REPAIR OF RADIATION DAMAGE IN MICROCOCCUS LUTEUS

Herstel van stralingsschade in *Micrococcus luteus*

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A.E. COHEN, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 25 OKTOBER 1972 TE KLOKKE 15.15 UUR

DOOR

CORNELIS ABRAHAM VAN SLUIS

GEBOREN TE ZIERIKZEE IN 1939

1972
BRONDER-OFFSET B.V. – ROTTERDAM

PROMOTOR: PROF. DR. A. RÖRSCH

STELLINGEN

I

Bij de bestudering van het excisie-herstel proces is onvoldoende aandacht geschonken aan de mogelijkheid dat verwijdering van pyrimidine dimeren uit DNA kan plaatsvinden in de vorm van langere oligonucleotiden, die niet in de zuuroplosbare fractie aantoonbaar zijn.

J.E. Cleaver en H.W. Boyer. Biochem. Biophys. Acta 262, 116 (1972)

II

De bewering van Setlow c.s. dat de *Haemophilus influenzae* mutant UV1 het UV-specifieke endonuclease mist, is aan bedenkingen onderhevig en dient door enzymologisch onderzoek te worden bevestigd.

J.K. Setlow en J.E. leClerc
Abstracts VI. International Congress on Photobiology, Bochum 1972
R.B. Setlow, J.K. Setlow en W.L. Carrier. J. Bacteriol. 102, 187 (1970)

III

De conclusie van Kitayama en Matsuyama, dat in *Micrococcus radiodurans* door gamma straling geinduceerde dubbelstreng breuken in DNA worden hersteld, berust op onvoldoende experimentele gegevens.

S. Kitayama en A. Matsuyama. Biochem. Biophys. Res. Commun. 33, 418 (1968)

IV

De resultaten verkregen bij sedimentatie van gealkyleerd DNA in alkalische sucrosegradiënten kunnen op foutieve wijze worden geïnterpreteerd en dienen te worden vergeleken met de resultaten verkregen met behulp van formamidegradiënten.

D. Gaudin en K.L. Yielding. Biochem. Biophys. Res. Commun. 47, 1396 (1972)

V

Bij het onderzoek naar herstel van beschadigd DNA dient men zich te realiseren, dat in veel gevallen de resultaten worden verkregen uit experimenten met een gemengde populatie van overlevende en lethaal beschadigde cellen.

C.E. Hildebrand en E.C. Pollard. Biophys. J. 9, 1312 (1969)

VI

De invloed van mutaties in microörganismen dient bij voorkeur te worden onderzocht in suppressor-vrije stammen, waarvan de genetische achtergrond goed is gedefinïeerd.

VII

Het verdient aanbeveling dat door de Overheid - naar analogie van de Nederlandse Consumentenbond - een Instituut wordt opgericht om de bruikbaarheid en duurzaanheid van kostbare wetenschappelijke instrumenten te beoordelen, teneinde door een gefundeerd aanschaffingsadvies de investeringen bij de door haar gesubsidieerde research- en onderwijsinstellingen te optimaliseren.

VIII

De Nederlandse automobilist dient zijn gedrag bij de nadering van een tegenligger te spiegelen aan de wijze waarop het zebrabarbeeltje (*Brachydanio rerio*) reageert op de nadering van een natuurlijke vijand.

L.M. Dill. Nature New Biology 236, 30 (1972)

C.A. van Sluis

Leiden, 25 oktober 1972

A luteus cell was UV hit
but lacked the enzyme to repair it
It did the incision
By nuclear fission
And that's how he got to survive it

To my parents
to Ineke

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ABBREVIATIONS

A_{xyz}	absorbance at xyz nm.
BSA	bovine serum albumin
DEAE-cellulose	di-aethyl-aminoethyl-cellulose
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid (di Na-salt)
FLR	fractions of UV lesions restored
Hcr^-	lacking host cell reactivation (phenotype)
HMP	10mM K-phosphate buffer (pH 7.0)
MitC	mitomycin C
4NQO	4-nitroquinoline-1-oxide
NB	nutrient broth
NBS	nutrient broth sucrose
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
pfu	plaque forming unit
PGY broth	peptone-glucose-yeast extract
RNA	ribonucleic acid
tRNA	transfer RNA (uncharged)
SDS	sodium dodecylsulphate
SMM	minimal medium Micrococcus luteus
SMS	minimal medium Micrococcus sodonensis
Tricine	N-Tris (hydroxymethyl)methylglycine
Tris	tri-(hydroxymethyl) aminomethane
TCA	trichloroacetic acid
T	thymine
$\widehat{TT}$	thymine dimer (pyrimidine dimer)
UV	ultraviolet (254 nm)
X-rays	röntgen radiation (8 keV_{eff})
RF DNA	replicative form DNA

The designation of bacterial mutations is according to the nomenclature as proposed by Demerec et al., (1966) and Taylor, (1970).

CHAPTER 1

GENERAL INTRODUCTION

Irradiation of living cells with short wave ultraviolet light (UV) leads to alterations in their DNA (Smith, 1966a). Pyrimidine base damage -such as the dimerization of thymine and cytosine- is the mayor chemical alteration which has been identified. Other photochemical reactions which have been observed, are the formation of heteroadducts of pyrimidines and the crosslinking of DNA and protein. A dose dependent relationship exists between the number of photochemical lesions and the decrease in the synthesis of protein and RNA and the replication of DNA (for reviews see Setlow, 1966; Howard-Flanders, 1968; Setlow, 1968; Strauss, 1968 and Hanawalt, 1969).

