C$ pyrimidine-C lesion (b) Clustering, isolation in pyrimidine tracts
7. Activation or Deactivation of UvrA or UvrB/C Gene Products	Deactivation by method of isolation, activation required for greater efficiency

of the UvrA, UvrB, or UvrC gene products (or cofactors) added to the reaction could be insufficient for maximal in vitro incision. The in vitro reaction may instead, completely lack some component that would improve the efficiency of incision. The addition of large quantities of the UvrA and UvrB/C protein preparations may be inhibitory in the in vitro reactions because of the possible effects of incorrect stoichiometry among the gene products or with the DNA or because of the possible inhibitory effects of other proteins in the partially-purified preparations. Also, if the DNA used in these reactions significantly differs from E. coli DNA by criteria such as the number and distribution of any sites to which the UvrABC gene products can preferentially bind, the kinetics (or stability) of the DNA-protein association and of incision may be significantly altered. The influence of the type of DNA may, therefore, be dependent upon whether the UvrABC gene products act in a processive or a distributive fashion. Another possibility is that the reaction conditions used for the assay of the UV-specific incision may not be optimal. The low efficiency of incision that is attained in vitro may be due to some limitation which limits incision to only a subset of the UV damage. Features of the damage that may determine this subset include the type of dimers ($\hat{T}T$, $\hat{T}C$, $\hat{C}T$, or $\hat{C}C$) and the location of the damage (proximity to other dimers or location in pyrimidine tracts). Finally, the method of isolation, the time involved in the isolation procedure, or the method of storage of the partially-purified UvrABC gene products might deleteriously alter them.

Certain conclusions can be drawn from the results of the first two experiments relative to these possible reasons for the low efficiency of incision with the partially-purified UvrABC gene products. The observation that the relationship between the amount of incision and the UV dose is linear strongly argues against the possibility that clustering (e.g., 2-3 dimers in close proximity) is required for incision because at higher doses of UV the probability of having dimer clusters is considerably greater than at low UV doses and the relationship between probability and dose is by no means linear. The observation that the efficiency of incision is the same whether there is one or many dimers per molecule argues against the possibility that location of the dimers in specific regions--such as pyrimidine tracts--is the efficiency-limiting factor--unless the target area represents 12-25% of the DNA molecule. Because this experiment is designed so that there are considerably more total dimers in the reactions in which the DNA is irradiated with higher UV doses, this observation also argues against the possibility that the amount of UvrABC gene products or cofactors is limiting per dimer. (It does not exclude, however, the possibility that the amount of gene products or cofactors may be limiting per molecule if the mechanism of action of the UvrABC gene products is not completely distributive.)

C. Amount of UvrABC Gene Products

A determination of the relationship between the amount of incision in a given time and the amounts of UvrABC gene products added to the reaction would address two non-exclusive possibilities:

(1) whether the incision reactions shown in Figures III-2 and III-3, pages 52 and 53, were limited by quantities of the gene products insufficient under these reaction conditions for incision to occur at every dimer and (2) whether the incision was limited due to inhibition of the reactions by some factor (other than the Uvr^+ gene products) which is added as part of the partially-purified protein preparations. The experiment performed to investigate these possibilities involved the addition of 150 picomoles of ^3H -labelled PM2 DNA (50.1 cpm/picomole; irradiated with a UV dose of $20 J/m^2$) to reaction mixtures consisting of 33 mM MOPS, pH = 7.5, 10.4 mM KCl, 12.5 mM $MgSO_4$, 1 mM EDTA, 0.7 mM DTT, 1 mM ATP, and 3.65 mM NMN (final concentrations). (The concentration of KCl varied with the amount of the protein preparations added but, as discussed below, did not affect the results of this experiment. The concentration shown above is for the reaction in which 0.010 ml each of the UvrA and UvrB/C protein preparation were added.) To these reactions were added equal quantities of between zero and 0.018 ml of UvrA and UvrB/C protein preparation (0.5 and 2.2 mg/ml protein, respectively). The 0.126 ml reactions were incubated 20 minutes at $37^{\circ}C$ and stopped by the addition of ice-cold EDTA to a final concentration of 45 mM and by chilling the tubes on ice. The centrifugation through alkaline sucrose gradients, the determination of the distribution of radioactivity, and the calculation of the number of breaks per molecule are as described in Chapter III, section J and Figure III-1, page 52. The results are shown in Figure III-4. The data shows that the amount of incision increases until approximately 0.010 ml each UvrA and

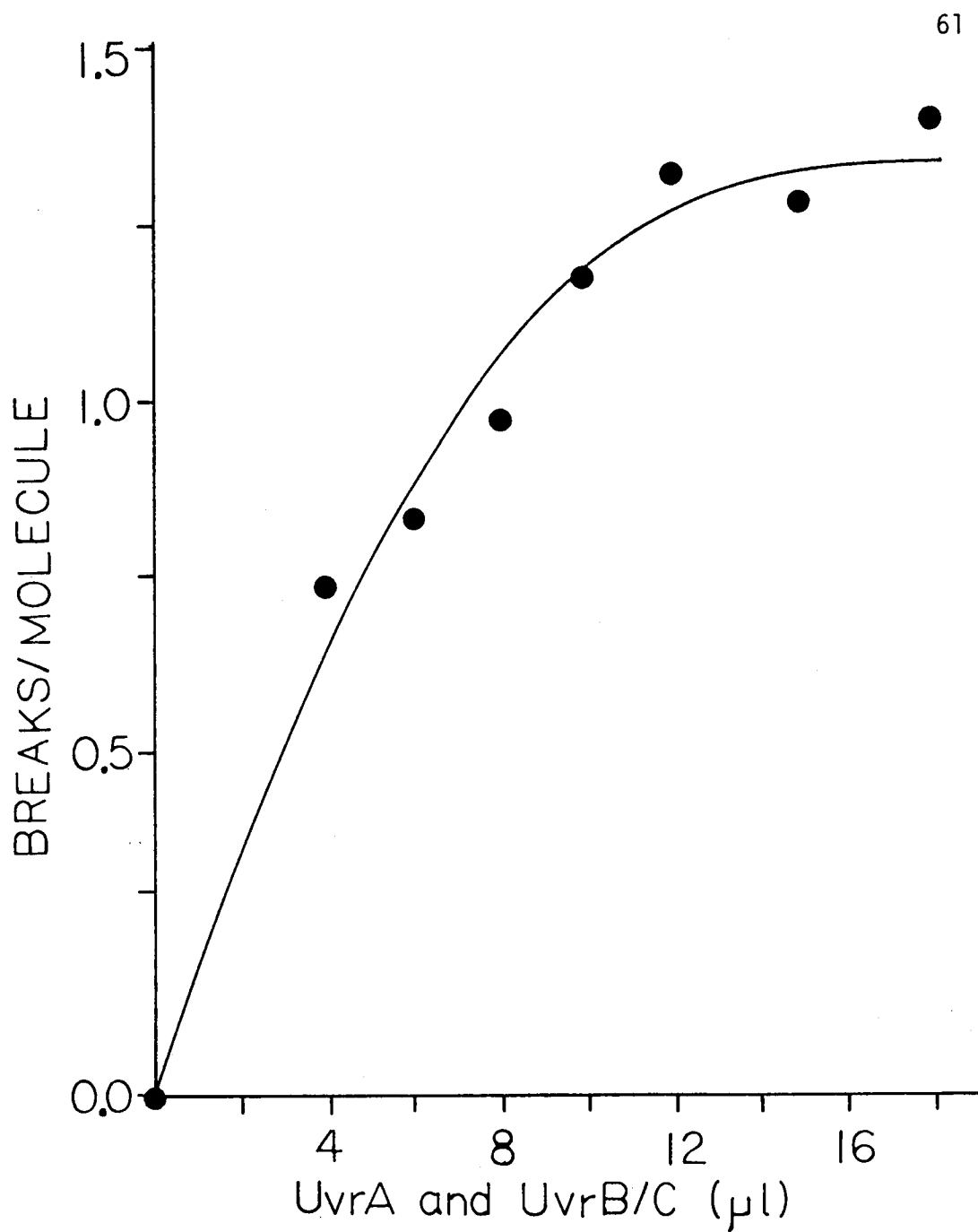


Figure III-4. Effect of the amount of UvrA and UvrB/C protein preparations on the amount of incision.

Equal volumes of UvrA and UvrB/C protein preparations were added in various quantities to reactions containing ^3H -labelled PM2 (irradiated 20 J/m^2). The reaction conditions and methods to determine the number of breaks per molecule were as described in Figure III-1 and the text. The data was corrected for non-UV-specific breakage of the DNA.

UvrB/C preparation (4 and 22 μg protein, respectively) have been added to the reaction. At this point a plateau apparently occurs in which the presence of greater quantities (0.012 to 0.018 ml each) of the protein preparations does not significantly alter the amount of incision. The number of breaks per molecule in this plateau region corresponds to incision at only 24% of the dimers. This indicates that the amount of UvrABC gene products added to the reaction is not the limiting factor.

Although the data of Seeberg (1981) showed that the range of KCl concentrations from 85 to 130 mM (which included the KCl concentrations of this experiment) would still result in greater than 90% of the optimal incisive activity, the experiment described in Figure III-4 was repeated maintaining a constant amount of KCl to determine whether under the reaction conditions used in this study, the KCl concentration would affect the results. The results of this experiment in which the KCl concentration was constant were the same as those seen in the experiment described in Figure III-4 (data not shown).

In order to investigate the effects of the ratio of partially-purified UvrA protein preparation to partially-purified UvrB/C protein preparation, the amount of UvrA protein preparation was increased from zero to 0.020 ml as the amount of UvrB/C protein preparation was decreased so that the total quantity of both protein preparations was 0.020 ml. The experimental conditions were the same as in the previous experiment except that the DNA was 50.1 cpm/picomole and was irradiated with 30 J/m^2 and the UvrA and UvrB/C protein

preparations were 0.5 and 1.3 mg/ml protein, respectively. The results are shown in Figure III-5. The data show the maximum amount of incision occurs when 0.008 ml and 0.012 ml of UvrA and UvrB/C protein preparations are added to the reaction, respectively. Although this experiment does not exclude the possibility that either of these protein preparations may be inhibitory when in excess in the reaction, the simplest explanation is that the amount of UvrA protein preparation limits the reaction when less than 0.008 ml (approximately 4 μ g protein from the UvrA preparation) are added and that the amount of UvrB/C protein preparation limits the reaction when less than 0.012 ml (approximately 16 μ g UvrB/C preparation) are added. These data are consistent with the data shown in Figure III-4.

D. Amount of DNA

It was decided to investigate the relationship between the amount of DNA and the amount of incision in these reactions. An advantage to this approach is that there are fewer serious technical limitations on the amount of DNA than there are (because of the KCl and glycerol) on the amounts of protein preparations that can be added to a reaction (although the specific activity of the DNA does limit how little DNA can be added to the reactions and still be detected). Also, this experiment would address whether, if enough DNA is added to the reactions, eventually a point will be reached when the amount of DNA and number of dimers in a reaction is in excess of the amount that the UvrABC gene products present can handle in a

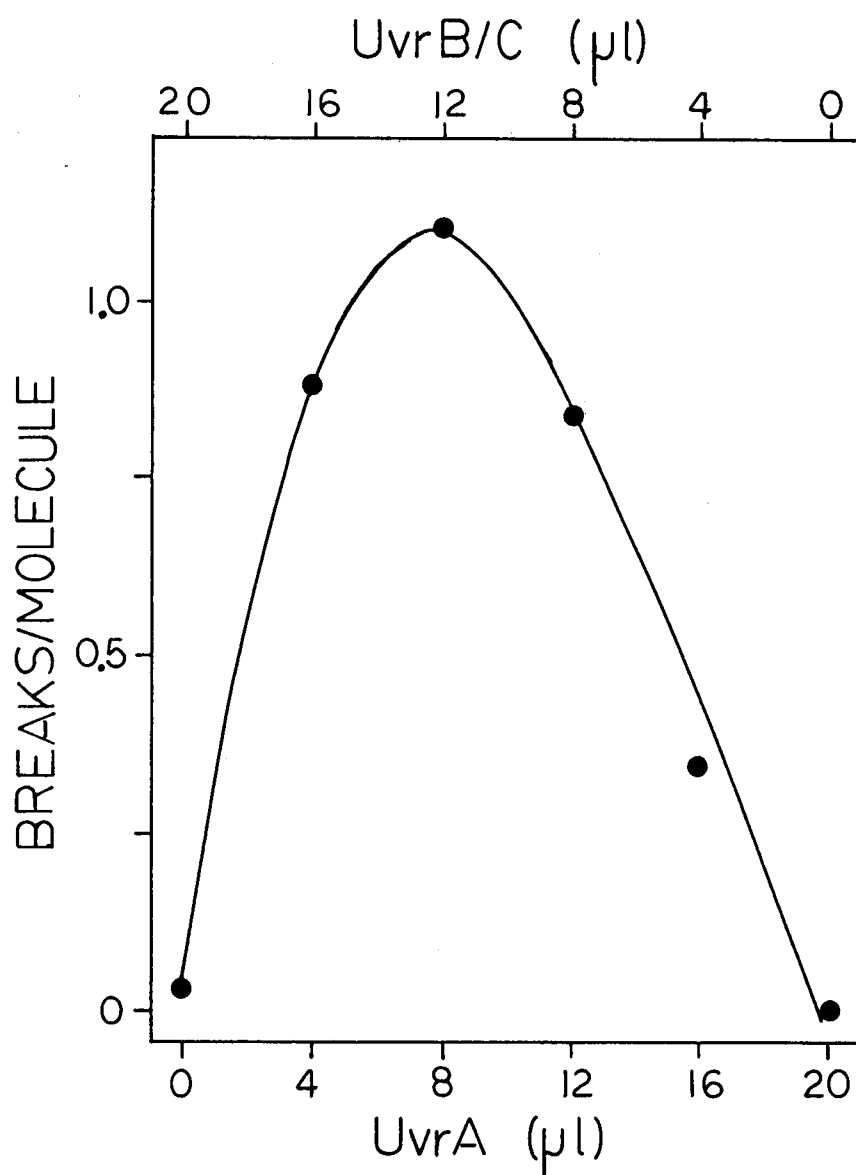


Figure III-5. Effect of the ratio of UvrA to UvrB/C protein preparation on the amount of incision.

