Mutagenesis and DNA repair in

Micrococcus radiodurans

by

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I hereby confirm that this thesis has been composed by myself and that all the work reported is my own.

Philip R. Tempest
Edinburgh
December, 1978
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SUMMARY

*Micrococcus radiodurans* wild-type is not mutated to rifampicin-resistance by UV radiation, MTC, DCMTC, BrMBA, ICR 191G, BrdUrd or 2-AP, and is only weakly mutated by hydroxylamine, nitrous acid, MMS, EMS and gamma-rays. In contrast, it is strongly mutated by MNNG and MNUr. The radiation-resistant bacteria, *M. roseus* ATCC 19172, *M. radiophilus* and *M. radioproteolyticus*, which are shown to be genetically related to *M. radiodurans*, and the unrelated Arthrobacter radiotolerans and P-30-A are all immutable by UV, whereas the more radiation-sensitive species, *M. luteus*, *M. sodonensis* and *Pseudomonas radiora* are UV-mutable.

Ten mutants of *M. radiodurans* with defective DNA repair are sensitive to the lethal effects of MNNG and three of these, namely 302, 262 and UV22 are hypermutable by MNNG.

Strain 302 is also very sensitive to the lethal effects of MTC and DCMTC, and slightly sensitive to the lethal effects of EMS, BrMBA, AAF and nitrous acid, but is resistant to the lethal effects of UV, MMS and hydroxylamine. It is also hypersensitive to the mutagenic effects of MMS, EMS, nitrous acid and hydroxylamine. It is defective in the initial recognition and/or incision step in the excision repair of BrMBA-damaged DNA. After treatment with MNNG wild-type can enzymically excise 7-methylguanine, 3-methyladenine and $\text{O}^6$-methylguanine residues from its DNA. 302 does not excise $\text{O}^6$-methylguanine residues. Strain 262 is phenotypically similar to strain 302.

Strain UV22 is sensitive to the lethal effects of MMS and UV and is hypermutable by MMS. It incises DNA damaged by UV but is defective in mending breaks and does not remove 3-methyladenine residues.

A UV-sensitive, MMS-resistant mutant, strain uvs10, has been
isolated as a putative pyrimidine dimer-excision-less mutant. It
does not incise UV-damaged DNA to the same extent as its parent
and is slightly UV-mutable. Mutants uvs10, 303 and UV47 are
mutator strains.
INTRODUCTION
1. The biology of *Micrococcus radiodurans*

For many years it was thought that resistant forms of bacteria only existed in the form of endospores. However, in 1956, a vegetative, non-sporing bacterium was isolated from cans of spoiled meat which had been subjected to gamma-radiation as part of the sterilization process (Anderson et al., 1956). The extreme resistance of this organism to ionizing radiations was then demonstrated as was later its resistance to ultraviolet (UV) light (Duggan et al., 1959). It is a red-pigmented, spherical bacterium and was designated *Micrococcus radiodurans* (Anderson et al., 1961).

Although *M. radiodurans* is a Gram-positive bacterium it is similar to a Gram-negative bacterium in that it has a cell envelope containing three distinct layers (Thornley et al., 1965). The chemical composition of the cell wall is also similar to a Gram-negative bacterium since it contains a lipoprotein-polysaccharide component (Work and Griffith, 1968) and considerable amounts of lipids, some of which are unusual in that they are composed of a large proportion of odd-numbered chain fatty acids (Knivett et al., 1965; Girard, 1971). However, in contrast to nearly all Gram-negative bacteria, *M. radiodurans* contains little or no oleic acid (Girard, 1971) and its peptidoglycan is not of a type found in any Gram-negative organism. In the electron microscope the peptidoglycan layer appears as a "holey" structure: an unusual feature not found in other bacteria (Thornley et al., 1965; Sleytr et al., 1973). Furthermore, the chemical composition of the peptidoglycan is unusual. In contrast to a typical *Micrococcus* which contains L-lysine as the diamino acid, *M. radiodurans* contains L-ornithine residues which are cross-
linked via two glycine molecules (Schleifer and Kandler, 1972).

Because the cell wall is not typical of a Gram-positive coccus and has many features in common with Gram-negative bacteria but also has some unique features it has been suggested that M. radiodurans be classified into a new taxon (Baird-Parker, 1965; Thornley et al., 1965; Sleytr et al., 1973).

The first isolation was not the only one. M. radiodurans was subsequently isolated near the canning factory although gamma-rays were used to select it since it was present only in very low numbers compared with the rest of the microbial population (Krabbenhoft et al., 1965). However, the prior exposure to gamma-rays has not been a prerequisite for its isolation since it was isolated in a hospital as an aerial contaminant (Murray and Robinow, 1958). This bacterium was initially of interest for its mode of cell division and it was only several years later that its resistance to UV and gamma-radiation was demonstrated in this strain designated M. radiodurans Sark. More recently, M. radiodurans has been isolated in Japan from mushroom sawdust culture but again could only be detected after gamma-radiation had eliminated the predominant microflora (Ito, 1977).

The existence of such an extraordinarily radiation-resistant organism is not unique. Several other bacteria, some of which may be generically related to M. radiodurans exhibiting similar resistance to radiation have been isolated from various gamma-irradiated sources. For example, Micrococcus roseus ATCC 19172 was isolated from gamma-irradiated haddock tissue (Davis et al., 1963). The cell envelope of this red-pigmented coccus is similar to that of M. radiodurans in that it also has a peptidoglycan containing L-ornithine with two glycine residues in the cross-link.
(Kandler, pers. commun), and contains odd-numbered fatty acids and little, if any, oleic acid in lipid containing material (Girard, 1971). But it does differ from *M. radiodurans* in its morphological, cultural and physiological characteristics.

Another red-pigmented bacterium of exceptional resistance to gamma-rays was that isolated from gamma-irradiated Bombay duck and was designated *Micrococcus radiophilus* (Lewis, 1971, 1973). This species was shown also to be extremely UV-resistant (Lewis and Kumta, 1972). More recently, *Micrococcus radioproteolyticus* has been isolated from the faeces of a llama (Kobatake et al., 1973). Thin sections of the cell envelopes of *M. radiophilus* and of *M. radioproteolyticus* have shown that they are similar to those from Gram-negative bacteria and *M. radiodurans* in that they contain three distinct layers. However, the outer surfaces of these two bacteria do not show the hexagonal pattern which is observed in *M. radiodurans* (Sleytr et al., 1976). Both *M. radiophilus* and *M. radioproteolyticus* also have peptidoglycan of the L-ornithine-(glycine)$_2$ type (Schleifer, unpublished results cited by Sleytr et al., 1976).

Another feature which distinguishes these bacteria from a typical *Micrococcus* is the state of the menaquinone system (vitamin K2) which in the radiation-resistant bacteria is identical with regard to the number and degree of saturation of the isoprenoid units (Yamada et al., 1977). These four radiation-resistant bacteria have a similar GC content of the their DNAs which varies between 64 and 71% (Moseley and Schein, 1964; Boháček et al., 1967; Kocur et al., 1971; Kobatake et al., 1973).

Two Gram-negative rod-shaped bacteria have also been
demonstrated to be radiation-resistant but to varying degrees. For instance, *Pseudomonas radiora* isolated from cereal grain is much more resistant to the lethal effects of gamma-rays than are other pseudomonads but it is considerably more sensitive than is *M. radiodurans* (Ito and Iizuka, 1971). However, a radioresistant bacterium designated *Arthrobacter radiotolerans* isolated from a radioactive hot-spring in Japan, was demonstrated to be substantially more resistant to gamma-rays than *M. radiodurans* (Yoshinaka *et al.*, 1973). A Gram-positive rod, slightly more resistant to gamma-rays than *M. radiodurans*, has been isolated from the faeces of a giant panda (Kobatake *et al.*, 1977).

The reasons for studying these radiation-resistant bacteria rests primarily on the ability of these organisms to overcome the lethal effects of UV and ionizing radiations. Most attention has been paid to *M. radiodurans*. Many reasons have been put forward to account for its extreme resistance. Kilburn *et al.* (1958) first suggested that the carotenoid pigment was important and was confirmed by reports that pigmentless mutants of *M. radiodurans* were more sensitive than the wild-type to X-rays (Moseley, 1963). However, other work found no difference in sensitivity of pigmentless mutants (Okazawa and Matsuyama, 1967; Moseley, pers. commun.). Furthermore, alteration of the pigment levels of both *M. radiodurans* and *M. radiophilus* had no effect on their sensitivity to gamma-radiation (Lewis *et al.*, 1974). The role of other intracellular radio-protective compounds has also been invoked. Bruce (1964) considered that the high sulphhydryl content of *M. radiodurans* may play a role and the extraction of a radiation-protection factor supported this view. More recently the association of the chromosome of *M. radiodurans* with manganese
ions has been suggested to reduce the yield of UV-photoproducts (Leibowitz et al., 1976). Although these factors may contribute in part to the extreme resistance of *M. radiodurans* to UV and ionizing radiations they are at most of minor importance since efficient DNA repair mechanisms appear to be the major reason for tolerance. Thus, Boling and Setlow (1966) observed the removal of UV-induced pyrimidine dimers from the DNA of *M. radiodurans* and the subsequent excretion of the dimers, in the form of small oligonucleotides, into the suspending medium. The presence of a recombination repair system has also been invoked indirectly by the isolation of a mutant which has very much reduced transformability and is also sensitive to DNA-damaging agents (Moseley and Copland, 1975). Other mutants have been isolated which are considerably more sensitive than the wild-type to the lethal effects of UV and/or ionizing radiations and to other DNA-damaging agents and has indicated the importance of cellular repair mechanisms (Moseley, 1967, 1969; Okazawa and Matsuyama, 1967; Suhadi et al., 1971; Kitayama and Matsuyama, 1975; Moseley and Copland, 1973, 1978) some of these mutants will be described more fully below.

Besides being able to repair such a large amount of damage sustained by its DNA such that lethal lesions are no longer present, *M. radiodurans* is able to accomplish this without appreciable error. Thus, *M. radiodurans* cannot be induced to mutate to trimetho-prim-resistance by gamma-rays, mitomycin C (MTC) or hydroxylamine and only weakly responds to mutagenesis by nitrous acid, \( \beta \)-propiolactone and ethyl methanesulphonate (EMS). It is, however, sensitive to a comparatively large extent by \( N \)-methyl-\( N' \)-nitro-\( N \)-nitrosoguanidine (MNNG) (Sweet and Moseley, 1976). From these results it was concluded
(Sweet and Moseley, 1976) that *M. radiodurans* lacks an error-prone pathway similar to the one operative in *Escherichia coli*.

Although some aspects of resistance can be explained by excision and post-replication repair it has not been clear why these modes of repair should be so very different from *E. coli* unless there was some other major factor(s) which enables *M. radiodurans* to survive excessive DNA damage. One very important observation recently reported is that *M. radiodurans* contains multiple copies of its genome (Hansen, 1978). By studying the rates of DNA renaturation Hansen has estimated that in rapid exponential growing cells, which have been used in most experiments, up to 10 copies of the chromosome are present. Thus, it may be envisaged that although a single cell of *M. radiodurans* may be able to sustain relatively large amounts of damage to its DNA if this amount is diluted amongst multiple genomes then by recombinational exchanges at least one intact genome could in theory be produced and result in a viable cell. Although no evidence is available as to whether a pre-replication form of recombination repair is operative it does seem that this hypothesis could account for many of the available data. For instance, the wild-type can repair some 1400 double-strand breaks in its DNA and although the role of the membrane in perhaps "holding together" in close proximity DNA ends has been implicated (Burrell *et al.*, 1971; Dardalhon-Samsonoff and Rebeyrotte, 1975) the precise mode of repair had not been envisaged. However, the findings that the repair of double-strand breaks in *E. coli* requires both a recombination function and an homologous chromosome (Krasin and Hutchinson, 1977) enables such a model to be directly applicable to *M. radiodurans*. 
1.1 DNA repair-deficient mutants of *M. radiodurans*

Several mutants of *M. radiodurans* have been isolated on the basis of their sensitivity to the lethal effects of radiations and chemical agents. Thus after MNNG mutagenesis two UV-sensitive mutants, UV17 and UV38, were isolated (Moseley, 1967). These were also shown to be sensitive to the lethal action of gamma-rays, MTC and MNNG. Both mutants were reported to be slow in removal of cytosine-thymine dimers (Moseley, 1969) and UV17 has been shown that some X-ray induced single-strand breaks are not repaired (Bonura and Bruce, 1974). Bonura and Bruce also showed that UV17 has a higher than wild-type nuclease activity which results in a defective polymerase-mediated form of repair. Other gamma-ray-sensitive mutants have been isolated and have been shown to be defective in the restoration of full molecular weight of their DNA after gamma-irradiation (Suhadi *et al.*, 1971, 1972; Kitayama and Matsuyama, 1975). Excessive nuclease activity in one of these mutants also occurred as well as reduced DNA polymerizing activity (Kitayama and Matsuyama, 1975). A mutant, rec3O, unable to be transformed has been presumptive evidence for the existence of a recombination repair function in the wild-type since the mutant is also sensitive to UV and gamma-radiation and is inactivated probably by 1 MTC-induced crosslink (Moseley and Copland, 1975).

Other genes involved in the repair of MTC-induced damage and in both MTC- and UV-induced damage have also been described (Moseley and Copland, 1978).
2. Aims of the present work

In *M. radiodurans*, *M. roseus* ATCC 19172, *M. radiophilus* and *M. radioproteolyticus* it is apparent from the fine structure of the cell wall, its chemical composition and the extreme resistance of these organisms to UV and ionizing radiations that they differ markedly from a typical Gram-positive micrococcus such as *Micrococcus luteus* and it has been suggested that they be reclassified into a new taxonomic group (Baird-Parker, 1965; Thornley et al., 1965; Sleytr et al., 1973, 1976). However, there have been no reports as to DNA homology within the radiation-resistant group. One approach would be direct DNA hybridization. Another might use the ability of at least some members of this group to be transformed by exogenous DNA. Such an approach has been made within the family Micrococccaceae. DNA from *M. radiodurans* was unable to transform auxotrophs of *M. luteus* to prototrophy (Kloos, 1969). *M. radiodurans* itself is capable of undergoing genetic transformation (Moseley and Setlow, 1968) and with relatively high frequencies (Tirgari, 1977). An attempt has been made in the work described here to establish taxonomic relationships between the radiation-resistant micrococci by interspecific transformations of *M. radiodurans*.

*M. radiodurans* is immutable by many DNA-damaging agents which are capable of exerting a mutagenic effect in *E. coli* (Sweet and Moseley, 1976). The possible range of mutagenic agents was extended in the work described here to include the methylating agents methyl methanesulphonate (MMS) and N-methyl-N-nitrosourethane (MNUR), the "frameshift mutagen" ICR-191 and the base analogues 2-aminopurine (2-AP) and bromouracil (BrUra).
Because of the response of \textit{M. radiodurans} to mutagenesis it has been concluded that it lacks an error-prone repair pathway (Sweet and Moseley, 1976). To see if this was a characteristic of the other radiation-resistant micrococci the ability of UV to induce rifampicin-resistant mutants of \textit{M. roseus} ATCC 19172, \textit{M. radiophilus} and \textit{M. radioproteolyticus} was examined to see if these bacteria possess error-prone repair. Other bacteria are known which also lack \textit{E. coli}-type error-prone repair but these are considerably more sensitive than is \textit{M. radiodurans} to the lethal effects of UV and ionizing radiations. On the other hand does extreme radio-resistance \textit{a priori} mean that error-prone repair is likely to be absent? This question was resolved by examining the UV mutability of \textit{Pseudomonas radiora}, \textit{Arthrobacter radiotolerans} and the asporogenous rod designated P-30-A.

Some features of the repair of UV-induced damage in \textit{M. radiodurans} was examined in the hope that these might yield information as to why error-prone repair is absent. Specifically, attempts were made to look at the size of the reinserted patch to see whether, as in \textit{E. coli}, there is both long and short patch repair. The use of nucleotide-permeable cells also might give an indication as to patch size and also indicate ATP requirements of any specific steps in repair.

Surprisingly, a strain of \textit{M. radiodurans} unable to excise pyrimidine dimers has not yet been reported. Such a strain, besides indicating the involvement of excision repair in total repair responses would also be useful to investigate other repair phenomena which are facilitated by the non-removal of pyrimidine dimers from the DNA. An attempt was made to isolate one such mutant.
In general, DNA repair studies in both bacteria and higher organisms have concentrated primarily on the repair of lethal damage. Only a limited amount of information is available on repair-deficient mutants responses to mutagenic damage by agents which do not act via error-prone repair. Because MNNG was the only mutagen capable of mutating *M. radiodurans* wild-type to any significant extent the sensitivity of some repair-deficient mutants routinely isolated in this laboratory over a period of several years, were examined for their sensitivity to the mutagenic effects of MNNG. Interesting mutants would then be examined by biophysical and biochemical means in order to establish why they differed in response to the wild-type which may further explain the mechanism of mutagenesis by at least some error-prone repair independent mutagens.

Some aspects of this work have already been reported (Tempest and Moseley, 1977, 1978).

The remainder of this introduction will put the work described in this thesis into perspective.
3. Heterologous transformation

The ability of DNA from one bacterial species to transform a competent recipient cell of another species is known as heterologous or interspecific transformation. Such interspecific genetic exchange has been demonstrated in nearly all transformable genera and include transfer between several species of *Haemophilus* (Leidy et al., 1956, 1959), between streptococci and *Diplococcus pneumoniae* (Bracco et al., 1957), within the family Neisseriae (Catlin, 1961) and between several species of *Bacillus* (Marmur et al., 1963).

The distinguishing feature of heterologous transformation is the relatively low frequency of occurrence compared with homologous transformation frequencies. The quantitative difference between heterologous and homologous transformation frequencies depends on the particular genes being transferred. For example, Dubnau et al. (1965) found that certain regions of the chromosome of some *Bacillus* species viz., those that confer resistance to streptomycin, erythromycin and to other antibiotics are transformed heterologously at much higher frequencies than other regions, such as those containing auxotrophic markers. Resistances to streptomycin and erythromycin are believed to involve changes in the ribosomes and such rRNA regions show higher levels of homology between species as measured by nucleic acid hybridization as if these ribosomal loci have been conserved in evolution between species (Doi and Igarashi, 1965; Dubnau et al., 1965; Chilton and McCarthy, 1969).

Three major explanations have been proposed to account
for the low frequency of heterologous transformation:

(1) discrimination against uptake of heterologous DNA as compared to homologous DNA; (2) after entry into the cell the heterologous DNA could be enzymically restricted; (3) differences in the nucleotide sequences of the donor and recipient DNAs leading to reduced recognition and therefore integration of the heterologous DNA.

Differential uptake of DNA does not appear to be important because radioactively-labelled DNA is taken up equally well by both homologous and heterologous strains of Haemophilus (Schaeffer, 1958). Furthermore, such discrimination does not occur in any systems showing measurable heterologous transformation (e.g. Marmur et al., 1963). Restriction of foreign DNA by specific enzymes is well recognized. Although crude extracts from H. influenzae (Smith and Wilcox, 1970), from B. subtilis (Wilson and Young, 1972) and from B. globigii (Harris-Warrick and Lederberg, 1978) are capable of degrading foreign DNA, site-specific cleavage of transforming DNA has not been demonstrated in vivo. Indeed, alteration of the restriction and modification phenotypes of Bacillus (Trautner et al., 1974), Streptococcus (Ravin and Chakrabarti, 1975) and Haemophilus (Stuy, 1976) has little or no effect on the frequencies of heterologous transformation.

Schaeffer (1958) first suggested that the lower frequency of heterologous transformation might be due to nucleotide sequence non-homology of the DNA molecules. Attempts were then made to correlate interspecific transformability with DNA base composition with genera such as Neisseria (Catlin and Cunningham,
1961) and Bacillus (Marmur et al., 1963). The overall base composition is a necessary but not sufficient requirement for heterologous transformation. Genetically hybrid DNA obtained from heterologous transformation is often intermediate in its transforming efficiency between the DNAs of the original donor and the recipient (Biswas and Ravin, 1971, 1976; Wilson and Young, 1972; Ravin and Chakrabarti, 1975; Harris-Warrick and Lederberg, 1978), which has been interpreted to be due to the production of non-homologous sequences. These results, together with the correlation between the extent of nucleic acid hybridization and heterologous transformation, argue strongly that sequence non-homology is the major barrier to interspecies transformation.

This mode of genetic exchange has been shown to be useful in measuring DNA homology between various species and can be used as a criterion in establishing taxonomic relationships where other means of interspecies genetic exchange are not available. Such approaches have been used to determine the closeness of species within genera such as *Neisseria*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Micrococcus* and *Haemophilus* (reviewed by Mandel, 1969).
4. Mechanisms of DNA repair

The genetic material is susceptible to many different kinds of damage which can be produced by spontaneous reactions and by external physical and chemical agents. Most of the known types of damage result in inactivation of the template and/or in alterations of template specificity. It is therefore not surprising that as early forms of life emerged into environments which were increasingly exposed to DNA damaging agents (predominantly solar UV) and also evolved larger genome sizes it was necessary for a parallel evolution of DNA repair mechanisms. Three basic types of DNA repair processes are known: photoreactivation repair (PR), excision repair and tolerance repair and are diagramatically represented in Figures 1 and 2. A general review of these repair mechanisms will not be attempted. Some aspects will be only briefly mentioned and others, more pertinent to the work reported in this thesis, will be discussed in more detail. More exhaustive information can be obtained from the reviews of Grossman et al., 1975; Hanawalt, 1975; Kondo, 1975; Harm, 1976; Witkin, 1976; Bridges, 1977; Lehmann and Bridges, 1977; Moseley and Williams, 1977.

4.1 Photoreactivation

With the exception of the sealing of certain single-strand breaks by polynucleotide ligase, photoreactivation is the only repair process that is dependent solely on a single enzyme. The PR enzyme binds specifically to pyrimidine dimers, a reaction which may occur in the dark. After the dimer-enzyme complex has absorbed sufficient photons the dimers are photochemically reversed to monomers in situ. Photoreactivation is almost universal amongst organisms but rather unexpectedly is absent from M. radiodurans.
Figure 1  Simplified diagrammatic representations of DNA repair mechanisms. A. Photoreactivation repair, B. nucleotide excision repair, C. base excision repair. Symbols: —, pyrimidine dimer; • , normal base; • , abnormal base; heavy lines, DNA polymerized during repair synthesis. The diagrams are not intended to show relative sizes of excised fragments or repair patches. After Witkin, 1976.
Figure 2  Simplified diagrammatic representations of DNA tolerance repair mechanisms. A. Recombination repair, B. replication repair. Symbols: --- pyrimidine dimer or some other non-instructive lesion; heavy lines, DNA polymerized in post-treatment DNA replication; dotted lines, DNA polymerized during repair synthesis. After Witkin, 1976; Hughes et al., 1976.
4. 2 **Excision repair**

Excision repair consists of a series of enzymic steps which result in the physical removal of the damage from the DNA. There are two types of this repair which differ in the nature of the excised damage. In nucleotide excision repair the damage is released in the form of a small oligonucleotide fragment. In base excision repair a specific glycosylase hydrolyses the bond between the damaged base and the deoxyribose moiety (Lindahl, 1976). The apurinic or apyrimidinic (AP) site may then be subject to nucleotide excision repair. Most work has concentrated on the excision of pyrimidine dimers from the DNA of *E. coli* which was first demonstrated by Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964a). The model that has emerged over the years seems to be applicable to the removal of other sorts of damage and to repair in other organisms. There would appear to be a minimum of four steps: (i) incision of the DNA near the damaged site by a damage-specific endonuclease; (ii) removal of the damage by the action of an exonuclease; (iii) DNA synthesis to fill in the excision gap; (iv) ligation of the newly synthesized DNA to the pre-existing DNA strand. Some of the methods involved in studying excision repair and the results obtained will be discussed.

(i) **Incision.** The principles outlined by McGrath and Williams (1965) have been extensively employed. This method depends on the sedimentation properties of DNA in alkaline sucrose. The incision of damaged DNA and the subsequent repair of the gaps can then be followed by observing the profile patterns.

Such studies in *M. radiodurans* have shown that DNA
damaged in vivo by UV is incised by (an) endonuclease(s) (Moseley, 1969). The low molecular weight DNA produced by these enzymes and by gamma-ray induced single-strand breaks have been shown to be restored to a high molecular weight form (Moseley, 1969; Kitayama and Matsuyama, 1975). Furthermore, in neutral sucrose the repair of double-strand breaks has been demonstrated (Kitayama and Matsuyama, 1968, 1971). The substrate specificities of the enzymes recognizing damage in the DNA of M. radiodurans is not known. In E. coli the endonuclease coded for by the uvrA,B gene products besides recognizing pyrimidine dimers (Braun and Grossman, 1974) is also involved in the repair of DNA damaged by MTC, nitrous acid, psoralen plus light, bifunctional mustards, decarbamoyl mitomycin C(DCMTC), 4-nitroquinoline 1-oxide (NQO), 7-bromomethylbenz[a]anthracene (BrMBA), N-acetoxy-2-acetylaminofluorene (AAAF) (Boyce and Howard-Flanders, 1964b; Kohn et al., 1965; Lawley and Brookes, 1965; Kondo et al., 1970; Otsuji and Murayama, 1972; Venitt and Tansy, 1972; Thielmann, 1976). The enzyme is probably recognizing some kind of gross distortion of the secondary structure of DNA (see review of Cerutti, 1975). Also both rat liver and Bacillus subtilis possess an endonuclease which can recognize DNA damaged by both UV, BrMBA and AAAF (Van Lancker and Tomura, 1974; Maher et al., 1974; Tanooka, 1977). However such spectra of specificities are not universal. For example, purified UV endonucleases from Micrococcus luteus do not degrade DNA damaged by BrMBA or psoralen plus light (Tomilin et al., 1976; Riazuddin and Grossman, 1977a) and T4 endonuclease V incises DNA damaged by UV but not by MTC or NQO (Friedberg, 1972). In mammalian tissues there are indications that repair of UV-induced damage and of damage
caused by AAAF and BrMBA occur through different pathways (Ahmed and Setlow, 1977; Amacher et al., 1977; Dipple and Roberts, 1977).

(ii) Specific removal of damage. The instances of the loss of damage from DNA in vivo has been mainly to loss of pyrimidine dimers since these are readily identifiable. Such excision has been demonstrated in *M. radiodurans* (Boling and Setlow, 1966), and in *M. radiophilus* (Lavin et al., 1976). Gamma-radiolysis products have also been shown to be specifically excised from the DNA of *M. radiodurans* (Hariharan and Cerutti, 1971, 1972) as well as from *E. coli* (Hariharan and Cerutti, 1974). In the latter case, the excision was independent of the *uvrA,B* gene products. Differential responses to excision of different BrMBA-DNA adducts has been demonstrated in human lymphocytes, Chinese hamster cells, HeLa cells and *E. coli* (Lieberman and Dipple, 1972; Dipple and Roberts, 1977; Venitt and Tarmy, 1972). The only other types of damage for which specific excision in vivo has been demonstrated is that of alkylation damage by monofunctional and some bifunctional alkylating agents which is discussed in further detail below.

The exonucleases possibly involved in the excision of damage have been reviewed by Grossman et al. (1975).

(iii) Filling in of excision gaps. The use of density labels has enabled the demonstration of a non-semiconservative type of DNA synthesis after damage to DNA. In *E. coli* the majority of excision gaps are subject to efficient reinsertion mechanisms since the size of most of the patches are generally 10 to 30 nucleotides in length (Setlow and Carrier, 1964; Cooper and Hanawalt, 1972; Ben-Ishai and Sharon, 1978). However, a minority are apparently repaired by a complex process requiring the *recA* gene product and produce tracts
of between 1000 to 3000 nucleotides long (Cooper and Hanawalt, 1972).

The permeabilization of *E. coli* with toluene has been useful in showing the involvement of the various DNA polymerases, the requirement for ATP during repair synthesis (reviewed by Hanawalt, 1975) and the requirement for ATP in UV-specific incision (Waldstein et al., 1974).

4.3 Tolerance repair

This may be defined as the ability to produce damage-free copies of DNA from damaged DNA templates (Kondo, 1975). The precise mechanisms of tolerance repair have not yet been elucidated but may be of two basic types: recombination repair where the damage can be by-passed in an error-free manner and a possibly more error-prone way when synthesis past a non-coding lesion can occur.

(i) **Recombination repair** A hypothetical but generally accepted model initially proposed by Rupp and Howard-Flanders (1968) is presented in Figure 2. They demonstrated that DNA synthesized in *E. coli* after irradiation contained breaks. The frequency of these breaks approximated to the number of pyrimidine dimers. From this relationship it was inferred that DNA synthesis proceeds until it encounters a non-coding lesion such as a pyrimidine dimer and a gap is produced. The size of this gap has been estimated to be about 1000 nucleotides (Iyer and Rupp, 1971). It is thought that DNA synthesis then begins at the next reinitiation site for Okazaki fragment formation. The discontinuous daughter strands are then filled in by recombinational exchanges with an homologous chromosome (Rupp et al., 1971).

(ii) **Replication repair** In *E. coli* at least it appears likely
that in some circumstances there is an inducible function which allows DNA synthesis to proceed past a pyrimidine dimer or other non-instructive lesion by random nucleotide insertion with concomitant high probability of error. This error-prone repair is discussed more fully below.

In mammalian cells there is evidence to suggest that post-repair replication does not necessarily involve recombinational exchanges (Lehmann, 1972). A model which could account for such observations has been proposed by Higgins et al. (1976) and is depicted in Figure 2. DNA synthesis proceeds until it is blocked by a lesion but replication proceeds on the complementary strand. There is then strand displacement and branch migration which puts the two daughter strands in close proximity which allows DNA synthesis to continue from the point at which it was inhibited. The replication fork can then move to its original position resulting in the lesion being by-passed in an error-free manner. Higgins et al. (1976) give some evidence that such repair might occur.

5. Mechanisms of mutagenesis

5.1 Error-prone repair (SOS repair, misrepair)

Prior to 1967 DNA repair processes were thought only to eliminate potential mutagenic damage, as indeed many do. This, however, is not always the case as Witkin (1967) found that certain
repair-deficient mutants i.e. recA and exrA (=lexA) which were sensitive to the lethal effects of UV were refractory to UV-induced mutagenesis. This was true whether the strains were excision-proficient or excision-deficient. Thus repair of potentially lethal damage could occur with no detectable error. She initiated the concept of error-free and error-prone repair pathways and suggested that UV-induced mutagenesis resulted from an error-prone form of post-replication repair.

Genes that resemble either recA or lexA in their action on sensitivity to the lethal effects and resistance to the mutagenic effects of UV and other DNA-damaging agents have been described in *Schizosaccharomyces pombe* (Nasim, 1968), *Saccharomyces cerevisiae* (Lemontt, 1973) and phage T4 (Green and Drake, 1974). In fact, if any organism is strongly mutated by UV light, the presence of an error-prone repair pathway may be inferred. However, such a misrepair process does not appear to be ubiquitous and is absent, at least in the form it occurs in *E. coli*, from *Proteus mirabilis* (Böhmé, 1963), *Methylococcus capsulatus* (Harwood et al., 1972), *Micrococcus radiodurans* (Sweet and Moseley, 1974, 1976), *Haemophilus influenzae* and *H. parainfluenzae* (Kimball et al., 1977) and *Diplococcus pneumoniae* (Tiraby, unpublished results cited by Drake and Baltz, 1976) since these species appear to be immutable by UV.

In *E. coli* many DNA-damaging agents depend for their mutagenic effectiveness on error-prone repair, i.e. the lesion is a necessary but not a prerequisite for mutagenesis. Thus, strains carrying mutations in either recA or lexA are immutable by UV, gamma-rays, thymine deprivation, NQO, MTC, DCMTC, MMS, dichlorvos
and BrMBA whilst exhibiting an increased sensitivity to the lethal effects of these agents (Witkin, 1967; Bridges et al., 1968; Kondo et al., 1970; Bridges et al., 1973; Murayama and Otsuji, 1973; Tarmy et al., 1973).

Because mutants of *E. coli* (and other organisms) with defective error-prone repair processes are invariably more sensitive to the lethal effects of the mutagens led Radman (1974) to introduce the term SOS repair. It was assumed that error-prone repair was a last ditch repair system for a lesion in DNA which could not be repaired by constitutive error-free mechanisms. A viable cell would be produced but at the expense of changes in the genetic script. However, some recent evidence suggests that the survival value in an individual cell conferred by the error-prone step is not as great as was first imagined. This idea comes from the findings that certain mutants of *E. coli* compared to *lexA* or *recA* have a much greater resistance to the lethal effects of UV (but not full wild-type resistance) whilst remaining completely refractory to mutagenesis (Volkert et al., 1976; Kato and Shinoura, 1977). It can therefore be envisaged that when cells were in a situation where error-prone repair was induced then it acted to cause specifically mutations with the result that any survivors might be able to cope better with the new environment. However, the tenet that SOS repair acts on lesions which cannot be repaired in an error-free manner seems to be true. What are these lesions?

Sedgwick (1976) has proposed that SOS repair acts when lesions introduced into DNA are in close proximity and yield a configuration in DNA that is difficult or impossible to repair in
an error-free way. He has presented evidence that overlapping daughter strand gaps produced as a consequence of replication on a dimer containing template may be such lesions. Long patches produced during excision repair (Cooper and Hanawalt, 1972) may also serve as sources of mutations if two patches are in close proximity or if a patch overlaps with a non-coding lesion in the other strand.