Since the isolation of a radiation sensitive mutant of E. coli B by Hill (1958), which showed that repair of radiation damage is genetically determined and therefore an enzymatic process, many laboratories have been involved in genetic and biochemical research to unravel the mechanism of repair. A great number of radiation sensitive mutants has been isolated in a variety of eukaryotes, prokaryotes and higher organisms.

Two experimental approaches have been used to obtain more information about cellular repair processes. Firstly, the isolated mutants were characterized by their radiation sensitivity and classified according to the location of the mutation on the bacterial chromosome. Secondly the fate of DNA in irradiated cells was studied by the examination of DNA degradation and synthesis. From the considerable amount of information which has been obtained at present, several mechanisms for the restoration of the damaged sites in DNA have been established.

(i) Exposure of UV irradiated cells to visible or long wave UV light, induces a photoreactivation reaction in which the pyrimidine dimers in the DNA are split in situ (Rupert, 1962).

(ii) In 1964, Setlow and Carrier and Boyce and Howard-Flanders, discovered that pyrimidine dimers in UV irradiated E. coli are removed from the DNA. The excised regions are repaired by repair replication using the undamaged strand as template. Finally rejoining occurs of newly formed DNA to the "old" strand. This process is called excision repair.

(iii) Repair by a recombinational process which occurs after the replication of UV damaged DNA (Howard-Flanders et al., 1968). This process is less efficient in the removal of pyrimidine dimers than either excision repair or photoreactivation.

Although our understanding of repair processes has considerably improved during the last decade, the identification of enzymes which are involved in repair is still far from complete.

The discovery by Strauss (1962) that extracts of M. luteus contain a nuclease activity which inactivates UV irradiated DNA but has no influence on native DNA, marked the beginning of the enzymological study of excision repair. In 1964 we started the investigation on repair of UV damage in M. luteus, which is the subject of this thesis. The results have been in part described elsewhere (Rörsch et al., 1967; Van de Putte and Van Sluis, 1969).

The aim of the present investigation was to obtain more insight into the relation of the phenotypical properties of various types of radiation sensitive mutants and the intracellular activity of nuclease which are suspected to be involved in repair.

The biochemical and analytical techniques as well as the selection of radiation-sensitive M. luteus mutants are described in chapter 2. In chapter 3 the radiation properties of the isolated mutants are described and a preliminary classification is given. The in vitro repair of biologically active DNA by M. luteus extract is described in chapter 4. The influence of mutations affecting UV sensitivity as well as the effect of repair inhibitors is studied. The excision of dimers from DNA in vitro is investigated in chapter 5, while the fate of intracellular DNA after UV irradiation of M. luteus cells is determined in chapter 6. The isolation of UV-endonuclease deficient mutants and their behaviour after irradiation has led to a reconsideration of the repair of radiation

damage in M. luteus.

In chapter 7, the results are discussed and several hypotheses are forwarded on the relative importance of different repair processes in M. luteus.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains

Two wild type strains of M. luteus were used (ML 1 and ML 8) and several radiation sensitive mutants were isolated (see chapter 3). ML 1 was obtained from the Culture Collection of the Microbiology Department of the Technological University, Delft and is probably the strain originally described by Fleming (1922).

ML 8 was isolated from spray-dried material manufactured by Miles Chem. Comp., Elkhart, U.S.A.

Strain ML 22 was obtained through the courtesy of Dr. Van de Putte and was isolated by Okubo et al, (1967) as ML 1312.

During the last five years various researchers have designated this organism as M. luteus as well as M. lysodeikticus. Althrough there exists some confusion about classification (Kloos 1969a), the designation M. luteus as proposed by Kocur and Martinec (1965) is used.

E. coli K12 parent type and several UV-sensitive mutants have already been described by Van de Putte et al., (1965). They were used in the DNA-spheroplast assay as described in 2.2.4.1.

The genotypes of these strains are given in table 2-1 and 2-2.

TABLE 2-1

E. coli K12 strains (MBL collection).

		Radiation phenotype	
Number	Mutation	Bacteria	Bacteriophage
KMBL 49	uvr^+	resistent	Hcr^+
KMBL 90	uvrB501	sensitive	Hcr^-
KMBL 91	uvrE502	sensitive	Hcr^+
KMBL 92	uvr-504	sensitive	Hcr^+
KMBL 99	uvrA503	intermediate	Hcr^-
KMBL 100	uvrC505	sensitive	Hcr^-
KMBL 101	uvrB506	sensitive	Hcr^-
KMBL 194	uvrE502 uvr-504		see KMBL 91
KMBL 196	uvrA6 uvr-504		see KMBL 90
KMBL 199	uvrA6 uvrE502		see KMBL 90
KMBL 202	uvrA503 uvrE502		see KMBL 90
KMBL 206/207	uvrA6 uvrC505		see KMBL 90
KMBL 212/222	uvrA6 uvrB5 uvrC505		see KMBL 90

For the radiation properties of the *E. coli* mutants, see Rörsch et al., (1964a); Van de Putte et al., (1965); Howard-Flanders et al., (1966) and Mattern, (1971). Hcr: host cell reactivation of UV irradiated phage.

TABLE 2-2

E. coli strains (received)

Number	Mutation	Other designation	Origin
(*E. coli* K12)			
KA15	uvr^+	-	Harm
KA16	uvrA516	-	Harm
KA46	uvr^+	AB 1157	Adler
KA64	uvrB5	AB 2434	H-Flanders
KA65	uvrC34	AB 2435	H-Flanders
KA66	uvrA6	AB 2436	H-Flanders
KA173	uvrA6	AB 1886	Devoret
(*E. coli* B)			
KMBL2003	uvrC500	B syn^-	Rörsch
KMBL2013	uvrB513 exr-11	B_{s-1}	Hill
KMBL2014	exr-12	B_{s-2}	Hill

For more details see the references given in table 2-1; Mattern et al., (1966) and Mattern, (1969).