^3H -labelled PM2 DNA (irradiated 30 J/m^2) was incubated in reactions containing increasing amounts of UvrA protein preparation (0-20 μl) and decreasing amounts of UvrB/C protein preparation (20-0 μl) such that the total volume of the protein preparations was 20 μl . The reaction conditions and alkaline sucrose gradient centrifugation method of determining the number of breaks per molecule were as described in the text. The data was corrected for non-UV-specific breakage.

given time. The relationship between the efficiency of incision and number of DNA molecules present in the reaction was investigated using these two protocols: (1) keeping the total number of dimers in the reaction constant by varying the number of dimers per molecule or (2) keeping the number of dimers per molecule constant and, thereby, altering the number of dimers in the reaction.

In the following experiment, the total number of dimers in the reaction was kept at a constant value of 4×10^{10} dimers per reaction. This was done by decreasing the amount of irradiation as the amount of DNA increased. For example, in the reactions containing 100 and 800 picomoles of PM2 DNA, the DNA was irradiated with doses of 40 J/m^2 ($= 12 \text{ dimers/molecule}$) and 5 J/m^2 ($= 1.5 \text{ dimers/molecule}$), respectively. The DNA (22.5 cpm/picomoles) was UV-irradiated at a constant concentration and then diluted as required into a reaction mix on ice consisting of 40 mM MOPS, pH = 7.5, 110 mM KCl, 15 mM MgSO_4 , 1.0 mM EDTA, 0.7 mM DTT, 1.3 mM ATP, and 3.65 mM NMN (final concentrations). Quantities of 0.012 ml each UvrA and UvrB/C partially-purified protein preparations (0.5 and 2.2 mg/ml protein, respectively) were then added. The reactions were incubated for 20 minutes at 37°C and stopped by the addition of EDTA to a final concentration of 45 mM and by chilling the reactions on ice. The number of breaks per molecule was determined by the standard method described in Chapter II, section J. The results are shown in Figure III-6a.

The relationship between the number of breaks per molecule and the number of molecules exhibits a smooth curve that resembles an inverse relationship. Because the design of the experiment fixed the

Figure III-6. Effect of the amount of DNA on the amount of incision (constant number of dimers per reaction).

Various amounts of ^3H -labelled PM2 DNA (irradiated at doses of UV inversely proportional to the amount of DNA such that the total number of dimers in the reaction remained constant at 4×10^{10}) was incubated with the partially-purified UvrA and UvrB/C gene products. The reactions were treated as described in Figure III-1 and the text. The data was corrected for non-UV-specific breakage of the DNA.

(a) The data were plotted as the amount of incision versus the amount of DNA in the reaction. (Inset) The efficiency of incision was calculated by dividing the UV-specific number of breaks per molecule by the number of dimers per molecule at that dose of UV-irradiation. (The number of dimers was determined by the experiment shown in Figure III-2.)

(b) The data was replotted as the amount of incision versus the dose of UV-irradiation applied to the DNA.

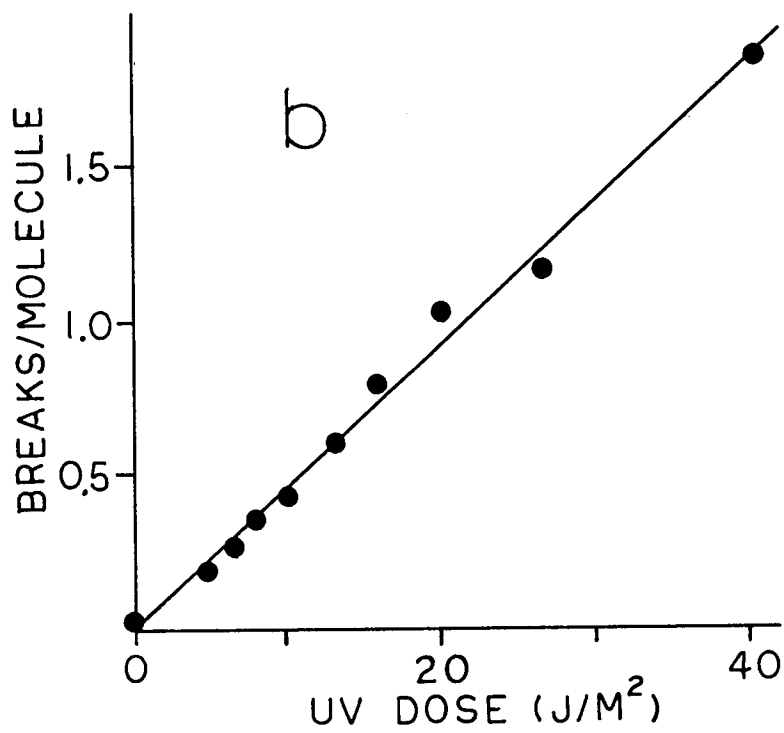
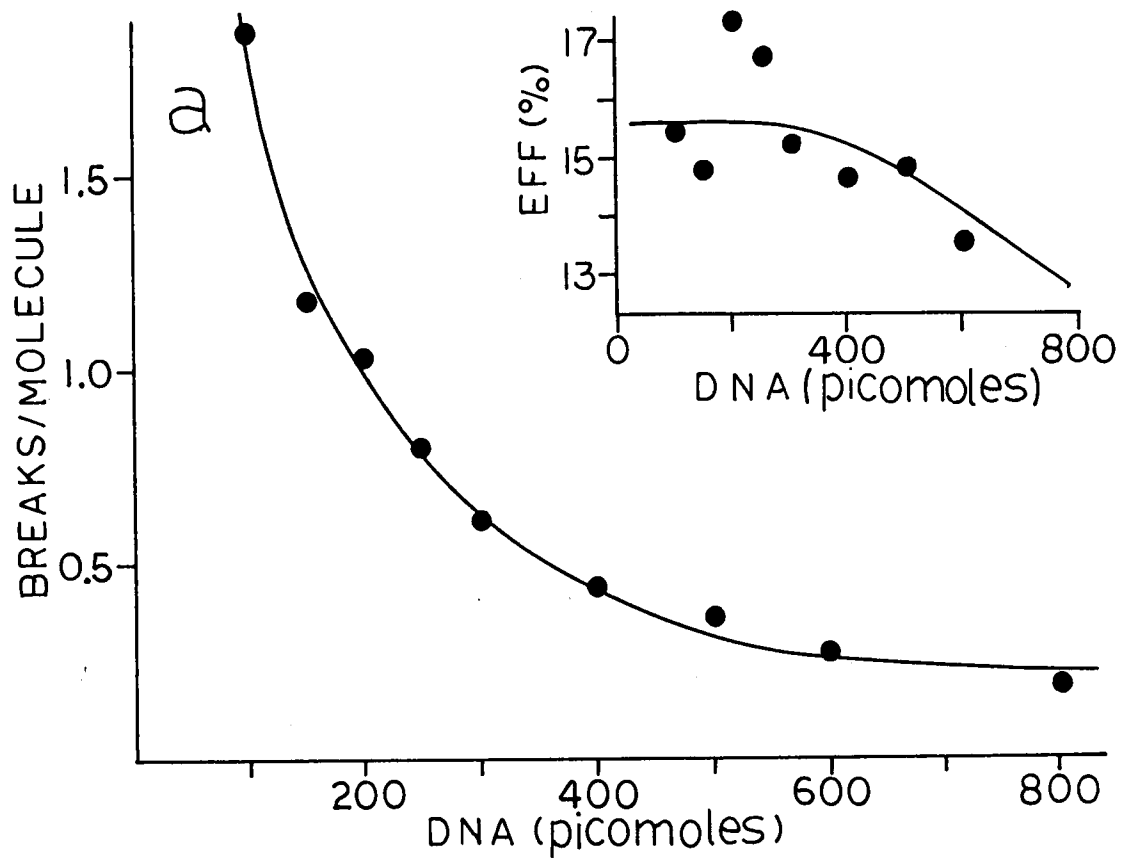


Figure III-6.

UV dose applied to the DNA as inversely proportional to the amount of DNA, the data were replotted (Figure III-6b) with respect to UV dose. This second plot shows a linear relationship with a slope of 0.047 breaks per molecule per J/m^2 --representing an efficiency of incision of 16%. The graph is linear from 5 J/m^2 (800 picomoles DNA) to 40 J/m^2 (100 picomoles DNA).

The percent efficiency of incision in these reactions was calculated by dividing the number of breaks per molecule (corrected for non-UV-specific breakage of the PM2 DNA which was determined by incubating 100 or 800 picomoles of unirradiated DNA in control reactions identical to the experimental ones) by the number of dimers per molecule expected from the applied UV dose (as determined in the experiment shown in Figure III-2, page 53). The results are shown in the inset of Figure III-6a. The calculation of the efficiency of incision is particularly sensitive to the correction for the non-UV-specific breakage of the DNA. The value experimentally determined (from the control reactions) for this non-specific breakage was the same as that defined by the intercept of the line in Figure III-6b. Therefore, the error associated with this correction factor is believed to be insignificant. The calculation of the percent efficiency for each reaction is also very sensitive to small variations in the number of calculated breaks per molecule. The variability in the number of breaks per molecule results from experimental error deriving from the UV dose applied to the DNA, from the measurement of the amounts of DNA, protein preparations, or components of the reaction mixture, or from the alkaline sucrose gradient-scintillation

spectrometric technique of determining the distribution of radioactivity and calculating the number of breaks per molecule. As can be expected, the variability seen in this experiment is greatest with small amounts of DNA and large UV doses. Despite the scatter of the calculated percent efficiency, the data support a relatively constant efficiency of 16% (breaks per dimer) if between 100 and 400 picomoles of DNA are added to the reaction. There does appear to be a decrease in efficiency as the amount of DNA in the reactions is increased from 400 to 800 picomoles of DNA. This decrease represents a change from 16% to 13% efficiency. Because the number of breaks per molecule is primarily a function of the UV dose applied to the DNA and because the design of this experiment was such that a large change in DNA concentration would correspond to only a small change in the UV dose applied to the DNA, there is less sensitivity to changes in the number of breaks per molecule at higher concentrations of DNA (and low doses of UV). This explains why the small decrease in the efficiency of incision as the amount of DNA increases (seen in the inset of Figure III-6a) is not distinguishable in the plot of the the number of breaks per molecule versus UV dose (Figure III-6b).

In the inset of Figure III-6a, the maximum efficiency determined by interpolation to 0 picomoles of DNA that can be supported by a subset of the data is only 20% (determined by the points at 200, 250, 300, and 400 picomoles of DNA per reaction). The two lower points (at 100 and 150 picomoles) argue against the possibility that the efficiency of incision increases markedly with less DNA. The following experiment was performed to consider whether the efficiency

of incision does increase significantly as the amount of DNA in the reaction is decreased. The same protocol as used in the experiment shown in Figure III-6 was employed because, although it is not the best for studying the effects of large amounts of DNA in the reaction, it is very sensitive for small amounts of DNA. The number of dimers per reaction was held constant at 1×10^{10} dimers per reaction. The DNA (22.5 cpm/picomole) was irradiated at a constant concentration of 0.015 mM and then diluted as appropriate so that between 20 and 200 picomoles of DNA were present in each 0.126 ml reaction. The reaction conditions were the same as described in the previous experiment. The results are shown in Figure III-7a. The relationship between the number of breaks per molecule and the amount of DNA added to the reactions is a smooth curve and characteristic of an inverse relationship. The data were replotted (Figure III-7b) with respect to UV dose and the results were linear (correlation = 0.996) with a slope corresponding to a 15% efficiency of incision.

The graph of the calculated percent efficiency of incision versus the amount of DNA in the reactions is shown in the inset of Figure III-7a. The line drawn through these points was determined by linear regression analysis of the data and represents one interpretation of the data. Despite the considerable scatter (probably due mainly to the inaccuracies resulting from the small amounts of DNA and, therefore, small amounts of reactivity), the data show no trends to support any conclusion except that the efficiency of incision is a relatively constant 14-15%. This is a very strong argument against the possibility that the amount of partially-purified UvrA

Figure III-7. Amount of incision of small amounts of DNA.

Various amounts of ^3H -labelled PM2 DNA (irradiated at doses of UV inversely proportional to the amount of DNA such that the total number of dimers in the reaction remained constant at 1×10^{10}) was incubated with the partially-purified UvrA and UvrB/C protein preparations. The reactions were treated as described in Figure III-1 and the text. The data were corrected for non-UV-specific breakage of the DNA.

(a) The data were plotted as the amount of incision versus the amount of DNA in the reaction. (Inset of a) The efficiency of incision was calculated by dividing the UV-specific number of breaks per molecule by the number of dimers per molecule at that dose of UV-irradiation. (The number of dimers per molecule was determined by the experiment shown in Figure III-2.)

(b) The data were replotted as the amount of incision versus the dose of UV-irradiation applied to the DNA.