The mutagenic step in SOS repair is assumed to be random insertion of nucleotide(s) opposite a non-coding lesion. UV-induced reversion studies of T4 II mutants have indicated that pyrimidines are the primarily affected base (Drake, 1963). Furthermore, UV has been shown to be capable of producing tandem base pair substitutions in both E. coli (Yanofsky et al., 1966; Coulondre and Miller, 1977) and in S. cerevisiae (Lawrence et al., 1974). A study of DNA synthesized intracellularly using UV-irradiated øX174 DNA as a template has indicated that dimers block replication. However, in the presence of SOS repair manifestation DNA synthesis can proceed past such lesions (Caillet-Fauquet et al., 1977). These results strongly suggest that SOS repair acts opposite non-coding lesions, such as pyrimidine dimers, although this need not necessarily be the only site.

Several mechanisms have been proposed to account for the error-prone step. For instance it could be the result of a terminal nucleotidyl transferase or a novel DNA polymerase but there is no evidence to support these postulates (see Witkin, 1976; Bridges, 1977). One attractive model has been proposed by Villani et al. (1978). These authors propose that at a non-coding lesion DNA polymerase "idles" due to the removal of mismatched bases put in by the polymerase activity opposite the
non-coding lesion by the 3' to 5' exonuclease activity. In the
presence of SOS repair manifestations, possibly induced as a
result of the large turnover of nucleoside triphosphates to
monophosphates, this exonuclease proof-reading is inhibited which
permits polymerization past the lesion. DNA polymerase III may be
the mutagenic enzyme (Bridges et al., 1976; Bridges and Mottershead,
1978).

UV, and several other DNA-damaging agents, are not able to
mutate M. radiodurans at several loci examined (Sweet and Moseley,
1974, 1976; Kerszman, 1975) and it has been inferred that
M. radiodurans lacks an error-prone repair process. In E. coli
it is probable that if SOS repair fails to act on a lesion which
cannot be repaired in an error-free way, then a lethal event occurs.
Thus, that Proteus mirabilis and Haemophilus influenzae lack
error-prone repair is reflected by their UV sensitivity, which is
greater than wild-type strains of E. coli. Simply put, these
bacteria die rather than mutate. However, M. radiodurans does not
mutate but does not die either since it can tolerate and repair
exceptionally high levels of DNA damage without the production of
errors. It may, therefore, be that the number of lesions
requiring error-prone repair are lower in M. radiodurans than in
E. coli or, alternatively, such lesions are produced frequently but
are repaired or tolerated by mechanism(s) not present in E. coli.

5.2 Misreplication

Ever since the structure of DNA was understood one
mechanism of mutagenesis proposed was the replication of DNA
containing bases in rare tautomeric states (Watson and Crick, 1953;
Topal and Fresco, 1976). Thus the classic mutagens BrUra and
2AP were proposed to exist more frequently in tautomeric states which would cause them to occasionally mispair with cytosine and guanine respectively (see Freese, 1971). Despite the iconoclastic report of Pietrzykowska (1973) that base analogue mutagenesis of lambda depended on error-prone repair other workers have concluded that BrUra mutagenesis of E. coli and lambda is independent of error-prone repair (Witkin and Parisi, 1974; Rydberg, 1977; Hutchinson and Stein, 1977). However, error-free repair processes are operative in reducing the lethal and mutagenic effects of BrUra (Pietrzykowska et al., 1975; Rydberg, 1977). Rydberg (1977, 1978) has presented evidence that an excision repair process is present in E. coli which recognizes mismatches containing BrUra residues since strains with defective heteroduplex repair are hypermutable by BrUra.

Hydroxylamine is another mutagen capable of exerting a mutagenic effect in the absence of error-prone repair (Ishii and Kondo, 1975; Hutchinson and Stein, 1977). In solution, hydroxylamine is able to modify predominantly cytosine but also to a lesser extent adenine to give $N^4$-hydroxycytosine and $N^6$-hydroxyadenine respectively. In in vitro synthesizing systems both these bases have been shown to cause mispairing (reviewed by Budowsky, 1976).

Nitrous acid-induced deamination of adenine and cytosine to hypoxanthine and uracil might be expected to cause transition mutations on simple (mis)pairing schemes. Apparently nitrous acid mutagenesis is more complex, even in phages. For example, nitrous acid mutagenesis of lambda depends on the host recA and exrA functions (Kerr and Hart, 1972) and in T4 on the presence of
a ligase function (Bernstein et al., 1976). DNA glycosylases have been identified in E. coli which can release uracil and hypoxanthine from DNA (Lindahl, 1974; Karran and Lindahl, 1978).

In her paper of 1967 Witkin first mentioned that MNNG was able to mutate an exr strain of E. coli. This mutagenicity of MNNG in the absence of error-prone repair was later confirmed and extended to include EMS (Kondo et al., 1970; Ishii and Kondo, 1975). The mutagenicity of these alkylating agents probably depends on their ability to produce certain alkylated bases which are capable of mispairing, although damage to non-DNA material may also play a contributory role. A more detailed discussion of alkylation mutagenesis is presented below.

DNA polymerases also play a crucial role in misreplication mutagenesis by chemical mutagens. For example, in T4 mutagenesis by 2AP, BrUra and to a lesser extent by EMS and nitrous acid are reduced by antimutator DNA polymerases (Drake and Greening, 1970). The role of DNA polymerase in nucleotide selection in vivo has been more directly invoked by the demonstration that the incorporation of 2AP into T4 DNA by mutator DNA polymerase is higher than by the wild-type enzyme which in turn is greater than by antimutator DNA polymerase (Goodman et al., 1977).

6. Alkylating agents

Alkylating agents are electrophilic reactants which in polar solvents donate an alkyl group or groups to nucleophilic sites of other molecules and include alkyl sulphates, alkyl sulphonates, alkyl halides, alkyl nitrosamines, alkyl nitrosamines,
alkyl nitrosamides, alkyl nitrosamidines, mustards, diazo compounds, lactones, epoxides and others. The chemical formulae of the alkylating agents used in this thesis are listed in the Appendix. That alkylating agents are capable of exerting biological effects stems from the observations made in the 1940s that mustard gas was capable of exerting a mutagenic and lethal effect in Drosophila (Auerbach and Robson, 1946). This was the first example of experimental mutagenesis by a pure chemical agent and essentially all of the above listed alkylating agents have been shown to be mutagenic in one or often several organisms, both prokaryotic and eukaryotic, and for mammals they constitute some of the most potent carcinogens.

These agents may be considered as to whether they donate a simple alkyl group eg. CH$_3$, CH$_2$CH$_3$ or whether the substituent contains an alkyl group to which is attached complex carbon rings eg. MTC, BrMBA. Another characteristic of an alkylating agent is its functionality ie. the number of alkyl groups that a single molecule can donate. For example, MTC has two sites open to attack by nucleophiles and consequently may react with a macromolecule such as DNA at two positions, thus producing a more complex lesion than that produced by simple substitution of a CH$_3$ group. Some of the chemicals mentioned above are not alkylating agents per se but require chemical conversion. For example, nitrosamines only yield an alkylating intermediate after metabolic activation of a form which is absent from bacteria. Nitrosamidines and nitrosamides decompose before yielding the eventual alkylating species. MTC has to be chemically reduced before it is able to react with biological macromolecules.
Before the biological effects of alkylating agents are discussed the nature of the reaction mechanisms will be considered since this is of fundamental importance and a knowledge of the mechanism can often give information as to what biological effects a chemical may have. Two principle types of nucleophilic substitution reactions have been recognized and are referred to as SN1 and SN2. The former unimolecular reaction type occurs in two steps. The first rate-determining step is the slow ionization to yield the carbonium ion: \[ RX \rightarrow R^+ + X^- \]. Different nucleophilic reagents (Y\(^-\)) may then exhibit different affinities to the carbonium ion and will react very quickly: \[ R^+ + Y^- \rightarrow RY \]. In contrast, SN2 reagents form a transition complex with the nucleophilic which then dissociates into the products:

\[ Y + RX \rightarrow Y...R...X \rightarrow YR + X. \]

The rate depends on the concentration of both the alkylating agent and the nucleophile and the kinetics are therefore bimolecular. The alkylating agents MMS and dimethyl sulphate (DMS) react via an SN2 mechanism whereas agents such as MNNG and N-methyl-N-nitrosourea (MNUA) react predominantly via an SN1 mechanism. Ethyl groups, compared with methyl groups, increase the stability of the carbonium ion and therefore EMS exhibits greater SN1 characteristics than MMS, although it still shows considerable SN2 reaction characteristics. Similarly, N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) and N-ethyl-N-nitrosourea (ENUA) will exhibit more SN2 characteristics than their methyl analogues. These reaction characteristics are important since as reactivity by an SN1 mechanism increases, the substrate factor \( s \) decreases and the relative ability to alkylate sites of low nucleophilicity \( eg. O \) atoms as compared to \( N \) atoms increases. These differences are
exemplified in Table 1 where ability to alkylate the $O^6$ and $N7$ positions of guanine in DNA is compared.

6.1 Reactions with nucleic acids

(i) Simple alkylating agents Since alkylating agents react with $O,N$ and $S$ atoms it is obvious that they will react with essentially all chemical components within a cell. For the most part attention has been narrowed to the reactions with nucleic acids since these are likely to be of fundamental importance when changes in the genetic script occur. However, alkylation and subsequent changes in the properties of proteins involved in nucleic acid metabolism could also have genetic consequences. Alkylation of bases in vitro has been documented but the reactions are quite different from those of nucleosides and nucleotides, which are the actual reactive components of nucleic acids. The reactions of alkylating agents with nucleosides has been reviewed by Singer (1975) and nearly all, or all, theoretically possible alkylation products have been identified.

The first attempts to identify alkylation products in nucleic acids were in the 1960s. Brookes and Lawley (1960) reacted $^{35}$S labelled mustard gas with both RNA and DNA in vitro and in vivo using nucleic acids from TMV, Bacillus megaterium and cells of Ehrlich ascites tumour. In such cases the only detectable reaction with both DNA and RNA was at the $N7$ of guanine. A similar result was found using DMS to methylate TMV RNA (Frankel-Conrat, 1961). Some time later Lawley and Brookes (1963) examined the products of alkylation after in vitro treatment of RNA with MMS and EMS and could further
Table 1

Relative extents of alkylation in DNA in relation to the reaction mechanism of alkylating agents
(from Lawley, 1974a, 1976a)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Reaction mechanism</th>
<th>$s^a$</th>
<th>Ratio $O^6$: N7 alkylation of DNA guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS</td>
<td>SN2</td>
<td>0.83</td>
<td>0.004</td>
</tr>
<tr>
<td>EMS</td>
<td>SN1/SN2</td>
<td>0.67</td>
<td>0.03</td>
</tr>
<tr>
<td>MNNG</td>
<td>SN1</td>
<td>0.42</td>
<td>0.1</td>
</tr>
<tr>
<td>MNUA</td>
<td>SN1</td>
<td>0.42</td>
<td>0.1</td>
</tr>
<tr>
<td>ENUA</td>
<td>SN1</td>
<td>0.26</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$^a$ is a substrate constant which expresses the sensitivity of an alkylating agent to changes in the nucleophilic strength of the nucleophile (for details see Lawley, 1974b).
identify 1-alkyladenine and 3-alkylcytosine, although compared with the major product 7-alkylguanine, these were present only in small amounts. These authors also treated salmon sperm DNA with MMS and could also identify 3-alkyladenine as well as those found in RNA. One year later Lawley and Brookes (1964) reported that in vitro treatment of DNA with DMS and diethyl sulphate (DES) gave 7-methyl and 7-ethyladenine as minor products of alkylation.

There then followed a period of several years when no new products of alkylation were reported until, in 1969, stimulus was provided by the findings of Loveless. Until this time no significant differences were found in the products of alkylation in a series of alkylating agents although it was realized that each agent has profoundly different biological effects. Loveless (1969) found that EMS, MNUA and ENUA all reacted with deoxyguanosine at the O6 position but that MMS did not. Much attention was then, and still is, focussed on this difference in order to try to correlate the ability of an agent to be mutagenic (or carcinogenic) and its ability to produce O6-alkylguanine. This difference was then extended to DNA where it was shown that about 7% of total alkylations were at the O6 position of guanine after in vitro or in vivo methylation with MNNG (Lawley and Thatcher, 1970). MNUA produced a similar spectrum of alkylation products to MNNG whereas MMS was unable to produce any O6-methylguanine (Lawley et al., 1973; Lawley et al., 1971/72). However, more refined analytical methods enabled the detection of a small proportion (0.0034) of MMS methylation products to be the O6 position of guanine (Lawley and Shah, 1972). The N3 of guanine was alkylated by MNUA and DMS to the same level and is thus a site not favoured by any class of agent (Lawley et al., 1971/72).
Two other minor products of MNNUA alkylation that have also been described are 3-methylthymine and _O_\(^4\)-methylthymine (Lawley et al., 1973; Lawley and Shah, 1973).

By far the majority of alkylated bases that were identified up to 1976 were alkylated purines because of the ease of liberation from DNA, their stability and the availability of authentic marker compounds. Recently, however, for the identification of other products, these difficulties have, in part, been surmounted and using enzyme digests of alkylated nucleic acids a host of new products have been identified (Singer, 1976). Not only were all the pyrimidine oxygens capable of being alkylated in neutral aqueous solution, but in the case of ENUA-or ENNG-treated single-stranded RNA, _O_\(^2\)-ethylcytidine and _O_\(^2\)-ethyluridine were present in greater amounts than _O_\(^6\)-ethylguanosine, and in double-stranded DNA treated with the same compounds the amount of _O_\(^2\)-ethylthymidine was equivalent to the amount of _O_\(^6\)-ethylguanosine (Singer, 1976).

The positions in bases which are attacked by these alkylating agents discussed are shown in Figure 3.

Besides oxygens in bases being attacked by alkylating agents the oxygens in phosphates are also open to alkylation. Bannon and Verly (1972) showed that phosphotriesters in DNA are quite stable and the amount produced depended on the nature of the alkylating agent. Thus when DNA is treated with EMS phosphate alkylation represents 15% of the total alkylation and only 1% when treated with MMS. Phosphotriesters in RNA are much less stable and result in chain breakage (Singer, 1975).

Another oxygen which is alkylated and therefore likely to be of some importance is the _2'-OH_ of ribose, unique to RNA.
Figure 3  The reported sites of attack by electrophilic reagents on DNA. The arrows point to positions found to be available for nucleophilic displacement and addition reactions. The approximate amounts (%) of reaction products are shown for some alkylating agents. References: Lawley and Brookes, 1963; Lawley and Thatcher, 1970; Lawley et al., 1973; Margison and O'Connor, 1973; Singer 1975, 1976; Lawley, 1976b.
Treatment of RNA in neutral aqueous solution with ENUA or ENNG gives a relatively large (10 - 15\%) amount of ethylation at this position (Singer and Kuśmierek, 1976).

(ii) Complex alkylating agents The formulae of the agents MTC, BrMBA and AAAF are shown in the Appendix. Although the sites of reaction of MTC with DNA are not precisely known guanine is the most readily alkylated base (Lipsett and Weissbach, 1964). Most alkylations are monofunctional, however a small amount of reactions, about 10-20\%, are bifunctional and lead to interstrand crosslinks (Weissbach and Lisio, 1965). Using space-filling models it seems that the $O^6$ atoms of guanine in opposite strands are the most likely sites for crosslink formation (Iyer and Szybalski, 1964). Alkylation at the N7 of guanine by MTC has been excluded (Tomasz, 1970).

In contrast to the simple alkylating agents which react predominantly with the ring N atoms the sites of attack by the arylalkylating carcinogen BrMBA in neutral aqueous solution are the extranuclear amino groups of predominantly guanine but also of adenine and of cytosine (Dipple et al., 1971).

The $N^2$ of the amino group is also a site of attack by the arylamidating carcinogen AAAF but is only about 8.5\% of reaction products. The major (88\%) site of attack is at the C8 of guanine (Westra et al., 1976). Figure 3 shows these sites.
6.2 Alkylation and mutagenesis

(1) Simple alkylating agents Since, historically, 7-alkylguanine was often the only detectable product of alkylation it was this lesion which was considered to exert the main biological effects. The first suggested mechanism for mutagenesis was based on the observation that 7-alkyldeoxyguanosine ionizes about 100 times more readily at pH7.0 than does deoxyguanosine (Lawley and Brookes, 1961). Although the degree of ionization in DNA is not known Lawley and Brookes suggested that tautomers of guanine lacking the proton at N1 might pair with thymine. Reversion analysis of T4 rII mutants produced by EMS and ethyl ethanesulphonate showed that most mutants were predominantly induced by GC→AT transitions (Bautz and Freese, 1960; Krieg, 1963a) and was consistent with this hypothesis for occasional mispairing of ethylated guanine with thymine. However, the strongest evidence that alkylation of guanine at the N7 position is not per se a mutagenic reaction has come from experiments with synthetic polynucleotides. Although methylation of poly (U,7-methylG) reduced the template activity for polypeptide synthesis there was no misincorporation of amino acids (Wilhelm and Ludlum, 1966). Similarly, at the level of transcription using the same polymer as a template for RNA polymerase showed that 7-methylguanine can still pair normally with cytosine (Ludlum, 1970). Furthermore, 7-methylGTP replaces only dGTP as a substrate for DNA polymerase albeit with lower efficiency (Hendler et al., 1970). Studies of mutagenesis in TMV showed that the extent of N7 alkylation of guanine was inversely related to mutation frequency (Singer and Fraenkel-
Conrat, 1969a,b).

The first demonstration that an alkyl base was directly capable of mispairing was the study of the template activity of copolymers of 3-methyl C and C. Rather non-specific incorporation of UTP, ATP and CTP was observed (Ludlum and Wilhelm, 1968; Singer and Fraenkel-Conrat, 1970). That such mispairings might be mutagenic in vivo came from the correlation between N3 alkylation of cytosine and mutation of TMV (Singer and Fraenkel-Conrat, 1969a,b).

The early studies of Loveless (1959) showed that EMS was an effective mutagen for extracellular phages but that MMS was ineffective and led to the suggestion that ethylated bases were mutagenic but that methylated ones were not. This hypothesis of the "uniqueness of ethylation" was disproved when it was found that MNUA was a potent mutagen for T2 (Loveless and Hampton, 1969). Since MMS produced at least as much, if not more, 7-alkylguanine than did EMS Loveless (1969) pointed out that alkylation of guanine at O6 was the most probable promutagenic reaction because this product could be found from deoxyguanosine and DNA treated with MNUA or MNNG but not when treated with MMS (Loveless, 1969; Lawley and Thather, 1970). The promutagenic status of O6-alkylguanine was strongly supported by the finding that in an in vitro RNA polymerase directed synthesis of a synthetic template containing O6-methylguanine led to the misincorporation of UMP and AMP into the product copolymer (Gerchman and Ludlum, 1973). Furthermore, studies of poly (O6-methyl G) and poly (O6-ethyl G) have shown that alkylation of O6 of guanine disrupts normal base pairing since neither polymer can form
helical structures with poly(C) (Mehta and Ludlum, 1976).

Lawley and Martin (1975) studied EMS-induced reversion of T4 rII mutants and estimated that at least one third of $O_6^\text{-ethylguanines}$ would miscode to induce mutations.

Alkylation of N3 of both adenine and guanine have also been suggested to be potentially mutagenic since these alkylated purines might be expected to pair with cytosine and thymine respectively (Krieg, 1963b; Lawley et al., 1971/72). However, since these products are formed to about equal extents by both SN1 and SN2 agents their involvement, if any, in direct mispairing mutagenesis must be relatively small. No misincorporation by *E. coli* DNA polymerase I into the product from a DMS-methylated poly(dA-dT) template occurred (Abbott and Saffhill, 1977) although the possibility of a small amount of illegitimate incorporation due to 3-methyladenine cannot be excluded.

After its identification as a product of MNUA alkylation $O_4^\text{-methylthymine}$ was suggested on theoretical grounds to be potentially mutagenic (Lawley et al., 1973). Misincorporation of dGMP by *E. coli* DNA polymerase I into MNUA-methylated poly (dA-dT) was ascribed to the miscoding potential of $O_4^\text{-methylthymine}$ since the amount of dGMP misincorporation was equal to the amount of this 0-alkylated pyrimidine.

Singer et al. (1978) also showed that $O_4^\text{-alkyluridine}$ mispairs with guanine and cytosine in an RNA polymerase-directed synthesis from a synthetic polynucleotide containing this residue. These authors have also demonstrated that the presence of $O_2^\text{-alkyluridine}$ also leads to the misincorporation of GMP and
CMP into the product copolymer. At variance with these results are those of Saffhill and Abbott (1978) which indicate that $O^{\text{2-}}$-methylthymine does not miscode during DNA synthesis. These conflicting results may reflect different steric factors in synthesis from an RNA and a DNA template.

A consequence of the alkylation of purines and pyrimidines at certain sites is the labilization of the glycosyl bond between the base and the sugar moiety and spontaneous loss of alkylated bases occurs from DNA (but not from RNA) at neutral pH leaving AP sites (Brookes and Lawley, 1960; Lawley and Brookes, 1963). Such AP sites could theoretically be sources of mutations if bases were added at random but there is no evidence that this occurs. EMS treatment of T4 and EMS and MMS treatment of transforming DNA have shown that the kinetics of mutation induction are linear with time, whereas depurination and depyrimidination are proportional to the square of time (Krieg, 1963a; Lawley and Martin, 1975; Rhaese and Boetker, 1973). Also, the full yield of mutants of T2 and T4 occurs immediately after EMS or DES treatment and on storage of alkylated phage lethal, but not mutagenic, events accumulate (Strauss, 1961; Lobbecke and Krieg cited by Krieg, 1963b). Furthermore, for T2 and T4 ethylation is generally more mutagenic than methylation yet methylated bases are more easily lost from DNA by spontaneous hydrolysis of the glycosyl bond. These results argue strongly that AP sites constitute lethal but not mutagenic lesions. It is, however, conceivable that if an AP site is replicated a single base-pair might be deleted and a frameshift mutation result (e.g., Yourno and Heath, 1969).
(ii) Complex alkylating agents  As outlined above certain agents depend for their mutagenic effectiveness on the presence of intact $\text{recA}$ and $\text{lexA}$ gene products and it has been implied that mutations may arise as errors in repair of lesions which per se are not mutagenic but may, or may not, be lethal. As a general rule to the more subtle changes produced by EMS and MNNG error-prone repair dependent mutagens are those which introduce large substituents into DNA which cause considerable distortion of the secondary structure of DNA. Thus, MTC, DCMTC and BrMBA depend on error-prone repair for mutagenesis in E. coli (Kondo et al., 1970; Murayami and Otsuji, 1973; Tarmy et al., 1973).

6.3 Localized mutagenesis at the replication fork

One feature of MNNG mutagenesis to which much attention has been paid is its apparent ability to cause localized damage. For example, MNNG causes preferentially mutations in the replication fork (Cerdá - Olmedo et al., 1968). This specificity has enabled the replication order of genes to be determined in synchronized cultures (Cerdá - Olmedo et al., 1968). Further evidence of extensive localized damage was obtained by Guerola et al. (1971). These authors showed that because MNNG induces mutations efficiently at the replication forks in E. coli there is a strong chance that closely linked mutations will result. Thus, of a particular class of mutants examined after MNNG treatment over 50% had a secondary mutation within 1.6 mapminutes. Such high frequencies of closely linked multiple mutations have also been demonstrated in Salmonella typhimurium (Oeshger and Berlyn, 1974). It has been calculated for E. coli that 81% of mutations are in the replicating regions (Guerola et al., 1971).
Cells of both *S. cerevisiae* and *Chlamydomonas reinhardii* in which DNA is replicating are also more sensitive than unreplicating cells to MNNG mutagenesis (Dawes and Carter, 1974; Lee and Jones, 1976). Also in mammalian cells MNNG-induced oncogenic transformation of mouse fibroblasts shows a cell cycle component (Bertram and Heidelberger, 1974). Further, excision repair of MNNG- and MNUA-induced damage occurs to a greater extent in replicating regions than in non-replicating regions. In contrast, repair of MMS-, AAAF- and BrMBA-induced damage occurs to the same extent in all parts of the chromosome (Scudiero and Strauss, 1976).

Other SN1 type reagents such as MNUA and ENUA possess this replication fork specificity in *E. coli* (Hince and Neale, 1974b). The phenomenon of increased susceptibility of replicating regions was thought to be only a property of mutagenesis by N-nitroso compounds. Thus, Guerola and Cerdá-Olmedo (1975) reported that mutations induced in *E. coli* by EMS (and UV) were distributed randomly over the chromosome. Similar conclusions were reached with continuous culture studies of EMS mutated cultures (Nestmann, 1975). However, at variance with these reports are those describing or implying a replication-fork component for EMS mutagenesis. Thus, EMS has been shown in yeast to produce multiple closely-linked mutations (Lindegren et al., 1968) and furthermore has been used to temporally map genes in *Anacystis nidulans* (Delaney and Carr, 1975). More extensive studies in the relation to the number of gene copies and number of replication forks have shown that EMS behaves in an identical manner to the N-nitroso compounds in that the number of mutations produced is in proportion to the number of replication forks and
not the number of gene copies contrasting with results obtained with MMS (Hince and Neale, 1977b).

The molecular basis of MNNG mutagenesis and, in particular, its special interaction with replicating regions remains a subject of speculation. In E. coli a proportion (20%) of mutants induced result from error-prone repair as discussed above. The remainder arise via replication errors and hypotheses have centred on three basic ideas: (i) the special molecular environment of the replicating region including the single-stranded nature of the DNA and the change in chemical environment caused by the presence of enzymes involved in DNA metabolism; (ii) alkylation and subsequent changes in the properties of enzymes involved in DNA repair or replication; (iii) the proximity of any potentially mutagenic alkylation product to the replication fork at which they may become fixed as mutations.

In vitro mutagenesis of TMV-RNA was shown to be considerably dependent on the conformation of the nucleic acid (Singer and Frankel-Conrat, 1967) and Cerdà-Olmedo et al., (1968) proposed that it was the single-strandedness of replication forks that allowed a more direct reaction of MNNG with the bases. This view was supported by the demonstration that MNNG produced a higher proportion of mutants in the β-galactosidase gene when it was derepressed than when it was in an inactive state (Brock, 1971). However, because the phenomena result solely from the single-strandedness of the nucleic acid cannot be substantiated because the single-stranded phage S13 treated extracellularly is not mutated by MNNG (Baker and Tessman, 1968). Also specific transcription of other genes in E. coli does not lead to an increased susceptibility of these genes to MNNG mutagenesis.
compared with when they are not being transcribed (Neale and Tristram, unpublished results cited by Neale, 1976). Baker and Tessman (1968) suggested that differences in response of S13 and T4 to MNNG mutagenesis were due to the differences in molecular environment at the replication point. A similar conclusion was reached by Bresler et al. (1972) since treatment of transforming DNA in formamide led to a greater yield of mutants compared with treating single-stranded DNA in aqueous solution and these authors suggested that the hydrophobic environment led to a special reactivity of MNNG with the bases of DNA. It could be envisaged that in vivo such a special reactivity could occur by the spatial organization of enzymes involved in DNA repair or replication.

It was further suggested that if such enzymes were rich in sulphydryl groups then these would further tend to enhance replication fork phenomena since thiols are known to accelerate the decomposition of MNNG (Lawley and Thatcher, 1970). However, MNUA decomposition is not catalysed by thiols (Lawley, 1974) but this compound still exhibits replication fork phenomena in both E. coli (Hince and Neale, 1977b) and human lymphoma cells (Scudiero and Strauss, 1976).

MNNG is known to react extensively with non-DNA material such as proteins (Sugimura et al., 1968). The idea that replication errors might directly involve enzymes active in DNA metabolism rather than primary DNA damage was suggested by the finding that pretreatment of host bacteria with MNNG gave an increased mutation frequency of subsequently infecting untreated phage (Kondo and Ichikawa, 1973). Experiments using concentrations of MNNG which were low enough not to have any
effect on bulk DNA synthesis showed that mutations were induced preferentially in a small fraction of the total population which had DNA synthesis inhibited (Jiménez-Sánchez and Cerdá-Olmedo, 1975). They suggested that MNNG may directly affect DNA polymerase III in such a way that it had drastically reduced fidelity and would replicate a small amount of DNA before being replaced by normal polymerase molecules. Such a model would explain why MNNG is generally a poor mutagen for DNA and phages treated in vitro. Evidence supporting this hypothesis came from the demonstration that in vitro treatment of DNA polymerase I with MNUA lead to a decreased activity of polymerase and 3'→5' exonuclease activities and also to the increased misincorporation of nucleotides (Saffhill, 1974). Similar inactivation of polymerase and exonuclease activities after in vitro MNNG treatment of DNA polymerase III was observed but reduced fidelity of replication could not be detected at doses well above those required for in vivo mutagenesis (Jiménez-Sánchez, 1976). Recently, Yamamoto et al. (1978a) have presented evidence for the involvement of a non-DNA factor in MNNG mutagenesis of lambda which acts synergistically with an increased probability of mispairing of a methylated base.

If repair of potentially miscoding bases was quite rapid in relation to the rate of semi-conservative DNA synthesis then some of the replication-fork phenomena could be accounted for by the proximity of a promutagenic alteration to a replication fork: if it was near then it may be replicated and fixed as a mutation instead of removed by an excision process. This possibility was first raised from the results of MNNG mutagenesis of Paramecium aurelia (Kimball, 1970). Similar conclusions were reached from
studies of mutation-fixation determined by transformation where the data suggested that most MNNG and N-nitrosocarbaryl (NC)-induced mutations were the result of replication of promutagenic lesions which were randomly distributed around the chromosome (Kimball and Setlow, 1974; Beattie and Kimball, 1974). Subsequent repair of these promutagenic lesions was shown to occur by the demonstration that the longer replicative DNA synthesis was inhibited (using a ts dna mutant) the greater was the reduction in final mutant yield. A small proportion of mutants produced at the restrictive temperature were ascribed to some special mechanism operating connected with the replication fork in some way rather than to previous proposals that it was due to errors in repair (Kimball et al., 1978).

Observations have been made in many bacterial systems (for example, Adelberg et al., 1965) that MNNG produces very few mutations in stationary phase cells. This could be interpreted to mean that the replication-fork dependent component is absent and that the residual mutations arise via error-prone repair of at least some alkylation products. Evidence supporting this hypothesis comes from MNUA-mutagenesis of repair-deficient strains of E. coli since it was found that MNUA did not mutate stationary phase lexA or recA strains, although both were highly mutable during exponential growth (Hince and Neale, 1974a).

Certain strains of Saccharomyces cerevisiae are completely refractory to MNNG mutagenesis (Prakash, 1974) but no strains of any organism have been reported which are hypersensitive to the mutagenic effects of MNNG.
6.4 Alkylation and lethality

Since all cellular components are subject to alkylation, death of an individual cell is likely to be the end result of a combination of damage to many components. It is therefore often difficult to decide which lesion is responsible for lethality but at least in some cases damage to DNA is implied.

As discussed, storage of alkylated phage results in the accumulation of lethal but not mutagenic events. Thus, processes subsequent to alkylation namely depurination and depyrimidination or strand breakage as a result of instability of the AP sites are responsible for the observed lethality.

Often alkylations per se constitute lethal lesions. This has best been demonstrated with agents that introduce large moieties into DNA. For example, interstrand crosslinkage induced by bifunctional mustards, mitomycin C or by psoralen plus light have been shown to be lethal in T7 DNA (Lawley et al., 1969), p2 RNA (Shooter et al., 1971), E. coli (Cole, 1971) and cultured Chinese hamster cells (Ben-Hur and Elkind, 1973). The introduction of bulky adducts to the extranuclear amino groups of guanine, adenine and cytosine also confers inactivation of the template of E. coli (Venitt and Tarmy, 1972), human lymphocytes (Lieberman and Dipple, 1972) and in the RNA-containing phage R17 (Dipple and Shooter, 1974). In the last case it was found that one molecule of BrMBA bound to an RNA molecule constituted a lethal lesion and was the result of blocking a site normally involved in the formation of Watson-Crick hydrogen bonding. Similarly with the simple alkylating agents MMS and DMS methylation at the N1 of adenine and N3 of cytosine, positions normally involved in hydrogen bonding, also conferred inactivation of R17 (Shooter et al.,
1974b). With MNUA and MNNG alkylation Shooter et al. (1974b) found it necessary to suggest that the presence of $O^6$-methylguanine was also a lethal event.

The biological effects of phosphotriester formation is less clear. The available data have shown that in the RNA containing phage R17 although a single phosphotriester group is not itself a lethal lesion (Shooter et al., 1974a) inactivation of TMV-RNA can result from phosphotriester formation per se and also indirectly as a result of chain breakage (Singer et al., 1975). When RNA is not the genetic material it is possible that breakage of RNA involved in protein synthesis may contribute to cell killing although no definitive data are available. It is known, however, that MNNG and $N,N'$-dimethylnitrosamine (DMN) inhibit protein synthesis in E. coli and rat liver respectively (Terawaki and Greenberg, 1965; Villa-Treviño, 1967). In DNA, however, where phosphotriesters are much more stable their presence could affect transcription as a result of the removal of the negative charge of the phosphodiester group. Where ethyl phosphotriesters have been studied in oligodeoxyribonucleosides the triester linkage is very stable to enzymes (Miller et al., 1971). Phosphate alkylation may also lead to changes in interaction with complementary nucleotides and conformation of polynucleotides (Miller et al., 1974).

