Studies on the Genetic and Biochemical Properties of a PUVA hyper-resistant mutant of *Escherichia coli*

Julie Holland B.Sc. (Hons)

Abstract

8-methoxypsoralen plus near ultraviolet light (PUVA) is used to treat psoriasis and other cutaneous skin disorders. Much of the lethal and theraputic effects of PUVA treatment are attributed to the drug's ability to covalently bind to DNA, forming crosslinks and thereby preventing its replication and transcription. Many organisms including bacteria have developed mechanisms to repair DNA crosslinking damage induced by PUVA. A mutant of *Escherichia coli* (SA270), hyper-resistant to PUVA, was shown to have simultaneously become hyper-resistant to the DNA crosslinking agents mitomycin C and nitrogen mustard,but not to monofunctional DNA damaging agents such as far ultraviolet light. SDS-Polyacrylamide gel electrophoresis of total protein extract showed that a 55kd protein was over-expressed in strain SA270, but not in its wild type parent strain KL16. A mutation (designated *puvR*), located at 57.2 minutes on the *E.coli* chromosome, concomitantly caused over-expression of the 55kd protein and PUVA hyper-resistance.

Studies with radiolabelled 8-methoxypsoralen showed that the PUVA hyper-resistant phenotype of SA270 was not due to a decreased permeability barrier. It has been previously suggested that the PUVA hyper-resistant phenotype of SA270 was due to an enhanced DNA repair mechanism involving *recA*; however, in an experiment showing induction of the RecA protein, no induction of the 55kd protein was detected. Therefore the *recA* gene was not directly involved in controlling expression of the 55kd protein. However, complementation studies showed that the 55kd protein was negatively regulated and may therefore be indirectly induced by DNA crosslinks. Evidence also suggested that the *recF* gene product may be involved in regulating expression of the 55kd protein. A number of DNA repair-deficient mutants of *E.coli* (*recA*, *uvrA*, *uvrC*, *recBC*, *recF* and *recN*) were all sensitive to PUVA. This implicated the UvrABC excision repair pathway and the RecBC and RecF post-replication recombination repair pathways in DNA crosslink repair.

Lambda DNA damaged with PUVA was degraded by total protein extract of strain SA270 with greater efficiency than with total protein extract of KL16. This phenomenon was dependent on the presence of the 55kd protein since total protein extract of a PUVA hyper-sensitive mutant did not degrade λ DNA. In contrast, DNA incubated with purified 55kd protein was not degraded, suggesting that the protein was dependent on other proteins for its activity.

Since the *uvrC* mutant derivative of SA270 was not hyper-sensitive to PUVA, the 55kd protein may form part of the UvrAB complex, thereby rendering its endonuclease activity specific for DNA crosslinks. Alternatively, there may exist a DNA repair pathway specific for crosslinked DNA damage which involves the 55kd protein in as yet an unknown role.

Studies on the Genetic and Biochemical Properties of a PUVA hyper-resistant mutant of *Escherichia coli*

By

Julie Holland B.Sc. (Hons)

A dissertation submitted to the CNAA in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Nottingham Polytechnic 1990

In collaboration with the Department of Genetics, University of Leicester.

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Contents

Declaration

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work presented in this thesis was carried out by the candidate. Due acknowledgement has been made of all assistance received.

signed Julie Holland.

Candidate

signed _____

Director of Studies

Notes on previous publications and abbreviations

Notes on previous publications

Part of the work presented in this thesis has been submitted for publication to Mutation Research.

An abstract entitled "DNA damage by 8-methoxypsoralen plus near ultraviolet (PUVA) and its repair, was presented at a meeting of the British Photobiology Society, London 1988.

Abbreviations

Abbreviations used in this thesis are those in common usage unless specifically indicated otherwise.

List of Tables and Figures

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Complementation studies showed that the 55kd protein was negatively regulated and may be indirectly induced by DNA crosslinks. Evidence suggested that the *recF* gene product may be involved in regulating expression of the 55kd protein. A number of DNA repair-deficient mutants of *E.coli* (*recA*, *uvrA*, *uvrC*, *recBC*, *recF* and *recN*) were all sensitive to PUVA. This implicated the UvrABC excision repair pathway and the RecBC and RecF post-replication recombination repair pathways in DNA crosslink repair.

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Chapter 1

INTRODUCTION

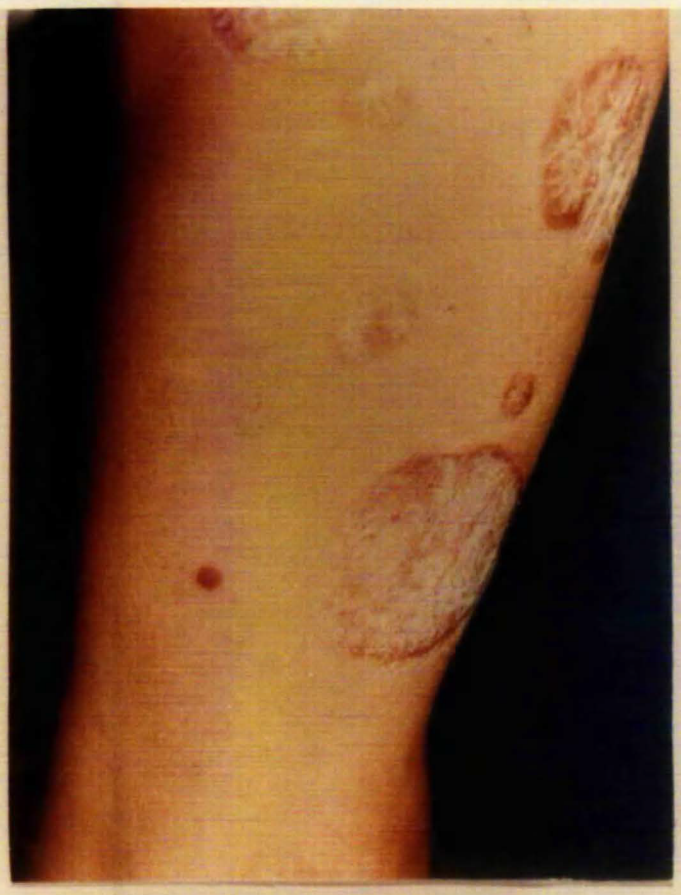
1.1 The History of Furocoumarins

Furocoumarins, which include the psoralen compounds, have been used for centuries, in the form of plant extracts, to treat skin disorders such as vitiligo (leukoderma). Psoralens are present in many plant species, including celery, parsnips, parsley and cloves, and preparations of such plants have been used to treat skin complaints. In ancient literature dating back to 1550 B.C., and in Indian and Chinese medical books from around 200 to 700 A.D., references were made to the seeds of the Bavachee plant, now known as *Psoralen corylifolia* which were used to treat vitiligo. Patients would ingest preparations of the seeds and expose themselves to sunlight. After a short time, an acute inflammatory response would occur, which would finally resolve in the repigmentation of the vitiligo.

In more recent times psoralens have been widely studied for their therapeutic properties, but it was not until the 1930's that a connection was made between the mode of action of psoralens and the requirement for ultraviolet light. Subsequently, El Mofty (1968) used orally administered psoralen compounds to treat vitiligo. The treatment was only successful if the patient was exposed to sunlight or ultraviolet light. By the late 1940's many psoralen compounds had been isolated and, in 1953, a combination of orally administered 8-methoxypsoralen and ultraviolet light was successfully used to treat vitiligo.

Lerner *et al.* (1953) suggested that psoralens could be used to treat other skin complaints such as psoriasis (fig.1). At a meeting held by the Academy of Dermatology in Chicago, Allyn (1962) reported

that there had been a favourable response when 8-methoxypsoralen was applied to psoriatic plaques followed by ultraviolet illumination of wavelength 320nm. The action spectrum for 8-methoxypsoralen was therefore noted to be in the UVA, near ultraviolet or long wave region of the electromagnetic spectrum (fig. 2). Subsequent studies showed that the combination of 8-methoxypsoralen, and indeed other psoralen compounds, with near ultraviolet light, caused inhibition of deoxyribonucleic acid (DNA) synthesis in mouse epidermis and human fibroblasts (Epstein & Fukuyama, 1970; Baden *et al.*, 1972). Thus, inhibition of DNA synthesis in the epithelial cells undergoing the abnormal proliferation characteristic of psoriasis (fig.3) was an ideal and obvious way to treat the disease. Parrish *et al.* (1974) successfully treated severe cases of psoriasis with orally administered 8-methoxypsoralen plus near ultraviolet light (PUVA), but it was not until 1982 that the treatment was approved by the Food and Drug Administration Authority, and even then, only for the most severe cases of psoriasis.



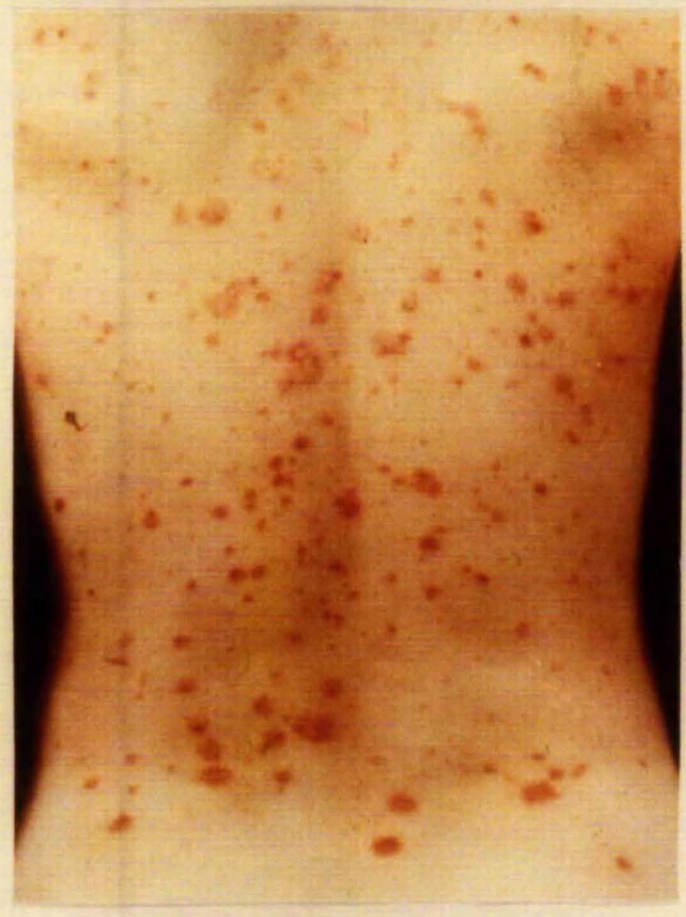


FIG.4. EXAMPLES OF PSORIASIS.

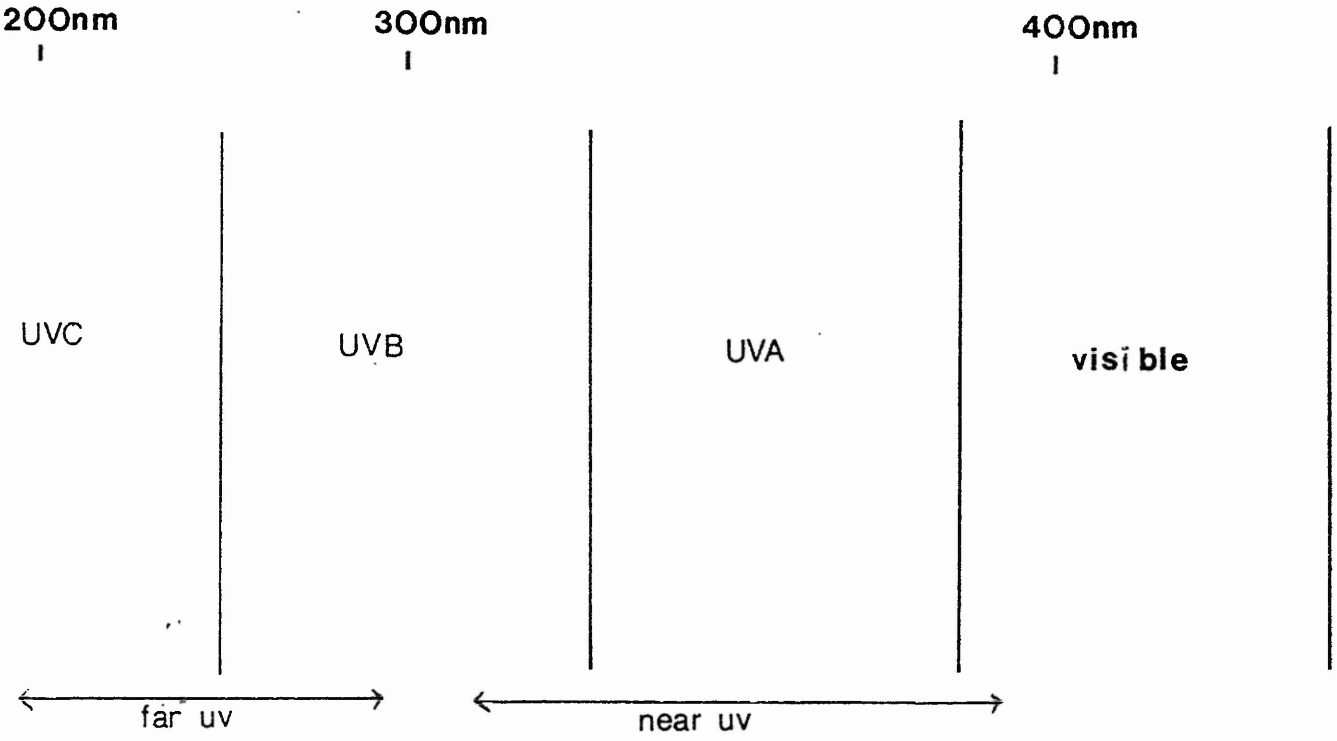


FIG.2. THE ELECTROMAGNETIC SPECTRUM SHOWING CLASSIFICATION OF THE ULTRAVIOLET PORTION.

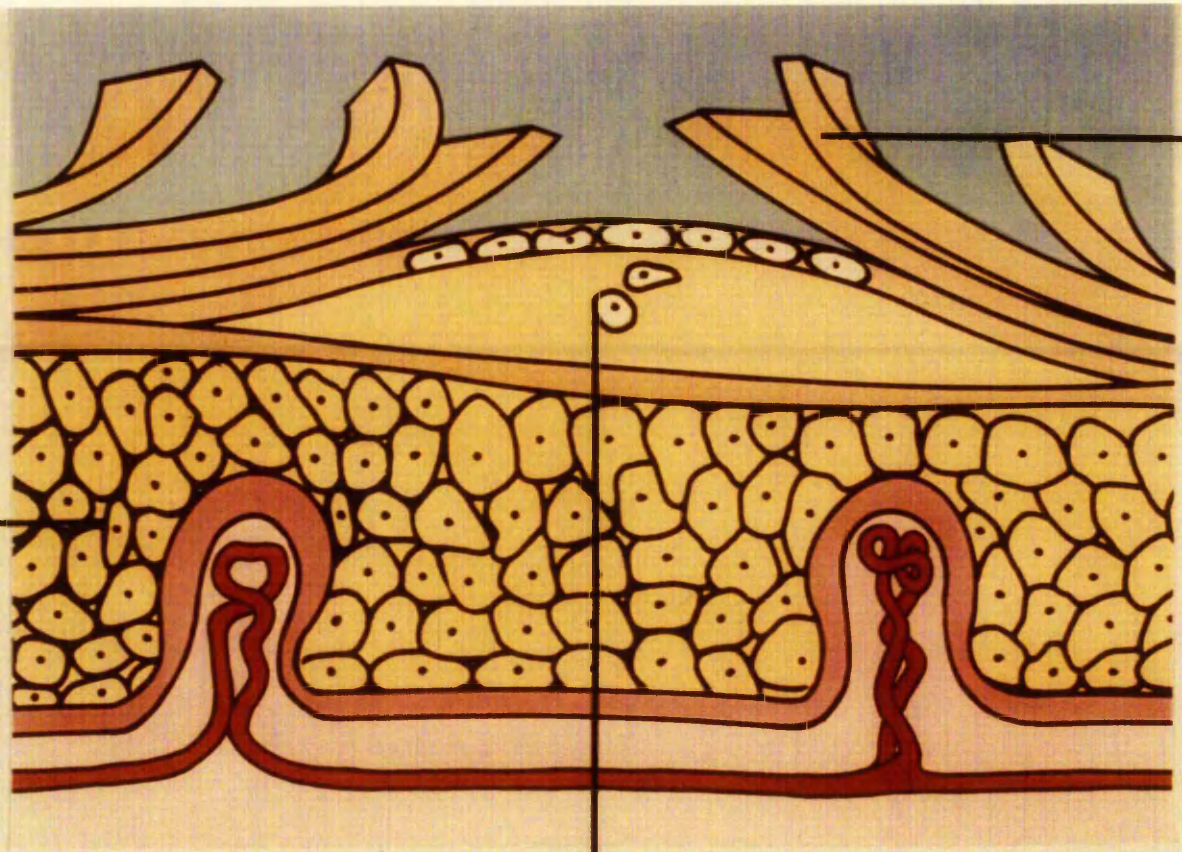


FIG.3. ABNORMAL PROLIFERATION OF PSORIATIC EPITHELIAL CELLS.

1.2 PUVA Therapy

Orally administered psoralen is readily absorbed into the body, with between one and six hours required for the drug to reach a peak concentration in the serum following ingestion (Goldstein *et al.*, 1982). Once absorbed by the cutaneous cells, the skin remains sensitive to near ultraviolet light for up to twelve hours, after which time 90% of the psoralen compound has been excreted. 8-methoxypsoralen is the drug most commonly used in PUVA therapy, although 5-methoxypsoralen has also been used in less severe cases of psoriasis since it is less phototoxic than 8-methoxypsoralen. As already described, the psoralen photochemotherapy action spectrum is within the near ultraviolet light region of the electromagnetic spectrum. The precise wavelength needed for maximum effectiveness of PUVA therapy is a matter for debate. Some workers have suggested that wavelengths of 330 to 360nm produce the best results (Pathak *et al.*, 1974), while others suggest a range between 320nm and 335nm (Fleming & Brody, 1985). Shorter wavelengths of near ultraviolet light are generally more effective and this should be considered by ultraviolet light source manufacturers when designing lamps for photochemotherapy.

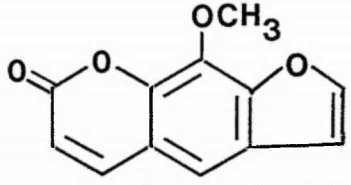
1.3 Mode of Action of Psoralens

The psoralens are linear furocoumarins composed of a furan ring fused to a coumarin molecule. An example of this is shown in fig.4. The most widely studied photoreaction of psoralens occurs with DNA, but it is worth noting that there are other target sites for these drugs within the cell. Other types of molecular lesion may also contribute to the effectiveness of PUVA therapy. Nevertheless, the

psoralen-DNA interaction is the most important consequence of PUVA treatment and has the greatest detrimental effect on the cell.

Since 8-methoxypsoralen is the compound used most in PUVA therapy its interaction with DNA will be discussed in detail. 8-methoxypsoralen causes damage to DNA when photolysed by near ultraviolet light. In this reaction, 8-methoxypsoralen first binds non-covalently to a pyrimidine base in the DNA. When irradiated with near ultraviolet light, a monoadduct is formed between the psoralen and the DNA base (fig.5). The base involved is usually a thymine molecule, although photoaddition to cytosine has also been reported (Calvin & Hanawalt, 1987). Absorption of a second photon of near ultraviolet light by a thymine monoadduct causes it to form a covalently bound adduct with another base in the complementary DNA strand (fig.5). This is known as interstrand crosslinking and, as will be discussed later, is of greater biological significance than monoadduct formation. It has been shown (D'all Acqua , 1977) that psoralen monoadducts and crosslinks result from cycloaddition between the 5,6-bond of a pyrimidine base and the 3,4-pyrone or 4,5-furan bond of the psoralen molecule. 30% of the 4,5-adducts photochemically react, via the 3,4-double bond, with a pyrimidine base on the complementary strand of DNA to form a crosslink. The capacity to form crosslinks varies with the psoralen compound involved. For example, 8-methoxypsoralen readily forms crosslinks, but angelicin, an angular furocoumarin (fig.6), can only form monofunctional adducts and its usefulness as a drug is dependent on the fact that it can form bonds with other cellular components ((D'all Acqua *et al.*, 1983).

Photobiological effects caused by PUVA, such as RNA and DNA synthesis inhibition, appear a short time after irradiation with near ultraviolet light. Such effects occur to the same extent whether the DNA contains monoadducts or crosslinks. Long term effects such as skin photosensitisation, are dependent on the DNA damage caused by crosslinks.



8-Methoxypsoralen

FIG.4. AN EXAMPLE OF A LINEAR FUROCOUMARIN.

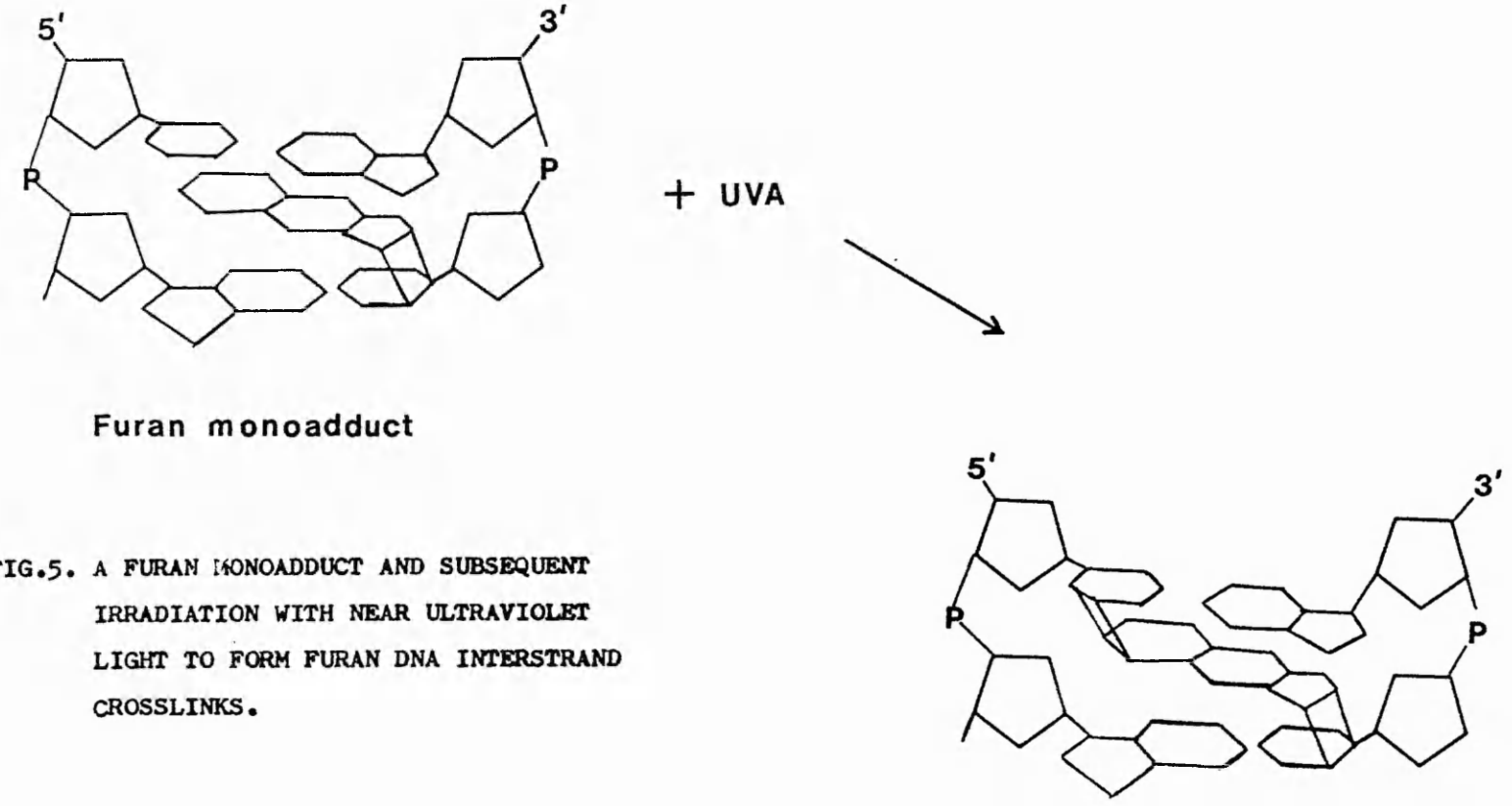
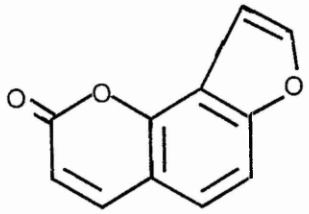


FIG.5. A FURAN MONOADDUCT AND SUBSEQUENT IRRADIATION WITH NEAR ULTRAVIOLET LIGHT TO FORM FURAN DNA INTERSTRAND CROSSLINKS.



Angelicin

FIG. 6. AN EXAMPLE OF AN ANGULAR FUROCOUMARIN.

1.4 Reaction of Psoralens with Cell Components Other than DNA

Consideration must also be given to the biological effects psoralens can induce in the cell when molecular lesions to cell components other than DNA are produced. Psoralens can also bind to cellular components such as lipids and proteins. Indeed Pathak & Kramer (1969) demonstrated that only 0.18% of topically applied psoralen bound to the DNA component of guinea pig skin, even under ideal conditions. Thus using radiolabelled psoralen to penetrate the epidermis, they found that 82% of the radiolabel was contained within the cytoplasm. From this fraction, 99.5% of the radiolabel was recovered in a low molecular weight fraction representing unreacted psoralen or psoralen bound to low molecular weight molecules such as lipids and amino acids. Frederickson & Hearst (1979) showed that treatment of HeLa cells with psoralen and near ultraviolet light caused an increased risk of RNA degradation attributed to the release of ribosomal enzymes. Lysis of lysozomes implied that damage had occurred to proteins or lipids in the lysosomal membrane. Work by Laskin *et al.* (1985) has shown that there are high affinity binding sites for psoralens on cellular membranes, while electron-microscopy, using fluorescence, clearly shows that photo-binding to target sites other than DNA occurs when cells are exposed to PUVA (Cech *et al.*, 1979).

A. Lipid-Psoralen Interactions

When simple fatty acids were mixed with 8-methoxypsoralen and irradiated with near ultraviolet light combined products were detected (Kittler *et al.*, 1986). It was demonstrated that some lipids play very important roles in mediating hormonal signals and

other processes within the cell. Psoralen-lipid interactions can seriously affect the pathways responsible for hormone production and consequently have a profound effect on cell processes. The binding of psoralens with cell membrane components is a significant biological interaction. 8-methoxypsoralen is known to bind to lectin binding sites (Laskin *et al.*, 1985) and morphological changes in the cell membrane have been observed when cells were treated with a high dose of PUVA (Wennersten, 1979). Psoralens have been shown to cause oxidation of fatty acids and lipid peroxidation leads to degradation of the cellular membrane and eventual cell death. Pathak & Joshi (1983) have suggested that lipid peroxidation might be as significant a component of the anti-proliferative effects of PUVA treatment as the cycloaddition of psoralens to DNA.

Caffieri *et al.* (1988) reported that PUVA can cause damage to cell membranes. This study showed that, both *in vitro* and *in vivo*, there was a photoreaction at 365nm between the methylesters of unsaturated fatty acids and various psoralen compounds. Psoralens were shown to bind covalently to epidermal unsaturated fatty acids and it was proposed that this reaction might also play a role in the skin phototoxic effect observed in patients treated with PUVA.

B. Protein-Psoralen Interactions.

Protein-Psoralen interactions have been widely studied and both "dark" and photoinduced binding has been reported. Psoralens have been shown to react with a number of proteins without the need for near ultraviolet light. These reactions, although slower than "dark" binding of psoralen to DNA are nevertheless critical in terms of their biological effect. Human and bovine lipoproteins in

particular, have been observed to non-covalently bind with psoralens (Melo *et al.*, 1984).

Interpreting the effects of psoralens plus near ultraviolet light on proteins *in vivo* has proved difficult. However, 8-methoxypsoralen plus near ultraviolet light has been observed to cause a decrease in the rate of glucose metabolism in brain and liver cells (Ali & Agarwala, 1974). Psoralens have been shown *in vitro* to covalently bind to proteins such as lysozyme, serum albumin, histone and ribonuclease in the presence of near ultraviolet light (Grossweiner, 1984). In addition to covalent binding psoralens plus near ultraviolet light may also cause amino acid oxidation (Nilsson *et al.*, 1972).

1.5 Side Effects Associated with PUVA Treatment

Besides the beneficial effects of PUVA therapy in the treatment of skin disorders, unwanted side effects are often encountered. The nature of PUVA treatment is such that psoralen is taken up into normal epithelial cells as well as into rapidly proliferating cells of the psoriatic lesion (fig. 7). When exposed to near ultraviolet light, normal healthy cells are subjected to the same phototoxic effects used to destroy the psoriatic cells. Binding of psoralen to DNA and other cellular components of normal epithelial cells can thus cause mutagenesis, changes in metabolism and even cell death. Side effects of PUVA treatment therefore include skin erythema, edema, vasiculation, hyper-pigmentation and carcinogenesis. Precise guide lines for the use of PUVA therapy have consequently been drawn up and, before such treatment is given, a detailed medical history is taken from each patient. For example, a history of exposure to

ionising radiation may increase the risk of developing cutaneous malignances following PUVA treatment. Some medical conditions, such as bullous disease, cataracts, severe cardiovascular, hepatic or renal disease and immunosuppression can be aggravated by PUVA therapy. Patients who show abnormalities in melanocytes during PUVA therapy (ie. increased synthesis of melanin or an increase in the size of melanocytes) must be screened for many years after PUVA treatment to monitor the development of any atypical or malignant lesions.

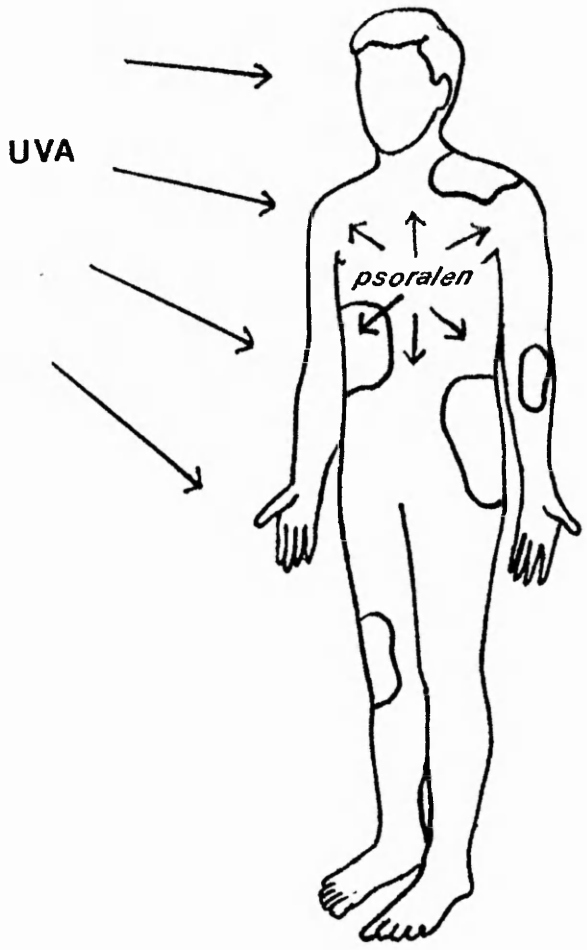


FIG.7. THE NATURE OF PUVA TREATMENT.

The formation of non-melanoma skin cancers following PUVA treatment has been studied by a number of groups. In a 2.1 year study of 1375 patients receiving oral PUVA treatment, Stern *et al.* (1982) found that 30 patients had cutaneous carcinomas and 48 had basal and squamous cell carcinomas. The incidence of these cancers was 2.6 times greater than in the untreated population control. Patients having certain skin types, *i.e.* those who burn and never tan following exposure to near ultraviolet light had an excess of skin cancers following PUVA treatment. Other studies have shown that patients who developed skin cancer [following PUVA therapy] often had a previous history of exposure to known carcinogens such as arsenic, ionising radiation or methotrexate (Honigsmann *et al.*, 1980). Following adjustments for exposure to ionising radiation and other factors, Stern *et al.* (1984) showed, in a 5.7 year study of 1380 psoriatic patients receiving PUVA treatment, that there was a dose dependent risk of the patients developing cutaneous squamous cell carcinoma. Compared with low dose PUVA treatment, patients exposed to a high dose had a 12.8 times higher risk of developing squamous cell carcinoma. Both exposure to other carcinogens and the intensity of PUVA treatment appears to determine the carcinogenic risk of PUVA therapy. PUVA therapy can therefore probably act as either a cocarcinogen or as an independent carcinogen in the development of cutaneous squamous cell carcinoma. It is consequently of vital importance that studies of PUVA-treated patients are carried out to monitor the long-term carcinogenic effects associated with PUVA treatment.

1.6 The Genotoxic Effects of PUVA

It is likely that the increased risk of cutaneous cell carcinoma found in patients receiving PUVA treatment is due to genotoxic effects produced by crosslinking of DNA in the cutaneous cells. DNA damaging agents are, in general, mutagenic and the psoralen compounds are no exception. Altenburg (1956) first studied the mutagenic effects of 8-methoxypsoralen in the polar caps of *Drosophila melanogaster* eggs. Genotoxicity has also been reported in bacteriophage T4 (Drake & McGuire, 1967), *Escherichia coli* (Igali *et al.*, 1970) and *Aspergillus sp.* (Alderson & Scott, 1970). Yatagai & Glickman (1986) studied the types of mutations generated by PUVA in the *lacI* gene of *E.coli*. They deduced that, following PUVA treatment, almost all classes of mutation, including base substitution, frameshift and deletions were produced. The variety of mutants generated by PUVA treatment is not particularly surprising since the DNA lesions can be either monoadducts or crosslinks. Studies have also shown that PUVA mutagenesis can occur in mammalian cell culture. Burger & Simmons (1979a;b) reported mutations in the genes of both Chinese hamster V79 cells and human fibroblasts. They also reported a good correlation between the mutation frequency and the concentration of psoralen used in the experiment.

Mutagenesis by psoralens alone, chiefly frameshift mutations, have been reported in *E.coli* (Bridges & Mottershead, 1977) and *Salmonella typhimurium* (Quinto *et al.*, 1984). 8-methoxypsoralen is administered to patients orally, and it has been suggested that this may cause a "dark" mutagenic effect. However, the reported occurrence of mutagenesis in skin cells exposed to psoralen only occurred at concentrations far above those which are present during

normal PUVA therapy; it would therefore seem unlikely that dark mutagenesis would pose a genetic risk to the psoriatic patient.

Mutagenesis in bacteria caused by psoralen monoadducts appears to occur as a result of error prone repair (see section 1.7). Psoralen monoadducts resemble the pyrimidine dimers produced by short wave ultraviolet light (235nm) in that they prevent base pairing. Every monoadduct will therefore have the potential for causing mutation, probably by a mechanism akin to that resulting from irradiation with ultraviolet light. In order to understand the possible mechanisms involved in the repair of PUVA damaged DNA, it is therefore appropriate to first consider the known mechanisms of repair available to the cell following DNA damage in general. It is beyond the scope of this thesis to consider DNA repair in all living organisms. Therefore the repair enzymes and mechanisms described in the following sections are those identified in bacteria, primarily in *E.coli*.

1.7 Types of DNA damage and Repair Mechanisms

Environmental conditions pose a continual threat to the genetic material of all living things. Ultraviolet light, ionising radiation and exposure to numerous chemicals can cause serious damage to the physical structure of DNA that, if not repaired, may lead to cell death. Spontaneous alterations to DNA can also occur as a consequence of base loss, chemical alteration of bases or changes in base sequence. These alterations also pose a threat to the cell's existence.

A. Types of DNA Damage

(i) Deamination products

Under normal physiological conditions the deamination of cytosine bases occurs at a high frequency in *E.coli*. Uracil is formed as a result and, if not repaired, will cause transition mutations (Duncan & Miller, 1980). Other deamination products also lead to transition mutations.

(ii) Depurination and Depyrimidination

Methylation of purines or pyrimidines leads to destabilisation of the N-glycosylic bond. Apurinic and apyrimidinic sites can be generated by a wide variety of N-glycosylases present in the cell (Lindahl, 1982) and can also be formed spontaneously.

(iii) Damaged Nucleotides

Chemicals such as hydroxylamine and hydrogen peroxide, or ionising radiation can damage nucleotides thereby generating compounds which have altered hydrogen-bonding characteristics.

(iv) Phosphodiester Cleavage

Phosphodiester bond cleavage is caused by agents which produce double or single strand breaks in the DNA. Ionising radiation has been shown to produce this type of damage (Téoule, 1987).

(v) Alkylation of DNA

Alkylating agents from the largest known class of mutagens include compounds such as methyl methane sulphonate and ethyl methane sulphonate. These agents transfer methyl or ethyl groups to

various positions on either the purine and pyrimidine rings or the phosphodiester backbone of DNA. Alkylation mainly occurs at the N7 group of guanine and the N3 group of adenine and, if unrepaired, can result in mutations arising through base mispairing.

(vi) Ultraviolet Light Induced DNA Damage

Ultraviolet light induces a variety of types of DNA damage, the best studied of which is the pyrimidine dimer which arises from formation of a cyclobutane ring across the 5,6 double-bond of adjacent pyrimidines. Thymine-thymine dimers are the most common dimer formed, but cytosine-thymine and cytosine-cytosine dimers have also been reported (Gorden & Haseltine, 1982). A second type of pyrimidine dimer has been identified between the 6 position on one pyrimidine and the 4 position on another; thymine-cytosine dimers are the most common photoproduct of this type found (Franklin *et al.*, 1982). Ultraviolet light is responsible for many types of mutation and it has been suggested that a variety of additional lesions other than dimer formation are generated when DNA is exposed to ultraviolet light (Kushner, 1987).

(vii) DNA Crosslinks

DNA crosslinking damage is caused by bifunctional alkylating agents such as psoralen plus near ultraviolet light, mitomycin C and mechloroethamine (nitrogen mustard). Damage to DNA by such agents will be discussed in detail elsewhere in this thesis. It is, however, worth noting that the formation of crosslinks is of great biological significance to the cell in that lesions which covalently join the two strands of the DNA helix present a special challenge to the *E.coli* repair system.

B. The SOS Response and Genes Involved in DNA Repair

The importance of accurate replication of DNA is exemplified by the numerous genes that are involved in DNA repair. Bacterial repair mechanisms are economic in that they make use of pre-existing functions. Gene products involved in both replication and recombination also participate in DNA repair, but, in addition, the cell encodes many enzymes whose role is limited to DNA repair only.

(i) The SOS Response

In *E.coli*, DNA damage or interruption of DNA replication by a variety of treatments results in the "SOS response" which increases the cell's capacity for DNA repair, cell filamentation, cessation of respiration, DNA degradation, induction of stable DNA replication, induction of resident prophages and enhances mutagenesis. These responses are due to the functions of more than 17 genes, designated *din* genes (damage inducible genes) (table 1).

Regulation of the SOS response is mediated through the *recA* and *lexA* genes. The LexA protein represses the SOS genes including *recA* and *lexA* (Walker, 1985). Following exposure to inducing treatments, signals are produced that activate the RecA protein. This is then capable of mediating LexA repressor cleavage. The cleaved LexA protein no longer functions as a repressor and various genes of the SOS regulon are consequently derepressed. Increased expression of these genes is responsible for enhanced DNA repair and the induction of the other physiological effects mentioned above.

Apart from replication and recombination functions, two other main types of activity have also been implicated in DNA repair:

endonuclease activity, which is responsible for strand cleavage, and glycosylase activity, which removes nitrogenous bases *in situ*.

TABLE 1

SOS-associated recombination and major DNA repair genes in E.coli

Gene	Function
lexA	SOS repressor
phr	Photolyase
recA	General recombination, LexA repressor cleavage, SOS mutagenesis. RecF pathway gene, DNA repair
recB, recC, recD	Exonuclease V, RecBC-dependent recombination, SOS signal generation
recF	RecF-dependent recombination, daughter strand gap repair, double strand break repair
recJ	RecF-dependent recombination
recN	RecF-dependent recombination, double strand break repair
recO	RecF-dependent recombination
recQ	RecF-dependent recombination, resistance to thymineless death
ruv	RecF-dependent recombination, daughter strand gap repair
sbcB	Exonuclease I, suppressor of *recBC*
sbcC	Suppressor of *recBC*
sulA (sfiA)	Cell division inhibitor
umuC, umuD	SOS mutagenesis
uvrA, uvrB, uvrC	Excision repair
uvrD	DNA helicase II, RecF dependent recombination, excision repair, methyl directed mismatch repair, DNA replication

(ii) Endonuclease Activity

Endonuclease activity is responsible for strand incision near the site of an altered or damaged nucleotide(s), thereby allowing the damaged nucleotide and several surrounding nucleotides to be subsequently removed. The *uvrA,B* and *C* genes have been implicated in the repair of a wide variety of DNA damage, including the "dark" repair of pyrimidine dimers and DNA crosslinks. Individually the *uvrA B* and *C* gene products have no endonuclease activity, but, as a complex, each protein performs partial reactions leading to a dual incision event. Sancar & Rupp (1983a)reported that incisions were made seven bases 5' to the lesion and three or four bases 3' to the lesion.

The *uvrA* gene has a single promoter which is overlapped at its 5' end by a consensus SOS box. The LexA protein has been shown to bind to this operator region *in vitro* (Sancar *et al.*,1982) Following induction by the SOS response the UvrA protein has been shown to increase from approximately 20 to 200 copies per cell. The *uvrB* gene is regulated in a more complex way. Three distinct promoter regions have been identified (Sancar *et al.*, 1982b). Two promoters, P1 and P2 are located close to each other, with P3 being 300 base pairs away upstream. Van den Berg *et al.* (1983) showed that both P1 and P2 had a LexA binding site. Little is known about P3 since transcription from this site has never been observed. Expression of the *uvrC* gene is also very complex and in contrast to other SOS genes induction is very slow. Four potential promoters in the 5' region of the *uvrC* gene have been identified. Van Sluis *et al.* (1983) suggested that transcription was initiated at a promoter site 0.4kb from the putitive translation start site. However, Sancar *et*

al. (1984) have produced evidence to suggest that transcription occurs predominantly from a promoter region, 1kb from the translation start site. Conflicting data regarding the control of expression of the *uvrC* gene exists. Van Sluis (1983) reported that the *uvrC* gene is induced *in vivo* and a putative SOS box was identified. It was later shown, however, that this site did not bind the LexA protein (Granger-Schnarr *et al.*, 1986). Foster & Strike (1988) failed to show any induction of the *uvrC* gene product following ultraviolet irradiation or mitomycin C treatment, and Van Houten (1990) has proposed that the *uvrC* gene is regulated so as to maintain expression at very low levels.

The functions associated with the UvrAB and C proteins are also complex. Several groups have cloned the *uvrAB* and *C* genes, enabling large amounts of the Uvr proteins to be purified (Sancar & Rupp, 1983b;Thomas *et al.*, 1985). Subsequently, detailed studies of the proteins have been undertaken and the incision events performed by the UvrABC endonuclease complex, elucidated.

The UvrA protein binds adenosine 5'-triphosphate (ATP) which increases its affinity for binding to damage induced deformities in the DNA helix. The UvrB protein, in association with the UvrA protein, forms a stable ternary complex with the DNA. This complex is recognised and bound by the UvrC protein, thereby generating a catalytically active endonuclease capable of nicking the DNA. It is important to note that the UvrABC endonuclease has a broad substrate specificity capable of incising many types of damage (Van Houten, 1990). Although it is not yet apparent how one protein complex can act on such a diverse set of DNA lesions, it is however known that damage can lead to changes in the dynamic structure of DNA. At least

six types of structural and conformational changes caused by various types of damage have been described (Van Houten, 1990) and it has been suggested that it is these alterations that are potential substrates for the UvrABC endonuclease complex.

(iii) Glycosylase Activity (Kushner, 1987)

The glycosylase enzymes are able to remove specific nitrogenous bases *in situ* from a polynucleotide chain, leaving the glycosyl bond between the base and the deoxyribose ring intact. These enzymes create apurinic or pyrimidinic sites (AP) depending on their specificity. Such sites produced by the action of glycosylase enzymes are subsequently recognised by endonucleases. AP endonucleases cleave the DNA backbone at the 5' side of the AP site. ExoIII (the *xthA* gene product) has endonuclease activity, termed EndoII, which can also cleave DNA on the 5' side of a lesion. EndoIV has the same activity. Three further endonucleases, EndoIII, EndoV and the endonuclease activity of the RecBC nuclease, appear to act non-specifically on damaged DNA.

Once a nick has been made by endonuclease activity, exonucleases remove damaged DNA. Following a 5' nick, the damage is removed by 5' -> 3' exonuclease activity. This 5' -> 3' activity can be mediated by the PolI exonuclease enzyme (the 5' -> 3' function of polymerase I), Exo VII (the *xseA* gene product) and Exo V (the RecBC exonuclease function). It is worth noting that these enzymes also have 3' -> 5' exonuclease activity. DNA repair employing N-glycosylases is summarised in fig. 8.

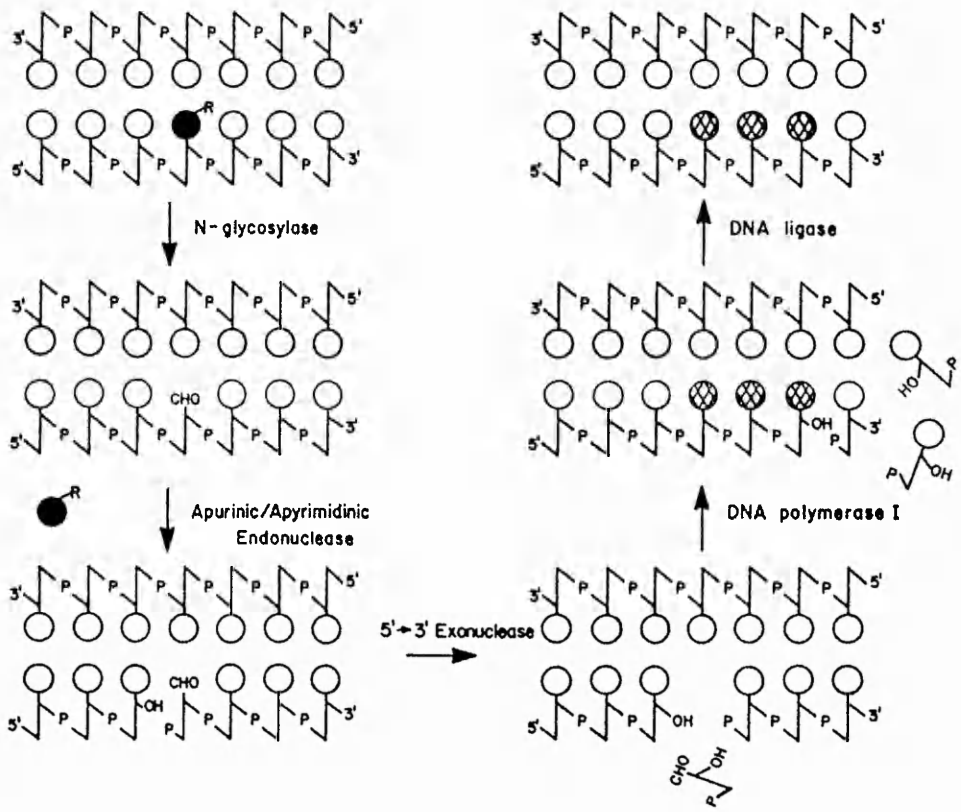


FIG.8. DNA REPAIR EMPLOYING N-GLYCOSYLASE ENZYMES.
(taken from Kushner, 1987)

C. DNA Repair Mechanisms

Having described the genes involved in DNA repair , it is now appropriate to consider the actual mechanisms of DNA repair.

(i) The Adaptive Response

The adaptive response is induced following exposure of cells to non-lethal concentrations of simple alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine. The adaptive response is *recA*-independent and is controlled by the *ada* gene product which, besides acting as a methyl-guanine-DNA methyltransferase (Teo *et al.*, 1984), acts as a regulatory protein in the response. Induction of at least four genes, *ada, alkA, alkB, and aidB* have been observed (Volkert & Nguyen, 1984) following exposure of cells to low concentrations of alkylating agents. The *alkA* gene product is a glycosylase enzyme which is thought to scan DNA and remove potentially mutagenic methyl groups (McCarthy *et al.*, 1985). Products of the *alkB* and *aidB* genes have not yet been identified. The *ada* gene product shows methyltransferase activity (Demple *et al.*, 1982) and is able to transfer either methyl or ethyl groups to a cysteine molecule located on the protein itself. It is thought that this activity converts the Ada protein to a positive regulator, which in turn binds to sequences in promoter regions of other adaptive response genes. Once the glycosylase activity of the Ada and AlkA proteins have produced an AP site, DNA polymerase I and DNA ligase complete the DNA repair process.

(ii) Mismatch Repair (Claverys & Lacks, 1986)

Occasionally an incorrect base is incorporated into DNA and is unable to form a hydrogen bond with the template base in the parental strand. A repair mechanism exists to correct such errors and is termed mismatch repair. A pair of non-hydrogen bonded bases is recognised and a polynucleotide segment is excised from one strand. The resulting gap is filled by PolI activity and the repair completed by DNA ligase. The mismatch repair system has been analysed both genetically and biochemically and the genes required for this type of repair include *dam, mutH, mutL, mutS and uvrD.* The product of the *dam* gene is a DNA adenosine methylase which recognised the degree of methylation of a DNA strand and preferentially excises nucleotides from the under-methylated strand. The *mutH, mutL and mutS* gene products have been purified (Claverys & Lacks, 1986). The *mutS* gene product binds to a single base pair mismatch and the *mutH* gene product is a site-specific endonuclease which cleaves (5') a phosphodiester bond on the under-methylated DNA strand. No activity for the purified *mutL* gene product has so far been obtained.

(iii) Other Pre-replicative Repair Processes

The remaining DNA repair processes can be conveniently divided into pre-replication and post-replication events. The following DNA repair processes are pre-replication events which function upstream of the replication fork.

a. Excision Repair

One of the best characterised and most widely studied DNA repair pathways in *E.coli* is nucleotide excision repair. This pathway consists of five basic steps: damage recognition, incision, excision, repair synthesis and ligation. Excision repair encompasses correction of the three main types of DNA damage: missing bases, base defects and structural alterations such as dimer formation. During damage recognition, one or several proteins bind to the damage induced distortion. The remainder of this section will concentrate on the UvrABC excision endocuclease and the excision repair events which occur following incision of the DNA strand.

A detailed account (section 1.7.B(ii)) has already been given of the enzymatic events involved in recognition of DNA damage and incision of the DNA strand 5' and 3' to the lesion. In the next step of excision repair, the damaged oligonucleotide is removed by exonuclease activity. In the case of *uvrABC* endonuclease activity, another protein is required, following excision, for the turnover of the protein complex (Yeung *et al.*, 1986). Kuemmerle & Masker (1980) observed that the UvrD protein (helicase II) stimulated UvrABC endonuclease activity within the cell and Husain (1985) found that the UvrD protein appeared to allow turnover of the UvrC subunit and stimulated the incorporation of nucleotides by polymerase I. It is conceivable that, unless UvrD protein is present during the polymerase step, UvrC will remain bound to the excised oligomer due to its high affinity for single stranded DNA.

It is interesting to note that exonucleolytic removal of damaged DNA by PolI exo5'-> 3' activity is concomitant with DNA polymerase repair synthesis of DNA.

DNA polymerase I will add sufficient nucleotides to enable sealing of the repair patch by DNA ligase.

Excision repair is completed by DNA ligase which ligates the 3' hydroxyl and the 5' phosphate groups of the nucleotides around the nick. The nick 3' to the original damage is identical to that formed by the repair endonuclease 5' to the DNA damaged site. It has been suggested that indiscriminate and premature ligation of incisions is blocked by removal of the terminal phosphate group by a DNA phosphatase or by the action of a specific DNA binding protein. The main features of excision repair are summarised in fig. 9.