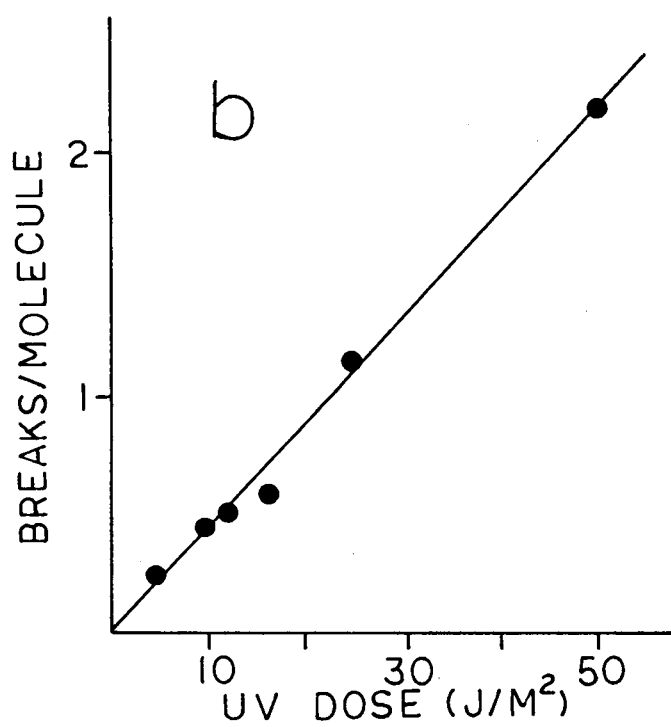
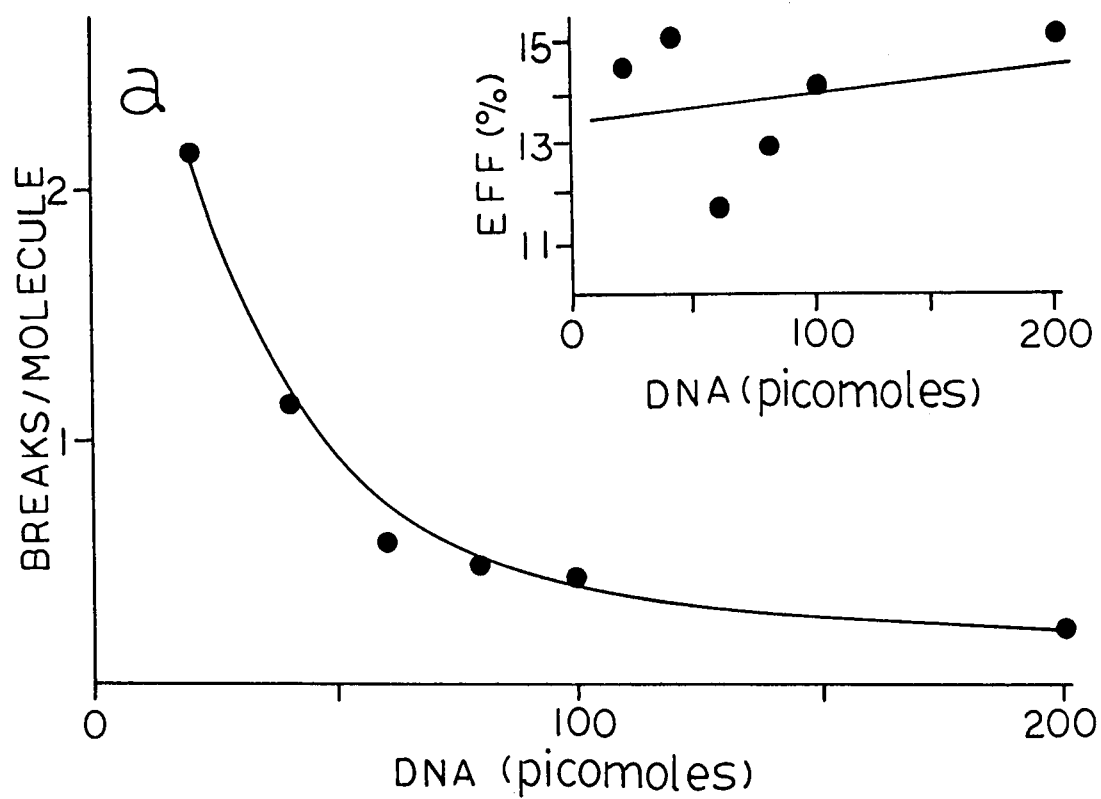


Figure III-7.

and UvrB/C protein preparations in these reactions may be limiting or inhibitory relative to the amount of DNA. Because the efficiency is the same in this experiment as in the previous one, although there are one-fourth as many dimers in the latter, these results also argue against the possibility that the amount of UvrABC gene products in the reaction may be limiting in relationship to the number of dimers in the reaction.

For the second protocol, PM2 DNA (22.5 cpm/picomole) was irradiated with a dose of 10 J/m^2 at a concentration of 0.015 mM. The irradiated DNA was added to reaction mixtures on ice such that between 100 and 800 picomoles of DNA were present in a reaction. Except for this uniform irradiation, the experiment was the same as that described for the experiment shown in Figure III-6, page 66. The results are shown in Figure III-8.

The results strongly support the relationship between efficiency and amount of DNA shown in the inset of Figure III-6a--that the number of incisions per reaction decreases if greater than 400 picomoles of DNA are present. The decrease in efficiency shown here is greater than that obtained under the first protocol (see inset of Figure III-6a). This might indicate an effect resulting from the greater number of dimers per reaction. These three experiments tend to indicate that the number of dimers per reaction is not a limiting factor in the range commonly used in the experiments presented here. In fact, the efficiency of incision of the UvrABC gene products appears to be independent of the number of DNA molecules in the reaction from a range of 20 to 400 picomoles, to be independent of the

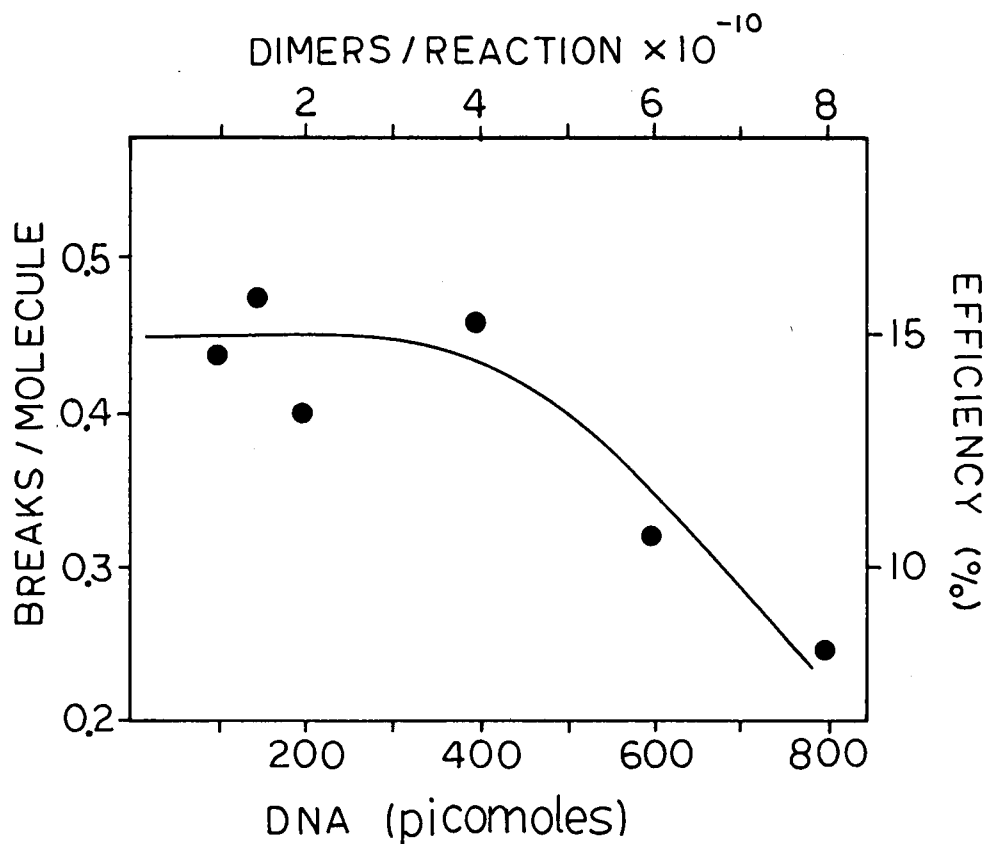


Figure III-8. Effect of the amount of DNA on the amount of incision (constant number of dimers per molecule).

Various amounts of ^3H -labelled PM2 DNA (irradiated with a dose of 10 J/m^2) were incubated with the partially-purified UvrA and UvrB/C protein preparations. The reaction conditions and alkaline sucrose gradient centrifugation method to calculate the number of breaks per molecule were as described in Figure III-1 and the text.

number of dimers per reaction in the range from 1 to 4×10^{10} , and to vary exclusively with the number of dimers per molecule. These results also support a processive mechanism of action of the UvrABC gene products.

E. Amount of ATP

The two cofactors known to be necessary for incision by the UvrABC gene products are ATP and Mg^{++} (Seeberg, 1981; Seeberg and Strike, 1976). In order to investigate whether the amount of incision in a reaction was limited by the amount of ATP, the following experiment was performed. A quantity of 180 picomoles of PM2 DNA (26.8 cpm/picomole, irradiated at a dose of 15 J/m^2) was added to a reaction mixture consisting of 50 mM MOPS, pH = 7.55, 90 mM KCl, 15 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 3.65 mM NMN and between 0 and 7 mM ATP. Quantities of 0.018 ml each UvrA and UvrB/C protein preparations (0.53 and 2.1 mg/ml protein, respectively) were gently stirred into the reaction. The reactions were incubated 20 minutes at 37°C , stopped and processed by the method described in Chapter II, section J. The results are shown in Figure III-9.

The data show a rapid increase in the number of breaks per molecule as the ATP concentration increases from 0 to 2 mM. There was, however, little difference in the amount of incision when between 3 and 7 mM ATP was present in the reaction. Results from other experiments suggest that the data point at 1 mM ATP is anomalously high. The data are not corrected for non-UV-specific breakage; this non-specific breakage (shown in the graph) corresponds to approximately

Figure III-9. Effect of the concentration of ATP on the amount of incision.

³H-labelled PM2 DNA was incubated with the partially-purified UvrA and UvrB/C gene products in reactions containing different concentrations of ATP. The details of the reaction conditions and method (using alkaline sucrose gradient centrifugation) to determine the number of breaks per DNA molecule are as described in Figure III-1 and the text.

(a) The amount of incision was plotted versus ATP concentration. The data were not corrected for non-specific breakage of the DNA.

(b) A double reciprocal plot of the amount of incision and ATP concentration is shown. The data were corrected for non-specific breakage of the DNA.

- Reactions containing PM2 DNA irradiated with 15 J/m².
- ▲ Reactions containing unirradiated PM2 DNA.

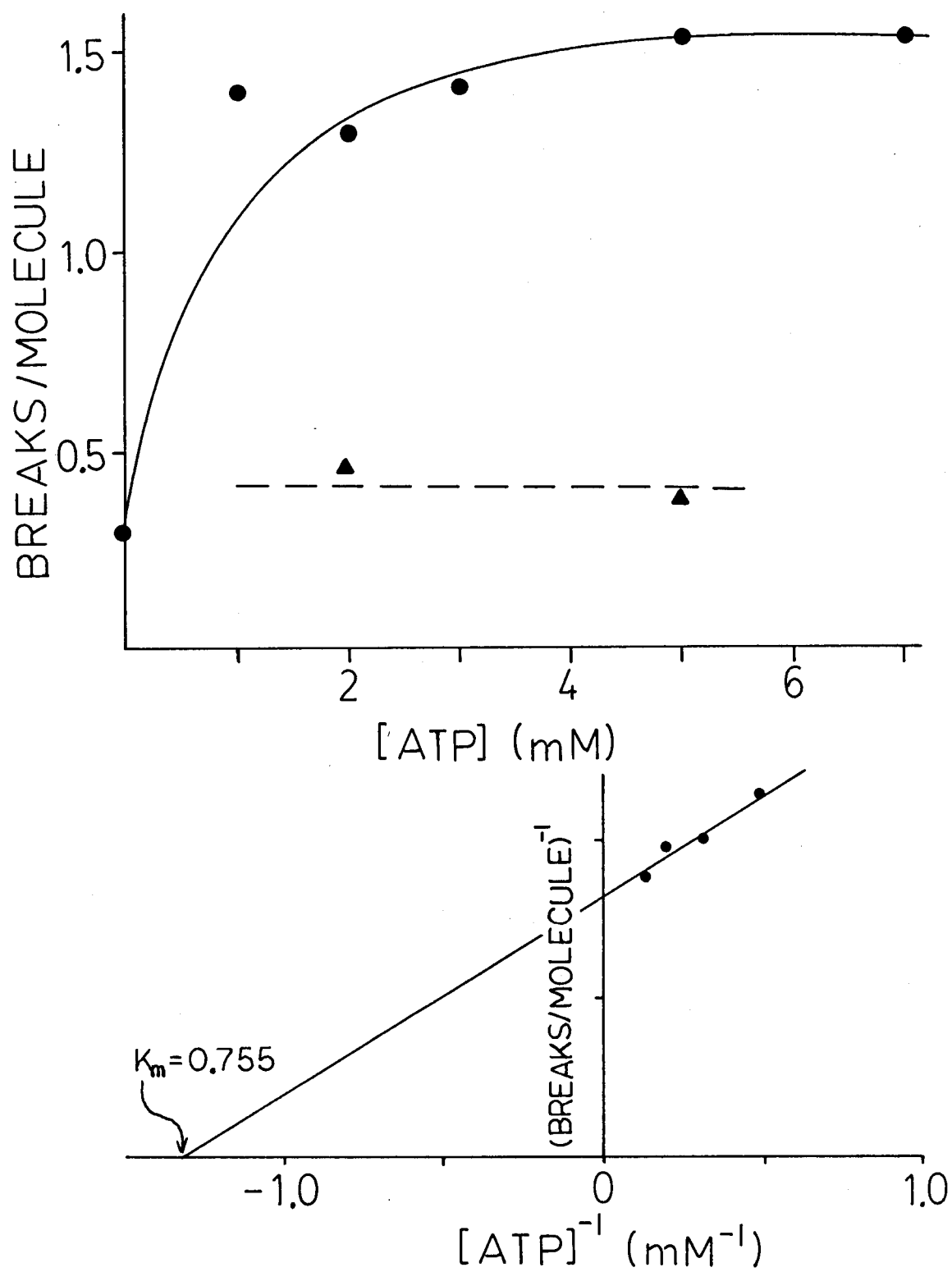


Figure III-9.

0.41 breaks per molecule. Figure III-9b shows a double reciprocal plot of the data from this experiment. The K_m determined from this data is 0.76 mM ATP. Interpolation of the data from either part of Figure III-9 shows that an infinite amount of ATP added to the reaction (assuming adequate magnesium is present) would result in only 27% efficiency of incision. The 1 mM ATP used in the previous experiment is clearly somewhat limiting, but the presence of 7 mM ATP will not enhance the efficiency to 100%.

The amount of magnesium in an ATP-dependent reaction can be limiting, therefore, it was decided to determine whether the presence of more $MgSO_4$ in the reactions would enhance incision. Results of these experiments demonstrated that the amount of magnesium was not limiting to these reactions (data not shown).