2.1.2 Bacteriophages

The micrococcal phage N5 was obtained from H. B. Naylor, New York. Bacteriophage was produced by the confluent lysis method (Adams, 1959). Titers of 10^{10} -10^{11} pfu/ml were obtained. Lysates were stored at 4^{o}C in PGY broth.

It was observed that the efficiency of plating of phage N5 differed on ML 1 and ML 8. To avoid this problem phage was always grown on the wild type strain related to the mutant under study.

Phage ØX 174 was obtained from Dr. P. H. Pouwels and was used to prepare the replicative form (RF) DNA, (see 2.2.3.1).

2.1.3 Culture media.

PGY broth (Field and Naylor, 1962) was used for the general cultivation of bacteria, and contained 1% Difco peptone, 0.5% Difco yeast extract, 0.5% sodium chloride and was supplemented with 0.2% glucose after autoclaving.

For solid medium 1.5% Difco Bacto agar was added and in soft agar overlays 0.7% agar was used.

In some early experiments two other complex media were employed (Steiner and Beers, 1961; and Kilburn et al.,1958). These media cause a long delay of growth after inoculation. Furthermore because sterilization of these media had to be done at 110°C to avoid the destruction of amino acids and yeast extract, it was difficult to avoid contaminations. Minimal medium, was used according to Wolin and Naylor (1957) and in later experiments according to Aaronson (1966) as described for M. sodonensis. They are disignated respectively as SMM and SMS. In several cases supplemented minimal medium was employed, because some of the isolated M. luteus mutants had acquired unknown growth requirements, which could be met by the addition of 0.1% Difco yeast extract and 0.1% Difco casamino acids. The generation time of several mutants could be decreased further by the addition of 10 μg/ml of a mixture of DNA and RNA bases, especially adenine.
Adenine dependency was spontaneously introduced into Micrococcus strains (Kloos and Schultes, 1969).

SPECIAL MEDIA FOR SPHEROPLAST-ASSAY.

Modified 3 XD (Fraser and Jerrel, 1951; Guthrie and Sinsheimer, 1960) was used to grow E. coli strains. The required amino acids and bases were added in a concentration of 50 μg/ml and 10 μg/ml respectively.

Nutrient-Broth-Sucrose (NBS) used to stabilize spheroplasts and to support the growth of ØX 174 phage contained: 1% Difco casamino acids, 1% Difco nutrient broth and 10% sucrose. After autoclaving 0.1% glucose and 0.1% $MgSO_4$ were added.

Zahler agar was used for the titration of ØX 174 phage and contained 1% Difco tryptone and 0.5% Difco yeast extract. For bottom agar 1.2% agar was added and for topagar 0.6%. The bottom layer was supplemented with 1.2% glucose after autoclaving.

Media were adjusted to pH 7.0-7.2 and sterilized for 15 min at 120°C. When heat labile substances were present, sterilization was done either by heating for 20 min at 110°C or by filtration.

Solutions which were used in some special experiments are described in the text concerning these experiments.

2.1.4 Chemicals.

Amino acids, bases, caffeine, proflavine and acriflavine were obtained from the British Drug Houses Ltd., England; deoxyadenosine from California Biochemicals, USA;
Tris and 2-mercaptoethanol from Koch-Light Labs, England;
EDTA from Siegfried AG., Switzerland;
Tricine and tRNA/E. coli from General Biochemicals, USA;
Chloramphenicol and streptomycin from Mycofarm, Delft;
Mitomycin C from Kyowa Hakko Kogyo, Tokyo;
Nitrosoguanidine from Aldrich Chem. Comp., USA;
4-Nitro-quinoline-1-oxide from K. & K. Labs, USA.
Tritiated thymine (TRK 15 and TRK 127), thymidine (TRK 300) from Radiochemical Centre, Amersham, England, (specific radioactivity 10-20 Ci/mmole);
Lysozyme (3 x cryst.) from Nutritional Biochemicals, USA;
Bovine serum albumin from Poviet, Amsterdam;
DEAE-cellulose (0.4-0.8 greq./gram) from Serva, Heidelberg, Germany;
Sephadex G-10, G-25, G-75 and G-100 from Pharmacia, Uppsala, Sweden.

Other chemicals were mostly analytical grade and were purchased from Merck AG., Germany or from the British Drug Houses Ltd., England.
Membrane filters were obtained from Millipore, USA or from Sartorius Membranfilter Gesellschaft GmbH., Germany. The following types were employed: Millipore HA045; Membranfilter MF 500-11301 (5 μ); MF 100-11305 (0.8 μ), 11306 (0.45 μ) and 11307 (0.2 μ).

2.1.5 Miscellaneous.

Low speed centrifugation was carried out in a Homef type LC-30 centrifuge or a Sorvall type M centrifuge.
Preparative high speed centrifugation was done in the B-20 International centrifuge and sucrose gradients were run in the Beckman Spinco L or L2-50 ultracentrifuge using SW 25.1, SW 27 or SW 50 rotors.
Ultrasonic treatment of bacterial extracts and suspensions of bacterial cells (see 2.2.1.3) was performed in a Raytheon 250 W 10 kcs sonic apparatus, type DF 101. The temperature of the vessel was maintained below 10^{o}C by circulating refrigerated ethanol. After each treatment of 30 sec. a cooling period of 15 sec was allowed. Samples of a small volume and sterile samples were soniced in polycarbonate tubes which were surrounded by a mixture of water and ice.
Absorption measurements in the 280/260 nm region were made with a Zeiss PMQ II spectrometer. Column effluents were monitored by a continuous flow unit (LKB 8300A) coupled to a logarithmic recorder (Vitatron UR 402).
The optical density of bacterial suspensions was measured in 10 mm cylindrical tubes in a Vitatron colorimeter (type UC200) fitted with a 700 nm filter.