### 6.5 Repair responses to alkylation damage

Bacteria and other organisms possess one or more systems which enable them to repair at least a portion of damage inflicted by alkylating agents. However, what is actually repaired has in
many instances not been clear due to the plethora of lesions produced. Alkylated DNA is degraded both spontaneously and enzymically and may be considered a mixture of alkylated bases, alkylated phosphates, AP sites and single-strand breaks.

(i) **Simple monofunctional alkylating agents** The limited number of bacteria which have been examined have shown that repair responses for the simple monofunctional alkylating agents are generally different from those involved in the repair of UV-induced damage, although this is not to say that some steps do not share common enzymes. Thus, **uvrA mutants of E. coli** are not sensitive to the lethal effects of MMS, EMS or MNNG and with the exception of EMS are not more sensitive to the mutagenic effects of these agents (Kondo et al., 1970; Ishii and Kondo, 1975). Similar pyrimidine dimer excision-less strains of **Haemophilus influenzae** and **Bacillus subtilis** are equally sensitive to mutagenesis by MMS, MNNG and NC as their excision-proficient derivatives (Kimball et al., 1971b; Beattie and Kimball, 1974; Beattie, 1975; Reiter and Strauss, 1965; Tanooka, 1977).

Some of the first evidence of specific repair of alkylation damage was provided by the work of Strauss and his colleagues. They found that crude extracts from **Micrococcus luteus** and **B. subtilis** could introduce single-strand breaks into DNA alkylated in vitro by MMS (Strauss, 1962; Reiter et al., 1967; Strauss and Robbins, 1968). An enzyme activity in E. coli was also shown to attack alkylated lambda DNA (Boyce and Farley, 1968). In some cases subsequent repair of the single-strand breaks produced was demonstrated for **B. subtilis** (Reiter et al., 1967; Prakash and Strauss, 1970), lambda-infected E. coli (Boyce and Farley, 1968), **H. influenzae** (Kimball et al., 1971a) and mouse
L-cells (Walker and Ewart, 1973). However, in none of these examples was the nature of the primary repairable lesions known. What are the lesions subject to repair?

The data from the storage of alkylated phage discussed above indicated that depurination, and by implication depyrimidination, leads to inactivation. The fact that DNA spontaneously loses undamaged purines and pyrimidines suggests that every organism is capable of repairing at least some AP sites. Endonucleases specific for AP sites in DNA have been isolated from a wide variety of sources and include E. coli (Verly and Paquette, 1972), calf thymus (Ljungquist and Lindahl, 1974), human cells (Brent, 1975), yeast (Chlebowicz and Jachmyczyk, 1977), other fungi and higher plants (Thibodeau and Verly, 1976). The excision of the AP site, together with a few undamaged nucleotides can occur in vitro via exonuclease III and the gap filled in by DNA polymerase I with subsequent sealing by ligase (Gossard and Verly, 1978).

The repair responses to phosphotriesters are largely unknown. In at least rat tissues both methyl and ethyl phosphotriesters are not susceptible to cellular DNA repair mechanisms (Shooter and Slade, 1977; Shooter et al., 1977).

Comparisons of the rates of depurination of DNA in vivo and in pseudo-physiological conditions in vitro necessitates the involvement of specific excision mechanisms for certain alkylation products. Surprisingly the major product of alkylation, 7-alkylguanine is not apparently excised in either E. coli (Lawley and Orr, 1970) or B. subtilis (Prakash and Strauss, 1970). The data of Lawley and Orr (1970) did however, show that E. coli
was able to excise enzymically 3-methyladenine and $\text{O}_6^\text{m}$-methylguanine residues after treatment with MNNG. The incision of \textit{E. coli} DNA damaged by a monofunctional sulphur mustard was shown to occur specifically at 3-alkyladenine residues but not at 7-alkylguanine residues (Papirmeister et al., 1970). Subsequent studies have indicated that \textit{E. coli} can also excise by an active process 3-methylguanine residues but not 7-methyladenine residues (Lawley and Warren, 1976). Analogous specific removal of ethylated purines also occurs in \textit{E. coli} (Lawley and Warren, 1975).

Treatment of small mammals with carcinogenic doses of nitrosamines and nitrosamides have been in general agreement with the limited data from bacterial systems (reviewed by Lawley, 1976b; Pegg, 1977). For instance, relative stabilities of 3-methyladenine and/or $\text{O}_6^\text{m}$-methylguanine in vivo have shown that active processes exist for excision from the DNA of rat liver, rat kidney (O'Connor et al., 1973; Kleihues and Margison, 1974; Craddock, 1975), various mouse tissues (Den Engelse, 1974; Frei and Lawley, 1975; Buechler and Kleihues, 1977) and the lung, liver and kidney of hamster (Margison et al., 1976). With regard to the loss of 7-methylguanine the rates of loss vary from between 1 - 4 days. In hamster liver the half-life is about 26h (Margison et al., 1976) but whether this reflects excision is not clear due to the incomplete knowledge of the precise intracellular ionic strengths and pH which will have a considerable effect on the stability of the glycosyl bond. Active excision of 7-methylguanine has been suggested to occur after chronic administration of DMN to mice (Nemoto and Takayama, 1974). A much more rapid rate of loss of 7-methylguanine (half-life of 10h) has been demonstrated in
Euglena gracilis (Olson and McCalla, 1969). In rat liver, however, loss of 7-methylguanine is much closer to the rate of depurination and an active mechanism need not be implicated (Craddock, 1969; Kleihues and Margison, 1974). What these collective data do show is that generally methylations at the N3 position of purines initiate a rapid response by both bacterial and mammalian cells and these lesions are in most cases rapidly removed. $\text{O}^6$-methylguanines appear to be less rapidly removed. In most instances active excision of 7-methylguanine is not believed to occur.

What are the mechanisms of repair? As previously mentioned early evidence showed that repair responses are generally different by at least one step from repair of UV-induced damage. The activities from crude extracts shown to be active on alkylated DNA were implied to be endonucleases but this was never shown to be true since the activities were never purified. Friedberg and Goldthwait (1969) partially purified an extract from E. coli that was able to incise alkylated DNA and designated this activity endonuclease II. An activity in this extract was subsequently shown to be able to incise depurinated DNA (Hadi and Goldthwait, 1971) and it was this activity that was presumed to be the same enzyme to be active on alkylated DNA. However, an endonuclease II preparation was able to release $\text{O}^6$-methylguanine, 3-methyladenine and 1-methyladenine from in vitro MN\textsuperscript{U}A-methylated DNA and thus acted as glycosylases (Kirtikar and Goldthwait, 1974). DNA containing methylbenz[a]anthracenyl residues and gamma-ray damage are also substrates for some activity in the preparation (Kirtikar et al., 1975a,b). However, subsequent findings from
other laboratories have shown that there is a specific glycosylase in *E. coli* which releases 3-methyladenine (and 3-ethyladenine) from DMS-methylated DNA but does not release $\beta$-methylguanine or degrade depurinated DNA (Riazuddin and Lindahl, 1978). The relationship between this enzyme and preparations of endonuclease II remains to be resolved. A similar 3-methyladenine-DNA glycosylase has also been found in *M. luteus* (Laval, 1977). This enzyme also does not act on DNA containing $\beta$-methylguanine or AP sites. Preliminary data has indicated that human lymphoblasts contain a glycosylase active on MMS-methylated DNA but what the nature of the product released is has not yet been reported (Brent, 1977). An activity in crude extracts of rat liver have been shown to excise $\beta$-methylguanine residues from *in vitro* methylated DNA (Pegg, 1978) and is probably a separate enzyme from that required to excise 3-methyladenine and other methylated purines (Pegg and Hui, 1978).

An inducible repair response occurs in *E. coli* which occurs during growth in the presence of low levels of MNNG and enables cells to survive better and to be less mutated than cells not induced (Samson and Cairns, 1977). This adaptive repair response occurs with other alkylating agents eg. MMS and EMS but not with UV or UV-mimetic agents (Jeggo *et al.*, 1977). This form of repair has been examined in many previously well-characterized DNA repair-deficient mutants and it was concluded by Jeggo *et al.* (1977, 1978b) that it represented error-free repair of lesions which could to some extent be dissociated according to the lethal and mutagenic effects. Although DNA polymerase I is implicated in the repair of lethal alkylation damage no mutants have been described with defective mutagenic responses (Jeggo *et al.*, 1977,
It has however, been shown that adapted bacteria are much more proficient than are unadapted bacteria in the ability to remove $O^6$-methylguanine residues, but not 3-methyladenine or 7-methylguanine residues, and strongly implicates $O^6$-methylguanine as an important potentially mutagenic lesion (Schendel et al., 1978).

To date no bacterial mutants have been reported which are defective in the removal of any methylated or ethylated base with the possible exception of *E. coli* B$_{s-1}$ (a uvrA lexA strain) which after doses of MNNG which result in very low survival show an inability to remove $O^6$-methylguanine (Lawley and Orr, 1970). Mutants of *E.coli* with defective endonuclease II have been reported (Kirtikar et al., 1977) and although these are MMS sensitive, their sensitivity to alkylating agent mutagenesis is not known. The slight hypermutability of uvrA strains of *E. coli* to EMS mutagenesis implies the presence of exciseable promutagenic lesions (Ishii and Kondo, 1975; Tarmy, unpublished results cited by Lawley, 1974a).

The ability of rat liver, kidney and brain to remove $O^6$-alkylguanine from DNA has been compared and the susceptibility to alkylating agent-induced carcinogenesis is inversely related to the ability to remove this lesion (Goth and Rajewsky, 1974; Kleihues and Margison, 1974). Defective removal of $O^6$-alkylguanine has also been demonstrated in xeroderma pigmentosum fibroblasts (Goth-Goldstein, 1977).

(ii) **Complex alkylating agents** The damage is in some instances recognized by an endonuclease which also recognizes pyrimidine dimers. However, this is not always the case (see Section 4.2.(i)).
Bacteria

The strains of *Micrococcus radiodurans* used and their relevant phenotypes are shown in Table 2. All other bacteria used are listed in Tables 3 and 4. Bacteria were stored on agar plates at 4°C and were subcultured onto fresh nutrient agar at 8 week intervals. They were grown in liquid cultures in approximately 20 ml amounts of media on an orbital shaker at 30°C except for *Escherichia coli* B/r and *Arthrobacter radiotolerans* which were grown at 37°C.

Media

TGY medium for the growth of *M. radiodurans*, *Micrococcus roseus* ATCC 19172, *Micrococcus radioproteolyticus* and P-30-A contained per 1 distilled water: 5 g tryptone (Difco Laboratories, Detroit, Michigan); 1 g D-glucose; 3 g yeast extract (Difco). Nutrient broth (NB) for the growth of *E. coli* B/r, *Micrococcus radiophilus*, *Micrococcus luteus*, *Micrococcus sodonensis*, *Pseudomonas radiola* and *A. radiotolerans* contained 25 g Oxoid Nutrient Broth No. 2 (Oxoid Ltd., Basingstoke, Hants.) per 1 distilled water. A supplemented minimal medium (SMM) for the growth of *M. radiodurans* T<sub>2</sub> (Little and Hanawalt, 1973) contained per 1 distilled water: potassium dihydrogen phosphate, 0.5 g; di-potassium hydrogen orthophosphate, 0.5 g; magnesium sulphate heptahydrate, 0.2 g; ferrous sulphate heptahydrate, 10 mg; manganous sulphate monohydrate, 7.6 mg; di-ammonium hydrogen orthophosphate 0.5 g; D-glucose, 5 g; Casamino acids (Difco), 1 g; L-glutamate (sodium salt), 0.5 g; L-methionine, 10 mg; thiamine hydrochloride, 10 µg; niacin, 250 µg; pyridoxine, 200 µg; cyanocobalamin, 200 µg; folic acid, 10 µg. It was further supplemented with thymine (2 µg ml⁻¹) or 5-bromodeoxyuridine (BrdUrd) (16 µg ml⁻¹) as required.
Table 2

Micrococcus spp. used

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<td>Moseley and Copland, 1978</td>
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<td>302</td>
<td>$\text{UV}^R\text{MTC}^S$</td>
<td>Moseley and Copland, 1978</td>
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<td>303</td>
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<td>rec30</td>
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<td>tsl</td>
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<td>$T_2^-$</td>
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<td>uvs10</td>
<td>$\text{MMS}^R\text{UV}^S$ derivative of 302</td>
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<tr>
<td>rif-t</td>
<td>Rif$^R$ transformant using DNA from $M. \text{ roseus}$ rif-1</td>
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Abbreviations: $\text{MMS}^R$, methyl methanesulphonate-resistant; $\text{MTC}^R$, mitomycin C-resistant; $\text{MTC}^S$, mitomycin C-sensitive; $\text{UV}^R$, ultraviolet-resistant; $\text{UV}^S$, ultraviolet-sensitive.
Other species of bacteria used

<table>
<thead>
<tr>
<th>Species</th>
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<tr>
<td>Pseudomonas radiora</td>
<td>IAM</td>
<td>Ito and Iizuka, 1971</td>
</tr>
<tr>
<td>Arthrobacter radiotolerans</td>
<td>IAM and M. Kobatake</td>
<td>Yoshinaka et al., 1973</td>
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<td>P-30-A</td>
<td>M. Kobatake</td>
<td>Kobatake et al., 1977</td>
</tr>
<tr>
<td>Escherichia coli B/r</td>
<td>D.M. Sweet</td>
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Abbreviation: IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo.
Solid media were made by the addition of 15 g agar (Difco) to 1 l of the appropriate liquid medium. Media were sterilized by autoclaving at 121°, 1.05 kg cm⁻² for 15 min. Vitamins, amino acids, bases and deoxynucleotides were filter-sterilized by passing through Millipore HAWP 0.45 μm pore size membranes (Millipore UK Ltd., Wembley, Middlesex). They were then added aseptically to the appropriate medium.

Buffers

Phosphate buffer, 0.1 M, pH 5.8 contained 14.35 g sodium dihydrogen phosphate dihydrate and 1.14 g di-sodium hydrogen orthophosphate per 1 distilled water. Phosphate buffer, 0.067 M, pH 7.0 contained 4.56 g potassium dihydrogen phosphate and 5.96 g di-sodium hydrogen orthophosphate dihydrate per 1 distilled water. Phosphate/EDTA buffer pH 7.5 contained 2.82 g di-potassium hydrogen orthophosphate, 0.52 g potassium dihydrogen phosphate and 3.36 g di-sodium ethylenediaminetetraacetaete(EDTA) per 1 distilled water. Acetate buffer, 0.1 M, pH 4.5 was made by adding 4.02 g sodium acetate and 2.95 g acetic acid per 1 distilled water. NET buffer consisted of 0.1 M sodium chloride, 10 mM EDTA and 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride/Tris base, pH 8.0. SSC pH 7.0 contained 0.15 M sodium chloride and 0.015 M tri-sodium citrate.

Chemical reagents

Routine chemical reagents were purchased from several sources. These were BDH Chemicals Ltd., Poole, Dorset; Koch-Light Laboratories, Colnbrook, Bucks.; May and Baker Ltd., Dagenham, Essex; Sigma Chemical Co., London. Amino acids, bases and deoxynucleoside
triphosphates (dNTPs) were purchased from Sigma. BrdUrd was obtained from Calbiochem (Bishop's Stortford, Herts.).

3-Methyladenine and $O^6$-methylguanine were kindly provided by Dr. P.D. Lawley, Institute of Cancer Research, Chalfont St. Giles, Bucks.

Rifampicin (Sigma) was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 10 mg ml$^{-1}$. MMS and EMS (Eastman Kodak & Co., Rochester, New York) were stored at room temperature in the dark. MNNG (Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in phosphate buffer pH 7.0 at a concentration of 400 or 1000 µg ml$^{-1}$, filter-sterilized and stored at $-20^\circ$C. Samples were never refrozen after use. MNU (K and K Laboratories Inc., Plainview, New York) was stored in the dark at $4^\circ$. MTC (Sigma) and DCMTC (a gift from Kyowa Hakko Kogyo Co. Ltd., Tokyo) were stored at $4^\circ$ either as solids or dissolved in phosphate buffer pH 7.0 at a concentration of 100 µg ml$^{-1}$. BrMBA (a gift from Dr. P.D. Lawley) and AAAF (a gift from Dr. R. Raineri, Frederick Cancer Center, Frederick, Maryland) were stored at $-20^\circ$ in a desiccator. Prior to use they were dissolved in DMSO. ICR 191G (a gift from the Chemotherapy Laboratory, Institute for Cancer Research, Fox Chase, Philadelphia) was dissolved in DMSO at a concentration of 1 mg ml$^{-1}$.

Radioisotopes were purchased from the Radiochemical Centre, Amersham. They had the following specific activities:

- $[6-^3\mathrm{H}]$thymidine (dThd), 25 and 27 Ci mmol$^{-1}$; $[2-^{14}\mathrm{C}]$dThd, 58 mCi mmol$^{-1}$; deoxy$[8-^3\mathrm{H}]$adenosine 5'-triphosphate (dATP), 29 Ci mmol$^{-1}$; $[\text{methyl}-^3\mathrm{H}]$thymidine 5'-triphosphate (dTTP), 30 Ci mmol$^{-1}$; 5-bromo-2'-deoxy$[1',2'-^3\mathrm{H}]$uridine, 27.5 Ci mmol$^{-1}$; $[N-^3\mathrm{H}]$methyl-$N'$-nitro-$N$-nitrosoguanidine, 115 mCi mmol$^{-1}$.

The purity of the purchased MNNG was not guaranteed by the Radiochemical
Centre. As a test of its radiochemical purity a sample was subjected to thin layer chromatography using as solvent n-hexane, di-ethyl ether, dichloromethane (2 : 5 : 2, by volume) (Martin, 1975). 9.6 µl of a solution of [methyl-³H] MNNG, freshly dissolved in ethanol at a concentration of 1.4 mg ml⁻¹, was applied to a 0.25 mm thick silica gel (Kieselguhr H) thin layer plate (kindly provided by Miss V. A. Letts). A marker of unlabelled MNNG was also applied to the plate. The plate was run for 50 min and then viewed under 254 nm light. Two UV-absorbing spots were visible and the $R_f$ values were identical. To measure radioactivity the sample strip was cut into 1 cm x 2 cm pieces which were scraped off the glass into scintillation vials to each of which was added 5 ml of a toluene-based scintillant. The result is shown in Figure 4. From the amount of radioactivity that coincided with the unlabelled MNNG spot it is evident that the radiochemical purity was at least 97%.

**Enzymes**

Egg white lysozyme (grade 1) and DNase I (from bovine pancreas) were purchased from Sigma. Pancreatic RNase (BDH) was heated at 80° for 2 min before use. Proteinase (Sigma, type V) was heated for 2 h at 37°, then for 2 min at 80° and cooled to -20°.

**Measurement of radioactivity**

Samples on glass-fibre disks or on paper were counted in either a commercially prepared toluene-based scintillant (NE 233, Nuclear Enterprises Ltd., Edinburgh) or in a mixture containing per 1 toluene: 4 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP). Aqueous samples were counted in either a commercially prepared 1,4-dioxan-based scintillant (NE 250) or in a mixture containing 1.1 toluene, 1.1
Figure 4  Thin layer chromatography of $^{3}$H-methyl] MNNG showing radiochemical purity. The bar represents the position of unlabelled pure MNNG. SF = solvent front.
Triton X-100 (BDH), 16 g PPO, 0.4 g POPOP. Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer model 3330 (Packard Instrument Ltd., Caversham, Berks.). When both $^3$H and $^{14}$C activities were measured in the same sample the data shown have been corrected for spillover from one channel to the other.

Measurement of growth rates

Growth rates were followed by measuring changes in the turbidities of the cultures. Bacteria were grown in conical flasks which had fitted sidearms through which the turbidities were measured in a nephelometer (Evans Electroselenium Ltd., Halstead, Essex) using an orange filter.

Chemical treatment of bacteria

Overnight cultures of bacteria were diluted into fresh growth medium and incubated for several generations until the bacteria were in the mid-exponential phase of growth. Cultures (10 or 20 ml) contained 1 to $2 \times 10^8$ viable units (vu) ml$^{-1}$, unless otherwise noted.

MNNG A sample of the culture was centrifuged, the bacteria washed and resuspended in half the original volume of phosphate buffer pH 7.0 at $30^\circ$. To this suspension was added an equal volume of phosphate buffer in which MNNG had been dissolved. The suspension was shaken at $30^\circ$ in the dark for up to 2.5 h. At appropriate times a sample (0.5 ml) was removed, the bacteria rapidly centrifuged in a Quickfit microcentrifuge (Corning Ltd., Stone, Staffs.), washed and resuspended in an equal volume of TGY medium from which samples were measured for viability and mutation frequency as described later.
When [methyl$^3$H]MNNG was used usually 4 l of a culture containing between 2 to 3 x $10^8$ vu ml$^{-1}$ were centrifuged at 2000 g for 25 min at 4°. The bacteria were washed in 50 ml ice-cold phosphate buffer pH 7.0 and resuspended in phosphate buffer at 30° at a concentration of between 5.2 x $10^9$ and 1.4 x $10^{10}$ vu ml$^{-1}$.

To the cell suspension was added [methyl$^3$H] MNNG, freshly dissolved in about 1 ml ethanol, to a final concentration of 68 μM (7.7 μCi ml$^{-1}$). The bacteria were exposed to the radioactive mutagen for 30 min with shaking at 30° in the dark and then centrifuged at 12000 g for 5 min at 4° and washed in 50 ml SSC. Some of the bacteria, usually one half, were resuspended in 25 ml butanol-saturated phosphate/EDTA buffer and the remainder in 25 ml TGY medium. These latter bacteria were added to TGY medium at a concentration of 2 to 3 x $10^8$ vu ml$^{-1}$ and incubated at 30° for up to 3 h. The bacteria were then collected by centrifugation, washed in SSC and resuspended in 25 ml butanol-saturated phosphate/EDTA buffer. DNA was isolated as described later.

MNUr Washed bacteria were exposed in phosphate buffer pH 7.0 at 30° to different concentrations of MNUr for 1 h. Samples were then removed, the bacteria washed and resuspended in TGY medium and viability and mutant frequency measured as described later.

MMS and EMS To a washed culture of bacteria in phosphate buffer pH 7.0 was added an equal volume of phosphate buffer in which the appropriate alkyl methanesulphonate was freshly dissolved. The suspension was shaken at 30° for up to 2 h. At appropriate times a sample was removed, the mutagen removed by centrifugation and viability and mutation frequency determined as described later.
MTC and DCMTC  Treatment with these mitomycins was identical to that for MMS and EMS treatment except that the bacteria were exposed in the dark. The chemicals were removed by centrifugation.

BrMBA and AAAF  For treatment with BrMBA and AAAF the bacteria were centrifuged, washed and resuspended in an equal volume of phosphate buffer pH 7.0. 0.1 ml amounts of DMSO containing BrMBA were added to 9.9 ml amounts of washed bacteria while 148 μl DMSO containing AAAF were added to 345 μl amounts of washed bacteria. Bacteria were exposed to these chemicals at 30°C for 20 min. Treatments were ended by at least 100-fold dilution into ice-cold TGY medium.

Nitrous acid  Treatment was carried out by suspending washed bacteria in acetate buffer pH 4.5 in which sodium nitrite was dissolved. The suspensions were shaken at 30°C for up to 16 min. At appropriate times samples were removed and treatments ended by 100-fold dilution into phosphate buffer pH 7.0 or into TGY medium for measurement of survival and mutant frequency respectively.

Hydroxylamine  Washed bacteria were exposed in phosphate buffer pH 5.8 to hydroxylamine monohydrochloride which was present with an equimolar amount of sodium chloride. At appropriate times, up to 90 min, samples were removed and the bacteria washed to remove the chemical before survival and mutation induction was measured.

2-AP and BrdUrd  The bacteria were diluted into fresh TGY medium at about 1 x 10⁷ vu ml⁻¹. They were incubated in TGY medium containing 2-AP or BrdUrd at the appropriate concentrations until they had usually entered the stationary phase. The frequency of mutants was then determined.
Irradiation of bacteria

UV  Exponentially growing cultures were centrifuged, the bacteria washed and resuspended in phosphate buffer pH 7.0 at the same concentration or at twice the original density. Bacterial suspensions (5 or 10 ml) were irradiated in a glass petri dish (9 or 11 cm diameter) and agitated by means of a magnetic stirrer. The UV source was an Hanovia model 12 germicidal lamp (Hanovia Lamps Ltd., Slough, Bucks.) giving an incident dose rate of 2.25 J m\(^{-2} \text{s}^{-1}\). After appropriate doses 0.1 ml or 1 ml samples were removed to determine viability and mutant frequency as described later.

Gamma-rays  Bacteria, in the exponential phase of growth, were irradiated in TGY medium in a \(^{60}\)Co source at a dose rate of 3.2 krad min\(^{-1}\). Oxygen was bubbled through the bacterial suspension during irradiation. Viability and mutant frequency were determined as described later.

Measurement of survival

After treatment of bacteria with physical or chemical agents 0.1 ml samples of the appropriate dilution were spread in duplicate or triplicate on TGY or NB agar. Colonies were counted after 1 day incubation at 37\(^{\circ}\) for E. coli; 2 days at 30\(^{\circ}\) for M. luteus and M. sodonensis; 3 days at 30\(^{\circ}\) for M. radiodurans, M. roseus ATCC 19172, M. radiophilus, M. radioproteolyticus and P-30-A; 6 days at 30\(^{\circ}\) for Ps. radiotolerans and 14 days at 37\(^{\circ}\) for A. radiotolerans.

Measurement of mutation induction

Samples (0.1 or 1 ml) of the treated cultures were diluted 100-fold into TGY or NB medium and shaken at 30\(^{\circ}\) or 37\(^{\circ}\) until they had entered the stationary phase. The frequency of mutants was
determined by plating 0.1 ml amounts of the appropriately diluted, or in some cases 10-fold concentrated, samples onto nutrient medium agar plates with and without rifampicin at 50 μg ml⁻¹ but for E. coli at 100 μg ml⁻¹. Rifampicin-resistant colonies were counted after 2 days incubation at 37° for E. coli; 4 days at 30° for M. luteus and M. sodonensis; 5 days at 30° for P-30-A; 6 days at 30° for M. radiodurans, M. roseus ATCC 19172, M. radiophilus and M. radioproteolyticus; 7 days at 30° for Ps. radiosa and 15 to 20 days at 37° for A. radiotolerans.

Genetic transformation

The method for genetic transformation of M. radiodurans used was that developed by Tirgari (1977). Recipient bacteria were grown overnight to a density of between 2 to 3 x 10⁸ cfu ml⁻¹. The bacteria from 10 ml of culture were collected by centrifugation and resuspended in 5 ml fresh TGY medium at 30°. Two ml of 0.1 M calcium chloride were added and in some cases DL-methionine was present at 100 μg ml⁻¹. Assay tubes contained 0.3 ml recipient bacteria and 0.06 ml DNA solution (1 mg ml⁻¹). Controls containing DNA only and bacteria only were included to check on DNA sterility and the spontaneous mutation frequency of the bacteria. The assay tubes were incubated, with gentle shaking, at 30° for 2 h. Then, 50 μl of a solution of DNase (3 mg ml⁻¹) was added and the mixture incubated at 30° for another 10 min. The mixtures were then diluted 10-fold with TGY medium and further dilutions, in TGY medium, were made. To 1 ml amounts of the appropriate dilution were added 10 ml quantities of molten agar at 46°. The bacteria were evenly distributed in the molten agar which was then allowed to solidify. The plates were incubated
at 30° for 5 h to allow phenotypic expression of the transformants. To isolate rifampicin-resistant transformants 10 ml molten agar containing 100 μg ml⁻¹ rifampicin was added as a top-layer to each plate. Transformants were counted after 4 days incubation at 30°.

Isolation of UV-sensitive mutants

An exponential culture of *M. radiodurans* 302 was grown to a density of 1 x 10⁸ vu ml⁻¹. To 10 ml of a washed suspension of bacteria in phosphate buffer pH 7.0 was added MNNG to a final concentration of 3.0 mM. The bacteria were incubated at 30° for 30 min. A sample (0.1 ml) was removed, the mutagen removed by centrifugation and the washed bacteria incubated in 10 ml TGY medium. When the culture was fully grown it was diluted and 0.1 ml amounts of the appropriate dilution spread on 60 agar plates to give about 100 colonies per plate. These plates were incubated for 36 h at 30° and replicas of each plate were made onto fresh TGY agar plates. These replica plates were irradiated with 1080 J m⁻² UV and incubated for 2 days at 30°. Colonies which failed to grow on the UV-irradiated plates were recovered from the master plates and were tested individually for UV-sensitivity.

DNA isolation

For transformation Bacteria were grown in TGY medium or NB as appropriate until they had entered the stationary phase of growth. For 2 l of culture the procedure was as follows. The bacteria were collected by centrifugation at 2000 g for 25 min, washed in 50 ml SSC and resuspended in 50 ml phosphate/EDTA buffer pH 7.5 saturated with butanol-1-ol. Butanol pretreatment sensitizes the cell wall to lysozyme degradation by removing lipid-containing material (Driedger and Grayston, 1970). After 45 min at room temperature the bacteria were centrifuged at 12000 g for 10 min.
and resuspended in 50 ml SSC/10. Five ml of a solution of lysozyme (20 mg ml$^{-1}$) were added and the mixture incubated at 37°C for 30 min. The bacteria were lysed by the addition of 5 ml 20% (w/v) sodium dodecyl sulphate (SDS). Purification of DNA then followed the procedures described by Marmur (1961). To the lysate was added 13 ml 5 M sodium perchlorate followed by 73 ml chloroform/isoamyl alcohol, 24:1 by volume. The mixture was shaken vigorously by hand in a round-bottomed flask for 30 min. The phases were separated by centrifugation at 15000 g for 30 min at 20°C. The aqueous (top) layer was removed and added to 1.7 volumes 95% ethanol. The nucleic acids which were precipitated were wound out on glass rods and allowed to dry in air. The nucleic acids were dissolved in 5 ml SSC and 0.5 ml of a solution of RNase (2.5 mg ml$^{-1}$) was added and the mixture incubated at 37°C for 1 h. To further purify the DNA an equal volume of chloroform/isoamyl alcohol was added and the mixture shaken for about 5 min. After centrifugation at 15000 g for 30 min the aqueous layer was removed and DNA precipitated in ethanol as before. The DNA was removed, dried, finally dissolved in sterile SSC at a concentration of approximately 1 mg ml$^{-1}$ and stored at 4°C.

The above procedure was used for the isolation of DNA from all bacteria except from *M. luteus* and *M. sodonensis*. In these cases the butanol pretreatment was dispensed with.

For analysis of methylated purines the phenol/m-cresol method of Kirby *et al.* (1967) was used for the isolation of DNA from bacteria after treatment with radioactively-labelled MNNG. Immediately after treatment with MNNG or after post-treatment incubation as described previously, the bacteria (usually 4 x 10$^{11}$ vu) were collected by centrifugation, washed in SSC and resuspended in 25 ml
butanol-saturated phosphate/EDTA buffer and spheroplasts made as described above. After treatment with lysozyme the bacteria were centrifuged and resuspended in 20 ml 6% (w/v) sodium 4-aminosalicylate. Lysis was achieved by adding 2 ml 20% SDS. An equal volume of phenol mixture (500 g phenol, 62 ml redistilled m-cresol, 62 ml water, 0.625 g 8-hydroxyquinoline) was added and the mixture shaken for 10 min. The phases were separated by centrifugation at 15000 g for 30 min at 80. The aqueous phase was removed and added to 1.5 volumes 2-ethoxyethanol. The DNA which was precipitated was wound onto glass rods, washed in 70% ethanol containing 2% (w/v) sodium acetate and, when dry, dissolved in about 5 ml 0.01 M sodium acetate. RNase was added to a final concentration of 50 μg ml⁻¹ and the solution was incubated at 370 for 30 min. The DNA was then re-extracted with 0.5 volume phenol mixture, the phases separated by centrifugation and the DNA precipitated in 2-ethoxyethanol as described above. The DNA was wound out, washed and dissolved in about 4 ml 0.01 M sodium acetate. Then, 0.1 volume 2.5 M sodium acetate was added followed by 0.1 volume 4 M sodium chloride and the mixture centrifuged at 82000 g for 30 min. The DNA was precipitated in 2-ethoxyethanol and finally dissolved in about 0.5 ml 0.01 M sodium acetate. If it was not used immediately it was stored at -700.

DNA concentrations were determined by absorbance at 260 nm measured in either a Pye Unicam SP 1800 or an SP6-500 ultraviolet spectrophotometer (Pye Unicam Ltd., Cambridge) using the relationship

\[ \varepsilon_{260,\text{nm}}^{1\text{mg} \cdot \text{ml}^{-1}} = 26. \]

Hydrolysis of DNA and chromatography of purines

DNA from bacteria treated with radioactively-labelled
MNNG was hydrolysed in dilute acid under conditions which liberate purines but do not cause substantial demethylation (Lawley and Thatcher, 1970). To each sample of methylated DNA dissolved in 0.01 M sodium acetate was added 1 M HCl to give a final concentration of 0.1 M HCl and the resultant mixture was heated at 70° for 30 min. Any residual material remaining was removed by centrifugation. Authentic methylated purines were added to the hydrolysate and the solution was applied to a 5 cm wide strip of Whatman 3MM chromatography paper (W. & R. Balston Ltd., Maidstone, Kent). Chromatograms were developed descending at 25° for 13 to 15 h using as solvent 2-methylpropan-2-ol, butan-2-one, aqueous ammonia (specific gravity 0.88), water, (4 : 3 : 2 : 1, by volume) (Maitra and Frei, 1975). The chromatograms were allowed to dry and were viewed under 254 nm light. The positions of UV-absorbing and UV-fluorescent spots were measured. The chromatograms were then cut into 5 mm strips which were added to 10 ml toluene-based scintillant for the measurement of radioactivity. Each sample was counted for a minimum of 20 min but in most cases for 40 min.