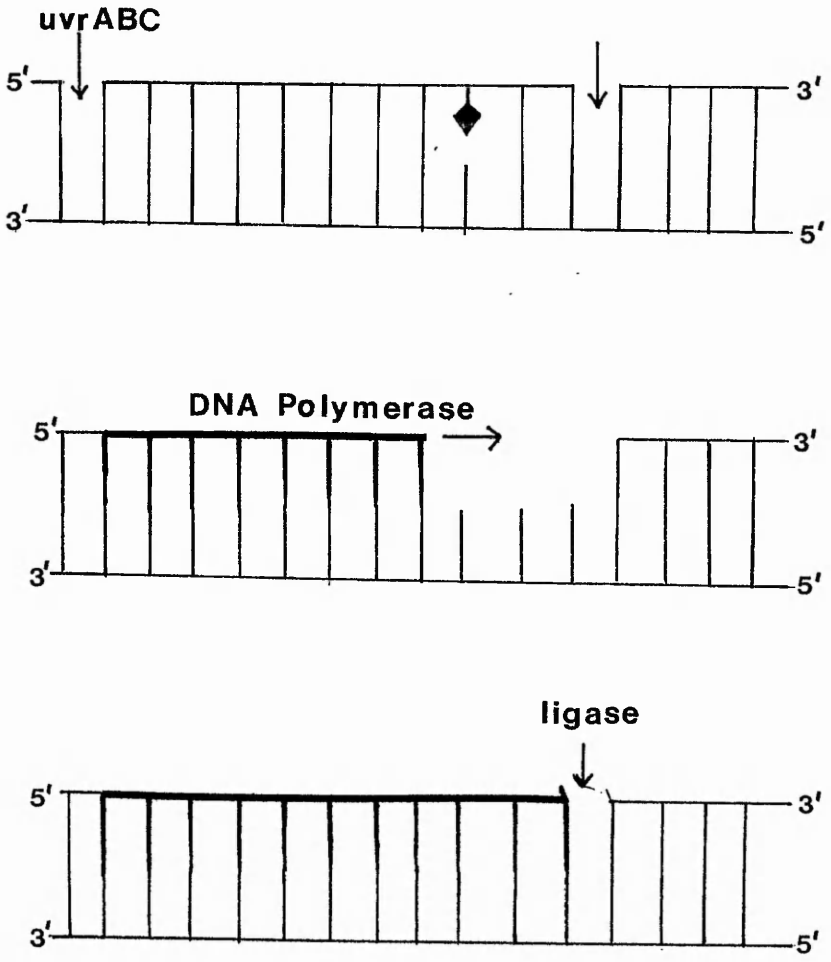
As already discussed, several of the enzymes involved in excision repair are under the control of the LexA repressor protein. These include UvrA, B, D and possibly UvrC. Polymerase I and DNA ligase are not controlled by LexA. Induction of the *uvrA B* and *D* gene products suggests that these proteins are involved in the rate limiting step of excision repair.

b. Photoreactivation

Photoreactivation was the first type of DNA repair recognised in *E.coli* and involves the enzymatic cleavage of cyclobutane pyrimidine dimers in the presence of visible light (330 to 600nm). It was observed that survival of ultraviolet light irradiated

bacteria was higher if the cells were exposed to intense visible light (Rupert *et al.*, 1958). It is now known that, in the presence of visible light, an enzyme identified as photolyase cleaves thymine dimers thereby removing DNA damage. Two photoreactivation enzymes have now been identified in *E.coli* and purified (Sutherland *et al.*, 1973; Snapka *et al.*, 1980; Sancar & Sancar, 1988). The enzyme purified by Sutherland, and later by Snapka, was termed photolyase R and had a molecular weight of 36,000 kd. This enzyme does not absorb light and it has been difficult to understand its function since visible light is a requirement in the photoreactivating repair process. The second enzyme, purified by Sancar & Sancar (1988), is now known to be the product of the *phrB* structural gene (Sancar & Rupert, 1978). The purified protein had an absorption maxima at 280 to 380 nm. Fig. 10 outlines the mechanism involved in photoreactivation.

FIG.9. EXCISION REPAIR OF A PSORALEN MONOADDUCT.



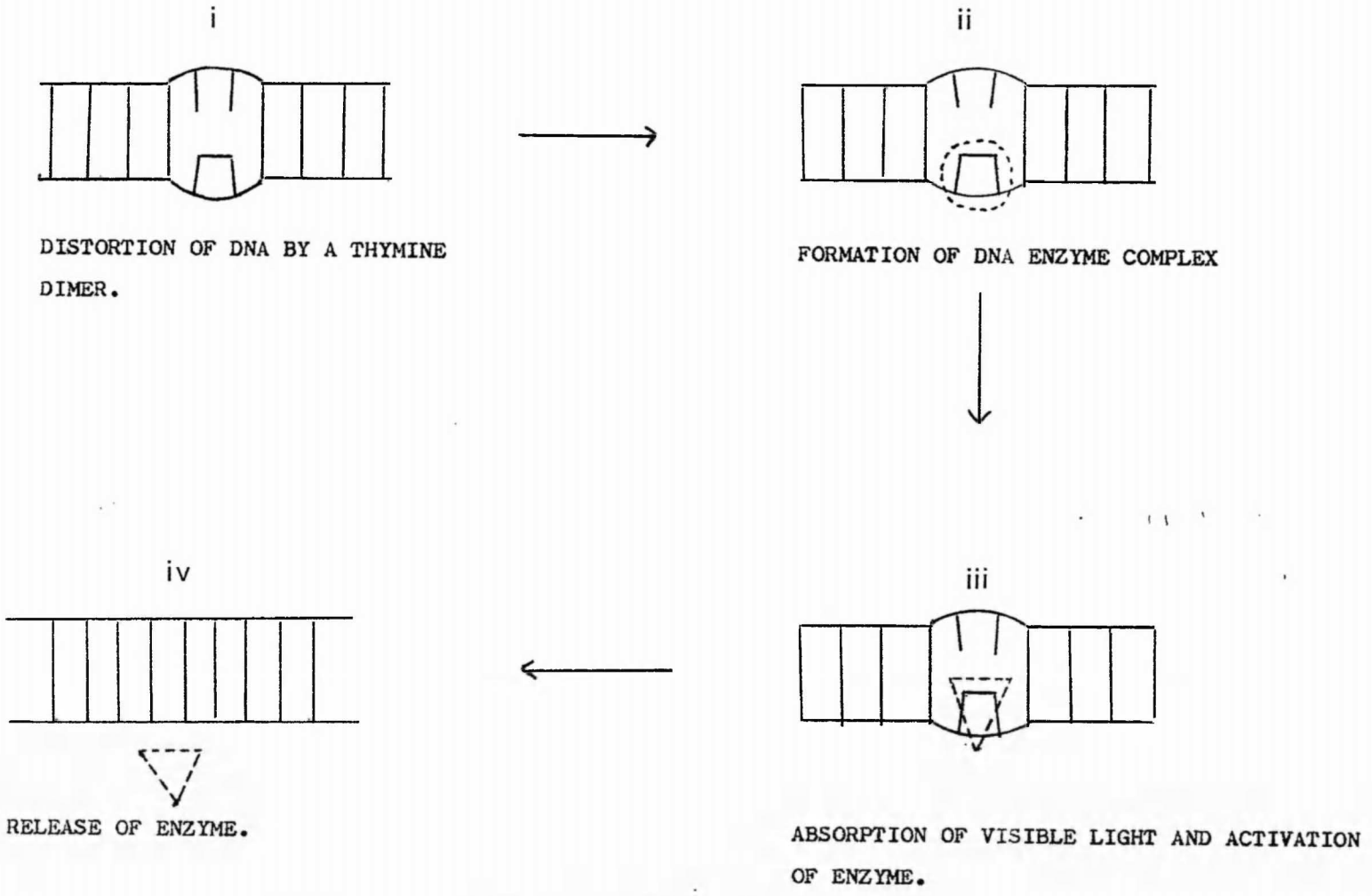


FIG.10. PHOTOREACTIVATION.

(iv) Post-Replication Repair

DNA damage has a major effect on DNA replication. During DNA replication, polymerase III may reach a pyrimidine dimer, or another lesion, which causes the cessation of DNA synthesis (Setlow *et al.*, 1963). An idling reaction occurs in which dNTP's are incorporated and removed from the DNA. After approximately five seconds the DNA polymerase resumes synthesis along the chromosome and an Okazaki fragment is initiated at the next available RNA primer site after the DNA damage. As a result, large gaps are found in the newly synthesised strand opposite the damage site. This type of process was first suggested by Rupp & Howard-Flanders (1968) who also hypothesised that the gaps or secondary lesions could then be filled by genetic recombination. Despite normal excision repair occurring in *recA* strains, cells were shown to be very sensitive to ultraviolet irradiation. Howard-Flanders & Boyce (1966) showed that *recA uvrA* double mutants were more sensitive to ultraviolet light than either of the single mutants, thereby suggesting a role of genetic recombination in the repair of ultraviolet light induced photoproducts.

Two main mechanisms have been proposed for post replication repair: daughter strand gap repair and error prone repair.

a. Daughter Strand Gap Repair

Fig. 11 shows the mechanism proposed for daughter strand gap repair. Repair of the secondary lesion requires both a functional recombination system and repair synthesis components.

General recombination proceeds via the RecBC-dependent pathway, which requires the *recA*, *recB* and *recC* genes. In a strain lacking the RecBC pathway, the RecF pathway mediates recombination, but only poorly. Its effectiveness can be increased by a mutation in either of two genes: *sbcA*, resulting in increased levels of exonuclease VII, or *sbcB*, resulting in the inactivation of exonuclease I (the *recBC* exo gene product). Both recombination pathways are controlled by the *recA* gene, but unlike the RecBC pathway, the RecF pathway is subject to LexA regulation and may therefore participate in DNA inducible recombination repair (Lovett & Clark, 1983). The RecF recombinational pathway is thought to augment the predominant role of the RecBC recombination pathway in *E.coli* by "helping" the cell to cope with additional recombinational substrates such as daughter strand gaps resulting from DNA damage. Gene components of the RecF pathway include *recF*, *recA*, *recN*, *recJ*, *recQ*, *recO* and *uvrD*. All have been implicated in post replication repair (Walker, 1985). Strains with mutations in RecF pathway genes display variable phenotypes depending on the type of DNA damaging agent encountered. For example, mutations in the *recA*, *recF*, *ruv* and *uvrD* genes confer sensitivity to both ultraviolet light (far ultraviolet) and mitomycin C, a DNA crosslinking agent (Walker, 1985). A mutation in the *recN* gene makes the cell sensitive to mitomycin C, but not to ultraviolet light (Lloyd *et al.*, 1983), whereas a mutation in the *recO* gene makes the cell sensitive to ultraviolet light, but not to mitomycin C (Kolodner *et al.*, 1985). This differential sensitivity of mutants in the same pathway suggests that different gene products are required to repair a given type of DNA lesion.

To summarise, the predominant mechanism of daughter strand gap repair is mediated through the RecF pathway of recombination. The *recA*, *recF* and *ruv* gene products all function directly in daughter strand gap repair (Walker, 1985) and mutations in these genes cause deficiencies in this repair mechanism.

In some cases, daughter strand gaps are not repaired and are converted to double strand gaps. Double strand gap repair is performed by the components of the RecF pathway and requires functional *recA* and *recN* genes (Picksley *et al.*, 1984). The introduction of a *sbcB* mutation into a *recBC* mutant strain (deficient in double strand gap repair) restores the cell's capacity to repair double strand gaps, presumably by depressing the RecF recombination pathway gene *recN* (Wang & Smith, 1985). Addition of a *recF* mutation to a *recBC sbcB* strain blocks repair, suggesting a role for *recF* in double strand gap repair. Wang & Smith (1986) have also described a double strand gap recombinational repair process involving the *recA* and *recB* genes, but this mechanism remains unclear.

FIG.11. DAUGHTER STRAND GAP REPAIR.

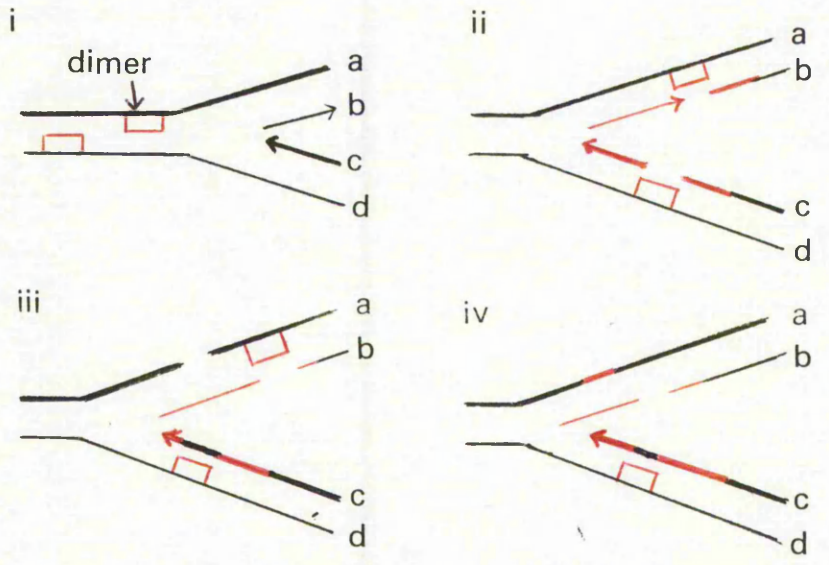


FIG.11. DAUGHTER STRAND GAP REPAIR.

i. A MOLECULE CONTAINING TWO THYMINE DIMERS IN STRANDS a AND d IS REPLICATED.

ii. A MOLECULE IS FORMED WHOSE DAUGHTER STRANDS b AND c HAVE GAPS.

iii. BY SISTER STRAND EXCHANGE A CONTINUOUS SEGMENT OF a IS EXCISED AND INSERTED INTO STRAND c.

iv. THE GAP IN a IS NEXT FILLED.

b. Error Prone Repair (Kushner, 1987)

As described earlier, the cessation of DNA replication caused by DNA damage elicits a pleiotropic set of effects in *E.coli* called the SOS response. A number of the genes induced by the SOS response are involved in error-free repair systems such as excision repair. Other SOS-inducible genes are, however, involved in error-prone repair. This is a bypass system that allows DNA replication to proceed past DNA damage. As in daughter strand gap repair, the replication of DNA is halted if damage is encountered at the replication fork. Unlike the daughter strand gap repair process, error-prone repair is able to mediate transdimer synthesis. Polymerisation proceeds across the dimer and, although fidelity of replication is lost, DNA chain growth can continue. Consequently, even though intact DNA strands are formed, the strands are often defective (survival with some loss of information is better than no survival at all) and mispaired bases are frequent, resulting in mutagenicity.

Following induction of the SOS response, the mutation frequency of *E.coli* is increased 100-fold. It is now known that the mutagenic events produced by error-prone repair result from a functional, and possibly physical, interaction between the DNA PolIII, RecA, UmuC and D proteins that allows replication across the site of a DNA lesion. Berckhardt *et al.* (1988) showed that the enhanced mutation rate following induction of the SOS response required the UmuC and D proteins. Although the genes encoding these proteins have been sequenced (Perry *et al.*, 1985), limited gene expression has meant that the biochemical nature of the UmuC and D proteins has not been widely studied. Berckhardt *et al.* (1988) were successful in

purifying the UmuD protein (15kd) and studies showed that this protein showed homology with the LexA protein. This was particularly interesting since the RecA protein was also shown to cleave the UmuD protein. This result suggested that the RecA protein had a function additional to its LexA regulatory role. Nohmi *et al.* (1988) showed that the RecA protein was also able to activate the UmuC protein.

In conclusion, the *umuC* and *umuD* gene products, induced by the SOS response, are of particular importance in error-prone repair. Current biochemical models suggest that these proteins affect the fidelity of DNA polymerase III, leading to reduced specificity in base pairing. The precise functions of the UmuC and D proteins are not well understood.

1.8 Repair of Psoralen Damaged DNA

A. The Repair of Psoralen Monoadducts

As with any other type of damage, damage to DNA by PUVA poses a genetic risk to the cell. Monoadducts are of less biological significance than crosslinks as the cells are able to repair damage to one DNA strand more readily than they can repair simultaneous damage to both strands of the DNA duplex. In repair proficient *E.coli* cells, the major pathway for the repair of psoralen monoadducts is by excision and resynthesis of the DNA. Averbeck & Moustacchi (1975) noticed that *Saccharomyces* cells deficient in excision repair were more sensitive than wild type cells to PUVA treatment. As with pyrimidine dimers, the *uvrABC* endonuclease recognises helical distortions in the DNA produced by the psoralen

adduct. Van Houten *et al.* (1987) used DNAase I foot-printing techniques to determine the parameters for the DNA-UvrABC protein interactions involved in damage recognition. They were able to show that the UvrA protein is the DNA recognition subunit and that the binding of the UvrB protein to the UvrA-DNA complex induces tighter binding accompanied by a conformational change in the DNA-enzyme complex. The incisions made by the UvrABC endonuclease either side of the psoralen adduct are approximately one helical turn of B-DNA apart. This may be significant when considering what determines the location of incision relative to the lesion. The distortion of the DNA created by the psoralen adduct is asymmetrical with respect to the helical axis of the DNA and this may facilitate preferential binding of the UvrA protein to the damaged strand. Alternatively, binding of the UvrABC endonuclease to the DNA may unwind the DNA helix, thereby creating denaturation regions approximately 12 nucleotides long. The enzyme complex may then incise the DNA at the boundaries of this conformation.

B. Repair of Psoralen Crosslinks

Removal of psoralen monoadducts from damaged DNA by excision repair requires an undamaged complementary DNA strand to act as a template in order to fill the resulting gap. However, this type of repair mechanism is unsuitable for the removal of psoralen crosslinks since both strands of the DNA are damaged and consequently there is no undamaged strand following an excision event to act as a repair template.

One of the most detailed studies of crosslink repair was made by Cole (1973). Using *E.coli* as a model system, Cole suggested

that the repair of crosslinks involved a combination of excision repair and recombinational repair (fig. 12). The UvrABC endonuclease recognises the crosslink and an incision is made on one strand either side of the damage. A nuclease then widens the gap and exposes a single stranded region. Ensuing strand exchanges between homologous duplexes inserts an intact base sequence complementary to the strand still carrying the partially excised crosslinking residue. The remaining arm of the crosslink is excised when the twin helical DNA structure is restored. The resulting gap in this repair process cannot be filled by simple polymerisation, but appears to be filled by recombination with a pre-existing intact duplex elsewhere in the cell. Cupido & Bridges (1985) reported that the repair of 8-methoxypsoralen induced DNA crosslinks in *E.coli* and lambda phage was associated with homologous recombination. They showed that there was no crosslink repair in *E.coli* cells containing a single genome.

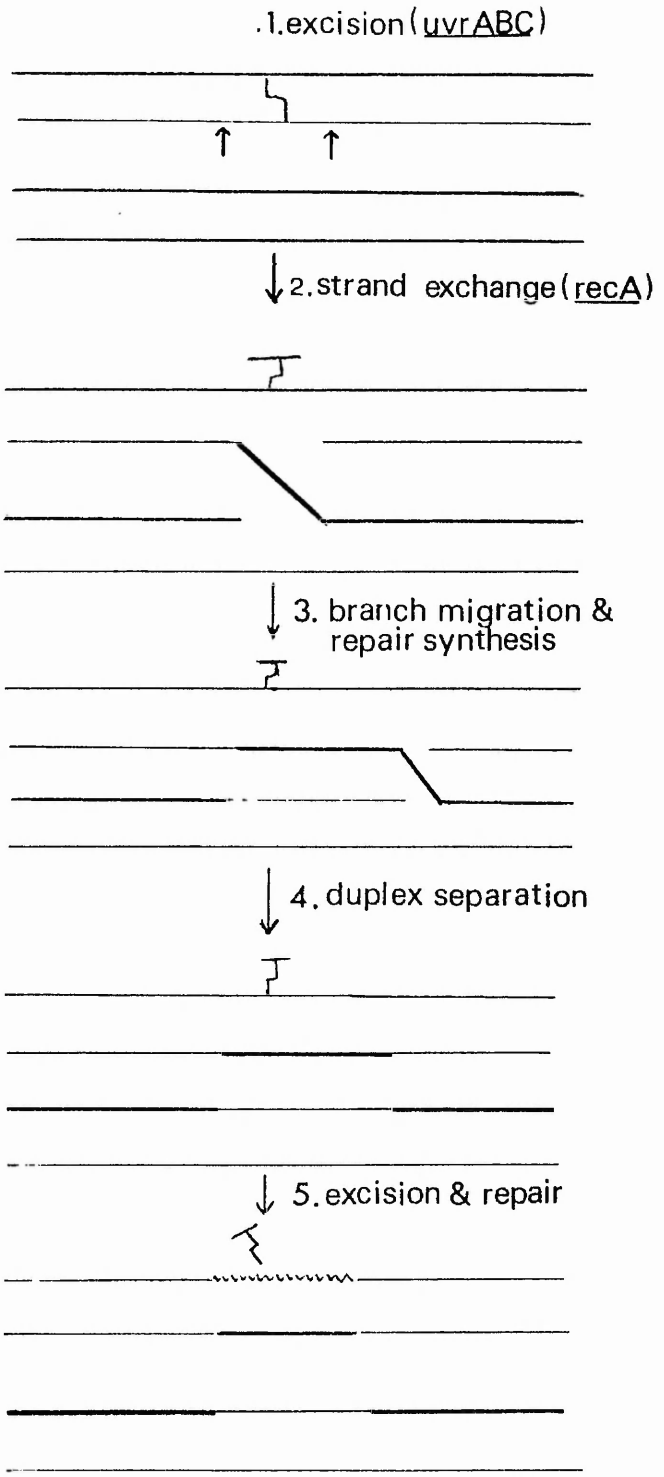


FIG.12. THE FIVE MAJOR STEPS OF PSORALEN CROSSLINK REPAIR AS PROPOSED BY COLE (1973).

More recently a similar repair mechanism for psoralen crosslinks has been proposed by Van Houten *et al.* (1986a) (fig. 13). The incision mechanism observed *in vitro* with a psoralen crosslink substrate, combined with previous *in vivo* studies, demonstrated that crosslink repair was absolutely dependent on a functional RecA protein. As with monoadduct repair, Van Houten *et al.* (1986a) suggested that the UvrABC endonuclease produces dual incisions in only the DNA strand which is covalently linked to the furan ring of the psoralen. The incision is made at the 9th and 3rd phosphodiester 5' and 3' respectively to the crosslink. This is a different pattern to that observed when the UvrABC endonuclease incises either side of monofunctional damage, and provides more evidence to suggest that the complex recognises the extent of helical distortion produced by different types of damage rather than the damage itself. Following incision, the excised oligomer is displaced by the RecA protein which mediates strand invasion by a sister duplex. Alternatively the oligomer may be displaced by the dual action of the UvrD protein and DNA polymerase I, which together mediate translesion DNA synthesis so as to fill in the gap. A triple strand intermediate is produced that has two fully complementary strands of DNA attached to the excised oligomer via the psoralen molecule. In a final step, endonucleases cut the DNA on either side of the attached psoralen molecule and the two oligomers, which are joined by the crosslink, are removed by the combined action of the UvrD protein and DNA polymerase I. Finally the excision gap is resynthesised and ligated.

FIG.13. A MECHANISM FOR THE REPAIR OF PSORALEN CROSSLINKS AS PROPOSED BY VAN HOUTEN ET AL. (1986b). SEE OVERLEAF.

1. THE UVRABC ENDONUCLEASE INCISES AT THE 9^{th} AND 3^{rd} PHOSPHODIESTER 5' AND 3' RESPECTIVELY.

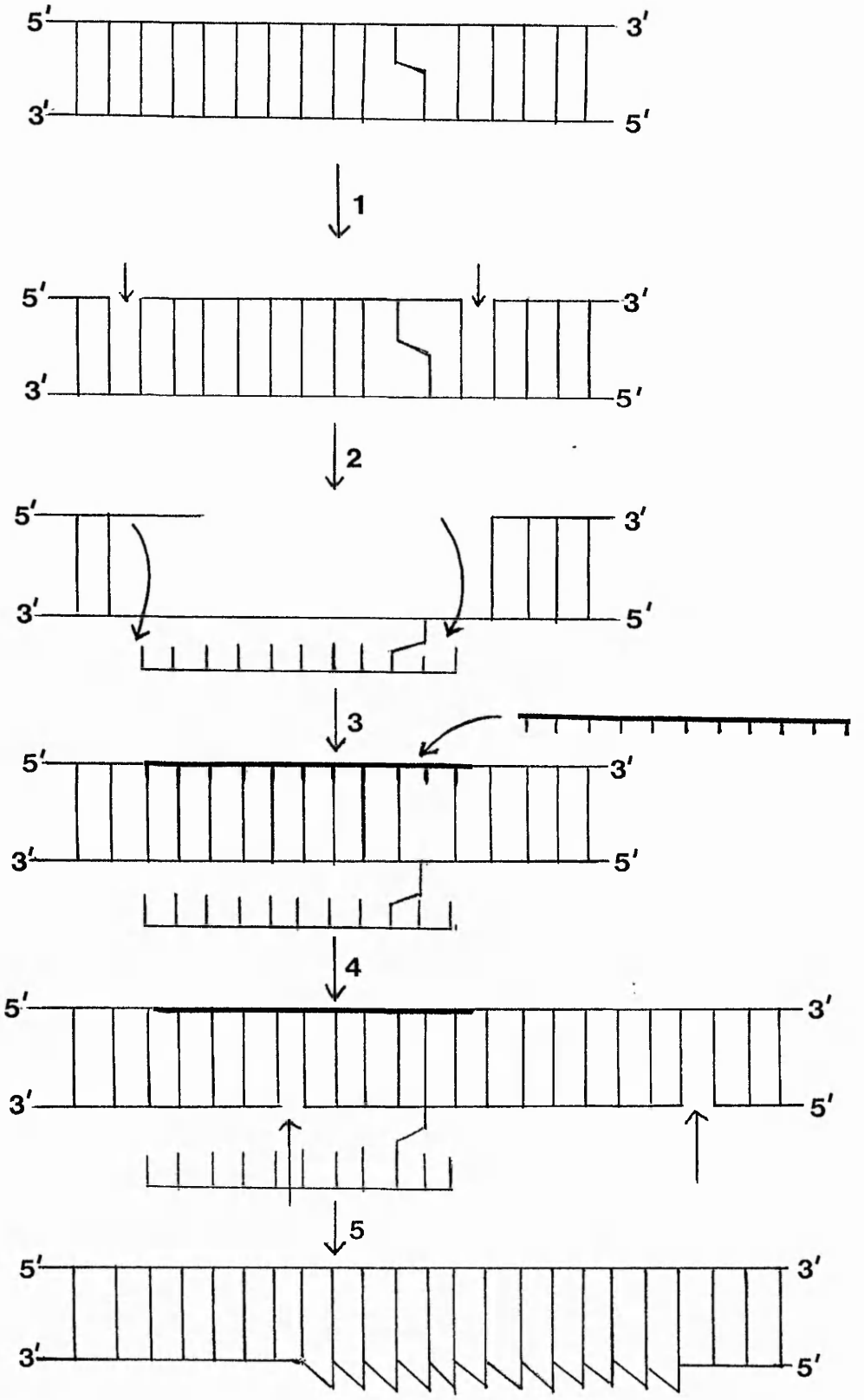
2. THE EXCISED OLIGOMER IS DISPLACED.

3. THE RECA PROTEIN MEDIATES STRAND INCISION BY A SISTER DUPLEX. A TRIPLE STRAND INTERMEDIATE IS PRODUCED.

4. ENDONUCLEASES CUT THE DNA ON EITHER SIDE OF THE PSORALEN MOLECULE AND THE TWO OLIGOMERS ARE REMOVED BY THE COMBINED ACTION OF UVRD AND POLI.

5. THE EXCISION GAP IS RESYNTHESISED AND LIGATED.

13. A MECHANISM FOR THE REPAIR OF PSORALEN CROSSLINKS PROPOSED BY VAN HOUTEN ET AL. (1986b).



Data produced by Jones & Yeung (1988) has shown that the UvrABC endonuclease is capable of incision on either the furan-DNA linked side of the psoralen molecule or the pyrone-DNA linked strand, although Van Houten *et al.* (1986a) observed only furan side incision. They suggested that the chemical structure of the crosslink is important and that a specific DNA-adduct conformation causes incision to be

i. mainly on the furan-DNA side
ii. mainly on the pyrone-DNA side
iii. the same on either strand

Cheng *et al.* (1988) tested for the presence of a post-recombination incision step and completion of repair as proposed by Van Houten *et al.* (1986a). A site specific crosslinked three stranded DNA molecule, generated through RecA mediated homologous pairing and psoralen photochemistry, was used to resemble the post-recombination substrate. The results obtained confirmed the model's prediction that UvrABC endonuclease will recognise a three-stranded complex and specifically incise the modified strand to release the crosslinked fragment. For psoralens lacking the 4' substituent, the initial incisions may occur on either strand, whereas for 4'-hydroxymethyl-4,5',8-trimethyl psoralen the furan side is recognised first. In both cases a three-stranded substrate may be required before the UvrABC endonuclease makes the second set of incisions (Cheng *et al.*, 1988). To excise the original duplex twice without replacing the excised fragments would induce a double strand break. Both the first and second incision steps have now been carried out *in vitro*, but the various interactions between the RecA protein, involved in a

RecA mediated recombination step, and the UvrABC endonuclease are still unknown.

Genetic and biochemical studies on *E.coli* mutants have been responsible for identifying the genes involved in the repair of psoralen crosslinks. Sinden & Cole (1978) studied the repair of crosslinked DNA in *E.coli* strains carrying mutations affecting DNA metabolism. Using wild type cells of *E.coli*, DNA strands cut by endonucleases during crosslink removal were rejoined during incubation to form high molecular weight molecules. The rejoining of DNA was dependent on gene products involved in genetic recombination. A correlation was found between recombination proficiency, the rate of strand rejoining and the formation of viable progeny after treatment with PUVA. Wild type and other *rec*$^{+}$ cells rejoined cut DNA strands at a rate of 0.8 +/- 0.1 per minute at 37 °C and survived 53 to 71 crosslinks per chromosome. Strains with *recB, recC, recBC, recF* or *polI* mutations showed reduced rates of strand rejoining and survived only 4 to 13 crosslinks per chromosome. Recombination-deficient strains carrying *recA, recBC sbcB recF, recB uvrD* or *lexA* mutations failed to rejoin DNA strands following crosslink removal and were unable to form colonies after treatment. Strand rejoining occurred normally in cells with mutations affecting DNA replication. In a *polI, polII, dnaE* strain, strand rejoining occurred at 37°C but not at 42°C, indicating that some DNA synthesis was required for formation of intact recombination molecules.

Sinden & Cole (1978) made the following generalizations:

i. Strains deficient in, or having greatly reduced levels of, recombination were most sensitive to PUVA treatment. Sensitive

strains included those carrying *recA*, *recBC sbcB recF*, *recB uvrD* or *lexA* mutations.

ii. *recB*, *recC*, *recBC*, *recF* or *polI* mutants survived about 4 to 13 crosslinks per chromosome.

These results suggested that repair of psoralen crosslinks proceeds through the RecBC and RecF recombination pathways, since single mutations in these genes reduced cell survival and the rate of strand rejoining after crosslink removal. No strand rejoining occurred when both pathways were blocked.

In conclusion, the repair of psoralen crosslinks has an absolute requirement for a recombinational step in *E.coli* cells.

C. Uvr-Independent Repair of Psoralen Crosslinks

Several studies have suggested that psoralen adducts, including DNA crosslinks, might be repaired in a manner different from the Uvr-Recombination pathway of repair. Bridges & Von Wright (1981) showed that a *uvrA rep* double mutant was more sensitive to the killing effects of psoralen crosslinks than a single *uvrA* mutation. It was suggested that the Rep protein, a DNA helicase, may work in conjunction with RecA to make the psoralen crosslink more accessible to repair enzymes such as glycosylases. Bridges (1983; 1984) showed that the repair of crosslinks occurred in *uvrA*, *uvrB* and *uvrA polI* mutants. In an attempt to ascertain whether this Uvr independent repair of crosslinks might be error-prone, mutation frequencies of *trp* gene mutations were determined in *uvrA* mutants treated with PUVA. A 12-fold increase in the mutation frequency was observed, suggesting that the residual crosslink repair in a *uvrA* mutant is

indeed error-prone. It has been proposed that *E.coli* might produce an alternative error-prone pathway that does not involve homologous recombination. Yatagi *et al.* (1987) suggested a pathway in which gap filling by DNA polymerase I, assisted by helicase II, can substitute for the recombination step.

Work by Zhen *et al.* (1986) and Sladek *et al.* (1988) has suggested the involvement of a novel glycosylase activity in the repair of monoadducts and crosslinks.

1.9 Aims of This Project

Molecular mechanisms involved in the repair of psoralen damage are of great significance if the response of cells to PUVA treatment is to be fully understood. Ahmad & Holland (1985) isolated a mutant strain of *E.coli* K12 that, compared to the parent strain showed enhanced resistance towards 8-methoxypsoralen plus near ultraviolet light. This PUVA resistant mutant, designated SA270, remained as sensitive as its parent wild-type strain for far ultraviolet light, a monofunctional damaging agent, suggesting that PUVA resistance was due to an enhanced repair mechanism unique to crosslinked damage. Using polyacrylamide gel electrophoresis to analyse proteins from total cell lysates of the PUVA resistant mutant, it was possible to show that a protein of approximately 55kd was synthesised in higher concentrations than in the wild type parent strain. Synthesis of this protein was greatly reduced in a *recA* derivative of the PUVA resistant mutant, indicating that the damage to DNA by PUVA is repaired by a mechanism under the control of the LexA/RecA inducible repair system.

Ahmad & Holland (1985) suggested that the PUVA resistant mutant resulted from a mutation affecting the regulation of the 55kd protein. It was suggested that over-expression of the 55kd protein was responsible for the increased resistance of the cell and that the 55kd polypeptide was specifically responsible for repairing PUVA-damaged DNA. It seems likely that the first step in such a repair process would be the identification of psoralen molecules bound to DNA and subsequent incision of the DNA at or near the damage. Perhaps a double strand specific endonuclease is responsible for the first step in this reaction, while the RecA/LexA inducible repair system completes the repair process. In a wild type *E.coli* the levels of the 55kd protein were low and this probably limits the repair of DNA damage. However, in the resistant strain, the removal of PUVA damage is facilitated due to enhanced levels of the 55kd protein perhaps acting as an endonuclease or a psoralen-binding protein. The low levels of the 55kd protein in the *recA* derivative of the resistant mutant suggested that the expression of the protein may be controlled by the RecA protein (Ahmad & Holland, 1985).

The aim of the work described in this thesis was to continue the study and elucidate the genetic and biochemical properties of the PUVA-resistant mutant, SA270. It was thereby hoped that the function of the 55kd protein and its role in the repair of psoralen crosslinks could be determined.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Bacteriophages

Origins and genotypes of the bacterial strains, plasmids and bacteriophages used in this study are listed in Table 2.

Long-term storage of bacterial strains was achieved in either sterile nutrient broth (NB) or minimal medium, both containing 20% glycerol, at -80°C.

When in use, strains were maintained on nutrient agar (NA) or minimal plates at 37°C or 30°C with frequent subculture.

2.2 Chemicals and Media

All chemicals used were of Analar grade (BDH Chemicals, Poole, UK.) or equivalent, or as specified in the methods. All solutions were made in distilled water unless stated otherwise. For genetic and biochemical work, all glassware was soaked for 2 hours in 12M hydrochloric acid and washed extensively in distilled water.

All media were sterilised by autoclaving at 121°C for 15 minutes at 15 lb/sq in. NB and NA were obtained from Difco and prepared according to the manufacturer's instructions.

Table 2

Bacterial strains, plasmids and bacteriophage

Strain		Genotype	Source
a. *E. coli*			
KL16	Hfr	*E. coli* K12 wild type	B. Bachmann
SA270	Hfr	PUVA hyper-resistant mutant derivative of KL16	S.I. Ahmad
AB1157	F⁻	*thi1,his4,proA2,argE3,thr1 leuB6,ara14,lacY1,galK2,xyl5,mtl1 tsx33,supE44,rpsl32.*	B. Bachmann
X121	F⁻	*galk35,λ⁻,pyrD34,trp45,his68,tyrA2, malA1,(λ^R),xyl7,mtl2,thi1*	B. Bachmann
JH108	F⁻	*nalB* mutant derivative of X121	This study
JH109	F⁻	*tyrA*::Tn*10,puvR* (*tyrA*⁻-->X121)	This study
KL711	F'(143)		B. Bachmann
N3087	F⁻	*tyrA*::Tn*10,IN(rrnD-rrnE)1*	R.G. Lloyd
PN10	F⁻	*phe,leu,met,pro,thi*	P.B. Nathan
JH110		PUVA hyper-sensitive mutant of SA270 carrying a Tn5*lac* insertion in the putative *puvP* gene	This study
JH111		PUVA hyper-sensitive mutant of KL16 carrying a Tn5*lac* insertion in the putative *puvP* gene	This study
SP254	F⁻	as AB1157, but *recN262*	R.G. Lloyd
SA256		*recBC,his,thi*	S.I. Ahmad
JC7623	F⁻	AB1157, but *sbcB15,recB21,recC22*	A.J. Clark
JC9239	F⁻	AB1157, but *recF143*	A.J. Clark
JC8111	F⁻	AB1157, but *sbcB15,recB21,recC22, recF143*	A.J. Clark
JC13013	F⁻	AB1157, but *recJ153*	A.J. Clark
SA316		*recA*::Tn*10* mutant derivative of SA270	S.I. Ahmad
RDK1540		*recF*::Tn5	R. Kolodner
N3024	F⁻	*uvrC279*::Tn*10* IN(*rrnD-rrnE*)1	R.G. Lloyd
BT12	F⁻	as AB1157, but *recF400*::Tn*5*, *uvrA215*::MudI(*lac*Ap)	A.J. Clark
JC12123	F⁻	as AB1157, but *recJ 284*::Tn*10*	A.J. Clark
SA78	Hfr	P4x *uvrA,pro,thi*	S.I. Ahmad
KL14	Hfr	*E.coli* K12 wild type	B. Bachmann
SA236		Far ultraviolet light hyper-resistant mutant derivative of KL14 *uvh,uvs*	S.I. Ahmad
SA305		*metB,thr,lys,ilv,thi,uvs*	S.I. Ahmad
SA273		*argB,thr,thi*	S.I. Ahmad
JH102		Rif^R SA236	This study
AB2569	F⁻	(*gpt-proA*)62,*lacY1,tsx29,supE44 galk2,λ⁻,hisG4 xyl5,mtl1 thi1 metA28*	B. Bachmann
AT753	F⁻	*lacY1,supE44,gal6,λ⁻,ilv1,rha1,metB1 argE1,thi1*	B. Bachmann
b. Strains of *E.coli* used to prepare plasmids and bacteriophages			
W3110		colE1 plasmid strain,F⁻, λ⁻,colE1⁺, IN(*rrnD-rrnE*)1	B. Bachmann
KT1087		MudI(*lac*⁺Ap^r),*gpt,proA,argF,rpsl*	K.J. Towner
SF800		(P1::Tn5lac) polI thy nal^r	K.J. Towner
c. Bacteriophage lysate			
Phage P1 vir			K.J. Towner

All media were adjusted to pH 7.0 unless otherwise stated and contained the following constituents:-

Minimal Medium (per litre):-

M9 X10	100ml
0.1M $MgSO_4$	10ml
0.01M $CaCl_2$	10ml
VitB1 (thiamine)	5ml
20% Glucose	20ml
(2% Agar No1 Difco)	

All solutions were autoclaved. Amino acids were added as required from autoclaved 4mg/ml stock solutions to a final concentration of 40µg/ml.

M9 X10 (per litre):-

Na_2HPO_4	60g
KH_2PO_4	30g
NaCl	5g
NH_4Cl	10g

dissolved in distilled water and autoclaved.

LB Medium (per litre):-

Bacto Tryptone (Difco)	10g
Bacto Yeast Extract (Difco)	5g
NaCl	10g

The pH was adjusted to 7.5 with sodium hydroxide and the medium autoclaved.

R-plates for P1 transduction (per litre):-

Bacto Tryptone (Difco)	10g
Bacto Yeast Extract	1g
NaCl	8g
Agar No1	12g

Following autoclaving, 5ml of 20%% glucose(sterile)was added to the medium. Soft top R-agar was prepared as above, but with 8g of Agar No 1 added instead of 12g.

2.3 Buffers and Antibiotics

a. Buffers

All serial dilutions and bacterial cell resuspensions were carried out in Genetic Buffer.

Genetic Buffer (per litre):-

Na_2HPO_4	7g
KH_2PO_4	3g
NaCl	4g
$MgSO_4.7H_2O$	0.2g

The solution was autoclaved.

λdil (TMG buffer) (per litre):-

Tris base	1.21g
$MgSO_4.7H_2O$	1.20g
Gelatin	0.10g

The pH was adjusted to 7.4 with HCl and the buffer sterilised by autoclaving.

b. Antibiotic stock solutions

Antibiotic stock solutions were prepared in water unless otherwise stated.

Ampicillin: Stock solution 25mg/ml, sterilised by filtration and stored at -20°C

Kanamycin: Stock solution 25mg/ml, sterilised by filtration and stored at -20°C

Streptomycin: Stock solution 25mg/ml, sterilised by filtration and stored at -20°C

Tetracycline: Stock solution 25mg/ml, sterilised by filtration and stored at -20°C in the dark.

2.4 Sensitivity Tests

a. Disc sensitivity test.

1ml of an overnight NB culture was added to 4ml soft-top NA in a sterile test tube. The contents of the tube were mixed well and immediately poured on to a plate of NA. A sterile antibiotic assay disc (Whatman, 6mm) impregnated with a DNA-damaging agent, was carefully laid on to the agar surface and the plate incubated overnight at 37°C. The zone of inhibition around the disc (diameter in mm) was recorded.

b. Sensitivity to far ultraviolet light.

Cultures were grown in NB medium. A drop of each culture to be tested was individually placed at one end of a plate of NA. The plate was then raised at that end so that the drop formed a line of culture across the plate. The cultures were allowed to dry on to the plate at room temperature. Using a piece of card, the cultures were then exposed to various doses of far ultraviolet light calculated as described in Appendix I, emitted by a Phillips type TUV, G15T8 (15W) ultraviolet lamp. The dose of far ultraviolet light required to kill the cells was recorded.

c. Sensitivity to psoralen plus near ultraviolet light (PUVA)

Cultures were grown overnight in NB medium and, as described above, applied to NA plates containing 25 µg 8-methoxypsoralen/ml.

The cultures were allowed to dry and, using a piece of card, were exposed to various doses of near ultraviolet light, calculated as described in Appendix I, emitted by a Phillips type HP 3148/A (240v) ultraviolet lamp (~360). The plates were incubated at 37°C for 12-24 hours and the dose of PUVA required to kill the cells recorded.

d. Measurement of cell survival.

1ml of an overnight culture in NB medium was diluted in 9ml genetic buffer containing the required concentration of DNA damaging agent. 0.1ml samples were withdrawn at known time intervals, or after exposure to a known dose of ultraviolet light, (depending on the experiment), serially diluted and plated on to NA. The number of colonies present after 12-24 hours incubation at 37°C was recorded. Experiments were repeated at least four times and the $\log_{10}$ percentage survival for each strain plotted against the concentration of damaging agent or exposure dose of ultraviolet light.

2.5 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed using a Protean II gel apparatus (Bio-Rad, Watford U.K.). Protein samples (0.2mg) in loading buffer were loaded into wells formed in a 4% stacking gel (pH6.8). The proteins were resolved using a 10% separating gel (pH8.8). The gel was run at 30mA constant current for 4 to 5 hours in running buffer. After this time, the gel was removed from the gel apparatus and immersed in fixative for 1 hour. The separated proteins were stained with 0.1% Coomassie blue (Sigma

U.K.) for 15 minutes and then destained overnight in methanol/glacial acetic acid (see below).

The reagents and gel preparations were prepared as follows;

Stacking gel 4%

Acrylamide (10%)	10ml
N'N'-Bis-methylene acrylamide	1.7ml
1M Tris-HCl pH 6.8	11.2ml
Distilled water	6.87ml
10% SDS (stock solution)	300µl
10% Ammonium persulphate (freshly made)	100µl
TEMED	10µl

Separating gel 10%

Acryamide (10%)	10ml
N'N'-Bis-methylene acrylamide	1.50ml
1M Tris-HCI pH 6.8	1.75ml
Distilled water	5.6ml
10% SDS (stock solution)	100µl
10% Ammonium persulphate (freshly made)	50µl
TEMED	5µl

Running Buffer (5x concentrate) per litre

Glycine	114g
Tris	30g
10% SDS	50mls
(adjust pH to 8.5)	

Loading Buffer

	Final concentration
DTT	0.1M
10% SDS	2%
1M Tris HCI pH6.8	80mM
Glycerol	10%
Bromophenol Blue	0.2% in ethanol

Fixative

Methanol	500mls
Glacial Acetic acid	100mls
Distilled water	500mls

Stain

As fixative but with 0.1% Coomassie blue.

Destain

Methanol	50ml
Glacial acetic acid	70ml

Made up to 1 litre with distilled water.

2.6 Preparation of Total Protein Cell Lysates

100ml of an overnight culture in LB medium were centrifuged at 6,000g for 10 minutes. Cells were washed and resuspended in 10ml Tris-HCl (pH 7.5). The cells were lysed by passing them through a French pressure cell (Moore & Sons, Birmingham, U.K.) at 10 tons/square inch. Cell debris was removed by centrifuging the lysate at 5,000g for 10 minutes at 4°C. The supernatant was retained and

kept on ice. A protein assay was performed on each sample as described in section 2.8.

For Polyacrylamide gel electrophoresis, 0.2mg of protein in loading buffer (50µl) was boiled for 3 minutes prior to loading on to the gel with a Hamilton syringe.

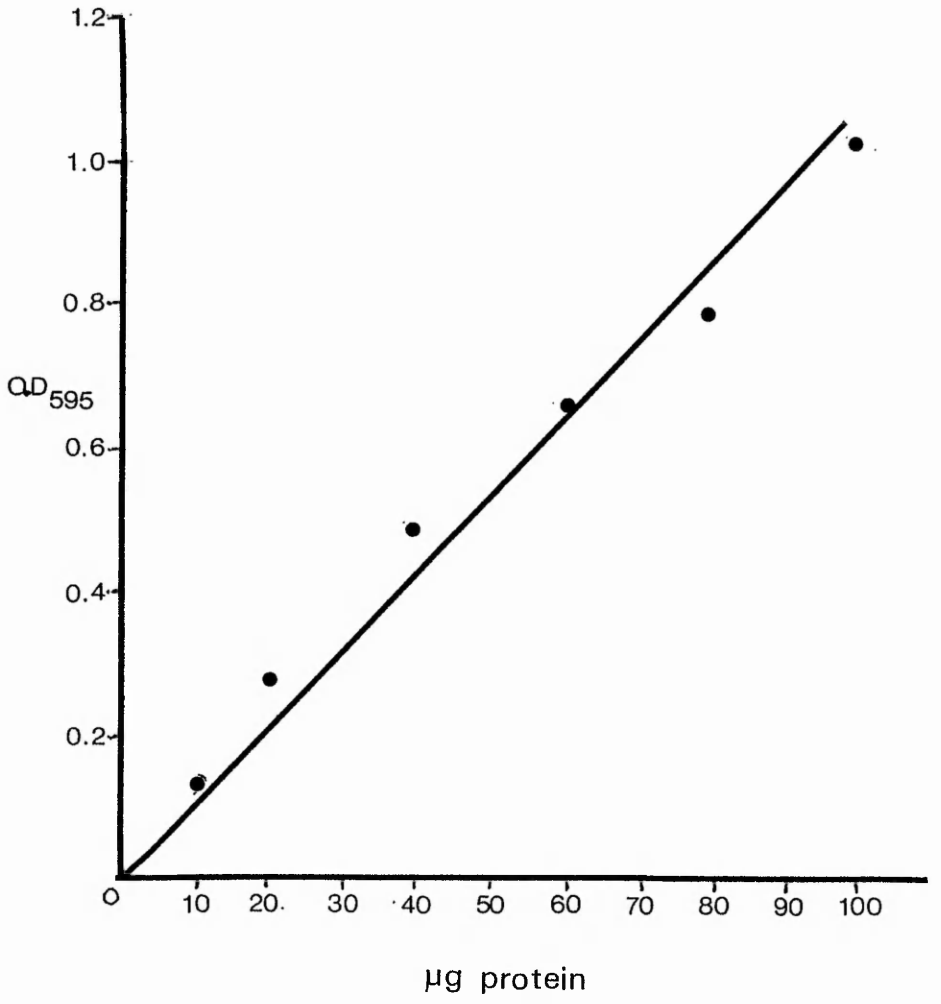
2.7 Determination of Protein Molecular Mass

Calibration proteins in the range 21.5kd to 200kd (Amersham) were run simultaneously with unknown protein samples. A calibration plot of $\log_{10}$ molecular mass against relative mobility (distance moved by the protein / distance moved by the tracking dye) was prepared for the standard proteins. This was then used to estimate the molecular mass of unknown proteins. A separate calibration plot was constructed for each gel.

2.8 Protein Assay

All protein assays were carried out using the Bio-Rad protein assay kit. A standard curve was prepared using known concentrations of bovine serum albumin (stock solution: 1mg/ml). 1ml of Biorad reagent was added to 0.5ml of each standard bovine serum albumin concentration and incubated at room temperature according to the manufacturer's instructions. The optical density at 595 nm was read after 20 minutes and a calibration curve plotted of optical density against protein concentration (fig 14). Unknown protein samples were similarly assayed using the BioRad protein assay kit and the optical densities measured. From the standard curve the concentration of protein present in each sample was calculated.

FIG. 14. CALIBRATION CURVE FOR PROTEIN CONCENTRATION.



2.9 Cell Fractionation: Separation of Cytoplasmic and Membrane fractions (Spratt, 1977)

1000ml of an overnight culture in LB medium were centrifuged at 6,000g for 10 minutes. Cells were resuspended in 8ml 10mM phosphate buffer (10mM KH_2PO_4/10mM K_2HPO_4) plus 0.5% 2-mercaptoethanol. The cells were lysed using a French pressure cell (see section 2.6) and cell debris removed by centrifuging the lysate at 8,000g for 20 minutes. The supernatant was carefully removed and centrifuged at 40,000g for 60 minutes to pellet the membrane fraction. The supernatant, containing the cytoplasmic fraction was retained on ice. The membrane pellet was resuspended in 2ml ice cold 10mM phosphate buffer, recentrifuged at 40,000g for 60 minutes, and finally resuspended in 0.5ml 10mM phosphate buffer. A protein assay was performed on each sample as described in section 2.8. For Polyacrylamide gel electrophoresis, 0.2mg of protein in 50µl of loading buffer was boiled for 3 minutes prior to loading on to the gel with a Hamilton syringe.

2.10 Bacterial Conjugation: Interrupted Mating Experiments

1ml of an overnight culture of strain SA270 (Hfr donor) was subcultured into 10ml NB medium and incubated with gentle shaking at 37°C for 1 hour. Similarly, 1ml AB1157 (recipient) was subcultured into 10ml NB medium and incubated with shaking at 37°C for 1 hour, at which point the cells were growing exponentially. 5ml AB1157 was then pipetted into a sterile 125ml conical flask and incubated with gentle shaking in a shaking water bath at 37°C. 5ml SA270 was added to the flask and 0.2ml of the mixture immediately withdrawn and vortexed vigorously. 0.1ml was plated on to a plate of NA containing

25µg 8-methoxypsoralen/ml and 0.1ml plated on a selective minimal plate without histidine. All plates contained 25µg streptomycin/ml to avoid remating. Further samples were withdrawn after 1,5,10,15,20,30,40 and 50 minutes, diluted as necessary, and plated on to 8-methoxypsoralen plates and selective minimal plates without histidine.