2.2 METHODS.

2.2.1 MICROBIAL TECHNIQUES.

2.2.1.1 Irradiation.

UV-irradiation was carried out in an internally blackened cabinet containing a Philips TUV 57413 P40 tube with reflector, emitting at least 90% of its energy at 254 nm. The samples were irradiated at a distance of 40 cm. The intensity varied from 25-40 erg/mm^2 x sec, depending on the age of the tube.

The intensity was frequently checked by a UV meter type J-221 (Ultraviolet Products, USA). More accurate calibrations were done every two or three months by the uranyl oxalate titration method described by Bowen, (1946).

Uranyl oxalate is photochemically decomposed by UV radiation according to the following reactions:

$$UO_2^{++} + h\nu \longrightarrow UO_2^{++*}$$

$$UO_2^{++*} + C_2O_4^{--} \longrightarrow UO_2^{++} + C_2O_4^{--*}$$

$$C_2O_4^{--*} + 2H^+ \longrightarrow CO_2 + H_2O + CO$$

The remaining oxalate can be determined oxidimetrically with permanganate. The quantum efficiency at 254 nm is 60%. The photodecomposition of oxalate has been reinvestigated recently (Volmand and Seed, 1964).

X-ray irradiation was performed with a watercooled Machlett OEG-60 tube fitted with a 1 mm beryllium window. The effective energy of the emitted radiation is 8 keV and 0.7 mm of water reduced the intensity to 50%. The tube was operated at 50 kV and 30 mA.
Liquid samples (0.6 ml) were irradiated under aerobic conditions. A 5 ml beaker placed at 10 cm from the tube, was agitated by a connection to a loudspeaker conus (25 cycles/sec). The dose was 600 rad/sec and was monitored by a Philips dosimeter, type 37463/01.
Bacteria plated on solid media were irradiated at a distance of 22 cm from the tube from which the 15 mm diaphragm had been removed. The intensity was 450 rad/sec.

2.2.1.2 Isolation of mutants.

Radiation sensitive mutants were isolated from the wild type strain ML 1, according to Van de Putte et al., (1965). ML 2 and ML 5 were subjected to a second NTG treatment resulting in the isolation of ML 2-1 to 2-4 and ML 5-

In further attemptsto isolate sensitive mutants from ML 1 and ML 8, difficulties were encountered due to the low mutagenic action of NTG on M. luteus. The inactivation of M. luteus during NTG treatment was determined and it was found that during the first 45 min incubation at 37°C there was no killing of bacteria. When the bacteria were irradiated with 2000 erg/mm^2 UV before the NTG treatment an exponential inactivation was observed.

The surviving cells after 120 or 180 min NTG treatment (0.1 M Na-acetate pH 5.0; 600 μg/ml NTG) were plated on PGY and the colonies were picked and replica-plated on plates which received 0, 300, 900 and 1500 erg/mm^2 to facilitate the discrimination into different classes. From 2000 colonies tested 25 were found

to be radiation sensitive.

After final examination several mutants were retained which were of the intermediate sensitivity type: ML 9, ML 10 and ML 12-15. The characteristics of the M. luteus mutants will be given in chapter 3.

2.2.1.3 Inactivation of colony formation by irradiation.

M. luteus-like other Micrococci- grows mostly in clumps consisting of two or four cells. When a suspension of cells is irradiated as such and plated, a significant reduction of the number of colony forming units will be only observed when the probability that all cells in one aggregate are lethally hit, approaches unity. This will result in survival curves with "false extrapolation numbers" (shoulders), which are not a result of intracellular processes determining survival after irradiation. Therefore precautions had to be taken to prevent that cells aggregated during or after irradiation.

Several methods have been attempted:

1. A suspension of washed bacterial cells, suspended in buffer or in synthetic medium (SMM of SMS) at 10^8 cells/ml is blended in the microattachment OM-2000 of a Sorvall Omnimixer for 3 min at 50,000 rpm.
   However excessive foaming occurs and sterile handling of the solution is difficult.

2. A more reliable method was introduced by Field and Naylor (1962), who sonicated the cells for 5 min and observed single cells and pairs.
   Their method was adopted in our experiments and somewhat refined.
   The optimal time of sonication was 4-8 min, resulting in a 5-fold increase of the viable count.

   An overnight culture in PGY broth is washed twice in minimal medium, diluted to 5×10^7 cells/ml and sonicated for 5 min at 0-5°C.
   Samples were irradiated in small petri dishes and 0.1 ml of dilutions were plated on PGY or YHC agar and counted after 3-5 days incubation at 30°C.
   In later experiments cells were diluted, plated and then directly irradiated on the plate. Contamination and re-aggregation of cells during irradiation was prevented by this modification.

3. When inactivation by X-rays was studied, irradiation after plating of the cells was too time consuming. Therefore cells were collected on 0.45 μ membrane filters, resuspended, sonicated and filtrated on 5 μ membrane filters to remove aggregates of three or more cells.
After a brief incubation period in broth (1 hr at 30°C) to get the cells again in an exponential phase, the formed aggregates were then removed by a second filtration. Subsequently samples were irradiated and dilutions were plated.