Alkaline sucrose gradients

DNA was radioactively labelled by growing bacteria in the exponential phase of growth for at least 7 generations in TGY medium containing [3H]dThd at 20 µCi ml⁻¹. Exponentially growing labelled bacteria (5 to 10 x 10⁷ vu ml⁻¹) were washed, resuspended in phosphate buffer pH 7.0 and treated with BrMBA, AAAF, MMS or UV as required. After treatment with BrMBA (7.6 µM) or AAAF (2.4 mM) for 20 min at 30°, or after treatment with 29.4 mM MMS for 30 min at 30° the bacteria were centrifuged, washed in ice-cold phosphate/EDTA buffer and resuspended in an equal volume of the
same buffer saturated with butanol. UV doses were either 270 or 540 J m\(^{-2}\) and irradiation was carried out at room temperature.

Immediately after irradiation or after post-irradiation incubation in TGY medium, the bacteria were collected by centrifugation and resuspended in ice-cold butanol-saturated buffer. The different samples were then treated in an identical way. After 4 min in ice the bacteria were centrifuged, washed and resuspended in phosphate/EDTA buffer containing lysozyme (2 mg ml\(^{-1}\)). After 25 min stationary incubation at 37\(^{\circ}\) 0.1 ml lysozyme-treated bacteria (less than 10\(^{7}\) spheroplasts) were layered into 0.2 ml 0.5 M NaOH on top of either 4.7 ml (Figures 21b and 24) or 4.5 ml (Figures 21c, 22, 23, 32, 33, 44, 45 and 50) alkaline sucrose gradient and allowed to stand at room temperature for 20 min to ensure complete lysis of the bacteria. Each gradient consisted of either 4.5 or 4.3 ml 5-20\(^{\circ}\) (w/v) sucrose gradient containing 0.7 M sodium chloride 0.3 M sodium hydroxide and 1 mM EDTA on top of a 0.2 ml shelf of 40\(^{\circ}\) sucrose. Sedimentation was at 30000 rpm for 105 min at 20\(^{\circ}\), unless otherwise noted, in a 6 x 5.5 ml swing-out rotor of an MSE Superspeed 65 ultracentrifuge (MSE Ltd., Crawley, Sussex).

The bottom of each tube was pierced with a hypodermic needle and 9 drop fractions were pumped out onto a 2.5 cm wide strip of Whatman \(3\MM\) chromatography paper. Each strip was given two 20 min washes in ice-cold 5\(^{\circ}\) (w/v) trichloroacetic acid (TCA) and two 10 min washes in ice-cold 95\(^{\circ}\) ethanol followed, in some cases, by a 5 min wash in acetone. When the strips were dry they were cut into segments and each segment placed in 10 ml of a toluene-based scintillant. Radioactivity was measured by liquid scintillation spectrometry.
DNA synthesis in permeabilized bacteria

The methods described by Kitayama and Matsuyama (1976) were used.

Preparation of permeable bacteria  The bacteria from an exponentially growing culture containing 1 to $2 \times 10^8$ vu ml$^{-1}$ were centrifuged, washed with 20 mM Tris-HCl/Tris base (pH 7.5), 50 mM NaCl, 2 mM EDTA and concentrated 20-fold in the same buffer. To the cell suspension was added 20% (v/v) Triton X-100 (Koch-Light) to give a final concentration of 1% and the bacteria were incubated at 37° for 5 min. The bacteria were then centrifuged, washed and resuspended in 50 mM Tris-HCl/Tris base (pH 8.4) to give a final density of $4 \times 10^9$ vu ml$^{-1}$. In some cases 10% (v/v) glycerol was present and if so the permeable bacteria were stored at −20°.

Assay for DNA synthesis  DNA synthesis in Triton-treated bacteria was measured by the incorporation of $[^3H]dTMP$ into TCA-precipitable material. Each reaction mixture (final volume 0.3 ml) contained 50 mM Tris-HCl/Tris base (pH 8.4), 2 mM MgCl$_2$, 5 mM 2-mercaptoethanol, 10% glycerol, 1 mM ATP, 25 μM each of dATP, dGTP, dCTP, $[^3H]dTTP$ (24.9 to 52.8 Ci mol$^{-1}$) and $4 \times 10^8$ Triton-treated bacteria. Reactions were initiated by the addition of the bacteria and the mixtures were incubated at various times at 37° unless stated otherwise. Reactions were terminated by the addition of 3 ml ice-cold 10% (w/v) TCA containing 0.1 M sodium tetra-pyrophosphate. After at least 20 min in ice TCA-insoluble material was collected on Whatman GF/C glass-fibre disks which were then washed twice with 5 ml ice-cold 5% TCA, once with 10 ml 5% TCA and once with 10 ml 0.01 M HCl. The disks were dried under an infra-red lamp, placed in scintillation vials and 10 ml toluene-based scintillant added for the measurement of radioactivity.
Inhibition of DNA synthesis induced by MNNG

Overnight cultures were diluted in TGY medium to give about 2 x 10^7 cfu ml\(^{-1}\). The cultures were shaken at 30\(^\circ\) until they reached between 7 to 8 x 10^7 cfu ml\(^{-1}\). The bacteria were centrifuged, washed and resuspended in phosphate buffer pH 7.0. MNNG was added to a final concentration of 68 \(\mu\)M and the bacteria were incubated for 30 min at 30\(^\circ\). The bacteria were then centrifuged, washed in TGY medium and resuspended in TGY medium containing 30 \(\mu\)Ci ml\(^{-1}\) \([^{3}H]\) dThd. A 75 \(\mu\)l sample was immediately removed and TCA-precipitable material collected as described below. The cultures were then incubated at 30\(^\circ\) for up to 3 h. At appropriate intervals 75 \(\mu\)l samples were removed, added to 3 ml ice-cold 10% TCA containing 0.1 M sodium tetra-pyrophosphate and allowed to stand in ice for at least 20 min. TCA-precipitable material was collected on glass-fibre disks which were processed and radioactivity measured as described previously.

DNA degradation induced by BrMBA and MNNG

Exponentially growing cultures were incubated for between 1.5 to 2 generations in TGY medium containing 20 \(\mu\)Ci ml\(^{-1}\) \([^{3}H]\) dThd to prelabel the DNA. The bacteria were centrifuged, washed and resuspended in phosphate buffer pH 7.0. In some cases BrMBA, freshly dissolved in DMSO, was added to a final concentration of 7.6 \(\mu\)M. The DMSO concentration was 1% (v/v). Bacteria were exposed in the dark at 30\(^\circ\) with shaking. At appropriate times up to 20 min a sample was removed, added to TCA and acid-insoluble material collected on glass-fibre disks as described before.

In other cases MNNG was added to give a final concentration of 68 \(\mu\)M. Samples (50 \(\mu\)l) were taken during the treatment and
TCA-precipitable material collected. After 30 min exposure to MNNG the bacteria were centrifuged, washed in cold phosphate buffer pH 7.0 and resuspended in TGY medium at 30°. Samples were then removed at 20 min intervals for up to 3h and TCA precipitable material measured for radioactivity as described before.

**Determination of repair replication**

In permeable cells of M. radiodurans wild-type. Bacteria were grown for about 15 h in 30 ml TGY medium containing 20 μCi [14C]dThd to about 2 x 10^8 cfu ml^-1. The bacteria were centrifuged, washed and resuspended in 3 ml 20 mM Tris-HCl/Tris base (pH 7.5) containing 50 mM NaCl and 2 mM EDTA. To the cell suspension was added 0.15 ml 20% Triton X-100 and after 5 min at 37° the mixture was cooled, the bacteria centrifuged, washed and resuspended in 50 mM Tris-HCl/Tris base (pH 8.4), containing 2 mM magnesium chloride, 5 mM 2-mercaptoethanol and 10% glycerol to give a cell density of 10^9 cfu ml^-1. Half the bacteria were then irradiated over ice with 540 J m^-2. The unirradiated bacteria were held in ice. Each of the two halves were further divided into two and solutions (0.1 ml) of dGTP, dCTP, BrdUTP and [3H]dATP (final specific activity of 200 Cimol^-1) were added to a final concentration of each of 25 μM. In some cases ATP at 1 mM was present. The mixtures were held at 0° and then transferred to 37°. At appropriate times reactions were stopped by the addition of 5 ml ice-cold NET buffer. Lysates were then made, with slight modifications, by the methods described by Hanawalt and Cooper (1971). The bacteria were centrifuged in the cold, washed in cold NET buffer and resuspended in 3 ml butanol-saturated NET buffer. After 4 min in ice the bacteria were centrifuged and resuspended in 1 or 2 ml NET/10 buffer containing lysozyme (2 mg ml^-1). After
25 min incubation at $37^\circ$ the bacteria were lysed by the addition of 25 or 50 μl 20% (v/v) Sarkosyl. Proteinase was added to a final concentration of 100 μg ml$^{-1}$. The lysates were incubated at $60^\circ$ for 30 min. Each lysate was then "whirlimixed" for 2 min or passed six times through a 25 gauge hypodermic needle. The volume of each lysate was adjusted to between 4.0 to 4.4 ml and solid CsCl was added to give a density of 1.73 g cm$^{-3}$. Densities were determined by measuring the refractive index on an Abbe"60" refractometer (Bellingham and Stanley Ltd., Tunbridge Wells, Kent).

The solutions were put in polycarbonate or polypropylene tubes which were filled to the top with liquid paraffin. Centrifugation was for 48 h at 35000 · rpm at $25^\circ$ in a 6 x 5.5 ml swing-out rotor of an MSE 65 ultracentrifuge. The gradients were then fractionated by piercing the bottom of each tube with a hypodermic needle and collecting 12 drop fractions by pumping liquid paraffin on the top of each gradient. Each fraction was collected on a continuous strip of Whatman 3MM chromatography paper which was processed as described previously and radioactivities were measured by liquid scintillation spectrometry.

In M. radiodurans $T_2$ M. radiodurans $T_2$ was grown in 40 ml SMM containing 2 μg ml$^{-1}$ thymine and 0.25 μCi ml$^{-1}$ $^{14}$C dThd to a density of $1.5 \times 10^8$ vu ml$^{-1}$. The bacteria were centrifuged, washed twice in phosphate buffer pH 7.0 and resuspended in an equal volume of buffer. The culture was divided into two parts. One part was held in ice to act as the unirradiated control. The remaining half was UV-irradiated over ice with a dose of 810 J m$^{-2}$. The bacteria, from both halves, were centrifuged and resuspended in equal volumes of SMM with thymine replaced $[^3$H] BrdUrd (16 μg ml$^{-1}$; 5 μCi ml$^{-1}$). Each sample was further
divided into two parts and these were incubated at 30° for either 20 or 40 min. At each of these times reactions were stopped by the addition of an equal volume of ice-cold NET buffer. Lysates (final volume 4.65 ml) were made and these were subjected to isopycnic centrifugation as described above. Gradients were fractionated as before except that 15 drop fractions were collected and these were directly added to 5 ml of dioxan-based scintillant and radioactivities measured by liquid scintillation spectrometry.
RESULTS
1. Mutagenesis of Micrococcus radiodurans wild-type

Previous mutation studies in M. radiodurans from this laboratory employed the forward mutation to trimethoprim resistance as the assay for mutation induction. However, the spontaneous mutation frequency is about $10^{-5}$ and such a high value limits the resolution when weak mutagens are used. To overcome this problem, a different marker was used in the experiments described, viz. resistance to rifampicin (50 µg ml$^{-1}$), for which the spontaneous mutation frequency is generally about 2 to $5 \times 10^{-8}$.

Before the range of possible mutagenic agents was extended the ability of some previously investigated DNA-damaging agents to induce rifampicin-resistant mutants was examined. Exponentially growing cultures of bacteria were treated with the various physical and chemical agents as described earlier. The data presented in Table 5 show that at doses of UV radiation up to 1350 J m$^{-2}$ there are few, if any, induced rifampicin-resistant mutants. The standard deviations are based on the number of mutant colonies counted in each experiment and do not include errors in estimating viable counts. The values are thus minimum estimates. The bifunctional alkylating agent MTC was also unable to induce rifampicin-resistant mutants (Table 6). Nitrous acid increased the number of rifampicin-resistant mutants to a frequency of about $2 \times 10^{-7}$ (Table 7). A similar 10-fold increase over the spontaneous level of mutants was obtained with hydroxylamine treatment (Table 8). With regard to the monofunctional ethylating agent EMS, at the doses used a 5 to 12-fold increase in the number of rifampicin-resistant mutants was obtained (Table 9). However,
### Table 5

The effect of UV on survival and mutation to rifampicin-resistance of *M. radiodurans* wild-type

The mean values from 2 experiments are shown together with their standard deviations (SD).

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors (SD)</th>
<th>+Caffeine (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>4.4 (0.8)</td>
<td>2.1</td>
</tr>
<tr>
<td>270</td>
<td>100</td>
<td>4.6 (1.0)</td>
<td>2.9</td>
</tr>
<tr>
<td>540</td>
<td>91</td>
<td>5.1 (1.4)</td>
<td>3.2</td>
</tr>
<tr>
<td>810</td>
<td>76</td>
<td>7.4 (1.8)</td>
<td>4.1</td>
</tr>
<tr>
<td>1080</td>
<td>37</td>
<td>6.3 (1.5)</td>
<td>4.6</td>
</tr>
<tr>
<td>1350</td>
<td>6</td>
<td>8.7 (2.1)</td>
<td>4.2</td>
</tr>
</tbody>
</table>

\(^{a}\) Caffeine (500 \(\mu\)g ml\(^{-1}\)) present in the post-irradiation growth medium.

### Table 6

The effect of MTC on survival and mutation to rifampicin-resistance of *M. radiodurans* wild-type

<table>
<thead>
<tr>
<th>MTC dose ((\mu)M h)</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>3.7</td>
</tr>
<tr>
<td>10</td>
<td>53</td>
<td>2.9</td>
</tr>
<tr>
<td>15</td>
<td>23</td>
<td>2.0</td>
</tr>
<tr>
<td>20</td>
<td>1.8</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Survival data from Sweet and Moseley (1976).
Table 7
The effect of nitrous acid on survival and mutation to rifampicin-resistance of *M. radiodurans* wild-type

<table>
<thead>
<tr>
<th>Nitrous acid dose (mM.min NaNO₂)</th>
<th>Survival (%)</th>
<th>Rif&lt;sup&gt;R&lt;/sup&gt; mutants per 10&lt;sup&gt;8&lt;/sup&gt; survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>7.8</td>
</tr>
<tr>
<td>170</td>
<td>98</td>
<td>10.2</td>
</tr>
<tr>
<td>510</td>
<td>95</td>
<td>17.0</td>
</tr>
<tr>
<td>680</td>
<td>92</td>
<td>12.0</td>
</tr>
<tr>
<td>850</td>
<td>98</td>
<td>22.4</td>
</tr>
<tr>
<td>1020</td>
<td>82</td>
<td>21.2</td>
</tr>
<tr>
<td>1190</td>
<td>76</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Survival data from Sweet and Moseley (1976).

Table 8
The effect of hydroxylamine on survival and mutation to rifampicin-resistance of *M. radiodurans* wild-type

<table>
<thead>
<tr>
<th>Hydroxylamine dose (M.h)</th>
<th>Survival (%)</th>
<th>Rif&lt;sup&gt;R&lt;/sup&gt; mutants per 10&lt;sup&gt;8&lt;/sup&gt; survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>6.5</td>
</tr>
<tr>
<td>0.05</td>
<td>100</td>
<td>8.8</td>
</tr>
<tr>
<td>0.10</td>
<td>100</td>
<td>12.1</td>
</tr>
<tr>
<td>0.15</td>
<td>97</td>
<td>14.0</td>
</tr>
<tr>
<td>0.20</td>
<td>81</td>
<td>26.2</td>
</tr>
<tr>
<td>0.25</td>
<td>30</td>
<td>25.1</td>
</tr>
<tr>
<td>0.30</td>
<td>5.2</td>
<td>19.9</td>
</tr>
</tbody>
</table>
the frequency of mutants induced after treatment with the methylating agent MNNG is up to 10000 times the spontaneous frequency with not more than 90% killing (Table 10). In contrast to its inability to induce sufficient numbers of trimethoprim-resistant mutants to be detectable gamma-rays induced up to 20 times more mutants than those arising spontaneously (Table 11).

1. 1 New mutagenic agents

In agreement with previous results (Sweet and Moseley, 1974; Kerszman, 1975) that only MNNG is capable of substantial mutagenesis in the wild-type, two other methylating agents namely MNUr and MMS were investigated for their ability to induce rifampicin-resistant mutants. The data presented in Figure 5 show that MNUr is as potent a mutagen as MNNG since it can induce rifampicin-resistant mutants at a frequency of 3 to 4 x 10⁻⁴. This is accomplished with a lower cytotoxic effect than is shown by MNNG. In contrast, MMS is comparatively weak in its mutagenic effect and only increased the number of mutants by between 5 to 25-fold (Table 12).

The base analogues form another class of potential mutagenic agents e.g. 2-AP and BrUra, analogues of adenine and thymine respectively. Bacteria were grown in the presence of 0, 1, 2 and 4 mg ml⁻¹ 2-AP. With increasing concentration the generation time of the bacteria increased (Table 13). After 27 h incubation the frequency of rifampicin-resistant mutants was determined. 2-AP had little or no mutagenic effect (Table 13). When Escherichia coli B/r was grown in the presence of 2 mg ml⁻¹ 2-AP for about 4 generations a 100-fold increase in the number of rifampicin-resistant mutants was found although the decrease in generation time was from 36 min to 137 min (data not shown). Thus
### Table 9

The effect of EMS on survival and mutation to rifampicin-resistance of *M. radiodurans* wild-type

<table>
<thead>
<tr>
<th>EMS dose (mM.h)</th>
<th>Survival (%)</th>
<th>Rif^R mutants per 10^8 survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2.5</td>
</tr>
<tr>
<td>97</td>
<td>100</td>
<td>8.6</td>
</tr>
<tr>
<td>194</td>
<td>100</td>
<td>11.4</td>
</tr>
<tr>
<td>389</td>
<td>100</td>
<td>30.1</td>
</tr>
<tr>
<td>486</td>
<td>100</td>
<td>24.1</td>
</tr>
<tr>
<td>583</td>
<td>74</td>
<td>22.0</td>
</tr>
<tr>
<td>680</td>
<td>75</td>
<td>15.0</td>
</tr>
<tr>
<td>778</td>
<td>3.5</td>
<td>25.6</td>
</tr>
</tbody>
</table>

### Table 10

The effect of MNNG on survival and mutation to rifampicin-resistance of *M. radiodurans* wild-type

<table>
<thead>
<tr>
<th>MNNG dose (µM.h)</th>
<th>Survival (%)</th>
<th>Rif^R mutants per 10^8 survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2.2</td>
</tr>
<tr>
<td>340</td>
<td>83</td>
<td>6.4 x 10^3</td>
</tr>
<tr>
<td>680</td>
<td>61</td>
<td>2.2 x 10^4</td>
</tr>
<tr>
<td>1020</td>
<td>43</td>
<td>1.9 x 10^4</td>
</tr>
<tr>
<td>1360</td>
<td>15</td>
<td>2.5 x 10^4</td>
</tr>
</tbody>
</table>

### Table 11

The ability of gamma-rays to induce rifampicin-resistance to mutants in *M. radiodurans* wild-type

<table>
<thead>
<tr>
<th>Gamma-ray dose (krad)</th>
<th>Rif^R mutants per 10^8 survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>100</td>
<td>28.6</td>
</tr>
<tr>
<td>200</td>
<td>21.2</td>
</tr>
<tr>
<td>300</td>
<td>41.2</td>
</tr>
<tr>
<td>400</td>
<td>38.5</td>
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<tr>
<td>500</td>
<td>42.7</td>
</tr>
<tr>
<td>600</td>
<td>46.9</td>
</tr>
<tr>
<td>700</td>
<td>43.3</td>
</tr>
<tr>
<td>800</td>
<td>47.4</td>
</tr>
</tbody>
</table>
Table 12
The effect of MMS on survival and mutation to rifampicin-resistance of M. radiodurans wild-type

<table>
<thead>
<tr>
<th>MMS dose (mM.h)</th>
<th>Survival (%)</th>
<th>Rif\textsuperscript{R} mutants per 10\textsuperscript{8} survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2.1</td>
</tr>
<tr>
<td>7.4</td>
<td>100</td>
<td>11.6</td>
</tr>
<tr>
<td>14.7</td>
<td>69</td>
<td>13.7</td>
</tr>
<tr>
<td>22.1</td>
<td>100</td>
<td>11.7</td>
</tr>
<tr>
<td>29.4</td>
<td>97</td>
<td>22.7</td>
</tr>
<tr>
<td>36.8</td>
<td>87</td>
<td>23.3</td>
</tr>
<tr>
<td>44.1</td>
<td>77</td>
<td>35.8</td>
</tr>
<tr>
<td>51.5</td>
<td>64</td>
<td>80.9</td>
</tr>
<tr>
<td>58.8</td>
<td>48</td>
<td>47.7</td>
</tr>
</tbody>
</table>

Table 13
The effect of 2-AP on the generation time (t) and mutation to rifampicin-resistance of M. radiodurans wild type

<table>
<thead>
<tr>
<th>2-AP concentration (mg ml\textsuperscript{-1})</th>
<th>t(h)</th>
<th>Rif\textsuperscript{R} mutants per 10\textsuperscript{8} viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.70</td>
<td>7.7</td>
</tr>
<tr>
<td>1</td>
<td>2.15</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>2.70</td>
<td>6.1</td>
</tr>
<tr>
<td>4</td>
<td>3.90</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Table 14
The effect of BrdUrd on generation time (t) and mutation to rifampicin-resistance of M. radiodurans wild-type

<table>
<thead>
<tr>
<th>BrdUrd concentration (mM)</th>
<th>t(h)</th>
<th>Rif\textsuperscript{R} mutants per 10\textsuperscript{8} viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.57</td>
<td>2.7</td>
</tr>
<tr>
<td>1</td>
<td>1.60</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>1.70</td>
<td>2.9</td>
</tr>
<tr>
<td>10</td>
<td>1.90</td>
<td>2.2</td>
</tr>
</tbody>
</table>
the treatment employed was potentially mutagenic.

Growth of cultures of *M. radiodurans* was followed for 9h in the presence of 1, 3 and 10mM BrdUrd. The data presented in Table 14 show that BrdUrd has a slight inhibitory effect on growth rate. The cultures were allowed to grow through about 6 generations before the frequency of mutants was determined. No induced mutants could be detected (Table 14). The data of Lett *et al.* (1970) show that *M. radiodurans* readily incorporates BrdUrd residues and by extrapolation from their data growth in the presence of 10mM BrdUrd for 9h should result in about 20% of dThd residues being replaced by BrdUrd residues.

Acridine compounds with alkylating side chains are a group of agents which are capable of producing predominantly frameshift mutations (see Drake, 1970). ICR 191G is such an acridine but it was unable to induce any rifampicin-resistant mutants in *M. radiodurans* (Table 15) although it did show a lethal effect. Treatment of *E. coli* under similar conditions did show that the treatment was mutagenic (data not shown).

Neither of the two alkylating agents BrMBA and DCMTC which introduce large substituents into DNA were able to induce rifampicin-resistant mutants (Tables 16 and 17).

2. Ultraviolet mutagenesis of radiation-resistant *Micrococcus*

The inability of UV radiation to induce forward mutation in *M. radiodurans* wild-type to streptomycin-resistance (Kerszman, 1975), to rifampicin-resistance (Table 5) or to trimethoprim-resistance or to revert a ts mutant to temperature-resistance
Table 15

The effect of ICR 191G on survival and mutation to rifampicin-resistance of M. radiodurans wild-type

<table>
<thead>
<tr>
<th>ICR 191G dose (µg.h.ml$^{-1}$)</th>
<th>Survival (%)</th>
<th>Rif$^R$ mutants per $10^8$ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>3.6</td>
</tr>
<tr>
<td>23</td>
<td>81</td>
<td>1.6</td>
</tr>
<tr>
<td>47</td>
<td>70</td>
<td>3.0</td>
</tr>
<tr>
<td>70</td>
<td>58</td>
<td>2.4</td>
</tr>
<tr>
<td>93</td>
<td>63</td>
<td>2.0</td>
</tr>
<tr>
<td>117</td>
<td>48</td>
<td>1.0</td>
</tr>
<tr>
<td>140</td>
<td>6</td>
<td>0.8</td>
</tr>
<tr>
<td>163</td>
<td>1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 16

The effect of BrMBA on survival and mutation to rifampicin-resistance of M. radiodurans wild-type

<table>
<thead>
<tr>
<th>BrMBA dose (µM.min)</th>
<th>Survival (%)</th>
<th>Rif$^R$ mutants per $10^8$ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>3.6</td>
</tr>
<tr>
<td>62</td>
<td>94</td>
<td>2.5</td>
</tr>
<tr>
<td>125</td>
<td>75</td>
<td>3.4</td>
</tr>
<tr>
<td>187</td>
<td>60</td>
<td>5.0</td>
</tr>
<tr>
<td>250</td>
<td>26</td>
<td>5.5</td>
</tr>
<tr>
<td>374</td>
<td>0.17</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Table 17

The effect of DCMTC on survival and mutation to rifampicin-resistance of M. radiodurans wild-type

<table>
<thead>
<tr>
<th>DCMTC dose (µM.h)</th>
<th>Survival (%)</th>
<th>Rif$^R$ mutants per $10^8$ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2.2</td>
</tr>
<tr>
<td>87</td>
<td>86</td>
<td>3.6</td>
</tr>
<tr>
<td>175</td>
<td>74</td>
<td>3.1</td>
</tr>
<tr>
<td>349</td>
<td>47</td>
<td>3.9</td>
</tr>
<tr>
<td>524</td>
<td>37</td>
<td>2.7</td>
</tr>
<tr>
<td>698</td>
<td>18</td>
<td>2.4</td>
</tr>
</tbody>
</table>
(Sweet and Moseley, 1974) has been demonstrated. In order to see whether this UV immutability is characteristic of the species or is a general property of other radiation-resistant micrococci the responses of Micrococcus roseus ATCC 19172, Micrococcus radiophilus and Micrococcus radioproteolyticus to UV-induced mutagenesis were examined and compared with the responses of some true members of the Micrococcaceae i.e. Micrococcus luteus and Micrococcus sodonensis. Firstly, the levels of UV-induced damage tolerated by these bacteria were determined. The survival curves are shown in Figure 6 together with data for E. coli B/r. All four red-pigmented micrococci, that is M. radiodurans, M. roseus ATCC 19172, M. radiophilus and M. radioproteolyticus were considerably more resistant than the other bacteria with a characteristic shoulder to the curves extending up to at least 1000 J m⁻² before exponential losses in survival occurred. M. radiodurans is the most sensitive of these radiation-resistant micrococci with a D₃₇ dose of 1080 J m⁻². The D₃₇ doses for M. roseus ATCC 19172, M. radiophilus and M. radioproteolyticus are 1100, 1440 and 1920 J m⁻² respectively.

M. luteus and M. sodonensis are more resistant than E. coli B/r to the lethal effects of UV but do not have the characteristic extended shoulder shown by the other micrococci (Figure 6). The D₃₇ doses of all the bacteria examined are given in Table 18.

The ability of UV radiation to induce rifampicin-resistant mutants was tested at biologically meaningful doses, i.e. those which result in at least between 1% and 10% survival. The resolution was such that a minimum of a four-fold increase over the spontaneous level of mutation could be detected. No detectable UV-induced rifampicin-resistant mutants could be found in either
Figure 6

UV survival curves of M. radiodurans, (Δ);
M. roseus ATCC 19172, (▲); M. radiophilus, (○);
M. radioproteolyticus, (●); M. luteus, (□);
M. sodonensis, (□); E. coli, (Φ).
Table 18

The sensitivity of some bacteria to UV-induced killing

<table>
<thead>
<tr>
<th>Species</th>
<th>( D_{37} ) (J m(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. radiodurans</td>
<td>1080</td>
</tr>
<tr>
<td>M. roseus ATCC 19172</td>
<td>1100</td>
</tr>
<tr>
<td>M. radiophilus</td>
<td>1440</td>
</tr>
<tr>
<td>M. radioproteolyticus</td>
<td>1920</td>
</tr>
<tr>
<td>M. luteus</td>
<td>235</td>
</tr>
<tr>
<td>M. sodonensis</td>
<td>310</td>
</tr>
<tr>
<td>Ps. radiora</td>
<td>250</td>
</tr>
<tr>
<td>A. radiotolerans</td>
<td>660</td>
</tr>
<tr>
<td>P-30-A</td>
<td>825</td>
</tr>
<tr>
<td>E. coli B/r</td>
<td>35</td>
</tr>
</tbody>
</table>

\( D_{37} \) dose is that required to reduce the population to 37\% of the initial number.

Table 19

The ability of UV radiation to induce rifampicin-resistant mutants of M. roseus ATCC 19172

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per (10^8) survivors</th>
<th>+Caffeine(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.6</td>
<td>1.3</td>
</tr>
<tr>
<td>270</td>
<td>100</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>540</td>
<td>69</td>
<td>3.8</td>
<td>1.4</td>
</tr>
<tr>
<td>810</td>
<td>64</td>
<td>2.5</td>
<td>2.9</td>
</tr>
<tr>
<td>1080</td>
<td>40</td>
<td>2.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(^a\) Caffeine (500 \(\mu\)g ml\(^{-1}\)) present in the post-irradiation growth medium.
or M. radiophilus (Table 20). In each case the spontaneous mutation frequency was approximately 1 to 4 x 10^{-8}.

In contrast to the lack of response of the four radiation-resistant micrococci to UV-induced mutagenises, M. luteus and M. sodonensis were, however, able to be mutated by UV to rifampicin-resistance. The data in Tables 22 and 23 show that UV can induce at least a 30 to 50-fold increase over the spontaneous levels of rifampicin-resistant mutants.

Since pyrimidine dimers are believed to be the premutagenic lesions after exposure to UV (see Witkin, 1976) the more dimers that remain in the DNA of a cell the more chance that the cell will be mutated. Thus, excisionless mutants of E. coli are hypermutable by UV (Hill, 1965). However, it is not possible to examine such mutants of M. radiodurans or of the other radiation-resistant micrococci since none have been reported. Another way to increase the mutant yield is to inhibit error-free repair so that dimers are chanelled into error-prone pathways of repair. Such an approach, has been used in E. coli by having caffeine present in the post-irradiation growth medium (Sideropoulos and Shankel, 1968). The presence of 500 µg ml⁻¹ caffeine in the manifestation medium did not increase the yield of mutants after UV-irradiation of M. radiodurans (Table 5), M. roseus ATCC 19172 (Table 19) or M. radiophilus (Table 20).

To exclude the possibility that the radiation-resistant micrococci could not be mutated at all to phenotypically rifampicin-resistant forms they were treated with MNNG or MNUr. The results in Table 24 indicate that all of them could be mutated
### Table 20

The ability of UV radiation to induce rifampicin-resistant mutants of *M. radiophilus*

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>(\text{Rif}^R) mutants per 10(^8) survivors</th>
<th>+Caffeine(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>270</td>
<td>100</td>
<td>3.6</td>
<td>2.6</td>
</tr>
<tr>
<td>540</td>
<td>100</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>810</td>
<td>82</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>1080</td>
<td>53</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>1350</td>
<td>17</td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Caffeine (500 \(\mu\)g ml\(^{-1}\)) present in the post-irradiation growth medium.

### Table 21

The ability of UV radiation to induce rifampicin-resistant mutants of *M. radioproteolyticus* (Values are averages of 2 experiments).