Samples spread on plates containing 8-methoxypsoralen were exposed to a dose of near ultraviolet light lethal to AB1157, but not to SA270. Plates were incubated for 12 hours at 37°C and the numbers of recombinants recorded. A mating curve was constructed for each gene locus, by plotting the number of recombinants obtained against the time of mating.

2.11 P1 Transduction (Miller, 1972)

a. Preparation of lysates.

$CaCl_2$ was added to an overnight culture in NB medium to a final concentration of 2mM. 0.5ml of the culture was placed in five sterile test tubes and incubated at 37°C in a water bath. After 15 minutes incubation, 0.1ml of a phage lysate (titre: 2×10^8 pfu/ml) was added to each of four tubes. The fifth tube was left as a control. After a further 20 minutes incubation at 37°C, 4ml of soft top R-agar was added to each tube and the whole contents immediately poured on to separate, well-dried R plates. The plates were incubated at 37°C for 12 hours.

Following incubation, the plates that showed confluent lysis were retained and the soft agar scraped into a sterile centrifuge tube. 5ml NB medium was added and the contents vortexed vigorously

for 30 seconds. Each centrifuge tube was allowed to stand at room temperature for 10 minutes, after which time the cell debris was removed by centrifuging at 5,000g for 10 minutes. The supernatant, containing phage P1, was transferred to a sterile Universal bottle and 10 drops of chloroform added. The lysate was mixed well and then recentrifuged at 5,000g to remove the chloroform and any remaining bacterial cell debris.

To avoid the necessity for titration, different dilutions of the phage lysate were used to transduce recipient cells.

b. Transduction with P1 lysate.

5ml of a fresh overnight culture of the recipient strain was centrifuged and resuspended in 5ml 0.1M $MgSO_4$/0.005M $CaCl_2$. The culture was shaken vigorously at 37°C for 15 minutes. 0.1ml portions of recipient cells were then added to each of five sterile test tubes. P1 lysate was added to each tube in the following dilutions:-

Tube	Cells (ml)	Phage P1 (ml)
1	0.1	0.1 undil.
2	0.1	0.1 10^{-1} dil.
3	0.1	0.1 10^{-2} dil.
4	0.1	0.1 10^{-3} dil.
5	0.1	___

A sixth tube containing 0.1ml of P1 lysate but no cells was prepared as a second control.

Phage P1 was preadsorbed by incubation for 20 minutes in a 37°C waterbath. 0.2ml 1M sodium citrate was then added to each tube to

prevent readsorption and the entire contents of each tube plated on to selective plates. The plates were incubated at 37°C for up to 48 hours.

2.12 Complementation Analysis

Over-night cultures of the donor KL711(F') and recipient JH109(F^-) in NB medium were subcultured 1:40 and 1:20 respectively into fresh NB and grown at 37°C until the donor had reached a density of $2x10^8$ cells/ml. A mating mixture was prepared by mixing 0.5ml of each strain in a small sterile flask. The flask was placed on a shaker (30rpm) at 37°C for 60 minutes, after which various dilutions of the mixture were plated on to selective plates and incubated at 37°C. Partial diploids were screened for their PUVA phenotype as described in Chapter 5.

2.13 Insertion Mutagenesis

Two methods were employed to isolate a PUVA hyper-sensitive insertion mutant. Method 1 uses MudI(*lac*Ap) phage; method 2 uses the Pl::Tn5*lac* phage.

A. Method 1

(i) Preparation of MudI(*lac*Ap) lysate.

A 5ml culture of the MudI(*lac*Ap) lysogen (KT1087) was grown over night in LB broth at 30°C with shaking. 0.05ml of an overnight culture was subcultured into 10ml LB broth and incubated with shaking until the cells reached early log phase (~0.2 OD_{600}). The culture was heat shocked for 20 minutes in a 42°C waterbath and then transferred to a shaking 37°C waterbath. Lysis of the cells was

observed after approximately 2 hours. 0.1ml chloroform was added to the lysate and vortexed. The cell debris was pelleted by centrifugation at 4,500g for 10 minutes and the supernatant transferred to a sterile screw-top tube. The lysate could be briefly stored at 4°C, but was only used for one experiment since Mu lysates become inactive during long term storage at 4°C.

(ii) Transduction with MudI(*lac*Ap)

A 5ml LB culture of KL16 was grown overnight at 37°C. The culture was then centrifuged at 1500g for 10 minutes and the pellet resuspended in a 2.5ml 10mM $MgSO_4$ containing 5mM CaCl2. The MudI(*lac*Ap) lysate was diluted to 10^{-2} and 10^{-3} in λ dil (see section 2.3). Four small test tubes were prepared as follows:-

Tube	Cells (ml)	MudI(*lac*Ap) (ml)
1	0.1	0.1 10^{-2} dil.
2	0.1	0.1 10^{-3} dil.
3	0.1	___
4	___	0.1 undiluted lysate

The tubes were mixed and incubated for 20 minutes at 30°C without shaking. The samples were then spread on LB plates containing 25µg ampicillin/ml and incubated at 30°C for 12 hours. Colonies were replica-plated on to LB plates and plates containing 25µg 8-methoxypsoralen/ml. The 8-methoxypsoralen plates were exposed to near ultraviolet light as described in section 2.4. 2000 colonies were tested and any which showed PUVA-sensitivity were further tested for their ultraviolet phenotype.

B. Method 2

(i) Preparation of Pl::Tn5*lac* lysate. (Kroos & Kaiser, 1984)

An overnight culture of SF800 (Pl::Tn5*lac*), grown at 30°C in LB broth plus 25µg kanamycin/ml, was subcultured (1:200 dilution) in fresh LB medium containing 25µg kanamycin/ml and 100mM $MgSO_4$. The culture was grown, with shaking at 30°C, until it had reached a density of approximately 5 x 10^7 cells/ml. The cells were then heat shocked in a 50°C water bath for 20 minutes and subsequently transferred to a 37°C shaking water bath until the cells lysed. Lysis was difficult to observe, but usually occurred after about 2 hours. 1ml chloroform was added to the culture and vortexed. Cell debris was removed by centrifugation at 5,000g for 15 minutes. The lysate was stored over a few drops of chloroform at 4°C, but was normally used as rapidly as possible.

(ii) Transduction with Pl::Tn5*lac*

A 10ml overnight culture of SA270 was centrifuged and resuspended in 1ml LB broth plus 5mM $CaCl_2$. 0.1ml of this culture was mixed with 0.1ml undiluted lysate and adsorbed for 30 minutes at 37°C (adsorption at 37°C prevented lysogenisation of SA270 since Pl::Tn5*lac* is temperature-sensitive). 0.1ml 1M sodium citrate was then added and the cells plated out on to MacConkey plates containing 25µg kanamycin/ml. The plates were incubated for up to 2 days at 37°C. Colonies were screened for their PUVA phenotype, with those that were PUVA hyper-sensitive being subsequently examined for their sensitivity to far ultraviolet light. ß-galactosidase activity was measured for the PUVA hyper-sensitive strains isolated, as described below.

2.14 Assay for ß-galactosidase (Miller, 1972)

Cells were grown to late exponential phase in minimal medium (section 2.2), cooled on ice and the following assay performed. The cell density was measured using a spectrophotometer (Unicam, SP1700) at 600nm. 1ml of cells were put in a test tube and one drop of toluene added to partially disrupt the membrane and allow o-nitrophenyl-ß-D-galactoside (ONPG) to diffuse into the cell. 0.2ml of a stock solution of ONPG (4mg/ml) was added to the cells and the tube vortexed for a few seconds. The reaction was carefully timed following the addition of ONPG. After 10 minutes, or at a time when sufficient yellow colour had developed, the reaction was stopped by adding 0.5ml 1M Na_2CO_3 solution.

The optical density of the assay mixture was measured at 420nm and 550nm. Using the following formula (Miller, 1972) the units of ß-galactosidase (which are proportional to the increase in OD_{600} per minute per bacterium) were calculated.

$$\text{Units of ß-galactosidase} = \frac{1000 \times OD_{420} - 1.75 \times OD_{550}}{t \times V \times OD_{600}}$$

OD_{420} and OD_{550} = optical densities read from the reaction mixture after incubation

OD_{600} = cell density before assay

t = time

V = volume of culture used in assay (1ml)

2.15 Induction Experiments with Mitomycin C

Strains to be induced were grown to exponential phase in minimal medium. Proteins were induced by incubating cells with 2µg mitomycin C/ml for 30 minutes (Finch *et al.*, 1985). Following exposure, cells were resuspended in NB. Viable counts were determined at this point. Cells were incubated at 37°C and 50ml samples withdrawn at 0,10,60 and 90 minutes. Cell lysates were prepared using the French pressure cell method and protein extracts resolved on a 10% SDS-Polyacrylamide gel. Protein extract from a wild type KL16 strain was run simultaneously as a control. In some experiments (using KL16 with a Tn*5lac* insertion making it PUVA hyper-sensitive) induced cells were also assayed for ß-galactosidase production (section 2.14). The amount of ß-galactosidase produced by induced cells was compared to that measured in uninduced cells.

2.16 Uptake of Radiolabelled Psoralen into the Cell

Strains were grown at 37°C to an OD_{600} of 1.0. The cells were centrifuged, weighed and resuspended at a concentration of 1mg cells/ml of minimal medium lacking glucose. A 1ml aliquot of cells was transferred to a vessel with a small magnetic stirrer and kept at 37°C. Radiolabelled ^{3}H-8-methoxypsoralen was added to the cells at a final concentration of 10µM and a specific activity of 29,000cpm/nmol (see Appendix I). 100µl samples were removed at intervals and placed on nitrocellulose filters under vacuum using the apparatus shown in fig. 15. The cells were washed with minimal medium (without glucose) to remove free ^{3}H-8-methoxypsoralen not taken up by the cells. The nitrocellulose filters were air dried and then dissolved in 1ml 2-ethoxyethanol in a scintillation vial.

Scintillation fluid (9ml) was added and the amount of radioactivity contained on the filter measured.

To measure the amount of ^{3}H-8-methoxypsoralen in spheroplasted cells, 0.1ml cultures containing 1nmol ^{3}H-8-methoxypsoralen were treated with 5µl lysozyme (2mg/ml) and 0.2ml 1.5mM EDTA in an eppendorf tube followed by centrifugation at high speed for 1 minute in a microcentrifuge (MSE) to separate the cells from the periplasmic contents. The spheroplasts were lysed by adding 0.5ml ice cold water and the amount of radioactivity measured. The amount of radiolabel contained in whole cells was measured in the same way to serve as a control.

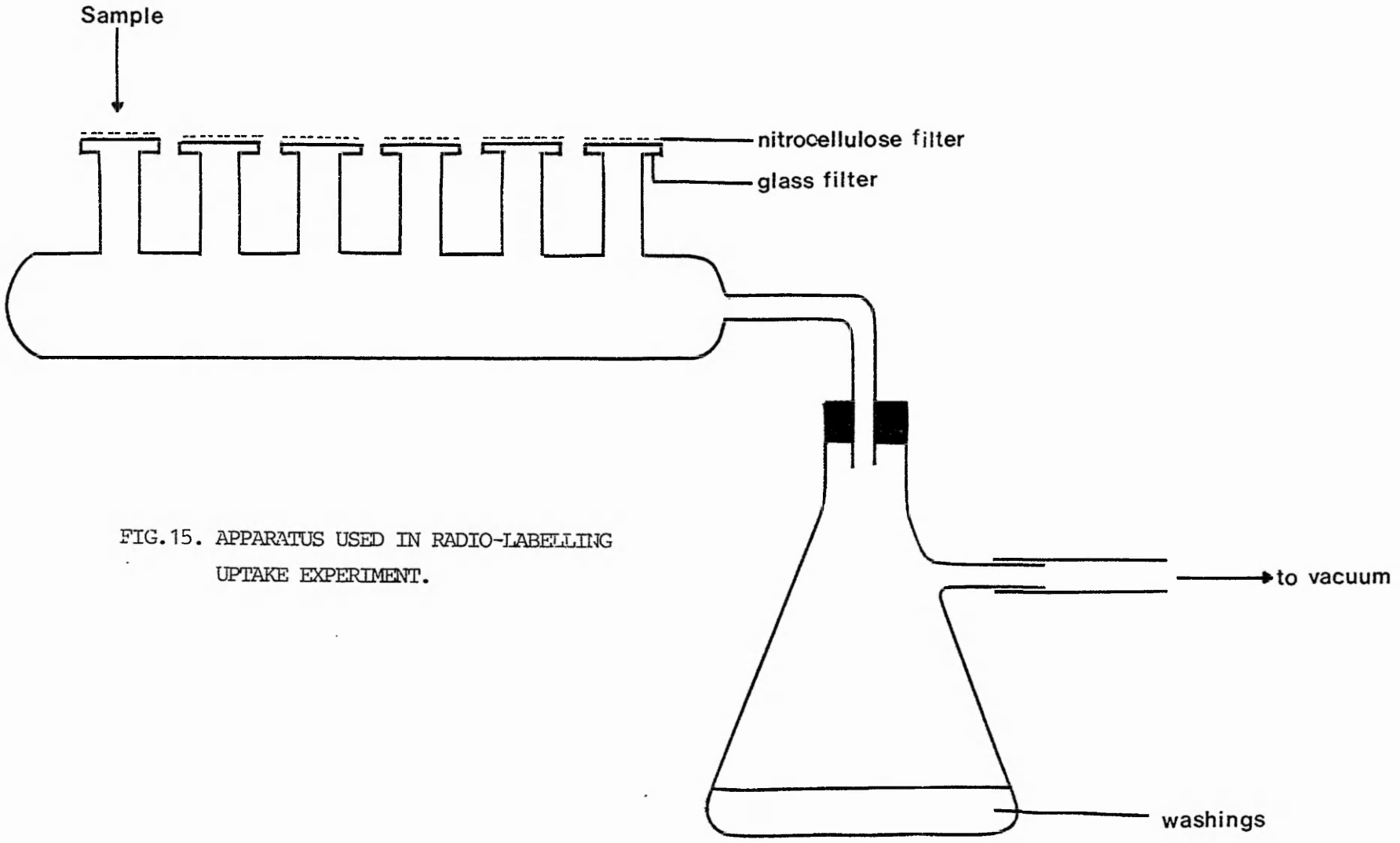


FIG. 15. APPARATUS USED IN RADIO-LABELLING UPTAKE EXPERIMENT.

2.17 Large Scale Isolation of *ColE1* Plasmid DNA (Maniatis *et al.*, 1982)

(i) Solutions used in preparation of colE1 plasmid DNA

STE	0.1M NaCl
	10mM Tris-HCl pH 7.8
	1 mM EDTA
Solution I	50mM Glucose
	25mM Tris-HCl pH 8.0
	10mM EDTA
Solution II	0.2N NaOH
	1% SDS

5M Potassium acetate

60ml	5M Potassium acetate
11.5ml	Glacial acetic acid
28.5ml	Distilled water

TE buffer	10mM Tris-HCl pH 8.0
	1mM EDTA

(ii) Growth of bacteria and amplification of the plasmid

10ml LB medium was inoculated with strain W3110 carrying the colE1 plasmid. The culture was incubated overnight at 37°C with vigorous shaking. 0.1ml of the overnight culture was subcultured in 25ml LB medium and incubated at 37°C, again with vigorous shaking, until the culture reached late log phase (OD_{600} = 0.6). The 25ml culture was then inoculated into 500ml LB medium (prewarmed to 37°C) and incubated with shaking for exactly 2.5 hours. 2.5ml of a

chloramphenicol stock solution (34mg/ml ethanol) was added to the culture to give a final concentration of 179µg chloramphenicol/ml. The culture was then incubated at 37°C with shaking for 12 hours.

(iii) Lysis of bacteria

The 500ml overnight culture was centrifuged at 5,000g for 10 minutes and the cells resuspended in 50ml ice cold STE. The cells were recentrifuged at 5,000g for 10 minutes and resuspended in 10ml solution 1 containing 5mg lysozyme/ml (added just before use). The solution was allowed to stand at room temperature for 5 minutes. 10ml of solution II was then added to the resuspended cells and the tube contents mixed and incubated on ice for 10 minutes. 15ml of an ice cold 5M potassium acetate solution (pH 4.8) was added and the contents of the tube mixed vigorously. The mixture was allowed to stand on ice for 10 minutes.

The mixture was centrifuged at 20,000g for 20 minutes to remove chromosomal DNA and debris. The supernatant was removed, mixed with 0.6x the volume of isopropanol, and left to stand at room temperature for 15 minutes. The plasmid DNA was recovered by centrifugation at 12,000g for 30 minutes at room temperature. The DNA pellet was washed with 70% ethanol and dried in a vacuum desiccator for 1 hour. The pellet was dissolved in 8ml TE buffer.

(iv) Purification of plasmid DNA by centrifugation on a caesium chloride gradient.

The plasmid DNA was purified on a caesium chloride gradient as described by Maniatis *et al.* (1982). Centrifugation was carried out in a Beckman Ultra centrifuge using a Beckman vertical rotor at

45,000 rpm for 36 hours at 20°C. Plasmid DNA was recovered using a hypodermic needle and the ethidium bromide removed by dialysis. RNase (0.1µg/µl) was added to the DNA and incubated at 37°C for 1 hour to remove RNA from the plasmid preparation. The plasmid DNA was precipitated with an equal volume of isopropanol and recovered by centrifugation as described above. The pellet was washed with 70% ethanol and dried in a vacuum desiccator for 1 hour. The plasmid DNA was then dissolved in TE buffer to a final concentration of 200 µg/ml and stored at -20°C.

2.18 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using a mini-sub cell unit (BioRad U.K.). 0.7% agarose gels were made using agarose (Sigma, electrophoresis grade) dissolved in TBE buffer. Following melting of the agarose and cooling to 50°C, ethidium bromide was added to a final concentration of 0.5 µg/ml and the agarose poured into the gel mold. Once the gel had completely set the comb was removed and electrophoresis buffer added to just cover the gel. Samples of DNA in a loading buffer were loaded into the slots of the submerged gel and electrophoresed for approximately 1 hour at 120V constant voltage. Once the dye front had migrated almost the full length of the gel, the DNA bands were observed using an ultraviolet transilluminator. (UVA-Products)

Solutions used in agarose gel electrophoresis.

TBE buffer (5x concentrate)

Tris Base	54g
Boric acid	27.5g
0.5 EDTA (pH 8.0)	20ml

Made up to 1000ml with distilled water

Loading buffer

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol in distilled water

2.19 Assay for Nuclease Activity (Methods adapted from Seeberg, 1981)

(i) Covalently closed circular ColE1 plasmid DNA was used as a substrate. The DNA was purified as described in section 2.17. PUVA damaged DNA was prepared by adding 25µg of 8-methoxypsoralen to 100µg of DNA dissolved in 1ml TE buffer. The mixture was exposed to 20J m^{-2} near ultraviolet light.

Reaction mixtures for assaying nuclease activity contained:

2mmoles ATP

0.8µg ColE1 DNA (either damaged or undamaged)

5µmoles MOPS (pH 7.7)

10µmoles KCl

2µmoles $MgSO_4$

0.1µmole EDTA

0.1µmole dithiothreitol

10µl total protein extract (100µg of protein)

in a total volume of 50µl

The samples were incubated in a water bath at 37°C and 5µl samples removed at timed intervals. These were mixed with 3µl of loading buffer (section 2.18), loaded on to a 0.7% agarose gel, and electrophoresed at 120 volts for approximately 1 hour. DNA was observed using an ultraviolet transilluminator.

(ii) A second method employing linear DNA was also used. For this method, PUVA damaged λ DNA was prepared by adding 25µg 8-methoxypsoralen to 100µg of DNA dissolved in 1ml TE buffer. (λDNA was obtained from Pharmacia). The mixture was exposed to near ultraviolet light as described above.

Reaction mixtures for assaying nuclease activity contained:

2mmoles ATP

0.8µg λDNA (either damaged or undamaged)

5µmoles MOP (pH 7.7)

10µmoles KCl

2µmoles $MgSO_4$

0.1µmole EDTA

0.1µmole dithiothreitol

10µl total protein extract (100µg of protein)

in a total volume of 50µl

The mixture was incubated in a water bath at 37°C for a timed period. 5µl samples were removed and mixed with 3µl loading buffer (section 2.18). The samples were loaded on to a 0.7% agarose gel and electrophoresed at 120 volts for approximately 1 hour. DNA was observed using an ultraviolet transilluminator.

In some experiments, reactions with DNA were carried out in TE buffer alone.

2.20 Radiolabelling of λ DNA with ^{3}H-8-methoxypsoralen

In order to determine whether psoralen was binding to λDNA, ^{3}H-8-methoxypsoralen was added to the λDNA prior to incubation with near ultraviolet light (section 2.19). The final concentration of 8-methoxypsoralen in the incubation mixture was 10µM (see Appendix I for calculations)

λDNA labelled with ^{3}H-8-methoxypsoralen was used in the experiment described in section 2.19. Following electrophoresis of

λDNA treated with protein extracts, the gel was incubated for 15 minutes in amplifier (Amplify, Amersham). The gel was thendried on to card using a gel dryer (BioRad U.K.) and the card exposed to Amersham Hyper film for 3 to 5 days at -70°C. The film was developed and fixed using Kodak LX24 developer and Kodak FX40 fixer.

2.21 Protein Purification using FPLC

Crude cell extracts of strains SA270 and KL16 were first prepared as described in section 2.6 and total protein assayed as described in section 2.8. Total protein extracts were freeze dried (Edwards Model EF03) and resuspended in elution buffer at a final concentration of 80mg/ml when required. Gel filtration was then used to separate proteins according to their molecular mass, followed by ion exchange chromatography to separate proteins according to their charge. Both Superose 12 and MonoQ columns used in the purification of the 55kd protein were packed according to the manufacturers' instructions.

(i) Gel filtration using the Superose 12 column (Pharmacia)

The Superose 12 column, used for separating proteins according to their molecular mass was calibrated using a mixture of proteins of known molecular mass. The column was equilibrated overnight with 20mM phosphate buffer (20mM K_2HPO_4:20mM KH_2PO_4) pH 7.1. This buffer was also subsequently used as the eluent in the experiment. 200µl of the standard protein mixture (at a concentration of 10mg/ml) was applied to the column and the proteins separated according to their molecular mass. An ultraviolet light protein detector (Pharmacia) linked to an integrator was used to monitor the elution of each protein and, by using the trace produced (fig. 57 Appendix I), the

retention time of each protein could be calculated as described in Appendix I.

In order to purify the 55kd protein over-expressed in strain SA270, a total protein cell extract was resuspended in 20mM phosphate elution buffer at a final concentration of 80mg/ml. 200µl of protein sample was loaded on to the Superose 12 column and the 0.5ml fractions collected every minute and retained on ice. Those fractions containing proteins of an estimated molecular mass range of 10kd to 100kd were pooled and freeze dried. This procedure was repeated with KL16 total protein extract. Samples of pooled protein extracts were examined for the presence of the 55kd protein using SDS-Polyacrylamide gel electrophoresis.

(ii) High resolution ion exchange

A MonoQ HR 5/5 anion exchange column (Pharmacia) was equilibrated overnight with 50mM Tris-HCl (pH 7.5). Pooled protein fractions from gel filtration were resuspended in 50mM Tris-HCl (pH 7.4) at a final concentration of 90µg/ml. 200µl of sample was loaded on to the column and separated using a sodium chloride gradient (fig. 59 Appendix I). 0.5ml fractions were collected every minute and retained on ice. Using the ultraviolet monitor and integrated protein scan profiles, eluted proteins from KL16 and SA270 were compared. Protein fractions of interest were electrophoresed using a 10% SDS-Polyacrylamide gel.

Chapter 3

PROPERTIES OF STRAIN SA270 IN RESPONSE TO MUTAGENIC AGENTS

3.1 Background

SA270, a mutant strain of *E.coli* K12, is more resistant to 8-methoxypsoralen plus near ultraviolet light (PUVA) than its parent wild type strain KL16 (Ahmad & Holland, 1985). In contrast, both strains were equally sensitive to far ultraviolet light (~254nm), which is a monofunctional damaging agent producing cyclobutane pyrimidine dimers in one strand of DNA. This Chapter describes studies carried out using strains SA270 and KL16 in order to determine whether SA270 was also resistant to other known crosslinking agents such as mitomycin C, mechloroethamine HCl (nitrogen mustard) and platosin. The sensitivity of strains SA270 and KL16 to other monofunctional DNA damaging agents was also determined. In addition Polyacrylamide gel electrophoresis of total protein extracts from SA270 and KL16 was performed in order to confirm the over-expression of the 55kd protein observed by Ahmad & Holland (1985) in strain SA270 and to determine the location of the protein within the cell.

Strains SA270 and KL16 were first exposed to a range of DNA damaging agents using the disc sensitivity method described in section 2.4. Treatments involving ultraviolet light irradiation were carried out using the method described in section 2.4. A range of DNA damaging agents causing both single strand damage and crosslinking damage were chosen for the tests.

3.2 Sensitivity of SA270 and KL16 to monofunctional DNA damaging agents

The sensitivity of SA270 and KL16 to the monofunctional damaging agents N-methyl-N'-nitro-N-nitrosoguanidine, ethylmethane

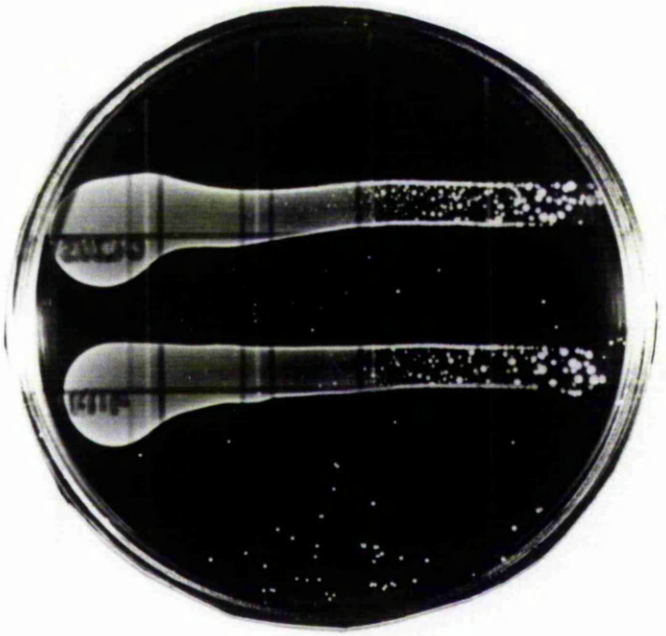
sulphonate, methyl methane sulphonate, far ultraviolet light (~254nm) and near ultraviolet light (~360nm) was determined using the disc sensitivity method and the plate exposure method (section 2.4). For each DNA damaging agent the zone of inhibition was measured around the antibiotic assay disc. For far ultraviolet light and near ultraviolet light exposure, the dose in Jm^{-2} at which the cells were killed was recorded. Strain SA270 was as sensitive as its parent wild type strain, KL16, towards N-methyl-N'-nitro-N-nitrosoguanidine, ethylmethane sulphonate and methyl methane sulphonate (table 3). In the dose given, SA270 was as sensitive to far and near ultraviolet light as KL16 (fig. 16). This result was confirmed quantitatively by measuring the percentage survival of SA270 and KL16 following exposure to an increasing dose of far ultraviolet light (fig. 17) and near ultraviolet light (fig.18).

Table 3

Sensitivity of SA270 and KL16 towards DNA alkylating agents

DNA damaging agent	Mean zone of inhibition (mm)	
	SA270	KL16
Nitrosoguanidine (1mg/ml)	18	17
Ethyl methane sulphonate	16	15
Methyl methane sulphonate	23	24

Far ultraviolet



0 200 400 600 800

Jm^{-2}

0 5520 11040 16560 22080

Near ultraviolet

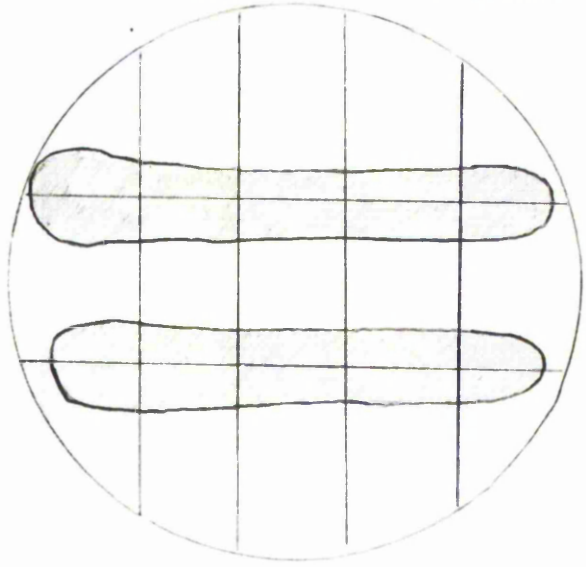


FIG.16. SENSITIVITY OF SA270 AND KL16 TOWARDS FAR AND NEAR ULTRAVIOLET LIGHT USING THE PLATE EXPOSURE METHOD.

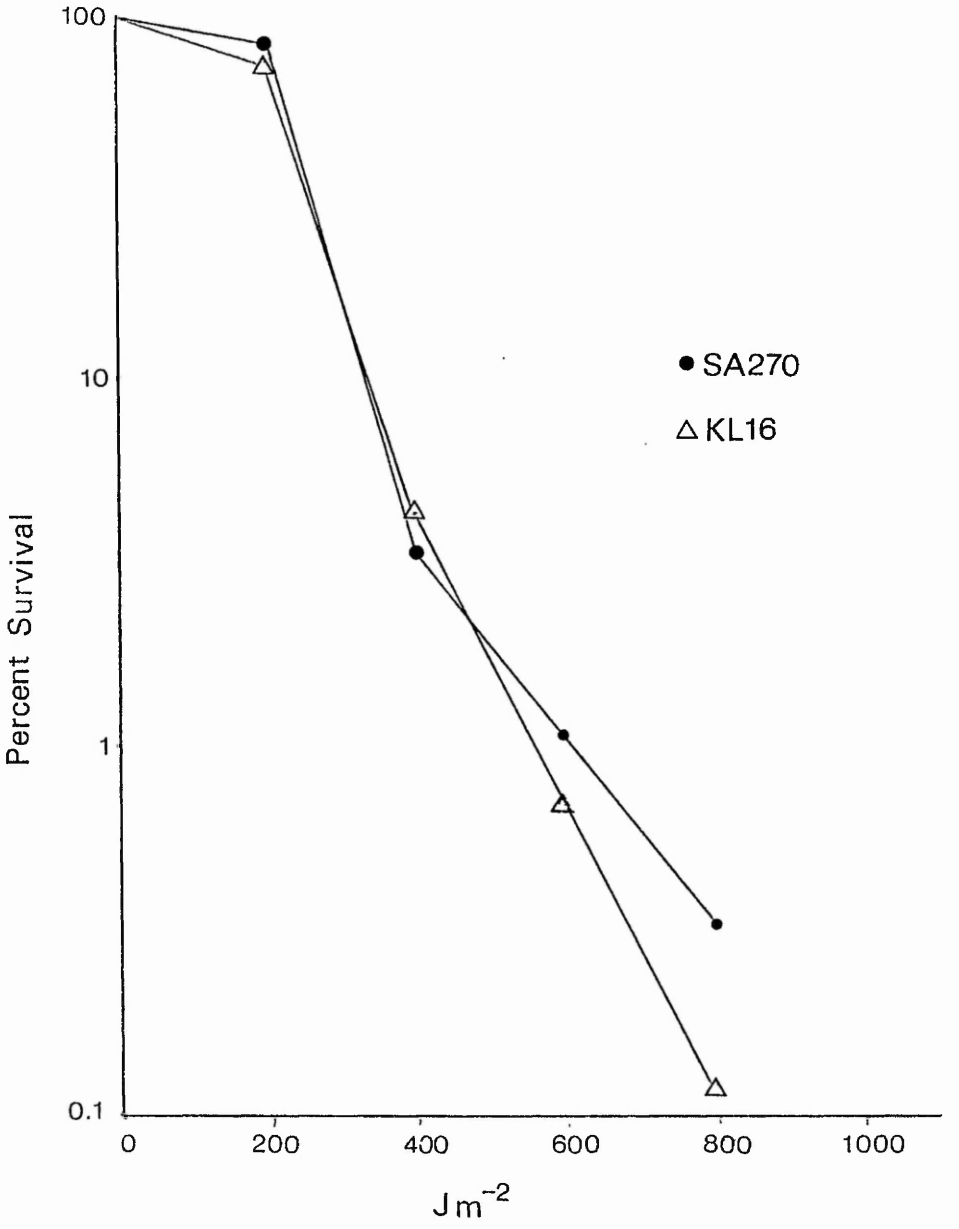


FIG.17. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF FAR ULTRAVIOLET LIGHT.

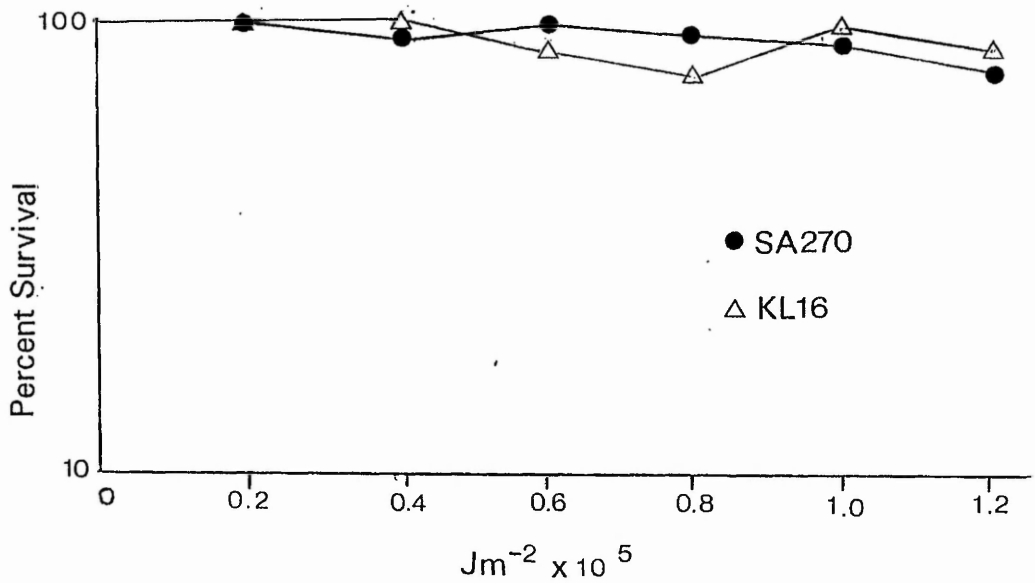


FIG.18. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF NEAR ULTRAVIOLET LIGHT.

3.3 Sensitivity of SA270 and KL16 to DNA crosslinking agents

In their previous study, Ahmad & Holland (1985) tested the sensitivity of strain SA270 towards PUVA. Compared to KL16, SA270 was found to be more resistant exclusively to PUVA. In order to confirm this result the PUVA hyper-resistant phenotype was first tested using the plate exposure method (section 2.4) with the result shown in fig. 19. The PUVA phenotype of SA270 and KL16 was then examined in more detail by producing a survival curve of each strain against 25µg 8-methoxypsoralen/ml plus an increasing dose of near ultraviolet light (fig. 20). Three other known DNA crosslinking agents, mitomycin C, mechloroethamine HCl (nitrogen mustard) and platosin were also tested against strain SA270 using the disc sensitivity test. SA270 showed resistance to mitomycin C and nitrogen mustard compared to wild type KL16 but the two strains showed equal resistance to platosin (table 4). Survival curves of strains SA270 and KL16, following exposure to mitomycin C (50µg/ml) or nitrogen mustard (50µg/ml) for 0, 5, 10 and 15 minutes confirmed that SA270 also had a hyper-resistant phenotype for these DNA crosslinking agents (fig. 21).

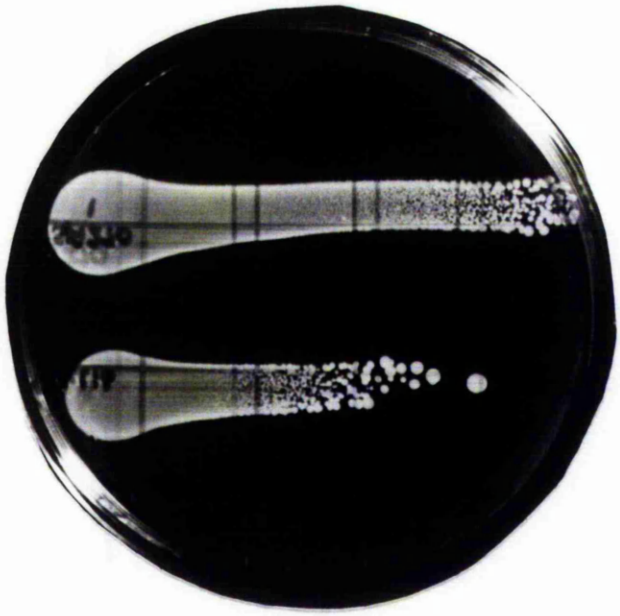


FIG. 19. SENSITIVITY OF SA270 AND KL16 TOWARDS PUVA MEASURED USING THE PLATE EXPOSURE METHOD.

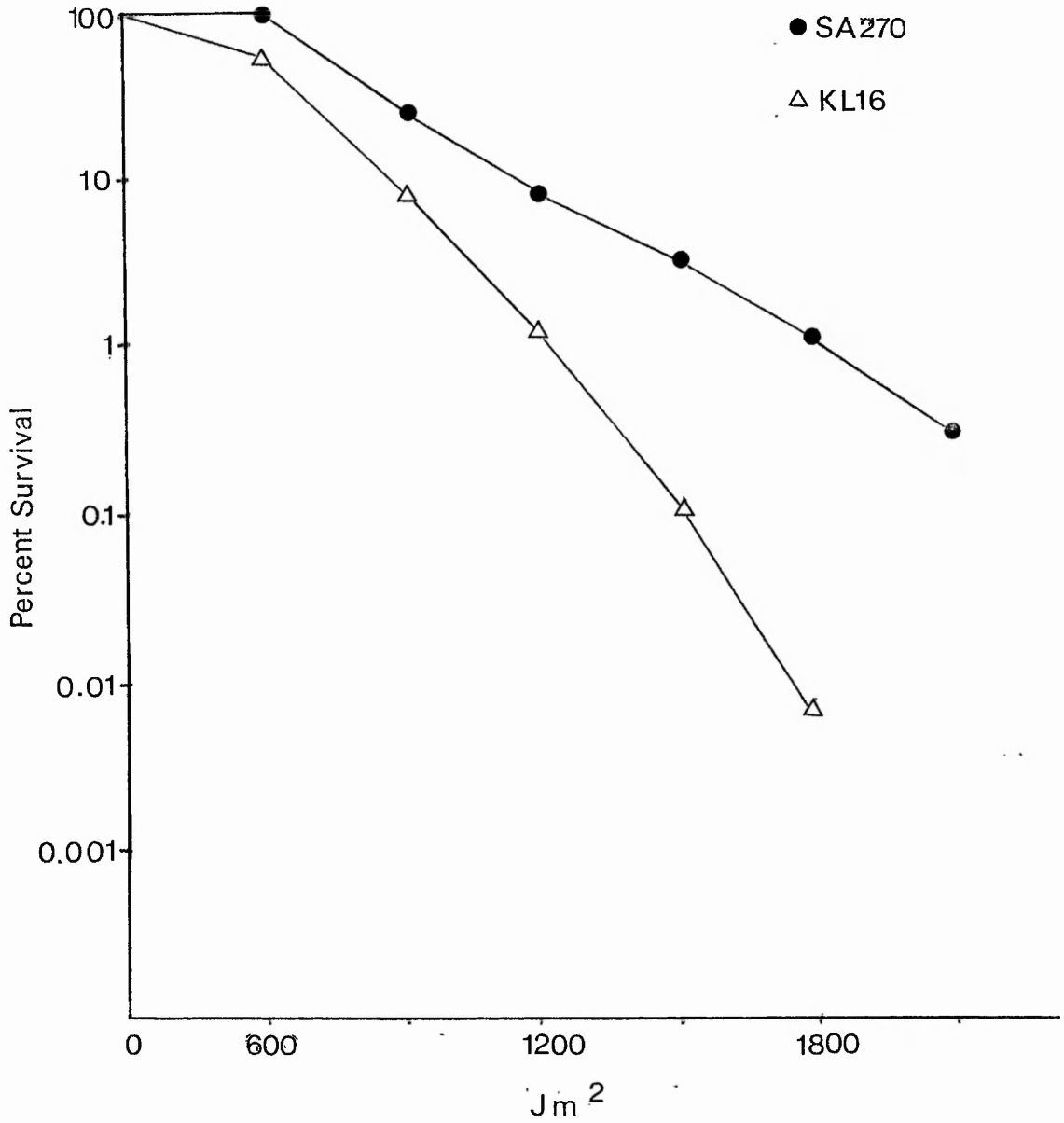
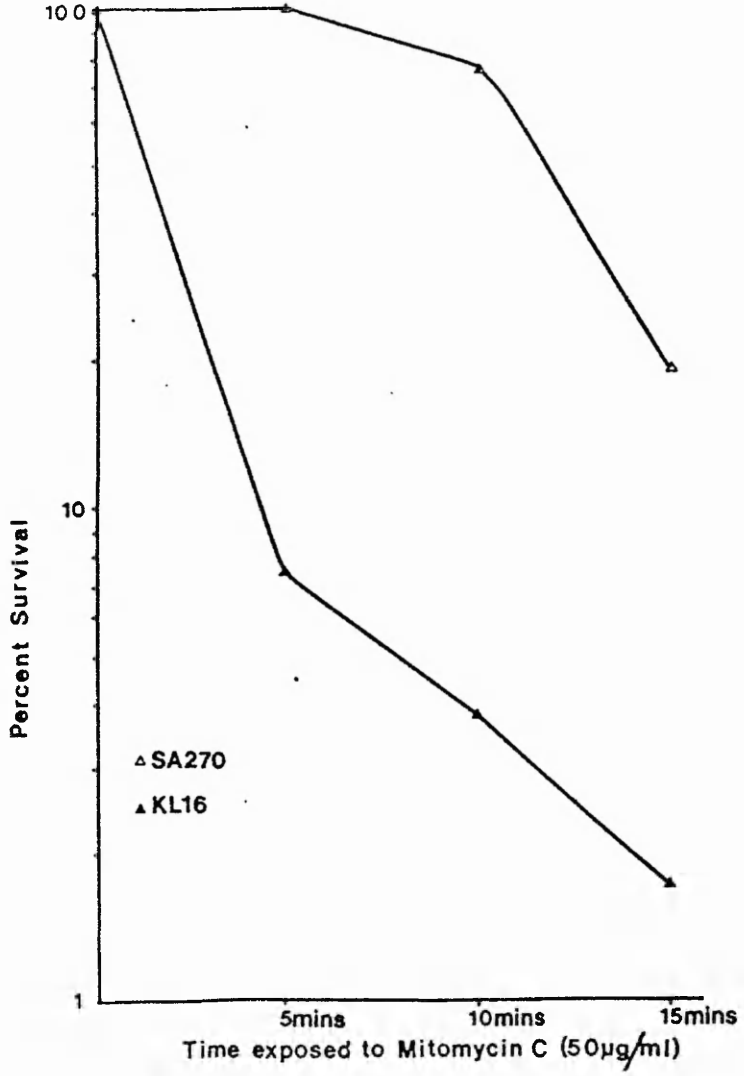


FIG 20. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF PUVA.

Table 4

Sensitivity of SA270 and KL16 towards DNA crosslinking agents

DNA damaging agent	Mean zone of inhibition (mm)	
	SA270	KL16
Mitomycin C (50ug/ml)	21	28
Nitrogen mustard (50ug/ml)	10	25
Platosin (50ug/ml)	12	12



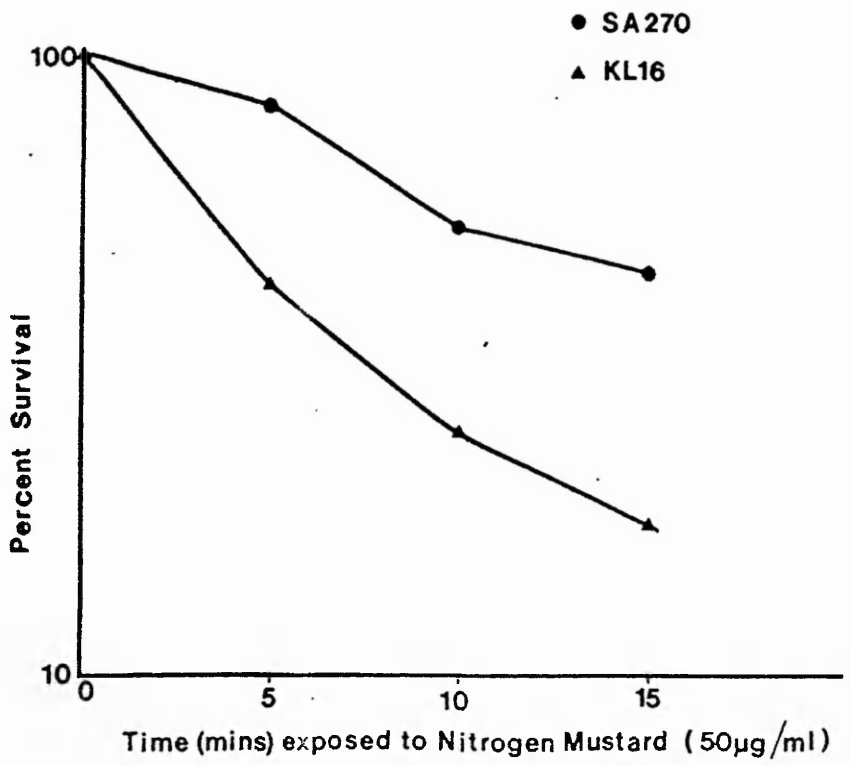


FIG.21. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF i. MITOMYCIN C AND ii. NITROGEN MUSTARD.

3.4 Sensitivity of SA270 and KL16 to hydrogen peroxide

In a disc sensitivity test SA270 appeared to be slightly more resistant to hydrogen peroxide than KL16. However, a survival curve for SA270 and KL16 following 10 minutes exposure to varying concentrations of hydrogen peroxide indicated that both strains were equally sensitive to this damaging agent (fig. 22).

The synergistic killing effect of near ultraviolet light plus hydrogen peroxide has been observed previously. Hartman & Eisenstark (1978) demonstrated that the number of single strand breaks formed in the DNA of wild type *E.coli* irradiated with near ultraviolet light, following exposure to hydrogen peroxide, was greater than the sum of single strand breaks caused by near ultraviolet light and hydrogen peroxide independently. SA270 was no more resistant to the damaging effects caused by near ultraviolet light plus hydrogen peroxide than its wild type parent strain KL16 (fig. 23).

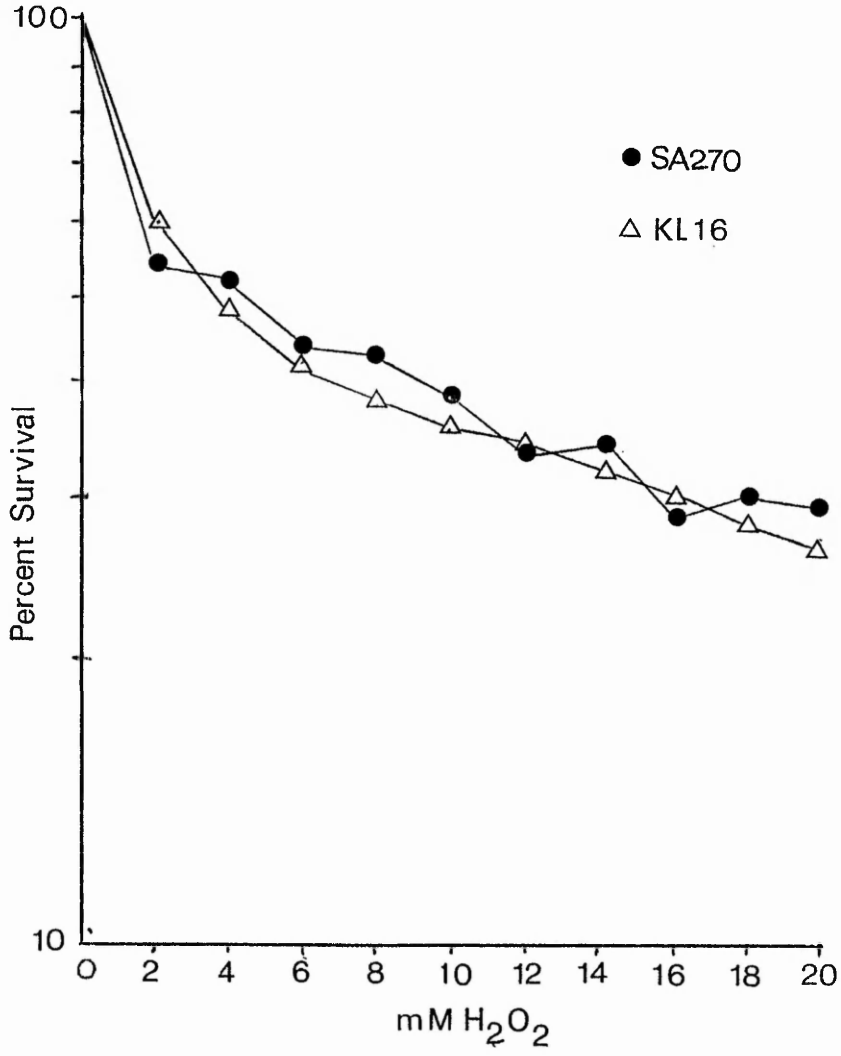


FIG 22. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF HYDROGEN PEROXIDE.

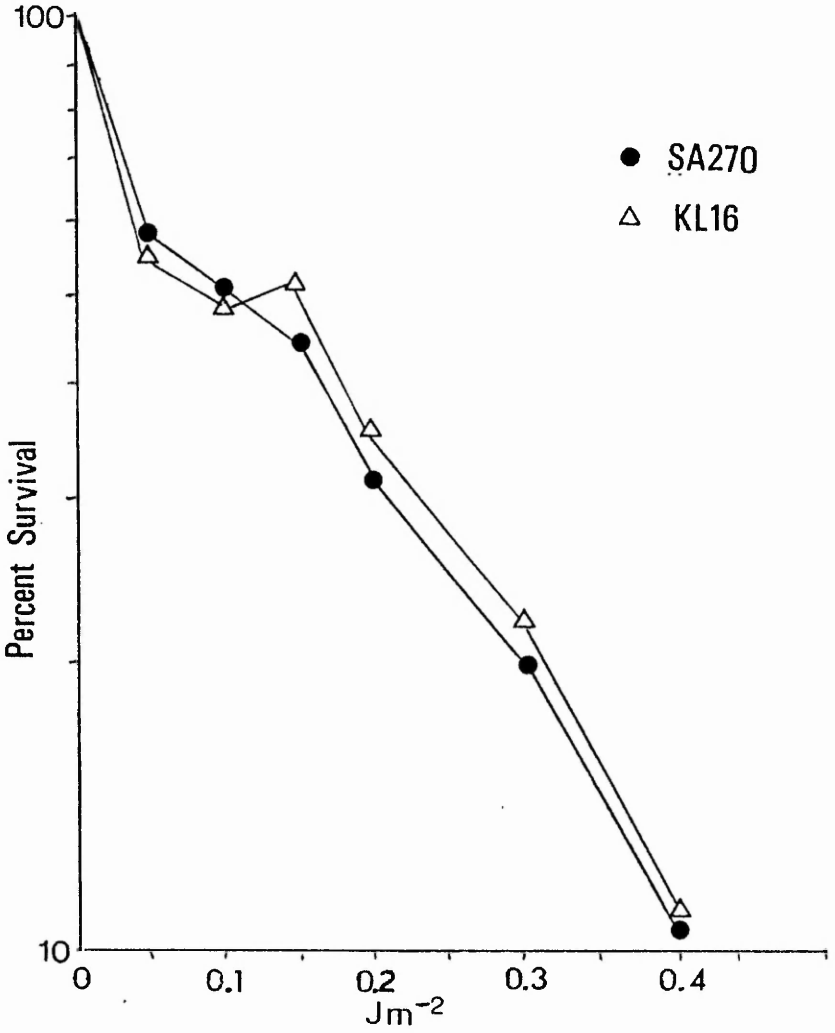


FIG.23. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF HYDROGEN PEROXIDE (10mM) PLUS NEAR ULTRAVIOLET LIGHT

3.5 Sensitivity of SA270 and KL16 to far ultraviolet light before and after exposure to near ultraviolet light

E.coli photolyase catalyses the light dependent repair of pyrimidine dimers induced in DNA by far ultraviolet light. The enzyme had an estimated molecular weight of 53,994d (Sancar & Sancar, 1988), similar to that of the over-expressed protein in strain SA270. In order to determine whether or not the 55kd over-expressed protein was photolyase, strains SA270 and KL16 were first irradiated with far ultraviolet light followed by exposure to near ultraviolet light. Results showed that the percentage survival of strains SA270 and KL16, following far ultraviolet light irradiation was equal after a dose of $600Jm^{-2}$. Following subsequent irradiation with near ultraviolet light, the percentage survival increased at the same rate for both strains (fig. 24), demonstrating that SA270 does not have a greater capacity for removing pyrimidine dimers due to over-expression of the 55kd protein, which is therefore not showing photolyase activity.