F. Addition of the Partially-Purified UvrD Gene Product

In order to determine whether the UvrD gene product would affect the efficiency of the incision of ultraviolet-irradiated DNA by the partially-purified UvrABC gene products in vitro, the following experiment was performed. A 300 picomole quantity of ^3H -labelled PM2 DNA (26.8 cpm/picomole, irradiated with 0 or 10 J/m² of 254 nm light) was added to a reaction mixture consisting of 40 mM MOPS, pH = 7.57, 100 mM KCl, 15.5 mM $MgSO_4$, 0.9 mM EDTA, 0.9 mM DTT, 2.0 mM ATP, and 3.6 mM NMN (final concentrations). In some reactions, 2.4 mM NAD^+ and 0.07 mM of each DNTP were added instead of the NMN. Into this, 0.020 ml each UvrA and UvrB/C protein preparation (0.55 and 2.1 mg/ml protein, respectively) and 0.025 ml of

partially-purified UvrD protein preparation were gently stirred. Control reactions contained buffer mixtures with concentrations of KCl and glycerol equivalent to those used in the preparation of the Uvr⁺ gene products. These 0.126 ml reactions were incubated at 37° C for 20 minutes then stopped by the addition of EDTA to a final concentration of 42 mM and by chilling the reactions on ice. The reactions were diluted 1:2.5 with ice-cold Tris-EDTA (10 mM Tris·HCl, 0.1 mM EDTA) in order to dilute the glycerol to a final concentration of 8%. A 150 µl portion of this diluted reaction was centrifuged through preformed alkaline sucrose gradients. Fractions were collected, the distribution of radioactivity determined, and the number of breaks calculated as described in Chapter II, section J, and Figure III-1, page 52. The results are shown in Tables III-2 and III-3. It can be seen from the data presented in Table III-2 that irradiation of the DNA alone does not break the DNA, but that the addition of the partially-purified UvrA and UvrB/C gene products to the reactions causes breakage at approximately 15% of the dimers. There is considerable non-UV-specific breakage in these reactions, presumably due to contaminating endonucleases in the protein preparations or buffer solutions. The addition of NAD⁺ (a cofactor necessary for break rejoining by *E. coli* ligase (Little et al., 1967) to the reactions results in fewer breaks in both unirradiated and irradiated DNA than in reactions with NMN. This argues that the protein preparations contain some polynucleotide ligase. It should be noted, however, that in these reactions, the addition of NAD⁺ only stimulated the closure of about half of the breaks in the UV-

TABLE III-2

EFFECT OF THE UvrD GENE PRODUCT IN INCISION BY THE PARTIALLY-PURIFIED
UvrA, UvrB, AND UvrC GENE PRODUCTS^a

UvrA (μ l)	UvrB/C (μ l)	UvrD (μ l)	NMN (mM)	NAD (mM)	Breaks per molecule			% Eff. ^d
					-UV ^b	+UV ^b	+ Δ ^c	
0	0	0	3.6	0.0	0.20	0.20	-	-
20	20	0	3.6	0.0	0.52	0.99	0.49	15.3
20	20	0	0.0	2.4	0.16	0.49	0.33	11.0
20	20	25	3.6	0.0	0.48	0.86	0.38	12.7

^aReaction conditions as described in the text.

^bAvg. number of breaks per molecule in unirradiated (-UV) and irradiated (+UV) DNA.

^cuv-specific breakage of the DNA = number of breaks per molecule in UV-irradiated DNA minus the number in unirradiated DNA.

^dPercent efficiency.

TABLE III-3
UV SPECIFIC AND UvrD-DEPENDENT BREAKS PER MOLECULE^a

	-UV ^b	+UV ^b	UV-specific ^c
-UvrD	0.52	0.99	0.47
+UvrD	0.48	0.86	0.38
UvrD-dependent	0.04	0.13	0.09

^a PM2 DNA was incubated with the partially-purified UvrABC gene products and either the partially-purified UvrD gene product or a buffer solution equivalent in KCl and glycerol to the UvrD preparation. Reaction conditions are as described in the text.

^b Average number of breaks per molecule in unirradiated (-UV) and irradiated (+UV) DNA.

^c UV-specific breakage of the DNA = number of breaks per molecule in UV-irradiated DNA minus the number in unirradiated DNA.

irradiated DNA. Because all of the non-UV-specific breaks (in the unirradiated DNA) are rejoined, this may indicate that the breaks formed by the UvrABC gene products are not ligatable. In contrast, it may mean that the amount of ligase present cannot rejoin all the breaks under these reaction conditions. The addition of the partially-purified UvrD gene product may result in fewer breaks per molecule. The number of UV-specific breaks per molecule formed in the DNA in the absence (-UvrD) and presence (+UvrD) of the UvrD gene product are shown in the right-hand column of Table III-3. The number of UvrD-dependent breaks for both unirradiated and irradiated DNA are shown in the bottom row. The UV-damage specific UvrD-dependent "reversal" of damage is thus 0.09 breaks per molecule or 20% of the number of UV-specific breaks per molecule formed in the absence of this gene product. This small amount of UvrD-dependent apparent rejoining may be a consequence of experimental variability among the reactions, may be due to interference by the UvrD gene product with UvrABC-dependent incision, or it may be due to a NAD^+ -independent break rejoining activity associated with the UvrD gene product. The latter is supported by other experiments characterizing the UvrD gene product (N. B. Kuemmerle and W. E. Masker, personal communication). However, clearly the addition of the UvrD gene product does not enhance the in vitro incision measured under these conditions.

G. Incubation Time

In order to determine whether the number of breaks per molecule was limited by the duration of the reactions in which the

partially-purified UvrABC gene products were incubated with the irradiated DNA, the following experiment was performed. A quantity of 180 picomoles of ^3H -labelled PM2 DNA (26.7 cpm/picomole, irradiated with 10 J/m^2 of 254 nm light) was added to a reaction mixture on ice consisting of 40 mM MOPS (pH = 7.57), 102 mM KCl, 15.5 mM MgSO_4 , 1 mM EDTA, 1 mM DTT, 2.0 mM ATP, and 3.65 mM NMN. Into this were gently stirred 0.020 ml UvrA protein preparation (0.55 mg/ml protein) and 0.018 ml UvrB/C protein preparation (2.1 mg/ml protein). The 0.126 ml reactions were incubated at 37°C for various lengths of time and stopped by the addition of ice-cold EDTA to a final concentration of 47 mM and by chilling the reactions on ice. The reactions were diluted with ice-cold Tris-EDTA (10 mM Tris-HCl, 0.1 mM EDTA) so that the final concentration of glycerol was 9% and 0.150 ml were centrifuged through alkaline sucrose gradients. The details of the procedure to determine the number of breaks per molecule in these reactions are presented in Chapter II, section J, and Figure III-1, page 52. The results are shown in Figure III-10.

Although the deviation of these data from a line is not so pronounced that the possibility of a linear relationship can be excluded, results from a similar experiment in which DNA (irradiated with 20 J/m^2) was incubated between 0 and 25 minutes and incision occurred at 22.6% of the dimers after a 20 minute incubation showed a more convex curve (data not shown). The amount of non-UV-specific breakage (as determined by incubation of the UvrABC gene products with unirradiated DNA) appears to be linear with increasing incubation time, but with a slope less than that for the breakage of irradiated

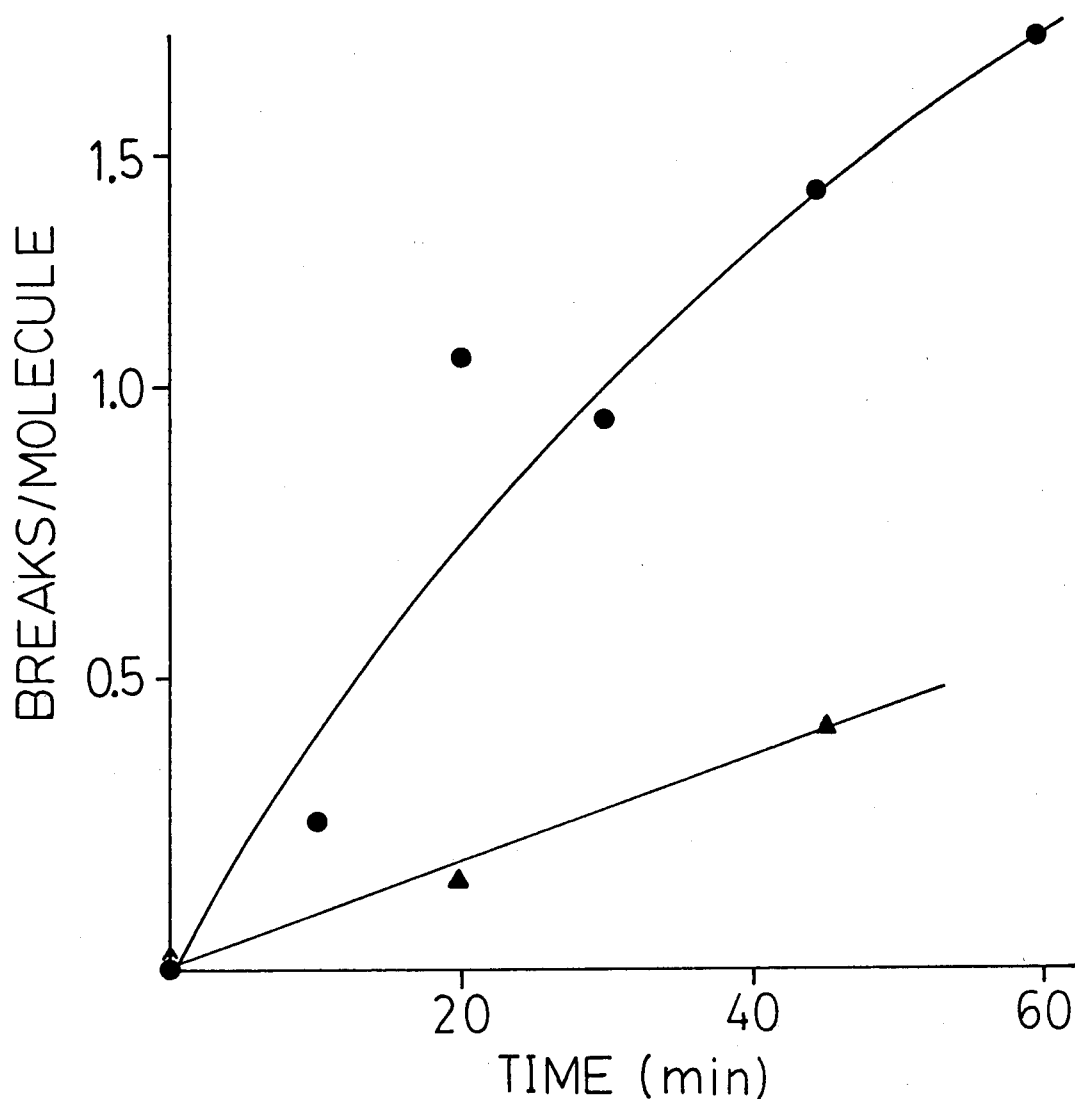


Figure III-10. Effect of the time of incubation on the amount of incision.

³H-labelled PM2 DNA was incubated with the partially-purified UvrA and UvrB/C gene products for various periods of time. The reactions were stopped, centrifuged through alkaline sucrose gradients, and the number of breaks per molecule determined. The details were as described in the text and Figure III-1. ● UV-irradiated PM2 DNA; ▲ unirradiated PM2 DNA.

DNA. This feature could prove useful for reactions containing DNA irradiated with low doses of UV light because long incubation times could amplify the difference between the total number of breaks in the irradiated molecule and the number of breaks in the unirradiated controls and thus give a more accurate measurement of the UV-specific breakage. The experimental error associated with these data (and resulting deviation of the data from the curve) and small number of data points preclude a very accurate drawing of the position of the curve describing the functional dependence of the number of breaks per molecule and the time of incubation. Calculations from the curve which is drawn to best fit these data support that the efficiency of incision after 20 and 45 minutes of incubation is approximately 17 and 35%, respectively. A maximum efficiency is obtained in these reactions when the slope of the curve of the number of breaks per irradiated PM2 DNA molecule is equal to the slope of the number of breaks per unirradiated PM2 DNA molecule versus time. (After the time when these slopes are equal, the efficiency of incision decreases because of an increased amount of non-UV-specific breakage relative to UV-specific breakage of the DNA). The curve shown in Figure III-10 supports that a maximum of 40% of the dimers would be incised if the incubation were continued for 90 minutes.

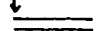
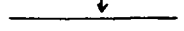
H. Mechanism of Action of the UvrABC Gene Products

The following experiment was performed to address whether pre-incubation of the UvrABC gene products would alter their activity and to further address whether the UvrABC gene products incise irradiated

DNA in a processive or distributive manner. The protocol for this experiment was based upon the addition of various combinations of the UvrABC protein preparations, UV-irradiated ^3H -labelled DNA, and UV-irradiated unlabelled DNA to the reaction mixtures at the beginning or 10 minutes into the incubation period. The quantities used include 0.012 ml each UvrA and UvrB/C protein preparation (0.51 and 2.2 mg/ml protein, respectively), 75 or 150 picomoles of ^3H -labelled PM2 DNA (22.5 cpm/picomoles), and 75 picomoles of unlabelled PM2 DNA. Both DNA mixtures were irradiated with a dose of 20 J/m². The reaction mixtures consisted of 42 mM MOPS (pH = 7.55), 121 mM KCl, 15.9 mM MgSO₄, 1 mM EDTA, 1 mM DTT, 1.3 mM ATP, and 4.65 mM NMN (final concentrations). The reactions were incubated 10 or 20 minutes and then stopped by the addition of ice-cold EDTA to a final concentration of 50 mM and by chilling the tubes on ice. A 0.110 ml portion of these reactions was layered onto preformed (5 to 20%) alkaline sucrose gradients. The gradients were centrifuged, fractions collected, distribution of radioactivity determined, and number of breaks per molecule calculated as described in Chapter II, section J, and Figure III-1, page 52. The results are shown in Table III-4.