<table>
<thead>
<tr>
<th>UV dose (J in (\mu) -2)</th>
<th>Survival (%)</th>
<th>(\text{Rif}^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.4</td>
</tr>
<tr>
<td>270</td>
<td>94</td>
<td>1.9</td>
</tr>
<tr>
<td>540</td>
<td>79</td>
<td>2.2</td>
</tr>
<tr>
<td>1080</td>
<td>78</td>
<td>3.6</td>
</tr>
<tr>
<td>1620</td>
<td>63</td>
<td>2.7</td>
</tr>
<tr>
<td>2160</td>
<td>33</td>
<td>2.1</td>
</tr>
<tr>
<td>2700</td>
<td>4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

### Table 22

The ability of UV radiation to induce rifampicin-resistant mutants of *M. luteus*

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>(\text{Rif}^R) mutants per 10 (\mu) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>68</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>135</td>
<td>82</td>
<td>87</td>
</tr>
<tr>
<td>203</td>
<td>78</td>
<td>150</td>
</tr>
<tr>
<td>270</td>
<td>7.4</td>
<td>180</td>
</tr>
</tbody>
</table>
### Table 23

The ability of UV radiation to induce rifampicin-resistant mutants of *M. sodonensis*

<table>
<thead>
<tr>
<th>UV dose (J m$^{-2}$)</th>
<th>Survival (%)</th>
<th>Rif$^{R}$ mutants per $10^8$ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>2.1</td>
</tr>
<tr>
<td>68</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>135</td>
<td>100</td>
<td>36</td>
</tr>
<tr>
<td>236</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>270</td>
<td>62</td>
<td>110</td>
</tr>
<tr>
<td>338</td>
<td>23</td>
<td>110</td>
</tr>
</tbody>
</table>

### Table 24

The ability of MNNG or MN$^{Ur}$ to induce rifampicin-resistant mutants of *M. radiodurans*, *M. roseus* ATCC 19172, *M. radiophilus* and *M. radioproteolyticus*

<table>
<thead>
<tr>
<th>Species</th>
<th>Mutagen</th>
<th>Survival (%)</th>
<th>Rif$^{R}$ mutants induced per $10^8$ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. radiodurans</em></td>
<td>MNNG(680 µM.h)</td>
<td>61.0</td>
<td>$2.2 \times 10^4$</td>
</tr>
<tr>
<td><em>M. roseus</em> ATCC 19172</td>
<td>MNNG(1020 µM.h)</td>
<td>56.3</td>
<td>$6.4 \times 10^1$</td>
</tr>
<tr>
<td><em>M. radiophilus</em></td>
<td>MN$^{Ur}$(1516 µM.h)</td>
<td>66.0</td>
<td>$2.9 \times 10^3$</td>
</tr>
<tr>
<td><em>M. radioproteolyticus</em></td>
<td>MN$^{Ur}$(758 µM.h)</td>
<td>87.0</td>
<td>$1.8 \times 10^3$</td>
</tr>
</tbody>
</table>
to rifampicin-resistance.

These results show that UV immutability is a character of the four UV-resistant micrococci whereas the more UV-sensitive \textit{M. luteus} and \textit{M. sodonensis} are UV-mutable.

3. Ultraviolet mutagenesis of other radiation-resistant species

In order to see whether the lack of UV-induced mutagenesis was characteristic of radiation-resistant bacteria in general and not just a feature of the radiation-resistant micrococci the susceptibility of \textit{Pseudomonas radiora}, \textit{Arthrobacter radiotolerans} and an asporogenous rod designated P-30-A to UV mutagenesis was examined. These three bacterial species have only been examined for their resistances to ionizing radiations. The UV dose-response curves for killing of these organisms are shown in Figure 7 and the \(D\) values in Table 18.

P-30-A is almost as resistant as \textit{M. radiodurans} to UV-induced killing and \textit{A. radiotolerans} is slightly more sensitive than \textit{M. radiodurans}. Both curves show the characteristic shoulder which extends up to at least 675 J m\(^{-2}\) before survival is reduced exponentially.

In contrast, \textit{Ps. radiora} is not strikingly UV-resistant and is approximately as resistant as \textit{M. luteus} (Table 18). Similarly its resistance to gamma-rays is considerably less than that shown by \textit{M. radiodurans} although compared with other pseudomonads it is much more resistant (Ito and Iizuka, 1971). The data shown in Table 25 indicate that \textit{Ps. radiora} is capable of being mutated by UV, the frequency of mutants increasing by nearly 100-fold after a UV dose of 405 J m\(^{-2}\).

In contrast no detectable UV-induced mutants of \textit{A. radiotolerans} or
Table 25

The ability of UV radiation to induce rifampicin-resistant mutants of *Ps. radiola*

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>68</td>
<td>71</td>
<td>9.8</td>
</tr>
<tr>
<td>135</td>
<td>47</td>
<td>5.8</td>
</tr>
<tr>
<td>270</td>
<td>30</td>
<td>19.5</td>
</tr>
<tr>
<td>304</td>
<td>22</td>
<td>41.9</td>
</tr>
<tr>
<td>338</td>
<td>12</td>
<td>54.8</td>
</tr>
<tr>
<td>405</td>
<td>0.7</td>
<td>90.5</td>
</tr>
</tbody>
</table>

Table 26

The ability of UV radiation to induce rifampicin-resistant mutants of *A. radiotolerans*

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>135</td>
<td>90</td>
<td>≦ 6(^a)</td>
</tr>
<tr>
<td>270</td>
<td>86</td>
<td>≦ 5(^a)</td>
</tr>
<tr>
<td>405</td>
<td>68</td>
<td>6</td>
</tr>
<tr>
<td>540</td>
<td>49</td>
<td>3</td>
</tr>
<tr>
<td>670</td>
<td>38</td>
<td>3</td>
</tr>
<tr>
<td>810</td>
<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>945</td>
<td>4.8</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\)No mutants detected. If there has been one colony then the frequencies would have been those shown.

Table 27

The ability of UV radiation to induce rifampicin-resistant mutants of *P-30-A*

<table>
<thead>
<tr>
<th>UV dose (J m(^{-2}))</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.4</td>
</tr>
<tr>
<td>270</td>
<td>91</td>
<td>1.2</td>
</tr>
<tr>
<td>540</td>
<td>69</td>
<td>2.1</td>
</tr>
<tr>
<td>810</td>
<td>42</td>
<td>1.6</td>
</tr>
<tr>
<td>1080</td>
<td>6.1</td>
<td>3.7</td>
</tr>
<tr>
<td>1350</td>
<td>0.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>
P-30-A could be found (Tables 26 and 27).

Thus, resistance to the lethal effects and resistance to the mutagenic effects of UV appear to be two associated characteristics for at least six organisms some of which undoubtedly belong to different genera. The data for \textit{Ps. radiora} do not contradict this relationship since although it is UV-mutable it is not particularly resistant to UV-inactivation.

4. Heterologous transformations

Possible taxonomic relationships between the radiation-resistant micrococci was examined by interspecies transformations. Although genetic transformation of \textit{M. roseus} ATCC 19172, \textit{M. radiophilus} and \textit{M. radioproteolyticus} using homologous DNA has not been reported such a genetic process has been demonstrated in \textit{M. radiodurans} (Moseley and Setlow, 1968) and occurs at frequencies comparable with other transformation systems (Tirgari, 1977).

Heterologous DNA extracted from spontaneously arising rifampicin-resistant micrococci was examined, therefore, for its ability to transform \textit{M. radiodurans} wild-type to rifampicin-resistance. Homologous DNA extracted from a rifampicin-resistant mutant of \textit{M. radiodurans} was included as a control on the competence of the recipient cultures used. The results from two experiments using different cultures of recipient bacteria and in some cases different DNA preparations are in Table 28. The data show that heterologous DNA from \textit{M. roseus} ATCC 19172, \textit{M. radiophilus} and \textit{M. radioproteolyticus} all transformed \textit{M. radiodurans} wild-type to rifampicin-resistance although, compared with homologous DNA, at considerably
<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Experiment</th>
<th>Frequency of transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. radiodurans rif-1</td>
<td>1</td>
<td>$1.9 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$2.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>M. roseus rif-1</td>
<td>1</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$2.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>M. roseus rif-2</td>
<td>2</td>
<td>$1.1 \times 10^{-6}$</td>
</tr>
<tr>
<td>M. radioproteolyticus rif-1</td>
<td>2</td>
<td>$8.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>M. radiophilus rif-1</td>
<td>1</td>
<td>$2.7 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$3.8 \times 10^{-7}$</td>
</tr>
<tr>
<td>M. sodonensis rif-1</td>
<td>2</td>
<td>$\leq 1.6 \times 10^{-8}$ a</td>
</tr>
<tr>
<td>M. luteus rif-1</td>
<td>1</td>
<td>$\leq 2.1 \times 10^{-8}$ a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$\leq 1.6 \times 10^{-8}$ a</td>
</tr>
<tr>
<td>M. radiodurans rif-t</td>
<td>1</td>
<td>$1.8 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

*No transformants detected. If there had been one colony then the frequencies would have been those shown.*
reduced frequencies. In contrast, no transformants were detected using DNA from rifampicin-resistant strains of M. luteus or M. sodonensis (Table 28). DNA was extracted from M. radiodurans (strain rif-t) which had been transformed with DNA from M. roseus rif-l. This DNA transformed M. radiodurans wild-type at a frequency comparable with DNA of entirely homologous origin (Table 28).

These data provide evidence that M. radiodurans is genetically related to M. roseus ATCC 19172, M. radiophilus and M. radioproteolyticus, but not to M. luteus and M. sodonensis. From the relative frequencies, M. roseus may be more closely related to M. radiodurans than is M. radioproteolyticus, which in turn may be closer to M. radiodurans than M. radiophilus. More precise estimates could be obtained by direct DNA-DNA hybridization.

5. Some aspects of MNNG mutagenesis

5.1 Repair-deficient mutants

Since MNNG along with MNUr and probably other nitrosamidines and nitrosamides are the only groups of mutagens which are capable of mutating the wild-type to any significant extent the sensitivity of some repair-deficient mutants of M. radiodurans to both the lethal and mutagenic effects of MNNG was examined to see if they showed different patterns from the wild-type. The mutants examined were those that have been isolated over several years from this laboratory and include UV2, UV17, UV22, UV38, UV47, UV50, 261, 262, 301, 302, 303 and rec30.
The survival curves for these mutants are shown in Figures 8, 9 and 10 and the $D_{37}$ values in Table 29. All the mutants examined were considerably more sensitive than the wild-type, their survival curves having reduced shoulders. Strain 302 was the most resistant, the most sensitive ones being UV17, UV47, 261, 303 and rec30 which have similar sensitivities.

301 is a double mutant containing mutations in the genes mtcA and uvsA whereas 302 and 303 are the single mutants defective in mtcA and uvsA respectively (Moseley and Copland, 1978). It is apparent that mtcA confers some resistance to MNNG-induced killing when uvsA is defective since 301 is more resistant than 303. The shape of the curve for UV2 is also found with other DNA-damaging agents and is characteristic of this strain (Moseley, pers. commun.).

The sensitivity of these mutants to the mutagenic effects of MNNG is shown in Figures 11 and 12. The pattern of response is different from the pattern of lethality since some of the mutants are more sensitive and some are less sensitive to MNNG mutagenesis. Because the mutants may be defective in the repair of lesions which contribute to mutation only, to lethality only or to both mutation and lethality it is not easy to compare the strains. Compared with the wild-type most of the strains (UV2, UV38, UV47, UV50, 261, 301, 303) induced a smaller number of mutants at their respective $D_{37}$ dose although two (262 and 302) induced more and one (UV22) induced about the same number. However, the $D_{37}$ doses of MNNG for the various strains are very different and the values in column 6 of Table 29 take into account both the relative sensitivity to mutation and the sensitivity to lethality. Thus, it is apparent that UV2, UV38 and UV50 produce less mutants per unit dose than does the wild-type. On the other hand UV22, 261, 262, 301, 302
Figure 8: MNNG survival curves of *M. radiodurans* UV2, (▲); UV22, (▼); UV38, (■); 302, (●). The curve of the wild-type (---) has been added for comparison.
Figure 9  MNNG survival curves of M. radiodurans UV17, (△); UV50, (●); 261, (▽); rec30, (□).
Figure 10 MNNG survival curves of *M. radiodurans* UV47, (■); 262, (○); 301, (▲); 303, (▼).
Table 29: The sensitivity of some repair-deficient strains of *M. radiodurans* to killing and mutation induced by MNNG

<table>
<thead>
<tr>
<th>Strain</th>
<th>( D_{37} ) dose (( \mu \text{M.h} ))</th>
<th>( \text{Rif}^R ) mutants per ( 10^8 ) survivors at ( D_{37} ) dose</th>
<th>Relative sensitivity to killing(a)</th>
<th>Mutation(m)</th>
<th>( \text{kxm} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1080</td>
<td>2.5 ( \times 10^4 )</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>UV2</td>
<td>45</td>
<td>2.7 ( \times 10^3 )</td>
<td>24</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>UV17</td>
<td>21</td>
<td>nt</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV22</td>
<td>61</td>
<td>1.5 ( \times 10^2 )</td>
<td>18</td>
<td>0.60</td>
<td>10.8</td>
</tr>
<tr>
<td>UV38</td>
<td>109</td>
<td>4.2 ( \times 10^2 )</td>
<td>10</td>
<td>0.017</td>
<td>0.17</td>
</tr>
<tr>
<td>UV47</td>
<td>23</td>
<td>2.5 ( \times 10^2 )</td>
<td>47</td>
<td>0.010</td>
<td>0.47</td>
</tr>
<tr>
<td>UV50</td>
<td>66</td>
<td>1.6 ( \times 10^2 )</td>
<td>16</td>
<td>0.006</td>
<td>0.10</td>
</tr>
<tr>
<td>261</td>
<td>21</td>
<td>4.5 ( \times 10^4 )</td>
<td>51</td>
<td>0.18</td>
<td>9.2</td>
</tr>
<tr>
<td>262</td>
<td>63</td>
<td>5.5 ( \times 10^3 )</td>
<td>17</td>
<td>2.2</td>
<td>37.4</td>
</tr>
<tr>
<td>301</td>
<td>32</td>
<td>4.9 ( \times 10^3 )</td>
<td>34</td>
<td>0.20</td>
<td>6.8</td>
</tr>
<tr>
<td>302</td>
<td>177</td>
<td>1.2 ( \times 10^3 )</td>
<td>6</td>
<td>4.8</td>
<td>28.8</td>
</tr>
<tr>
<td>303</td>
<td>18</td>
<td>2.4 ( \times 10^3 )</td>
<td>60</td>
<td>0.096</td>
<td>5.8</td>
</tr>
<tr>
<td>rec30</td>
<td>21</td>
<td>nt</td>
<td>51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Relative sensitivity to killing is the ratio of the \( D_{37} \) dose for the wild-type to the \( D_{37} \) dose for the mutant.

b. Relative sensitivity to mutation is the ratio of number of mutants induced at the \( D_{37} \) dose of the mutant to the number of mutants induced at the \( D_{37} \) dose of the wild-type.

c. nt means not tested.
Figure 11

MNNG mutagenesis of *M. radiodurans* wild-type, (⊙);
UV2, (▼); UV38, (◆); UV22, (▲); 302(□).
Figure 12

MNNG mutagenesis of *M. radiodurans* UV47,(▼);
UV50,(□); 261,(▲); 262,(●); 301,(▼); 303,(Φ).
and 303 are hypermutable by MNNG since these strains produce more mutants than the wild-type per unit dose. A more detailed examination of these mutants, in particular UV22, 262 and 302, is presented in later sections. Some further experiments are also described with UV47 and 303 since these appear to be mutators.

5.2 MNNG mutagenesis after growth in the presence of low concentrations of MNNG

Recently, Samson and Cairns (1977) demonstrated that growth of *E. coli* in the presence of low levels of MNNG increases the resistance of the bacteria to both the lethal and mutagenic effects of subsequent higher doses. They presented evidence that this adaptive response is due to the operation of an inducible, previously uncharacterized, repair pathway. To test whether this error-free repair pathway is present in *M. radiodurans* an experiment was done using concentrations of MNNG as described by Samson and Cairns (1977) but no adaptation was detected (data not shown). However, because *M. radiodurans* is more resistant to MNNG-induced inactivation than is *E. coli* (Sweet and Moseley, 1976) subsequent experiments employed higher doses of MNNG. To an exponentially growing culture of *M. radiodurans* in TGY medium MNNG was added to a final concentration of 13.6 μM. The bacteria were incubated at 30°C with shaking and growth monitored by nephelometry. There was no difference either in the rate of increase of turbidity or in the increase of viable numbers compared with a culture containing no MNNG (results not shown). However, although 13.6 μM MNNG had no net effect on the growth rate it was capable of producing a mutagenic effect (Table 30). At appropriate times during growth in the presence of MNNG samples (250 μl) were added to 750 μl MNNG solution (1 mg ml⁻¹ in phosphate
Table 30

*Mutagenesis during growth in the presence of 13.6 μM MNNG and after challenges of 5.1 mM MNNG for 10 min.*

<table>
<thead>
<tr>
<th>Exposure to 13.6 μM MNNG (min)</th>
<th>Rif$^R$ mutants per 10$^8$ cells during growth in the presence of 13.6 μM MNNG</th>
<th>Rif$^R$ mutants per 10$^8$ cells after challenges of 5.1 mM MNNG for 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.6</td>
<td>1.54 x 10$^4$</td>
</tr>
<tr>
<td>20</td>
<td>23.4</td>
<td>1.59 x 10$^4$</td>
</tr>
<tr>
<td>40</td>
<td>44.2</td>
<td>1.89 x 10$^4$</td>
</tr>
<tr>
<td>60</td>
<td>42.5</td>
<td>1.90 x 10$^4$</td>
</tr>
<tr>
<td>80</td>
<td>75.2</td>
<td>1.89 x 10$^4$</td>
</tr>
<tr>
<td>100</td>
<td>67.0</td>
<td>1.51 x 10$^4$</td>
</tr>
</tbody>
</table>
buffer, pH 7.0) to give a final concentration of 5.1 mM MNNG. After exposure to this challenging concentration for 10 min at 30\(^\circ\) the numbers of mutants induced was measured using the standard method (Table 30). Thus, growth in the presence of 13.6 \(\mu\)M MNNG for up to 100 min did not increase the resistance of the bacteria to much higher concentrations of MNNG. The number of bacteria which were killed was not determined because the challenging dose does not cause extensive killing.

It is conceivable that different adapting and challenging doses might be required to produce an effect similar to that observed in \textit{E. coli}. It is technically difficult to challenge \textit{M. radiodurans} for brief periods with high concentrations of MNNG because of the limited solubility of MNNG in aqueous solutions. From the results it may be tentatively concluded that \textit{M. radiodurans} does not possess an inducible pathway for the repair of potentially mutagenic lesions.

5.3 MNNG mutagenesis of stationary phase bacteria

In many systems it is observed that MNNG is a weak mutagen for non-replicating DNA. For instance, phages treated extracellularly and stationary phase bacteria are only poorly mutated, if at all, by MNNG. A culture of \textit{M. radiodurans} wild-type was allowed to reach a turbidity of 1.70 (5 \times 10^8 cells ml\(^{-1}\)) and was treated with MNNG as described previously. The data in Figure 13 show that MNNG is only capable of producing a weak mutagenic effect for stationary phase cells. Compared with the exponentially growing bacteria stationary phase cells are about 100 times less mutable. These data do not, however, indicate whether the lowered susceptibility is due
MNNG mutagenesis of exponential (○) and stationary phase (○) cells of *M. radiodurans* wild-type.
to the lack of replicating DNA, to the increased time available for repair of potentially mutagenic lesions or to a combination of these, or other, phenomena.

6. *M. radiodurans* 302

The nature of the defect in this strain was examined by a combination of biochemical and biophysical techniques. The strain originated after MNNG mutagenesis of the wild-type and was isolated as a mutant sensitive to the lethal action of MTC but as resistant as the wild-type to the lethal effects of UV (Moseley and Copland, 1978). The relatively high sensitivity of *M. radiodurans* wild-type to MNNG mutagenesis is exceeded in 302 by a factor of about 50 (Figure 11). This hypersensitivity is not mirrored by the lethal effects of MNNG since 302 is only 6 times more sensitive than the wild-type (Figure 9, Table 29). The defect in 302 was examined in relation to the repair of lethal and mutagenic effects of many DNA-damaging agents.

**MMS**

Strain 302 is as resistant as the wild-type to MMS-induced killing (Figure 14a) with a $D_{37}$ dose of 66 mM.h. The sensitivity of 302, compared with the wild-type, to MMS mutagenesis is shown in Figure 15a. 302 is hypersensitive to MMS mutagenesis and yields between 70 to 400 times more mutants than the wild-type depending on the dose.

**EMS**

The $D_{37}$ dose for EMS inactivation is 260 mM.h compared with
Figure 14 Survival curves of M. radiodurans wild-type (●) and 302 (○) treated with MMS, (a); EMS, (b); MNNG, (c).
Figure 15 The sensitivity of M. radiodurans wild-type (⊗) and 302 (○) to mutation to rifampicin-resistance induced by MMS, (a); EMS, (b); MNNG, (c).
a value of 725 mM·h for the wild-type (Figure 14b). The shoulder of the curve for 302 has been substantially reduced whereas the rate of exponential loss in survival is similar to wild-type killing. The sensitivity of 302 to EMS mutagenesis is shown in Figure 15b. As with methylation, 302 also exhibits hypermutability by this ethylating agent. The yield of rifampicin-resistant mutants is about 200 times higher in 302 than in the wild-type.

**BrMBA**

The data for BrMBA inactivation of *M. radiodurans* wild-type shown in Table 16, are presented graphically in Figure 16a. The D_{37} dose for the wild-type is 223 μM·min compared with 1373 μM·min for *E. coli* (Venitt and Tarmy, 1972). BrMBA is the first DNA-damaging agent used to which *M. radiodurans* is more sensitive than *E. coli*. 302 is three times as sensitive as the wild-type (Figure 16a). The D_{37} of 302 of 78 μM·min is similar to that of a *uvrA exprA* strain of *E. coli* which is unable to excise any BrMBA residues from its DNA (Venitt and Tarmy, 1972). 302 is similar to the wild-type in that it is not mutated to rifampicin-resistance by BrMBA (Table 31).

**AAAF**

Strain 302 is also three times as sensitive as the wild-type to AAAF inactivation (Figure 16b). The survival curve has a reduced shoulder and the final slope of the curve is steeper.

**DCMTC**

Strain 302 is 40 times as sensitive as the wild-type to the lethal effects of the bifunctional alkylating agent MTC (Moseley and Copland, 1978) and 50 times as sensitive to the lethal effects of the monofunctional agent DCMTC (Figure 16c). However, on a
Figure 16  Survival curves of M. radiodurans wild-type (●) and 302 (○), treated with BrMBA, (a); AAF, (b); DCMTC, (c); nitrous acid, (d). Broken line is the wild-type data from Sweet and Moseley, 1976.
molar basis MTC is much more effective in killing both the wild-type and 302 (compare Tables 6, 17, 32 and 33). 302 is not mutated to any detectable extent by either DCMTC or MTC (Tables 32 and 33).

Nitrous acid

Compared with *E. coli* B/r, *M. radiodurans* wild-type is extremely resistant to the lethal effects of nitrous acid with a $D_{37}$ of 2.0 M.min (Sweet and Moseley, 1976). 302 is more sensitive to the lethal effects of nitrous acid with a $D_{37}$ of 0.81 M.min (Figure 16d). Nitrous acid is a weak mutagen in its ability to induce trimethoprim-resistant mutants (Sweet and Moseley, 1976) and rifampicin-resistant mutants (Table 7, Figure 17a) 302 yielding about four times as many mutants as the wild-type (Figure 17a).

Hydroxylamine

Strain 302 is as sensitive as the wild-type to the lethal effects of hydroxylamine (Figure 18a). The $D_{37}$ value of 0.24 M.h is 3.5 times lower than that previously reported (Sweet and Moseley 1976). 302 is also hypermutable by hydroxylamine (Figure 17b) giving 10 to 20 times the yield of the wild-type.

ICR 191G

Both wild-type and 302 show a similar sensitivity to ICR 191G-induced killing (Figure 18b), and both are immutable by ICR 191G (Tables 15 and 34).

Gamma- and UV-radiation

Strain 302 is only very slightly more sensitive than the wild-type to gamma-ray induced killing (Moseley and Copland, 1978). It is not, however, more sensitive to gamma-ray mutagenesis (Figure 17c).

The ability of UV to induce rifampicin-resistant mutants of
Table 31
The sensitivity of *M. radiodurans* 302 to mutagenesis by BrMBA

<table>
<thead>
<tr>
<th>BrMBA dose (µM.min)</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.7</td>
</tr>
<tr>
<td>25</td>
<td>86</td>
<td>0.8</td>
</tr>
<tr>
<td>50</td>
<td>62</td>
<td>3.7</td>
</tr>
<tr>
<td>75</td>
<td>37</td>
<td>0.6</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>2.6</td>
</tr>
<tr>
<td>125</td>
<td>1.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 32
The ability of DCMTC to induce rifampicin-resistant mutants of *M. radiodurans* 302

<table>
<thead>
<tr>
<th>DCMTC dose (µM.h)</th>
<th>Survival (%)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.</td>
<td>100</td>
<td>2.3</td>
</tr>
<tr>
<td>4.4</td>
<td>68</td>
<td>3.6</td>
</tr>
<tr>
<td>8.7</td>
<td>46</td>
<td>2.7</td>
</tr>
<tr>
<td>13.1</td>
<td>29</td>
<td>2.9</td>
</tr>
<tr>
<td>17.5</td>
<td>18</td>
<td>3.0</td>
</tr>
<tr>
<td>26.2</td>
<td>6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 33
The ability of MTC to induce rifampicin-resistant mutants of *M. radiodurans* 302

<table>
<thead>
<tr>
<th>MTC dose (µM.h)</th>
<th>Rif(^R) mutants per 10(^8) survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.6</td>
</tr>
<tr>
<td>0.1</td>
<td>3.8</td>
</tr>
<tr>
<td>0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>0.3</td>
<td>4.4</td>
</tr>
<tr>
<td>0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>0.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>
Figure 17  The sensitivity of M. radiodurans wild-type (●) and 302 (○) to mutation to rifampicin-resistance induced by nitrous acid, (a); hydroxylamine, (b); gamma-rays, (c).
Figure 18 Survival curves of *M. radiodurans* wild-type (•) and 302 (○) treated with hydroxylamine, (a); ICR 191G, (b).
302 is shown in Table 35. 302 may be very slightly mutable by UV but the standard deviations of the values shown are minimum estimates as discussed previously.

2-AP

Strain 302 was grown in TGY medium containing 2-AP at 0,1,2 and 4 mg ml\(^{-1}\). With increasing concentration the generation time increased (Table 36) to those levels observed for wild-type (see Table 13). When the bacteria had gone through about 5 generations, (2 to 3 generations with 4 mg 2-AP ml\(^{-1}\)) the frequency of mutants was determined. The spontaneous mutation frequency to rifampicin-resistant in this experiment was higher than normal. The results (Table 36) show that 2-AP is unable to mutate 302 to any significant extent.

Alkylation mutagenesis of stationary phase M. radiodurans 302

Previous results (Figure 13) showed that stationary phase cells of wild-type are about 100 times less mutable than exponentially growing cells. A culture of 302 was grown to a density of \(5.5 \times 10^8\) vu ml\(^{-1}\) and exposed to MNNG. Its mutability was compared with that of an exponential culture (Figure 19). In contrast to the wild-type stationary phase 302 is strongly mutated by MNNG but is about 10 times less sensitive than an exponential culture.

Stationary phase 302 cells are also about 10 times less mutable than exponential phase cells to MMS mutagenesis (compare Figures 15a and 20). In contrast, stationary phase wild-type are not mutated at all by MMS (Figure 20).

Mutability of MTC-resistant revertants

The sensitivity of 302 to the lethal effects of MTC is due to a mutation in a single gene, designated \textit{mtcA} (Moseley and Copland,
Table 34

The ability of ICR 191G to induce rifampicin-resistant mutants of *M. radiodurans* 302

<table>
<thead>
<tr>
<th>ICR 191G dose (μg.h ml⁻¹)</th>
<th>Survival (%)</th>
<th>Rif⁺R mutants per 10⁸ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1.9</td>
</tr>
<tr>
<td>23</td>
<td>94</td>
<td>3.0</td>
</tr>
<tr>
<td>47</td>
<td>65</td>
<td>3.8</td>
</tr>
<tr>
<td>70</td>
<td>35</td>
<td>0.4</td>
</tr>
<tr>
<td>93</td>
<td>37</td>
<td>4.5</td>
</tr>
<tr>
<td>117</td>
<td>22</td>
<td>4.5</td>
</tr>
<tr>
<td>140</td>
<td>8.4</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Table 35

The ability of UV radiation to induce rifampicin-resistant mutants of *M. radiodurans* 302. The values shown are the means from 3 experiments

<table>
<thead>
<tr>
<th>UV dose (J m⁻²)</th>
<th>Survival (%)</th>
<th>Rif⁺R mutants per 10⁸ survivors (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>3.4 (1.4)</td>
</tr>
<tr>
<td>270</td>
<td>100</td>
<td>4.0 (2.4)</td>
</tr>
<tr>
<td>540</td>
<td>92</td>
<td>4.9 (1.5)</td>
</tr>
<tr>
<td>810</td>
<td>77</td>
<td>7.1 (2.6)</td>
</tr>
<tr>
<td>1080</td>
<td>37</td>
<td>8.4 (3.5)</td>
</tr>
<tr>
<td>1350</td>
<td>6</td>
<td>11.8 (2.6)</td>
</tr>
</tbody>
</table>

Table 36

The effect of 2-AP on generation time (t) and mutation to rifampicin-resistance of *M. radiodurans* 302

<table>
<thead>
<tr>
<th>2-AP concentration (mg ml⁻¹)</th>
<th>t (h)</th>
<th>Rif⁺R mutants per 10⁸ cells (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.65</td>
<td>9.4 (1.5)</td>
</tr>
<tr>
<td>1</td>
<td>2.10</td>
<td>8.6 (2.1)</td>
</tr>
<tr>
<td>2</td>
<td>2.68</td>
<td>10.2 (1.7)</td>
</tr>
<tr>
<td>4</td>
<td>4.30</td>
<td>22.0 (8.2)</td>
</tr>
</tbody>
</table>
In order to establish that the same mutation is responsible for the hypersensitivity to mutagenesis by the monofunctional alkylating agents some spontaneously arising MTC (0.05 μg ml⁻¹)-resistant revertants of 302 were examined for their mutability by MMS and/or MNNG. The results are shown in Table 37. Of four revertants of independent origin examined all showed wild-type resistance to either or both methylating agents.

**Alkaline sucrose gradients**

Strain 302 is sensitive to many DNA damaging agents which in other organisms produce damage subject to excision repair. The ability of 302 to incise DNA damaged in vivo by various DNA-damaging agents was therefore examined by changes in the molecular weight of the DNA sedimented through alkaline sucrose (McGrath and Williams, 1966), and compared with incision in the wild-type. Because 302 is as sensitive to the lethal effects of BrMBA as a *uvrA* *exrA* strain of *E. coli* which is unable to excise any BrMBA-DNA adducts (Venitt and Tarmy, 1973) the incision of this kind of damage was first determined. When the wild-type was treated with 7.6 μM BrMBA for 20 min its DNA had a lower molecular weight than that from untreated controls (Figure 21a,b). Since BrMBA-DNA adducts are chemically stable in alkali (Thielmann, 1976) this result indicates that in the wild-type the damaged DNA is subject to the action of an endonuclease or glycosylase to yield single-strand breaks or alkali-labile sites respectively. When 302 was similarly treated with BrMBA the DNA sedimented close to control positions (Figure 21a,b) which indicates that no single-strand breaks or alkali-labile sites are present in such damaged DNA in 302. Thus 302 is defective in excision repair of BrMBA-induced damage.
### Table 37

Sensitivity of MTC-resistant revertants of *M. radiodurans* 302 (strains R1 - 4) to mutagenesis by MMS and MNNG

<table>
<thead>
<tr>
<th>Strain</th>
<th>Frequency of Rif^R mutants per survivor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMS (44mM.h)</td>
</tr>
<tr>
<td>R1</td>
<td>2.4 x 10^-7</td>
</tr>
<tr>
<td>R2</td>
<td>3.8 x 10^-7</td>
</tr>
<tr>
<td>R3</td>
<td>nt</td>
</tr>
<tr>
<td>R4</td>
<td>nt</td>
</tr>
<tr>
<td>wild-type</td>
<td>3.2 x 10^-7</td>
</tr>
<tr>
<td>302</td>
<td>8.0 x 10^-5</td>
</tr>
</tbody>
</table>

^a nt means not tested
Figure 21 Sedimentation profiles in alkaline sucrose gradients of DNA from BrMBA- and AAAF-treated M. radiodurans wild-type (●) and 302 (○). (a) Untreated controls, (b) BrMBA-treated (7.6 μM for 20 min), (c) AAAF-treated (2.4 mM for 20 min).
Because the profiles were examined after 20 min treatment with BrMBA, there was the possibility that incisions did take place in 302 but were quickly sealed by polynucleotide ligase thus restoring the DNA to its original high molecular weight state. The profiles were therefore examined at 2, 5, 10 and 20 min during treatment with 7.6 μM BrMBA. In the wild-type, strand breaks or alkali-labile sites were detectable after 2 min and the amount of incision increased with time up to 20 min (Figure 22). However, in 302 no reduction of the molecular weight of the DNA occurred (Figure 23). Indeed, the DNA sedimented faster at 2, 5 and 10 min. The profiles, however, do seem unusual, in that most of the radioactivity appears in one fraction. If this represented unlysed cells then the radioactivity would be in the first fraction. At 20 min a "normal" profile is obtained but even this seems to be of a higher molecular weight than control DNA (Figures 21a, b and 23).

A total of 3 experiments were done and in each case a similar pattern occurred and although the precise fraction in which all the DNA appeared was different in each case it was always ahead of the control DNA peak. It is conceivable that these profiles represent attempted recombinational-repair of unexcised lesions and reflects either DNA-DNA or alkali-stable DNA-membrane associations. Recombination repair is involved in the repair of BrMBA-damaged DNA (see later).