4. The previous method could not be used for UV irradiation because removal of broth by washing the cells and resuspension in buffer was always accompanied by new aggregation. In this case the cells were brought into an exponential phase by incubating the plated cells for 1 hr prior to the UV irradiation.

2.2.1.4 Inactivation of phage N5 by UV irradiation.

Phage N5 grown on ML 1 or on ML 8 was diluted to 10^7 pfu/ml in 0.01 M K-phosphate buffer pH 7.0 (HMP) and irradiated at room temperature. After dilution in PGY broth, one tenth millilitre samples were mixed with 0.25 ml of an exponential culture of the indicator strain and plated in duplicate on PGY agar. Plates were incubated for 2 days at 30°C, after which no new plaques appeared.

2.2.2 ENZYMOLOGICAL TECHNIQUES.

2.2.2.1 Preparation of bacterial extracts.

Large amounts of bacterial cells were grown at 30°C with shaking. Two litre flasks, containing 750 ml PGY or YHC medium supplemented with 0.2% glucose were inoculated to an optical density at 700 nm of 0.05. In 18-24 hours an A_{700} of 8-12 was reached. Cultures were then chilled and cells were collected by centrifugation for 5 min at 7500 rpm. The wet cell paste was washed twice with HMP and either stored at -20°C or used to prepare bacterial extracts. About 8-12 gram of wet cells was obtained from a one liter culture.

Five grams of wet cell paste were resuspended in 100 ml buffer and

lysozyme was added to a final concentration of 25 μg/ml. If lysis was not complete after 20 min incubation at 37°C, the same amount of lysozyme was added and the incubation was continued for 15 min.

The viscous dark yellow lysate was sonicated for 60 sec after addition of 1 mM 2-mercaptoethanol. Cell debris was removed by centrifugation for 20 min at 12,000 rpm. The resulting extract was neither turbid nor viscous.

In some experiments with the parent type strain ML 8, extracts were prepared from spray dried cells (Miles Chem. Comp., Elkhart, USA), according to the method described by Carrier and Setlow (1966). The properties of these extracts did not differ significantly from those prepared by the procedure described above.

Extracts could be kept at 4°C for several days without loss of activity but with a great risk of bacterial contamination. Therefore extracts were divided into small portions and stored at -20°C.

2.2.2.2 Partial purification of UV-endonuclease activity.

All manipulations were carried out in a cold room at 4-6°C.

Nucleic acid was removed by precipitation with streptomycin. Concentrated K-phosphate buffer pH 7.5 was added to a final concentration of 0.05 M. A 30% solution of streptomycin was introduced gradually untill a concentration of 2% was reached. The mixture was left overnight with gentle stirring. The precipitate was removed by centrifugation. Ammonium sulphate was added to 40% saturation and after 30 min the precipitate was removed and discarded. The precipitate collected after saturation to 70% was dissolved in a small volume of 0.01 M K-phosphate buffer pH 7.0 or 0.02 M Tris buffer (pH 7.5) containing 0,001 M 2-mercaptoethanol.

Dialysis proved to be insufficient to remove the remaining streptomycin and ammoniumsulphate. Therefore chromatography on Sephadex G-50 or G-75 in 0.05 M K-phosphate or Tris buffer pH 7.5 was used. The enzymatic activity was eluted in the bulk of the protein.

Most experiments were carried out using either the crude extract or a preparation collected from a Sephadex column. In some cases partial pu-

rification was attempted. Chromatography on DEAE-cellulose and fractionation on some other materials are described in section 4-3.

2.2.3 ISOLATION OF NUCLEIC ACIDS.

2.2.3.1 ØX 174 RF-DNA.

The double stranded form of ØX 174 DNA is formed as an intracellular replicative intermediate during the synthesis of new single stranded phage DNA. RF DNA can be isolated from infected E. coli C bacteria, in which phage development has been arrested by chloramphenicol (Hayashi et al., 1963).

The isolated DNA is found in two different configurations: RF I and RF II, of which the latter has one or more single strand breaks (Jansz and Pouwels, 1965).

DNA was isolated according to the method of Hayashi et al., (1963) and subsequently separated from host cell by fractionation on methylated albumin kieselguhr columns according to Mandell and Hershey (1960). ^{32}P-labelled RF DNA was prepared by the same method but the incubation time of the ØX-infected cells was reduced to minimize intracellular radiation damage (see also Pouwels et al., 1966).

For experiments in which the conversion of RF I into RF II was investigated, preparations containing high amounts of RF I were prepared by either a second chromatography according to Mandell and Hershey (1960) or by separation on neutral sucrose gradients as described in 2.2.5.

Later the preparation technique for RF DNA was adopted from Jansz et al., (1966), that makes use of the reversible denaturability of RF I, while RF II and contaminating host cell DNA are irreversibly denatured (Pouwels et al., 1966). Double stranded DNA (RF I) was separated from denatured DNA by filtration on 0.2 μ membrane filters at high ionic strength.

DNA was stored at 4°C in 1 M NaCl. When it was necessary to avoid the conversion of RF I into RF II, stringent precautions were taken to maintain sterility and to avoid contamination by endonucleases.

2.2.3.2 E. coli DNA.

E. coli strains KMBL 2912 or KMBL 49 were labeled with tritiated thymine or thymidine by growing them overnight in M9 minimal medium, sup-

plemented with 0.2% glucose, 2 μg/ml thymine and 1-4 μCi/ml of the labeled precursor. After collection, cells were washed and DNA was extracted by the method of Marmur (1961).
After treatment with RNase, the low molecular weight material was removed by chromatography on a Sephadex G-100 column in a buffer of high ionic strength.