In order to address whether the activity of the UvrABC gene products is altered by incubation prior to the addition of the DNA, reactions in which the UvrABC gene products are added simultaneously were compared to reactions in which the gene products were preincubated at 37° C for 10 minutes before the DNA was added. This was done for reactions containing both 75 (Table III-4, reactions A and C) and 150 (Table III-4, reactions B and D) picomoles of ^3H -labelled

TABLE III-4
THE ACTIVITY OF THE UvrABC GENE PRODUCTS AND EFFECT OF PREINCUBATION^a

Sample	Symbolic Representation ^b Time (Min) 0 10 20	Components at T=0			Added at T=10 Min.				% Form I DNA	Breaks Per Molecule ^c
		³ H-DNA (pmoles)	Cold DNA (pmoles)	UvrABC (μ l each)	³ H-DNA (pmoles)	Cold DNA (pmoles)	UvrABC (μ l each)	EDTA (μ l)		
A		75		12				10	32.5	1.12
B		150		12				10	37.8	0.97
C				12	75				22.6	1.49
D				12	150				26.6	1.33
E		75		12					25.2	1.38
F		75		12			12		14.0	1.97
G		75					12		34.5	1.07
H			75	12	75				27.2	1.30
I		75		12		75			15.9	1.84
J		75	75	12					18.4	1.69

^aReaction conditions are as described in the text.

^b $\downarrow$ = Addition of Uvr + UvrB/C gene products; — ³H-labelled PM2 DNA; ~ Unlabelled (cold) DNA; T = Time.

^cThis is not corrected for non-UV-specific.

DNA. It can be seen that preincubation of the UvrABC gene products does considerably enhance (by approximately 40%) the number of breaks per molecule. Another experiment (data not shown) confirmed this and supported that this enhancement primarily was due to the preincubation of the UvrA gene product, but that preincubation of the UvrB/C gene product also increases the amount of incision. A comparison of the results of reactions A with G indicates that preincubation of the DNA for 10 minutes before the addition of gene products does not affect the number of breaks per molecule.

To determine whether a second addition of the UvrABC gene products will increase the number of breaks per molecule, the UvrABC gene products and 75 picomoles of ^3H -labelled DNA were incubated for 10 minutes and then an additional 0.012 ml each UvrA and UvrB/C protein preparation was added (compare reaction F with E). The second addition increased the number of breaks per molecule by approximately 50%. If the results of reaction E (in which the DNA is incubated for 20 minutes after the addition of the UvrABC gene products) and the results of reaction G (in which the UvrABC gene products are added 10 minutes after the addition of the DNA) are corrected for nonspecific breakage (1.38-0.53 and 1.07-0.4, respectively), the resulting sum ($0.85 + 0.67$) is 1.52. The corrected value for the data of reaction F is $(1.97 - 0.53 - (0.53 - 0.4)) = 1.32$. The slight difference between the two values (which may be within experimental error) suggest that exposing the DNA second time to the UvrABC gene products results in a number of breaks per molecule slightly less than the sum of the single exposures. This could mean that there

are only a finite low number of molecules or sites at which incision can occur and that the reaction with two exposures of the UvrABC gene products (and, therefore, more gene products) is limited by this. The greater quantity of gene products resulting from the double exposure (reaction F) may account for some of the increased amount of incision--but because the data presented in Figure III-3, page 56, indicate that the addition of 0.012 ml or more of each of the Uvr⁺ protein preparations causes equally efficient incision, these results are probably not due simply to the presence of twice as much enzyme in the reaction.

It was possible that the relatively low efficiency of incision obtained with the UvrABC gene products was due to sequestering of part of this complex on a fraction of the UV-irradiated DNA molecules. Reaction H involves the incubation of the UvrABC gene products with the unlabelled UV-irradiated DNA for 10 minutes before the labelled DNA was added. The fact that the number of breaks per molecule found with the labelled DNA is equal to the number in a reaction in which the UvrABC gene products are not exposed to any DNA until after 10 minutes of incubation (reaction D) argues that the UvrABC gene products are not sequestered on the DNA or inactivated after an incision. These results are consistent with those shown in Figure III-10, page 83, in which incision is seen to occur after greater than 60 minutes of incubation. The question of whether the mechanism of action of the UvrABC gene products is processive or distributive can be addressed by a comparison of the results of a reaction similar to I in which a large excess of unlabelled DNA is added to

the reaction after incubation with labelled DNA has occurred for a period of time. If the action of the UvrABC enzyme is distributive, the amount of incision obtained would closely approximate the results of reaction A because the unlabelled DNA would immediately be competitive for incision. If the mechanism of action of the UvrABC gene products were processive, however, the amount of incision should be intermediate between that obtained in reactions A and E--with the amount reflecting the time the UvrABC gene products remain on the DNA molecules. A quantity of 180 picomoles of ^3H -labelled DNA (26.8 cpm/picomole, irradiated 10 J/m^2) was added to a reaction mixture which consisted of 38 mM MOPS, pH 7.55, 100 mM KCl, 15 mM MgSO_4 , 1 mM EDTA, 1 mM DTT, 1.9 mM ATP, and 3.4 mM NMN (final concentrations). Into this was gently stirred 0.020 ml of UvrA and 0.018 ml of UvrB/C protein preparations (0.51 and 2.4 mg/ml protein, respectively). One-half of these reactions were incubated at 37°C for increments of time from 0 to 20 minutes and the reactions were stopped by the addition of ice-cold EDTA to a final concentration of 50 mM and by chilling the reactions on ice. (The reactions of this first set are analogous to reactions A (10 minute point) and E (20 minute point).) The other set of reactions were incubated at 37°C for periods of time from 0 to 20 minutes; then 1020 picomoles of unlabelled PM2 DNA (irradiated 10 J/m^2) was added. The incubation of these reactions was continued until a total time of 20 minutes incubation had elapsed. This set of reactions was stopped by EDTA and chilling as above. (The reactions of this second set are analogous to reaction J (0 minute point) and I (10 minute point).) Both sets

of reactions were centrifuged through alkaline sucrose gradients and the number of breaks per molecule determined as described in Chapter II, section J, and Figure III-1, page 52. The results are shown in Figure III-11. It can be seen that in the reactions with ^3H -labelled DNA alone which were stopped at various times of incubation, that the relationship between the number of breaks per molecule and the time of incubation is approximately linear. In contrast, the reactions to which the unlabelled DNA was added and the incubation was continued for 20 minutes show a discontinuous profile such that the number of breaks per molecule is a constant if an excess of unlabelled DNA is added to the reaction at any time between 2 and 12 minutes or between 14 and 20 minutes after incubation of the ^3H -labelled molecule is begun. This can be interpreted to mean that the UvrABC gene products act by a processive mechanism and that the average time of association of at least one of these gene products with a PM2 DNA molecule irradiated with 10 J/m^2 is approximately 10-12 minutes. This estimate is supported by the sudden change in the amount of otherwise constant amounts of incision (presumed to result from the association of the UvrABC gene products with new DNA molecules after 12 minutes of incubation). The apparent processivity of action of the UvrABC endonuclease cannot be proved nor the time of association of the Uvr⁺ gene products with the DNA absolutely ascertained by this experiment alone because of experimental error inherent to the procedure (as demonstrated by the scatter of the data) and because of the absence of an obvious explanation as to why the curves do not intersect at a time corresponding to 20 minutes of incubation of

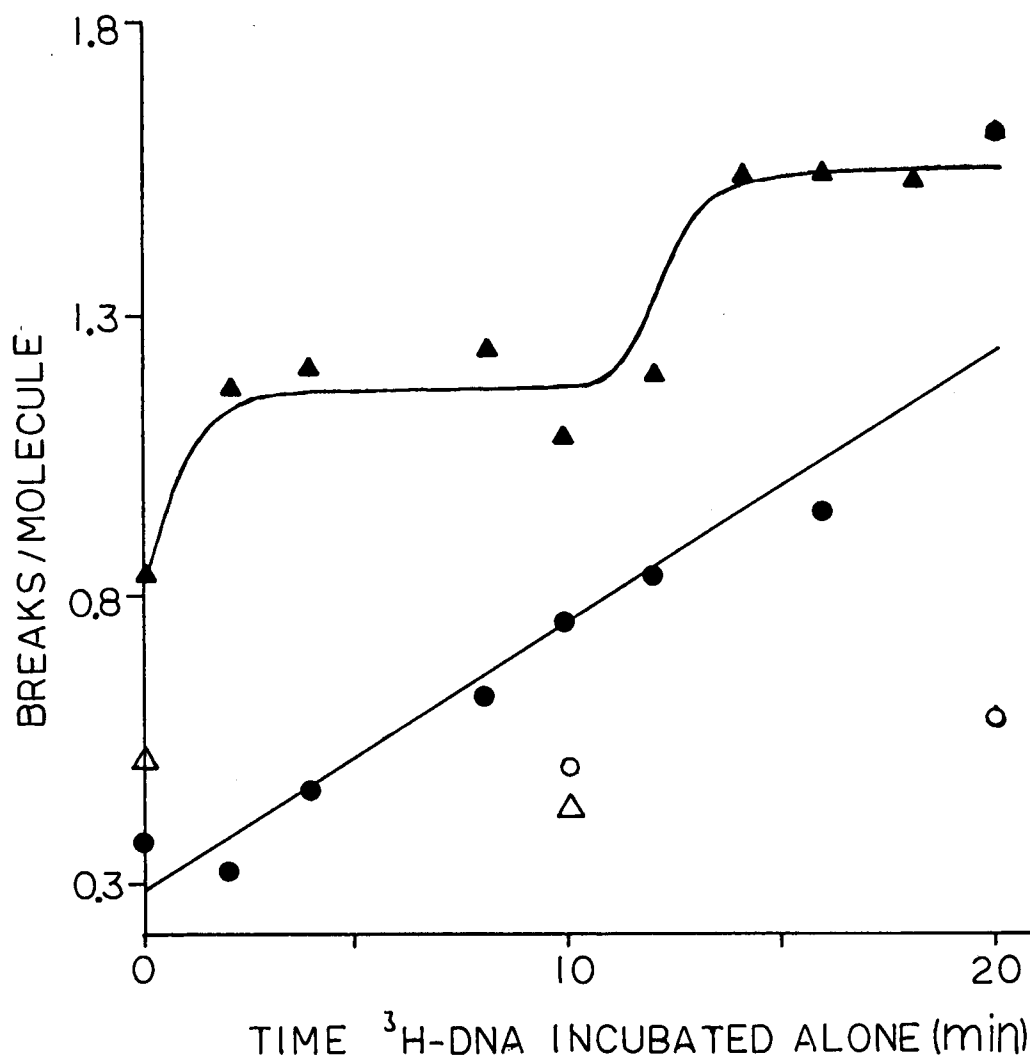


Figure III-11. Investigation of processivity of incision by the UvrABC endonuclease.

A quantity of 180 picomoles of ^3H -labelled PM2 DNA (irradiated 10 J/m^2) was incubated at 37°C with the partially-purified UvrABC gene products and a reaction mixture as described in the text. At various times the reactions were stopped with EDTA and by chilling the tubes on ice (●) or 1020 picomoles of unlabelled PM2 DNA (an amount in excess of the amount this quantity of UvrABC gene products can efficiently incise) was added, incubation was continued until a total of 20 minutes, and the reactions were stopped with EDTA and by chilling the tubes on ice (▲). Both sets of reactions were centrifuged through alkaline sucrose gradients and the number of breaks per molecule calculated as described in Figure III-1 and the text. Open symbols refer to reactions treated as above but containing unirradiated DNAs.

the ^3H -labelled DNA alone. The fact that in one set of reactions, the ^3H -labelled DNA was incubated for a total of 20 minutes (although diluted to 15% of the total DNA after addition of unlabelled DNA at various times of incubation) is probably important for why the two graphs shown in Figure III-11 do not intersect at the 0 and approximately 12 minute points. The continued incubation would permit a small amount of additional incision (UV-specific and non-UV-specific) as compared to the reactions stopped with EDTA which corresponded to them. The amount of additional incision would presumably depend upon the length of time the ^3H -labelled DNA was incubated alone before the unlabelled DNA was added. The experimental error associated with these data and the small number of unirradiated control reactions for this experiment do not allow for definite conclusions concerning whether the amount of non-UV-specific breakage is linear with the time of incubation of the ^3H -DNA alone. Thus, in order for the data to be corrected for non-UV-specific breakage of the DNA, a much more detailed quantitation of the functional dependence of non-specific-breakage and time of incubation under these reaction conditions would have to be done. The relatively linear relationship between breaks per molecule and time of incubation for short (20 minute) periods observed in this Figures II-10, page 84, and Figure III-11 support that there is a constant rate of incision. If this is so and if the data are corrected for the added amount of incision due to the extended incubation time, then the reactions saturated by an excess of unlabelled DNA added between 2 and 12 minutes after the start of incubation show an amount of UV-specific incision

approximately equal to what was observed in the reactions with the ^3H -DNA alone after 10-12 minutes of incubation. The fact that the curves do not intersect at the 20 minute incubation points is consistent with the data shown in Table III-4, page 87, in which the addition of unlabelled DNA after 10 minutes of incubation of the reaction increased the amount of incision. The observation of a probable processive nature for at least one component of the UvrABC endonucleolytic activity may figure prominently in the difficulty to observe high (>50%) efficiencies of incision of UV-irradiated PM2 DNA in vitro under the reaction conditions chosen in this study.

CHAPTER IV

INVESTIGATION OF THE IN VITRO REPAIR OF BACTERIOPHAGE T7 DNA

The first step toward the investigation of the in vitro repair of UV-irradiated bacteriophage T7 DNA using enzymes and extracts obtained solely from E. coli was an optimization of the reaction conditions for the incision of bacteriophage T7 DNA in the presence of a crude extract formed by sucrose plasmolysis of E. coli. This step also serves to help characterize the action of the UvrABC gene products.