When the wild-type was treated with 2.4 mM AAAF for 20 min the profiles showed that single-strand breaks or alkali-labile sites were present. Since AAAF-DNA adducts are also chemically stable in alkali (Thielmann, 1976) this indicates that AAAF-treatment of the wild-type leads to the action of endonuclease(s)
Figure 22

Sedimentation profiles in alkaline sucrose gradients of DNA from *M. radiodurans* wild-type after treatment with 7.6 μM BrMBA for 0 min, (●);
2 min, (■); 5 min, (▲); 10 min, (◆); 20 min, (▼).
Figure 23

Sedimentation profiles in alkaline sucrose gradients of DNA from M. radiodurans 302 after treatment with 7.6 μM BrMBA for 0 min, (○); 2 min, (□); 5 min, (△); 10 min, (◇); 20 min, (★).
Relative distance sedimented
or glycosylase(s) (Figure 21c). Similar strand-breaks or alkali-labile sites were present in the DNA from AAAF-treated 302 (Figure 21c). However, there is the possibility that this DNA is of a slightly higher molecular weight than that from the wild-type since the two profiles are not identical, thus indicating that some lesion(s) remain unexcised in 302.

The profiles obtained after MMS-treatment of the wild-type and 302 are shown in Figure 24. The molecular weight of the two DNAs were identical. Although alkylated bases are stabilized in alkali (Kohn and Spears, 1967), alkylated bases are lost spontaneously and enzymically from alkylated DNA with the resultant production of alkali-labile AP sites and single-strand breaks.

Removal of methylation products from the DNA of M. radiodurans 302 after treatment with [methyl-\(^{3}H\)] MNNG

Since it was apparent that 302 was defective in excision repair of BrMBA-DNA adducts it could be inferred that the sensitivity to mutagenesis by methylating and ethylating agents is due to non-removal of (a) certain alkylation products(s). The ability of wild-type and 302 to remove the three principle products of alkylation by MNNG, namely 7-methylguanine, \(\text{O}^{6}\)-methylguanine and 3-methyladenine was therefore examined. Bacteria were treated with radioactively-labelled MNNG, washed to remove the mutagen and divided into two parts. From one sample the DNA was extracted immediately. The bacteria from the other sample were incubated in growth medium for 3h to allow repair of alkylation damage before the DNA was extracted. Isolated DNA was hydrolysed and methylated purines released were separated by paper chromatography (Maitra and Frei, 1975). The coincidence of radioactivity with authentic marker compounds viewed under 254 nm light was taken as
Figure 24
Sedimentation profiles in alkaline sucrose gradients of DNA from *M. radiodurans* wild-type (■) and 302 (□) after treatment with 29.4 mM MMS for 30 min.
Relative distance sedimented
a measurement of that particular methylated purine.

When 302 was treated with labelled MNNG and the DNA analysed immediately after treatment 7-methylguanaine was the major alkylation product comprising 75% of total methylations identified (Table 38). Alkylation at N3 and N7 of adenine and O6 of guanine were 1.9% and 8.1% respectively (Table 38). The chromatogram from which these results were obtained is shown in Figure 25. The distribution of the products of methylation after 3h post-treatment incubation in growth medium was different (Figure 25). 7-Methylguanaine consisted of 34% of the total products and O6-methylguanaine 20.6% (Table 38). The low level of radioactivity in the 3-methyladenine plus 7-methyladenine spot could not be determined with any accuracy since the total counts were close to the background level. During the incubation in TGY medium the amount of DNA increased by a factor of 1.6 as judged by the increase in optical density of the culture. Therefore, before comparisons could be made the values at 3h were multiplied by this number to make them equivalent to the zero time sample. This is shown in Table 38. When the amounts of each product are compared it is apparent that during the 3h incubation the amount of 7-methylguanaine has been reduced by about 77% but whether the amounts of 3-methyladenine and/or 7-methyladenine were reduced cannot be determined with any accuracy. In contrast the amount of O6-methylguanaine remained constant during the 3h incubation.

M. radiodurans wild-type was similarly treated with [3H]MNNG. In two experiments the DNA could not be successfully isolated immediately after treatment with MNNG for unknown reasons. However, at 3h the DNA could be isolated and methylation
Table 38  Distribution of methyl groups in the DNA of M. radiodurans 302 extracted immediately and 3h after treatment with [methyl-$^3$H] MNNG

<table>
<thead>
<tr>
<th>Product of methylation</th>
<th>Time after MNNG treatment</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oh</td>
<td>3h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cpm (% total)</td>
<td>µmol/mol DNA P</td>
<td>cpm (% total)</td>
</tr>
<tr>
<td>7-Methylguanine</td>
<td>989 (75.3)</td>
<td>58.4</td>
<td>160 (34.3)</td>
</tr>
<tr>
<td>3-Methyladenine</td>
<td>25 (1.9)</td>
<td>1.5</td>
<td>17 (3.6)</td>
</tr>
<tr>
<td>7-Methyladenine+</td>
<td>106 (8.1)</td>
<td>6.3</td>
<td>96 (20.6)</td>
</tr>
<tr>
<td>6-Methylguanine</td>
<td>76 (5.8)</td>
<td>4.5</td>
<td>34 (7.3)</td>
</tr>
<tr>
<td>Origin</td>
<td>76 (5.8)</td>
<td>4.5</td>
<td>34 (7.3)</td>
</tr>
</tbody>
</table>

* a Values have been corrected for a 1.6-fold increase in DNA during the 3h incubation. DNA content of M. radiodurans was assumed to be 3.4 x 10^7 nucleotides per cell (Driedger, 1970).
Figure 25 Chromatogram of hydrolysed DNA extracted from *M. radiodurans* 302,0 and 3h after treatment with [methyl-^3H]MNNG.
products were identified. The results shown in Table 39 are taken from the chromatogram (Figure 26). In contrast to the results obtained with 302 7-methylguanine was the only product of alkylation that was detectable. There were no significant peaks of activity in either the 3-methyl-/7-methyladenine or the $\text{O}^6$-methylguanine positions. In fact there were more cpm present in 7-methylguaninane than in the 3h chromatogram of strain 302 DNA shown in Figure 25 and thus if $\text{O}^6$-methylguanine was not removed counts should be readily measured. This was not the case.

The half-life of hydrolysis of the glycosyl bond between 7-methylguanine and deoxyribose in DNA in pseudo-physiological conditions is about $10^5$h (Lawley and Warren, 1976) but shorter half-lives of 1 to 4 days in the livers of small mammals have been reported. The data presented here show that about 77% of 7-methylguanine residues in 302 are lost in 3h and this undoubtedly implies an active enzymic excision process. Since this lesion is found in the wild-type after 3h incubation at approximately the same level as in 302 implies that the wild-type also excises this residue. With regard to 3-methyladenine and 7-methyladenine the amount of the former following in vitro alkylation of DNA by MNNG is about 11% (Lawley and Thatcher, 1970) and the amount of 7-methyladenine after MNUA treatment of DNA is about 2% (Lawley et al., 1973) but here at time zero both products together only consist of about 2% of the methylation products. The half-life of release of 3-methyladenine from DNA is about $38^5$h in vitro and because the yield is lower than expected the data may be interpreted to mean that this residue is removed during the 30 min treatment time, a situation which occurs during MNNG-and DMS-treatment of
Table 39

Distribution of methyl groups in the DNA of M. radiodurans wild-type extracted 3h after treatment with [methyl-\(^3\)H] MNNG

<table>
<thead>
<tr>
<th>Product of methylation</th>
<th>cpm (% total)</th>
<th>μmol/mol DNA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Methylguanine</td>
<td>363 (51.3)</td>
<td>6.5</td>
</tr>
<tr>
<td>3-Methyladenine</td>
<td>26 (3.9)</td>
<td>0.5</td>
</tr>
<tr>
<td>7-Methyladenine</td>
<td>26 (3.9)</td>
<td>0.5</td>
</tr>
<tr>
<td>(\text{O}^6)-Methylguanine</td>
<td>24 (3.6)</td>
<td>0.4</td>
</tr>
<tr>
<td>Origin</td>
<td>57 (8.6)</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 26 Chromatogram of hydrolyzed DNA extracted from *M. radiodurans* wild-type 3h after treatment with [methyl- H] MNNG.
E. coli (Lawley and Orr, 1970; Lawley and Warren, 1976). Both wild-type and 302 can apparently excise 3-methyladenine residues. No definitive statements can be made as to the minor product 7-methyladenine due to its low and in these experiments, undetectable amounts. \( ^6 \)-Methylguanine is stable in DNA and loss must therefore necessitate active excision. 302, unlike the wild-type, is unable to remove this lesion from its DNA.

Perturbations in DNA metabolism after MNNG treatment

The dose of MNNG (68μM for 30 min) used in the experiments described above is non-lethal for both M. radiodurans wild-type and 302. After such treatment DNA synthesis in the wild-type is unaffected. However, in 302 DNA synthesis is inhibited for approximately 20 min before it proceeds at a rate slightly below that of wild-type (Figure 27).

A more dramatic difference between these strains was seen when bacteria were prelabelled with \(^3\)HdThd, exposed to MNNG and loss of TCA-insoluble counts determined during the treatment time and during subsequent post-treatment incubation in TGY medium. The results are shown in Figure 28. In the wild-type about 1% of DNA was degraded during the treatment time, increasing to about 30% after 3h in TGY medium. In contrast, in 302 about 56% of the DNA was degraded during the 30 min treatment time with no increase on subsequent incubation.

7. Some aspects of repair of BrMBA-damaged DNA

7. 1 DNA degradation during BrMBA treatment

The amount of DNA degraded in M. radiodurans wild-type and
Figure 27  Inhibition of DNA synthesis in *M. radiodurans* wild-type (●), 302 (▲) and UV22 (■) induced after treatment with 68 μM MNNG for 30 min.
Figure 28 DNA degradation in M. radiodurans wild-type (○), 302 (□) and UV22 (△) induced during and after treatment with 68 μM MNNG. The arrow indicates the end of the treatment with MNNG when the bacteria were washed and incubated in TGY medium.
302 during treatment with 7.6 μM BrMBA was measured. Bacteria, prelabelled with [³H]dThd were washed and resuspended in buffer containing DMSO (1%) in which the BrMBA was dissolved. At appropriate times samples were removed, TCA-insoluble material collected and the radioactivity counted as described earlier. The results are shown in Table 40. In neither wild-type nor 302 was there evidence of substantial DNA degradation during the 20 min treatment time.

7.2 Involvement of recombination in repair of BrMBA-induced DNA damage

The BrMBA dose-response curve for 302 has a shoulder to it which is indicative of repair. What is the nature of this repair? The alkaline sucrose gradient data suggest that the excision repair which is defective in 302 is the only form of excision repair for BrMBA-damage (but not for AAAF damage). The possible involvement of recombination in the repair of BrMBA damage was investigated by examining the dose-response curve of rec30. The result is shown in Figure 29. The D₃₇ value is 38 μM.min compared with values of 220 μM.min and 73 μM.min for wild-type and 302 respectively. Thus, recombination is involved in repair of BrMBA-DNA adducts and contributes to survival of the wild-type more than the excision repair process defective in 302.

7.3 DNA repair synthesis in permeable cells after BrMBA treatment

If M. radiodurans wild-type is capable of the excision repair of BrMBA-induced damage there must be some DNA synthesis to replace the excised region but none in 302. The amounts of repair synthesis was measured by incorporation of [³H]dTMP into the acid-insoluble fraction of nucleotide-permeable cells. Bacteria
Table 40

Degradation of DNA of *M. radiodurans* wild-type and 302 during treatment with 7.6 μM BrMBA

<table>
<thead>
<tr>
<th>Strain</th>
<th>BrMBA treatment (min)</th>
<th>% Counts remaining TCA insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>302</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>89</td>
</tr>
</tbody>
</table>
Figure 29  BrMBA survival curve of *M. radiodurans* rec30.
were made permeable as described previously and exposed to 0, 3.6, 7.2, 10.8 and 14.4 μM BrMBA for 20 min. The BrMBA was removed by centrifugation and the bacteria incubated in the assay mixtures for DNA synthesis as described. At appropriate times reactions were stopped and the amount of acid-insoluble radioactivity determined. The results are shown in Figure 30. In both the wild-type and 302 the incorporation of dTMP decreases as the BrMBA concentration increases. Because the lowest concentrations used in the wild-type was a non-lethal dose there was the possibility that the Triton X-100 treatment also increased the permeability of the bacteria to BrMBA resulting in increased damage to DNA and to other cellular components. The incorporation of dTMP was therefore followed after much lower doses of BrMBA, viz., 0.29, 0.72 and 1.44 μM for 20 min. The results are shown in Figure 31. In the wild-type these low concentrations did not inhibit incorporation of dTMP. However, neither was there any BrMBA-stimulated DNA synthesis. Since 302 does not excise BrMBA-DNA adducts the higher doses of BrMBA (Figure 30b) probably inhibit normal replicative synthesis as in E. coli (Thielmann, 1976). However, in E. coli BrMBA does have a stimulating effect on DNA synthesis in uvrB+ although not in uvrB- cells. For M. radiodurans wild-type two explanations may account for the observations: either the size of the inserted patch is very small and is masked, at higher BrMBA doses, by the relatively larger inhibition of semi-conservative DNA synthesis or the Triton X-100 treatment inhibits excision of BrMBA-DNA adducts with the result that no excision gaps are produced. An experiment, described below, to determine the ATP requirement of the incision step supports the latter.
Figure 30 The effect of BrMBA on DNA synthesis in nucleotide-permeable M. radiodurans wild-type (closed symbols) and 302 (open symbols). BrMBA concentrations were 0 μM (●, ○), 3.6 μM (▲, △), 7.2 μM (■, □), 10.8 μM (▼, ▼), 14.4 μM (◆, ◆) and bacteria were exposed for 20 min at 30°C. The BrMBA was removed and DNA synthesis was followed as described.
Figure 31 DNA synthesis in nucleotide-permeable M. radiodurans wild-type after exposure for 20 min at 30°C to BrMBA at concentrations of 0 μM (○), 0.29 μM (△), 0.72 μM (□), 1.44 μM (▽).
Incision is difficult to study in vivo as an independent event because the single-strand breaks are quite rapidly repaired. However, incision can be followed independently of subsequent repair events in vitro in permeable cells by omitting the deoxyribonucleoside triphosphates (dNTPs) to prohibit repair replication. Such methodology has shown that the incision of both UV- and BrMBA-damaged DNA in E. coli requires ATP (Waldstein et al., 1974; Thielmann, 1976).

The ATP requirement of the incision of BrMBA-damaged DNA in M. radiodurans wild-type was examined. Bacteria, prelabelled with \(^{3}H\) dThd, were made permeable and incubated in the appropriate buffer in the presence of ATP and BrMBA (7.6 \(\mu\)M for 20 min). Spheroplasts were prepared as described and these were lysed on top of alkaline sucrose gradients and centrifuged. An initial experiment (data not shown) indicated that the process of making the cells permeable causes the DNA to be of a low molecular weight form, in agreement with the observations of Kitayama and Matsuyama (1976). Therefore centrifugation times were extended to 3h. The gradient profiles are shown in Figures 32 and 33. In the presence or absence of ATP, BrMBA treatment has no effect on the molecular weight of the DNA.

These results therefore do not give any indication as to the ATP requirement of the incision of BrMBA-damaged DNA. Whether the apparent lack of incision in vitro is a reflection of more stringent conditions required for the enzymes involved in incision of BrMBA-damaged DNA or whether it is due to damage of enzymes by the Triton X-100 treatment remains unknown.
Figure 32 Sedimentation profiles in alkaline sucrose gradients of DNA from nucleotide-permeable M. radiodurans wild-type treated with (●) or without (○) 7.6 μM BrMBA for 20 min in the absence of ATP.
Figure 33

Sedimentation profiles in alkaline sucrose gradients of DNA from nucleotide-permeable *M. radiodurans* wild-type treated with (▲) or without (△) 7.6 μM BrMBA for 20 min in the presence of 1 mM ATP.
M. radiodurans 262 is similar to 302 in its sensitivity to the lethal effects of MTC, gamma-rays and its resistance to UV and is mutant in a gene designated mtcB (Moseley and Copland, 1978). The sensitivity of 262 to the lethal and mutagenic effects of some alkylating agents was examined.

**MNNG**

The sensitivity of 262 to the lethal effects of MNNG is shown in Figure 10 and Table 29. It is 17 times as sensitive as the wild-type and 3 times as sensitive as 302. Strain 262 is also more sensitive than the wild-type to the mutagenic effects of MNNG (Figure 12) being about as hypersensitive as 302.

**MMS**

Strain 262 is slightly more sensitive than the wild-type to the lethal effects of MMS (Figure 34) since the $D_{37}$ dose is 41 mM.h compared with 66 mM.h for the wild-type and is about 100 times as sensitive as the wild-type to MMS mutagenesis (Figure 34).

**EMS**

The sensitivity of 262 to the lethal and mutagenic effects of EMS is shown in Figure 35. The $D_{37}$ dose of 170 mM.h is 4.3 and 1.5 times lower than the values for the wild-type and 302 respectively. Strain 262 is also hypermutable by EMS (Figure 35) being almost as sensitive as 302 and over 100 times as sensitive as the wild-type. For example, after a dose of 200 mM.h EMS 262 yields rifampicin-resistant mutants at a frequency of $1.5 \times 10^{-5}$ compared with a value of $1.2 \times 10^{-7}$ for the wild-type.

**DCMTC**

Extreme sensitivity of 302 to the lethal effects of an
Figure 34

The effect of MMS on survival (●) and mutation to rifampicin-resistance (▲) of *M. radiodurans* 262.
agent is only observed with MTC and DCMTC (Moseley and Copland, 1978; Figure 16c). The $D_{37}$ value for DCMTC inactivation of 262 is 10.3 μM.h (Figure 36) and identical to that for 302.

Removal of methylation products from the DNA of *M. radiodurans* 262 after treatment with $[^{3}\text{H}]$ MNNG

The ability of 262 to remove methylated bases or nucleotides from its DNA was examined after treatment with radioactively-labelled MNNG. DNA isolated immediately and 3h after treatment with MNNG was hydrolysed in dilute acid and the released purines were separated by paper chromatography as described previously. The distribution of radioactivity in the chromatograms is shown in Figure 37 and 38. Immediately after treatment with the mutagen 7-methylguanine was the major product containing 76% of the total radioactivity (Table 41). In this experiment the $O^6$-methylguanine spread out for several cm as indicated by the dashed lines in Figures 37 and 38. The amount of $O^6$-methylguanine present could therefore not be determined. From the amount of activity that was in the UV-absorbing spot corresponding to 3-methyladenine it is evident that this purine and 7-methyladenine contained 4% of the total activity (Table 41). The amount of methylation of the N7 of guanine is the same as found for 302 (see Tables 38 and 41). Similarly after 3h incubation the amount of 7-methylguanine in 262 has been reduced by 74%. No peaks of activity coincided with the 3-methyladenine marker but the activity represents less than 2.5 μmol of this purine per mol DNA P.

It can be concluded that 262, like wild-type and 302, is proficient in the excision of 7-methylguanine. Also it seems likely that 262 can excise 3-methyladenine since at time zero the amount of this purine is lower than the level expected from *in vitro* levels as is the case with 302 and is interpreted to mean rapid excision during the 30 min treatment time.
Figure 36: DCMTC survival curves of *M. radiodurans* 262 (△), 301 (□), 303 (○).
Figure 37 Chromatogram of hydrolysed DNA of M. radiodurans 262 extracted immediately after treatment with [methyl-\(^{3}\)H] MNNG.
Figure 38 Chromatogram of hydrolysed DNA of *M. radiodurans* 262 extracted 3h after treatment with [*methyl* $^3$H] MNNG.
Table 41 Distribution of methyl groups in the DNA of *M. radiodurans* 262 extracted immediately and 3h after treatment with [methyl-$^3$H] MNNG

<table>
<thead>
<tr>
<th>Product of methylation</th>
<th>Oh</th>
<th>Time after MNNG treatment</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm(% total)</td>
<td>µmol/mol DNA P</td>
<td>cpm(% total)</td>
</tr>
<tr>
<td>7-Methylguanine</td>
<td>1618 (76.4)</td>
<td>54.6</td>
<td>140 (22)</td>
</tr>
<tr>
<td>3-Methyladenine</td>
<td>92 (4)</td>
<td>3.1</td>
<td>24 (3.7)</td>
</tr>
<tr>
<td>7-Methyladenine +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_6$-Methylguanine</td>
<td>NM$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Origin</td>
<td>78 (3.7)</td>
<td>2.6</td>
<td>54 (8.4)</td>
</tr>
</tbody>
</table>

$^a$Values have been corrected for a 1.45-fold increase in DNA during the 3h incubation in growth medium.

$^b$NM = not measured, see text.
whether 262 can remove $\text{O}^6$-methylguanine cannot be answered but it is tempting to think that it cannot because 262 is phenotypically very similar to 302. Indeed in spite of the poor chromatograms there appears to be quite a lot of radioactivity under the position of $\text{O}^6$-methylguanine in Figure 38.

9. *M. radiodurans* UV22

The experiments on MNNG mutagenesis of repair-deficient mutants described previously showed that UV22, along with 302 and 262, was more sensitive to the mutagenic effects of MNNG. However, it is different from 302 and 262 in this sensitivity and it is also different in being sensitive to the lethal effects of UV.

**MNNG**

The data in Figure 8 and Table 29 show that UV22 is 18 times as sensitive as the wild-type to MNNG-induced killing. Also Table 29 and Figure 11 show that although UV22 is hypermutable by MNNG compared with the wild-type it is not as hypersensitive as 302 or 262.

**MMS**

The dose-response curve for MMS-induced killing and mutation of UV22 is shown in Figure 39. The $D_{37}$ dose is 13.2 mM.h which is about 5 times lower than the wild-type value. However, UV22 is much more sensitive to the mutagenic effects of MMS and yields about 20 times more mutants than the wild-type (Figure 39). It is thus considerably less sensitive than 302 or 262 to MMS mutagenesis.
Figure 39. The effect of MMS on survival (○) and mutation to rifampicin-resistance (■) of *M. radiodurans* UV22.
Removal of methylation products from the DNA of *M. radiodurans* UV22 after treatment with [*methyl-^3^H*] MNNG

*M. radiodurans* UV22 was treated with 68 µM MNNG, isolated DNA was hydrolysed and methylated purines were identified as previously described. The distribution of radioactivity in the chromatograms from one experiment (Expt. A) is shown in Figures 40 and 41 and in numerical form in Table 42. Immediately after treatment of UV22 with MNNG the level of methylation, as measured by the amount of 7-methylguanine, was the same as found in 302 and 262 and thus rules out the possibility that the mutant has increased membrane permeability. Clear peaks of radioactivity corresponding to 3-methyladenine and 6-methylguanine were not present but the amounts of radioactivity in the UV-absorbing and UV-fluorescent spots correspond to 7% of 3- and 7-methyladenine and 5% 6-methylguanine. After 3h incubation in growth medium about 88% of 7-methylguanine residues have been lost, similar to results with strains 302 and 262. In contrast, there was no loss of 3- and/or 7-methyladenine. The amount of 6-methylguanine has been reduced during the 3h incubation but since the counts are so low the actual amount cannot be determined with accuracy.

The results from a second experiment are shown graphically in Figures 42 and 43 and numerically in Table 42. Because the yield of 7-methylguanine was higher than the previous experiment it is assumed that an error was made in weighing out the MNNG. Some radioactivity was present in the 3-methyladenine and 6-methylguanine regions but because the total amount of DNA chromatographed was small the yield of these methylated purines was low and could not be determined with any accuracy. After 3h post-treatment incubation in TGY medium the amount of 7-methylguanine was reduced by 73%. A
Figure 40 Chromatogram of hydrolysed DNA of *M. radiodurans* UV22 extracted immediately after treatment with [methyl-3H] MNNG.
Chromatogram of hydrolysed DNA of M. radiodurans UV22 extracted 3h after treatment with [methyl-³H] MNNG.
Table 42 Distribution of methyl groups in the DNA of *M. radiodurans* UV22 extracted immediately and 3h after treatment with \(^{3}\text{H}\)MNNG

<table>
<thead>
<tr>
<th>Product of methylation</th>
<th>Time after MNNG treatment</th>
<th>Expt A</th>
<th>Expt B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oh</td>
<td>3h</td>
</tr>
<tr>
<td></td>
<td>cpm (% total)</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td></td>
<td>µmol/mol DNA P (% total)</td>
<td>µmol/mol DNA P (%) total</td>
<td>µmol/mol DNA P (%) total</td>
</tr>
<tr>
<td>7-Methylguanine</td>
<td>854 (61.4)</td>
<td>58.8</td>
<td>105 (30.7)</td>
</tr>
<tr>
<td>6-Methylguanine</td>
<td>70 (5.0)</td>
<td>4.8</td>
<td>40 (11.7)</td>
</tr>
<tr>
<td>3-Methyladenine</td>
<td>98 (7.1)</td>
<td>6.7</td>
<td>84 (24.6)</td>
</tr>
<tr>
<td>7-Methyladenine</td>
<td>83 (6.0)</td>
<td>5.7</td>
<td>20 (5.8)</td>
</tr>
<tr>
<td>Origin</td>
<td>83 (6.0)</td>
<td>5.7</td>
<td>20 (5.8)</td>
</tr>
</tbody>
</table>

*Values have been corrected for a 1.3-fold increase in DNA during the 3h incubation.*
Figure 42 Chromatogram of hydrolysed DNA of *M. radiodurans* UV22 extracted immediately after treatment with [methyl-\( ^{3} \text{H} \)] MNNG.
Figure 43  Chromatogram of hydrolysed DNA of *M. radiodurans* UV22 extracted 3h after treatment with 3-MeAdo and 7-MeGua.
substantial amount of radioactivity was present in the position corresponding to 3- and 7-methyladenine whereas no significant amount could be detected under $\text{O}^6$-methylguanine.

When the data from these experiments are taken together and with the in vitro rates of loss of methylated purines as discussed above, it is apparent that UV22 is proficient in excision of 7-methylguanine. In contrast, the amount of radioactivity corresponding to 3- and 7-methyladenine remains constant during the 3h incubation. 7-Methyladenine is expected to contribute only about 2% of total alkylation products. Using the values in Expt B (Table 42) that have the greatest amount of activity it is apparent that the ratio of 3-methyladenine: 7-methylguanine is 14.8 : 107.5 i.e. 0.14, similar to the ratio in in vitro methylated DNA. Thus UV22 is unable to remove 3-methyladenine residues. However, in contrast with the results with strain 302 $\text{O}^6$-methylguanine is efficiently removed during the 3h incubation time.

**DNA synthesis and degradation following MNNG treatment**

Exponentially-growing cells of UV22 were treated with 68 $\mu$M MNNG for 30 min at 30$^\circ$ and were centrifuged to remove the mutagen. The bacteria were incubated in TGY medium containing $[^3\text{H}]\text{dThd}$ and at appropriate times TCA-insoluble material was collected on glass-fibre disks which were processed and measured for radioactivity as described previously. DNA synthesis is inhibited after such treatment, resumes after about 100 min and then proceeds at a rate comparable to that in the wild-type (Figure 27).

The release of acid-soluble material from prelabelled cells during, and subsequent to, MNNG treatment is shown in Figure 28.
There is extensive degradation of DNA during the 30 min treatment time 46% of radioactivity becoming acid-soluble. On subsequent incubation in growth medium this increases to 56% during the first 20 min but then degradation ceases for up to 3h.

Alkaline sucrose gradients of UV-irradiated UV22

UV22 has a $D_{37}$ dose for UV of 84 J m$^{-2}$ which is about 13 times lower than the wild-type dose (Moseley, pers. commun.). Because UV22 seemed to be defective in the removal of 3-methyladenine residues from its DNA it seems likely that it must also be unable to remove some form of UV-induced damage. To determine whether this was an inability to incise DNA, or some other factor, profiles of UV-damaged DNA sedimented through alkaline sucrose were examined. Bacteria were irradiated with 270 J m$^{-2}$ UV and either spheroplasts were made immediately or after the bacteria had been incubated for 15 min in TGY medium. The profiles of DNA from wild-type after such treatment are shown in Figure 44. The nicks that have been produced as a result of UV-damage are repaired partly during the 15 min post-irradiation incubation as shown by the movement of the peak toward control molecular weight.

The data shown in Figure 45 indicate that UV22 can incise UV-damaged DNA at least as well as the wild-type and is thus not defective in the incision step. However, at least one step subsequent to incision must be defective since these gaps remain open at least for 15 min after irradiation because the profile at this time is the same as that immediately after irradiation.
Figure 44 Sedimentation profiles in alkaline sucrose gradients of DNA from *M. radiodurans* wild-type: (○), unirradiated control; (▲), UV-dose of 270 J m\(^{-2}\); (■), UV-dose of 270 J m\(^{-2}\) plus incubation 15 min post-irradiation in TGY medium.
Figure 45  Sedimentation profiles in alkaline sucrose of DNA from *M. radiodurans* UV22: (○), unirradiated control; (△), UV-dose of 270 J m\(^{-2}\); (□), UV-dose of 270 J m\(^{-2}\) plus 15 min post-irradiation incubation in TGY medium.
Relative distance, sedimented

% Total cpm, O

% Total cpm, △, □
10. Excision of 7-methylguanine

All the experiments described above involved quantification of methylated purines at the beginning and end of a 3h incubation period to allow the repair of methylation products. The presence of alkylation products was therefore examined after 1h and 2h incubation following treatment of *M. radiodurans* wild-type labelled MNNG. The distribution of radioactivity in the chromatograms is shown in Figure 46 and in Table 43. Sufficient DNA was available only to examine the presence of the major product i.e. 7-methylguanine. The data from experiments with 302, 262, UV22 and wild-type presented earlier have been combined and are shown in Table 44. In order that the strains can be compared directly the differences in amount of total DNA degraded during the treatment have been taken into account (see Figure 28). This has been done in Table 44 and the actual yields have been plotted in Figure 47. The excision of 7-methylguanine shows a biphasic response with most of them removed during the first hour after treatment with a half-life of 37 min or less. After 1h, the rates of loss of 7-methylguanine is much slower.

Crude extracts of *M. radiodurans* wild-type were prepared and tested for ability to release radioactivity from *in vitro* \[^{3}H\text{M}	ext{N}	ext{N}	ext{G} \]methylated calf thymus DNA. The nature of the released products might then give an indication as to which methylated purines residues are released by endonuclease or glycosylase action. However, none of these enzyme activities could be detected in crude extracts under a variety of assay conditions. The release of label which did occur was shown to be due to non-specific nuclease action (data not shown).
Figure 46  Chromatograms of hydrolysed DNA of *M. radiodurans* wild-type extracted 1h and 2h after treatment with \([\text{methyl-}^3\text{H}]\text{MNNG}\).
Table 43

The amount of 7-methylguanine present in the DNA of *M. radiodurans* wild-type extracted at different times after treatment with [methyl-\(^3\)H] MNNG

<table>
<thead>
<tr>
<th>Time after MNNG treatment (h)</th>
<th>cpm</th>
<th>(\mu)mol 7-MeGua/mol DNA P</th>
<th>Corrected values for increases in DNA</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Actual value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>139</td>
<td>16.0</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>12.0</td>
<td>15.6</td>
<td></td>
</tr>
<tr>
<td>3(^a)</td>
<td>363</td>
<td>6.5</td>
<td>12.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Value from Table 39

\(^b\) Increases in DNA compared to zero value: 1h, 1.1; 2h, 1.3; 3h, 1.9

Table 44

The amount of 7-methylguanine present in the DNA of *M. radiodurans* wild-type, 302, 262 and UV22 extracted at different times after treatment with [methyl-\(^3\)H] MNNG

<table>
<thead>
<tr>
<th>Strain</th>
<th>% DNA degraded</th>
<th>(\mu)mol 7-MeGua/mol DNA P post-treatment incubation(h)</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment time</td>
<td>1h 2h 3h</td>
<td>0 1 2 3</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1 6.6 18.2 30</td>
<td>18.8 19.1 17.7</td>
<td></td>
</tr>
<tr>
<td>302</td>
<td>56 0 132.7 13.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>262(^a)</td>
<td>56 0 124.1 14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV22</td>
<td>46 0 110.7 10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>199.1 33.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Amount of DNA degradation in 262 is assumed to be the same as in 302.
Figure 47 In vivo loss of 7-methylguanine from the DNA of M. radiodurans wild-type (▼), UV22 (○, ●), 262 (▲), 302 (■).
Of many UV-sensitive mutants of *M. radiodurans* wild-type isolated over many years from this and other laboratories all have been excision-proficient and able to remove pyrimidine dimers. From studies in other bacteria it is often the case that the endonuclease initiating excision repair of pyrimidine dimers is not involved in the repair of MMS damage. Since strain 302 is extremely mutable by MNNG an attempt was made to isolate some UV-sensitive mutants which would then be tested for MMS resistance. A culture of 302 was treated with MNNG and after replica-plating 12 UV-sensitive mutants were isolated from about 6000 colonies screened. The UV and MMS dose-response curves for one of these mutants, designated uvs10, are shown in Figures 48 and 49. The mutant has a $D_{37}$ dose for UV of $90 \, \text{J m}^{-2}$ and is thus 12 times as sensitive as the wild-type. For MMS the $D_{37}$ is $59 \, \text{mM.h}$ and is thus as resistant as the wild-type. Strain uvs10 therefore represents a putative excision-less mutant. However, compared with the equivalent *E. coli* strains it is much more resistant and in fact is more resistant than wild-type *E. coli* B/r.