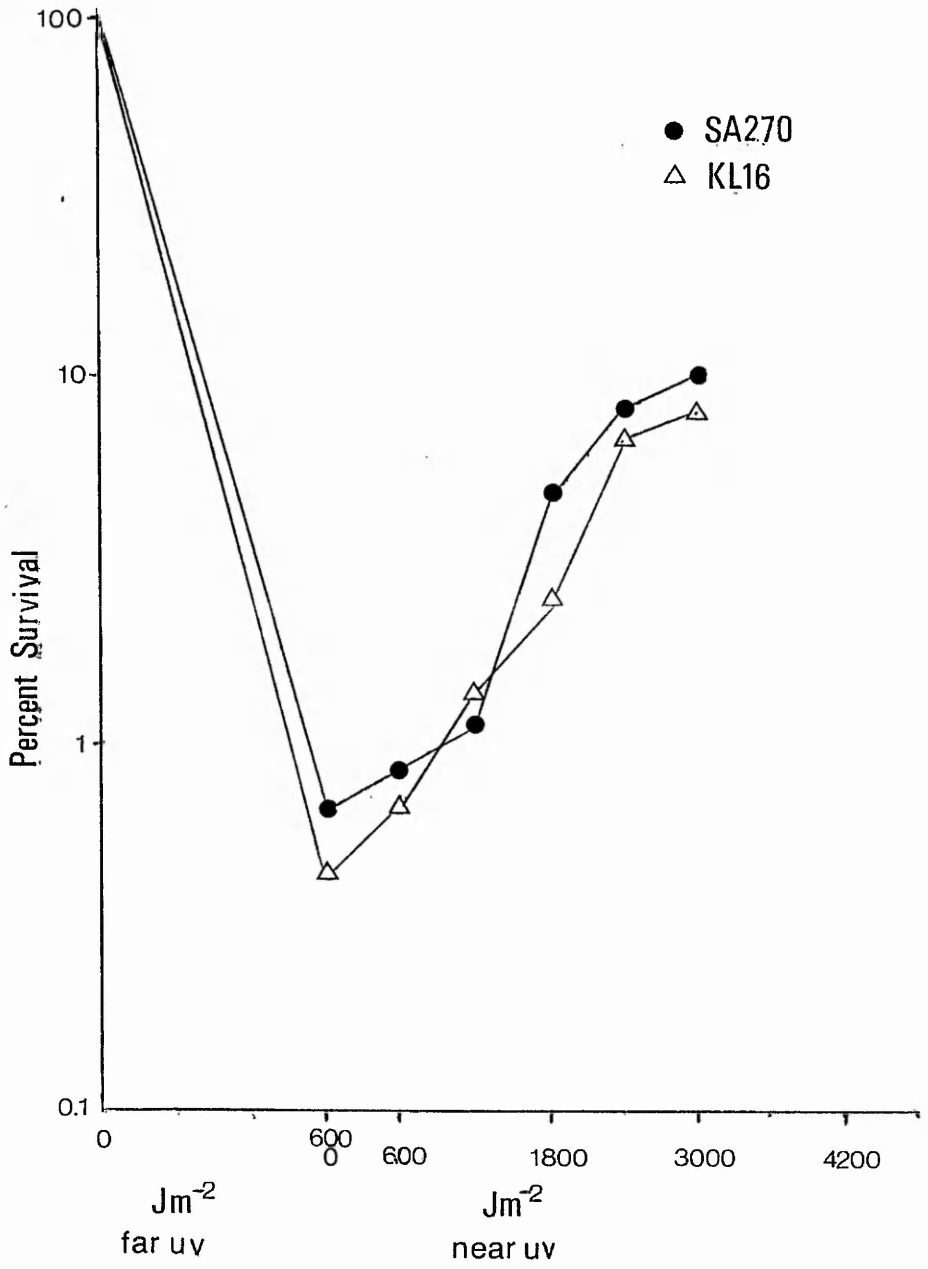


FIG. 24. BACTERIAL KILL CURVES OF SA270 AND KL16 IN THE PRESENCE OF NEAR ULTRAVIOLET LIGHT FOLLOWING EXPOSURE TO 600 Jm^{-2} FAR ULTRAVIOLET LIGHT.

3.6 SDS-Polyacrylamide gel electrophoresis studies

SDS-Polyacrylamide gel electrophoresis studies of total protein cell extracts of strains KL16 and SA270 confirmed that the mutant strain was producing a protein of 55kd molecular weight in higher concentrations than the wild type parent strain KL16 (fig. 25).

3.7 Location of the 55kd protein in the *E.coli* cell

An experiment designed to isolate the membrane fraction (containing both inner and outer membrane proteins) from the cytoplasmic membrane fraction (Spratt, 1977 section 2.9), was carried out to determine the position of the 55kd protein in the bacterial cell. SDS-Polyacrylamide gel electrophoresis of the cytoplasmic and membrane fractions of SA270 showed that the protein was located in the cytoplasmic fraction(fig. 26).

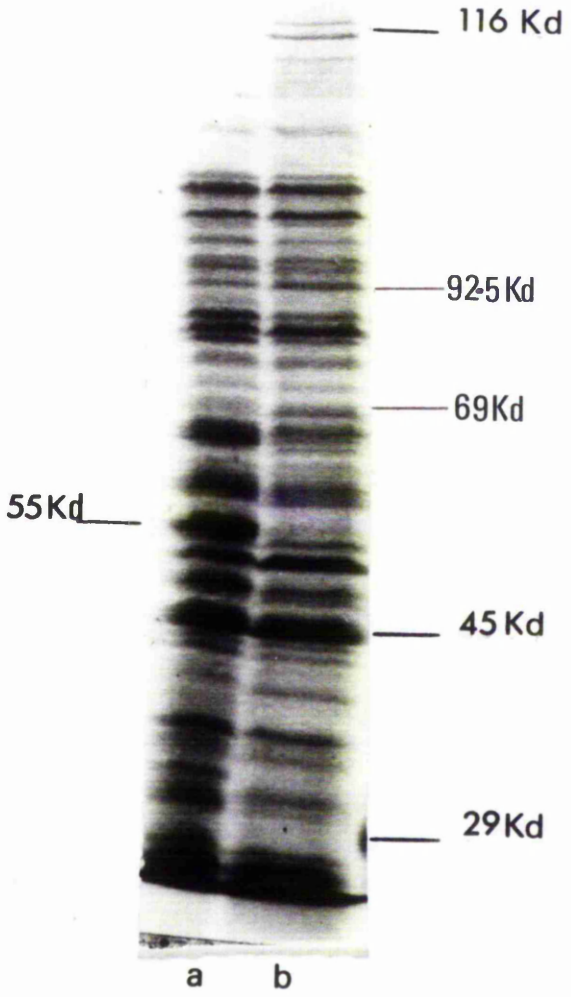


FIG.25. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TOTAL PROTEIN EXTRACTS OF a. SA270 AND b. KL16.

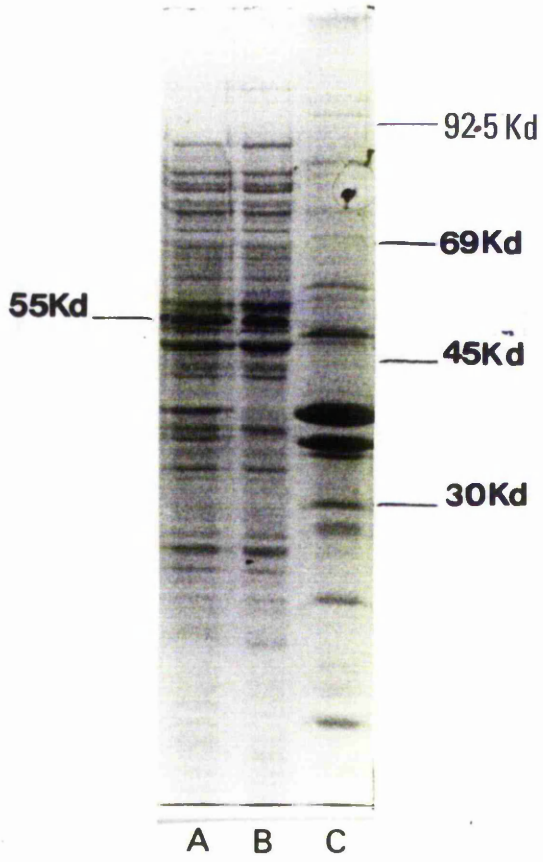


FIG 26. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF SOLUBLE AND MEMBRANE PROTEIN FRACTIONS OF SA270.

A. SA270

B. SOLUBLE FRACTION

C. MEMBRANE FRACTION.

3.8 Discussion

The results in this Chapter confirmed that strain SA270 was hyper-resistant to PUVA and also demonstrated its resistance towards the DNA crosslinking agents mitomycin C and nitrogen mustard. SA270 showed wild type sensitivity to all other DNA damaging agents to which it was exposed.

Mitomycin C is an anti-bacterial and anti-neoplastic agent used in chemotherapy that reacts with DNA in a similar way to psoralens in that it is able to form DNA interstrand crosslinks (Iyer & Szybalski, 1963). Unlike psoralens, mitomycin C does not require near ultraviolet light to form DNA diadducts. Enzyme catalysed chemical reduction of the mitomycin C molecule is however necessary for the formation of crosslinks (fig. 27). These occur mainly between guanine and cytosine bases. Nitrogen mustard is a bifunctional alkylator capable of producing interstrand DNA crosslinks, and, as with mitomycin C does not require near ultraviolet light to form DNA diadducts. Approximately 4% of the nitrogen mustard molecules bound to DNA form interstrand crosslinks, mainly between the N^7 positions of guanine molecules on opposite DNA strands (fig. 28) (Kohn *et al.*, 1966).

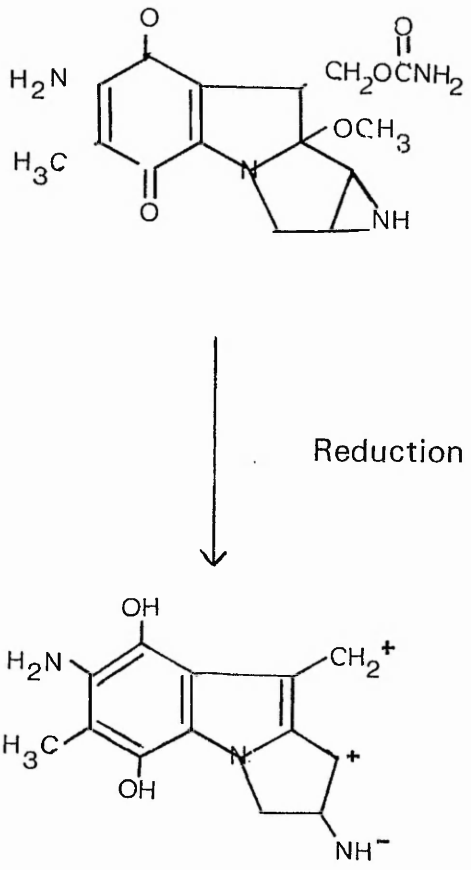


FIG. 27. CHEMICAL REDUCTION OF MITOMYCIN C.
THE SITE OF THE MITOMYCIN C-DNA REACTION HAS NOT YET BEEN FULLY ELUCIDATED. HOWEVER, A PREFERENCE FOR CROSSLINKING BETWEEN G AND C BASES HAS BEEN NOTED.

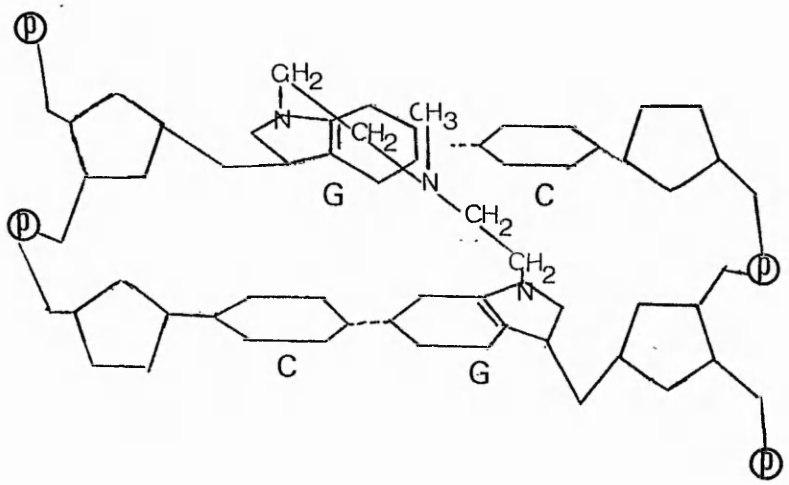


FIG.28. NITROGEN MUSTARD DNA INTERACTION.

SCHEMATIC REPRESENTATION OF THE CROSSLINKING OF DNA BY NITROGEN MUSTARD THROUGH THE N POSITION OF TWO GUANINES.

The repair of DNA interstrand crosslinks formed by mitomycin C and nitrogen mustard in *E.coli* proceeds via the excision and post-replication repair process. Both of these mutagenic agents are known to induce the SOS response in *E.coli* (Fram *et al.*, 1986). It is also known that *recA* and *uvrA* mutant strains are rendered extremely sensitive to both mitomycin C and nitrogen mustard (Fram *et al.*, 1986). The hyper-resistance of SA270 to these agents therefore suggests that a mutation(s) directly or indirectly involved in repairing DNA damaged by interstrand crosslinking agents is carried by this strain. Polyacrylamide gel electrophoresis of total cell protein from SA270 confirmed the existence of the over-expressed 55kd protein observed by Ahmad & Holland (1985). These authors assumed that enhanced production of this protein in SA270 was responsible for the increased resistance of the cell towards PUVA. The evidence in this Chapter now suggests that this protein is not involved solely in the repair of 8-methoxypsoralen induced diadducts, but that it is also concerned in the repair of crosslinks produced by mitomycin C and nitrogen mustard. Ahmad & Holland (1985) also suggested that the 55kd protein may be a double-strand-specific endonuclease; results in this Chapter suggest that, if this is the case, the protein probably recognises the distortion to DNA produced by the bulky adduct rather than the actual chemical conformation of the crosslink, since this is different depending on the type of crosslinking agent used.

Platosin is a bifunctional DNA damaging agent which covalently binds to DNA primarily at the N^7 positions of two guanine bases in two opposite strands (Eastman, 1983). A current model for the DNA binding mechanism of platosin postulates a two-step reaction between

the platinum compound and DNA. First, biavalent fixation at N^7 (guanine) and O^6 (guanine) produces a mutagenic, metastable intermediate; second, a slower reaction forms toxic DNA interstrand crosslinks by fixation at the N^7 position of a second purine base (Marquet *et al.*, 1983).

The wild type sensitivity of strain SA270 towards platosin was interesting, but not altogether surprising, since Roberts & Pascoe (1972) demonstrated that the formation of platosin crosslinks in DNA was a much rarer event than the formation of crosslinks by 8-methoxypsoralen, mitomycin C or nitrogen mustard. Perhaps more interestingly, it has been reported that the excision repair process, which plays an integral part in the removal of crosslinks, contributes very little to the repair of platosin crosslinks (Roberts & Thompson, 1979). Robins *et al.* (1983) reported that the adaptive response enzyme, O^6-methyltransferase, which is induced in *E.coli* by the exposure of cells to low concentrations of alkylating agent, can prevent formation of interstrand crosslinks as well as removing O^6-(guanine) alkyl lesions. These authors proposed that the enzyme acts by removing the alkyl group that is temporarily fixed to the O^6-guanine as an intermediate of the platosin DNA crosslinking reaction. In contrast, Germanier *et al.* (1984) reported the existence of a repair mechanism for platosin-DNA lesions which was independent of the adaptive response and the excision repair response. One interpretation of the wild type sensitivity of SA270 towards platosin is that the resistance of SA270 to PUVA, mitomycin C and nitrogen mustard is due to an enhanced repair mechanism, either in excision or recombination repair. Alternatively, since interstrand crosslinking by platosin is not

considered to be an important event *in vivo*, the extent of diadduct formation in this study may therefore have been very low. Consequently the observed zone of inhibition may have been caused by other cytotoxic events, besides DNA crosslinking, which occurred to the same extent in both SA270 and KL16.

In their original study Ahmad & Holland (1985) showed that strain SA270 remained as sensitive to far ultraviolet light as the parent wild type strain KL16. Results in this Chapter have shown that SA270 is similarly no more resistant to a wide range of monofunctional and single strand DNA damaging agents than wild type KL16. Alkylating agents such as N-methyl-N'nitro-N-nitrosoguanidine, ethyl methane sulphonate and methyl methane sulphonate all cause methylation to DNA bases in *E.coli*. Damage of this kind is subjected to error prone repair and mismatch repair, two mechanisms giving rise to mutagenesis. Since SA270 is no more resistant to alkylating agents than its parent strain KL16, it can be suggested that it does not have an enhanced error prone or mismatch repair mechanism.

It also seems unlikely that the PUVA hyper-resistance of SA270, apparently conferred by over-expression of the 55kd protein, is due to enhanced efficiency of the excision repair pathway, since results in this Chapter showed that SA270 was as sensitive to far ultraviolet light and near ultraviolet light as wild type KL16. Far and near ultraviolet irradiation produces pyrimidine dimers which, in turn, are repaired by the exicision repair mechanism. Studies by Webb & Brown (1982) showed that near ultraviolet light can produce cyclopyrimidine dimers in sufficient yield to account for many of the biological effects, such as mutagenesis, that are observed in *E.coli* following irradiation. However, even cells with *uvrA* mutations

have a great capacity to repair near ultraviolet light induced cyclobutane dimers; consequently the contribution of dimers to cell death is, even at high doses, below the limits of detection (Webb & Brown, 1982). These observations would account for the result obtained in this study which showed that a much higher dose of near ultraviolet light than far ultraviolet light was needed to kill strains KL16 and SA270.

At this point it is interesting to note that when strains SA270 and KL16 were exposed to N-methyl-N'nitro-N-nitrosoguanidine, ethyl methane sulphonate or methyl methane sulphonate in the disc sensitivity test, an inner zone of very slight growth was observed for each strain. This may result from induction of the adaptive response, following exposure of *E.coli* to low concentrations of these alkylating agents (Jeggo *et al.*, 1977).

Near ultraviolet light combined with other agents can also have a variety of indirect effects on DNA. Hydrogen peroxide is a photosensitiser which becomes excited following absorption of photons of near ultraviolet light, and it has been shown that near ultraviolet light irradiation of L-tryptophan yields hydrogen peroxide (Ananthaswamy & Eisenstark, 1979). Many research groups have noted single strand breaks produced following irradiation with near ultraviolet light (Tyrrell *et al.*, 1974). In every case the induction of single strand breaks was shown to have a substantial oxygen requirement. Hydrogen peroxide and hydrogen peroxide plus near ultraviolet light cause single strand breaks in DNA (see Appendix II D), probably due to the hydroxyl radical OH· produced when hydrogen peroxide reacts with ferrous ions attached to DNA (Mello-Filhi *et al.*, 1984).

Hagensee & Moses (1989) proposed that damage caused by hydrogen peroxide and hydrogen peroxide plus near ultraviolet light induces a repair response involving DNA polymerase I and DNA ligase. They also proposed that damage could be repaired by two mechanisms: one pathway utilises DNA polymerase I, probably with the aid of other unknown components; the other uses exonuclease III, polymerase III and polymerase I. Strain SA270 was equally sensitive to hydrogen peroxide and hydrogen peroxide plus near ultraviolet light as its parent wild type strain KL16, thereby suggesting that the mutant was unable to repair single strand breaks any more efficiently than the wild type strain KL16. It is also important to note that Hagensee & Moses (1989) proposed that there was no direct role for the RecA protein in repairing hydrogen peroxide induced DNA damage, while in contrast, Ahmad & Holland (1985) suggested that a functional *recA* gene was required for over-expression of the 55kd protein. Combined with the results in this Chapter, these observations indicate that repair of PUVA-induced DNA damage in SA270 is not more efficient due to an enhanced single strand break repair mechanism.

In summary, compared to the wild type parent strain KL16, SA270 is hyper-resistant to the DNA interstrand crosslinking agents mitomycin C and nitrogen mustard as well as 8-methoxypsoralen. However, SA270 shows wild type sensitivity to monofunctionalDNA damaging agents and agents producing single strand breaks in DNA. Over-expression of the 55kd protein in strain SA270 may therefore

play a significant role in crosslink repair, but does not seem to have any function in general excision repair of single strand damage in either the repair of secondary lesions formed as a result of dimer removal, in mismatch repair of damage caused by alkylating agents or in repair of DNA single strand breaks. Although it seems likely that the role of this protein is not directly concerned with controlling cell permeability to 8-methoxypsoralen (Ahmad & Holland, 1985) or similar molecules, a possible functioncould include a role as a DNA binding protein, perhaps influencing the degree of drug intercalation (Hansen, 1982). Alternatively the protein may act as a specific repair enzyme independent of the UvrABC mediated pathway of repair for DNA crosslinks. The following Chapters document genetic and biochemical studies designed to investigate the precise role of the 55kd protein in the repair of DNA damage induced by crosslinking agents.

Chapter 4

STUDIES ON THE UPTAKE OF RADIOLABELLED PSORALEN INTO CELLS OF SA270

4.1 Background

Several previous studies have indicated the possible involvement of penetration barriers in the determination of psoralen resistance. It was suggested by Bridges & Mottershead (1979) that differences in the DNA accessibility of bacteria might account for large differences in sensitivity between strains. Hansen (1982) considered that the cell envelope of some strains might form a permeability barrier to psoralens and therefore limit the amount of photo-induced psoralen damage. A mutation in *E.coli* (designated *acrA*) has been shown to confer PUVA sensitivity as a result of an alteration in the environment of the cellular DNA that allowed increased interaction and photobinding of psoralen (Hansen, 1982).

In order to investigate whether the PUVA hyper-resistant strain SA270 had developed a mechanism by which it could either directly or indirectly control uptake of 8-methoxypsoralen into the cell, a series of experiments were designed to compare the rate of uptake of ^{3}H-8-methoxypsoralen into SA270 and the wild type strain KL16. These experiments aimed to determine whether strain SA270 had developed a permeability barrier to psoralens which limited the uptake of the molecules and consequently rendered the cell resistant to PUVA treatment. The apparatus used in these experiments (fig. 15) was specially designed to enable radioactivity measurements to be taken for bacterial cells only.

4.2 Uptake of ^{3}H-8-methoxypsoralen by whole cells of SA270 and KL16

Table 5 shows the typical results of three experiments designed to measure the relative amounts of 8-methoxypsoralen absorbed by the

resistant mutant SA270 and the wild type strain KL16. No repeatable differences were observed and it seemed that ^{3}H-8-methoxypsoralen was absorbed by strains SA270 and KL16 with the same efficiency. This suggested that the resistance of SA270 to DNA crosslinking agents was not due to an increased outer permeability barrier. Uptake of psoralen by the cells was very rapid and it was possible to calculate from the results that between 33% and 50% of the total amount of 8-methoxypsoralen added to the incubation mixture (3.3 to 5.0nmol out of 10nmol added) was taken up by the whole cells. It is important to emphasise that this amount also included any 8-methoxypsoralen molecules bound to the cell surface.

Table 5

Relative amounts of ^{3}H-8-methoxypsoralen absorbed by whole cells of SA270 and KL16 over a known time course.

Expt 1	Time(minutes)	KL16 counts per minute	SA270 counts per minute
	1	9582	6637
	2	12271	11732
	5	9580	12041
	7	11691	11116
	10	7982	11003

Expt 2	Time(seconds)	KL16 counts per minute	SA270 counts per minute
	15	11949	10568
	30	11926	11494
	45	12560	11414
	60	10343	12723
	300	12604	13185
	600	11306	14403
	900	10878	14779
	1200	11040	14163

Expt 3	Time(seconds)	KL16 counts per minute	SA270 counts per minute
	5	12638	11505
	10	12925	12106
	15	12941	13368
	20	11872	12798
	25	14248	13151
	30	12631	14258
	35	12331	13964
	40	13847	13733
	50	13345	12025
	60	13871	13629

Between 33% and 50% of the total amount of ^{3}H-8-methoxypsoralen added to the incubation mixture was absorbed by the whole cells.

4.3 Distribution of radiolabelled psoralen

Previous studies have observed that 8-methoxypsoralen may bind to the outside of the bacterial cell or remain in the cell envelope. It is therefore possible that SA270 may protect its DNA from psoralen photobinding by limiting the uptake of 8-methoxypsoralen across the inner cell membrane. Following fractionation of the bacterial cells, the amount of ^{3}H-8-methoxypsoralen present in the cytoplasmic fraction was only 5% of the total amount of ^{3}H-8-methoxypsoralen measured for the whole cell (Table 6). This amount represented only 0.2nmol of ^{3}H-8-methoxypsoralen in 1g of cells, compared with approximately 4.8nmol absorbed by 1g of whole cells, with the remaining 95% presumably contained within the envelope fraction, including the periplasm, or bound to the outer cell wall. Nevertheless, no significant difference was observed between cells of SA270 and KL16. It was therefore concluded that reduced penetration into the cytoplasmic fraction was not a significant factor in the PUVA resistance of SA270.

All calculations performed in this study are shown in Appendix I.

Table 6

Relative amounts of ^{3}H-8-methoxypsoralen contained within the cytoplasmic fractions of KL16 and SA270 (Time of incubation = 5 minutes).

	KL16 whole cells counts per minute	KL16 cytoplasmic fraction counts per minute
	12313	609
	12847	672
	12436	662
mean value	12532	626

The amount of ^{3}H-8-methoxypsoralen contained within the cytoplasmic fraction of KL16 after 5 minutes incubation was 5.1% of the total amount of label contained within the whole cell.

	SA270 whole cells counts per minute	SA270 cytoplasmic fraction counts per minute
	12362	692
	11988	538
	12485	612
mean value	12278	614

The amount of ^{3}H-8-methoxypsoralen contained within the cytoplasmic fraction of SA270 after 5 minutes incubation was 5% of the total amount of label contained within the whole cell.

4.4 Discussion

The results in this Chapter indicated that resistance of SA270 to PUVA was not due to a lack of ability by the cell to take up psoralen across the cell envelope. It is clear from the results of the ^{3}H-8-methoxypsoralen uptake experiment that the compound can enter the cells of strain SA270 with an efficiency similar to that of the wild type strain KL16. There was no evidence to suggest that strain SA270 had developed a mechanism whereby psoralen was actively pumped out of the cell in a similar manner to that seen in *E.coli* for efflux of tetracycline (Levy, 1984).

Psoralens accumulate in the bacterial cell very quickly, probably by simple diffusion through the outer membrane. In contrast, D'all Acqua (personal communication) has proposed that uptake of psoralen molecules across the inner membrane may be controlled by an active uptake mechanism. The results in this Chapter demonstrated that only a very small amount of psoralen was present in the cytoplasmic fraction, which, of course, also includes those molecules which may be caught up in the lipopolysaccharide web of the outer membrane or bound to membrane proteins or lipids (see section 1.4). The amount of radioactivity measured in the spheroplasted cells was essentially the same for both SA270 and KL16.

The studies of psoralen uptake and penetration into the cell described in this Chapter produced results similar to those of Pathak & Kramer (1969). These authors showed that only a small portion of the total psoralen taken up by mammalian epithelial cells was recovered in the cytoplasmic fraction. From the results obtained

in this Chapter it seems unlikely that the PUVA resistance of strain SA270 can be attributed to a permeability change in the cell envelope; however it is important to note that it could conceivably be the immediate environment of the cellular DNA which is altered by over-expression of the 55kd protein. Binding of psoralens has been shown to be influenced by the presence of DNA binding proteins which can hinder access to the DNA (Weishahn *et al.*, 1977). Similarly, over-expression of the 55kd protein could also affect psoralen binding. A possible relationship of this type was reported by Hansen (1982), who showed that an *acrA* mutant strain of *E.coli* was hypersensitive to PUVA due to increased photobinding of radiolabelled psoralen.

It was concluded from the experiments described in this Chapter that the 55kd protein is not involved in controlling psoralen uptake into the bacterial cell, although a possible role as a protein affecting the immediate environment of cellular DNA cannot be dismissed at this stage.

The following Chapters describe genetic experiments designed to map the SA270 gene(s) involved in PUVA-resistance and to investigate the possible interaction of the PUVA-resistance mutation(s) contained in SA270 with other known DNA repair genes. In this way it was hoped to investigate an additional possibility: that over-expression of the 55kd protein results in enhancement of an already existing DNA repair mechanism.

Chapter 5

GENETIC MAPPING, COMPLEMENTATION ANALYSIS AND INDUCTION STUDIES WITH THE PUVA RESISTANCE GENE

5.1 Background

This Chapter describes experiments designed to map the position of the gene(s) responsible for the PUVA hyper-resistant phenotype of strain SA270. Ahmad & Holland (1985) suggested that the PUVA hyper-resistant phenotype of strain SA270 was due to a mutation, or mutations, giving rise to the over-expression of a 55kd protein. In order to study the mutation(s), its precise location on the *E.coli* chromosome must first be determined. Once the site of the mutation determining PUVA resistance has been found, experiments can then be performed to check that the genetic data correlate with the biochemical evidence suggesting that over-expression of the 55kd protein is responsible for the hyper-resistant PUVA phenotype of SA270.

Ahmad & Holland (1985) also proposed that the method used to isolate a PUVA hyper-resistant mutant might have produced conditions which selected a mutation affecting regulation of a gene responsible for the synthesis of the 55kd protein. This Chapter therefore also includes complementation tests with a diploid PUVA-wild type/PUVA hyper-resistant strain in order to test this proposal.

A further suggestion was that the RecA protein might control the synthesis of the putative crosslink-specific repair protein. The SOS response induces a number of repair proteins when DNA damage occurs in *E.coli* (Peterson *et al.*, 1988). Since mitomycin C, a crosslinking agent, is able to induce known SOS repair proteins such as RecA, UvrA and RecN (Finch *et al.*, 1985) it can be hypothesised that PUVA damage may do the same. If this is correct, then the 55kd protein may be inducible by crosslink DNA damage in the wild type

strain KL16. This Chapter therefore includes experiments to test this possibility.

5.2 Conjugation Experiments

The PUVA hyper-resistant strain SA270 was derived from the wild type Hfr strain KL16. Preliminary conjugation experiments were carried out using the strain SA270 as a donor (Hfr) and an F⁻ auxotrophic strain AB1157 as a recipient. Samples of mating mixture were spread on to plates containing psoralen (25µg/ml) and streptomycin (25ug/ml). The plates were then exposed to 7000 Jm^{-2} of near ultraviolet light (lethal to AB1157, but not to SA270). The number of PUVA hyper-resistant recombinants was scored after 12 hours incubation at 37°C. The time of entry of the *his* gene marker was also determined by spreading samples of the mating mixture on minimal medium plates containing streptomycin and unselected auxtrophic requirements.

Table 7 shows the number of transconjugants obtained at each time interval. The PUVA hyper-resistant phenotype was detected in AB1157 after only 5 minutes incubation of the mating mixture. Fig. 29 shows interrupted mating curves for each marker. Extrapolation of each curve gave the estimated time of entry for each marker. Results showed that the gene mutation responsible for the PUVA hyper-resistant phenotype in SA270 (referred to from now on as *puvR*) was located at approximately 58 minutes on the linkage map of the *E.coli* chromosome.

Table 7

Results obtained in a typical conjugation experiment

Results shown are those obtained in a typical conjugation experiment between SA270 and AB1157. Selection for recombinants was performed as described in the text.

Time(minutes)	Number of PUVA-resistant recombinants	Number of *his*$^+$ recombinants
0	0	0
1	0	0
5	4	0
10	9	0
15	44	1
20	137	22
30	170	73
40	179	96
50	194	156

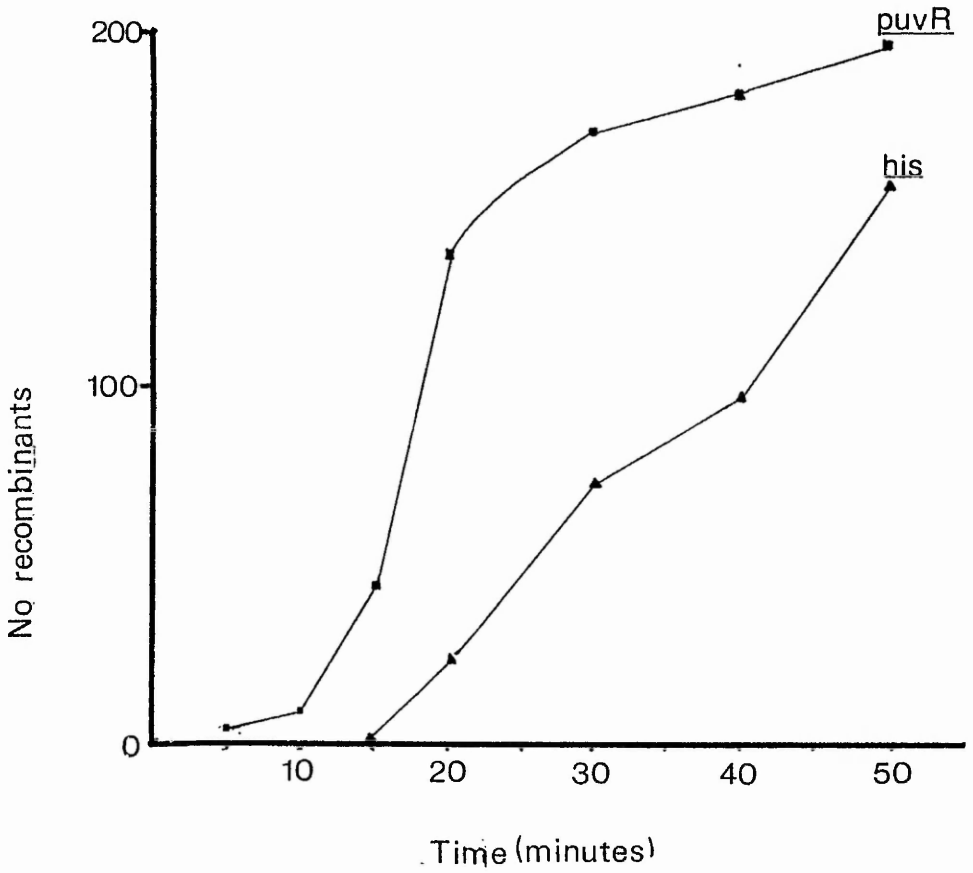


FIG. 29 AN INTERRUPTED MATING CURVE OBTAINED IN EXPERIMENT 2.10.

Table 8

Fine structure genetic mapping of the *puvR* gene using P1 transduction

Results of a typical transduction experiment between SA270(*puvR*$^-$,*tyrA*$^+$) and JH108(*puvR*$^+$,*tyrA*$^-$,*nalB*). Selection was made for transduction of the *tyrA*$^+$ marker to JH108, followed by screening of resulting transductants for *nalB* and *puvR* as described in the text. 95% confidence intervals (95% CI) were calculated as described in Appendix I.

Transduction Data

Donor	Recipient	*tyrA*$^+$	*nal-r* *puvR*$^-$	*nal-s* *puvR*$^-$	*nal-r* *puvR*$^+$	*nal-s* *puvR*$^+$
SA270	JH108	129	46	7	76	0

Cotransduction frequency between *tyrA* and *nalB* = 5.4%±3.8% (95%CI)

Cotransduction frequency between *tyrA* and *puvR* = 41%±8.4% (95%CI)

5.3 Fine genetic mapping of the *puvR* gene using P1 transduction

Fine genetic mapping of the gene responsible for PUVA hyper-resistance in SA270 was carried out using P1 transduction experiments as described in section 2.11. SA270 was used as the donor strain and a *tyrA nalB* mutant (strain JH108) as the recipient strain. Selection was made for transduction of the *tyrA*$^+$ marker (located at 56.7 minutes on the *E.coli* chromosome) by plating onto minimal medium plates supplemented with trp, his, thi and cytidine. Transductants were examined for cotransduction of the *nalB* and *puvR* markers by screening for nalidixic acid sensitivity or PUVA resistance (as described in section 2.4). Data (table 8) showed that the cotransduction frequency between *tyrA* and *nalB* was 5.4% +/- 3.8% and between *tyrA* and *puvR* was 41% +/- 8.4%. No transductants with a *tyrA*$^+$ *puvR*$^+$ *nalB*$^+$ phenotype were obtained. It was therefore concluded that the *puvR* gene was located between *tyrA* and *nalB* on the linkage map of *E.coli* (Bachmann 1983), since the *tyrA*$^+$ *puvR*$^+$ *nalB*$^+$ class of recombinants would require four crossovers and would therefore be extremely rare. Using the formula devised by Wu (1966) the position of the *puvR* gene mutation on the *E.coli* chromosome was calculated as 57.2 +/- 0.1 minutes.

In order to confirm this conclusion, P1 transduction experiments were also carried out with SA270 as the donor and a *pheA* auxotroph (strain PN10) as the recipient. Selection was made on minimal medium plates supplemented with leu, met, pro and thi. *pheA*$^+$ transductants were screened for *puvR* as described above. Data (table 9) showed that the cotransduction frequency between *pheA*$^+$ and *puvR* was 30% $^+$/- 12%. Using the formula of Wu (1966) this result mapped the *puvR* mutation at a distance of 0.66 $^+$/- 0.15 minutes from the *pheA* gene locus. The co-transduction data from these experiments is summarised in fig. 30.

5.4 SDS-Polyacrylamide gel electrophoresis of total cell lysate from a *puvR* transductant

To confirm that the *puvR* mutation located at 57.2 minutes on the *E.coli* chromosome was solely responsible for both the PUVA hyper-resistant phenotype of SA270 and for over-expression of the 55kd protein, a *tyrA*$^+$ *puvR* transductant was analysed for the presence of the over-expressed 55kd protein in a total protein cell lysate using SDS-Polyacrylamide gel electrophoresis. A *tyrA*$^+$ *puvR*$^+$ total protein cell lysate was examined as a control. Results showed (fig. 31) that the 55kd protein was over-expressed in the *tyrA*$^+$ *puvR* transductant, but not in the *tyrA*$^+$ *puvR*$^+$ transductant. This confirmed the correlation between the *puvR* mutation, the PUVA hyper-resistant phenotype of SA270 and the over-expression of the 55kd protein.

Table 9

Fine structure genetic mapping of the *puvR* gene using P1 transduction

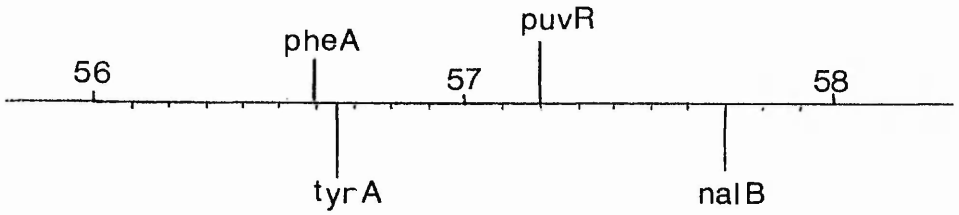
Results of a typical transduction experiment between SA270(*puvR*$^-$,*pheA*$^+$) and PN10(*puvR*$^+$,*pheA*$^-$). Selection was made for transduction of the *pheA*$^+$ marker to PN10, followed by screening of resulting transductants for *puvR* as described in the text. 95% confidence intervals (95% CI) were calculated as described in Appendix I.

Transduction Data

Donor	Recipient	*pheA*$^+$	*pheA*$^+$ *puvR*$^-$	*pheA*$^+$ *puvR*$^+$
SA270	PN10	50	15	35

Cotransduction frequency between *pheA* and *puvR* = 30%±12% (95% CI)

FIG.30. CALCULATED MAP POSITION OF THE puvR GENE ON THE E.COLI CHROMOSOME.



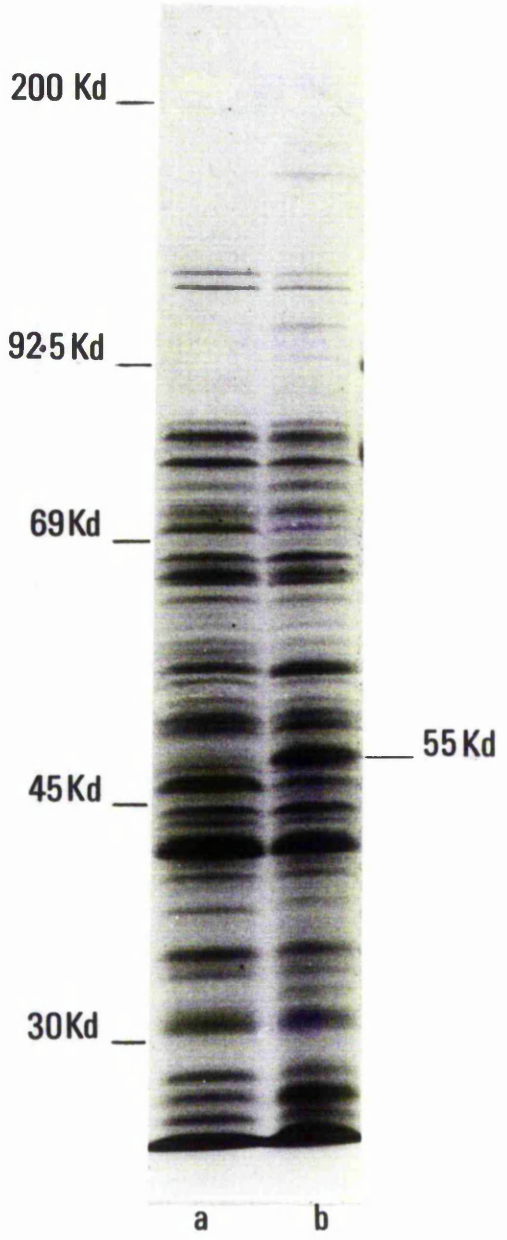


FIG. 31. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TOTAL PROTEIN EXTRACTS OF A *puvR*$^{+}$ *tyrA*$^{+}$ (a), AND A *puvR*$^{-}$ *tyrA*$^{+}$ (b), TRANSDUCTANT.

5.5 Complementation analysis of the *puvR* gene

An F' plasmid carrying *tyrA*$^+$ and *puvR*$^+$ was introduced into an F$^-$ strain (JH109) with the *tyrA* and *puvR* mutations. This was done by mating strain KL711 (F'143, *tyrA*$^+$, *puvR*$^+$) with strain JH109 (F$^-$ *tyrA*::Tn10, *puvR*) in a conjugation experiment and selecting for the transfer of *tyrA*$^+$ on minimal medium plates containing tetracycline (15µg/ml). Resulting transconjugants were tested for their PUVA phenotype as described previously. Of 100 transconjugants tested, 85% had wild type levels of PUVA sensitivity. Those which retained PUVA hyper-resistance were retested for their *tyrA*$^+$ phenotype, but were found to be *tyrA*$^-$, suggesting that the F' had been lost from these cells. It would be expected that at least some of the *tyrA*$^+$ transconjugants might result from recombination of the F' with the host cell chromosome. However, the fact that all of the proven *tyrA*$^+$ transconjugants tested had wild type levels of PUVA-sensitivity (combined with the observed instability in 15% of "transconjugants") suggested that the *puvR*$^+$ gene on the F' was producing a gene product acting *in trans* to reverse the effect of the original *puvR* mutation located on the chromosome. Fig. 32 shows typical survival curves of F', F$^-$ and transconjugant strains.

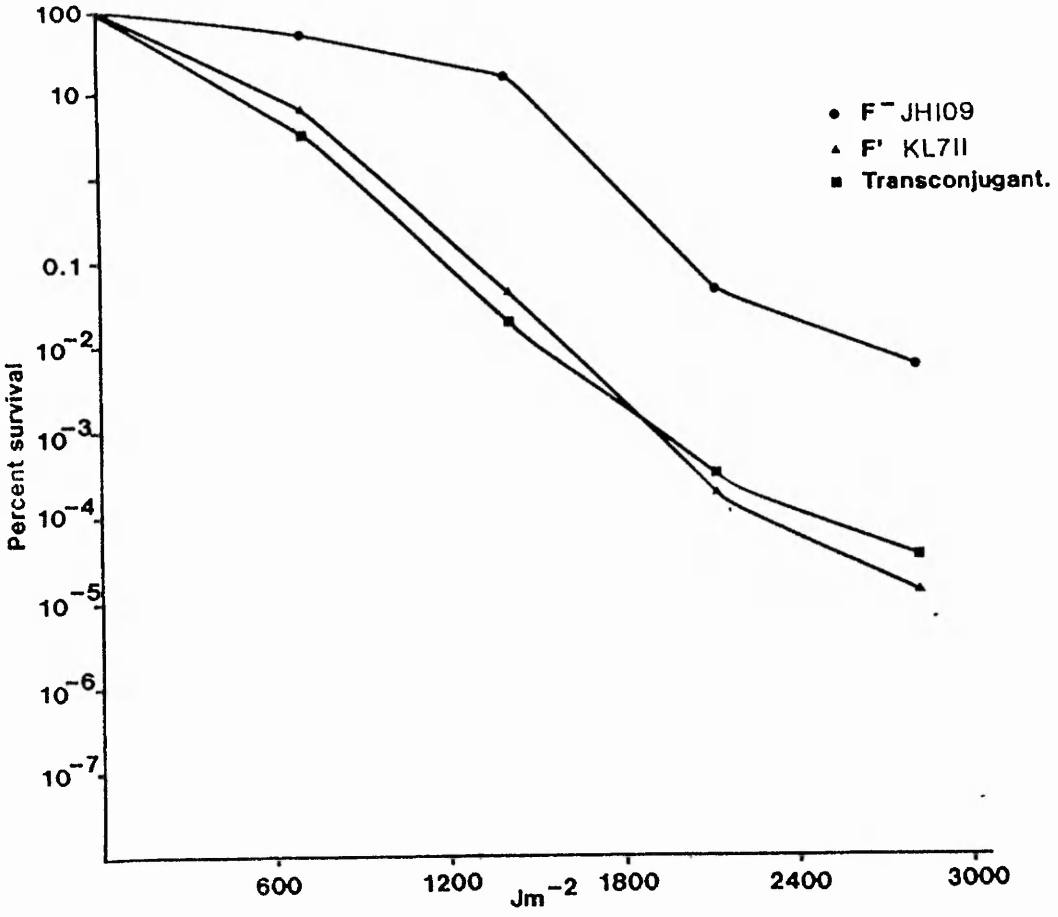


FIG. 32. BACTERIAL KILL CURVES OF KL711, JH109, AND A TRANSCONJUGANT IN THE PRESENCE OF PUVA.

5.6 Isolation of a PUVA-hypersensitive mutant by insertional mutagenesis

When a cell is infected with bacteriophage MudI(*lac*,Ap) the phage integrates into the bacterial chromosome at random sites. Although the phage carries a *lacZ* structural gene, it does not carry a promoter capable of initiating transcription. In an initial experiment, MudI*lac*(Ap) phage was used to infect wild type strain KL16 in the hope of obtaining a PUVA hyper-sensitive strain with a mutation in the structural gene encoding synthesis of the 55kd protein. As the Mu phage carries the *lacZ* structural gene, but no promoter, the *lacZ* gene can only be expressed (and ß-galactosidase produced) if the phage integrates into an active operon. Selection was made on LB plates containing 25µg ampicillin/ml as described in section 2.13. A total of 2000 colonies were then screened on 8-methoxypsoralen and 8-methoxypsoralen plus near ultraviolet light plates. Two PUVA hyper-sensitive mutants were isolated and tested for their sensitivity to far ultraviolet light in comparison with KL16 and SA270. Both of the KL16 insertion mutants were found to be hyper-sensitive to far ultraviolet light (results not shown) and it was therefore concluded that the Mu phage had not inserted into the same gene that was responsible for PUVA hyper-resistance in SA270, since over-expression of the 55kd protein did not increase resistance to far ultraviolet light (see section 3.2). There was no evidence that these insertion mutations were in genes solely concerned with repair of PUVA damage, particularly in view of their hyper-sensitivity to far ultraviolet light. Although interesting, they were therefore not further investigated in this thesis.

In a second experiment, P1::Tn5*lac* (Kroos & Kaiser, 1984) was used in an attempt to isolate a PUVA hyper-sensitive mutant from SA270 that showed wild type levels of sensitivity to far ultraviolet light. P1::Tn5*lac* is a defective phage which is used simply as a method of introducing Tn5*lac* into the cell, where it inserts into the chromosome at random locations. Following selection as described in section 2.13 on MacConkey agar plates, 200 colonies with a deep red colour were examined and a PUVA hyper-sensitive mutant was isolated (fig. 33) which showed wild type sensitivity to far ultraviolet light (fig. 34) and hyper-sensitivity to the DNA crosslinking agent mitomycin C (fig. 35).