A. Ultraviolet Dose Dependence

To investigate the ultraviolet dose dependence of the incision of bacteriophage T7 DNA by the partially-purified UvrA and UvrB/C protein preparations, the following experiment was performed. A quantity of 400 picomoles of ³H-labelled bacteriophage T7 DNA (14 cpm/picomole, UV-irradiated as appropriate) was added to an ice-cold reaction mixture consisting of 45 mM Tris, pH = 7.5, 98 mM KCl, 16 mM MgSO₄, 6 mM 2-mercaptoethanol, 2.0 mM ATP, and 3.6 mM NMN (final concentrations). To this, 0.018 ml each of UvrA and UvrB/C protein preparations (0.54 and 2.1 mg/ml protein, respectively) and 0.040 ml of an extract prepared by sucrose plasmolysis of E. coli strain JC6720 (recB,C) were gently added. These 0.126 ml reactions were incubated at 35⁰ C for 40 minutes and stopped by the addition of EDTA to a final concentration of 48 mM and by chilling the reactions

on ice. The reactions were centrifuged through alkaline sucrose gradients, the fractions collected, the distribution of radioactivity determined, and the weight-average molecular weight determined as described in Chapter II, section K. The results are shown in Figure IV-1. The distributions of radioactivity show that the sizes of the DNA fragments in each of the reactions with irradiated DNA appear to be normally distributed with similar variances. This is interpreted to mean that, under these reaction conditions and time of incubation, the incision of the dimers is random over the molecules and that no subset of the molecules is incised preferentially. The insert in Figure IV-1 shows that the number of breaks per molecule is (like for PM2 DNA, see Figure III-3, page 56) linear with UV dose. The slope of the line corresponds to a constant efficiency of incision of 29.4%.

The use of a biological endpoint to monitor the in vitro repair of bacteriophage T7 DNA made it critical that exonucleases in the reaction be eliminated (or minimized). The amount of time that the reactions could be incubated without significant degradation of the DNA was studied. It was found that the number of UV-specific breaks per molecule increased with longer times of incubation of the reactions at 35° C, however, the number of breaks per molecule in the unirradiated DNA (non-UV-specific breakage) was basically constant for incubation times between 20 and 40 minutes (data not shown). Therefore, it was determined that the reactions could be incubated for as long as 40 minutes without pronounced degradation.

Figure IV-1. Effect of UV dose on the amount of incision of bacteriophage T7 DNA.

³H-labelled bacteriophage T7 DNA (irradiated with various doses of 254 nm light) was incubated in a reaction mixture containing partially-purified UvrA and UvrB/C protein preparations and an extract from sucrose plasmolyzed *E. coli* strain JC6720 (recB,C). After these reactions were centrifuged through alkaline sucrose gradients, the distribution of radioactivity was determined and the weight average molecular weight was calculated. Details are as described in the text (Chapter II, section K, and this section).

○, 0 J/m²; ■, 10 J/m²; △, 20 J/m²; ●, 40 J/m².

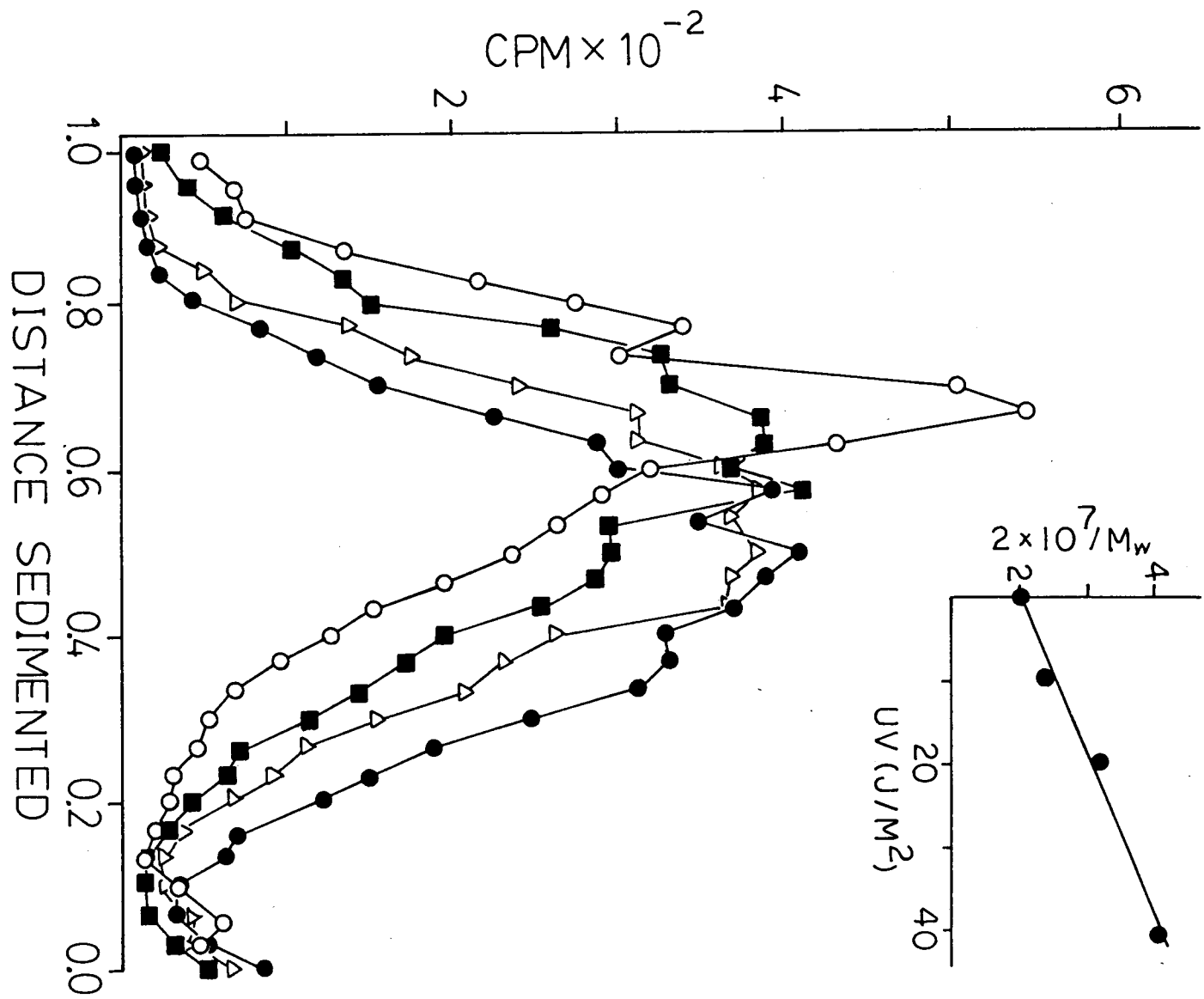


Figure IV-1.

The UvrA protein was isolated from E. coli strain JC6720 (because of its recB,C genotype) in order to reduce the levels of endonuclease V in the in vitro repair reactions. Because the UvrA protein isolated from strain JC6720 has less activity than that isolated from strain KLC658 (in which the uvrA⁺ gene is located on a plasmid), twice as much of the former was used in a reaction. The efficiency of UV-specific incision in the reactions with a double quantity of the UvrA protein isolated from strain JC6720 was slightly less than those reactions with a single quantity of the UvrA protein isolated from strain KLC658, but the amount of non-UV-specific breakage in the former was only 25% of the latter (data not shown).

B. Investigation of the Activity of the UvrABC Protein

Preparations and the "Repair" Extract in the

In Vitro Repair Process

In order to determine whether there were other components in the partially-purified UvrA and UvrB/C protein preparations which could complete repair and whether the extract prepared from sucrose-plasmolyzed E. coli could significantly incise UV-irradiated DNA, the following experiment was done. ³H-labelled bacteriophage T7 DNA was treated as in the experiment described in Chapter IV, section A (and Figure IV-1) with the exception that, in some cases, 0.24 mM NAD⁺ and 0.07 mM of each dNTP were substituted for the NMN in order to allow the completion of repair. Control reactions contained buffer mixtures with concentrations of KCl (and glycerol) equivalent to those of the Uvr⁺ gene products or the extracts. The results are

shown in Table IV-1 and Figure IV-2. Figure IV-2a shows the reactions containing both the UvrABC gene products and the extract prepared from sucrose-plasmolysis of *E. coli*. It can be determined from this, that in the presence of NMN, the irradiated DNA (reaction D) is reduced to approximately half its original molecular weight (reaction C, see Table IV-1). The presence of NAD^+ and dNTPs in the reaction (E) restores the molecular weight of the irradiated DNA back to that of the unirradiated control (reaction C).

The data plotted in Figure IV-2b represent those reactions containing either the UvrABC gene products (reaction A, + NMN; reaction B, + NAD^+ + dNTPs) or the *E. coli* crude extract (reaction F, + NMN). By comparing the reaction with the UvrABC gene products but no extract (reaction A) to the reaction also containing the extract (reaction D), it can be seen that there are considerably more breaks per molecule (about twice as many) in the former. This may indicate that some component in the extract either inhibits incision or, more likely, can rejoin breaks even in the presence of NMN.

A comparison of the distribution of radioactivity in the reactions with the UvrABC gene products and NAD^+ + dNTPs but no extract (reaction B) with the reaction with the UvrABC gene products, NAD^+ , dNTPs, and the crude extract from *E. coli* (reaction E) shows that, in the presence of NAD^+ (+ dNTPs), the breaks formed by the UvrABC gene products can be rejoined, but the original molecular weight is not completely restored unless the extract is also present in the reaction. From the molecular weights of the DNAs in these reactions (see Table IV-1), it can be calculated that in the absence of the

TABLE IV-1

INVESTIGATION OF THE REPAIR PROPERTIES OF THE UvrA AND UvrB/C GENE PRODUCTS AND
THE EXTRACT PREPARED FROM SUCROSE PLASMOLYZED *E. COLI*^a

Reaction	UV ^b	NMN ^c	UvrABC proteins	Uninfected extract	T7 _{3,5,6} -infected extract	M _w ⁷ (x2x10 ⁻⁷ Daltons)	bks/mol ^d	% efficiency
A	+	+	+	-	-	1.448	2.24	67.5
B	+	-	+	-	-	3.142	0.50	14.9

C	-	+	+	+	-	4.698	0	-
D	+	+	+	+	-	2.172	1.16	35.0
E	+	-	+	+	-	4.574	0.03	0.8
F	+	+	-	+	-	4.008	0.17	5.2

G	-	+	+	-	+	3.181	0	-
H	+	+	+	-	+	1.931	0.65	28.8
I	+	-	+	-	+	3.270	-0.03	-
J	+	+	-	-	+	2.426	0.31	13.8

^aReaction conditions are as described in the text.

^bUV dose = 40 J/m²

^cNMN prevents ligation, in the reactions without NMN, NAD⁺ and dNTPs were added to allow the completion of repair.

^dThe number of breaks per molecule are calculated with respect to reaction C.

Figure IV-2. Investigation of the activity of the UvrABC gene products and the "repair" extract in the in vitro repair reactions.

³H-labelled bacteriophage T7 DNA was incubated with the partially-purified UvrA and UvrB/C protein preparations, an extract prepared from sucrose plasmolysis of E. coli strain JC6720, and either NAD⁺ and dNTPs or NMN. The reaction conditions, alkaline sucrose gradient centrifugation, and determination of the distribution of the radioactivity are as described in the text.

(a) Reactions containing both UvrABC gene products and the extract from sucrose plasmolyzed E. coli.

- C ■ UV = 0 J/m², + NMN
- D ● UV = 40 J/m², + NMN
- E ○ UV = 40 J/m², + NAD⁺ + dNTPs

(b) Reactions containing either the UvrABC gene products or the extract from sucrose plasmolyzed E. coli, UV = 40 J/m².

- A ■ + UvrABC, + NMN
- B ○ + UvrABC, + NAD⁺ + dNTPs
- F ● + Extract, + NMN

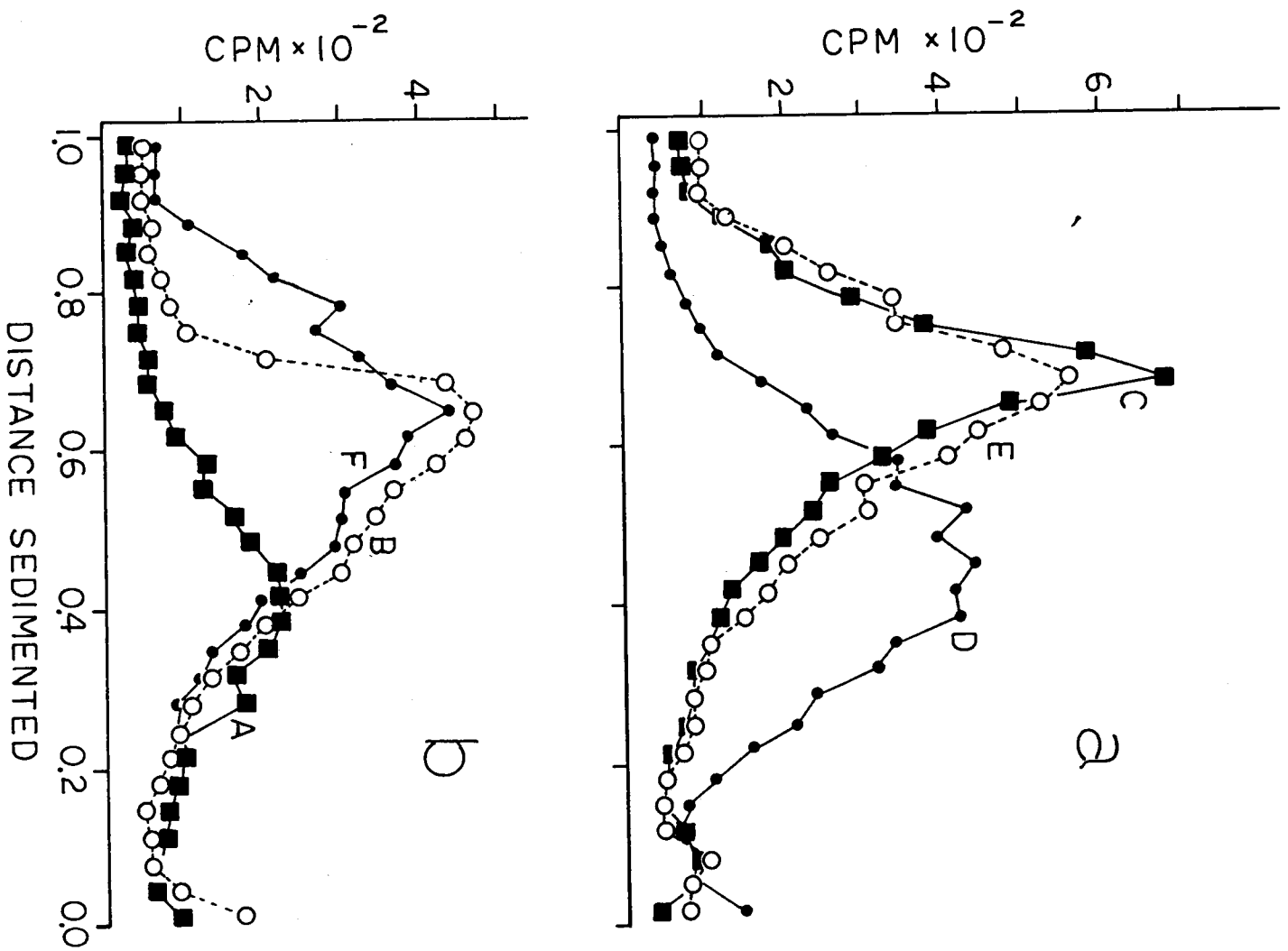


Figure IV-2.

extract approximately one-fourth of the incision breaks were not re-joined by components of the UvrABC protein preparations (in the presence of NAD^+ and dNTPs). These results suggest that the partially-purified UvrABC protein preparations may contain some polynucleotide ligase--or other components for enzymatic repair (the procedure used will not distinguish whether these rejoined breaks represent repaired sites or sites where "abortive ligation" (ligation of the incised DNA such that the damage remains) has occurred). These results are consistent with those of the experiment in which the effect of the UvrD gene product was investigated (Chapter III, section F, and Tables III-2 and III-3, pages 80 and 81).