**Alkaline sucrose gradients of UV-irradiated uvs10**

The molecular weights of DNA present after irradiation of uvs10 and its parent strain 302 with a UV dose of $270 \, \text{J m}^{-2}$ were determined by sedimentation in alkaline sucrose gradients. The sedimentation profiles are shown in Figure 50. Compared with the unirradiated controls it is apparent the both strains can incise UV-damaged DNA since both profiles show a reduction in the molecular weight of the DNA. However, after UV-irradiation uvs10 possesses DNA of an average molecular weight greater than that
Figure 48: UV survival curve of *M. radiodurans* uvs10.
Figure 49  The effect of MMS on survival (■) and mutation to rifampicin-resistance (□) of *M. radiodurans* uvs10.
Figure 50  Sedimentation profiles in alkaline sucrose gradients of DNA from unirradiated (triangles) and UV-irradiated (540 J m\(^{-2}\)) (circles) *M. radiodurans* 302 (closed symbols) and uvs10 (open symbols).
present in 302 as shown by the broader distribution of radioactivity. These data suggest that uvs10 may be defective in incision of UV-irradiated DNA but whether it is unable to remove some pyrimidine dimers or other UV-photoproduct is not known.

**Spontaneous mutation frequency**

Five cultures of uvs10 were grown each from an inoculum consisting of about $10^4$ cells. The cultures were shaken at $30^\circ$ for between 17-18 generations and the frequency of rifampicin-resistant mutants determined. The results in Table 45 show that uvs10 is a weak mutator with an approximate 5-fold increase over the wild-type frequency.

**UV mutagenesis of uvs10**

The ability of UV radiation to mutate uvs10 to rifampicin-resistance was determined. The results, shown in Table 46, are from two experiments and indicate that uvs10 is very slightly mutable by UV and gives an approximate 2-fold increase over the spontaneous level. The number of mutants induced appears to reach a saturation level even after a low dose of $11 \text{ J m}^{-2}$ or less.

**BrMBA inactivation of uvs10**

Exponentially growing cells of uvs10 were exposed to different concentrations of BrMBA for 20 min at $30^\circ$. The survival curve is shown in Figure 51. The $D_{37}$ dose is $78 \mu M\cdot\text{min}$ which is identical to the value for 302. At a dose of $92 \mu M\cdot\text{min}$ uvs10 is considerably more sensitive than 302 but since this is based on only one point it could be due to experimental error.

**UV-stimulated DNA synthesis in permeabilized cells**

If uvs10 were unable to incise at least one kind of UV-induced damage then the lesion would not be removed to produce an excision gap and there would thus be a reduction in the amount of
Figure 51: BrMBA survival curve of *M. radiodurans uvs10.*
Table 45

The spontaneous mutation frequency to rifampicin-resistance of M. radiodurans uvs10

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Rif&lt;sup&gt;R&lt;/sup&gt; mutants per 10&lt;sup&gt;8&lt;/sup&gt; cells (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.8 (1.7)</td>
</tr>
<tr>
<td>2</td>
<td>7.2 (0.4)</td>
</tr>
<tr>
<td>3</td>
<td>9.3 (0.9)</td>
</tr>
<tr>
<td>4</td>
<td>13.6 (2.2)</td>
</tr>
<tr>
<td>5</td>
<td>9.2 (1.0)</td>
</tr>
</tbody>
</table>

Table 46

The ability of UV to induce rifampicin-resistant mutants of M. radiodurans uvs10

<table>
<thead>
<tr>
<th>UV dose (J m&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>Survival (%)</th>
<th>Rif&lt;sup&gt;R&lt;/sup&gt; mutants per 10&lt;sup&gt;8&lt;/sup&gt; survivors (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>11.3 (0.8)</td>
</tr>
<tr>
<td>11.25</td>
<td>98</td>
<td>17.9 (2.6)</td>
</tr>
<tr>
<td>22.50</td>
<td>96</td>
<td>19.3 (2.4)</td>
</tr>
<tr>
<td>33.75</td>
<td>92</td>
<td>22.7 (3.7)</td>
</tr>
<tr>
<td>45.00</td>
<td>81</td>
<td>22.8 (1.7)</td>
</tr>
<tr>
<td>56.25</td>
<td>73</td>
<td>23.0 (0.7)</td>
</tr>
<tr>
<td>67.50</td>
<td>64</td>
<td>19.5 (1.8)</td>
</tr>
<tr>
<td>90.00</td>
<td>24</td>
<td>20.1 (2.9)</td>
</tr>
<tr>
<td>112.50</td>
<td>6.4</td>
<td>18.7 (3.5)</td>
</tr>
</tbody>
</table>
repair synthesis. Before the amount of UV-induced DNA synthesis was measured it was necessary to examine the pattern of DNA synthesized after UV-irradiation of the wild-type.

**Wild-type**

The amount of DNA synthesized in 30 min in UV-irradiated *M. radiodurans* wild-type is shown in Figure 52. The results, in contrast to those obtained with *E. coli* show that with UV-irradiation the amount of DNA synthesized increases after non-lethal doses of UV to a level about 22% higher than unirradiated controls. With increasing UV doses the level of DNA synthesis, as in the case of *E. coli*, remains constant. More experiments on the nature of the DNA synthesized after UV-irradiation are presented below.

**uvs10**

Cells of uvs10 were made permeable, UV-irradiated or not and incubated in the presence of dNTPs and appropriate cofactors. The amount of dTMP incorporated as a function of UV dose is shown in Figure 53. The overall pattern of DNA synthesis is similar, if not identical, to the wild-type, with a plateau level 17% higher than unirradiated controls. However, it cannot be concluded that this is significantly lower than the wild-type level due to the experimental errors.

Further complications arise because it is not clear whether the 122% represents all repair-synthesis, or nearly all, or whether it is composed of normal semi-conservative (100%) plus repair synthesis (22%). Certainly in non-Triton treated cells UV radiation inhibits DNA synthesis, as judged by the uptake of [³H] dThd in a dose dependent way (Setlow and Boling, 1965). A
Figure 52 The effect of UV-irradiation on DNA synthesis in permeable cells of *M. radiodurans* wild-type. The results from two independent experiments are shown. 100% is equivalent to the incorporation of 532 pmol (O) and 551 pmol dTMP (△).
Figure 53 The effect of UV-irradiation on DNA synthesis in permeable cells of *M. radiodurans uvs10.*
temperature-sensitive mutant of *M. radiodurans*, strain tsl, which is unable to incorporate dThd immediately it is put at the restrictive temperature (39°C) was used in order to see if semi-conservative and repair synthesis could be separated. However, the result (data not shown) was that at 39°C incorporation of dTMP proceeded for 30 min before it ceased. The reason for these apparently conflicting results *in vivo* and *in vitro* is not clear and was not further investigated.

### 12. Repair replication

Experiments described above did not clearly indicate whether the DNA synthesis observed after UV irradiation was predominantly semi-conservative synthesis, predominantly repair synthesis or a mixture of both types. An attempt was made to resolve this problem by separating the two components by using a density label (Pettijohn and Hanawalt, 1964). Further, experiments could give an indication of the size of the reinserted fragments required to fill the excision gaps to see if there is a heterogeneity of patch size which is known to occur in *E. coli* (Cooper and Hanawalt, 1972) but which has not been demonstrated in any other bacterium.

Initially, experiments with *M. radiodurans* were done using nucleotide-permeable cells so that the ATP requirements of the polymerization steps could be determined. In these experiments the DNA was prelabelled with [¹⁴C] dThd, the cells then made permeable, UV-irradiated or not and then incubated in a reaction mixture with the four dNTPs (³H-labelled dATP) with dTTP replaced by BrdUTP.
In some cases ATP was absent. Lysates were made and the DNA centrifuged to equilibrium in CsCl gradients as described previously.

The pattern of DNA synthesized in 30 min in the presence of ATP is shown in Figure 54. The results were unexpected. Figure 54a shows DNA synthesized in an unirradiated control. All the DNA sedimented at one position and thus appeared to be of uniform density. It was expected that there would have been some DNA of parental density with no associated $^3$H activity which represents unreplicated DNA. Semi-conservative synthesis would produce DNA of hybrid density which would necessarily contain some $^3$H counts. When the bacteria were irradiated with a UV dose of 540 J m$^{-2}$ the pattern of DNA synthesized is shown in Figure 54b. The distribution of radioactivity was similar to that of unirradiated controls in that all the DNA sedimented at uniform density. However, the ratio of $^3$H:$^{14}$C counts is higher in the irradiated bacteria indicating that UV has caused a stimulation of DNA synthesis which agrees with the data on the incorporation of dTMP as described above. In the absence of ATP the amount of DNA synthesized in unirradiated or UV-irradiated cells was very much reduced (Figure 55).

Hansen (1978) has estimated that the time to replicate the DNA of *M. radiodurans* is between 60 to 80 min. Using a different method, Moseley (pers. commun.) has estimated that the replication time is between 50 to 60 min. Therefore the 30 min time used here would still be expected to show unreplicated parental DNA.

In these experiments the DNA was only "whirlimixed". There was the possibility that the DNA fragments were quite large
Figure 54 Isopycnic analysis of DNA synthesis in Triton-treated *M. radiodurans* wild-type in the presence of 1 mM ATP, (a) unirradiated control, (b) 540 J m⁻².
Figure 55  Isopycnic analysis of DNA synthesis in Triton-treated
M. radiodurans wild-type in the absence of ATP, (a) unirradiated
control, (b) 540 J m$^{-2}$.
and most of uniform density. In a second experiment the DNA was sheared by passing through a hypodermic needle. The pattern of DNA synthesized in unirradiated cells at 20 min and 40 min is shown in Figure 56. The results were as before with no clear separation of DNA into bands of different densities. In the absence of ATP there was little or no DNA synthesized.

A separate experiment (data not shown) indicated that in the absence of BrdUTP the amount of DNA synthesized was drastically reduced to a level similar to that of DNA synthesized in the absence of dGTP. Thus the incorporation of $^3$H activity is accompanied by the incorporation of BrdUMP.

Experiments described previously have indicated that the process of making the cells permeable to nucleotides causes extensive damage to DNA by an unknown mechanism. The results obtained could have conceivably been due to an artefact as a consequence of the Triton treatment. Permeable cells were therefore no longer used. These results have, however, shown that normal replicative DNA synthesis requires ATP and after UV-irradiation there is a UV-induced stimulation of DNA synthesis. They also show that in the absence of ATP in irradiated cells there is little or no DNA synthesized which may mean that the incision step of UV-damaged DNA requires ATP and/or the repolymerization of the excision gap also requires the presence of ATP.

Further experiments were carried out to resolve the problem presented by these unexpected profiles using non-Triton treated cells in experiments similar to those described by Hanawalt and Cooper (1971) and Cooper and Hanawalt (1972). In these experiments a thymine-requiring mutant of M. radiodurans, strain T$_2^-$ (Little and Hanawalt, 1973), was used. Little and
Figure 56  Isopycnic analysis of DNA synthesis in unirradiated Triton-treated *M. radiodurans* wild-type, (a) 20 min, +ATP (b) 40 min, +ATP (c) 20 min, -ATP.
Hanawalt (1973) reported that with this strain they were able to demonstrate repair replication but did not give any details in their publication.

The DNA was prelabelled with $^{14}$C\textit{dThd} by growing the bacteria in a chemically defined medium (SMM) containing exogenously supplied \textit{dThd}. UV-irradiated and unirradiated bacteria were incubated for 20 min or 40 min in SMM with \textit{dThd} replaced by $^3$H\textit{BrdUrd}. The results of isopycnic analysis of DNA synthesized in 20 min is shown in Figure 57 and in 40 min in Figure 58. The results were similar to the previous ones in that no DNA was synthesized which had a density detectably different from parental DNA. The permeabilization treatment was thus not responsible for the observed effects. It could be postulated that these results are indicative of an unusual form of DNA synthesis in \textit{M. radiodurans} but Little's results (pers. commun.) for unirradiated cells gave DNA of two distinct densities and argues against this possibility. Why the results presented here do not show this is not clear. One possibility is that the \textit{CsCl} gradients were too steep and could not separate clearly DNA of hybrid and normal density. In fact, DNA in which no incorporation of density label occurred sedimented at about the same place as DNA which was known to contain some hybrid DNA. It was therefore not possible to look at patch size since DNA containing only repair patches could not be separated from semi-conservatively replicated DNA.

13. \textit{M. radiodurans} UV47 and 303

The data presented in Figures 12 and 59 show that UV47 and 303 have a high spontaneous mutation frequency to rifampicin-resistance and are about 50 to 100 and about 10 times higher than the wild-type
Figure 57  Isopycnic analysis of DNA synthesized in *M. radiodurans* in 20 min, (a) unirradiated, (b) 810 J m⁻².
Figure 58. Isopycnic analysis of DNA synthesized in *M. radiodurans* in 40 min, (a) unirradiated, (b) 810
respectively.

Strain UV47 is the most UV-sensitive mutant isolated. Certain UV-sensitive mutator mutants of *E. coli* are hypermutable by certain mutagens. However, UV47 is not mutable by either UV or 2-AP (Tables 47 and 48).

303 is defective in the repair of UV- and MTC-induced damage and is mutant in a gene designated *uvsA* (Moseley and Copland, 1978). The data in Figure 36 show that 303 is also very sensitive to DCMTC, similar to 302 being 50 times as sensitive as the wild-type. The data in Figure 59 show that 303 is also very sensitive to the lethal effects of MMS being about 21 times as sensitive as the wild-type but is not mutable by MMS.
Table 47

The effect of UV on survival and mutation to rifampicin-resistance of M. radiodurans UV47

<table>
<thead>
<tr>
<th>UV dose (J m⁻²)</th>
<th>Survival (%)</th>
<th>Rif⁰ mutants per 10⁸ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>4.5</td>
<td>100</td>
<td>270</td>
</tr>
<tr>
<td>9.0</td>
<td>92</td>
<td>190</td>
</tr>
<tr>
<td>13.5</td>
<td>66</td>
<td>190</td>
</tr>
<tr>
<td>18.0</td>
<td>27</td>
<td>230</td>
</tr>
<tr>
<td>22.5</td>
<td>11</td>
<td>180</td>
</tr>
<tr>
<td>33.75</td>
<td>1.2</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 48

The effect of 2-AP on generation time and mutation to rifampicin-resistance of M. radiodurans UV47

<table>
<thead>
<tr>
<th>2-AP concentration (mg ml⁻¹)</th>
<th>t (min)</th>
<th>Rif⁰ mutants per 10⁸ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92</td>
<td>180</td>
</tr>
<tr>
<td>1.85</td>
<td>148</td>
<td>190</td>
</tr>
</tbody>
</table>
Figure 59  The effect of MMS on survival (○) and mutation to rifampicin-resistance (▲) of *M. radiodurans* 303.
DISCUSSION
1. Mutagenesis of M. radiodurans wild-type

A previous investigation showed that in general Micrococcus radiodurans was not mutated to trimethoprim-resistance by agents which in Escherichia coli exert their mutagenic effect through error-prone repair pathways (Sweet and Moseley, 1976). For instance, UV light, gamma-rays, MTC and hydroxylamine were unable to induce any mutants and nitrous acid and EMS were only weakly mutagenic. In contrast, MNNG was potent in its mutagenicity. The data presented here on the ability of these agents to induce rifampicin-resistant mutants of M. radiodurans are in agreement with those of Sweet and Moseley (1976) with the exception of gamma-rays and hydroxylamine which can induce a small number of rifampicin-resistant mutants whereas neither were able to induce, to a detectable level, any trimethoprim-resistant mutants. This discrepancy probably reflects the increased resolution obtained using the rifampicin-resistance marker.

In E. coli, Schizosaccharomyces pombe, Saccharomyces cerevisiae, phage T4 and, no doubt, other organisms, UV radiation depends on a misrepair process for its mutagenicity (Witkin, 1976; Nasim, 1968; Lemontt, 1973; Green and Drake, 1974). The ability of UV radiation to cause mutation is known to be due to the production of pyrimidine dimers. Genetic evidence suggests that at least one of the mutational steps is the incorporation of incorrect nucleotides opposite pyrimidine dimers since UV radiation produces tandem base pair substitution mutations both in E. coli (Yanofsky et al., 1966; Coulondre and Miller, 1977) and in S. cerevisiae (Lawrence et al., 1974). Pyrimidine dimers are unable to form Watson-Crick hydrogen bonds and cannot be replicated by E. coli DNA polymerases (e.g. Caillet-Fauquet et al., 1978). UV radiation is unable to induce forward mutation of M. radiodurans to resistance to trimethoprim,
streptomycin or rifampicin and is unable to revert a _ts_ mutant to temperature-resistance (Sweet and Moseley, 1974; Kerszman, 1975; Table 5). Thus, _M. radiodurans_, unlike _E. coli_, is able to repair a substantial amount of UV-induced damage without detectable error. It must be concluded that error-prone repair is absent from _M. radiodurans_. It is highly improbable that any DNA polymerase in _M. radiodurans_ could replicate past pyrimidine dimers in an error-free manner. Further discussion on the lack of need for error-prone repair is presented later.

MTC is an alkylating agent which forms both mono- and diadducts in DNA (Weissbach and Lisio, 1965) and depends, in _E. coli_, on error-prone repair for its mutagenic effect (Kondo _et al._, 1970). Furthermore, it is also dependent on a functional excision repair mechanism in both _E. coli_ and _Bacillus subtilis_ (Kondo _et al._, 1970; Tanooka, 1977) since an unexcised crosslink prohibits strand separation and is lethal, so that any potential mutants would not survive. The bulky monoadducts result in non-instructive nucleotides some of which are likely to cause substantial structural distortion of the DNA. Studies of psoralen plus light-induced killing and mutation of _E. coli_ indicates that monoadducts are responsible for most mutations whereas crosslinks are primarily responsible for killing (Seki _et al._, 1978). In this case, and with MTC, the mutations are believed to be due to the need for replication past monoadducts and, less frequently, past partly excised crosslinks which must be an error-prone step. The immutability by MTC is again further evidence that error-prone repair is absent from _M. radiodurans_.

The nitrous acid-induced deamination of cytosine, adenine and guanine leads to the production of uracil, hypoxanthine and xanthine respectively. The first two of these products in DNA are capable of
causing transition mutations on simple pairing schemes (Freese, 1971). However, in E. coli specific glycosylases exist for the removal of these bases from DNA (Lindahl, 1974; Karran and Lindahl, 1978) and the available data suggest that nitrous acid mutagenesis is not as straightforward as might be expected from such direct (mis)pairings (see Introduction). Compared with E. coli, M. radiodurans is extremely resistant to the lethal effects of nitrous acid (Sweet and Moseley, 1976) but is only weakly mutated by it. It seems likely that this mutational response is due to the replication of at least one of the deaminated bases.

Hydroxylamine is a mutagen which in E. coli and phage lambda is independent of error-prone repair (Ishii and Kondo, 1975; Hutchinson and Stein, 1977). Hydroxylation at the N⁴ and N⁶ positions of cytosine and adenine respectively produce potentially miscoding bases (Budowsky, 1976). Such a simple mutational misreplication mechanism may occur in phages (Drake, 1970; Freese, 1971). However, in more complex organisms such as E. coli, a large number of deletions are produced, independent of error-prone repair, and it has been suggested that hydroxylamine may induce errors as a result of damage to the replication machinery (Ishii and Kondo, 1975). Either mechanism could explain the mutability of M. radiodurans by hydroxylamine but compared with other organisms the effect is relatively weak.

As discussed previously alkylation of bases at certain sites by EMS and MNNG is likely to lead to mispairings of these modified residues. Both compounds are extremely mutagenic in E. coli, even in recA and exrA strains (Kondo et al.; Ishii and Kondo, 1975). In M. radiodurans EMS is only a weak mutagen although it is as toxic as in E. coli. It seems likely that this mutagenicity is due to the replication of some ethylated bases although these may be repaired
more efficiently than in *E. coli*. However, *M. radiodurans* is very much more sensitive to MNNG mutagenesis supporting the view that it acts via a misreplication mechanism. Whether in *M. radiodurans* there is a differential repair response to methylated and ethylated bases or whether some other factor plays a role in MNNG mutagenesis is unknown.

Although most work on gamma-rays has concentrated on the repair of single-strand breaks, since these are readily measurable, ionizing radiations cause many different types of base damage. At least one kind of damage to bases per se is known to constitute a lethal lesion in phages (Freifelder, 1968). Damage to bases are also sources of mutations in phages (Bresler et al., 1975). Since in most instances mutagenesis depends on host error-prone repair functions the mutations in the phage probably arise during error-prone repair of gaps produced during excision repair of damaged bases. However, a small number of phage mutants arise in a host *exr* mutant possibly because of replication errors, although other explanations are available (Bresler et al., 1975). In *E. coli* all gamma-ray mutagenesis depends on error-prone repair (Bridges et al., 1968). However, gamma-ray-induced reversion of a ØX174 mutant has been reported not to require the host *recA* gene product (Bleichrodt and Verheij, 1973). Also, host repair functions are irrelevant to gamma-ray mutagenesis of T7 (Bleichrodt et al., 1977) although in this case the phage may possess its own error-prone pathway. Direct damage to the replication complex cannot be excluded as a source of mutation because Saffhill (1974) has shown that *in vitro* gamma-irradiation of *E. coli* DNA polymerase I causes both a reduction in its activity and its fidelity. In *M. radiodurans* the weak mutability by gamma-rays may be ascribed to misreplication caused by the presence of miscoding
nucleotides or by damage to a DNA polymerase.

MNUr is a nitrosamide which is expected to produce a similar spectrum of methylation products to MNNG. Although not widely used it is known to be a potent mutagen for S. pombe and Neurospora crassa (Guglielminetti et al., 1966; Auerbach and Ramsay, 1968). In M. radiodurans, it is also an extremely potent mutagen, at least as efficient as MNNG, and therefore acts via a misreplication mechanism.

In contrast, the methylating agent MMS is weak in its mutagenic effect in M. radiodurans. In E. coli it has been reported that MMS is dependent on error-prone repair for mutagenesis (Kondo et al., 1970; Bridges et al., 1973). However, the finding that MMS produces a low level of alkylation at the O^6 position of guanine (Lawley and Shah, 1972) implies that it should be able to produce some mutations by a misreplication mechanism. This is substantiated by the finding that a minor part of MMS mutagenesis of T4 inducing GC to AT transitions is independent of error-prone repair (Green and Drake, unpublished results cited by Drake and Baltz, 1976). Further, a subsequent report showed that MMS can induce mutations in an _exrA_ strain of E. coli (Green et al., 1976). These data indicate that the weak mutability of MMS in M. radiodurans is likely to be due to the misreplication of certain methylated bases rather than to an error-prone repair mechanism.

The presence in DNA of bases in rare tautomeric or isomeric forms leads to the increased probability of anomalous base-pairing at replication. The bases 2-AP and BrUra have a greater tendency to exist in their tautomeric forms than their normal analogues. As discussed earlier, BrUra and probably 2-AP mutagenesis of phage and E. coli is independent of error-prone repair. However in
Haemophilus influenzae when 20% of dThd residues are replaced by BrdUrd residues no mutants are induced (Kimball and Perdue, 1977). Kimball and Perdue (1977) thought it likely that the replication complex probably always treats BrUra as thymine. They supposed a similar explanation could account for the lack of 2-AP mutagenesis in H. influenzae. A similar phenomenon may apply for M. radiodurans so that BrUra and 2-AP never get incorporated opposite guanine and cytosine respectively. If a base is in the wrong tautomeric configuration it may be rejected by the base selection mechanism, or, if after incorporation it takes up the rare tautomeric form it may be efficiently excised by a 3'→5' exonuclease activity of a DNA polymerase. These factors could account for the inhibitory effect on growth rate which is observed with BrdUrd and more markedly with 2-AP.

The precise mechanism of action of frameshift mutagens is not known. The immutability of M. radiodurans by ICR 191G might be due to differences in the repair of strand breaks or in the repair of misannealed DNA, believed to be intermediates in frameshift mutagenesis, (Drake, 1970) if indeed these are formed in M. radiodurans.

BrMBA introduces large aryl substituents at the extranuclear amino groups of guanine, adenine and cytosine (Dipple et al., 1971), sites normally involved in Watson-Crick hydrogen bonding. Such adduct formation produces lesions some of which are likely to be non-instructive. Similarly, DCMTC modifies bases so that they can no longer form base pairs. Thus, in E. coli mutagenesis by both compounds depends on recA+/lexA+ dependent misrepair and is enhanced by uvrA mutations (Tarmy et al., 1973; Murayama and Otsuji, 1973). Again, M. radiodurans is immutable by BrMBA and DCMTC which is expected with agents that produce non-pairing as opposed to mispairing, nucleotides.
Those results confirm the general pattern of weak- or non-mutability of *M. radiodurans* by most potential mutagens. Apart from showing the absence of certain mutagenic pathways the results also suggest that other factors such as efficient repair and stringent discriminatory properties of enzymes involved in DNA metabolism are important in maintaining the integrity of the genetic script.

2. Lack of error-prone repair in radiation-resistant bacteria

A vast source of literature has accumulated in recent years reporting the ability of UV to induce mutations in a wide variety of organisms. However, certain bacteria, namely *Proteus mirabilis*, *Methylococcus capsulatus*, *M. radiodurans*, *H. influenzae* and *H. parainfluenzae* are immutable by UV (Böhme, 1963; Harwood *et al.*, 1973; Sweet and Moseley, 1974; Kimball *et al.*, 1977) and it may be concluded that these bacteria lack error-prone repair. The data presented here show that the radiation-resistant micrococci i.e. *Micrococcus roseus* ATCC 19172, *Micrococcus radiophilus* and *Micrococcus radioproteolyticus* are all immutable by UV. Attempts were made to increase the frequency of any UV-induced mutants by adding caffeine to the post-irradiation growth medium. Such treatment of UV-irradiated *E. coli* results in an enhanced mutation frequency (Sideropoulos and Shankel, 1968) because caffeine binds tightly to the *uvrA,B* endonuclease (Braun and Grossman, 1974) thereby inhibiting the removal of pyrimidine dimers which are channelled into more error-prone repair pathways. Caffeine did not increase the mutant yield of any of the radiation-resistant micrococci but it is not known if any steps in the excision of pyrimidine dimers from the DNA of these
bacteria are caffeine-sensitive. Since all the bacteria could be mutated by mutagens which act via a misreplication mechanism it would seem that these bacteria lack a misrepair mechanism for at least UV-induced damage.

It was conceivable, because of the genetic relatedness of the four red-pigmented micrococci (see later), that lack of error-prone repair was only a common generic feature and no more. However, the data on the mutability of *Arthrobacter radiotolerans* and the asporogenous rod designated P-30-A showed that these bacteria are also immutable by UV and therefore lack a misrepair mechanism of mutagenesis. These two bacterial species differ substantially from the four micrococci in their morphology, cultural and physiological characteristics (Yoshinaka *et al.*, 1973; Kobatake *et al.*, 1977) and undoubtedly belong to different genera from the micrococci. Therefore, resistance to the lethal effects of UV and gamma-radiation and lack of error-prone repair appear to be two associated phenomena in at least six bacterial species, belonging to three different genera.

Why should this be so? Evidence from *E. coli* suggests that an error-prone function is needed to repair lesions which cannot be repaired by constitutive mechanisms. Sedgwick (1976) has presented evidence that overlapping daughter strand gaps in two homologous chromosomes, produced as a consequence of the replication of pyrimidine dimer-containing DNA, may be such a lesion. He also proposed that any non-pairing base, be it a pyrimidine dimer, an alkylated base, a gamma-ray damaged base, opposite a gap or a non-coding base cannot be repaired in an error-free manner and necessitates random nucleotide insertion for survival. In bacteria which do not exhibit extreme resistance to the lethal effects of radiations the lack of error-prone repair is reflected in the sensitivity of these
organisms to killing. However, in the radiation-resistant bacteria that lack error-prone repair either such lesions are produced infrequently, or they are repaired by a mechanism(s) not present in E. coli. M. radiodurans possesses three DNA polymerases, only one of which has associated 3′ → 5′ exonuclease activity (Kitayama et al., 1978). This activity could result in polymerase idling so that pyrimidine dimers, or other non-instructive lesions could not be replicated in an error-free way, unless a replication repair pathway was present (Higgins et al., 1976). There is no evidence that such repair is present in any bacterium. One possibility which could account for the lack of need for error-prone repair is that the size of gaps produced when DNA synthesis reinitiates after encountering a dimer is much smaller than the 1000 or so base pair gap produced in E. coli (Iyer and Rupp, 1971). Also, excision repair of dimers may be more efficient not only in the rate of excision (Boling and Setlow, 1966) but also the size of the patches may be small so that no long patches are produced (Cooper and Hanawalt, 1972). An attempt was made to examine patch size but it was unsuccessful.

The recent report of Hansen (1978) demonstrating that M. radiodurans contains multiple genomes makes it possible that large gaps occur frequently but because of the greater number of homologous chromosomes such lesions could be repaired by recombinational exchanges. It would need very large doses of UV, for instance, to produce strand gaps that overlapped in all (ten) chromosomes. Before this could happen the high UV dose may result in non-DNA damage as the ultimate lethal event (Setlow and Boling, 1965). Whether there is redundancy of genetic material in the other bacteria, especially in the two unrelated rod-shaped bacteria remains to be determined.
3. Heterologous transformations

The experiments reported here showed that DNA from *M. roseus* ATCC 19172, *M. radiophilus* and *M. radioproteolyticus* could transform *M. radiodurans* from rifampicin-sensitivity to resistance although compared with DNA of homologous origin, at very much reduced frequencies i.e. lower by several orders of magnitude. This reduction in frequency occurs in all transformation systems in which interspecies genetic exchange has been demonstrated. As discussed earlier, most evidence suggests that the reduced frequency is due to the non-homology of the transforming and recipients DNAs. This is the most likely explanation for *M. radiodurans* for two reasons. Firstly, *M. radiodurans* probably lacks a restriction-modification system since its DNA contains no biomethylated bases (Schein *et al.*, 1972) and it thus could not restrict foreign DNA. Secondly, DNA from a strain of *M. radiodurans* obtained after being transformed with DNA from *M. roseus* ATCC 19172, transformed *M. radiodurans* wild-type at a frequency comparable with DNA of homologous origin. This phenomenon occurs in many systems and is due to the production of genetically hybrid DNA, which is usually intermediate in transforming efficiency, but this is not always the case (e.g. Wilson and Young, 1972).

Certain regions of the chromosome, notably those involved in ribosome structure and function, are transformed heterologously at much higher frequencies than other regions. In *E. coli* rifampicin-resistant mutants have an altered RNA polymerase (Ezekiel and Hutchins, 1968) and it seems likely that this may also occur in *M. radiodurans*. Genes involved in ribosome structure and function appear to have been conserved to varying extents during evolution and the ability of *M. radiodurans* to be transformed with DNA from
rifampicin-resistant mutants of the radiation-resistant micrococci but not from such mutants of Micrococcus luteus indicates some degree of DNA homology, and therefore genetic relatedness, between the four radiation-resistant micrococci. In contrast, there appears to be no genetic relationship between M. radiodurans and M. luteus, in agreement with the data of Kloos (1969).

These results, together with those on the structure and chemical composition of the peptidoglycan and the cell wall, the state of the menaquinone system, the extreme radiation-resistance and the lack of error-prone repair, are evidence that these four radiation-resistant bacteria form a different taxonomic group from the genus Micrococcus. The information in Table 49 indicates the similarities and differences between some of the bacteria examined.

4. Aspects of MNNG mutagenesis

All the DNA repair-deficient mutants of M. radiodurans isolated in this laboratory have been shown to be defective in the repair of MNNG-induced damage. There are thus at least 10 genes involved. However, in most instances the defects in these strains are not known. Recombination is involved as shown by the sensitivity of rec30 to MNNG-induced killing. Similarly recombination repair is involved in both E. coli and H. influenzae (Kondo et al., 1970; Kimball et al., 1971b). Genes, which are defective in 302 and 262, are involved in the repair of MNNG-induced damage but not UV-induced damage. Since repair pathways often share common steps this suggests that these two genes (mtcA and mtcB) are involved in initial repair events. A similar gene affecting sensitivity to MNNG, but not UV,
<table>
<thead>
<tr>
<th>Species</th>
<th>Colour and gross morphology</th>
<th>GC content of DNA (%)</th>
<th>Heterologous transformation of <em>M. radiodurans</em></th>
<th>Error-prone repair</th>
<th>Menaquione system</th>
<th>Peptidoglycan type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. radiodurans</em></td>
<td>Red coccus</td>
<td>67.0 (3)</td>
<td></td>
<td>No(8)</td>
<td>MK-8 (5)</td>
<td>L-Orn-Gly₂(4)</td>
</tr>
<tr>
<td><em>M. roseus</em> ATCC 19172</td>
<td>Red coccus</td>
<td>68.0 (1)</td>
<td>Yes(8)</td>
<td>No(8)</td>
<td>MK-8 (5)</td>
<td>L-Orn-Gly₂(7)</td>
</tr>
<tr>
<td><em>M. radiophilus</em></td>
<td>Red coccus</td>
<td>64.4 (1)</td>
<td>Yes(8)</td>
<td>No(8)</td>
<td>MK-8 (5)</td>
<td>L-Orn-Gly₂(6)</td>
</tr>
<tr>
<td><em>M. radioproteolyticus</em></td>
<td>Red coccus</td>
<td>70.5 (1)</td>
<td>Yes(8)</td>
<td>No(8)</td>
<td>MK-8 (5)</td>
<td>L-Orn-Gly₂(6)</td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td>Yellow coccus</td>
<td>66.3 (2)</td>
<td></td>
<td>Yes(8)</td>
<td>MK-7,MK-8(H₂)(5)</td>
<td>L-Lys(4)</td>
</tr>
<tr>
<td>P-30-A</td>
<td>Red rod</td>
<td>65.7 (5)</td>
<td></td>
<td>No(8)</td>
<td>MK-8 (5)</td>
<td>L-Lys(5)</td>
</tr>
</tbody>
</table>

References: (1) Kobatake et al., 1973; (2) Kocur et al., 1971; (3) Moseley and Schein, 1964; (4) Schleifer and Kandler, 1972; (5) Yamada et al., 1977; (6) Schleifer, unpublished results cited by Sleytr et al., 1976; (7) Kandler, pers. commun.; (8) This thesis.
has recently been identified in *E. coli* (Yamamoto et al., 1978b).