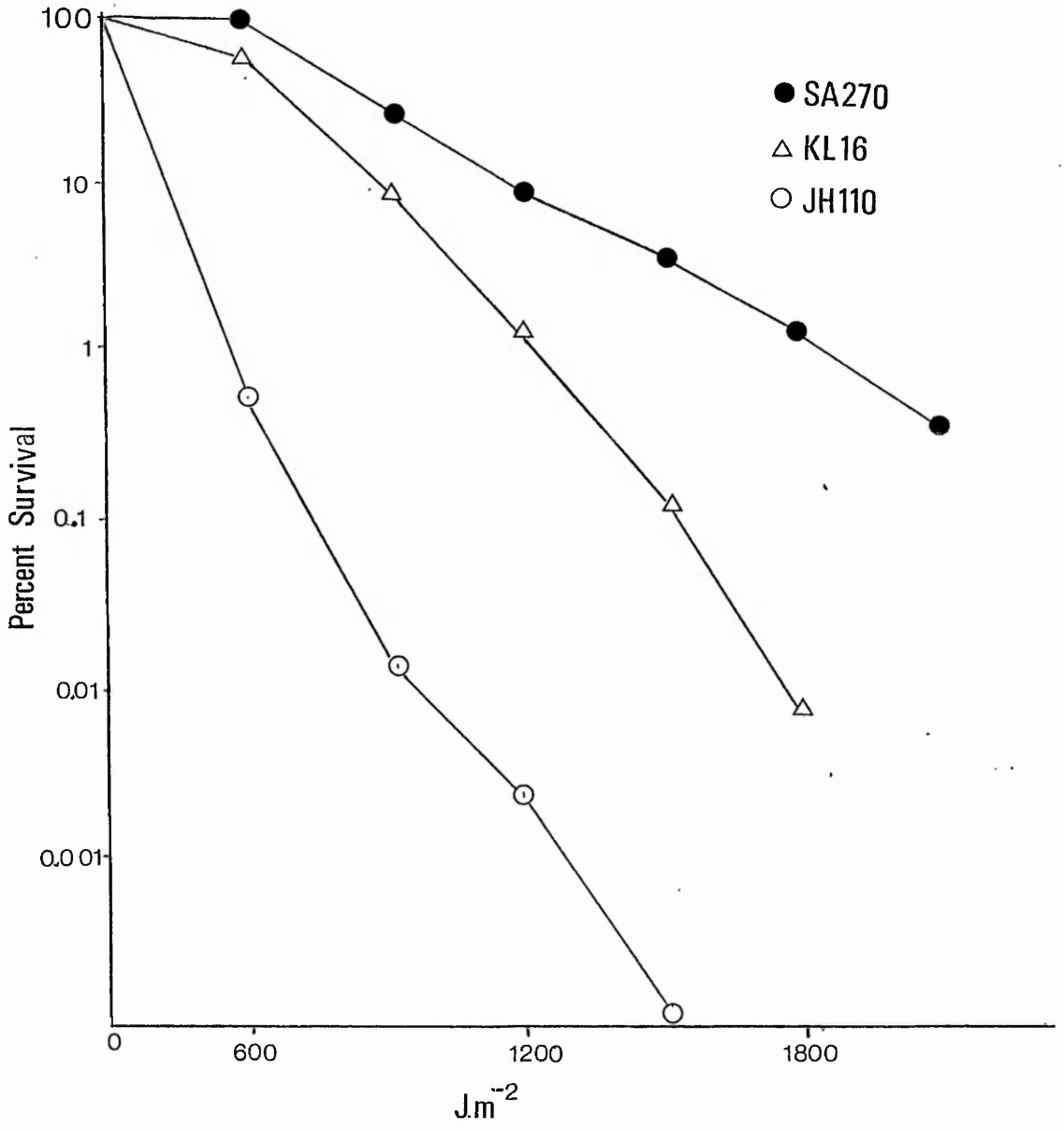


FIG.33. BACTERIAL KILL CURVE OF A PUVA HYPER-SENSITIVE MUTANT (JH110) IN THE PRESENCE OF PUVA.

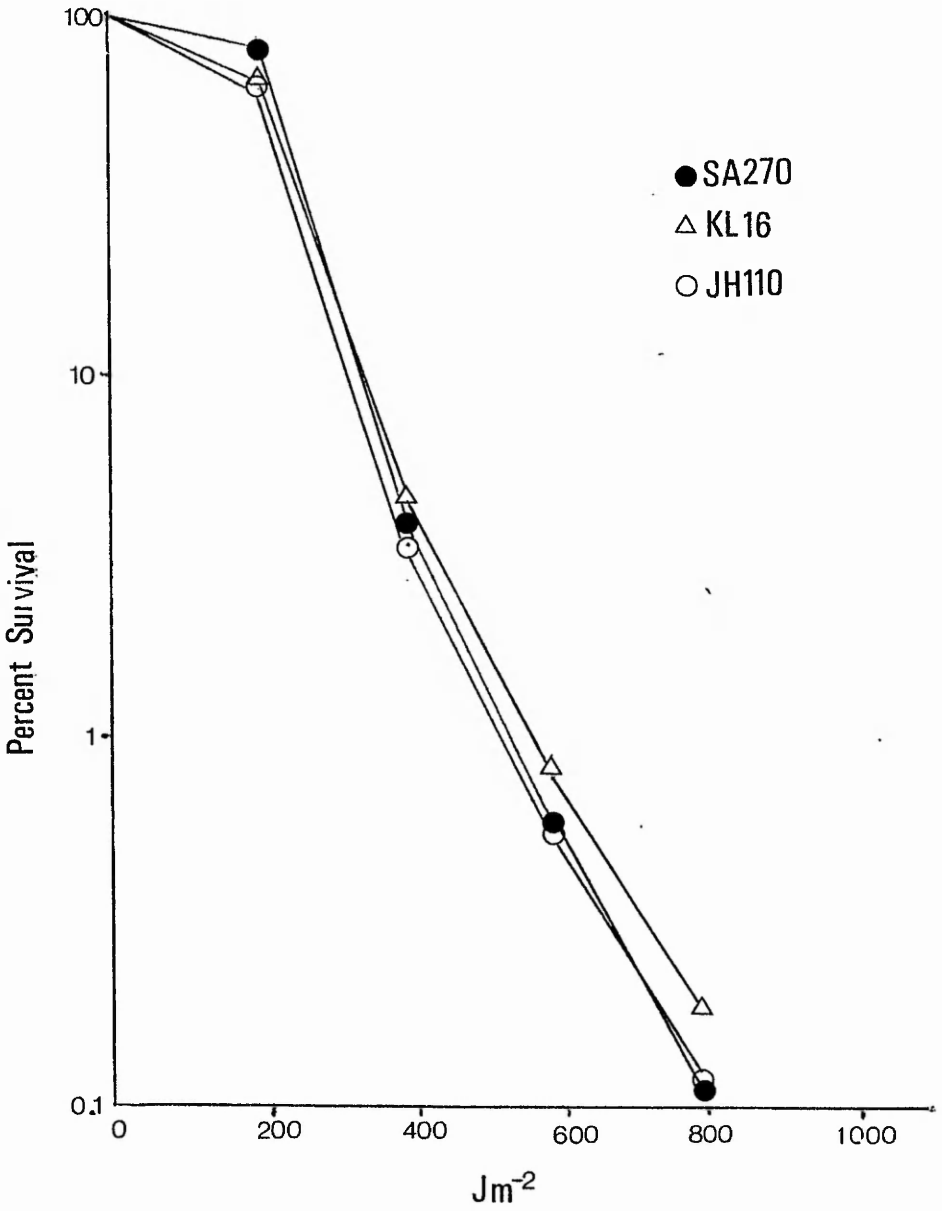


FIG.34. BACTERIAL KILL CURVE OF A PUVA HYPER SENSITIVE MUTANT (JH110) IN THE PRESENCE OF FAR ULTRAVIOLET LIGHT.

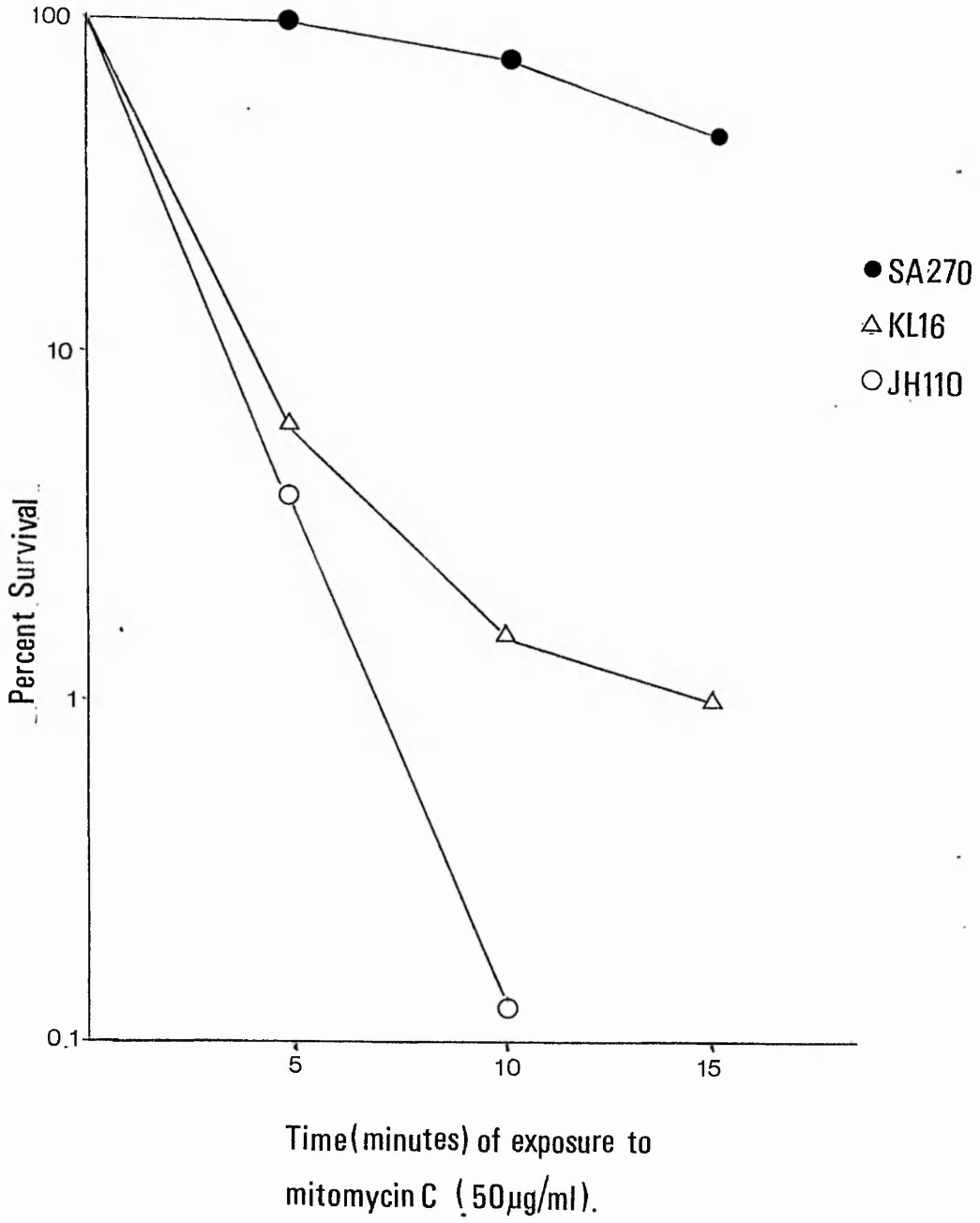


FIG.35. BACTERIAL KILL CURVE OF A PUVA HYPER SENSITIVE MUTANT (JH110) IN THE PRESENCE OF MITOMYCIN C.

When a B-galactosidase assay was performed on JH110, it was found that high constitutive levels (Miller, 1972) of~800 units of β-galactosidase were being produced. This indicated that the Tn5lac had inserted into (and inactivated) a gene which was being constitutively expressed with a high rate of transcription.

It was hoped that Tn5*lac* had inserted into the structural gene encoding the 55kd protein (now designated *puvP*). To determine whether this was the case, a normal P1 transduction experiment was carried out which used the Tn5*lac* insertion mutant as a donor strain and a *tyrA nalB* mutant (JH108) as a recipient strain. Selection was made on minimal medium plates containing trp, his, thi and cytidine for transduction of *tyrA*$^+$. Resulting transductants were screened for hyper-sensitivity to PUVA and nalidixic acid resistance.The data showed that the cotransduction frequency between PUVA hyper-sensitivity and *tyrA*$^+$ was 29% $^+$/- 9.3% (table 10). Using the formula of Wu (1966) the insertion mutation was therefore located at 0.67 $^+$/- 0.1 minutes from the *tyrA* gene. While this was not a statistically significant difference from the map position of the *puvR* gene (leading to over-expression of the 55kd protein) described in 5.3, the insertion mutation examined here was not *puvR* since it exhibited hyper-sensitivity to PUVA. This gene could be the 55kd protein structural gene, designated *puvA*, but could also be a third gene concerned with PUVA hyper-sensitivity that cotransduced with *tyrA*$^+$ and was therefore, like *puvR*, located close to *tyrA* on the chromosome (fig. 36).

Table 10

Fine structure genetic mapping of the *PUVA hyper-sensitive* gene mutation using P1 transduction

Results of a typical transduction experiment between a PUVA hyper-sensitive mutant (*puvP*$^-$,*tyrA*$^+$) and JH108(*puvP*$^+$,*tyrA*$^-$,*nalB*). Selection was made for transduction of the *tyrA*$^+$ marker to JH108, followed by screening of resulting transductants for *nalB* and the PUVA phenotype, as described in the text. 95% confidence intervals (95% CI) were calculated as described in Appendix I.

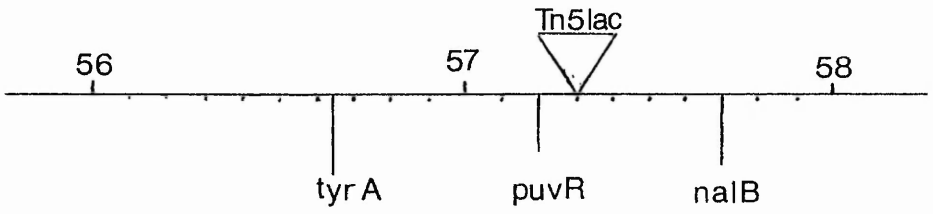
Transduction Data

Donor	Recipient	*tyrA*$^+$	*nal-r* PUVA-hyper sensitive	*nal-s* PUVA-hyper sensitive	*nal-r* PUVA-wt	*nal-s* PUVA-wt
SA270	JH108	92	22	5	65	0

Cotransduction frequency between *tyrA* and *nalB* = 5.4%±4.6%

Cotransduction frequency between *tyrA* and *puvP* = 29%±9.3%

FIG.36. CALCULATED MAP POSITION OF THE Tn5lac INSERTION ON THE E.COLI CHROMOSOME CONFERRING PUVA HYPER-SENSITIVITY.



5.7 Induction Studies

Strain KL16 was incubated at 37°C with 2µg mitomycin C/ml for 30 minutes and then resuspended in fresh nutrient broth and incubated on a rotary shaker at 37°C. Samples (10mls) were harvested and lysed after 0, 10, 30, 60, and 90 minutes incubation. Total protein cell extracts were then run on a Polyacrylamide gel to determine whether the 55kd protein, over-expressed in SA270, was inducible in KL16. No induction by mitomycin C was observed; however, it was interesting to note that the RecA protein was present, even at 0 minutes, suggesting that damage caused by mitomycin C treatment was effective in switching on the SOS response in wild type *E.coli* (fig. 37).

When KL16 was exposed to PUVA following exposure to 2µg mitomycin C/ml for 30 minutes, there was a significant increase in the PUVA-resistance of the strain (fig. 38). This suggested that exposure of the wild type strain KL16 to a DNA crosslinking agent does induce PUVA resistance, but that the 55kd protein is not normally inducible to the levels observed in SA270. Alternatively this observation might be the result of the induction of other repair genes also able to mediate crosslink repair.

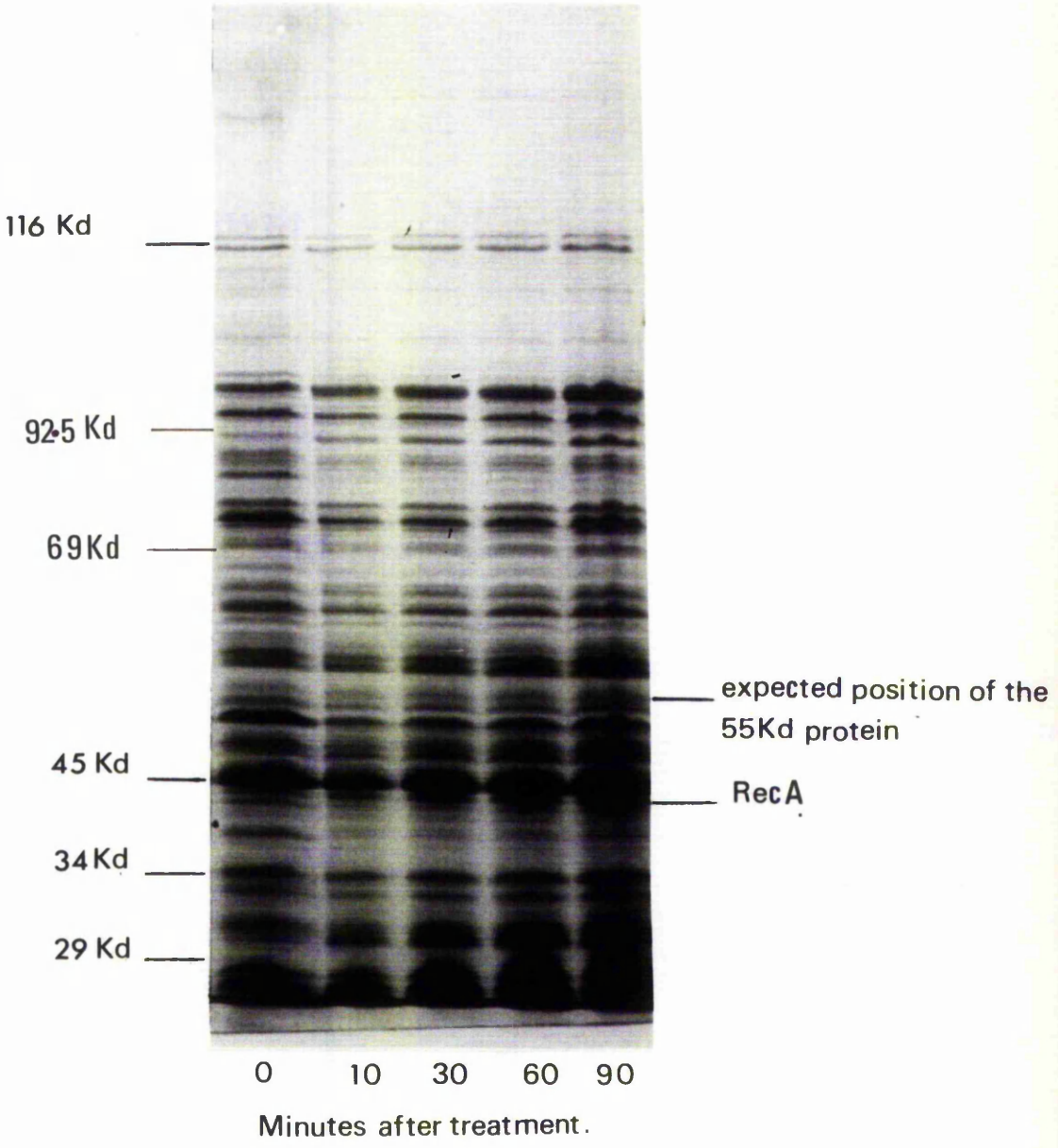


FIG.37. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TOTAL PROTEIN EXTRACTS OF KL16 FOLLOWING 30 MINUTES INCUBATION WITH MITOMYCIN C (2ug/ml).

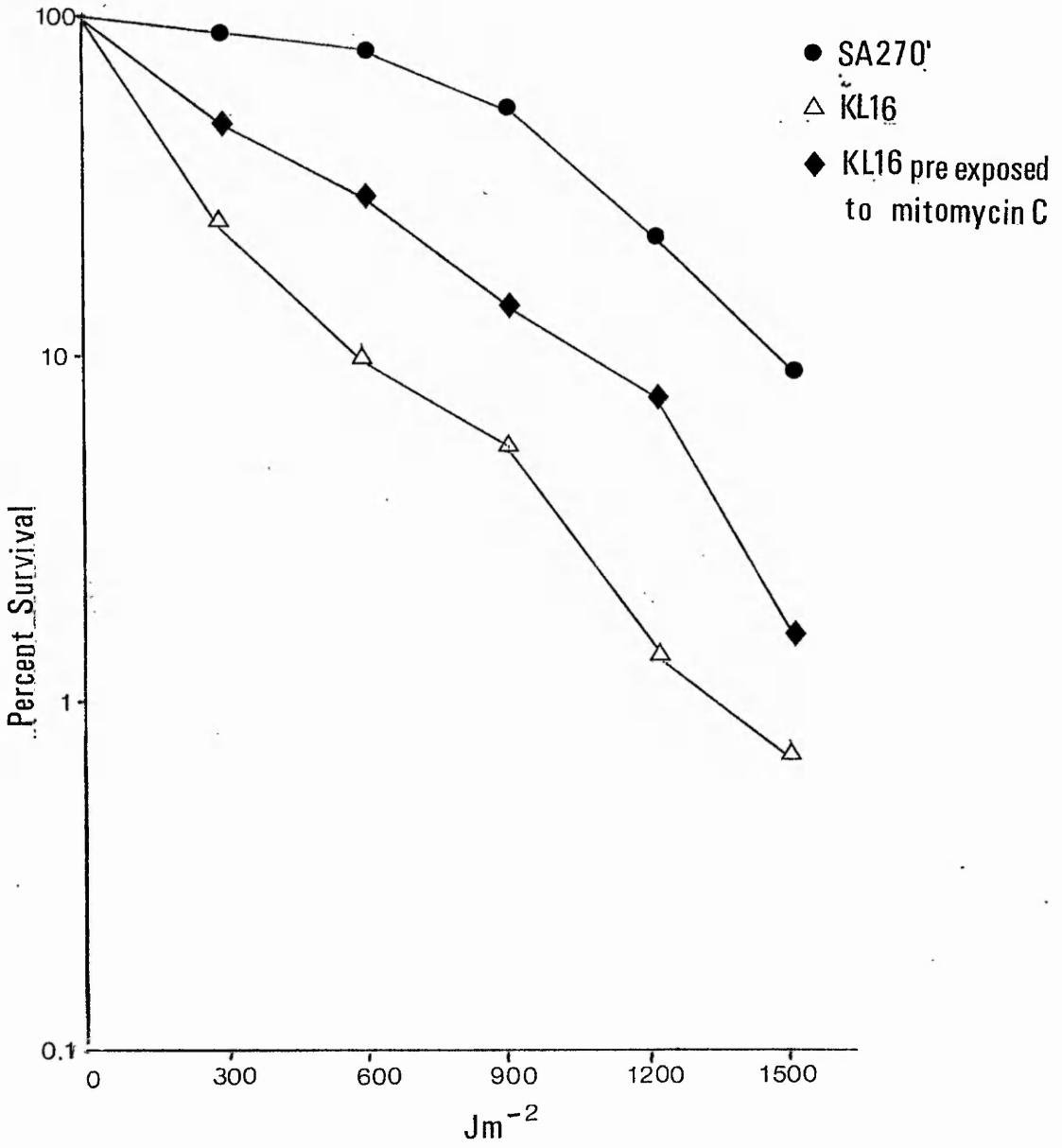
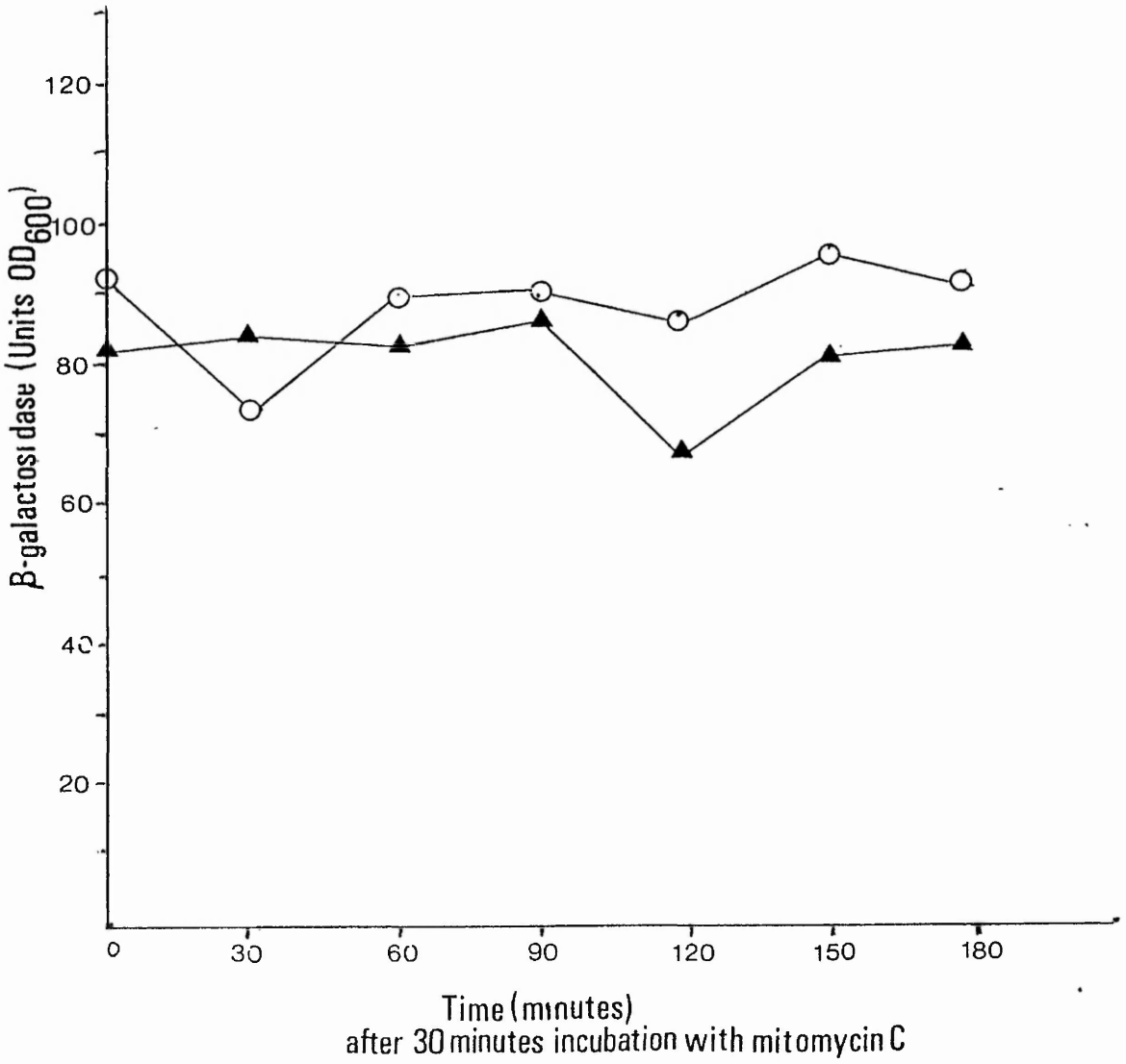


FIG.38. BACTERIAL KILL CURVES OF SA270, KL16, AND KL16 PRE-EXPOSED TO MITOMYCIN C (2ug/ml), IN THE PRESENCE OF PUVA.

To measure the induction of β-galactosidase activity, it was necessary to construct a strain with Tn5lac inserted into the *puvA* gene while still retaining the *puvR*$^+$ allele.

Phage P1 was grown on the PUVA hyper-sensitive Tn5*lac* mutant isolated from SA270 and transduced into the wild type strain KL16, selecting for kanamycin-resistant colonies, on MacConkey plates containing25μgkanamycin/ml. Two types of colony were observed; pale pink and dark red. The ratio of pale pink colonies to dark red colonies was approximately 10%. Dark red colonies probably resulted from cotransduction of Tn5*lac* and the *puvR* mutation. Pale pink colonies were isolated, as these presumably had the *puvR*$^+$ gene, and were tested for their PUVA phenotype to confirm PUVA hyper-sensitivity. All the PUVA hyper-sensitive transductants screened also retained wild type sensitivity to far ultraviolet light. A transductant showing low levels of ß-galactosidase activity and PUVA hyper-sensitivity was used in an induction experiment. Following exposure to 2μg mitomycin C/ml for 30 minutes (see section 2.15) the level of ß-galactosidase in the cells was measured 0,30, 60, 90, 120, 150, 180 and 210 minutes after treatment. Results showed (fig. 39) that the level of ß-galactosidase in the cells did not increase in the induced cells. It was therefore concluded that the gene inactivated by insertion of Tn5*lac* (leading to PUVA hyper-sensitive) in SA270 was not directly inducible by the DNA damage caused by mitomycin C.

FIG.39. RESULTS OF AN INDUCTION EXPERIMENT SHOWING THE EFFECT OF MITOMYCIN C ON THE INSERTION MUTATION IN JH111.



5.8 Discussion

The mutation site conferring PUVA hyper-resistance in strain SA270 was located at 57.2±0.1 minutes on the *E.coli* chromosome. PUVA hyper-resistant transductants also showed over-expression of the 55kd protein, thereby confirming the correlation between the PUVA hyper-resistant phenotype and the high level of expression of this protein.

Ahmad & Holland (1985) suggested that the resistance of strain SA270 to PUVA damage was due to a mutation in a gene regulating transcription of the 55kd protein. Inactivation of this gene would lead to constitutive expression of this protein in the cell. The complementation study described in this Chapter provided further evidence for this hypothesis, since the wild type gene (designated *puvR*$^+$) was dominant *in trans* over the mutant derivative. From the results obtained it can be proposed that, in the partial diploid strain, the *puvR*$^+$ gene located on the F' encodes a diffusible repressor protein which regulates transcription of the structural 55kd protein gene (designated *puvA*)(fig. 40).

As already described (see section 1.7) the regulation of a number of DNA repair genes is under the control of the RecA/LexA SOS regulon which causes their induction following DNA damage. Ahmad & Holland (1985) showed a need for a functional *recA* gene in the repair of PUVA induced DNA damage and observed that the cells of a *recA* derivative of SA270 were not only very sensitive to PUVA, but that expression of the 55kd protein was returned to basal wild type levels. They proposed that, following DNA damage by a crosslinking agent, the RecA protein might cause derepression of a regulatory

gene that increased the levels of the 55kd protein. Results in this study showed that wild type strain KL16 became more resistant to PUVA damage following exposure to a sublethal dose of mitomycin C, although the level of resistance observed in strain SA270 was never attained. Although Polyacrylamide gel electrophoresis (fig. 37) showed expression of the RecA protein, no induction of the 55kd protein could be detected. From these results it can be proposed that expression of the protein might only be increased by small undetectable amounts following crosslinking DNA damage. Since complementation studies suggested that the mutation causing over-expression of the 55kd protein was in a regulatory gene this result was somewhat surprising, but may indicate that it is not the presence of the DNA crosslinks that actually induces the expression of the 55kd protein. It would be interesting in future experiments to determine what factors are responsible for induction of the 55kd protein, particularly since the Tn5*lac* insertion in a gene concerned with PUVA resistance was also non-inducible.

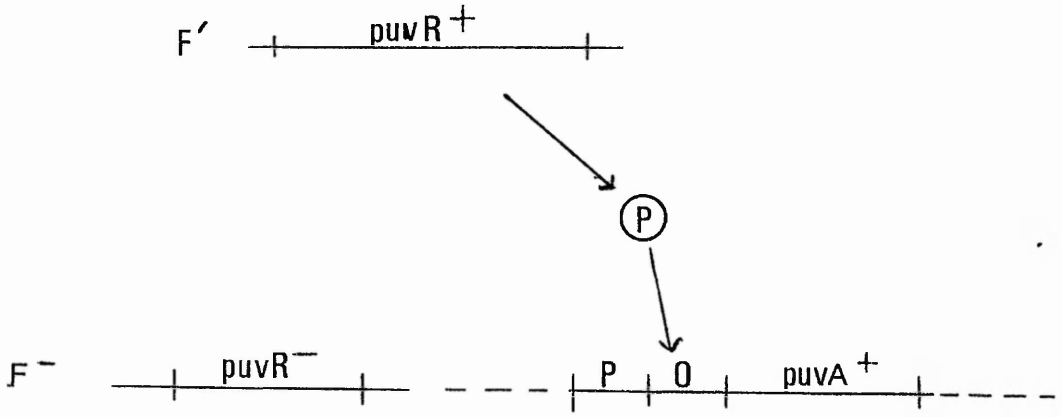


FIG.40. REGULATION OF TRANSCRIPTION OF THE 55Kd PROTEIN BY A REPRESSOR PROTEIN IN A PARTIAL DIPLOID STRAIN.

Although no evidence was obtained to suggest that the 55kd protein was directly inducible by damage caused by DNA crosslinks, complementation studies suggested that the protein was under regulatory control. Ahmad & Holland (1985) proposed that the RecA protein may control synthesis of the putative crosslink specific repair protein (see section 1.9); however, at this point it must be emphasised that, as determined in this thesis, the *recA* gene locus maps within 1 minute of the *puvR* gene mutation site. Thus, in their attempt to transduce a *recA*::Tn10 insertion mutation into strain SA270, these authors may have cotransduced a *puvR* wild type gene, thereby leading to expression of basal levels of the 55kd protein.

Isolation of a PUVA hyper-sensitive mutant derivative of SA270 was successfully achieved using P1::Tn5*lac*. A ß-galactosidase assay of the hyper-sensitive mutant showed a high constitutive level of ~800 units (Miller, 1972), compared with low level production in a *puvR*$^+$ regulated transductant derived from the PUVA hyper-sensitive strain. The fact that transduction of the Tn5*lac* insertion into KL16 produced approximately 10% *puvR*$^+$ regulated mutants confirmed that *puvR* and the Tn5*lac* insertion were closely linked on the chromosome.

Polyacrylamide gel electrophoresis of the PUVA hyper-sensitive insertion mutant derived from SA270 did not confirm the absence of the 55kd protein, although the high constitutive levels of expression seen in the PUVA hyper-resistant strain were no longer observed. It is, however, often difficult to detect even wild type levels of the 55kd protein in a total protein cell extract on a one dimensional Polyacrylamide gel and it would be advisable to use a

two dimensional gel system to screen for PUVA hyper-sensitive mutants in future experiments.

On the basis of the genetic data described in this Chapter, it is possible to propose a model for the control of expression of the 55kd protein (fig. 41). In a wild type strain (fig. 41a) the level of expression of the 55kd protein may be repressed by a protein binding to a site in the operator region of the *puvA* gene. It is proposed that there is a repressor protein acting in this way since complementation data suggests that a *puvR*$^{+}$ gene is dominant *in trans* in a partial diploid. The *puvR*$^{+}$ regulatory gene product may control transcription of the *puvA* gene and even when bound to the operator region, "leaky" transcription may be observed, in a similar way to that seen with the *lac* operon (Riggs *et al.*, 1970), so that low levels of the 55kd protein are produced.

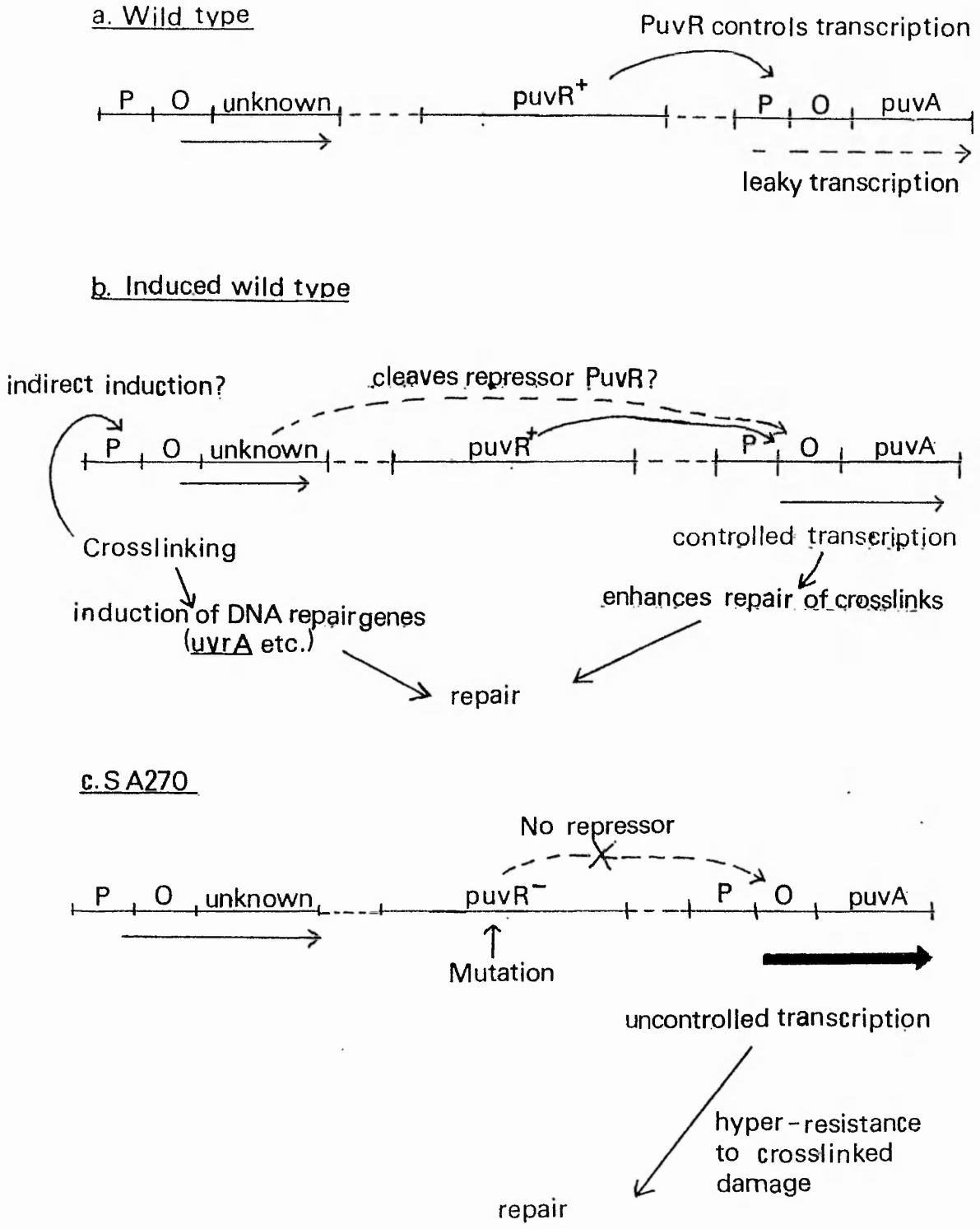


FIG.41. PROPOSED MODEL FOR THE CONTROL OF EXPRESSION OF THE 55Kd. PROTEIN.

When DNA crosslinking damage occurs, the RecA protein is modified and cleaves the LexA protein allowing transcription of repair genes such as *uvrA, uvrB* and *recN.* It seems unlikely, considering the results described in this thesis, that the regulatory protein preventing transcription of the *puvA* gene is also cleaved by RecA in response to crosslinking DNA damage. However, the possibility of the *puvR* gene product being cleaved by an unknown protein, induced indirectly in response to crosslinking damage, cannot be ruled out. Consequently the expression of the 55kd protein may be increased in a controlled manner, but not to the levels detected in SA270 (fig. 41b).

In the PUVA hyper-resistant strain SA270(fig. 41c), a mutation in the *puvR* gene probably results in loss of the putative PuvR repressor protein. Uncontrolled transcription occurs and enhanced repair of crosslinks is mediated. Since the *recA* gene has multiple roles in DNA repair and recombination it is not difficult to explain why the *recA* derivative of thePUVA hyper-resistant mutant became hyper-sensitive to PUVA induced DNA damage.

Indeed, the most likely role of the *recA* gene may be to indirectly control transcription of the *puvA* gene, perhaps by controlling an unknown gene which is needed for transcription initiation, or even by preventing attenuation of the *puvA* gene. The next Chapter examines in more detail the interactions of *puvR* with *recA* and other genes known to be involved in DNA repair.

Chapter 6

INTERACTIONS OF THE PUVA RESISTANCE GENE WITH OTHER GENES INVOLVED IN DNA REPAIR

6.1 Background

Possible mechanisms involved in the repair of PUVA-induced crosslinks have already been investigated in *E.coli* (Van Houten *et al.*, 1986a; 1986b; Jones & Yeung, 1988; Cheng *et al.*, 1988). The current proposed mechanism (described in detail in section 1.8) involves two incision steps with a RecA-mediated recombination event. In order to obtain an overall view of the way in which the known genes and gene products involved in the repair of DNA crosslinks might interact with the 55kd protein, the experiments described in this Chapter introduced known DNA repair deficient mutations into SA270 and examined the properties of the resulting recombinants.

6.2 Sensitivity of known DNA repair deficient mutants to PUVA

Strains of *E.coli* carrying mutations in the *recA*, *recBC*, *recF*, *recN*, *uvrA*, *uvrC*, *recJ*, *recBC sbcB* or *recBC sbcB recF* genes (see table 2; section 2.1) were exposed to PUVA and their percentage survival at various doses determined. The results showed that strains carrying mutations in the *recA*, *recBC*, *recF*, *recN*, *uvrA*, *uvrC*, or *recBC sbcB recF* genes were all hyper-sensitive to PUVA, whereas *recJ* or *recBC sbcB* mutants showed wild type sensitivity to PUVA (fig. 42). SA270 was considerably more resistant than any of the mutant strains. It was concluded from the results that the gene products of the mutant strains showing hyper-sensitivity to PUVA must be directly or indirectly involved in the repair of PUVA induced DNA damage.

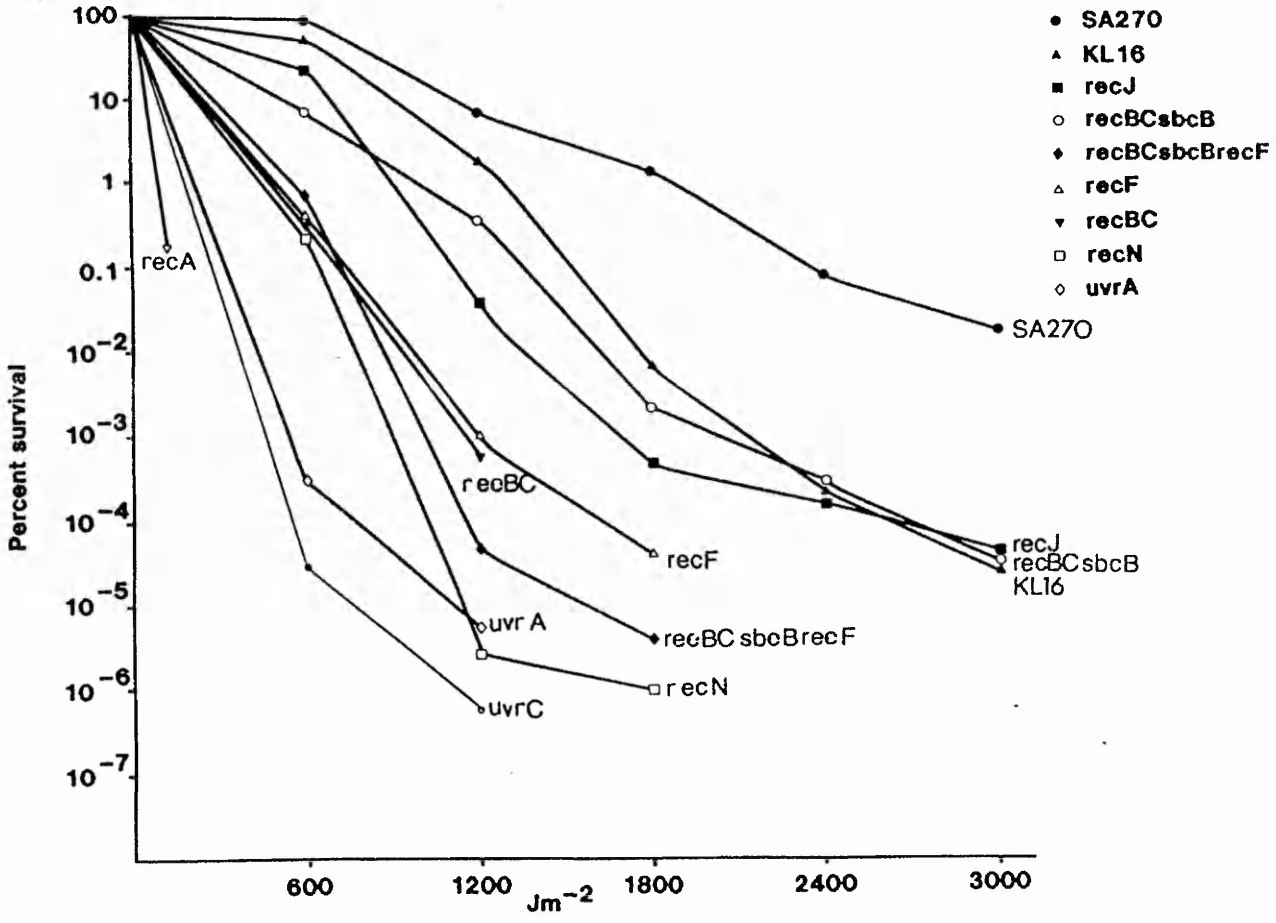


FIG.42. BACTERIAL KILL CURVES OF DNA REPAIR-DEFICIENT MUTANT STRAINS IN THE PRESENCE OF PUVA.

6.3 PUVA sensitivity of SA270 derivatives carrying known mutations affecting DNA repair

A number of mutations in known DNA repair genes, produced by transposon insertion mutagenesis, were transduced into the PUVA hyper-resistant strain SA270 by P1 transduction. Selection for transductants was made on nutrient agar plates containing the appropriate antibiotic required for selection of the inserted transposon gene.

In the case of the putative *uvrC* transductants, the transductants were far ultraviolet hyper-sensitive as expected. In contrast, the putative *uvrA* transductants were resistant to PUVA, but showed wild type sensitivity to far ultraviolet light, indicating that transfer of the *uvrA* mutant gene had not occurred. The remaining transductants were exposed to PUVA treatment and a survival curve constructed for each derivative strain (fig. 43). The results showed that *recA*, *recN* and *recF* derivatives of SA270 were all, to varying degrees, more sensitive to PUVA treatment than the wild type strain KL16, and considerably more sensitive than the original SA270 strain. The *uvrC* derivative of SA270 showed wild type sensitivity to PUVA. In contrast, the *recJ* derivative of SA270 was as resistant to PUVA treatment as SA270 itself.

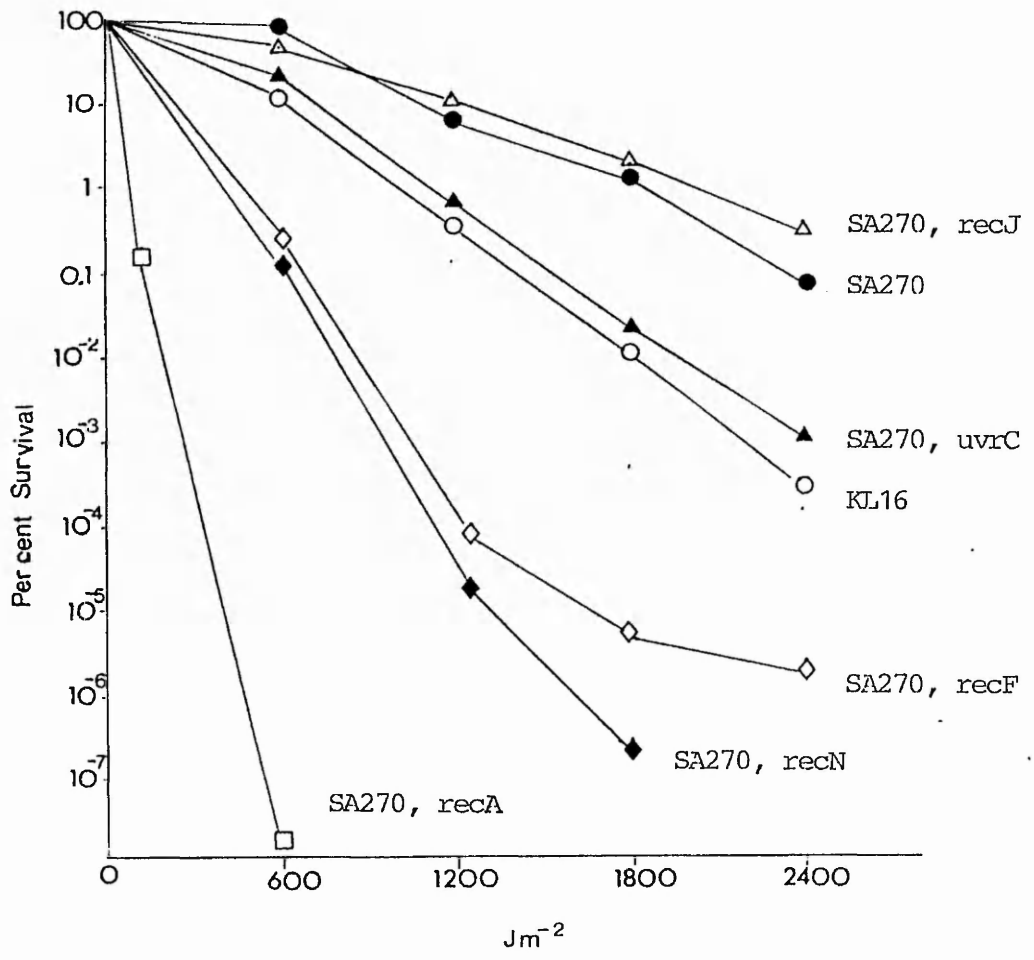


FIG.43. BACTERIAL KILL CURVES OF DNA REPAIR-DEFICIENT MUTANT DERIVATIVES OF SA270 IN THE PRESENCE OF PUVA.

6.4 SDS-Polyacrylamide gel electrophoresis of SA270 derivatives carrying mutations in other DNA repair genes

Total protein cell lysates of SA270 derivatives carrying mutations in other DNA repair genes were examined on a 10% SDS-Polyacrylamide gel to determine what effect, if any, the newly introduced mutations had on the expression of the 55kd protein. The results (fig. 44) demonstrated that *recF* and *recA* mutant derivatives of SA270, both of which showed varying degrees of hyper-sensitivity to PUVA (section 6.3) had 55kd protein levels (tracks b and g) reduced to that of the wild type strain KL16 (track f). The *recN* mutant derivative of SA270 also showed a PUVA hyper-sensitive phenotype, but continued to over-express the 55kd protein (track a). The *uvrC* mutant derivative of SA270 showed a similar level of PUVA resistance to the wild type strain KL16 and also continued to over-express the 55kd protein (track d). The *recJ* mutation had no apparent effect on the hyper-resistant phenotype of SA270 and the 55kd protein remained over-expressed (track c).

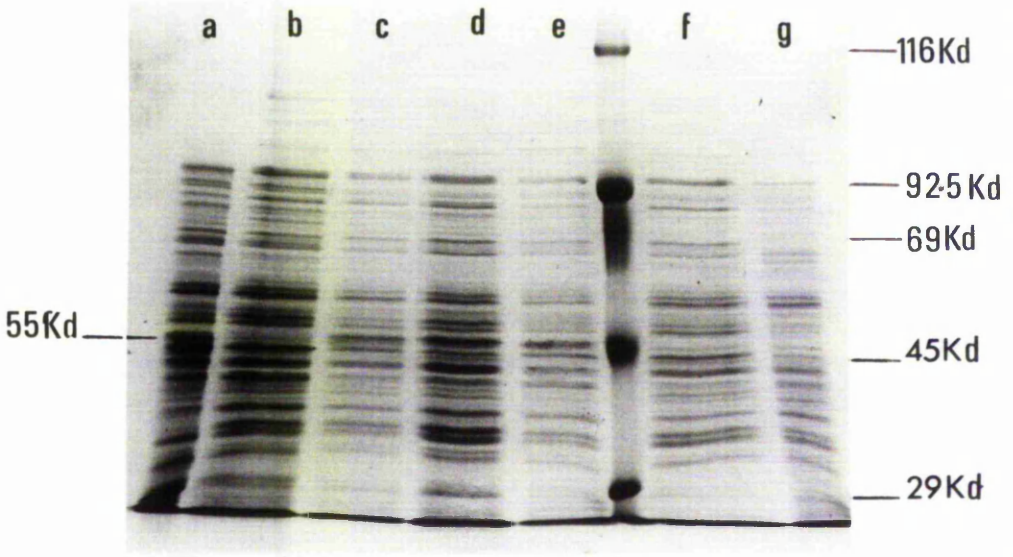


FIG.44. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TOTAL PROTEIN EXTRACTS OF DNA REPAIR DEFICIENT MUTANT DERIVATIVES OF SA270.

a. puvR recN

b. puvR recF

c. puvR recJ

d. puvR uvrC

e. puvR (SA270)

f. puvR (KL16)

g. puvR recA

6.5 Discussion

A model for DNA crosslink repair has been proposed by Van Houten *et al.* (1986b) that involves UvrABC excision endonuclease activity. Although the early incision steps of the pathway have been elucidated, little is known about the recombinational events that are also necessary for crosslink repair. During crosslink repair, excision of the psoralen adduct on one strand leaves the second adduct attached to a single stranded region. Not only do single stranded lesions provoke recombination, but recombination is crucial for repair since it converts these lesions to a double stranded form that is susceptible to a second excision repair step.