In later experiments the PAS/TIPNS method was employed as developped by Lohman (1969) from the procedures of Kirby (1965) and Parish and Kirby (1966). From one gram of cells 1-1.5 mg of DNA (containing 10 percent of the initially added radioactivity) was usually obtained. Preparations were stored in standard saline citrate (SSC) at 4°C or at -20°C. Storage for longer than two months was avoided due to the possible induction of radiochemical changes and the depolymerization of the DNA caused by radioactive decay.

2.2.4 DETERMINATION OF REACTIVATING ACTIVITY.

2.2.4.1 Assay of biologically active RF DNA on E. coli spheroplasts.

Preparation of spheroplasts.

The method is essentially the same as outlined by Guthrie and Sinsheimer (1960 and 1963).

E. coli K12 radiation sensitive mutants (see table 2-1 and 2-2) were inoculated in 3 XD medium and incubated overnight at 37°C without aeration. Three ml was diluted into 22 ml medium and incubated for 2.5 hrs with shaking. Cells were centrifugated and the sediment as well as the interior of the tube were dried with sterile filterpaper. The pellet was resuspended in 0.1 ml 0.25 M Tris buffer pH 8.1. Subsequently the following solutions were added: 0.35 ml 0.5 M sucrose and 0.04 ml lysozyme (2 mg/ml). After 1 min incubation at 30°C, 0.02 ml 0.1 M EDTA pH 6.25 was added and incubation was continued for another 8.5 min with occasional shaking. Two ml NBS containing 2% BSA were introduced, mixed gently and the spheroplasts were allowed to stand for 5 min at 30°C. Finally the mixture was diluted with 7.5 ml NBS. Spheroplasts could be kept for several hours at room temperature. When the effect of one of the various inhibitors for repair (e.g. caffeine) was studied, it was added to the culture of E. coli cells during the last hour of growth.

Incubation of UV irradiated DNA and extract.

Bacterial extracts were usually diluted in HMP containing 0.002 M EDTA to a protein concentration of 10-100 μg/ml and 0.1 ml was added to 0.1 ml (0.01 μg) ØX 174 RF DNA, which had been inactivated to a survival of 10^{-3} (measured on Hcr^- spheroplasts). The mixture was subsequently incubated for 30 min at 37°C and the reaction was terminated by 10 min heating at 65°C. This temperature caused inactivation of the enzymatic activity of the extract, but did not alter the configuration nor the biological activity of the DNA.

Absorption of DNA to E. coli K12 spheroplasts and titration of phage.

Spheroplasts (0.2 ml) were introduced into the incubation mixture and absorption of DNA took place within 5-10 min at 37°C. Finally NBS was added up to 2 ml and incubation was continued for 2.5 hrs at 37°C with aeration. Dilutions were made in tryptone broth containing 0.5% NaCl and were plated on Zahler agar with E. coli C (KMBL 2901 or 2902) as indicator strain. The biological activity of a given DNA preparation is dependent on the capacity of a spheroplast preparation to absorb DNA; this capacity varied from day to day. Therefore data could only be compared in terms of survival. Each determination of biological activity was performed in duplicate. Generally both irradiated and unirradiated DNA were incubated with extract to correct for inactivation of the biological activity by aspecific nucleases. It was assumed that these nucleases act in the same way on both kinds of DNA, disregarding of course that irradiated DNA may have a partly different configuration.

2.2.4.2 Quantitative estimation of reactivation.

The capacity of an extract of M. luteus to "repair" UV lesions in the dark can be deduced from the difference in the survival of the biological activity of control DNA and DNA treated with extract. The incubation with extract acts on the DNA as a dose reducing factor (DRF). The calculation of the fraction of UV lesions reactivated (FLR) has certain advantages from an enzymatic point of view. The reactivating activity is then directly correlated to substrate changes, i.e. the modification of UV lesions.

The calculation of the fraction of UV lesions reactivated (FLR) is based on the assumption that the number of lethal UV lesions introduced in DNA

by UV irradiation, is linearly related to the UV dose, and that the inactivation of the biological activity is exponential. When S_o is the surviving fraction of the biological activity of RF DNA after a given dose D_o and S_e is the survival after incubation with extract, the apparent dose D_e can be determined by interpolation as indicated in fig 2-3.

$$DRF = \frac{D_e}{D_o} = \frac{-k.\log S_e}{-k.\log S_o}$$

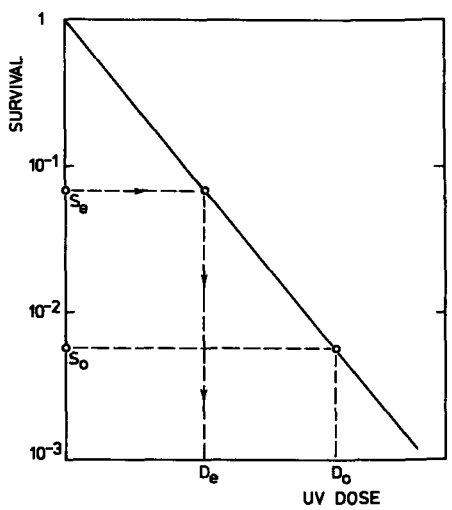

Fig. 2-3 Graphical estimation of FLR from the surviving biological activity of RF DNA.

The number of UV lesions in DNA at dose D_o is $C.D_o$ (C is a photochemical factor); so we get for the fraction of UV lesions restored:

$$FLR = \frac{c.D_o - c.D_e}{c.D_o} = 1 - \frac{D_e}{D_o} = 1 - \frac{1}{DRF}$$

To facilitate the estimation of FLR a nomogram was constructed, which is shown in fig 2-4. The absolute number of UV lesions restored was not calculated, but standardization was attempted by employing a fixed amount of RF DNA (0.01 µg) in all incubations and a UV dose (1500-2000 erg/mm^2) giving a survival of 10^{-3}.