For the reactions containing NMN and the extract, a comparison of the radioactivity distributions of the reaction without the UvrABC gene products (reaction F) with the reaction which also contained the UvrABC gene products (reaction D), shows that the extract alone can cause very few breaks in the irradiated DNA (approximately 15% as many as in the reactions which also contain the UvrABC gene products).

C. In Vitro Repair of Bacteriophage T7 DNA

In order to investigate the completion of repair as measured by a biological as well as a physicochemical endpoint, ^3H -labelled bacteriophage T7 DNA (14.9 cpm/picomole) was used in the following experiment. A quantity of 1250 picomoles of ultraviolet-irradiated bacteriophage T7 DNA was diluted into a reaction mixture consisting of 48 mM Tris·HCl, pH = 7.5, 95 mM KCl, 16 mM MgSO_4 , 6 mM 2-

mercaptoethanol, 2.0 mM ATP, 0.25 mM NAD^+ , and 0.07 mM of each dNTP (final concentrations). To the experimental ("repaired") reactions were added 0.045 ml each UvrA and UvrB/C protein preparation (0.58 and 1.8 mg/ml protein, respectively) and 0.100 ml crude extract prepared by the sucrose-plasmolysis of *E. coli* strain JC6720 (*recB,C*). To the control ("not repaired") reactions were added 0.045 ml each of solutions of chromatography buffer B (see Chapter II, section F) containing concentrations of KCl and glycerol equivalent to those in the UvrA and UvrB/C protein preparations and 0.100 ml of the lysis buffer used to make the extract from the *E. coli* cells. These 0.315 ml reactions were incubated for 40 minutes at 35° C and stopped by the addition of ice-cold EDTA to a final concentration of 45 mM and by chilling the reactions on ice. (Smaller (0.126 ml) reactions of the same proportions but containing 3.65 mM NMN instead of the NAD^+ and dNTPs were treated as above and used to monitor physico-chemically the formation of incision breaks in the DNA by the UvrABC endonuclease.) The reactions were dialyzed for 6 hours each against 2000 X volume Tris-EDTA (25 mM Tris-HCl and 0.1 mM EDTA) and 25 mM Tris-HCl, pH = 7.0. Immediately thereafter the *in vitro* packaging step was performed. The 0.030 ml *in vitro* packaging reactions consisted of 0.21 μl of an extract prepared from T7_{4ts}-infected cells (Dodson et al., 1982), 4.28 mg/ml BSA, 3.7 μM MgCl_2 , 2.9 μM spermidine, 15 μl of an extract prepared from bacteriophage T7-infected *E. coli* cells and partially-fractionated by sedimentation through neutral sucrose, 5 μl capsids plus dextran 10 (see Chapter II, section I) and 6.4 μl DNA. The reactions were incubated for

1 hour at 30° C, and stopped by the addition of 1 ml of ice-cold low-salt T7 diluent. The reactions were diluted and plated using E. coli strain TN207 (uvrA) as the indicator bacteria. The plates were incubated at 43° C for 3 hours and the number of plaques which resulted were determined. Because of possible dilution and loss of DNA in the dialysis step of this procedure, the number of plaques counted for each reaction was corrected by a dilution factor obtained by determining the amount of radioactivity associated with a 0.120 ml volume of the in vitro repair (or control) reactions after filtration of the acid-precipitated DNA through glass fiber filters (this procedure is described in Chapter II, section L). The results of the in vitro packaging experiment are shown in Figure IV-3. From this it can be seen that there is no significant difference in the survival of the UV-irradiated in vitro repaired DNA and the unrepaired DNA. In other attempts to determine whether there would be enhanced survival of UV-irradiated bacteriophage T7 DNA packaged in vitro after repair in vitro with the partially-purified UvrABC gene products, the discontinuity in the survival of unrepaired DNA as shown in Figure IV-3 did not occur. For this reason and because the graphs of repaired and nonrepaired DNA are so similar in shape and magnitude as to be considered within experimental error, this discontinuity is not believed to be biologically significant. In general for the experiments investigating the in vitro repair of bacteriophage T7 DNA by this procedure, the number of phage resulting from the packaging of unrepaired DNA was approximately twice as many (regardless of UV dose) as the number resulting from the packaging

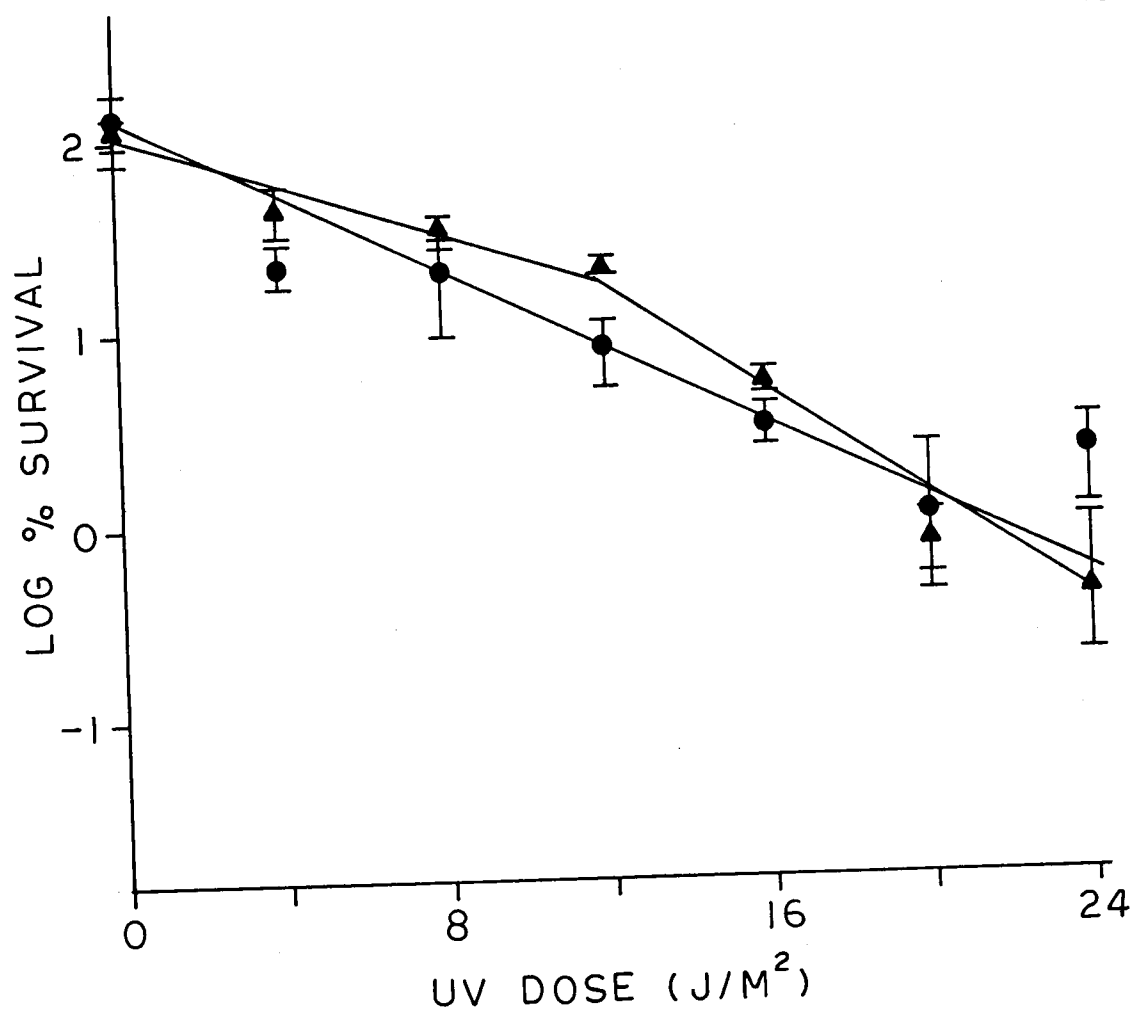


Figure IV-3. Biological endpoint for monitoring in vitro repair of T7 DNA.

³H-labelled bacteriophage T7 DNA was incubated in reactions containing the partially-purified UvrA and UvrB/C gene products and an extract of *E. coli* strain JC6720 (*recB,C*) prepared by sucrose plasmolysis. The details are described in the text in Figure I-2, Chapter II, section K, and this section. The DNA, after extensive dialysis, was packaged in vitro. The resulting phage were plated using *E. coli* strain TN207 (*uvrA*) as the indicator bacteria. The number of resulting phage was corrected for dilution of the DNA during dialysis by a method described in the text. The data represent averages of four in vitro packaging reactions (X2 plates each) (and the standard deviations) for each condition.

"Control reactions" containing irradiated DNA but buffer solutions instead of the UvrABC protein preparations or extract.

"Repaired reactions" containing irradiated DNA, the UvrA and UvrB/C protein preparations, and the extract.

of DNA exposed to the UvrA and UvrB/C protein preparations and extract from sucrose-plasmolyzed E. coli. (This is not evident in Figure IV-3 because the data are plotted as the logarithm of percent survival relative to the repaired or unrepaired unirradiated DNA, as appropriate). The reactions to which the partially-purified UvrA and UvrB/C protein preparations and the extract prepared from sucrose plasmolyzed E. coli were added contained nucleases which completely degraded the DNA within 30 hours. This nuclease activity probably contributed to the lower number of viable phage produced in these reactions.

To determine whether the molecular weight of the DNA in this experiment was restored to its original value after being incubated with the partially-purified UvrA and UvrB/C gene products and extract from sucrose plasmolyzed E. coli, the DNA was centrifuged through alkaline sucrose gradients (as described in Chapter II, section K). The resulting distributions of radioactivity for DNA exposed to 16 J/m^2 ultraviolet light are shown in Figure IV-4. In this figure, it can be seen that incubation of the irradiated DNA with the UvrA and UvrB/C gene products, the extract from sucrose-plasmolyzed E. coli, and NMN to prevent rejoining of the incisions formed in the DNA (reaction C) caused breakage of the DNA such that the average molecular weight was half of that of the control reaction in which buffer solutions were added instead of the gene products or extract (reaction A). One-half of the weight average molecular weights for the DNA in reaction A ("not repaired"), B ("repaired"), and C ("incised") were 5.55×10^6 , 4.92×10^6 , and 2.71×10^6 , respectively. The efficiency of incision calculated from these results is 66.7%. By

Figure IV-4. Biophysical endpoint for monitoring in vitro repair of T7 DNA.

³H-labelled bacteriophage T7 DNA was incubated with the partially-purified UvrA and UvrB/C gene products and an extract of E. coli strain JC6720 (recB,C) prepared by sucrose plasmolysis. The details are as described in the text in Figure I-2, in Chapter II, section K, and in this section. The DNA after extensive dialysis, was centrifuged through alkaline sucrose gradients. The distribution of radioactivity and calculation of the weight average molecular weight are as described in Chapter II, section K.

A ● "Control reactions" containing buffer solutions (with appropriate salt and glycerol concentrations) instead of the UvrABC gene products or extract.

B ○ "Repaired reactions" containing the UvrABC gene products and the extract from sucrose plasmolyzed E. coli.

C ■ "Incised reaction"--the same as B except containing NMN instead of NAD^+ + dNTPs.