The crucial importance of this recombination stage in the repair process was demonstrated by the results in this Chapter which showed that a *recA*-deficient mutant of *E.coli* was very sensitive to PUVA-induced DNA damage. This was not a surprising result in view of the known importance of the RecA protein in the induction of some DNA repair enzymes and in recombination repair events in the cell. Mutations in the *recA* gene abolish homologous recombination in all known recombination pathways and consequently block all recombinational repair processes (Peterson *et al.*, 1988). Van Houten (1986b) proposed that there is an absolute requirement for a functional RecA protein in the recombinational events of crosslink repair (section 1.8). Following the dual incision activity of the UvrABC endonuclease, a single-stranded gap is produced which is subsequently repaired by a recombinational event. The RecA protein promotes strand transfer with a homologous region of DNA in another DNA molecule and strand invasion by a sister duplex is mediated. The recombinational function of the RecA protein has been extensively

studied and the strand transfer reaction has been demonstrated *in vitro*. In *E.coli*, single-stranded DNA (ssDNA) is complexed with a single-strand binding protein (SSB) which is required to allow the formation of a RecA ssDNA complex. The SSB protein is then released to permit production of longer RecA-ssDNA nucleo-protein filaments that are needed for strand pairing and recombinational repair (Moreau, 1988). Regulation of the RecA protein by the LexA protein is of utmost importance in that the RecA protein is induced following DNA damage.

At least two pathways for genetic recombination in *E.coli* have been elucidated, both dependent on a functional *recA* gene. One pathway requires the *recB* and *recC* genes that encode subunits of the exonuclease V protein. The RecBC proteins form part of a multifunctional complex, RecBCD, that has both endonuclease and exonuclease activity against single-stranded and double stranded DNA. The enzyme complex also has an unwinding activity for double stranded DNA with double strand breaks, but requires the ends of the DNA to be blunt or nearly so (Simmon & Lederberg, 1972). This suggests that the RecBCD protein complex may be important in recombinational repair of double strand breaks in DNA; indeed Wang & Smith (1983) showed that the RecBCD complex played no role in the repair of daughter strand gaps, but was required, together with a functional RecA protein, for the recombinational repair of double strand breaks. In this Chapter it was demonstrated that a *recBC* mutant of *E.coli* was sensitive to PUVA damage, thereby also suggesting the involvement of these gene products in the repair of crosslinks.

In *recBC* mutants there is a significant level of residual recombination, due to another pathway controlled by the *recF* gene (Horii & Clark, 1973). In *recBC* proficient cells, only 1% of recombination is controlled by the *recF* gene. However, in a *recBC sbcB* mutant this minor pathway becomes the only recombination pathway available to the cell. It is important to note that the recombination deficiency of *recBC* mutants is suppressible by a mutation in the *sbcB* gene (exonuclease I); hence enhancement of recombination in these double mutants has been interpreted to mean that a DNA intermediate of the *recF* pathway is sensitive to degradation by this nuclease (exonuclease I). Results in this Chapter showed that a *recBC sbcB* double mutant has wild type sensitivity towards PUVA, thereby implicating the *recF* gene in crosslink repair. This proposal was supported by the observation that both a *recBC sbcB recF* triple mutant and a *recF* single mutant were sensitive to PUVA. The triple mutant was more sensitive to PUVA than either the *recBC* or *recF* single mutant. This suggested that both recombination pathways were involved in the PUVA-induced damage repair process.

The RecF pathway, like the RecBC pathway, requires a functional *recA* gene for recombination to occur. Apart from the *recF* gene itself, several other genes are known to form part of the pathway. The RecF pathway becomes more active in cells exposed to DNA damage (Lovett & Clark, 1983) and, because mutations in RecF pathway genes have little effect on recombination in wild type cells, it has been suggested that the major role of the RecF pathway is in DNA repair (Lovett & Clark, 1985). Mutations belonging to the RecF class were first identified in *recBC sbcB* strains of *E.coli*. To date, no role

has been assigned to the RecF protein, although Walker (1987) has proposed that it might function by modifying the activity of the RecA protein, either in strand transfer or by regulation of gene expression.

The predominant mechanism for daughter strand gap repair seems to be mediated via the RecF pathway, and both the RecA and RecF proteins are directly involved in repair of this type of lesion (Walker, 1985). This may explain why single mutations in the *recA* and *recF* genes make cells hyper-sensitive to PUVA (section 6.2) since PUVA induced DNA damage frequently results in the formation of DNA lesions of this type.

The RecF recombination pathway has eight well identified gene members *recA, recF, recN, recJ, recO, recQ, ruv* and *uvrD*. The *recN, recQ, ruv* and *uvrD* genes are all part of the SOS regulon, but the status of the remaining genes is unknown. Single mutants of genes in the RecF pathway display variable phenotypes when treated with different DNA damaging agents (section 1.7), and this differential sensitivity may reflect variations in the proteins required to repair a given type of DNA lesion. As described in this Chapter, a *recN* single mutation in wild type *E.coli* made the cells hyper-sensitive to PUVA. Even though the RecBC protein is important in recombinational repair of double strand breaks, it is also known that inducible repair of such lesions can be mediated by components of the RecF pathway requiring a functional *recN* gene (Picksley *et al.*, 1984). The relationship between the *recN*-dependent and *recBC*-dependent repair mechanisms for double strand breaks remains unclear, but it is apparent from the studies in this thesis that

both the *recN* and *recBC* gene products are required to repair DNA lesions resulting from PUVA exposure.

In contrast, the experiment described in section 6.2 showed that a strain with a single mutation in the *recJ* gene had wild type sensitivity to PUVA damage, indicating that a functional *recJ* gene product is not required to repair PUVA induced DNA damage. The role of the RecJ protein is not yet clear, although it is certainly necessary for recombination events in a *recBC sbcB* background (Wang & Smith, 1988). Thoms & Wackernagal (1988) showed that there was suppression of the ultraviolet sensitive phenotype of *recF* mutants if the *recA* alleles *srf* and *tif* and a functional RecJ protein were present, indicating that there may be an alternative recombination pathway independent of both the *recA* and the *recF* gene products.

Considering the role of the UvrABC endonuclease in the repair mechanism of DNA crosslinks, it was not surprising that both the *uvrA* and *uvrC* mutant strains of *E.coli* were very sensitive to PUVA damage (section 6.2). The two incision steps mediated by the UvrABC protein complex (Van Houten, 1990) are required for the repair of psoralen-induced crosslinks, whereas the intervening RecA-mediated recombination event can probably proceed via different pathways, depending on the type of lesions produced. This would explain why single mutants of certain recombinational genes, such as *recB*, *recN* and *recF*, were not as sensitive to PUVA as single mutations in genes required for the incision step in crosslink repair. Although some investigations of post damage incision in *uvrC* mutants have suggested that the *uvrC* gene product is not absolutely required for the UvrAB complex to mediate strand incision, Walters (1988) has demonstrated conclusively that a *uvrC*::Tn10 mutant is deficient in

excision repair of ultraviolet light induced DNA damage and suggested that other *uvrC* mutants were "leaky".

To summarise the discussion to this point, the results obtained in this Chapter confirmed that the *recA*, *recBC*, *recF*, *recN*, *uvrA* and *uvrC* genes were either directly or indirectly involved in PUVA repair. Further experiments were carried out to determine what role the 55kd protein might play in PUVA repair, and to determine whether its function was dependent or independent of some of the genes listed above. A number of single repair gene mutations were therefore transduced into strain SA270 and the resulting PUVA phenotype and expression levels of the 55kd protein studied.

As described in section 6.3, the *recA* derivative of SA270 (designated SA316) was extremely sensitive to PUVA, indicating that the *recA* gene product must have an important role in the repair of PUVA-induced crosslinks. As already discussed, the RecA⁻ protein plays a critical role in recombination repair and also regulates the expression of other repair genes such as *recN*. Since SA316 showed wild type levels of the 55kd protein this could suggest, as pointed out by Ahmad & Holland (1985) that the RecA protein might control expression; however, as outlined in Chapter 5, the *puvR*⁺ gene may have been cotransduced with *recA* into strain SA270, resulting in wild type expression of the protein.

A *recF* mutant derivative of SA270 was also hyper-sensitive to the PUVA and the over-expression of the 55kd protein was reduced to wild type basal levels. However, since the *recF* gene maps at 83 minutes on the *E.coli* chromosome, it is not possible to suggest that the *puvR*⁺ gene had been cotransduced into strain SA270. It can

therefore be concluded that the *recF* gene is required in strain SA270 for over-expression of the 55kd protein. Unfortunately very little is known about the *recF* gene and its protein. Interestingly, studies by Thoms & Wackernagel (1987;1988) revealed a close relationship between the *recA* and *recF* genes. They showed that the SOS response was impaired in *recF* mutants. In a *uvrA*::MudI(*lac,Ap*) fusion strain, the introduction of a *recF*400 mutation impaired ß-galactosidase production, compared with levels seen in the *uvrA*::MudI(*lac,Ap*) strain, following induction with far ultraviolet light. It can therefore be proposed that, in a wild type strain treated with a DNA crosslinking agent, the presence of a *recF* mutation may inhibit transcription of the *recA* gene. It is likely that the *recF* gene product may be required for transcription of the *puvA* gene, and that a *recF* mutation could indirectly inhibit transcription of the *puvA* gene.

Alternatively, since little is known about the function of the RecF protein, it is also a possibility that the protein directly affects the transcription of the *puvP* gene. Perhaps the transcription of the *puvP* protein is enhanced by binding of the RecF protein. In a *recF* mutant derivative of SA270 this does not occur, and the protein is consequently not over-expressed. These proposals are summarised in fig. 45. As already described, the *recF* gene product is involved in crosslink repair and is necessary for daughter strand gap repair. It is therefore not surprising that a *recF* mutant derivative of SA270 is hyper-sensitive to PUVA.

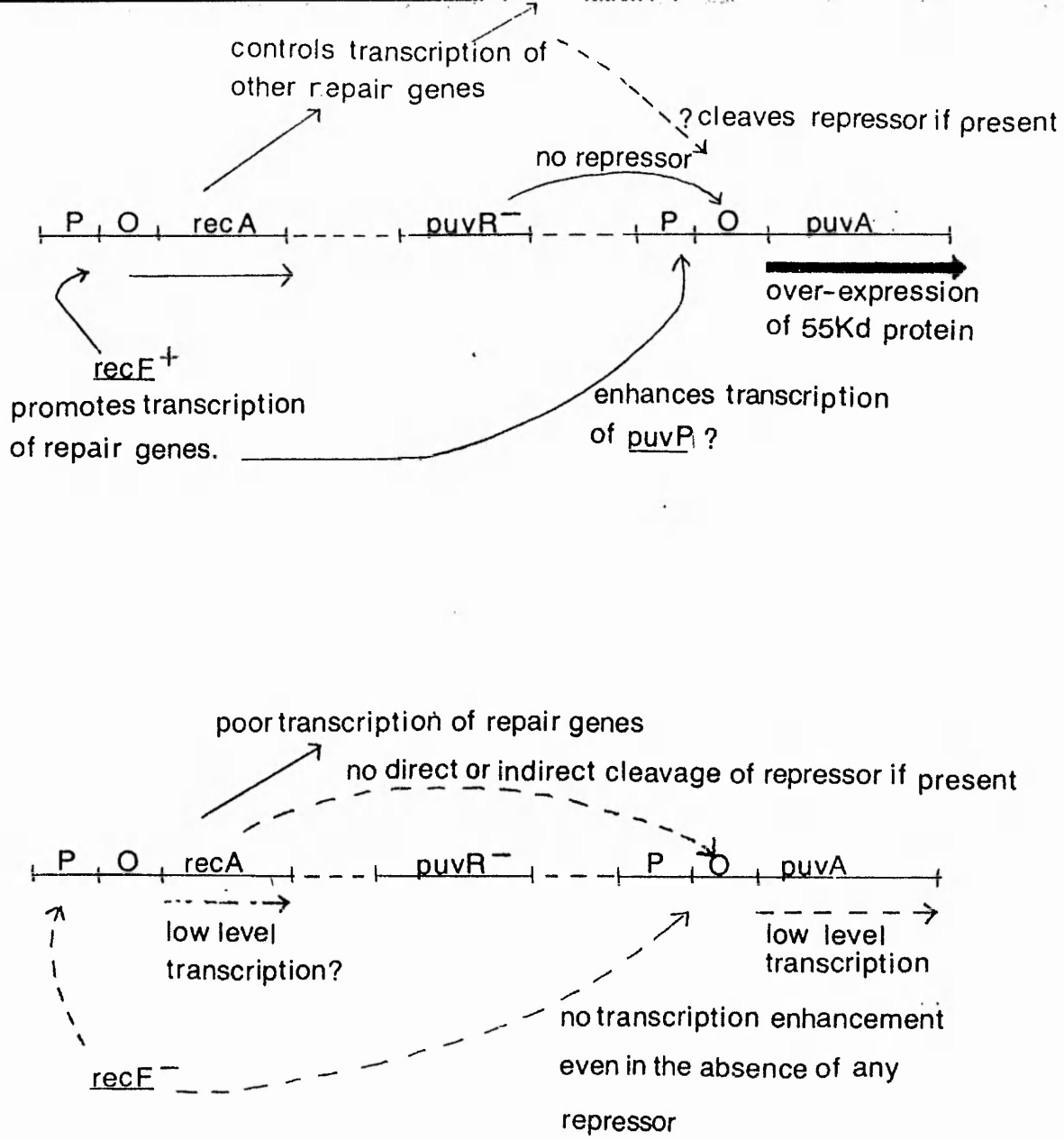


FIG.45. PROPOSED MODEL FOR THE CONTROL OF EXPRESSION OF THE 55Kd. PROTEIN INVOLVING THE recF GENE.

The introduction of a *uvrC* mutation into SA270 led to loss of PUVA resistance, but not to the level of sensitivity found in a wild type strain with a single *uvrC* mutation. The *uvrC* derivative of SA270 also showed continued expression of the 55kd protein. As described in detail below, this is a very important result, particularly since the new strain was very sensitive to far ultraviolet light.

The events leading to incision of crosslinked DNA strands by the UvrABC endonuclease are already well understood (section 1.8). The UvrA protein binds to the DNA in a damage dependent manner (Seeberg & Steinum, 1982) and the UvrB protein stabilises this association by forming a complex (Strike & Rupp, 1985). The UvrC protein binds to complete the complex, resulting in incision of the DNA (Yeung *et al.*, 1986). In an SOS induced cell the UvrA and UvrB proteins are present in vast excess over the UvrC protein (Yoakum & Grossman, 1981). A possible interpretation of the results described in this thesis is that the 55kd protein may bind to the UvrAB complex and mediate a different type of strand incision that is specific for DNA crosslinks. In strain SA270 the amount of 55kd protein is greatly elevated and the protein may possibly be in competition with the UvrC protein. In a *uvrC* derivative of SA270 the 55kd protein may bind to the UvrAB complex and mediate only crosslink repair since the strain is rendered very sensitive to far ultraviolet light. An obvious further experiment would be to introduce *uvrA* or *uvrB* mutations into SA270 and examine their effect on the PUVA hyper-resistant phenotype.

The precise role of the 55kd protein is not yet clear, but it could function as an independent endonuclease specific for DNA

crosslink damage, whose activity is minimal in a wild type strain, but increased in strain SA270. On the other hand, the protein may not necessarily be an endonuclease, but could be a component of a repair pathway which provides the cell with an alternative method for repairing crosslinked DNA lesions. No firm conclusion can be drawn from the results until the function of the 55kd protein has been elucidated. However, the PUVA phenotype of the *uvrC* mutant derivative of SA270 may provide a vital clue as to why strain SA270 is hyper-resistant to DNA crosslinking damage.

It should be noted at this stage that a *recN* mutant derivative of SA270 is sensitive to PUVA, but still retains over-expression of the 55kd protein. The *recN* gene maps very close to the *puvR* gene (at 56.5 minutes on the *E.coli* chromosome) and its gene product has a similar molecular weight (60kd). As the 55kd protein is still over-expressed in the *recN* mutant derivative of SA270 it can be concluded that it is not the product of the *recN* gene. It is interesting to note that, although the map positions of the *puvR* and *recN* genes are very close on the chromosome, cotransduction of the *puvR* and *recN* genes was not observed. The RecN protein is part of the RecF pathway of recombination repair and is involved in the repair of DNA double strand breaks (Picksley *et al.*, 1984). As proposed above, over-expression of the 55kd protein may enhance a particular step in the repair of PUVA-induced DNA crosslinks, but because the cell is *recN* it is unable to repair double strand breaks formed during the later stages of repair. As a result the strain is sensitive to PUVA damage due to incomplete overall repair.

In contrast, as described earlier, a *recJ* mutation does not make wild type *E.coli* sensitive to PUVA-induced DNA damage,

suggesting that the RecJ protein plays no role in repairing DNA crosslinks. In support of this conclusion, a *recJ* mutant derivative of SA270 was as resistant to PUVA as SA270 and continued to over express the 55kd protein. As Thoms & Wackernagal (1988) have proposed that the RecJ protein may be part of an alternative recombination pathway suppressing ultraviolet-sensitivity, it would be interesting to construct a mutant strain carrying *recF, recA, sif puvR* with and without *recJ*, and elucidate its phenotype for PUVA.

In conclusion, the results presented in this Chapter indicated that the probable role of the 55kd protein is as an adjunct to existing DNA repair pathways. It is also clear that the final outcome of its action is specific for the repair of crosslink DNA damage. As proposed above, one of the most likely ways in which it could operate would be as an endonuclease specific for DNA crosslinks, and this hypothesis would seem to be a suitable starting point for further studies on its mode of action.

The next Chapter describes experiments designed to purify the 55kd protein and an initial investigation of its possible function as an endonuclease.

Chapter 7

STUDIES WITH TOTAL PROTEIN CELL EXTRACTS AND THE PURIFIED 55KD PROTEIN IMPLICATED IN PUVA RESISTANCE

7.1 Background

The experiments already described in this thesis have suggested that the PUVA hyper-resistant mutant SA270 may have developed a more efficient repair mechanism for DNA crosslinks mediated by the 55kd protein. This Chapter describes further experiments carried out in an attempt to determine the precise biological role of the protein and to elucidate whether the protein itself had endonuclease activity specific for DNA crosslinks as proposed by Ahmad & Holland (1985).

In its normal state, superhelically twisted colE1 plasmid DNA can be relaxed by the introduction of nicks in the DNA. If the 55kd protein is an endonuclease, or enhances the action of a pre-existing nuclease, it may be possible to show that supercoiled ColE1 DNA molecules treated with PUVA are relaxed with greater efficiency in the presence of total protein extract obtained from SA270, compared with ColE1 DNA molecules treated with a total protein extract from the wild type parent strain KL16.

This Chapter also includes experiments designed to purify the 55kd protein using FPLC. Subsequent analysis of the purified protein, in an attempt to determine whether it had an endonuclease activity itself, is also described.

7.2 Effect of total protein extracts from strains SA270 and KL16 on PUVA damaged and undamaged ColE1 DNA

Following isolation and purification (as described in section 2.17) ColE1 DNA was exposed to near ultraviolet light plus 8-

methoxypsoralen (section 2.4). The PUVA-damaged plasmid DNA was then incubated with total protein extract from either strain SA270 or KL16. If SA270 had greater endonuclease activity specific for DNA crosslinks, then the supercoiled ColE1 molecules should relax with greater efficiency in the presence of protein extract obtained from this strain. Plasmid DNA treated with total protein extracts was therefore examined using agarose gel electrophoresis.

In fig. 46, lanes 2 and 3 represent the PUVA-damaged colE1 plasmid at 0 minutes incubation with protein extract. Two bands are visible: the top faint band consists of relaxed circular DNA and the lower band consists of supercoiled DNA. Following incubation with protein extracts from strain SA270 and KL16 for 60 minutes, there was an increase in the relaxed circular form of DNA which may indicate a local disruption of the helix by endonuclease activity. After 60 minutes there was no more relaxed circular DNA in the SA270 protein extract incubation mixture than in the KL16 protein extract incubation mixture (lanes 4 and 5). Thus the ColE1 plasmid molecules had not relaxed with greater efficiency in the presence of protein extract obtained from the PUVA hyper-resistant mutant SA270.

LANE 1 PLASMID ONLY
2 PLASMID + KL16, 0 MINUTES
3 PLASMID + SA270, 0 MINUTES
4 PLASMID + KL16, 60 MINUTES
5 PLASMID + SA270, 60 MINUTES
6 KL16 PROTEIN EXTRACT ONLY
7 SA270 PROTEIN EXTRACT ONLY
8 λ DNA, Hind III DIGEST

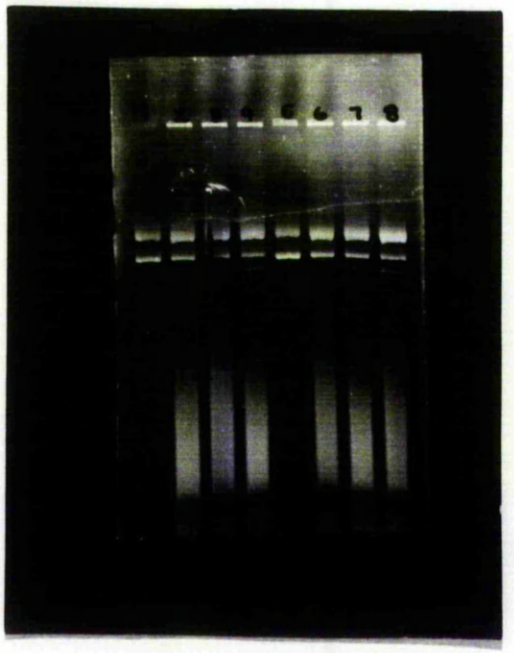
FIG.46. INCUBATION OF PUVA DAMAGED ColE1 PLASMID WITH TOTAL PROTEIN EXTRACTS OF KL16 AND SA270 FOR 0 AND 60 MINUTES.

One possibility was that, after 60 minutes, the formation of relaxed circular DNA had reached a maximum with protein extracts from SA270 and KL16. The rate of plasmid relaxation could therefore be important. However fig. 47 shows that the ColE1 plasmid treated with SA270 protein extract did not form relaxed open circles at a quicker rate than ColE1 plasmid treated with KL16 protein extract. Also shown in fig. 47 are experiments carried out with undamaged ColE1 plasmid, but the results obtained were similar to those shown for damaged plasmid DNA.

In order to test whether there were any differences in the efficiency of plasmid relaxation over a longer time scale, colE1 DNA was incubated with KL16 and SA270 total protein extracts and examined with agarose gel electrophoresis after 60, 180 and 300 minutes incubation (fig. 48). Again no obvious difference in the amount or rate of formation of relaxed circular DNA was seen and the result was consistent for both PUVA-damaged and undamaged plasmid DNA.

It was interesting to note that an extra band of DNA appeared below the supercoiled form of the plasmid after 20 minutes incubation with protein extract from both SA270 and KL16. After 300 minutes incubation a further band was detected. The precise nature of these moieties is unclear, but one possibility is that these bands represent single-stranded or partially digested DNA molecules formed by exonuclease activity. The ColE1 plasmid forms relaxed open circles following endonuclease activity. This is the result of the production of a nick in one strand of DNA. Subsequent exonuclease action may remove bases to produce single-stranded DNA molecules. The formation of these DNA bands was no more efficient in

the presence of protein extract obtained from SA270, suggesting that this particular enzyme activity was not responsible for PUVA hyper-resistance in the mutant strain. Again, this phenomenon was observed with both PUVA-damaged and undamaged plasmid DNA. Further work is required to elucidate the precise nature of these molecular events.



LANE 1 DAMAGED PLASMID + KL16
O MINUTES.

2 DAMAGED PLASMID + KL16
20 MINUTES

3 DAMAGED PLASMID + KL16
40 MINUTES

4 DAMAGED PLASMID + KL16
60 MINUTES

5 UNDAMAGED PLASMID + KL16
O MINUTES

6 UNDAMAGED PLASMID + KL16
20 MINUTES

7 UNDAMAGED PLASMID + KL16
40 MINUTES

8 UNDAMAGED PLASMID + KL16
60 MINUTES

LANE 1 DAMAGED PLASMID + SA270
O MINUTES

2 DAMAGED PLASMID + SA270
20 MINUTES

3 DAMAGED PLASMID + SA270
40 MINUTES

4 DAMAGED PLASMID + SA270
60 MINUTES

5 UNDAMAGED PLASMID + SA270
O MINUTES

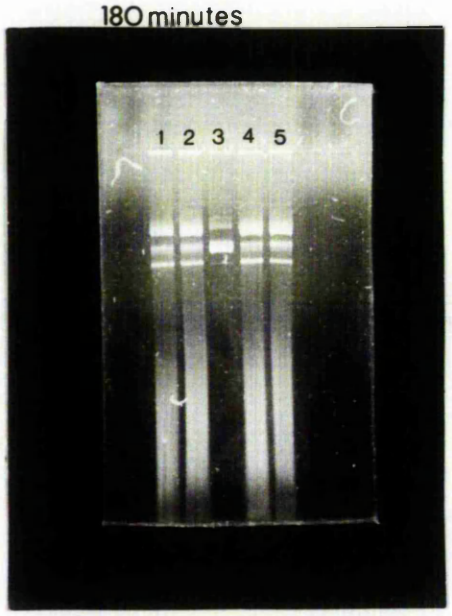
6 UNDAMAGED PLASMID + SA270
20 MINUTES

7 UNDAMAGED PLASMID + SA270
40 MINUTES

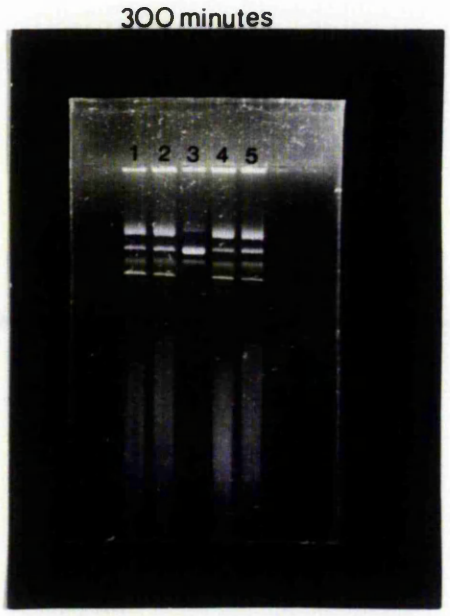
8 UNDAMAGED PLASMID + SA270
60 MINUTES

FIG.47. INCUBATION OF PUVA DAMAGED AND UNDAMAGED ColE1 PLASMID WITH TOTAL PROTEIN EXTRACTS OF KL16 AND SA270 FOR 0, 20, 40, AND 60 MINUTES.

LANE 1 DAMAGED PLASMID+KL16
2 UNDAMAGED PLASMID + KL16
3 PLASMID ONLY
4 DAMAGED PLASMID + SA270
5 UNDAMAGED PLASMID + SA270



LANE 1 DAMAGED PLASMID + KL16
2 UNDAMAGED PLASMID + KL16
3 PLASMID ONLY
4 DAMAGED PLASMID + SA270
5 UNDAMAGED PLASMID + SA270



LANE 1 DAMAGED PLASMID + KL16
2 UNDAMAGED PLASMID + KL16
3 PLASMID ONLY
4 DAMAGED PLASMID + SA270
5 UNDAMAGED PLASMID + SA270

FIG.48. INCUBATION OF PUVA DAMAGED AND UNDAMAGED ColE1 PLASMID WITH TOTAL PROTEIN EXTRACTS OF SA270, AND KL16 FOR 60, 180 AND 300 MINUTES.

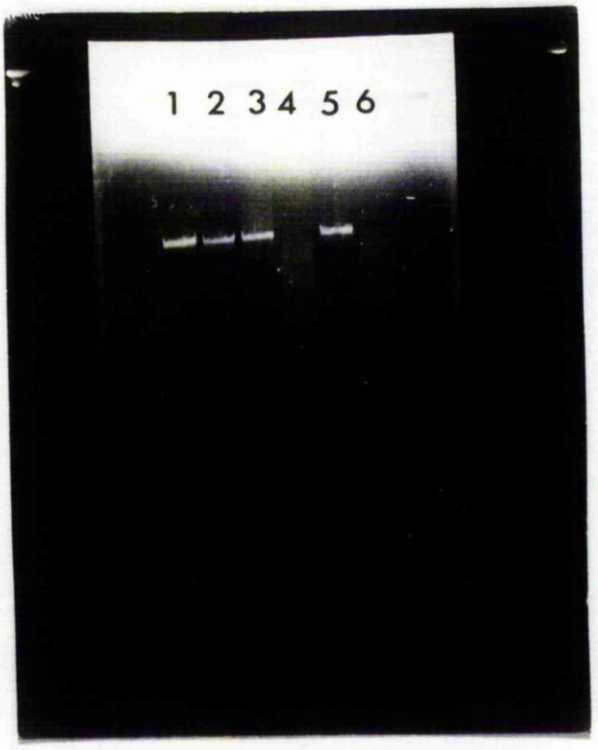
7.3 Analysis of PUVA-damaged and undamaged λ DNA following incubation with total protein extracts from strains SA270 and KL16

Since there was no indication of an enhanced relaxation efficiency when supercoiled ColE1 plasmid DNA was incubated with total protein extract from the PUVA hyper-resistant mutant SA270, it was decided to perform similar experiments with PUVA-damaged and undamaged linear λ DNA.

Fig. 49 shows that there was only slight degradation of the PUVA-damaged λ DNA following incubation for 60 minutes with total protein extract from strain KL16. In contrast, total degradation was observed following incubation of PUVA-damaged λ DNA with total protein cell extract from strain SA270.

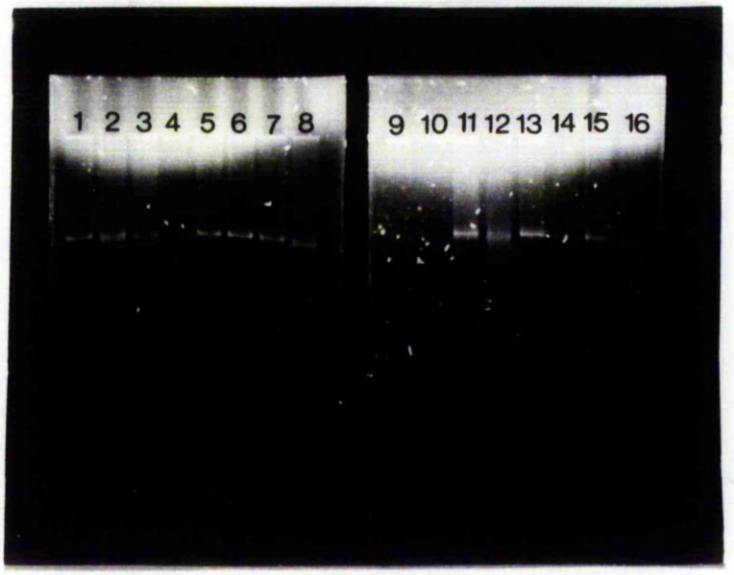
Total protein extracts were prepared of repair-deficient mutant derivatives of SA270. Results showed that degradation of the λ DNA was more efficient when the 55kd protein was over-expressed in the cell. Protein extracts of *recN*, *recJ* and *uvrC* mutant derivatives of SA270 all showed over-expression of the 55kd protein, and degraded λDNA after 60 minutes incubation at 37°C. Protein extract from the *recF* mutant derivative of SA270 caused wild type levels of λDNA degradation; since this strain had basal levels of the 55kd protein it was concluded that the 55kd protein plays some role in degrading the DNA (fig. 50).

To provide more evidence that the 55kd protein was involved in mediating λ DNA degradation, λ DNA was incubated with total protein extract from the PUVA hyper-sensitive mutant, JH110. After 60 minutes incubation there was no degradation of λ DNA (fig. 51).



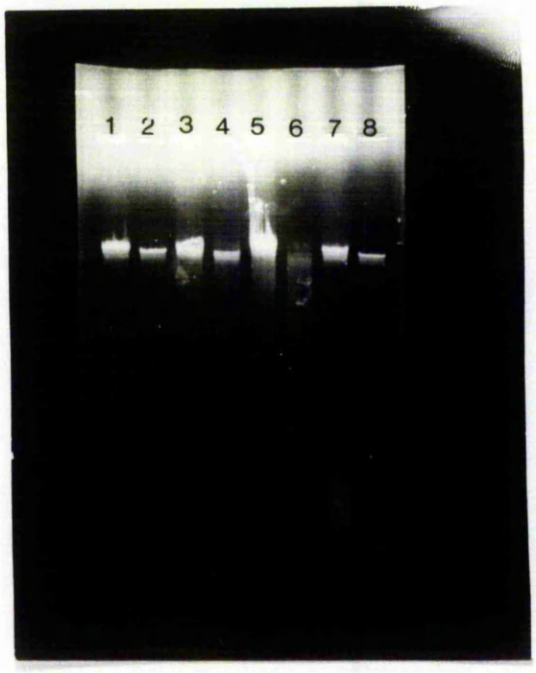
LANE 1 λ DNA + KL16, 0 MINUTES
2 λ DNA + KL16, 60 MINUTES
3 λ DNA + SA270, 0 MINUTES
4 λ DNA + SA270, 60 MINUTES
5 λ DNA + SA270, 0 MINUTES
6 λ DNA + SA270, 60 MINUTES

FIG.49. INCUBATION OF PUVA DAMAGED λ DNA WITH TOTAL PROTEIN EXTRACTS OF KL16 AND SA270 FOR 0 AND 60 MINUTES.



LANE 1 λ DNA + KL16, 0 MINUTES
2 λ DNA + KL16, 60 MINUTES
3 λ DNA + SA270, 0 MINUTES
4 λ DNA + SA270, 60 MINUTES
5 λ DNA + JH110, 0 MINUTES
6 λ DNA + JH110, 60 MINUTES
7 λ DNA + recF, SA270, 0 MINUTES
8 λ DNA + recF, SA270, 60 MINUTES
9 λ DNA + recN, SA270, 0 MINUTES
10 λ DNA + recN, SA270, 60 MINUTES
11 λ DNA + recJ, SA270, 0 MINUTES
12 λ DNA + recJ, SA270, 60 MINUTES
13 λ DNA + uvrC, SA270, 0 MINUTES
14 λ DNA + uvrC, SA270, 60 MINUTES
15 λ DNA + recA, SA270, 0 MINUTES
16 λ DNA + recA, SA270, 60 MINUTES

FIG.50. INCUBATION OF PUVA DAMAGED λ DNA WITH TOTAL PROTEIN EXTRACTS OF DNA REPAIR DEFICIENT MUTANT DERIVATIVES OF SA270 FOR 0 AND 60 MINUTES.



LANE 1 UNDAMAGED λ DNA + KL16
0 MINUTES

2 DAMAGED λ DNA + KL16
0 MINUTES

3 UNDAMAGED λ DNA + SA270
0 MINUTES

4 DAMAGED λ DNA + SA270
0 MINUTES

5 UNDAMAGED λ DNA + SA270
0 MINUTES

6 DAMAGED λ DNA + SA270
0 MINUTES

7 UNDAMAGED λ DNA + JH110
0 MINUTES

8 DAMAGED λ DNA + JH110
0 MINUTES

LANE 1 UNDAMAGED λDNA + KL16
60 MINUTES

2 DAMAGED λ DNA + KL16
60 MINUTES

3 UNDAMAGED λ DNA + SA270
60 MINUTES

4 DAMAGED λ DNA + SA270
60 MINUTES

5 UNDAMAGED λ DNA + SA270
60 MINUTES

6 DAMAGEDλDNA + SA270
60 MINUTES

7 UNDAMAGED λ DNA + JH110
60 MINUTES

8 DAMAGED λ DNA + JH110
60 MINUTES

FIG.51. INCUBATION OF PUVA DAMAGED AND UNDAMAGED λDNA WITH TOTAL PROTEIN EXTRACTS OF KL16, SA270 AND JH110 FOR 0 AND 60 MINUTES.

It was also interesting to note that the same result was produced when the above experiments were also carried out in TE buffer alone. This suggested that the activity of the 55kd protein in mediating degradation of λ DNA was ATP and Mg^{2+}-independent. Results obtained for undamaged λ DNA were the same as for PUVA-damaged λ DNA.

in order to determine whether or not 8-methoxypsoralen was binding to λ DNA, ^{3}H-8-methoxypsoralen was added to a solution of λDNA and then irradiated with near ultraviolet light. PUVA-damaged λ DNA was incubated for 60 minutes with total protein extracts from SA270, KL16, JH110 and all the repair mutant derivatives of SA270. The DNA was then examined on an agarose gel. The gel was then dried and exposed to X-ray film for 3-5 days. Fig. 52 shows that 8-methoxypsoralen had indeed bound to λ DNA, and that protein extracts from strains over-expressing the 55kd protein degraded the DNA more efficiently than protein extracts from strains with wild type expression of the 55kd protein.

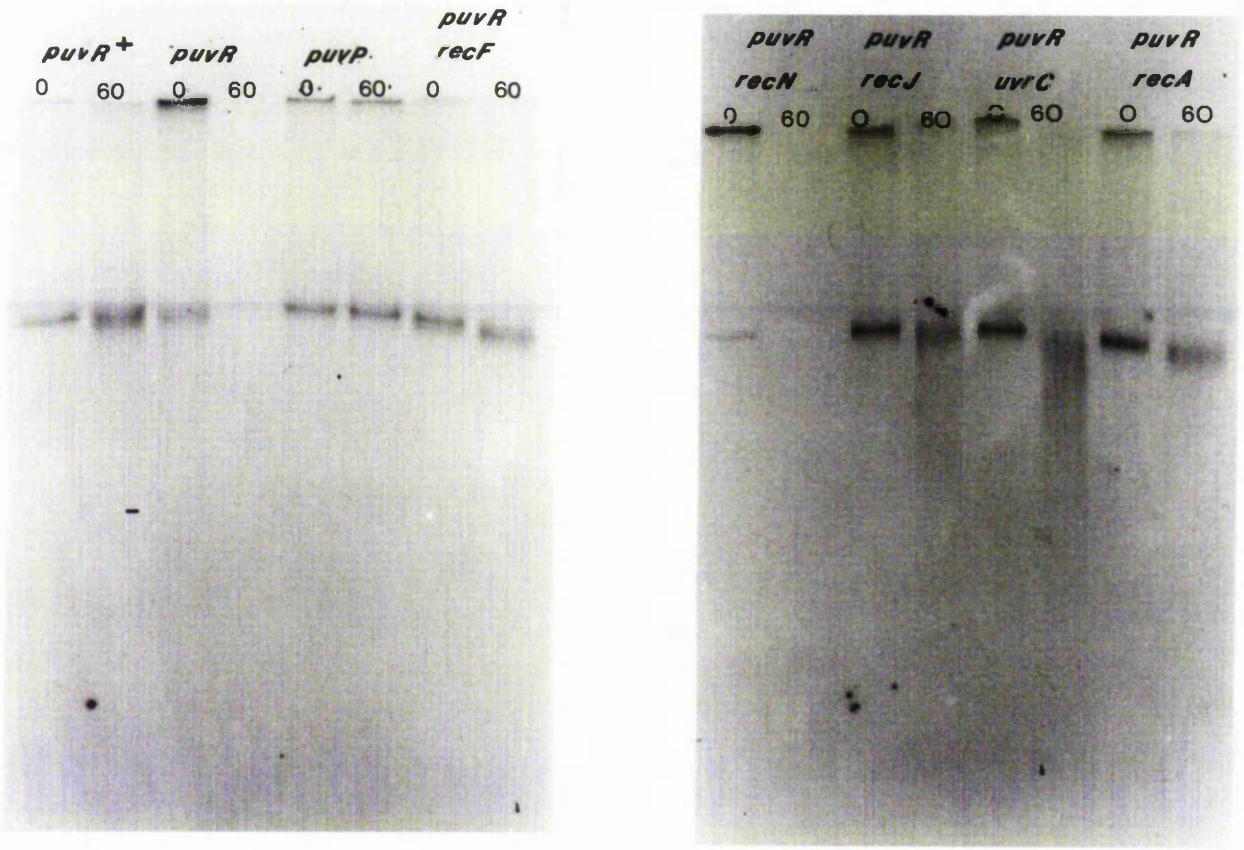


FIG.52. FLUOROGRAPH OF PUVA DAMAGED λ DNA INCUBATED WITH TOTAL PROTEIN EXTRACTS OF KL16, SA270, JH110 AND DNA REPAIR DEFICIENT MUTANT DERIVATIVES OF SA270.

7.4 Purification of the 55kd protein using FPLC

Crude cell extracts were prepared as described in section 2.6. Gel filtration techniques were used to exclude high and low molecular mass proteins (section 2.21) and fractions containing proteins with a molecular mass between 10kd and 100kd were collected and pooled. The pooled fractions were freeze dried and concentrated. This technique was performed on both strains SA270 and KL16. Concentrated fractions containing a known amount of protein (section 2.21) were loaded on to a monoQ 5/5 ion exchange column and the proteins separated using a sodium chloride gradient. 0.5ml fractions were collected every minute and retained on ice.

Using an ultraviolet light protein monitor, a scan of the proteins eluted from the column was obtained (fig. 53). Profiles of proteins eluted from the KL16 and SA270 samples were compared and, since no assay for the 55kd protein was available, any peaks showing a greater amount of protein in SA270 than in KL16 were noted. The corresponding fractions containing proteins in greater concentration in SA270 than in KL16 were collected and electrophoresed on a 10% SDS-Polyacrylamide gel. Fraction 25 was shown to contain the 55kd protein in an almost pure form (fig. 54).

This fraction was retained and used to treat PUVA-damaged and undamaged λ DNA for 60 minutes at 37°C. Agarose gel elecrophoresis showed that the 55kd protein did not mediate λ DNA degradation in its purified form (fig. 55).

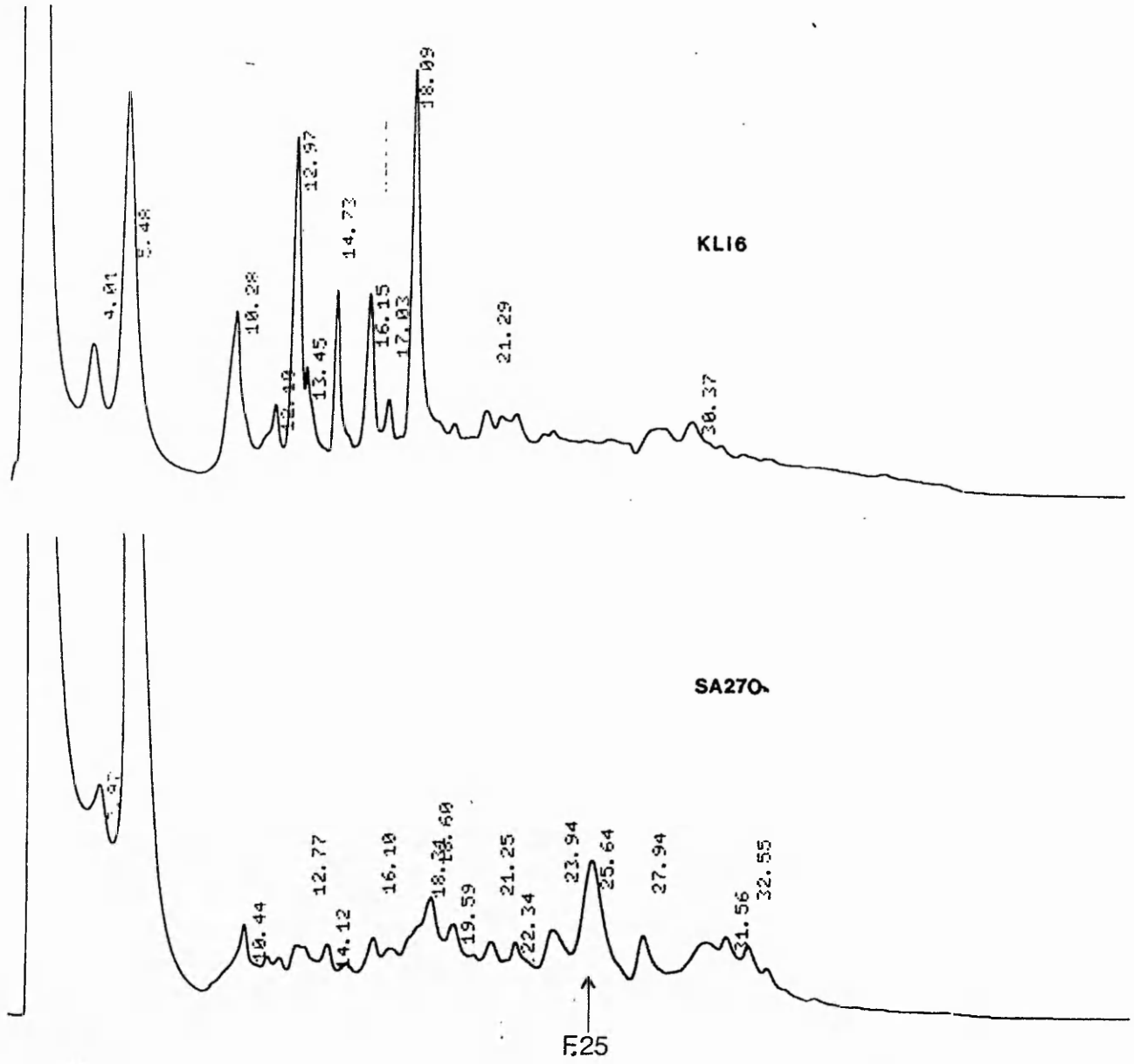


FIG.53 . FPLC ELUTION PROFILES OF PROTEIN EXTRACTS OF KL16 AND SA270.

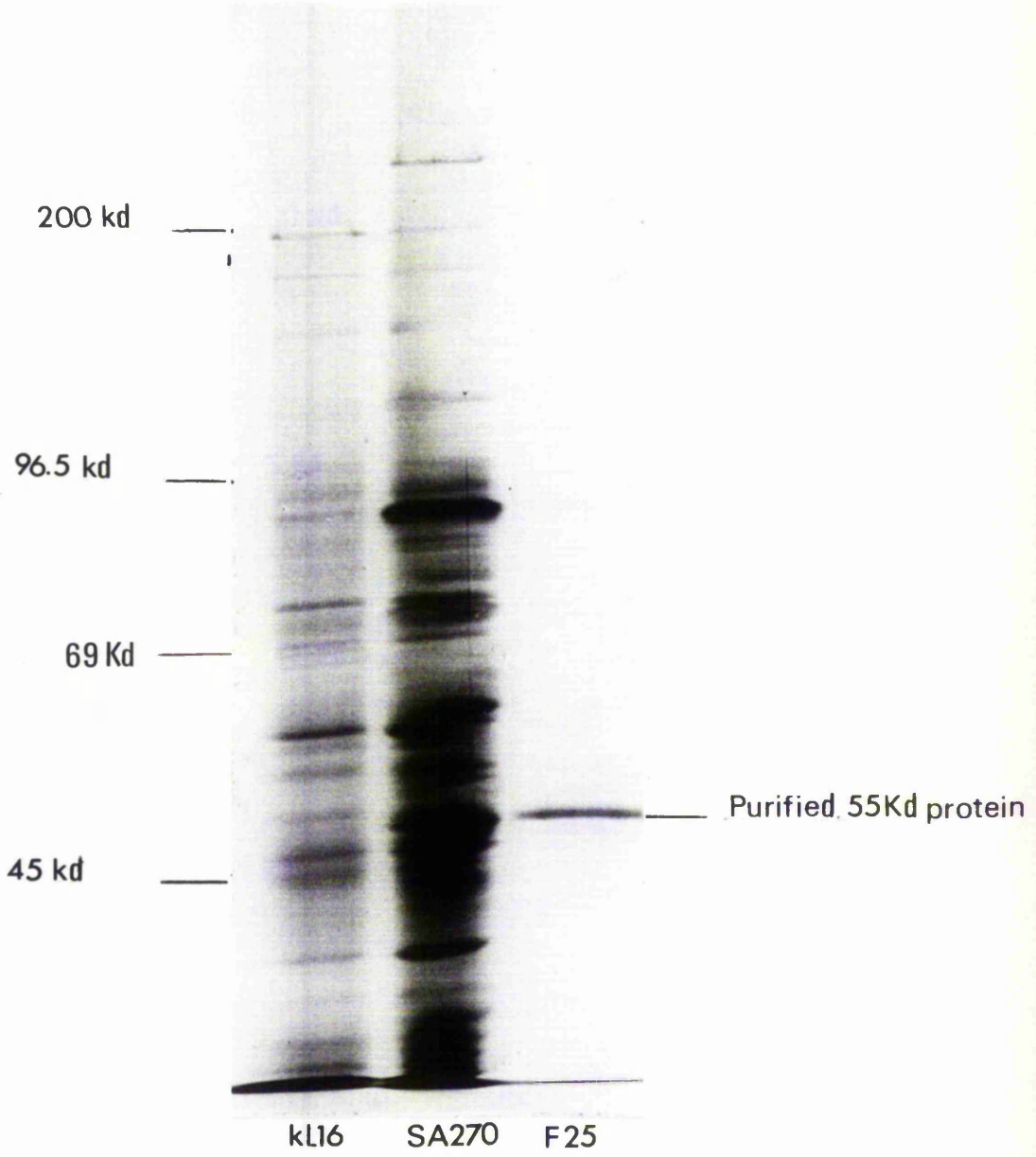
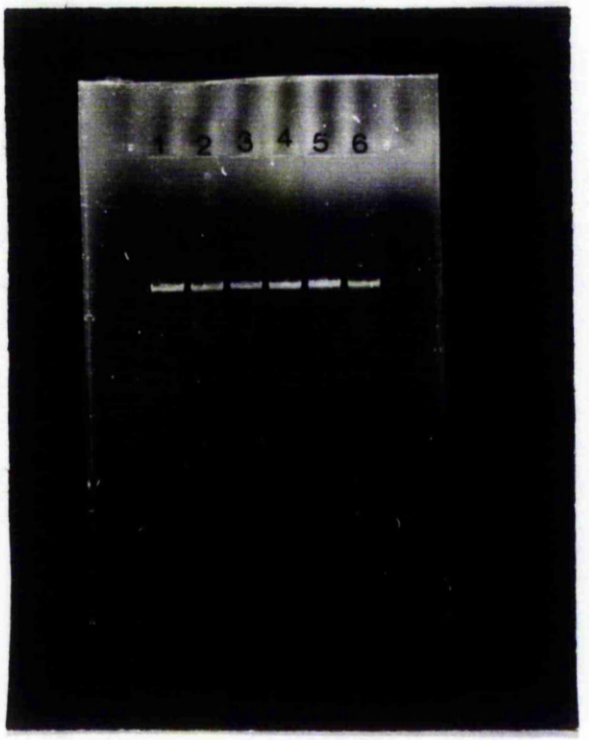


FIG.54. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS SHOWING THE 55Kd PROTEIN IN AN ALMOST PURE FORM.



LANE 1 λ DNA + KL16, 0 MINUTES
2 λ DNA + KL16, 60 MINUTES
3 λ DNA + 55Kd PROTEIN, 0 MINUTES
4 λ DNA + 55Kd PROTEIN, 60 MINUTES
5 λ DNA + 55Kd PROTEIN, 0 MINUTES
6 λ DNA + 55Kd PROTEIN, 60 MINUTES

FIG.55. INCUBATION OF PUVA DAMAGED λ DNA WITH PURIFIED 55Kd PROTEIN FOR 0 AND 60 MINUTES.

7.5 Discussion

Analysis of PUVA-damaged ColE1 plasmid DNA on agarose gels following treatment with total protein extracts from SA270 and KL16 showed that the supercoiled ColE1 molecules formed relaxed open circles at the same rate. If strain SA270 had a more efficient endonuclease activity than wild type KL16, it would have been expected that plasmid relaxation would occur at a greater efficiency with total protein extract from the mutant bacteria. However, there was no obvious increased activity in strain SA270 causing such an effect. Since the same result was obtained using undamaged plasmid DNA treated with protein extract from SA270 and KL16, it was concluded that there was also no difference in activity that was specific for repairing 8-methoxypsoralen crosslinked DNA.