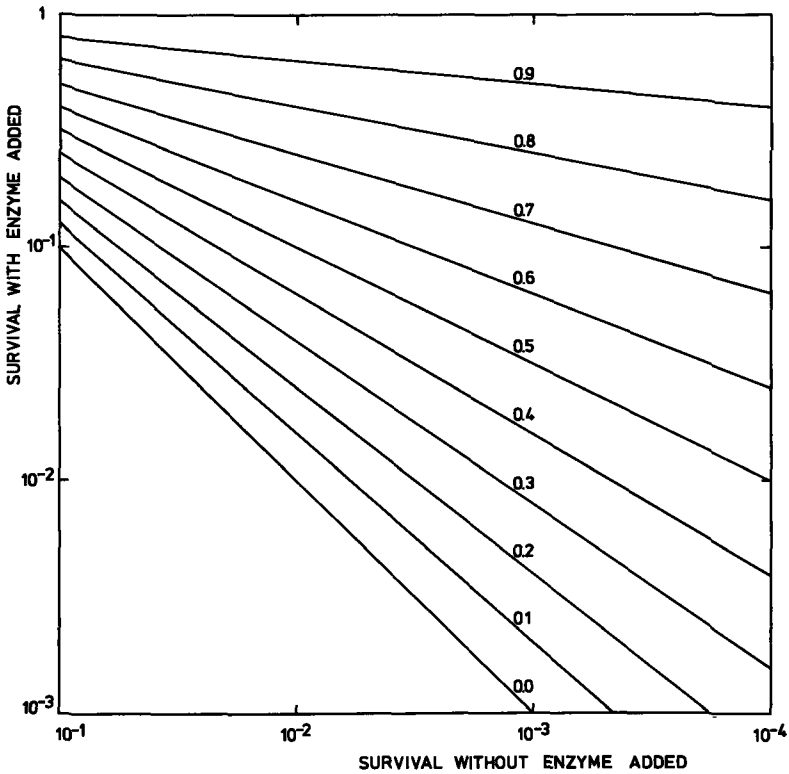

Fig. 2-4 Nomogram for the determination of the FLR from the surviving biological activity of RF DNA with and without incubation. From a point on the vertical axis, corresponding to the survival with incubation a horizontal line is drawn. The intersection of a vertical line from the point on the horizontal axis, representing the survival without irradiation, gives the fraction of UV lesions restored.

2.2.5 REACTIONS WITH DNA IN VITRO.

2.2.5.1 Conversion of ØX RF I into RF II (incision).

As was already mentioned in 2.2.4.1 the conversion of RF I into RF II DNA alters the physical properties, which can be used to separate RF I and RF II molecules (Pouwels et al., 1966).

In a volume of 1.5 ml were mixed:

0.50-0.75 ml	^{32}P-labeled RF DNA (0.5-2 μg)
0.10-0.25 ml	enzyme preparation
0.50-0.90 ml	0.01 M K-phosphate buffer (pH 7.0)
	0.002 M EDTA.

A complete experiment consisted of four tubes. Unirradiated as well as irradiated DNA were incubated with buffer and with enzyme preparation. After incubation for 30 min at 37°C, the reaction was terminated by heating for 5-10 min at 65°C.

Separation of RF I and RF II DNA on neutral sucrose gradients.

A sample of the incubation mixture was used to determine the biological activity and the fraction of UV lesions restored (FLR) as indicated in 2.2.4., while a 1.0 ml sample was placed on a 28 ml 5%-20% (w/v) linear sucrose gradient, prepared in an apparatus described by Van der Schans et al., (1969).

The buffer in which the sucrose was dissolved was usually 0.01 M K-phosphate (pH 7.0) 0.001 M Na-citrate or 0.001 M EDTA.

Gradients were centrifuged for 16 hrs at 22,500 rpm at 5°C in a SW 25.1 rotor. Fractionation was achieved through special caps placed on top of the gradient tube, by displacing the sucrose solution upwards with carbontetrachloride that entered through a needle at the bottom of the tube (Van der Schans et al., 1969). Usually 0.4 ml of the collected fractions was used to determine radioactivity and these fractions were also assayed for biological activity (see 2.2.4) after dilution with buffer. In preparative runs of ^{32}P-RF I and RF II DNA only 0.05 ml of the fractions was used to locate the RF I peak. The fractions were pooled and dialyzed against buffer under sterile conditions.

Separation of RF I and RF II DNA by membrane filtration.

In later experiments the separation of RF I from RF II DNA was facilitated by the rapid renaturation of RF I following heating (Heijneker, pers. comm.). In this case the reaction mixtures were stored in ice, heated for 3

min in a boiling waterbath and then rapidly cooled in ice. The drop in temperature to below 20°C required less than 10 sec.
A 5 M NaCl solution in 0.01 M Tris buffer (pH 8) 0.001 M EDTA was added to a final concentration of 1 M and the mixture was filtered slowly on a cellulose-nitrate membrane filter (0.2 μ), which had been soaked in 1 M NaCl Tris-EDTA buffer. Under these conditions the renatured double stranded RF I DNA is not retained by the filter while the RF II DNA remains in a single-stranded form and is absorbed.

The filtrate containing the double stranded RF I DNA was collected in a liquid scintillation counting vial and the filter was washed with several ml of 1 M NaCl buffer.