The response of the mutants of *M. radiodurans* to the mutagenic effects of MNNG is quite different from most other organisms. For instance, *uvrA*, *polA* and *recA* mutants of *E. coli* are equally mutable by MNNG (Ishii and Kondo, 1975). Similarly strains of *H. influenzae* defective in excision and recombination repair are also equally susceptible to MNNG mutagenesis (Kimball et al., 1971b). However, in *M. radiodurans* the majority of the mutants do not show wild-type sensitivity either at the same MNNG dose or at the appropriate *D*$_{37}$ dose. Some of the strains yield less mutants than the wild-type, others more. Because MNNG introduces a plethora of lesions in DNA these results may be explained by differential repair of lesions which contribute to lethality only, to mutation only, or to both lethality and mutation. Thus, strains UV2, UV38 and UV50 which are hypomutable might be defective in the repair of potentially lethal lesions or in the repair of potentially mutagenic lesions in which repair is abortive and lethality results. The hypermutable mutants may be defective in the removal of lesions which contribute predominantly to mutation. These mutants are discussed more fully below.

*M. radiodurans* wild-type does not show any inducible adaptive mutagenic response to low levels of alkylation damage, as *E. coli* does (Samson and Cairns, 1977; Jeggo et al., 1977). If at least a part of this response is due to proficiency at removal of $O^6$-alkylguanine (Schendel et al., 1978) then it appears that *M. radiodurans* constitutively possesses such a pathway, at least for the excision of $O^6$-methylguanine residues. In spite of the potent mutagenicity of MNNG this repair pathway is very efficient since it can remove at least 98% of the mutagenic lesions.
MNNG and nitrosamides generally have a weak mutagenic effect on non-replicating DNA. Thus stationary phase E. coli cells are only weakly mutable by MNNU and since stationary phase recA and lexA strains are non-mutable, the mutational response of the wild-type may be ascribed to error-prone repair (Hince and Neale, 1977a). Similarly, the mutability of stationary phase M. radiodurans is very much reduced (less than 1% of exponential cultures). These results are consistent with the idea that stationary phase cells have a longer period of time available for repair of promutagenic lesions. The slight mutability of stationary phase cells may due to some lesions which are not removed before being replicated either because they are relatively near the replication fork or they are in DNA which is in a conformational state that prohibits excision repair.

5. M. radiodurans 302

M. radiodurans 302 is mutant in a single gene designated mtcA which plays a major role in the repair of MTC damage but not UV damage (Moseley and Copland, 1978). The alkaline sucrose gradients indicate that 302 is defective in the excision repair of BrMBA-DNA adducts. It seems unlikely that 302 is performing the incision step but that the breaks are being sealed by polynucleotide ligase, since in UV-irradiated uvrC mutants of E. coli, which probably behave in this way, single-strand breaks are detectable (Kato, 1972; Seeberg and Rupp, 1975). The most likely explanation is that 302 is unable to incise BrMBA-damaged DNA because it lacks a correctional endonuclease or glycosylase activity. By inference, the defect in 302 must also be involved in the repair of lethal and mutagenic lesions to which 302 is hypersensitive. It could be that the mtcA gene is
involved in the control of several enzymes initiating repair of a wide range of defects or it could be a single enzyme capable of recognizing some common feature of a range of differently modified bases or nucleotides.

In *E. coli* BrMBA-modified residues are excised by a process initiated by the uvrA,B endonuclease (Venitt and Tarmy, 1972; Thielmann, 1976) although in vitro an endonuclease II preparation incises DNA damaged by 7-bromomethyl-12-methylbenz[a]anthracene and releases via glycosylase action methylbenz[a]anthracenyl-purine adducts (Kirtikar et al., 1975a). In contrast to its inability to incise DNA containing any BrMBA-damaged residues, 302 does incise DNA damaged by AAAF although probably not to the same extent as the wild-type, suggesting that in 302, some, but not all, AAAF-modified residues are excised. In *E. coli* all such excisions are mediated by the uvrA,B endonuclease (Thielmann, 1975). The nature of the product(s) excised in *E. coli* is not known but in rat liver DNA C-8 substituted guanines are excised whereas substitutions at N² of guanine persist (Kriek, 1972; Westra et al., 1976). It is possible that the same relative sensitivity of 302, compared with the wild-type, to killing by AAAF and BrMBA reflects an inability to excise a common modified product i.e. N² substituted guanine.

Although 302 is very sensitive to the mutagenic effects of MMS, EMS and MNNG it is not correspondingly sensitive to their lethal effects and is, in fact, as resistant to MMS-induced killing as the wild-type. These results provide further evidence for suggestions that at least some of the lethal and mutagenic lesions produced by methylating and ethylating agents are separate entities (Hill et al., 1972; Hince and Neale, 1974a; Ishii and Kondo, 1975; Skavronskaya and Smirnov, 1975). Compared with the wild-type, 302
is between 100 and 400 times more mutable by MMS and EMS which means that the wild-type is capable of repairing, without error, at least 99% of the mutagenic lesions caused by these agents.

The hypermutability of 302 could be explained by the non-removal of an alkylated base(s) which is then able to misrepair during replication. Thus 302, unlike the wild-type is unable to remove O⁶-methylguanine residues from its DNA although it does remove 7-methylguanine and 3-methyladenine residues. Can the persistence of O⁶-alkylguanine account for the hypersensitivity of 302 to mutagenesis? In 1969, Loveless suggested that alkylation at the O⁶ position of guanine was likely to account for the differences between SN1 and SN2 agents in their ability to induce mutations in phages and to induce cancer in higher eukaryotes due to the possibility that O⁶-alkylguanine could pair with thymine. Genetic analysis of the types of mutations induced by those agents which produce relatively large amounts of O⁶-alkylguanine show that most are due to GC to AT transitions (Krieg, 1963a,b; Drake, 1970; Coulondre and Miller, 1977). This evidence that O⁶-alkylguanine is promutagenic is somewhat circumstantial and more direct evidence has been provided by the work of Ludlum and his colleagues. Thus, Gerchman and Ludlum (1973) showed that in an RNA polymerase-directed synthesis from a polymer containing O⁶-methylguanine misincorporation of UMP and AMP into the product copolymer occurred. Such misincorporation was not inhibited by the presence of CTP indicating that cytosine is not a normal partner for O⁶-methylguanine. Furthermore, poly(O⁶-methylG) and poly(O⁶-ethylG) complex with poly(U) but not with poly(C) (Mehta and Ludlum, 1976). Theoretical calculations of the interaction energies of base-pairing indicate that (mis)pairing of O⁶-methylguanine with uracil is much more likely to occur than for 3-methyladenine,
7-methylguanine or guanine (Abdulnur and Flurry, 1976).

However, the available data on the effects of \( O^6 \)-alkylguanine in DNA in vivo is very limited. Genetic evidence, as mentioned above, suggests that if \( O^6 \)-alkylguanine mispairs it is rather specific, contrasting with in vitro studies, which may be a reflection of the different steric requirements of DNA and RNA polymerases. The demonstration that E. coli cells which have been adapted to the lethal and mutagenic effects of alkylating agents have an increased ability, compared with unadapted bacteria, to remove \( O^6 \)-methylguanine strongly implicates this lesion as a source of mutation (Schendel et al., 1978).

In unadapted bacteria, after MNNG treatment, DNA synthesis is inhibited for about 20 min after the mutagen has been removed whereas in adapted bacteria DNA synthesis resumes almost immediately (Jeggo et al., 1978a). Also, in M. radiodurans it would appear that DNA containing \( O^6 \)-methylguanine cannot be replicated without problem since after exposure of 302 to low levels of MNNG DNA synthesis is inhibited for about 20 min whereas such treatment of wild-type has little or no effect on DNA synthesis. \( O^6 \)-methylguanine in DNA may cause some distortion of the DNA helix (Mehta and Ludlum, 1976) and adversely affect the binding and movement of enzymes. Alternatively, the delay may be due to a by-passing of unexcised lesions by recombination repair or by some other mechanism. The data constitute strong evidence that mispairing of \( O^6 \)-methyl- and \( O^6 \)-ethylguanine is the major source of mutation in 302. However, the possibility that 302 is unable to remove some other potentially promutagenic base cannot be excluded.

If the mutability of 302 by MMS is due to the persistence of \( O^6 \)-methylguanine then it is apparent that \( O^6 \)-methylguanine is not a lethal lesion since 302 is as resistant to MMS-induced killing as the wild-type, contrasting with the data of Shooter et al. (1974b) who
suggested that $\text{O}^6$-alkylguanine is a lethal lesion in the RNA-containing phage R17. In 302, EMS produces approximately as many mutants as MMS does, yet 302 is sensitive to the lethal effects of EMS. This could reflect the presence of a lethal lesion in 302, excisable in the wild-type, which is produced more frequently by SN1 than SN2 agents but to a lower extent than $\text{O}^6$-alkylguanine, such as an $\text{O}^2$-alkylated pyrimidine residue, so that very few are present in 302 after treatment with MMS. Alternatively, one must postulate that ethylated bases, perhaps because of their larger size, are more lethal than methylated bases. 302 is even more sensitive than the wild-type to the lethal effects of MNNG and this could be explained by the greater SN1 character of MNNG compared with EMS.

Also, because of the exceptionally high levels of mutagenesis obtained with MNNG it might be that lethal mutations are the cause of the observed inactivation.

Because $\text{O}^6$-alkylguanine is chemically stable in DNA (Lawley and Thatcher, 1970) its removal necessitates an active excision process. Such excision, first demonstrated in E. coli (Lawley and Orr, 1970) has subsequently been shown to occur in various organs of rodents after treatment with nitrosamides and nitrosamines (O'Connor et al., 1973; Goth and Rajewsky, 1974; Kleihues and Margison, 1974; Margison et al., 1976; Buecheler and Kleihues, 1977; Pegg and Hui, 1978). The first indication of the nature of the repair process was that in E. coli a glycosylase activity of endonuclease II released in vitro $\text{O}^6$-methylguanine and 3-methyladenine from methylated DNA (Kirtikar and Goldthwait, 1974). However, subsequent reports have shown that both M. luteus and E. coli possess
a 3-methyladenine-DNA glycosylase unable to release $O^6$-methylguanine (Laval, 1977; Riazuddin and Lindahl, 1978). *M. radiodurans* also possesses different steps in the repair of these two methylated purines, but the nature of the excision product containing $O^6$-methylguanine is not known in either *M. radiodurans* or in any of the mammalian organs.

*M. radiodurans* 302 is the first bacterial mutant reported which is unable to remove $O^6$-methylguanine from DNA and demonstrates the promutagenic status of this alkylated base in vivo. Xeroderma pigmentosum-derived fibroblasts are partially defective in the removal of $O^6$-alkylguanine residues (Goth-Goldstein, 1977) and although such cells are resistant to the lethal effects of MNNG (Maher et al., 1978) there have been no reports on their sensitivity to MNNG mutagenesis. The persistence of $O^6$-alkylguanine in DNA may be one factor in alkylating agent-induced carcinogenesis since in rats and mice the ability of different organs to remove this $O^6$-alkylated base is inversely correlated to the susceptibility of these organs to tumour formation (Goth and Rajewsky, 1974; Kleihues and Margison, 1974). This is what would be expected if a somatic mutation was required as a step in the initiation of carcinogenesis.

After treatment of 302 with 68 μM MNNG for 30 min, a non-lethal dose, there is excessive DNA degradation with about 56% rendered acid-soluble during the 30 min treatment time. The reason for this is not known. It is conceivable that when the replication complex encounters an $O^6$-methylguanine residue the distortion produced may result in loss of control of a nuclease activity. Or, it could be due to DNA metabolism during recombination repair. However, if this were the case then it is not apparent why unexcised BrMBA-DNA adducts do not stimulate such degradation. It is
surprising that such a large amount of DNA can be degraded without a loss of cell viability. If, however, multiple copies (up to 10 in this case) of the genome are present such degradation could occur without loss of genetic information since by recombinational exchanges at least one intact genome could be restored.

In contrast to mutagenesis of stationary phase cells of wild-type, stationary phase 302 is very sensitive to MNNG- and MMS-induced mutation, which is what is expected if 302 does not remove any promutagenic methylated bases. However, these results do implicate another factor in alkylation mutagenesis because stationary phase 302 cells are not as sensitive as exponentially growing cells. One of several explanations may account for this phenomenon. Firstly, some factor as a consequence of DNA replication in the presence of MMS and MNNG enhances mutation. This could conceivably be due to damage (direct or indirect) to the replication machinery so that certain unexcised methylated bases, which are excisable in the wild-type, are much more likely to be misreplicated. A similar explanation has been proposed by Yamamoto et al. (1978a) to account for enhancement of MNNG-induced mutation of phage lambda when assayed in MNNG-treated host+ cells. Secondly, the increase in time in stationary phase cells before the onset of DNA replication allows a greater time for recombination repair so that unexcised promutagenic lesions are perhaps distributed randomly throughout all but one chromosome, similar to the fate of unexcised pyrimidine dimers in E. coli (Ganesan, 1974).

Strain 302 is unable to repair a major proportion of mitomycin-induced monoadducts since DCMTC, unlike MTC, is a monofunctional alkylating agent (Otsuji and Murayama, 1972). However, MTC is much more effective in killing both 302 and the wild-type than is DCMTC,
having the same effect as in _E. coli_ (Otsuji and Murayama, 1972). This indicates that crosslinks are more lethal than monoadducts and are the cause of inactivation of both wild-type and 302 by MTC. In _M. radiodurans_ most crosslinks and monoadducts are recognized by the damage-specific enzyme whose activity is defective in 302. However, 302 can repair some 7 crosslinks (Moseley and Copland, 1978) and therefore may have at least two excision repair pathways, as it seems to have for AAAF-damaged DNA. In _E. coli_ both kinds of adduct are recognized predominantly, if not exclusively, by the _uvrA,B_ endonuclease (Boyce and Howard-Flanders, 1964b; Otsuji and Murayama, 1972). However, recently a mutant of _E. coli_ has been reported to be UV-resistant but sensitive to MTC (Yamamoto _et al._, 1978b). This could explain the ability of _uvrA_ mutants to apparently excise some 6 psoralen-induced crosslinks (Cole, 1973). There could thus be two excision pathways for repair of crosslink damage in _E. coli_. Further, one of these may be initiated by a glycosylase since a mutant defective in an AP endonuclease activity is slightly MTC-sensitive, although this may be due to lack of exonuclease III activity which this mutant also lacks (Yajko and Weiss, 1975).

What is the cause of sensitivity of 302 to the lethal and mutagenic effects of nitrous acid? As discussed earlier uracil and hypoxanthine in DNA may be considered to be mutagenic since these bases would lead to transition mutations (Vielmetter and Schuster, 1960; Freese, 1971). Xanthine in DNA, and subsequent crosslink formation, may constitute lethal lesions (Vielmetter and Schuster, 1960; Becker _et al._, 1964). It may also be mutagenic since poly(X) is very versatile in its pairing properties, being able to complex with poly(A), poly(I), poly(U) and poly(X) but not with poly(C) (Michelson and Monny, 1966). However, there is no evidence to
support its ability to mispair in vivo. If 302 could not remove uracil and hypoxanthine one might expect a much higher sensitivity to nitrous acid mutagenesis than is observed. Therefore, it may be that in 302 xanthine is not removed and this does occasionally mispair. Also as a result of depurination of xanthine crosslinks are produced (Burnotte and Verly, 1971) which may explain the sensitivity of 302 to the lethal effects of nitrous acid.

The experiments with hydroxylamine give another example of the separation of mutational and lethal lesions produced by mutagens since 302 is hypermutable by hydroxylamine but is as resistant as the wild-type to killing. It is thus likely that _M. radiodurans_ has an excision repair pathway for an hydroxylamine-modified base which has the potential to mispair. Conceivably, either \( \mathbb{A}^4 \)-hydroxycytosine or \( \mathbb{A}^6 \)-hydroxyadenine are bases which remain unexcised in 302.

The sensitivity of 302 to killing by gamma-rays (Moseley and Copland, 1978) must reflect the presence of a lesion which in the wild-type is excised by a process initiated by an endonuclease or glycosylase. This undefined lesion is not, however, capable of mispairing. The elevated mutability of a _uvrA_ strain of _E. coli_ to the frameshift mutagen ICR 191 implies the presence of excisable lesions (Newton _et al._, 1972) but such damage is not recognized by the repair enzyme(s) defective in 302.

There are very few reports of mutants which are hypermutable by agents which act via a misreplication mechanism. A _polA_ strain of _E. coli_ is hypermutable by EMS. This sensitivity has been ascribed to increased error-prone repair of single-strand breaks in the absence of DNA polymerase I (Bridges _et al._, 1973). _E. coli_ _uvrA_ mutants exhibit a weak hypersensitivity to EMS mutagenesis.
(Ishii and Kondo, 1975; Tarmy, unpublished results cited by Lawley, 1974a) implying the presence of exciseable mutagenic lesions. A much stronger hypersensitivity to EMS mutagenesis has been found in dam strains of E. coli (Glickman et al., 1978). These mutants are also hypermutable by 2-AP and BrUra and are believed to be defective in mismatch repair. The lack of adenine methylation does not enable the parental strand to be distinguished by repair enzymes. Strain 302 is not likely to be defective in such mismatch repair since it is immutable by 2-AP. However, the results of Glickman et al. (1978) do raise a point of interest: how does M. radiodurans differentiate between parental and daughter bases in a mismatched pair, since general biomethylation of adenine and cytosine is absent (Schein et al., 1972)?

If the defect in M. radiodurans 302 is due to lack of activity of a correctional enzyme then it is similar, in some respects, to endonuclease II from E. coli, which has both endonuclease and glycosylase activity, since the enzymes from both organisms act on DNA containing O6-methylguanine, BrMBA-adducts and some form of gamma-ray-induced base damage (Kirtikar and Goldthwait, 1974; Kirtikar et al., 1975a,b). More recently an endonuclease preparation has been obtained from M. luteus, with properties very similar to the putative enzyme defective in 302, recognizes DNA bases modified by N-alkyl-N-nitrosoureas, alkyl methanesulphonates, alkyl sulphates, nitrogen mustard, BrMBA, AAAF and also AP sites (Hecht and Thielmann, 1978). The enzyme preparation may well contain glycosylase activity in addition to the endonuclease activity.

What common feature is the enzyme(s) defective in 302 recognizing in the wild-type? It has been supposed that the introduction of large molecules in DNA causes substantial structural
distortion. However, some lesions can be accommodated within the confines of a helix. For instance, substitutions by AAAF and probably BrMBA at the N² of guanine cause little or no distortion (Yamasaki et al., 1977; Beland, 1978) although they may affect the movement and binding of enzymes. The "302 enzyme(s)" may recognize a residue modified at certain O atoms e.g. O⁶-methylguanine, MTC-crosslinks between O⁶ atoms of guanine (Szybalski and Iyer, 1964) and certain modified extranuclear N atoms e.g. N-hydroxylated bases, deaminated guanine.

6. M. radiodurans 262

Strain 262 is mutant in a gene designated mtcB and is similar to 302 in its sensitivity to the lethal effects of MTC, its sensitivity to gamma-rays and its resistance to UV (Moseley and Copland, 1978). The similarity of these two strains is also shown by their sensitivity to the mutagenic and lethal effects of MNNG, MMS, EMS and DCMTC. However, they are not identical since 262 is about 1.5 times more sensitive to the lethal effects of both MMS and EMS and slightly more so to the lethal effects of MNNG. These results, however, do support the proposition (Moseley and Copland, 1978) that the mtcA and mtcB genes code for subunits of the same enzyme or for enzymes involved in the same repair pathways.

Since strain 262 can remove both 7-methylguanine and 3-methyladenine residues from its DNA as efficiently as the wild-type, these lesions do not account for the observed hypermutability. Although it was not possible to show that 262 was unable to remove O⁶-methylguanine residues, the similarities between 262 and 302 would indicate that it is a likely possibility. The discussions of
the hypermutability of 302 presented earlier are equally applicable for 262.

7. **M. radiodurans UV22**

Strain UV22 is sensitive to the lethal effects of MMS, MNNG and UV and to the mutagenic effects of MMS and MNNG. It is able to excise pyrimidine dimers (Moseley, pers. commun.), 7-methylguanine and \( \delta^6 \)-methylguanine residues but not 3-methyladenine residues, which may form an effective block to DNA replication and stimulate excessive DNA degradation. UV22 can incise UV-damaged DNA as well as the wild-type but is defective in restoration of the DNA to full molecular weight. If all these phenomena are the result of a single gene change how can these results be accounted for?

A mutant of *E. coli* with a defective 3-methyladenine-DNA glycosylase has been isolated as an MMS-sensitive mutant (Karran, Lindahl, Öfsteng and Seeberg, unpublished results cited by Karran and Lindahl, 1978). Therefore, the sensitivity of *M. radiodurans* to the lethal effects of MMS and MNNG could be due, at least, to the persistence of 3-methyladenine. Krieg (1963b) suggested that 3-methyladenine might mispair and a mispairing scheme has been presented by Lawley (1974a). However, the N3 of adenine is a major site of alkylation by both SN1 and SN2 agents and thus could not account for the major difference between the agents in mutagenic effectiveness. Also, in vitro assays for misincorporation by *E. coli* DNA polymerase I shows that 3-methyladenine mispairs infrequently, if ever, although it might reduce template activity (Abbott and Saffhill, 1977). Because the N3 alkylation of adenine
does not vary greatly between different alkylating agents it would appear that 3-methyladenine is not the only source of mutation, if at all, since UV22 is very much more sensitive to MNNG mutagenesis than it is to MMS mutagenesis. This may mean that UV22 is defective in removal of a promutagenic methylated base which is produced more frequently by SN1 agents, such as $\text{O}^4\text{-methylthymine}$ for instance. Since UV22 excises thymine dimers as well as the wild-type it is therefore defective in the removal of a minor, as yet unidentified UV-photoproduct.

In *M. luteus* and *E. coli* a specific glycosylase exists for which the only known substrate is 3-alkyladenine (Laval, 1977; Riazuddin and Lindahl, 1978). If the same defect in UV22 responsible for the non-removal of 3-methyladenine residues is also responsible for the sensitivity to UV-induced killing then it seems unlikely that 3-methyladenine and the UV photoproduct are removed by a glycosylase because the alkaline sucrose gradient profiles indicate that UV22 can incise UV-damaged DNA at least as well as the wild-type. The defect appears to be after an incision step since DNA with single-strand breaks is not restored to a high molecular weight form at the same rate as that in the wild-type. The failure to repair these gaps may also contribute to lethality.

8. *M. radiodurans* uvs10

Strain uvs10 is partially defective in the incision of UV-irradiated DNA. This could signify that it is deficient in an endonuclease which incises UV- but not MMS-damaged DNA. If this were the case then at least one more UV-specific endonuclease must be present. This is probably the case in most other organisms.
Even in *E. coli* where most, if not all, dimers are recognized by the *uvrA,B* endonuclease (Braun and Grossman, 1974), another endonuclease has been isolated which can in *vitro* incise UV-damaged DNA (Radman, 1976), and, in *vivo*, single-strand breaks are produced in UV-irradiated *uvrB* mutants (Youngs and Smith, 1976). In *M. luteus* there appear to be at least five separable enzyme activities that can incise UV-damaged DNA (Riazuddin and Grossman, 1977a). Although some may represent enzymes specific for minor UV-photoproduts at least two of the activities have pyrimidine dimers as substrates but it appears that the two enzymes act on DNA in different conformational states *in vitro* and both are required for full repair *in vivo* (Riazuddin and Grossman, 1977b; Riazuddin et al., 1977).

It is conceivable that the more resistant an organism is to UV-induced damage the more efficient its excision repair mechanism must be. Thus different endonucleases may be required to excise dimers in different configurations in DNA. If *M. radiodurans* contains several UV-specific endonucleases this could explain why an *E. coli* *uvr* type mutant has never been isolated since it would require several mutations to be totally excision-less. It is not yet known whether the *uvslO* gene is involved in the repair of some minor UV-photoproduction or whether it acts in concert with other pyrimidine dimer-specific endonucleases.

It has been demonstrated that strains of *E. coli* and *M. luteus* which do not excise pyrimidine dimers show a lower level of repair synthesis measured in nucleotide-permeable cells (Moses and Moody, 1975; Riazuddin et al., 1977). If *uvslO* exhibits a lower amount of repair synthesis then the reduction is relatively small.

Strain uvslO is a weak mutator showing an approximate 5-fold
increase in the frequency of rifampicin-resistant mutants. In other organisms certain mutants unable to excise pyrimidine dimers also exhibit mutator phenotypes e.g. in *S. cerevisiae* (Brychcy and von Borstel, 1977) and *Rhizobium trifolii* (Cunningham, pers. commun.). The molecular basis of these mutators is not known. One possibility is that the enzymes involved in the excision of pyrimidine dimers are also involved in the excision of lesions which are produced spontaneously as a result of the inherent instability of DNA. Such unexcised lesions may then be repaired in an error-prone way or they may be capable of direct mispairing upon replication. The latter possibility may arise in uvs10.

Strain uvs10 is very slightly mutable by UV. It is unlikely that this represents error-prone repair activity but is likely to be due to the presence of a minor UV-photoproduct which is capable of being misreplicated. The nature of such a lesion is not known. This excision repair pathway does not appear to be involved in the repair of MMS- or BrMBA-induced damage.

9. Excision of 7-methylguanine

It is surprising that *M. radiodurans* can excise a lesion which in most organisms evokes no repair response. For example, there is evidence that neither *E. coli* (Lawley and Orr, 1970; Lawley and Warren, 1976) nor *B. subtilis* (Prakash and Strauss, 1970) can remove this lesion. Similarly, in rat liver the rate of loss of 7-methylguanine is near to the rate of spontaneous depurination and an active repair mechanism does not need to be implicated (Craddock, 1969; O'Connor et al., 1973; Kleihues and Margison, 1974). However, a much faster loss in vivo (half-life of 26 h) occurs from the liver of hamster
(Margison et al., 1976), but due to lack of a detailed knowledge of the intracellular environment the presence of an active repair mechanism cannot be stated with confidence. An active repair mechanism has been suggested to occur in the liver cells of mice after treatment with a carcinogenic dose of DMN (Nemoto and Takayama, 1974). However, other evidence suggests that mice are incapable of excising this lesion (Den Engelse, 1974). The most rapid rate of excision of 7-methylguanine (half-life of 10 h) has been reported to occur from the DNA of *Euglena gracilis* after treatment with N-methyl-N-nitroso-p-toluenesulphonamide (Olson and McCalla, 1969). The excision of 7-methylguanine from the DNA of *M. radiodurans* shows a two-component response. Initially, up to 1 h, most are excised with a half-life of 37 min or less, then there is a much less rapid loss of this lesion. At higher doses of MNNG the loss of 7-methylguanine appears to be slightly inhibited which may reflect either saturation of repair enzymes or damage to the enzymes involved in excision.

7-Methylguanine does not mispair *in vitro* in either transcriptional or translational systems (Wilhelm and Ludlum, 1966; Ludlum 1970) and the deoxynucleoside triphosphate replaces only dGTP in a DNA-synthesizing system (Hendler et al., 1970). Thus, 7-methylguanine is not likely to play a role in direct mispairing mutagenesis. In *B. subtilis* DNA containing many methyl groups (most presumably at N-7 of guanine) can be replicated without problems (Prakash and Strauss, 1970). Most evidence suggests that 7-methylguanine is biologically inert.

However, there is a little evidence that alkylation at N7 of guanine *per se* does have some effect. For instance, the presence of 7-methylguanine in templates for protein synthesis does lower their efficiency and this was ascribed to the presence of the methyl group at a sterically important site (Wilhelm and Ludlum, 1966). On the
contrary, in the RNA polymerase directed synthesis the efficiency of a poly (U,7-methylG) template was equal to that of a poly (U,G) or poly(U) template (Ludlum, 1970). In the DNA synthesizing system of Hendler et al. (1970) the efficiency of replacement of dGTP by 7-methyldGTP was lower and was believed to be due to the increased ionization of the latter so that the negative charge in the hydrogen-bonding region of the analogue precludes the bonding to the template. However, the pK value of 7-methyldeoxyguanosine in DNA is raised and few ionized species may be present, although any that are present may be unable to form base pairs. Some in vivo data available does indicate that formation of 7-alkylguanine is a lethal event in the RNA of TMV (Singer et al., 1975).

Alkylation at the N7 position labilizes the glycosyl bond and 7-methyldeoxyguanosine spontaneously depurinates. Thus although primary damage may be insignificant secondary reactions do lead to the production of AP sites and therefore strand breaks.

10. Mutator strains of M. radiodurans

In most cases the molecular basis of mutator mutants is not known. Strain UV47 is UV-sensitive and exhibits a 50- to 100-fold increase in the spontaneous frequency to rifampicin-resistance. In E. coli uvrE mutants (reviewed by Cox, 1976) and a mutant of Neisseria meningitidis (Jyssum, 1968) are UV-sensitive strains which exhibit strong mutator properties. The uvrE gene is believed to function in mismatch repair and uvrE mutants are hypermutable by base analogues (Rydberg, 1978). However, UV47 is not mutated by 2-AP (or UV).
Strain 303 is a much weaker mutator exhibiting only a 10-fold increase in the yield of rifampicin-resistant mutants. It is defective in a gene designated uvsA (Moseley and Copland, 1978) and is very sensitive to killing by UV, MTC, DCMTC, MNNG and MMS. Other mutants of *E. coli* that are sensitive to the lethal effects of many DNA-damaging agents are weak mutators e.g. *polA* strains with defective polymerase and 3'→5' exonuclease activities (see Cox, 1976). Conceivably strain 303 could be defective in either activity.

Although considerable more work is needed these results show that the spontaneous mutation rate in *M. radiodurans* is under genetic control. They are also in concordance with other studies showing that DNA repair processes and spontaneous mutation are closely interrelated and provide evidence for the basic mechanistic similarities of these processes in *M. radiodurans* and *E. coli*.

11. General conclusions

The results presented in this thesis explain, at least in part, why *M. radiodurans* is resistant to the mutagenic (and lethal) effects of most DNA-damaging agents. Of the possible mutagenic pathways, one of them, namely *E. coli*-type error-prone repair is absent from *M. radiodurans* which may well be a prerequisite for extreme radioresistance. Resistance to mutation is also a consequence of efficient repair mechanisms for lesions which alter the template specificity of DNA replication since repair-deficient mutants have been isolated which are very much more sensitive than the wild-type to certain mutagens, in particular monofunctional alkylating agents. These mutants have also highlighted the biological significance of certain lesions in DNA e.g. the data constitute one of the strongest pieces of evidence to
date that $^6$-methylguanine mispairs in vivo. The results have also indicated the distinctness of lesions, produced by one agent, which contribute toward lethality and mutation.

The advantage of studying mutagenesis in *M. radiodurans* is that mutagens can be examined for their role in direct misreplication mutagenesis independently of error-prone repair. The benefit arises because *M. radiodurans* behaves, with regard to mutagenesis, like a recA or lexA mutant but has the property of being able to tolerate very much more damage than these mutants so that more potentially mutagenic lesions can be introduced.

Why *M. radiodurans* should possess such efficient DNA repair/tolerance mechanisms is not clear. It is possible that this bacterium represents one of a group of organisms whose ancestors had similar efficient repair mechanisms needed to combat the higher levels of DNA-damaging agents, in the environment than are present today, e.g. solar UV radiation. However, as the UV fluence was reduced the need for such efficient repair mechanisms disappeared so that most organisms may have reduced their overall repair capacity. For some reason this did not occur in all organisms. Indeed, it might be considered disadvantageous to be too efficient because mechanisms of mutagenesis might be necessary to provide the required genetic diversity for species survival. Thus, although *M. radiodurans* mutates spontaneously at frequencies comparable with other bacteria the general induced immutability may have necessitated a slow evolutionary process to form the present-day organism.
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Structural formulae of some electrophilic reagents

\[
\text{Dimethyl sulphate} \quad \text{CH}_3\text{-O-S-O-CH}_3
\]

\[
\text{Methyl methanesulphonate} \quad \text{CH}_3\text{-S-O-CH}_3
\]

\[
\text{Ethyl methanesulphonate} \quad \text{CH}_3\text{-S-O-CH}_2\text{CH}_3
\]

\[
\text{N-methyl-N'-nitro-N-nitrosoguanidine} \quad \text{O=N-N}_3\text{ch}_3\text{H} \quad \text{C-N} \quad \text{NH} \quad \text{NO}_2
\]

\[
\text{N-methyl-N-nitrosourethane} \quad \text{O=N-N}_3\text{ch}_3\text{C-O-CH}_2\text{CH}_3
\]

\[
\text{N-methyl-N-nitrosourea} \quad \text{O=N-N}_3\text{ch}_3\text{C-NH}_2
\]
Mitomycin C

Decarbamoyl mitomycin C

7-Bromomethylbenz[a]anthracene

N-acetoxy-2-acetylaminofluorene