Apart from the formation of relaxed circular DNA, two extra bands were observed below the supercoiled DNA band following incubation of plasmid with total protein extracts. These bands were formed at the same rate following incubation with either the KL16 or SA270 protein extracts and may represent single-stranded or partially digested DNA molecules formed by the action of non-specific exonucleases following relaxation (nicking) of the DNA by endonucleases. It was clear, however, that strain SA270 did not appear to have any increased exonuclease activity in this experiment. A similar result was obtained for undamaged colE1 DNA treated with total protein extracts, suggeting that the proposed exonuclease activity observed was not specific for PUVA-damaged DNA.

In contrast, λ DNA damaged with PUVA was degraded by total protein extract from strain SA270 with greater efficiency than with

total protein extract from KL16. It is proposed that this phenomenon was dependent on the presence of the 55kd protein, since protein extract from the PUVA hyper-sensitive mutant JH110 did not degrade the λ DNA. Protein extracts prepared from DNA repair-deficient mutant derivatives of SA270 showed that digestion of λ DNA was more efficient when the 55kd protein was over-expressed.

The precise role of the 55kd protein in repairing DNA crosslinks remains a matter for hypothesis. λ DNA did not show any degradation following incubation with purified 55kd protein, suggesting that the protein itself does not have any activity. It is possible that the protein has lost activity during the purification procedure. The protein could therefore be a subunit of a protein complex specific for incision of DNA containing crosslinking damage. Considering the phenotype of the *uvrC* mutant derivative of SA270, and the fact that DNA was degraded when incubated with protein extract from this strain, it can be proposed that the 55kd protein may form a complex with the UvrA and UvrB proteins that is specific for mediating incision of DNA containing crosslinks. Against this hypothesis is the fact that no enhanced endonuclease activity was observed when colE1 plasmid DNA was treated with total cell extract, but this could be due to the conformation of the plasmid not allowing strand incision to occur. However, this result cannot be entirely dismissed and perhaps suggests another role, besides endonuclease activity, for the 55kd protein in DNA crosslink repair.

Several possible alternatives can be suggested. Degradation of DNA was only observed when total protein extract from a strain over-expressing the 55kd protein was incubated with λ DNA molecules.

Since λ DNA is a linear molecule with double-strand overlapping ends, this may provide a clue as to the biological role of the 55kd protein. Zhen *et al.* (1986) proposed that *E.coli* may have a recombination-independent mechanism for the repair of DNA crosslinks as the cells used in their experiments were not thought to contain homologous DNA to a psoralen crosslinked plasmid. These authors hypothesised that recombination-independent repair of crosslinks could be mediated by a glycosylase which would remove the DNA damage and produce two apyrimidinic sites (fig. 56). It was also proposed that full fidelity of the repair could be obtained when the pyrimidinic sites were excised (3'→5') and gap-filled in succession, one strand at a time, thereby avoiding double strand scissions. It can therefore be proposed that the 55kd protein may be involved in recombination-independent repair, where it could have any one of a number of roles.

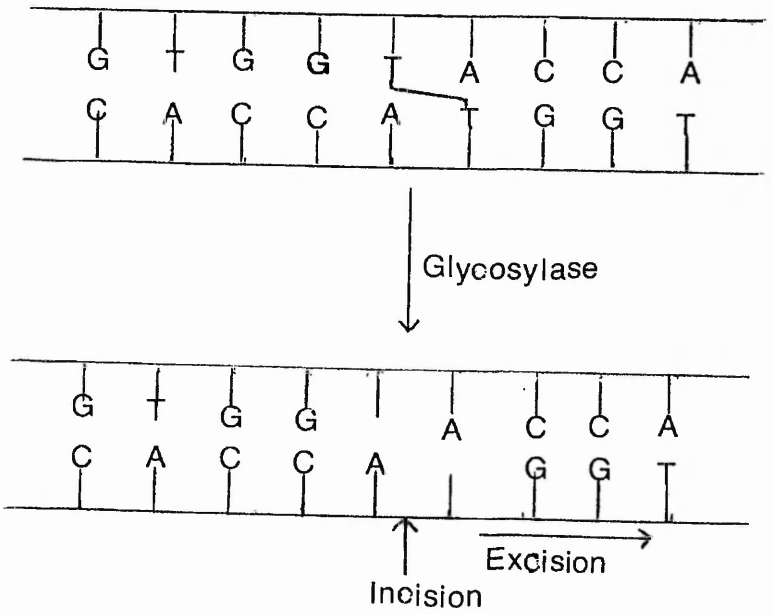


FIG.56. AN ALTERNATIVE PATHWAY FOR REPAIRING DNA CROSSLINKS.

One of these putative roles could be as a DNA unwinding enzyme making crosslinks more accessible to enzymes, such as the glycosylase proposed by Zhen *et al.* (1986), that is able to incise the DNA diadduct. Perhaps λ DNA is degraded because the double strand ends allow the 55kd protein to unwind the DNA in a similar way as the protein product of the *rep* gene (Bridges & Von Wright, 1981). Since plasmid DNA does not have exposed double strand ends the protein may not be able to function in this way. However, the experiments in this Chapter demonstrated that the λ DNA molecules were completely digested when incubated with total protein extract from strains over-expressing the 55kd protein, perhaps suggesting that the protein may play a more critical role in actual removal of DNA damage. It is however, possible that enhanced DNA unwinding may mediate exonuclease activity in damaged or undamaged λ DNA which may account for the phenomenon observed.

At this point it is particularly interesting to note that the *puvR* mutation maps within 0.2 minutes of the *grpE* gene on the *E.coli* chromosome. This gene, comprising 1,582 base pairs, encodes a 25kd heat shock protein (Lipinska *et al.*, 1988) which is inducible by mitomycin C (R. Lloyd, personal communication). The *grpE* gene product is involved in phage λ replication and host DNA synthesis. It is thought to control DnaK protein activity, which appears to function during a prepriming step in DNA replication, a step that involves the transfer of DnaB helicase on to single-stranded DNA near to the origin of replication. The possibility that the 55kd protein could be involved in phage λ DNA processing cannot be ruled out.

It is unlikely that the 55kd protein is the putative glycosylase enzyme required in recombination-independent DNA crosslink repair, since the digestion of undamaged DNA is also more efficient when incubated with total protein extract from SA270. The protein may, however, have endonuclease or exonuclease activity in conjunction with other proteins. As hypothesised by Zhen *et al.* (1986), a glycosylase could remove DNA crosslinking damage and putative endonucleases may then nick the DNA at unknown sites. It is unlikely that the 55kd protein is an endonuclease of this type since no elevated endonuclease activity was observed with purified protein or when total protein extract from SA270 was added to plasmid DNA. Zhen *et al.* (1986) proposed that this endonuclease activity may be independent of the UvrABC endonuclease; indeed a study by Sladek *et al.* (1988) found a Mg^{2+}-independent activity that was able to process psoralen damage under conditions when the UvrABC system would be inoperative. It is interesting to note that degradation of λ DNA by protein extract from SA270 was ATP and Mg^{2+}-independent, possibly suggesting that UvrABC-independent endonucleases may produce incisions in the DNA.

Zhen *et al.* (1986) hypothesised that, following formation of two apyrimidinic sites in the DNA, exonuclease activity was required to remove the secondary damage. The 55kd protein may have a similar exonuclease activity initiated by strand breaks in DNA induced by unidentified endonucleases. It can be hypothesised that this endonuclease activity may not be present in in vitro tests, explaining why ColE1 plasmid DNA is not digested in the same way as

λDNA. With λ DNA, exonuclease activity could be permitted by the presence of double strand ends, so that digestion proceeds whether or not there is DNA damage. Such an exonuclease could have 3'→5' or 5'→3' activity and could be uncontrolled *in vitro* (section 1.7B). It is not clear why the purified 55kd protein cannot act on its own, but this suggests that it is dependent on other proteins for its activity (i.e. in a similar way to the RecBCD proteins). It will clearly be important to elucidate the details of the UvrABC recombination-independent repair mechanism before any further attempts can be made to determine whether the 55kd protein over-expressed in SA270 has a major role in a novel crosslink specific repair pathway.

Chapter 8

FINAL CONCLUSIONS

The primary aim of this project was to study a mutant strain of *E.coli* K12 (SA270) that exhibited hyper-resistance to PUVA-induced DNA damage. Studies using a variety of DNA-damaging agents showed that SA270 was also resistant to other DNA-crosslinking agents besides PUVA, but not to monofunctional DNA damaging agents. It was therefore concluded that the mutant strain had developed a mechanism by which it could repair or protect itself more efficiently than the wild type strain from the lethal effects of crosslink DNA damage. Over-expression of the 55kd protein was shown to play a major role in conferring PUVA hyper-resistance in SA270. This conclusion was supported by the mapping studies which showed that the responsible mutation, located at 57.2 minutes on the *E.coli* chromosome, concomitantly caused constitutive expression of the 55kd protein and conferred PUVA hyper-resistance.

From the evidence presented in this thesis it was concluded that the 55kd protein was not involved in regulating the uptake of psoralen or other crosslinking agents into the bacterial cell. It is therefore proposed that the protein is involved in enhancing the efficiency of a DNA repair pathway. Complementation analysis showed that the wild type allele was dominant *in trans* in a partial diploid. This implied that expression of the 55kd protein was negatively regulated. Ahmad & Holland (1985) proposed that the RecA protein had a regulatory effect on the expression of the 55kd protein and hypothesised that the 55kd protein was likely to be inducible by DNA crosslinking damage. The results in this thesis were, however, unable to demonstrate induction of the protein following exposure to mitomycin C. Perhaps induction of the protein is not directly determined by the crosslinking damage, but by some

indirect response of the cell to such damage. A similar indirect response has been implicated in the repair of oxidative DNA damage (Demple, 1990). It was, however, clearly apparent from the results in this thesis that the *recF* gene product is involved in regulating expression of the 55kd protein. The *recF* mutant derivative of SA270 was sensitive to PUVA and expression of the 55kd protein returned to what appeared to be wild type basal levels. It was not clear whether the *recA* gene product was also involved in regulating expression of the 55kd protein, since cotransduction may have taken place between the *recA* gene and *puvR*$^+$. However, in an experiment showing induction of the RecA protein, no induction of the 55kd protein was detected, suggesting that the *recA* gene is not directly involved in controlling expression of the *puvA* gene. Further studies will be required to finally elucidate the mechanism by which expression of the 55kd protein is regulated.

From the sensitivity of the *recF, recN, recA, uvrA, uvrC* and *recBC* mutant bacterial strains to PUVA, it can be postulated that both excision repair and post replication recombination repair operate to repair psoralen adducts in DNA. From the results in this thesis, it can be proposed that, in wild type *E.coli*, psoralen-induced DNA damage is repaired by three major pathways:- (i) *uvrABC*-mediated excision repair; (ii) *recBC*-dependent; and (iii) *recF*-dependent post replication recombination repair. In addition to these mechanisms, it is proposed that there may exist a further pathway, perhaps showing glycosylase activity, which may involve the 55kd protein in an, as yet, unknown role. It is suggested that this pathway is specific for repairing DNA crosslinks. Experiments with λDNA suggested that strain SA270 may

have an enhanced exonuclease activity compared to wild type KL16. It is interesting to note that nuclease activity is still elevated in a *uvrC* mutant derivative of SA270. Alternatively, it can be proposed that the 55kd protein might form part of the UvrAB protein complex, thereby rendering its endonuclease activity specific for DNA crosslinks. In order to clarify this point it would be necessary to carry out experiments using the 55kd protein, which shows no endonuclease ability on its own, in association with other gene products such as the UvrA and UvrB proteins.

The results described in this thesis have considerably expanded knowledge of the way in which the mutant strain SA270 has developed a crosslink specific hyper-resistant phenotype. Many interesting questions have been answered, but at the same time the complexity of the system has also been demonstrated. It would be interesting to sequence the *puv* gene region of the *E.coli* chromosome and study the regulation and transcription of the *puvA* gene in a more detailed way. It would also be particularly important to study further the biological role of the 55kd protein and decipher its relationship with PUVA-damaged DNA at a molecular level by elucidating possible protein-DNA interactions. In the long term, a search for an analogous gene and gene product in eukaryotic cells might have subsequent implications for PUVA therapy in humans.

There is still much to be learned about DNA repair mechanisms in *E.coli* and higher organisms. Current knowledge indicates that *E.coli* has evolved many sophisticated and inter-linked repair processes, one of which involves the 55kd protein, in order to protect its genetic integrity and ensure its survival when exposed to DNA crosslinking agents. The work described in this thesis has

provided an insight into the complexities of the repair of PUVA induced DNA damage. Studies of this type although carried out in prokaryotes, can add considerably to an understanding of molecular mechanisms for mammalian DNA repair. Already human excision repair genes ERCC-1, 3 and 6 have been cloned (Höeijmakers*et al.*, 1990) and their protein products been demonstrated to have striking evolutionary similarities with the RAD proteins of yeast and, in part, with the UvrA and UvrC proteins of *E.coli*. Wood (1990) reported that protein extracts from human cell lines can perform repair synthesis in plasmid DNA damaged with PUVA. Thus, when the purified *E.coli* UvrABC proteins were incubated with PUVA-damaged plasmid DNA, extracts from human xeroderma pigmentosum cells were able to complete repair. (Xeroderma pigmentosum syndrome is a disease where the ability to tolerate DNA damage is lost.) Clearly the work carried out on excision repair in *E.coli* may enable the basis of the condition to be elucidated. Similarly, the mutant strain SA270 still has much to offer towards gaining an understanding of the mechanisms involved in the repair of PUVA induced DNA damage. It is hoped that the work described in this thesis will provide a secure basis for further studies of this complex repair system.

Addendum

Following completion of the work described in this thesis, additional experiments performed by other workers (S.I.Ahmad, personal communication) have indicated that the 55kd protein is inducible following treatment with MMC at high concentrations and prolonged subsequent incubation (up to 5 hours).

Appendix I

CALCULATIONS

This appendix contains details of the calculations performed to determine certain parameters referred to in the main section of this thesis.

1. Calculation of intensity of ultraviolet lamps used in this project.

The intensities of the near ultraviolet and and far ultraviolet lamps used in this study were calculated using a radiometer UVX-25 (UV-Products Ltd). The reading on the meter was measured in mW/cm^2.

Example of calculation:

the meter reading was 9.2 mW/cm^2

to convert this into mW/m^2

$9.2 \times 100 \times 100 = 92000 mW/m^2$

$1mW = \frac{1}{1000} W$

Therefore 92000 mW/m^2 = 92 W/m^2

1 watt = 1 joule/sec

Therefore 92 w/m^2 = 92 $J/sec/m^2$

Therefore in 10 secs the ultraviolet lamp emitted

920 J/m^2

2. Calculation of map distance using cotransduction frequencies and the formula devised by Wu (1966).

Cotransduction frequency = $(1 - d/L)^3$

where

d = distance between the selected and unselected markers in minutes.

L = represents the size of the transducing DNA fragment in minutes. The value of 2 is used.

3. Calculation of a confidence interval for each cotransduction frequency obtained.

Considering each recombinant as an independent trial (total of n independent trials) with a probability p of "success" (i.e. cotransfer of unselected marker) and a probability of (1-p) of "failure" (i.e. no cotransfer) the confidence interval for each postulated map position was calculated.

If $n > 30$, the number of "successes", x, has a distribution which is approximately normal. If x "successes" are observed in n trials (where $n > 30$), the estimated standard error of the observed sample proportion ($\hat{p} = x/n$) is therefore $\sqrt{[\hat{p}(1-\hat{p})/n]}$ and a confidence interval for p is $\hat{p} \pm z(\alpha)\sqrt{[\hat{p}(1-\hat{p})/n]}$, where $\pm z(\alpha)$ are the α% points of the normal distribution curve.

Example.

In the experiment designed to map the *puvR* gene mutation, the following data were obtained.

No. of transductants tested(recombinants) = 129

No of *puvR*$^-$*tyrA*$^+$ transductants = 53

Therefore observed $p = \frac{53}{129} = 0.41$

The estimated standard error observed

$= \sqrt{[\hat{p}(1-\hat{p})/n]}$

$= \sqrt{[0.41(1-0.41)/129]}$

$= 0.043$

$\pm z(\alpha) = 1.96$ for 95% confidence and therefore a 95% confidence interval for $p = \hat{p} \pm z(\alpha)\sqrt{[\hat{p}(1-\hat{p})/n]} = p \pm 1.96 \times 0.043 = p \pm 0.084$.

Therefore the cotransduction frequency between *tyrA*$^+$ and *puvR* = 41% ± 8.4%.

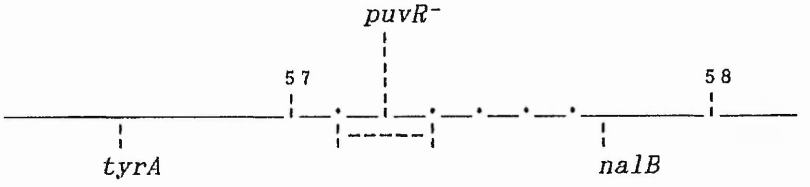
This can be converted to map distance using the formula of Wu(1966).

A cotransduction frequency of 41% ≡ map distance of 0.51 mins.

A cotransduction frequency of 41% + 8.4% = 49.4% which is equal to a map distance of 0.41 mins.

A cotransduction frequency of 41 - 8.4% = 32.6% which is equal to a map distance of 0.62 mins.

Therefore the position of the *puvR* gene mutation on the *E.coli* chromosome was calculated to be 57.2 mins ± 0.1 min.



4. Calculations used in radio-labelled 8-methoxypsoralen uptake experiments and radio-labelling of λ DNA

a. 8-methoxypsoralen is added to 1ml of cells at a final concentration of 10μM (molecular mass of 8-methoxypsoralen = 214).

10μl ^{3}H-labelled stock solution (Amersham), containing 25.68ng ^{3}H-8-methoxypsoralen, was added to 90μl of a solution containing 0.47 mg unlabelled 8-methoxypsoralen/ml. The 100μl mixture therefore contained a total of 42.325μg, or 197.78nmols, of 8-methoxypsoralen.

5μl of this stock was added to 1ml of cells (1mg dry weight resuspended in minimal medium) in the incubation chamber. This gave a final concentration of~10μM 8-methoxypsoralen.

In a control experiment 5μl of stock (10μl labelled plus 90 μl of unlabelled 8-methoxypsoralen) was added to 1ml minimal medium. The amount of radioactivity in 100μl of this stock was determined and a mean value of 29,000 cpm recorded.

In 5μl of stock there was 2.11μg of 8-methoxypsoralen equivalent to 10nmols.
Therefore the 1ml incubation mixture also contained 10nmols of 8-methoxypsoralen and in a 100μl sample (incubation mixture) there was 1nmol or 0.211μg of 8-methoxypsoralen.

Since each 100μl sample generated 29,000 cpm ≡ 1nmol, the specific activity = 29,000 cpm/nmol.

b. A stock solution of ^{3}H-8-methoxypsoralen plus unlabelled 8-methoxypsoralen was prepared as described in 4.a. 5µl of this stock was added to 1ml λDNA incubation mixture prior to near ultraviolet light irradiation to give a final concentration of 10µM 8-methoxypsoralen.

5. Calculation for FPLC

Fig. 57 shows a typical trace produced by FPLC showing the elution peaks for proteins of known molecular mass. From the data the retention time (k_{av}) of each protein was calculated.

(a) Calculated k_{av} for proteins of known molecular mass

Bovine serum albumin (BSA) (66kd)

V_o = 0.2 mls x 33.34

= 6.668 mls

V_t = 24 mls

V_e = 55.37 x 0.2 = 11.074

$$k_{av} = \frac{11.074 - 6.668}{24 - 6.668} = 0.254$$

Ovalbumin (43kd)

V_o = 6.668

V_t = 24

V_e = 11.845

$$k_{av} = \frac{11.854 - 6.668}{24 - 6.668} = 0.299$$

Cytochrome C (12.5kd)

V_o = 6.668

V_t = 24

V_e = 17.69

$$k_{av} = \frac{17.69 - 6.668}{24 - 6.668} = 0.635$$

A graph of $\log_{10}$ molecular mass against k_{av} was constructed (fig. 58). From the graph the molecular mass of an unknown protein with a known retention time could be estimated.

(b) Fig 59 shows a laboratory record showing the parameters used in the experiment described in section 2.21.

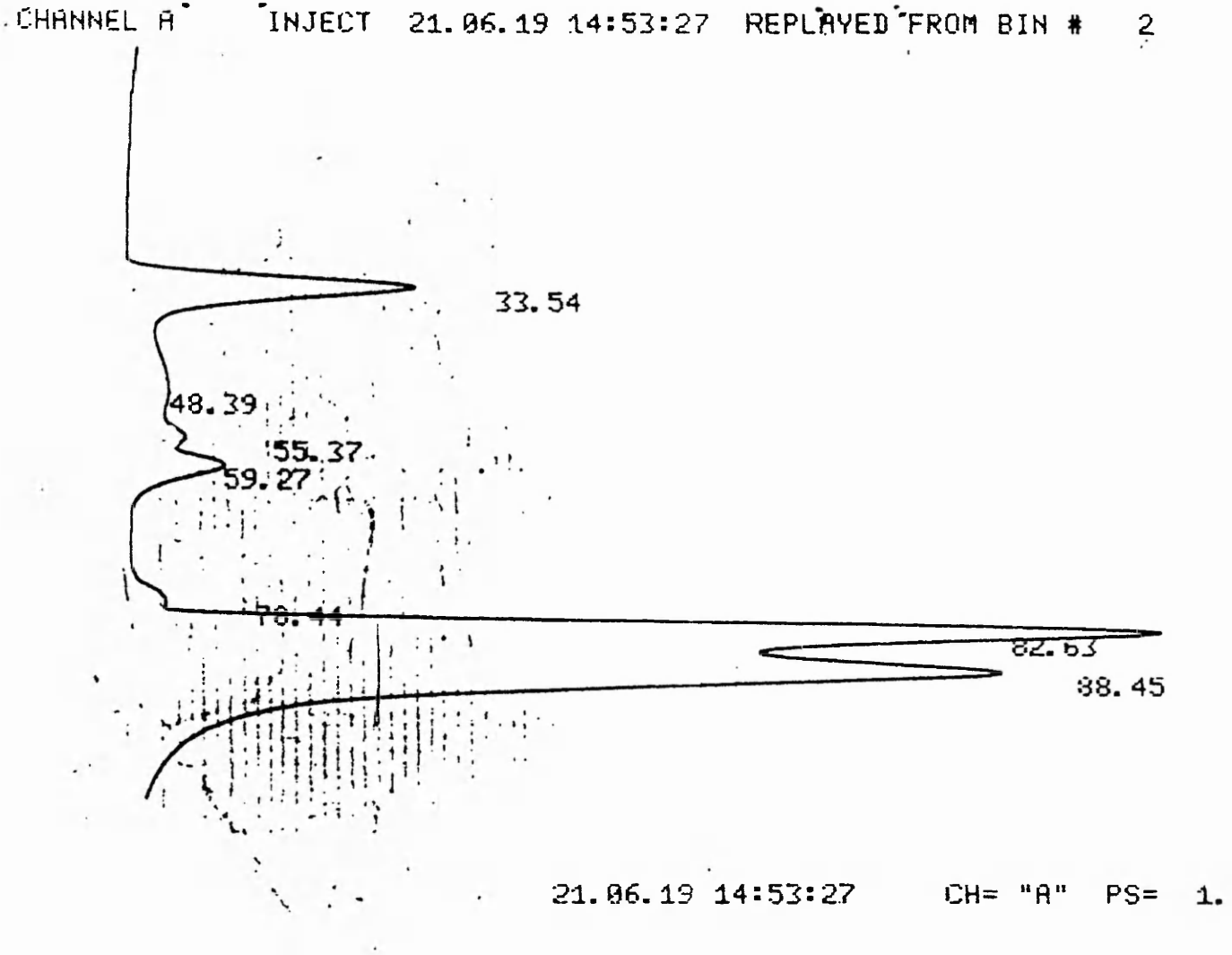


FIG.57. FPLC ELUTION PROFILE OF KNOWN MOLECULAR MASS PROTEINS.

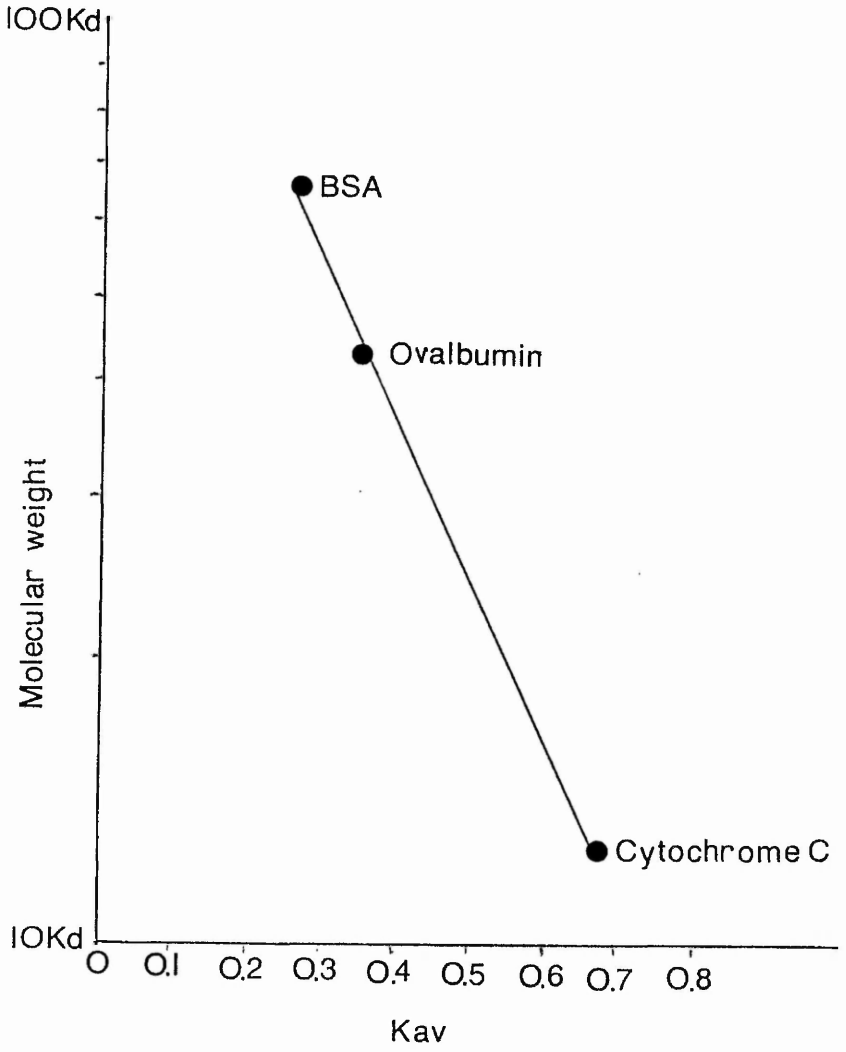


FIG.58. CALIBRATION CURVE FOR DETERMINING THE MOLECULAR MASS OF UNKNOWN PROTEINS SEPARATED BY FPLC.

LABORATORY
RECORD

OPERATOR [illegible] DATE 3.7.89

SAMPLE Pooled fractions from Gel filtration. —

GEL	COLUMN SIZE	TEMP.
Mono Q	HR 5/5	Ambient. °C

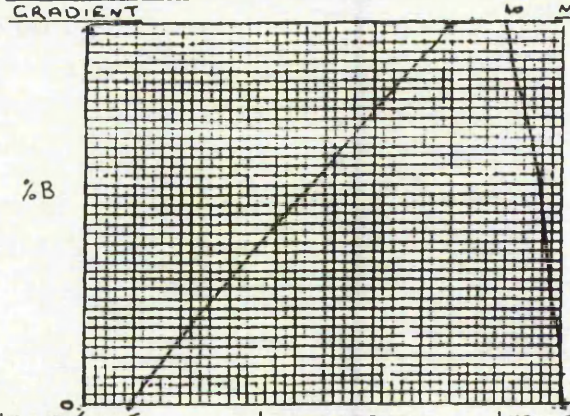
ELUENT A: 50mM Tris pH 7.5
B: " " " + 0.5M NaCl

FLOW	PRESSURE
1.0 (ml/min)	1.5 psi bar (MPa)

SAMPLE SIZE	SAMPLE VOLUME (LOADING)
89 mg/ml µg/ml	200 µl ml

DETECTOR	SENSITIVITY	CHART SPEED
280nm	0.05	cm/min mm/hr

GRADIENT



METHOD

0.5ml samples taken.

TIME/VOLS	INSTRUCTION	VALUE	NOTE
0.0	conc % B	0.0	
0.0	ml/min	1.0	
0.0	cm/min	0.5	
0.0	port set	6.1	
0.0	valve position	1.2	
0.0	monitor	1.0	
0.0	Level %	0.0	
0.0	min/mark	2.0	
0.0	integrate	1.0	
5.0	conc % B	0.0	
35.0	conc % B	100	
40.0	conc % B	100	
45.00	conc % B	0.0	
50.00	" "	0.0	
5.000	port set	6.0	
50.00	valve pos	1.1	
50.00	monitor	0.0	
50.00	integrate	0.0	

FIG.59. LABORATORY RECORD OF EXPERIMENT 2.21 (ii).

Appendix II

Study of an *E.coli* mutant

hyper-resistant to a number of

DNA damaging agents

1. Background

During this project studies were also carried out on a mutant of *E.coli* that was hyper-resistant to far ultraviolet light and a number of other DNA damaging agents. These investigations were peripheral to the main subject of this thesis, but are included in this Appendix for the benefit of the interested reader.

Ahmad *et al.* (1980) isolated a far ultraviolet light hyper-resistant strain, SA236, and showed that it was also resistant to thymineless death, mitomycin C, nalidixic acid and fluorouracil compared with its parent wild type strain KL14. Strain SA236 was not resistant to hydrogen peroxide or chloramphenicol. It was proposed that, since SA236 had enhanced resistance against various DNA damaging agents, the mutant cells might be able to repair DNA damage more efficiently than the wild type strain KL14. Ahmad *et al.* (1980) assayed for activity of six repair enzymes: DNA polymerase I, exonuclease I, exonuclease II, exonuclease III, endonuclease I and DNA ligase in both the mutant and wild type strain. In SA236 DNA polymerase activity was increased 35 fold and endonuclease I activity was increased 12 fold compared with wild type KL14. It was suggested that the large increase in expression of these enzymes might result in a more efficient repair mechanism for damage caused by a variety of agents. As already discussed in Chapter 1, the DNA polymerase I protein is involved in replacing missing bases in DNA strands during excision repair, post replication recombination repair and SOS repair. Endonuclease I, a major cellular protein, is able to degrade DNA excised from the damaged molecule. Consequently, free bases can be incorporated back into newly synthesised DNA.

In a more recent study, Ahmad & Van Sluis (1987) further examined the mutant SA236 to elucidate why the strain had become hyper-resistant to far ultraviolet light. Genetic analysis showed that SA236 carried at least two mutational sites, one conferring far ultraviolet hyper-resistance on the cell (designated *uvh*) and the other conferring far ultraviolet hyper-sensitivity (designated *uvs*) on the cell. It was proposed, as a result of experiments carried out to study the regulation of the *polI* gene, that the gene product of the *uvh* gene was responsible for repression of the *polI* operon in *E.coli*. A mutation in the *uvh* gene resulted in either inactivation or reduced activity of the gene product, with a consequent increase in the levels of polymerase I. Ahmad & Van Sluis (1987) introduced a recombinant plasmid carrying the DNA sequence from the *polI* promotor region linked to the *galK* structural gene into the mutant strain SA236. As a result of this experiment, the levels of galactokinase were higher in SA236 than those observed in wild type KL14. In addition, it was proposed that the *polI* gene may have two putative promoter regions and two operator regions which are affected by different repressor proteins.It was elucidated that one promoter region was repressed by the DnaA protein, whose function is to initiate a new round of replication in *E.coli*, since a plasmid with a *dna* box deleted showed no repression ofthe *polI* gene in a wild type strain. The second promoter region was presumably repressed by the gene product of the *uvh* gene and, as a result of a mutation in this gene, polymerase I is expressed constitutively. Other data (Ahmad & Van Sluis, 1987) showed that the mutation in the gene (*uvs*) conferring far ultraviolet hyper-sensitivity on the strain had no effect on the activity of the *polI*

promoters, and it was proposed, therefore, that the *uvs* mutation in SA236 had no significant role in regulating the *polI* gene.

In the study outlined in this Appendix, experiments were carried out to confirm some of the results obtained for strain SA236 with regard to its resistance to other DNA damaging agents. Experiments were also performed to map the mutational sites responsible for the far ultraviolet hyper-resistant and hyper-sensitive phenotypes of SA236.

2. Results

Strains SA236 and KL14 were tested against a range of DNA damaging agents using the disc sensitivity method (section 2.4). Treatments involving ultraviolet light irradiation were carried out using the plate exposure method (section 2.4). As in the main body of this thesis, a range of DNA damaging agents causing both single-strand and crosslinking damage were chosen for the tests.

A. Sensitivity of strains SA236 and KL14 towards DNA damaging agents.

Table 11 shows that strain SA236 was more resistant to mitomycin C, hydrogen peroxide and fluorouracil than its wild type parent strain KL14. Survival curves were produced for strains SA236 and Kl14 following exposure to mitomycin C and hydrogen peroxide to confirm the disc sensitivity results in a quantitative manner (figs. 60 & 61). SA236 was no more resistant to near ultraviolet light than KL14 in the given dose (fig. 62), but when exposed to near ultraviolet light plus a low concentration of hydrogen peroxide, the mutant strain was more resistant than its parent strain (fig. 63).

The far ultraviolet light hyper-resistant phenotype of SA236 was confirmed by comparing its survival curve with that of KL14 (fig. 64): resistance to 8-methoxypsoralen plus near ultraviolet light was also determined (fig. 65). The results showed that the mutant strain was more resistant to PUVA than its parent strain KL14.

Table 11

Sensitivity of SA236 and KL14 towards DNA damaging agents

DNA damaging agent	Mean zone of inhibition (mm)	
	SA236	KL14
Mitomycin C (50µg/ml)	25	32
Hydrogen peroxide	14	17
Fluorouracil (1mg/ml)	18	21

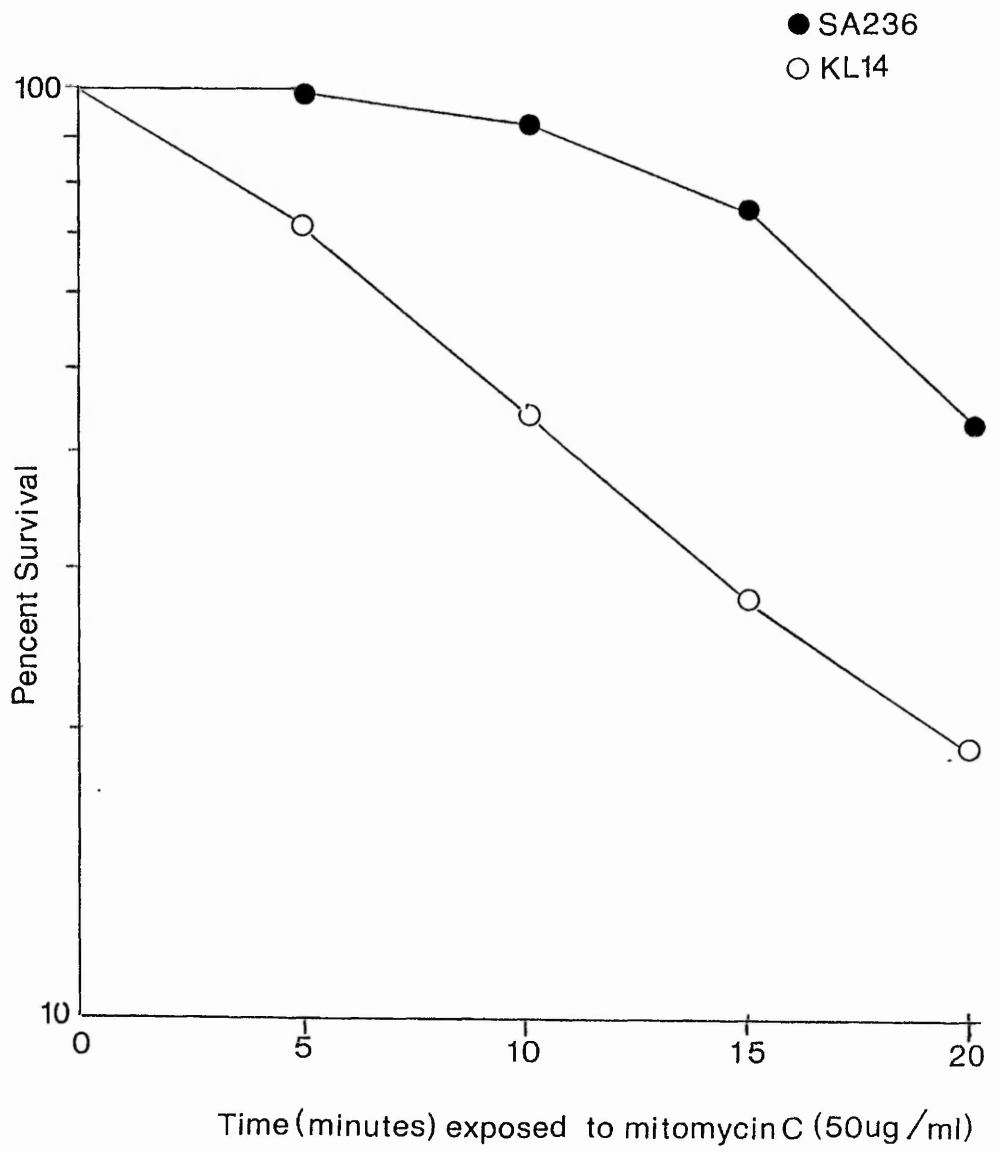


FIG.60. BACTERIAL KILL CURVES OF SA236 AND KL14 IN THE PRESENCE OF MITOMYCIN C.

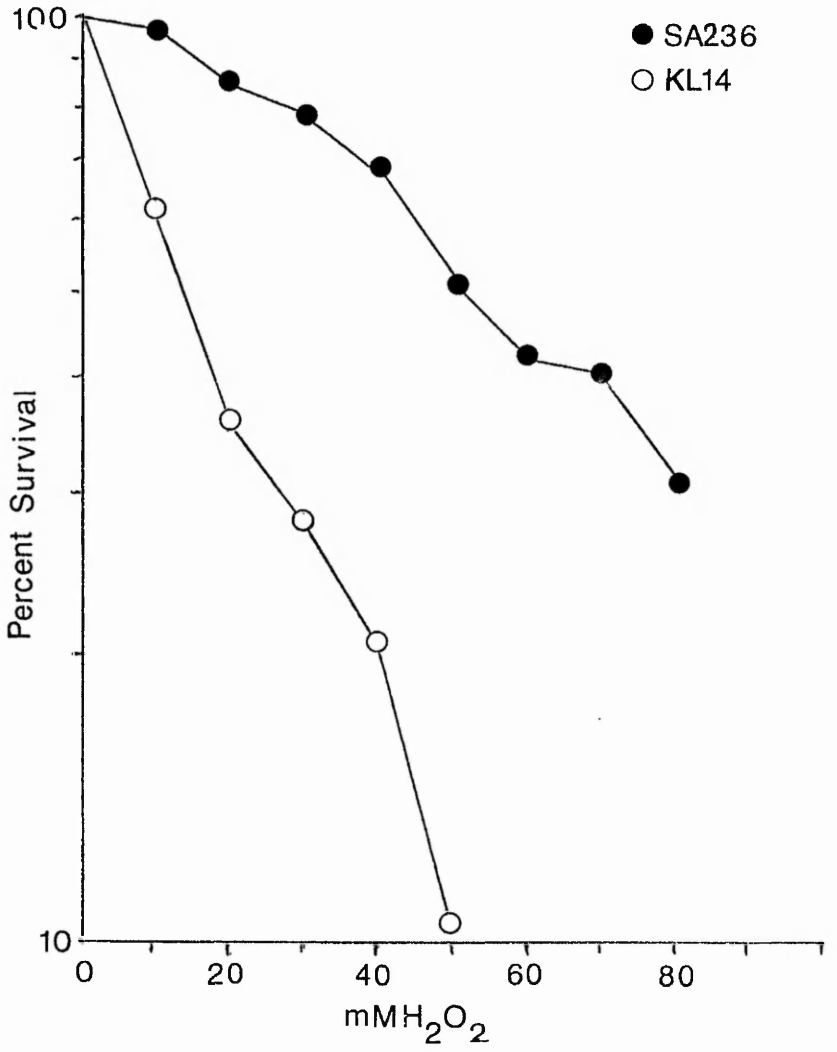


FIG.61. BACTERIAL KILL CURVES OF SA236 AND KL14 IN THE PRESENCE OF HYDROGEN PEROXIDE.

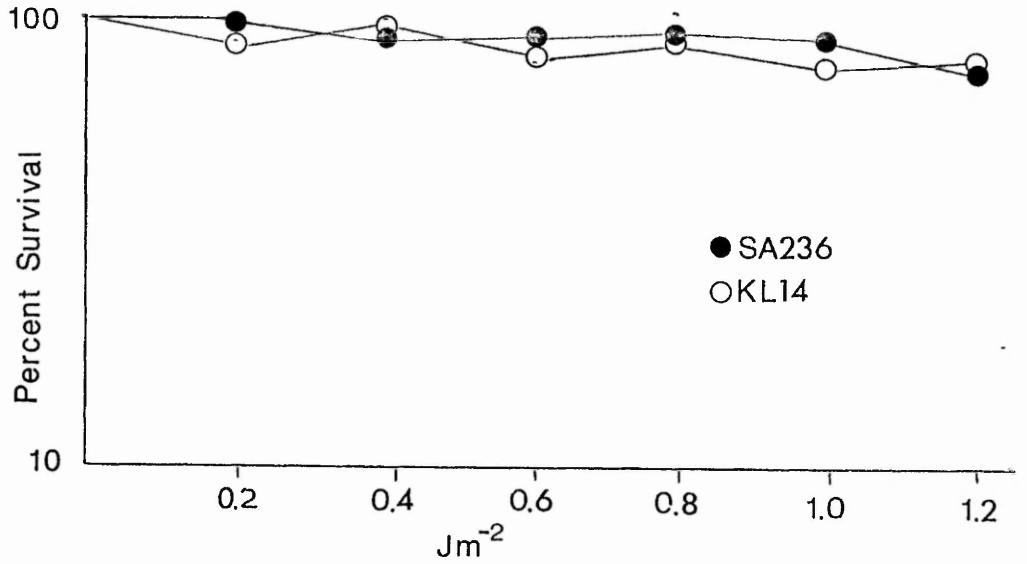


FIG. 62. BACTERIAL KILL CURVES OF SA236 AND KL14 IN THE PRESENCE OF NEAR ULTRAVIOLET LIGHT.

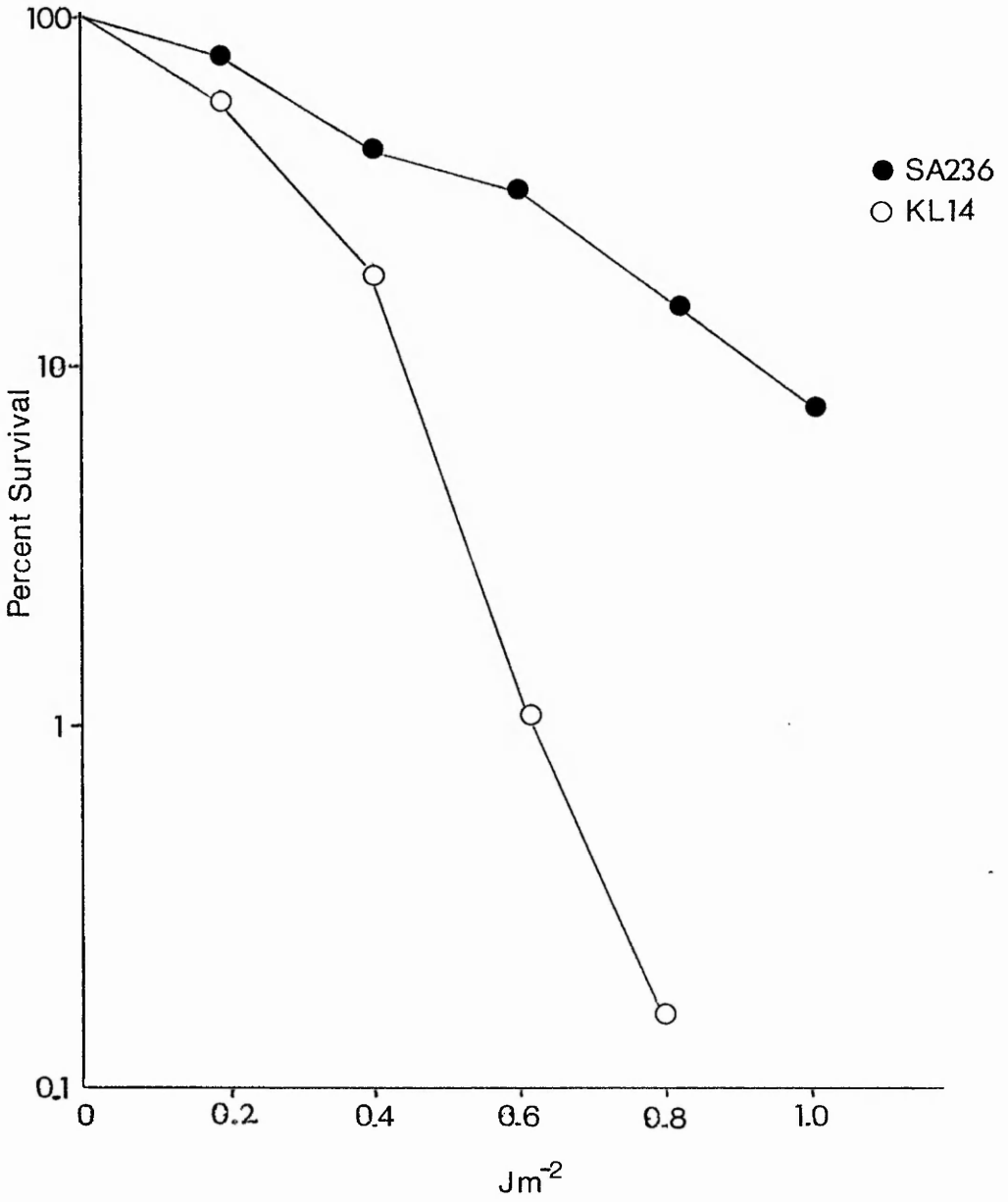


FIG. 63. BACTERIAL KILL CURVES OF SA236 AND KL14 IN THE PRESENCE OF HYDROGEN PEROXIDE PLUS NEAR ULTRAVIOLET LIGHT.

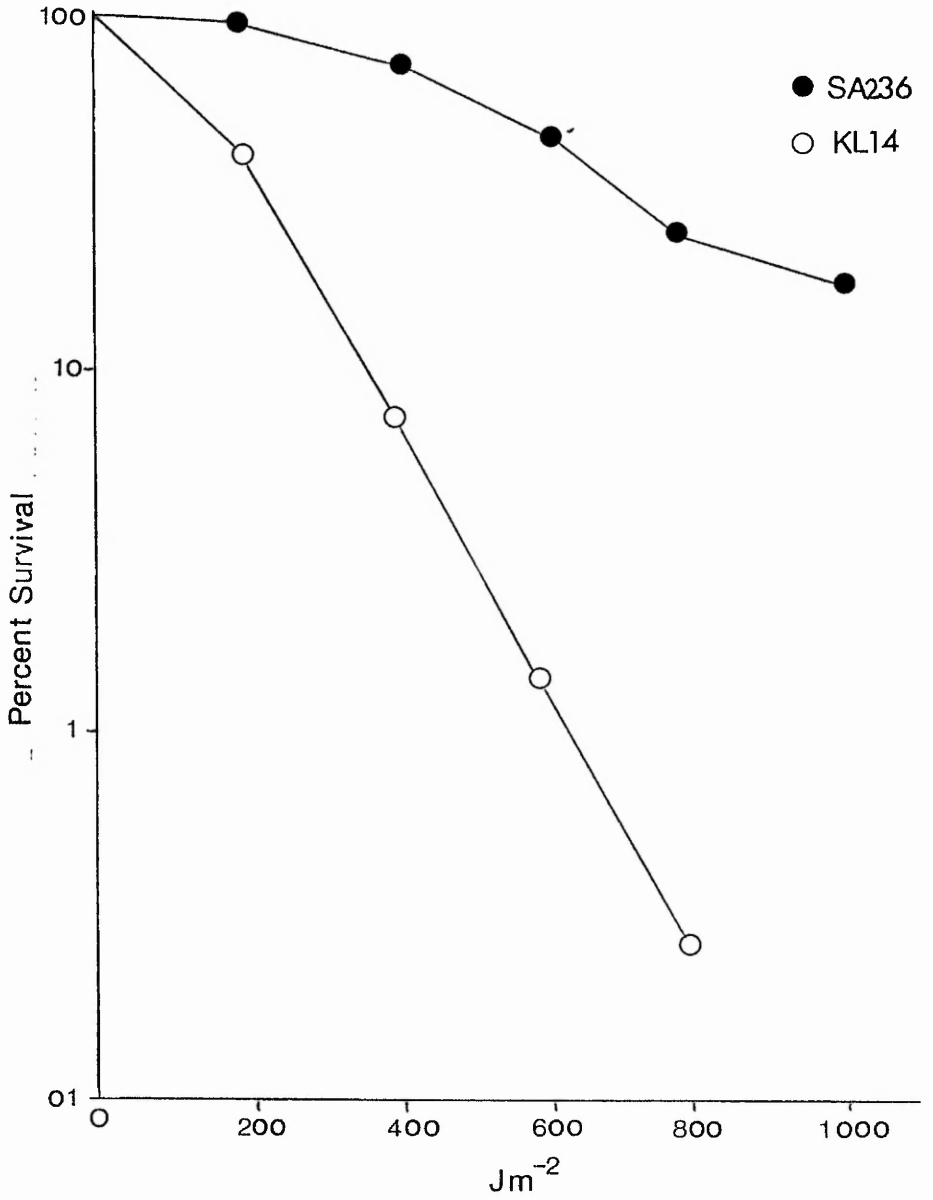


FIG.64. BACTERIAL KILL CURVES OF SA236 AND KL14 IN THE PRESENCE OF FAR ULTRAVIOLET LIGHT.