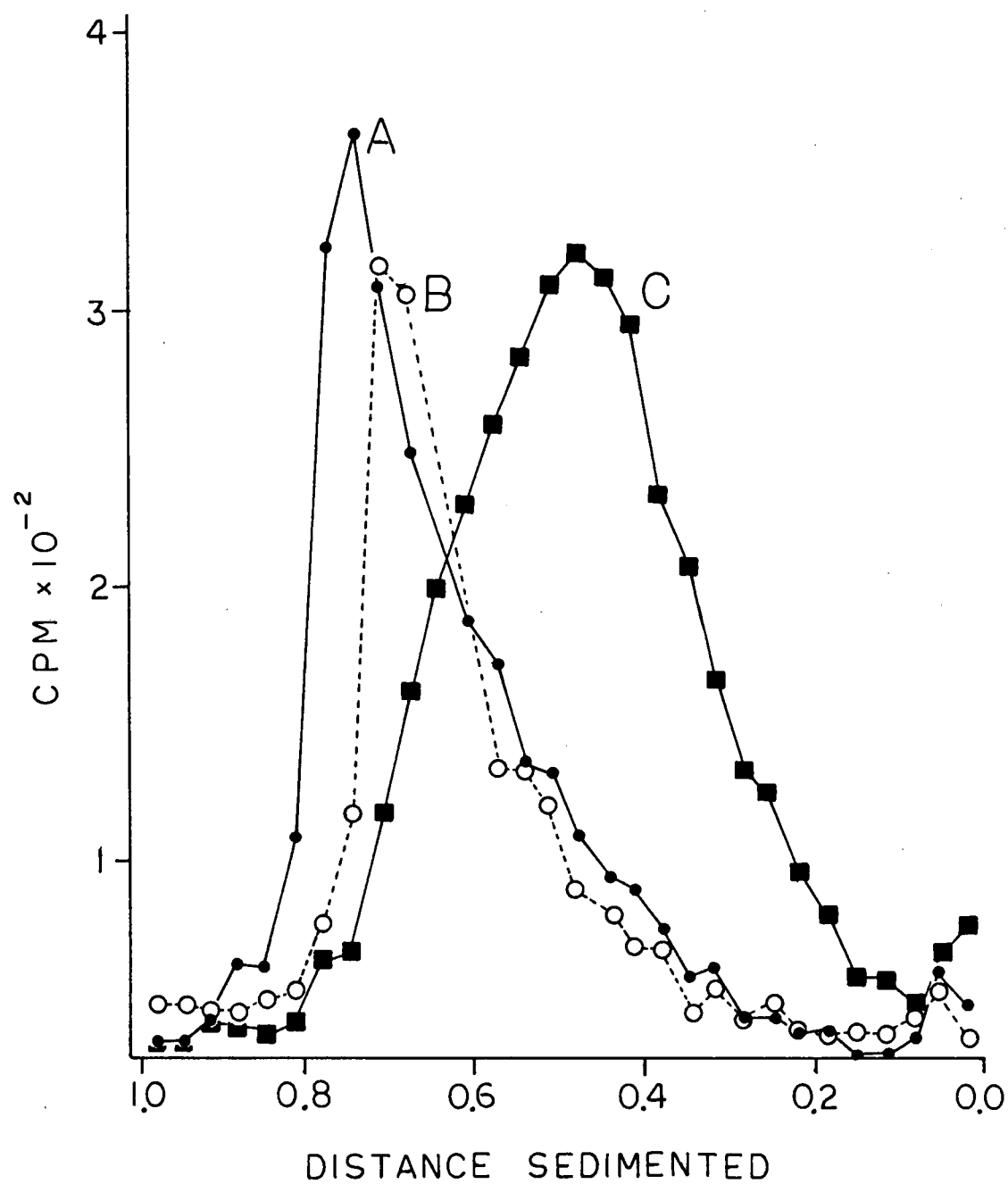


Figure IV-4.

comparing the "repaired" (B) to the "unrepaired" reaction (A), it can be seen that the molecular weight is 90% restored after incubation of the DNA with the UvrABC gene products and an extract prepared by sucrose plasmolysis of E. coli. The inability (under these reaction conditions) of this in vitro repair procedure to restore the biological activity of UV-irradiated bacteriophage T7 DNA despite the relatively efficient incision and considerable break rejoining as seen in Figure IV-3 and IV-4 argues that the reactions are probably limited by some factor other than incision.

CHAPTER V

DISCUSSION

In Chapter III, section B and Table III-1, page 57, a number of possible reasons were outlined for the observation that the efficiency of incision (breaks per dimer) of UV-irradiated PM2 DNA incubated with partially-purified UvrA and UvrB/C protein preparations is only about 20-40% (e.g., see Figure III-2, page 53). During the course of this investigation of the incision in vitro by the UvrABC gene products, most of these points were addressed. It was found that under the reaction conditions employed in this project, variation in the amount of DNA (between 20 and 400 picomoles), the amount of UvrABC gene products (between 0.012 and ≥ 0.018 ml each UvrA and UvrB/C protein preparation), the amount of ATP (between 2 and ≥ 7 mM), the amount of Mg^{++} and KCl, or the number of dimers per reaction (between 1 and 4×10^{10}) per 0.126 ml reaction apparently did not influence the amount of incision performed by the partially-purified UvrA and UvrB/C gene products (Figures III-4, 6, 7, 8, and 9, pages 61, 66, 71, 73, and 76, respectively). In a reaction in which 180 picomoles of PM2 DNA and 0.018 ml of the UvrA protein preparation are added, calculations show that there are 275 UvrA protein molecules per PM2 DNA molecule--corresponding to 1.3×10^5 UvrA protein molecules per E. coli genome equivalent. (This assumes that the recoveries and protein concentrations obtained as part of this study for partial purification are close to those obtained by Seeberg and Steinum (1982) in their purification of the UvrA protein.) The

requirement for such a large amount of protein, when compared to the fact that in vivo there are only 20-100 copies of the UvrA protein per E. coli cell (Sancar et al., 1981a; Seeberg and Steinum, 1982), reflects the limitations of the in vitro repair reaction in mimicking the situation in vivo.

One possible explanation of the need for a large amount of the UvrABC gene products may be that the method and time that it takes to partially-purify these gene products and the method of storing them may result in a significant decrease in their activity. In support of this, the UvrA protein was found to have an approximately 20% greater activity (in complementing the UvrB/C gene products in vitro to incise irradiated DNA) with 6 hours of isolation. (The complementation activities of the UvrA and UvrB/C protein preparations were, however, constant for greater than one month after storage in glycerol in a -20°C freezer or liquid nitrogen, respectively.) A second possible explanation for the need for a large amount of the partially-purified UvrA and UvrB/C protein preparations may be that the incision reactions may be limited by some component or cofactor other than the UvrABC gene products, Mg^{++} or ATP. The data shown in Tables III-2 and 3, pages 80 and 81, indicates that the UvrD gene product does not enhance the amount of incision when added to these in vitro reactions. The data (not shown for reactions with PM2 DNA; Figure IV-2 and Table IV-1, pages 102 and 103, respectively for T7 DNA) indicate that the addition of a crude extract prepared by sucrose-plasmolysis of E. coli cells does not enhance incision either. However, the reaction conditions and

procedures used in this investigation may not be optimal for the identification of a missing component or cofactor, if there is one.

One possible reason for the low efficiency of incision which might also be dependent on the amount of UvrA and UvrB/C protein preparations added to the reaction was that there are inhibitory factors in the protein preparations. The data shown in Figures III-4 to 8, pages 61, 64, 66, 71, and 74, do not support this. The data shown in Figure III-4, page 61, support the idea that there is no decrease in incision when up to 0.018 ml each of the UvrA and UvrB/C protein preparations are added to the reactions used to monitor incision. The data shown in Figures III-6-8, pages 66, 71, and 74, argue more strongly against the presence of inhibition factors in the protein preparations because the efficiency of incision is not decreased at low concentrations of DNA (when the ratio of the amounts of gene products and, therefore, possible inhibitory factors for DNA is greatly increased).

It was also found that preincubation of the UvrABC gene products (particularly the UvrA gene product) resulted in a greater amount of incision in the reactions (see Table III-4, page 87). The increase in efficiency occurred whether the DNA was in the reaction during the preincubation or not and the amount of incision was approximately 40% greater than the amount observed for reactions in which the DNA and UvrABC gene products were incubated together for the same period of time without preincubation of the gene products. These observations argue that the enhanced incision is not due just to the increased time that the individual gene products have (because

of preincubation) in order to associate with the DNA or locate the damage. They also support the suggestion that it is more likely that the enhanced incision is due to some interaction between the various components of the UvrABC endonuclease. There are several postulates concerning the ways in which the gene products can interact to alter the efficiency. One way is that one or more of the gene products may be modified (perhaps proteolytically) by one of the other gene products. In support of this is the fact that Sancar and his colleagues (1981a) observed two protein bands in agarose gels when purifying the UvrA gene product. These two similar "forms" of the UvrA protein differed in their amino termini. Although the two forms might represent an artifact of the isolation procedure, Sancar and his coresearchers proposed that the forms might instead represent proteins with different activities. Another possibility that could affect the efficiency of incision is the order and kinetics by which the gene products associate to each other or to the DNA. Preincubation might, in consideration of this possibility, be a result of an increased time for the gene products to associate with each other.

The two parameters which influenced the amount of incision obtained in these reactions most significantly were the time of incubation and the UV dose applied to the DNA. From the shape of the curve in Figure III-10, page 84, it can be determined that incubation of the reactions for approximately 90 minutes will result in incision at a maximum of approximately 40% of the dimers. The linear relationship between UV dose and the amount of incision indicates

that a constant proportion of the dimers formed in the PM2 DNA are being incised in these in vitro reactions.

The results presented in this study suggest that the UvrABC gene products act by a processive mechanism. The major data supporting this are shown in Figure III-11, page 92. In this experiment ^3H -labelled PM2 DNA was incubated for various times before a large excess of unlabelled (also irradiated) DNA was added. It was found that the addition of the excess DNA 2 to 12 minutes after the start of the incubation of the ^3H -labelled DNA did not affect the amount of incision in the reaction but rather the number of breaks per molecule remained constant over this time period. When the ^3H -labelled DNA incubated between 14 and 20 minutes before the addition of the unlabelled DNA, the amount of incision in each reaction represents a second constant value. The existence of a second constant region argues that the first constant level of incision (2-12 minutes) is not due just to a long period of time before the components of the UvrABC endonuclease can associate with each other and/or with the DNA nor due to some possible requirement for activation of one or more of the gene products in order to increase the efficiency of incision. For PM2 DNA (irradiated with 10 J/m^2) under these conditions, these data suggest that the time the UvrABC gene products remain associated with a DNA molecule may be an average of approximately 10-12 minutes. The length of this time could be dependent upon several factors--including the size and shape (circular or linear, superhelical or relaxed) of the DNA or on the dose of ultraviolet light applied to this DNA. The importance of the latter depends on

whether the primary rate-limiting step of incision is the location of the damage or the actual incision. In order to address whether the amount of time that UvrABC gene products are associated with a DNA molecule is dependent on the UV dose applied to the DNA, an experiment similar to the one shown in Figure III-11, page 92 but in which the UV doses applied to the DNA were also varied could be performed. One possible explanation for the linearity observed for the relationship between the amount of incision and the UV dose applied to the DNA (see Figures III-2 and 3 and IV-1, pages 53, 56, and 97, respectively) after long (more than 20 minutes) incubations is that the factor limiting the efficiency of incision is the amount of time that it takes the UvrABC gene products to find the damage. The interdimer distance in DNA irradiated with higher doses of UV is less than in DNA irradiated with lower doses--which, if the time it takes the gene products to scan the DNA molecule for damage is limiting, may result in greater amounts of incision. It should be noted that the UV dose dependence of the amount of incision of a processive endonucleolytic activity is linear probably only if the time of incubation of the reactions is long (approximately three times the average length of time the enzyme is associated with a DNA molecule (at shorter incubation times, the functional dependence between breaks per molecule and UV dose is expected to be concave upward such that the amount of incision increases more rapidly at high UV doses (and short interdimer distances) than at low UV doses). This expected relationship for an enzyme that acts processively to show a nonlinear functional dependence between the amount of

incision and the UV dose applied to the DNA when the reactions are incubated for short times (less than twice the length of time of association of the enzyme with the DNA molecule) can be used to investigate whether the mechanism of action of an enzyme is processive or distributive. To do this two experimental parameters are varied--time of incubation and UV dose applied to the DNA--and the amount of incision determined. The question of processivity of action of the UvrABC gene products can be more directly investigated using agarose gel electrophoresis to separate Form I, Form II, and Form III DNA (Lloyd et al., 1980).

The in vitro repair of bacteriophage T7 DNA using enzymes and extracts originating solely from its host, E. coli, was investigated using both a biological (enhanced ability of repaired DNA to form viable phage when packaged in vitro into phage heads) and a biophysical (restoration of the mode molecular weight) endpoint. The fact that in vitro repair of bacteriophage T7 DNA as determined by a biological endpoint was not achieved may reflect any of several possibilities. The molecular weight of the repaired DNA was not completely restored indicating either that some of the damage was not repaired or that exonucleolytic activity contaminating the UvrABC protein preparations had partially degraded the DNA. When DNA which had been incubated with the UvrABC gene products, the extract prepared from sucrose-plasmolyzed E. coli, and NMN was packaged in vitro, no viable phage resulted. This argues that single strand breaks in the DNA are lethal. Thus, if either the reactions did not complete repair or the size of the DNA was decreased by nucleases, the number

of surviving phage would be considerably decreased. UV-specific incision was performed in bacteriophage T7 DNA at an efficiency of sometimes as high as 70%. This argues that the defect in these repair reactions might be in the completion of the repair not in the incision step. The methods used in this study did not directly monitor the excision of dimers, so that it is possible that the restored molecular weight seen in these reactions reflects resealing at incised dimers ("abortive ligation"). Abortive ligation in these reactions would be consistent with the data shown in Figures IV-3 and 4, pages 107 and 109--which indicate that there is no difference in the biological activity of irradiated DNA incubated with the UvrABC gene products and an extract from sucrose-plasmolyzed or without E. coli although the molecular weight of the DNA is restored. Recently, however, Rupp and his coresearchers (1982) have determined that the purified UvrABC gene products excise a 12 base pair segment from the DNA by making cuts in the DNA on both sides of the damage. In this preliminary report, they do not give details of the mechanism or kinetics associated with the two breaks in the DNA. It is difficult to reconcile this report with the data obtained here from the reactions investigating the in vitro repair of bacteriophage T7 DNA.

Repair in these in vitro reactions may be incomplete because of the use of extracts prepared from sucrose-plasmolyzed E. coli. This could be tested by the use of extracts prepared by a different method (e.g., by the method used by Masker and Kuemmerle (1977)). The completeness of the repair could be tested by use of the glycosylase/AP endonuclease of M. luteus to monitor the removal of

endonuclease sensitive sites by the UvrABC gene products. It could also be done by monitoring repair resynthesis.

The data presented in Chapter III (particularly in Figure III-11, page 92) support that the UvrABC gene products incise PM2 DNA by a processive mechanism (discussed above). The reaction conditions used for the experiments with bacteriophage T7 DNA are not such that this can be addressed directly because the reactions were incubated for 40 to 45 minutes (an incubation time that might be sufficient for the gene products to associate more than two times with the DNA) and because there was not a large amount of DNA in these reactions (400 picomoles DNA per 0.126 ml reaction is probably not in excess of that at which the amount of UvrA and UvrB/C gene products present in the reaction can incise). The question of a processive mechanism of action could be addressed using T7 DNA if the incubation of the reactions was stopped at short (less than 15 minutes) lengths of time or if a large amount (perhaps greater than 1 nanomole per 0.126 ml reaction) of DNA was used. Both of these could be expected to show a bimodal distribution of radioactivity if the mechanism of action of the UvrABC endonuclease is processive and a uniform distribution of radioactivity if the mechanism is distributive.

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