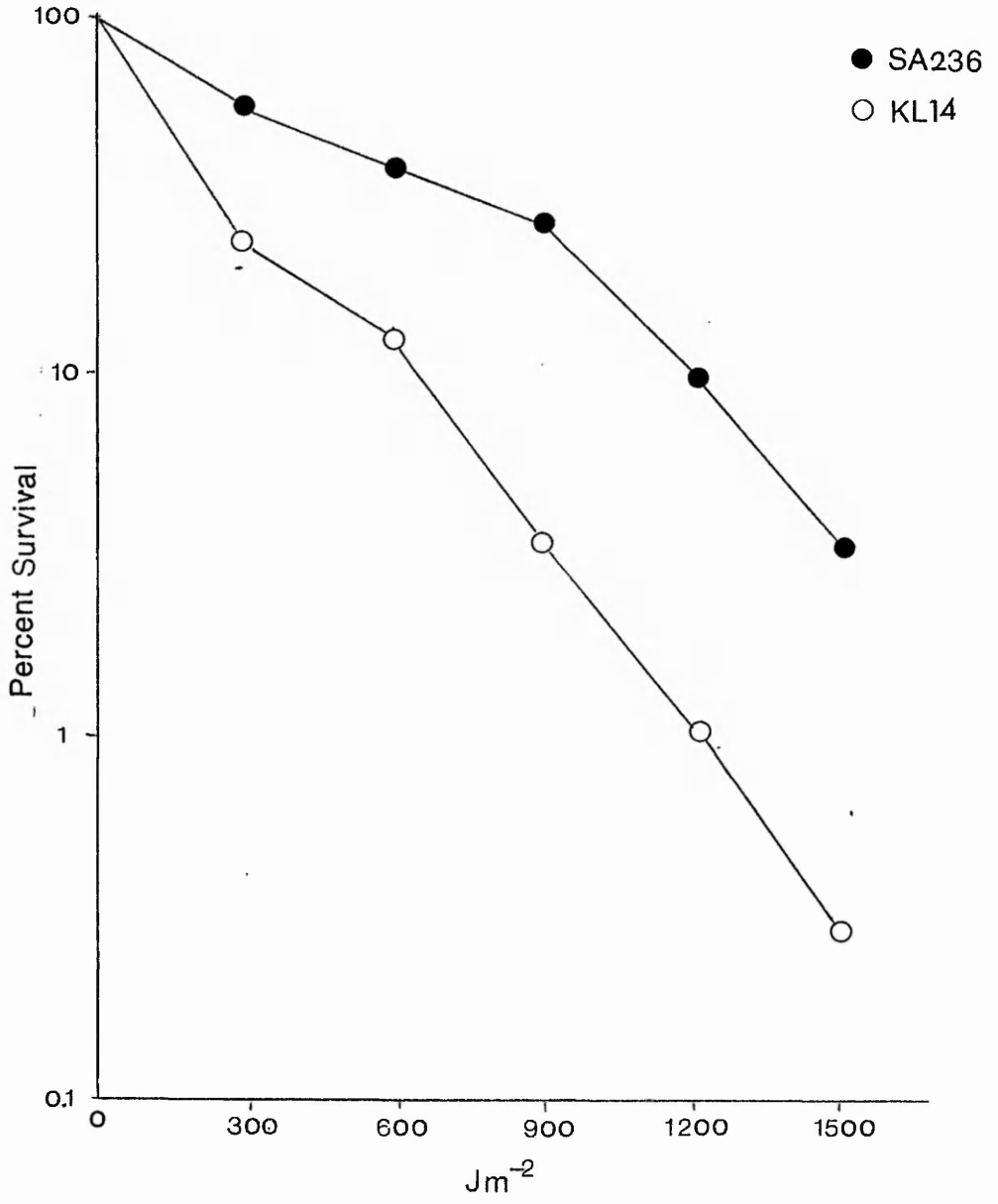


FIG.65. BACTERIAL KILL CURVES OF SA236 AND KL14 IN THE PRESENCE OF PUVA.

B. SDS-Polyacrylamide gel electrophoresis of total cell extracts from SA236 and KL14.

Total protein extracts were prepared from strains SA236 and KL14 as described in section 2.6. The proteins were electrophoresed on a 10% SDS-Polyacrylamide gel for 5 hours at 30mA constant current. From the results obtained (fig. 66), it was observed that a protein of approximately 23kd molecular mass had reduced expression in the SA236 protein extract compared to wild type levels of expression in KL14. It is interesting to note that this protein is of a similar molecular weight to the LexA protein and that other workers have observed this phenomenon in strain SA236 (S.I. Ahmad, personal communication).

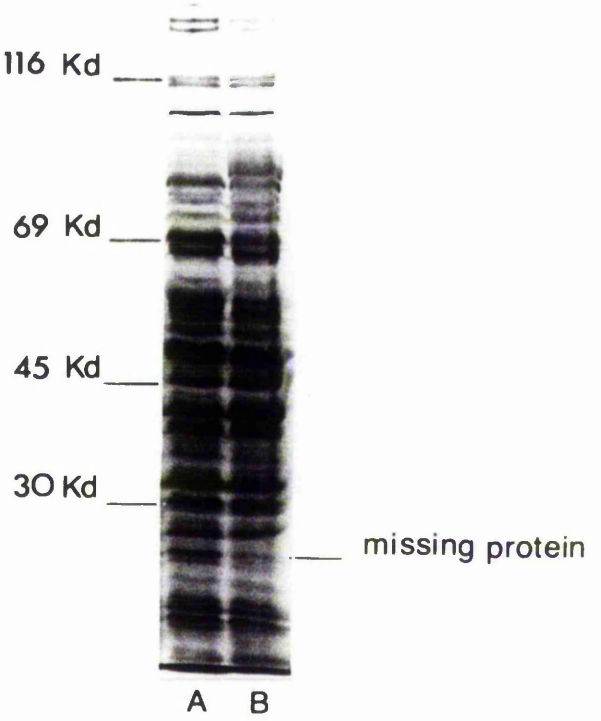


FIG.66. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TOTAL PROTEIN EXTRACTS OF A. KL14 AND B. SA236.

C. Fine genetic mapping of the *uvh* and the *uvs* genes using P1 transduction.

Preliminary experiments have shown that both of these mutational loci were cotransducible with the *argECBH* operon and were therefore located in the 88 to 90 minute region on the *E.coli* chromosome. (Ahmad & Van Sluis, 1987). Using P1 transduction the mutational loci were precisely mapped.

(i) P1 transduction using SA236 as a donor strain and AT753 as a recipient strain.

Phage P1 was grown on SA236 (*uvh, metB*$^+$, *argE*$^+$) and used to transduce AT753 (*uvh*$^+$, *metB, argE*) selecting for *metB*$^+$ colonies. The transductants were tested for their *arg* and far ultraviolet phenotypes. Data showed that the cotransduction frequency between *argE* and *metB* was 30% ± 9.4% and between *metB* and *uvh* was 11% ± 6.4%. Using the formula of Wu (1966), the *uvh* mutation was located at 90.04 ± 0.16 minutes on the *E.coli* chromosome. Cotransduction of the *uvs* allele from SA236 was not taken into account.

(ii) P1 transduction using SA236 (rifampicin-R) as a donor and AB2569 as a recipient.

Phage P1 was grown on a rifampicin-resistant derivative of SA236 (*rpoB, uvh, metA*$^+$) and used to transduce SA2569 (*rpoB*$^+$, *uvh*$^+$, *metA*), selecting for *met*$^+$ colonies. The transductants were tested for their resistance to rifampicin and far ultraviolet light phenotype. Data showed that the cotransduction frequency between *rpoB* and *metA* was 35% ± 8.9% and between *metA* and *uvh* was 32% ± 8.7%. Using the formula of Wu (1966) the *uvh* mutation was located at 89.8 ± 0.1 minutes on the *E.coli* chromosome.

It has been suggested by S.I. Ahmad (personal communication), that the *uvs* mutation maps at approximately 88.5 minutes on the *E.coli* chromosome. The following transduction experiment was therefore carried out to map its precise location.

(iii) P1 transduction using SA305 (*uvs*) as a donor and SA273 as a recipient.

Phage P1 was grown on SA305 (*uvs*, *metB*, *argB*$^+$) and used to transduce SA273 (*uvs*$^+$, *metB*$^+$, *argB*) selecting for *arg*$^+$ colonies. Transductants were tested for their *met* phenotype and for their far ultraviolet light phenotype. Data showed that the cotransduction frequency between *argB* and *metB* was 35% ± 9.3% and between *argB* and *uvs* was 40% ± 9.6%. Using the formula of Wu (1966) the position of the *uvs* mutation was located at 89.1 ± 0.1 minutes on the *E.coli* chromosome.

Data from the cotransduction experiments is summarised in table 12 and postulated map positions are shown in fig. 67.

Table 12

Fine structure genetic mapping of the *uvh* and *uvs* genes using P1 transduction

Results of typical transduction experiments to map the *uvh* and *uvs* genes. See text for details of each experiment.

A.

			Transduction Data			
			argE$^+$	*argE*$^-$	*argE*$^+$	*argE*$^-$
Donor	Recipient	*metB*$^+$	*uvh*$^-$	*uvh*$^-$	*uvh*$^+$	*uvh*$^+$
SA270	AT753	90	4	6	23	57

Cotransduction frequency between *metB* and *argE* = 30%±9.4% (95% CI)

Cotransduction frequency between *metB* and *uvh* = 11%±6.4% (95% CI)

B.

			Transduction Data			
			rpo$^-$	*rpo*$^+$	*rpo*$^-$	*rpo*$^+$
Donor	Recipient	*metA*$^+$	*uvh*$^-$	*uvh*$^-$	*uvh*$^+$	*uvh*$^+$
SA236	AB2569	110	12	24	27	4

Cotransduction frequency between *metA* and *rpo* = 35%±8.9% (95% CI)

Cotransduction frequency between *metA* and *uvh* = 32%±8.7% (95% CI)

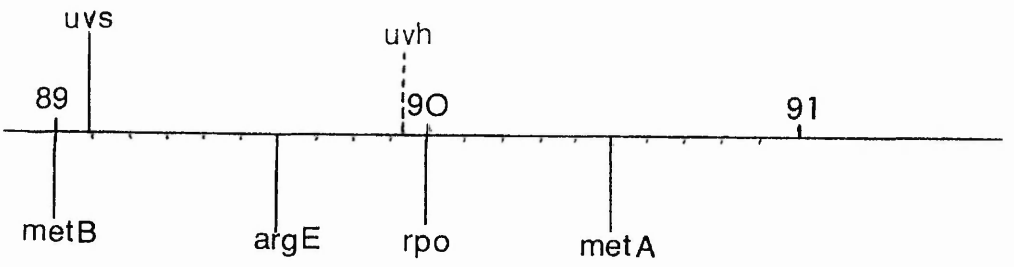
C.

			Transduction Data			
			metB$^-$	*metB*$^+$	*metB*$^-$	*metB*$^+$
Donor	Recipient	*argB*$^+$	*uvs*$^-$	*uvs*$^-$	*uvs*$^+$	*uvs*$^+$
SA305	SA273	100	5	35	30	30

Cotransduction frequency between *argB* and *metB* = 35%±9.3% (95% CI)

Cotransduction frequency between *argB* and *uvs* = 40%±9.6% (95% CI)

FIG.67. CALCULATED MAP POSITIONS OF *uvh* AND *uvs* ON THE *E.COLI* CHROMOSOME..



D. Discussion

As shown by Ahmad *et al.* (1980), strain SA236 is more resistant to certain DNA damaging agents than its wild type parent strain KL14. Interestingly, SA236 was resistant to all the DNA damaging agents tested in this study apart from near ultraviolet light. Ahmad et al. (1980) showed an increased activity of polymerase I and endonuclease I in strain SA236 and, considering their known roles in repair mechanisms, it is not surprising that their enhanced expression makes DNA repair more efficient.

As already described, the UvrABC incision complex is the major incision enzyme for both monofunctional and difunctional DNA damage. DNA damaging agents, such as far ultraviolet light, give rise to pyrimidine dimers which are recognised and incised by the UvrABC complex. It has been shown *in vitro* that the DNA-protein complex produced by the the UvrABC endonuclease is very stable and that the turnover of the UvrA protein is limited (see Chapter 1). The production of UvrD and DNA polymerase I (which is over-expressed in SA236) alters the stability of the endonuclease complex and has a significant effect on the DNA incision events (Husain *et al.*, 1985). However, the complete reaction involving the UvrABC and D proteins plus DNA polymerase I is not associated with an increased initial rate of incision (Husain *et al.*, 1985), although in SA236 the efficiency of this reaction may be increased. Having stimulated the release of 12 to 13 bases, in a damage-containing DNA fragment, the DNA polymerase enzyme mediates the synthesis of DNA in the resulting gap (Caron *et al.*, 1985). Presumably, endonuclease I activity is increased in strain SA236 to deal with the large amounts of excised DNA produced by an enhanced repair mechanism.

Strain SA236 was more resistant than its parent strain KL14 to mitomycin C and PUVA, both of which are DNA damaging agents which induce DNA crosslinks. Jones & Yeung (1988) showed that the uvrABC endonuclease was absolutely necessary for the incision step in the crosslink repair mechanism proposed by Van Houten *et al.*, (1986b) and that the involvement of DNA polymerase I in association with the UvrD protein was necessary to stimulate the release of the endonuclease complex from DNA. DNA polymerase I is also important in post replication recombination repair, a feature of crosslink repair that is necessary for mediating synthesis of DNA strand gaps. Again, it is therefore not surprising that enhanced expression of polymerase I leads to increased efficiency of the DNA crosslink repair mechanism.

The result obtained in this study confirmed that SA236 was resistant to 5-fluorouracil. This compound is incorporated into DNA as fluorodeoxyuridylate and fluorodeoxycytidylate and, although the damage caused by 5-fluorouracil can be removed by direct action of uracil-DNA glycosylase, it can also be indirectly removed during base excision repair of uracil containing regions. Warner & Rockstroh (1980) have shown that the exonuclease III enzyme, which has an associated endonuclease activity that is specific for apurinic and apyrimidinic sites in DNA, can directly mediate the excision repair of 5-fluorouracil-induced DNA damage. They proposed that the repair synthesis of DNA is mediated by the action of DNA polymerase I. Interestingly, in SA236 the activity of exonuclease III was increased two-fold (Ahmad *et al.*, 1980) and may account, in association with the enhanced activity of DNA

polymerase I, for the increased resistance of mutant strain SA236 to 5-fluorouracil-induced DNA damage.

The results obtained with hydrogen peroxide were not in agreement with those reported by Ahmad *et al.* (1980). These authors stated that strains SA236 and KL14 were equally sensitive to hydrogen peroxide. Since survival curves are more sensitive than disc sensitivity tests, it is suggested that the method used by Ahmad *et al.* (1980) to expose the strains to hydrogen peroxide may be the cause of such discrepancies. The present study found that SA236 was more resistant to hydrogen peroxide than the wild type strain KL14. Hydrogen peroxide is known to have several effects on DNA. Hagensee & Moses (1989) showed that hydrogen peroxide causes strand cleavage of DNA and induces a repair response involving DNA polymerase I and DNA ligase. They reported that two repair pathways existed for the repair of hydrogen peroxide-induced damage; one pathway utilises DNA polymerase I alone, or with other unknown components, while the other utilises endonuclease III, DNA polymerase III and DNA polymerase I. In an earlier study, Ananthanswamy & Eisenstark (1977) showed that a strain carrying a mutation in the *polI* gene, which controls the synthesis of the DNA polymerase I protein, was considerably more sensitive to the effects of hydrogen peroxide compared to its wild type parent strain. They proposed that DNA damage, in the form of single-strand breaks, is not efficiently repaired in mutants lacking *polI* gene activity. Hagensee & Moses (1986) concluded that damage caused by hydrogen peroxide is of a DNA incision nature, and that resynthesis of DNA following hydrogen peroxide treatment demonstrated a strict requirement for DNA polymerase I. The increased level of

polymerase I, and probably exonuclease III, observed in the mutant strain SA236 could account for the resistance of the mutant towards hydrogen peroxide.

The given dose of near ultraviolet light had no significant effect on the survival of SA236 and KL14. This is somewhat surprising since damage induced by near ultraviolet light is of a similar nature to that produced by hydrogen peroxide. Near ultraviolet light in the presence of oxygen is known to yield hydrogen peroxide as a toxic photoproduct (Ananthaswamy & Eisenstark, 1977) which, as already described, produces single-strand breaks in DNA. Following exposure to near ultraviolet light, DNA polymerase I enzyme is responsible for repairing up to 80% of these breaks (Jagger, 1981).

One possibility as to why SA236 and KL14 were not killed by near ultraviolet light in the experiments described in this Appendix is that the lamp may not have emitted sufficient energy at the necessary wavelength (~365nm) to cause significant cell death. In future experiments it may be important to use filters to exclude wavelengths different from 365nm. An additional factor is that the dose rate of the near ultraviolet lamp used in this study was considerably lower than that used in other studies. Lang *et al.* (1986) noted that the fluence rate was important in the observed effect of near ultraviolet light on *E.coli*, with a short exposure to a high intensity source having a different biological effect from a long exposure to a lower intensity source.

SA236 was more resistant than wild type KL14 to the combined effects of near ultraviolet light and hydrogen peroxide. As demonstrated in this study, near ultraviolet light alone, or low

concentrations of hydrogen peroxide (10mM), have little or no effect on the survival of the two strains, but when combined there is a synergistic killing effect mediated by the two agents. The synergistic killing effect of near ultraviolet light and hydrogen peroxide has been investigated previously. Hartman & Eisenstark (1978) demonstrated that the number of single-strand breaks in the DNA of wild type *E.coli* irradiated with near ultraviolet light following treatment with hydrogen peroxide was greater than the sum of the number of breaks caused by both damaging agents acting independently. Hartman & Eisenstark (1978) described a system which would quantitatively measure the degree of synergism between near ultraviolet light and hydrogen peroxide. The dose enhancement factor (D.E.F.) is calculated as the ratio between the dose required to reduce cell survival to 37% by near ultraviolet light alone and the dose required to reduce cell survival to 37% by near ultraviolet light and a given concentration of hydrogen peroxide. It was not possible to calculate the D.E.F. for SA236 and K114 in this study, since no significant decrease in cell survival was obtained when the strains were treated with near ultraviolet light alone. Interestingly, Hartman & Eisenstark (1978) calculated the D.E.F. for a wild type strain as 16.4 and a *polI* mutant strain of *E.coli* as 11.1. It was concluded that the results reflected the increasing importance of excisable lethal photoproducts in cell killing of the strains. From the result (fig. 63) obtained in this study, it can be concluded that polymerase I, exonuclease III and, perhaps to a lesser degree, endonuclease I may have a significant role in repairing DNA damage induced by near ultraviolet light plus hydrogen peroxide.

Polyacrylamide gel electrophoresis of total cell protein from SA236 and KL14 showed that the mutant strain had reduced levels of a protein of low molecular mass (fig. 66). This protein may be part of the SOS system. It is proposed that the mutation in the *uvh* gene may have an effect on this control mechanism. It is suggested that the gene product of *uvh* is a positive effector of the *lexA* gene. In normal wild type conditions the LexA protein functions as an active repressor. In SA236 a mutation in the *uvh* gene causes the levels of LexA to drop and it can no longer act as a repressor molecule. Consequently gene expression under the control of the LexA protein is increased and repair efficiency of the cell is elevated.

Clearly, further research must be performed on the mutant strain SA236 in order to fully understand the mechanisms of repair at a genomic level. No attention has been paid to the *recA* gene and its role in SA236 and KL14, although preliminary experiments with *recA* derivatives of both strains have shown them to be very sensitive to various types of DNA damage (data not shown).

This aspect might reward further study since a distinction between *recA*-repairable and *recA* non-repairable damage has been demonstrated in terms of near ultraviolet light and hydrogen peroxide synergystic killing (Hartman & Eisenstark, 1978). Hagensee & Moses (1989) reported no direct role for the RecA protein in the repair of hydrogen peroxide damaged DNA, in contrast to Imlay & Linn (1987). Obviously there is a need to clarify the involvement of different gene products in response to different types of DNA damage.

Mapping data outlined in this Appendix suggested that the mutation responsible for conferring a far-ultraviolet light hyper-resistant phenotype to SA236 was located between 89.8 and 90 minutes on the *E.coli* chromosome. The location of the mutation conferring ultraviolet light hyper-sensitivity on the cell was mapped at 89 minutes on the *E.coli* chromosome. Ahmad & Van Sluis (1987) estimated that the distance between the *uvh* gene and the *uvs* gene was 1.5 minutes. From the results obtained in this study the distance between the genes was estimated to be 1 minute on the *E.coli* chromosome. It is important to note that approximations and assumptions are made when applying the formula devised by Wu (1966) to P1 transductions. It is assumed that the bacterial fragments transduced by P1 are 2.0 minutes in length, although P1 can actually accommodate up to 2.4 minutes. This can lead to error in determining the precise map positions of unknown genes. Wu (1966) also assumed that the transducing fragments are cut at random from bacterial chromosomes. The theoretical frequency of cotransduction of two markers is simply a function of the physical distance between the markers and of the consequent probability that both markers will be contained in the same fragment. Bachmann *et al.* (1976) noted different values for reciprocal cotransduction frequencies, suggesting that transducing fragments may not be produced randomly. The data of reciprocal crosses is therefore generally averaged to calculate a gene position. Obviously, more mapping data for the *uvh* and *uvs* genes must be produced.

In summary, the mutant strain SA236 shows a greater DNA repair efficiency than its wild type parent strain KL14. The strain carries two mutations: *uvh* which is *trans* dominant and *uvs* which is *trans*

recessive. The *uvh* mutation plays a part in determining the ultraviolet phenotype of the strain, probably because its gene product is involved in regulating the *polI* structural gene and also the LexA protein. The part played by the *uvs* gene is unknown and requires further elucidation.

Bibliography

Ahmad, S.I., Atkinson, A., and Eisensterk, A. 1980.
Isolation and characterisation of a mutant of *Escherichia coli* K12 synthesising DNA polymerase I and endonuclease I constitutively.
J. Gen. Microbiology. 117: 419-422.

Ahmad, S.I., and Holland, I.B. 1985.
Isolation and analysis of a mutant *Escherichia coli* hyper-resistant to near-ultraviolet light plus 8-methoxypsoralen
Mutation Res. 151: 43-47.

Ahmad, S.I., and Van Sluis, C.A. 1987.
Inducible DNA polymerase I synthesis in a UV hyper-resistant mutant of *Escherichia coli*.
Mutation Res. 151: 77-81

Alderson, T., and Scott, B.R. 1970.
The photosensitising effect of 8-methoxypsoralen on the inactivation and mutation of *Aspergillus* conidia by near ultraviolet light.
Mutation Res. 9: 569-571.

Ali, R., and Agarwala, S.C. 1974.
In vitro and *in vivo* effect of normal and irradiated psoralen on glucose oxidation of brain and liver.
Enzyme. 18: 321.

Allyn, B. 1962.
Studies on phototoxicity in man and laboratory animals.

Presented at the annual meeting of the American Academy of Dermatology, Chicago, Il.

Altenburg, E. 1956.
Studies of the enhancement of mutation rate by carcinogens.
Tex. Rep. Biol. Med. 14: 481-483.

Ananthaswamy, N.H., and Eisenstark, A. 1976.
Near-UV-induced breaks in phage DNA: Sensitization by hydrogen peroxide (a tryptophan photoproduct).
Photochen. Photobiol. 24: 439-442.

Ananthaswamy, H.N., and Eisenstark, A. 1977.
Repair of hydrogen peroxide-induced single strand breaks in *E.coli* DNA.
J. Bacteriol. 130: 187-191.

Averback, D., and Moustacchi, E. 1975.
8-methoxypsoralen plus 365nm light effects and repair in yeast.
Biochim. Biophys. Acta. 395: 393-396.

Bachmann, B.J., Low, K.B., and Taylor, A.L. 1976.
Recalibrated linkage map of *Escherichia coli* K12.
Bacteriol. Rev. 40: 116-167.

Bachmann, B.J. 1983.
Linkage map of *Escherichia coli* K12. Edition 6.

Microbiol. Rev. 44: 1-56.

Berckhardt, S.E., Woodgate, R., Scheuermann, R., and Echols, H. 1988.
UmuD mutagenesis protein of *E.coli*: overproduction, purification and cleavage by RecA.
Proc. Natl. Acad. Sci. USA. 85: 1811-1815.

Baden, H.P., Parrington, J.M., Delhanty, J.D.A., and Pathak, M.A. 1972.
DNA synthesis in normal and xeroderma pigmentosum fibroblasts following treatment with 8-methoxypsoralen and long-wave ultraviolet light.
Biochim. Biophys. Acta. 262: 247-255.

Bridges, B.A., and Mottershead, R.P. 1977.
Frameshift mutagenesis in bacteria by 8-methoxypsoralen (methoxalen) in the dark.
Mutation. Res. 44: 305.

Bridges, B.A., and Mottershead, R.P. 1979.
Inactivation of *Escherichia coli* by near ultraviolet light and 8-methoxypsoralen: different responses of strain B/r and K12.
J. Bacteriol 139: 454-459.

Bridges, B.A., and Von Wright, A. 1981.
Influence of mutations at the *rep* gene on survival of *Escherichia coli* following ultraviolet light irradiation or 8-methoxypsoralen photosensitization. Evidence for a *recA*$^+$ *rep*-dependent pathway for repair of DNA crosslinks.
Mutation Res. 82: 229-238.

Bridges, B.A. 1983.
Psoralens and serendipity: aspects of the genetic toxicology of 8-methoxypsoralen.
Environ. Mutagen. 5: 329-339.

Bridges, B.A. 1984.
Further characterisation of repair of 8-methoxypsoralen crosslinks in UV-excision-defective *Escherichia coli*.
Mutation Res. 132: 153-160.

Burger, P.M., and Simons, J.W. 1979a.
Mutagenicity of 8-methoxypsoralen and long-wave ultraviolet irradiation in V79 Chinese hamster cells.
Mutation Res. 60: 381-385.

Burger, P.M., and Simons, J.W. 1979b.
Mutagenicity of 8-methoxypsoralen and long-wave ultraviolet irradiation in diploid human skin fibroblasts.
Mutation Res. 63: 371-374.

Caffieri, A., Schoonderwoerd, S.A., Beijersbergen, G.M.J., Daga, A., Dall'acqua, F. 1988.
Photodamage to unsaturated fatty acids by furocoumarins both *in vitro* and *in vivo* in rat skin.

Abstract presented at the meeting of the British Photobiology Society, London, 1988.

Calvin, N.M., and Hanawalt, P.C. 1987.
Photo-adducts of 8-methoxypsoralen to cytosine in DNA.
Photochem. Photobiol. 45: 323-330.

Caron. P.R., Kushner, S.R., and Grossman, L. 1985.
Involvement of helicase II (*uvrD* gene product) and DNA polymerase I in excision mediated by the UvrABC protein complex.
Proc. Natl. Acad. Sci. USA. 82: 4925-4929.

Cech, T., Pathak, M.A., and Biswas, R.K. 1979.
An electron microscopic study of the photochemical crosslinking on DNA in guinea-pig epidermis by psoralen derivatives.
Biochem. Biophys. Acta. 562: 342-347.

Cheng, S., Van Houten, B., Gamper, H.B., Sancar, A., and Hearst, J.E. 1988.
Use of psoralen-modified oligonucleotides to trap three-stranded RecA-DNA complexes and repair of these cross-linked complexes by ABC excinuclease.
J. Biol. Chem. 263: 15110-15117.

Claverys, J.P., and Lacks, S.A. 1986.
Heteroduplex deoxyribonucleic acid base mismatch repair in bacteria.
Microbiological Reviews 50: 133-165.

Cole, R.S. 1973.
Repair of DNA containing interstrand crosslinks in *Escherichia coli*: sequential excision and recombination.
Proc. Natl. Acad. Sci. USA. 70: 1064-1068.

Cupido, M., and Bridges, B.A. 1985.
Uvr-independent repair of 8-methoxypsoralen crosslinks in *Escherichia coli*: evidence for a recombinational process.
Mutation Res. 146: 135-141.

Dall'acqua, F. 1977.
New chemical aspects of the photoreaction between psoralens and DNA.

In Castellani, A.(ed.), Research in Photobiology. New York. Plenum Press. pp245-255.

Dall'acqua, F., Vedaldi, D., Bordin, F., Baccichetti, F., Carlassare, F., Tamaro, M., Rodighiero, P., Pastorini, G., Guiotto, A., Recchia, G., and Cristofolini, M. 1983.
4'-methylangelicins: new potential agents for photochemotherapy of psoriasis.
J. Med. Chem. 26: 870-876.

Demple, B., Jacobsson, A., Olsson, M., Robins, P., and Lindahl, T. 1982.
Repair of alkylated DNA in *Escherichia coli*. Physical properties of O^6-methylguanine-DNA methyltransferase.
J. Biol. Chem. 257: 13776-13780.

Demple, B. 1990.
Oxidative damage and its repair.

Abstract presented at the meeting of the Society for General Microbiology. Warwick. April 1990.

Drake, J.W., and McGuire, J. 1967.
Properties of r mutants of bacteriophage T4 photodynamically induced in the presence of thipyronin and psoralen.
J. Virol. 1: 260-264.

Duncan, B.K., and Miller, J.H. 1980.
Mutagenic deamination of cytosine residues in DNA.
Nature (London). 287: 560-561.

Eastman, A. 1983.
Characterisation of the adducts produced in DNA by cis-diamminedichloroplatinum(II) and cis-di-chloro (ethylenediammine) platinum(II).
Biochemistry 22: 3927-3933.

El Mofty, A.M. 1968.
Vitiligo and psoralens.

Oxford: Pergamon Press.

Epstein, J.H. and Fukuyama, K. 1970.
A study of 8-methoxypsoralen (8-MOP) induced phototoxic effects on mammalian epidermal macromolecular synthesis *in vivo*.
J. Invest. Dermatol. 54: 350-351.

Finch, P.W., Chambers, P., and Emmerson, P.T. 1985.
Identification of the *Escherichia coli recN* gene product as a major SOS protein.
J. Bacteriol. 164: 653-658.

Fleming, M.G., and Brody, N. 1985.
Biologic effectiveness of black light fluorescent lamps for PUVA.
J. Am. Acad. Dermatol. 12: 894-899.

Foster, J.W., and Strike, P. 1988.
Analysis of the regulatory elements of the *Escherichia coli uvrC* gene by construction of operon fusions.
Mol. Gen. Genet. 211: 531-537.

Fram, R.J., Sullivan, J., and Marinus, M.G. 1986.
Mutagenesis and repair of DNA damage caused by nitrogen mustard, N,N'-bis(2-chlorethyl)-N-nitrosourea (BCNU), streptozotocin, and mitomycin C in *E.coli*.
Mutation Res. 166: 229-242.

Franklin, W.A., Lo, K.M., and Haseltine, W.A. 1982.
Alkaline lability of novel fluorescent photoproducts produced in ultraviolet light irradiated DNA.
J. Biol. Chem. 257: 13535-13543.

Frederickson, S., and Hearst, J.E. 1979.
Binding of 4'-aminomethyl-4,5',8-trimethyl-psoralen to DNA, RNA and protein in Hela cells and *Drosophila* cells.
Biochim. Biophys. Acta. 563: 343-348.

Germanier, M., Defais, M., Johnson, N.P., and Villani, G. 1984.
Repair of platinum-DNA lesions in *E.coli* by a pathway which does not recognise DNA damage caused by MNNG or UV light.
Mutation Res. 145: 35-41.

Goldstein, D.P., Carter, D.M., Lyunggren, B., and Burkholder, J. 1982.
Minimal phototoxic doses and 8-MOP plasma levels in PUVA patients.
J. Invest. Dermatol. 78: 429-433.

Gorden, L.K., and Haseltine, W.A. 1982.
Quantitation of cyclobutane pyrimidine dimer formation in double and single stranded DNA fragments of defined sequence.
Radiat. Res. 89: 99-112.

Granger-Schnarr, M., Scharr, M., and Van Sluis, C.A. 1986
In vitro study of the interaction of the LexA repressor and the UvrC protein with a *uvrC* regulatory region.
FEBS Lett. 198: 61-65.

Grossweiner, L.I. 1984.
Photochemistry of proteins: a review.
Curr. Eye Res. 3: 137-145.

Hagensee, M.E., and Moses, R.E. 1986.
Repair response of *Escherichia coli* to hydrogen peroxide DNA damage.
J. Bacteriol. 168: 1059-1065.

Hagensee, M.E., and Moses, R.E. 1989.
Multiple pathways for repair of hydrogen peroxide induced DNA damage in *Escherichia coli*.
J. Bacteriol. 171: 991-995.

Hansen, M.T. 1982.
Sensitivity of *Escherichia coli acrA* mutants to psoralen plus near ultraviolet radiation.
Mutation Res. 106: 209-216.

Hartman, P.S., and Eisenstark, A. 1978.
Synergistic killing of *E.coli* by near-UV radiation and hydrogen peroxide: distinction between *recA*-repairable and *recA*-nonrepairable damage.
J.Bacteriol. 133: 769-774.

Höeijmakers, J.H.J., Weeda, G., Koken, M.H.M., Troelstra, C., Van der Eb, A.J., and Bootsma, D. 1990.
Isolation and characterisation of mammalian DNA-repair genes.

Abstract presented at the meeting of the Society for General Microbiology. Warwick April 1990.

Hönigsmann, H., Wolff, K., Gschnait, F., Brenner, W., Jaschke, E. 1980.
Keratoses and nonmelanoma skin tumors in long-term photochemotherapy (PUVA).
J. Am. Acad. Dermatol. 3: 406-414.

Horii, Z., and Clark, A.J. 1973.
Genetic analysis of the RecF pathway of genetic recombination in *Escherichia coli* K12: isolation and characterisation of mutants.
J. Mol. Biol. 80 : 327-344.

Howard-Flanders, P., and Boyce, R.P. 1966.
DNA repair and genetic recombination: studies on mutants of *Escherichia coli* defective in these processes.
Radiation Res., Suppl. 6: 156-184.

Husain, I., Van Houten, D.C., Thomas, M., Abdel-Monem, and Sancar, A. 1985.
Effect of DNA polymerase I and DNA helicase II on the turnover rate of UvrABC excision nuclease.
Proc. Natl. Acad. Sci. USA. 82: 6774-6778.

Igali, S., Bridges, B.A., Ashwood-Smith, M.J., and Scott, B.R. 1970.
Mutagenesis in *E.coli* IV. Photosensitisation to near ultraviolet light by 8-methoxypsoralen.
Mutation Res. 9: 21-25.

Imlay, J., and Linn, S. 1987.
Mutagenesis and stress response induced in *E.coli* by hydrogen peroxide.
J. Bacteriol. 169: 2967-2976.

Iyer, V.N., and Szybalski, W. 1963.
A molecular mechanism of mitomycin C action: linking of complementary DNA strands.
Proc. Natl. Acad. Sci. USA. 82: 255-361.

Jagger, J. 1981.
Near-UV radiation effects on microorganisms.
Photochem. Photobiol. 34: 761-768.

Jeggo, P., Defais, M., Samson, L., and Schendel, P. 1977.
An adaptive response of *E.coli* to low levels of alkylating agents: comparison with previously characterised DNA repair pathways.
Mol. Gen. Genet. 157: 1-9.

Jones, B.K., and Yeung, A.T. 1988.
Repair of 4,5',6-trimethylpsoralen monoadducts and crosslinks by the *Escherichia coli* UvrABC endonuclease.
Proc. Natl. Acad. Sci. USA. 85: 8410-8414.

Kittler, L.K., Midden, W.R., and Wang, S.Y. 1986.
Interactions of furocoumarins with subunits of cell constituents. Photoreaction of fatty acids and aromatic amino acids with TMP and 8-MOP.
Stud. Biophys. 114: 139-145.

Kohn, K.W., Spears, C.L., and Doty, A., 1966.
Interstrand crosslinking of DNA by nitrogen mustard.
J. Mol. Biol. 19: 288.

Kolodner, R., Fishel, R.A., and Howard, M. 1985.
Genetic recombination of bacterial plasmid DNA: Effect of RecF pathway mutations on plasmid recombination in *Escherichia coli*.
J. Bacteriol. 163: 1060-1066.

Kroos, L., and Kaiser, D. 1984.
Construction of Tn5*lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*.
Proc. Natl. Acad. Sci. USA. 81: 5816-5820.

Kuemmerle, N.B., and Masker, W.E. 1980.
Effect of the *uvrD* mutation on excision repair.
J. Bacteriol. 142: 535-546.

Kushner, S.R., 1987.
DNA repair.

In: Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umbarger, H.E. (eds.),
Escherichia coli and *Salmonella typhimurium*, cellular and molecular biology. Vol. 2. American Society Microbiology.

Lang, H., Risenberg, D., Zimmer, C., and Bergler, F. 1986.
Fluence-rate dependence of monophotonic reactions of nucleic acids *in vitro* and *in vivo*.
Photochem. Photobiol. 44: 565-570.

Laskin, J.D., Lee, E., Yurkow, E.J., Laskin, D.L., and Gallo, M.A. 1985.
A possible mechanism of phototoxicity not involving direct interaction with DNA.
Proc. Natl. Acad. Sci. USA. 82: 6158-6162.

Lerner, A.B., Denton, C.R., Fitzpatrick, T.B. 1953.
Clinical and experimental studies with 8-methoxypsoralen in vitiligo.
J. Invest. Dermatol. 20: 299-314.

Levy 1984.
Resistance to the tetracyclines.

In: Antimicrobial drug resistance. Bryan, L.E.,(ed.), Academic Press London pp191-240.

Lindahl, T. 1982.
DNA repair enzymes.
Annual Review of Biochemistry. 51: 61-87.

Lipinska, B., King, J., Ang, D., and Georgopoulos, C. 1988.
Sequence analysis and transcriptional regulation of the *Escherichia coli grpE* gene, encoding a heat shock protein.
Nucleic Acids Res. 16: 7545-7562.

Lloyd, R.G., Picksley, S.M., and Prescott, C. 1983.
Inducible expression of a gene specific to the RecF pathway for recombination in *Escherichia coli* K12.
Mol. Gen. Gent. 190: 162-167.

Lovett, S.T., and Clark, A.J. 1983.
Genetic analysis of regulation of the RecF pathway of recombination in *Escherichia coli* K12.
J. Bacteriol. 153: 1471-1478.

Lovett, S.T., and Clark, A.J. 1985.
Cloning of the *Escherichia coli recJ* chromosomal region and identification of its encoded proteins.
J. Bacteriol. 162: 280-285.

Macquet, J.P., Butour, J.L., and Johnson, N.P. 1983.
Physicochemical and structural studies of the *in vitro* interactions between platinum(II) compounds and DNA.

In: S.J. Lippard (Ed.), Platinum, gold and other metal chemotherapeutic agents: Chemistry and Biochemistry,American Chemical Society. pp 75-100.

Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982.
Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

McCarthy, T.V., and Lindahl, T. 1985.
Methyl phosphotriesters in alkylated DNA are repaired by the Ada regulatory protein of *E.coli*.
Nucleic Acids Res. 13: 2683-2698.

Mello-Filhi, A.C., Hoffman, M.E., and Meneghini, R. 1984.
Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron.
J. Biochem. 218: 273-275.

Melo, T.S., Morliere, P., Goldstein,S., Santus, R., Dubertret, L., and Lagrange, D. 1984.
Binding of 5-methoxypsoralen to human serum low density lipoproteins.
Biochem. Biophys. Res. Commum. 120: 670-675.

Miller, J.H., 1972.
Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Moreau, P.L. 1988.
Overproduction of single-stranded-DNA-binding protein specifically inhibits recombination of UV-irradiated bacteriophage DNA in *Escherichia coli*.
J. Bacteriol. 170: 2493-2500.

Nilsson, R., Merkel, P.B., and Kearns, D.R. 1972.
Unambiguous evidence for the participation of singlet oxygen in photo-dynamic oxidation of amino acids.
Photochem. Photobiol. 16: 117-123.

Nohmi, T., Battista, J.R., Dodson, L., Walker, G.C. 1988.
RecA mediated cleavage activates UmuC for mutagenesis: mechanistic relationship between transcriptional derepression and post-translational activity.
Proc. Natl. Acad. Sci. USA. 85: 1816-1820.

Ossanna, N., Peterson, K.R., and Mount, D.W. 1986.
Genetics of DNA repair in bacteria.
Trends in Genetics, Reviews February.

Parrish, J.A., Fitzpatrick, T.B., Tanebaum, L., and Pathak, M.A. 1974.
Photochemotherapy of psoriasis with oral methoxsalen and long-wave ultraviolet light.
N. Engl. J. Med. 291: 1207-1211.

Pathak, M.A., and Kramer, D.M. 1969.
Photosensitisation of skin *in vivo* by furocoumarins (psoralens).
Biochim. Biophys. Acta. 195: 197-201.

Pathak, M.A., and Kramer, D.M., and Fitzpatrick, T.B. 1974.
Photobiology and photochemistry of furocoumarins (psoralens).

In: Fitzpatrick, T.B., Pathak, M.A., Harber, L.C., Seiji, M., and Kukita, A. (eds). Sunlight and man. Tokyo: University of Tokyo Press. 335-368.

Pathak, M.A., and Joshi, P.C. 1983.
The nature amd molecular basis of cutaneous photosensitivity reactions to psoralens and coaltar.
J. Invest. Dermatol. Supplement 66.

Perry, K.L., Elledge, S.J., Mitchell, B.B., Marsh, L., and Walker, G.C. 1985.
umuDC and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology.
Proc. Natl. Acad. Sci. USA. 82: 4331-4335.

Peterson, K.R., Ossanna, N., Thliveris, A.T., Ennis, D.G., and Mount, D.W. 1988.
Derepression of specific genes promotes DNA repair and mutagenesis in *Escherichia coli*.
J. Bacteriol. 170: 1-4.

Picksley, S.M., Attfield, P.V., and Lloyd, R.G. 1984.
Repair of DNA double-strand breaks in *Escherichia coli* K12 requires a functional *recN* product.
Mol. Gen. Genet. 195: 267-274.

Quinto, I., Averbeck, D., Moustacchi, E., Hrisoho, Z., and Moron, J. 1984.
Frameshift mutagenicity in *Salmonella typhimurium* of furocoumarins in the dark.
Mutation Res. 136: 49-51.

Riggs, A., Suzuki, H., and Bourgeois, S. 1970.
lac Repressor-Operator interaction. I. equilibrium studies
J. Mol. Biol. 48: 67-83.

Roberts, J.J., and Pascoe, J.M. 1972.
Crosslinking of complementary strands of DNA in mammalian cells by antitumor platinum compounds.
Nature (London). 235: 282-284.

Roberts, J. J., and Thompson, A.J. 1979.
The mechanism of action of anti-tumour platinum compounds.
Progress in Nucleic Acid Research and Molecular Biology. 22: 71-129.

Robins, P., Harris, A., Goldsmith, I., and Lindahl, T. 1983.
Crosslinking of DNA binding by chloroethyl-nitrosourea is prevented by O^6-methylguanine DNA methyltransferase.
Nucleic Acids Res. 11. 7743-7758.

Rupert, C.S., Goodgal, S.H., and Herriot, R.M. 1958.
Photoreactivation *in vitro* of ultraviolet inactivated *Haemophilus influenzae* transforming factor.
J. Gen. Physiol 41: 451-471.

Rupp, W.D., and Howard-Flanders, P. 1968.
Discontinuities in the DNA synthesized in an excision defective strain of *Escherichia coli* following ultraviolet irradiation.
J. Mol. Biol. 31: 291-304.

Sancar, A., and Rupert, C.S. 1978.
A general method for isolation of repair deficient mutants.

In: Hanawalt, P.C., Freidberg, E.C., and Fox, C.F. (eds.), DNA repair mechanisms. Academic Press, Inc., New York. pp 101-104.

Sancar, A., Sancar, G.B., Rupp, W., Little, J.W., and Mount, D.W. 1982a.
LexA protein inhibits transcription of the *E.coli uvrA* gene *in vitro*.
Nature (London) 298: 96-98.

Sancar, G.B., Sancar, A., Little, J.W., and Rupp, W.D. 1982b.
The *uvrB* gene of *Escherichia coli* has both LexA repressed and LexA-indepenent promoters.
Cell. 28: 523-530.

Sancar, A., and Rupp, W.D. 1983a.
A novel repair enzyme: UVRABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region.
Cell. 33: 249-260.

Sancar, A., and Rupp, W.D. 1983b.
Cloning the *uvrA* and *uvrB* genes of *E.coli* and the use of the Maxicell procedure for detecting gene expression.

In: E.C. Freidberg and P.C. Hanawalt (eds.), DNA repair: a laboratory manual of research procedures, vol2. Marcel Dekker, Inc., New York.

Sancar, G.B., Sancar, A., and Rupp, W.D. 1984.
Sequences of the *Escherichia coli uvrC* gene and protein
Nucleic Acids Res. 12: 4593-4608.

Sancar, A., and Sancar, G. 1988.
DNA repair enzymes.
Annu. Rev. Biochem. 57: 29-67.

Seeberg, E. 1981.
Strand cleavage at psoralen adducts and pyrimidine dimers in DNA caused by interaction between semi-purified *uvr*$^+$ gene products from *Escherichia coli*.
Mutation Res. 82: 11-22.

Seeberg, E., and Steinum, A.L. 1982.
Purification and properties of the *uvrA* protein from *Escherichia coli*.
Proc. Natl. Acad. Sci. USA. 79: 988-992.

Setlow, R.B., Swenson, P.A., and Carrier, W.L. 1963.
Thymine dimers and inhibition of DNA synthesis by ultraviolet irradiation of cells.
Science 142: 1464-1466.

Simmon, V.F., and Lederberg, S. 1972.
Degradation of bacteriophage λ deoxyribonucleic acid after restriction by *Escherichia coli* K12.
J. Bacteriol. 112: 161-169.

Sinden, R.R., and Cole, R.S. 1978.
Repair of cross-linked DNA and survival of *Escherichia coli* treated with psoralen and light: effects of mutations influencing genetic recombination and DNA metabolism.
J. Bacteriol. 136: 538-547.

Sladek, F.M., Dynlacht, B., and Howard-Flanders, P. 1988.
Characterisation and partial purification of a novel activity from *E.coli* that acts on psoralen monoadducts.
J. Cell. Biochem. Suppl. 12a: 276.

Snapka, R.M., and Sutherland, B.M. 1980.
Escherichia coli photoreactivity enzyme: purification and properties.
Biochemistry 19: 4201-4208.

Spratt, B.G. 1977.
Properties of the penicillin-binding protein of *Escherichia coli* K12.
Eur. J. Biochem. 72: 341-352.

Stern, R., Zierler, S., and Parrish, J.A. 1982.
Psoriasis and the risk of cancer.
J. Invest. Dermatol. 78: 147-149.

Stern, R.S., Laird, N., Melski, J., Parrish, J.A., Fitzpatrick, T.B., and Bleich, H.L. 1984.
Cutaneous squamous-cell carcinoma patients treated with PUVA
N. Engl. J. Med. 310: 1156-1161.

Strike, P., and Rupp, W.D. 1985.
Crosslinking studies with the UvrA and UvrB proteins of *E.coli*.
Mutation Res. 145: 43-48.

Sutherland., B.M., and Chamberlin, M.J. 1973.
Deoxyribonucleic acid photoreactivating enzyme from *Escherichia coli*.
J. Bacteriol. 138: 333-338.

Teo, I., Sedgwick, B., Demple, B. and Lindahl, T. 1984.
Induction of resistance to alkylating agents in *E.coli*: the *ada*$^+$ gene serves both as a regulatory protein and as an enzyme for repair of mutagenic damage.
EMBOJ. 190: 2151-2157.

Téoule, R. 1987.
Radiation-induced DNA damage and its repair
Int. J. Radiat. Biol. 51: 573-589.

Thomas, D.C., Levy, M., and Sancar, A. 1985.
Amplification and purification of UvrA, UvrB and UvrC proteins of *Escherichia coli*.
J. Biol. Chem. 260: 9875-9883.

Thoms, B., and Wackernagel, W. 1987.
Regulatory role of *recF* in the SOS response of *Escherichia coli*: impaired induction of SOS genes by UV irradiation and nalidixic acid in a *recF* mutant.
J. Bacteriol. 169: 1731-1736.

Thoms, B., and Wackernagel, W. 1988.
Suppression of the UV-sensitive phenotype of *Escherichia coli recF* muants by *recA(Srf)* and *recA(Tif)* mutations requires *recJ*$^+$.
J. Bacteriol. 170: 3675-3681.

Tyrrell, R.M., Webb, R.B., and Ley, R.D. 1974.
Induction of single strand breaks in bacterial and phage DNA by near UV (365nm) radiation.
Photochem. Photobiol. 20: 395-398.

Van den Berg, E.A., Geerse, R.H., Pannekoek, H., and Van de Putte, P. 1983.
In vivo transcription of the *E.coli uvrB* gene: both promoters are inducible by UV.
Nucleic Acids Res. 11: 4355-4363.

Van Houten, B., Gamper, H., Holbrook, S.R., Hearst, J.E., and Sancar, A. 1986a.
Action mechanism of ABC excision nuclease on a DNA substrate containing a psoralen crosslink at a defined position.
Proc. Natl. Acad. Sci. USA. 83: 8077-8081.

Van Houten, B., Gamper, H., Hearst, J.E., and Sancar, A. 1986b.
Construction of DNA substrate modified with psoralen at a unique site and study of the action mechanism of ABC excinuclease on these uniformly modified substrates.
J. Biol. Chem. 261: 14135-14141.

Van Houten, B., Gamper, H., Sancar, A., and Hearst, J.E. 1987
DNaseI Footprint of ABC Excinuclease.
J. Biol. Chem. 262: 1-8.

Van Houten, B. 1990.
Nucleotide Excision Repair in *Escherichia coli*.
Microbiological Reviews 54: 18-51.

Van Sluis, C.A., Moolenaar, G.F., and Backendorf, C. 1983.
Regulation of the *uvrC* gene of *Escherichia coli* K12: localization and characterisation of damage - inducible promoter.
EMBOJ. 2: 2313-2318.

Volkert, M.R., and Nguyen, D.C. 1984.
Induction of specific *E.coli* genes by sublethal treatments with alkylating agents.
Proc. Natl. Acad. Sci. USA. 81: 4110-4114.

Walker, G.C. 1985.
Inducible DNA repair systems.
Annu. Rev. Biochem. 54: 425-457.

Walker, G.C. 1987.
The SOS response of *Escherichia coli*.

In: Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umbarger, H.E. (eds.) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.

Walters, R.G., Wilbraham, H.O., Strike, P., and Forster, J.W. 1988.
A transposon insertion in the *Escherichia coli uvrC* gene; UvrC protein is absolutely required for the incision step in excision repair.
J. Gen. Microbiology. 134: 403-412.

Wang, T.V., and Smith, K.C. 1983.
Mechanism for the *recF*-dependent and *recB*-dependent pathways of post replication repair in UV-irradiated *Escherichia coli uvrB*.
J. Bacteriol. 156: 1093-1098.

Wang, T.V., and Smith, K.C. 1985.
Mechanism of *sbcB*-suppression of the *recBC*-deficiency in post replication repair in UV-irradiated *Escherichia coli* K12.
Mol. Gen. Genet. 201: 186-191.

Wang, T.V., and Smith, K.C. 1986.
recA (Srf) suppression of *recF* deficiency in the postreplication repair of UV-irradiated *Escherichia coli* K12.
J. Bacteriol. 168: 940-946.

Wang, T.V., and Smith, K.C. 1988.
Different effects of *recJ* and *recN* mutations on the postreplcation repair of UV-damaged DNA in *Escherichia coli* K12.
J. Bacteriol. 170: 2555-2559.

Warner, R.H., and Rockstroh, A. 1980.
Incorporation and excision of 5-fluorouracil from deoxyribonucleic acid in *Escherichia coli*.
J. Bacteriol. 141: 680-686.

Webb, R.B., and Brown, M.S. 1982.
Genetic damage in *Escherichia coli* k12 AB2480 by broad-spectrum near-ultraviolet radiation.
Science. 215: 991-993.

Wennersten, G. 1979.
Membrane damage caused by 8-MOP and UVA-treatment of cultivated cells.
Acta. Derm. Venereol. 59: 21-26.

Weishahn, G.P., Hyde, J.E., and Hearst, J.E. 1977.
The photoaddition of trimethylpsoralen to *Drosophila melanogaster* nuclei: a probe for chromatin substructure.
Biochemistry 16: 925-932.

Wood, R.D. 1990.
The biochemistry of DNA repair deficiencies in man.

Abstract presented at the meeting of the Society for General Microbiology. Warwick, April 1990.

Wu, T.T. 1966.
A model for three-point analysis of random general transduction.
Genetics 54: 405-410.

Yatagai, F., and Glickman, B.W. 1986.
Mutagenesis by 8-methoxypsoralen plus near-UV treatment: analysis of specifity in the *lacI* gene of *Escherichia coli*.
Mutation Res. 163: 209-224.

Yatagai, F., Horfall, M.J., and Glickman, B.W. 1987.
Defect in excision repair alters the mutational specificity of PUVA treatment in the *lacI* gene of *Escherichia coli*.
J. Mol. Biol. 194: 601-607.

Yeung, A.T., Mattes, W.B., and Grossman, L. 1986.
Protein complexes formed during the incision reaction catalysed by the *Escherichia coli* UvrABC endonuclease.
Nucleic Acids Res. 14: 2567-2582.

Yoakum, G.H., and Grossman, L. 1981.
Identification of *E.coli uvrC* protein.
Nature (London) 292: 171-173.

Zhen, W.P., Jeppesen, C., and Nielsen, P.E. 1986.
Repair in *Escherichia coli* of a psoralen-DNA interstrand crosslink site specifically introduced into $T_{410}A_{411}$ of the plasmid pUC19.
Photochem. Photobiol. 44: 47-51.