The radioactivity of the combined liquids was determined in a liquid scintillation counter, using the Cerenkov method (Clausen, 1968). Filters were dried and counted on planchets. In later experiments the Cerenkov method was also used for filters which were placed in 5 ml water. Counting efficiences for ^{32}P in solution and on the filter did not differ significantly. In this way the ratio RF I to RF II could easily be calculated.

2.2.5.2 Degradation of irradiated DNA in vitro.

In a volume of 1 ml were mixed:

0.1 ml	E. coli DNA (1-3 μg) 40,000-60,000 erg/mm2
0.1 ml	0.5 M phosphate buffer or Tris buffer pH 7-8
0.1 ml	0.1 M $MgCl_2$ or 0.01 M EDTA pH 7.5
0.1 - 0.5 ml	micrococcal extract (200-2000 μg protein)
0.2 - 0.6 ml	water

The mixture was incubated for 1 - 4 hours at 37°C and the reaction was terminated by the addition of 1.2 ml ice cold 10% TCA (Moriguchi and Suzuki, 1966). Thymus DNA (200 μg) was added to ensure complete precipitation of acid insoluble material. After 30 min standing in ice, the tubes were centrifuged for 20 min at 4000 rpm in the cold. Samples (0.4 or 0.5 ml) were counted in a liquid scintillation counter, using either a dioxane-naphathalene or a toluene-TRITON X-100 scintillation mixture (see also 2.2.7.1). The total radioactivity of DNA present in the mixture was measured after hydrolysis of the DNA for 90 min at 85°C.

2.2.5.3 Excision of pyrimidine dimers from E. coli DNA.

Incubation was done essentially as described in 2.2.5.2, but the quantity of DNA and the volume were increased. After sedimenting the acid insoluble material, the sediment as well as the TCA supernatant were assayed for pyrimidine dimers.

Sediment: (DNA)

The material was washed once with 1 ml cold 5% TCA and subsequently the remaining TCA was extracted by washing twice with 2 ml 96% ethanol. The pellet was dried at 80°C and dissolved in 0.3-0.5 ml 90% formic acid or trifluoroacetic acid.

Protein, which was coprecipitated with the DNA, interfered with the hydrolysis of DNA and the subsequent paperchromatography. In the case that large amounts of protein were present in the incubation mixture, a different procedure was employed. After termination of the reaction by heating for 10 min at 65°C, the solution was chilled and extracted twice with phenol. After removal of phenol by extraction with ether, DNA was precipitated in the usual way by the addition of TCA and the normal procedure was followed. The removal of protein was not required when the hydrolysis products were separated by gelchromatography on Sephadex G-10 columns.

Supernatant: (oligonucleotides)

TCA was removed by two extractions with ether and the remaining ether was evaporated at 40°C. The solution was dried at 60°C with blowing air and lyophilized in later experiments. The dry material was dissolved in 0.3-0.5 ml of formic acid or trifluoroacetic acid.

Hydrolysis:

In most cases hydrolysis was done in formic acid by heating in an oven for 60-90 min at 175°C. Material was transferred to thick walled glass tubes and the sealed vials were placed in small metal cylinders to decrease the risk of damage and contamination in the case of explosion. In later experiments the vials were heated in a silicone oil bath in which the temperature control was

more reliable. Trifluoroacetic acid was only used in some cases as its high toxicity required special attention.

To avoid waste of material when the tubes were opened, the contents were frozen in liquid nitrogen. The formic acid and the trifluoroacetic acid were removed by heating at 60°C or by lyophilization. The dry material was dissolved in 0.05 ml or 0.5 ml 0.01 N HCl and carrier thymine (15 μg) and thymine dimer (35 μg) were added.

Separation of pyrimidine dimers and thymine in the hydrolysate.

The separation was carried out according to Bollum (1962), Setlow et al., (1963) and Boyce and Howard-Flanders (1964) by the paper-chromatography method using Whatman no. 1 or DE 81 paper or on Schleicher and Schull type 2043 b. The following solvents and elution times were used:

a. Whatman no. 1	n-butanol-acetic acid-water	80:12:30 (v/v)
S. & S. no 2043 b.	(16 hours)	
b. Whatman DE81	0.25 M ammoniumhydrocarbonate	
	(6 hours)	
c. Whatman no. 1	n-butanol-water	86:14 (v/v)
	(16 hours)	
	ammonium sulphate-Na-acetate-isopropanol	
	(6 hours)	40: 9: 1 (v/v)

For the one dimensional paper chromatography the material was usually applied as a thin streak of 2 cm wide and spreading of material during the development of the chromatograms was prevented by cutting out "separation lanes". The dried chromatograms were irradiated with 50,000 erg/mm^2 to split the dimers into thymine. This treatment facilitated the location of the dimer ($\widehat{TT}$) besides thymine (T). The $\widehat{TT}$ and T regions were divided into pieces of 1.5-3 cm, placed in scintillation vials and eluted with 0.5 ml water (method a and c) or with 0.5 ml 1 M ammoniumhydrocarbonate (method b).

In early experiments a dioxane-naphtalene scintillator (Setlow et al., 1963) was used; later a toluene-TRITON-mixture was employed and the volume of eluant was increased to 1 ml.

In case streaking of thymine into the dimer containing region of the chromatogram was observed (system a), corrections were made by running

parallel hydrolysates of unirradiated DNA in which the same quantity of protein and salt was present during the incubation. More reliable results were obtained by separation of the bases in the hydrolysate according to their molecular weight on Sephadex G-10 (Smith, 1966b). Columns of 50 x 1 cm were used and the material was applied in a volume of 0.5 ml. The columns were eluted with water or with 10^{-3}M acetic acid at a rate of 30 ml/hr and fractions of 1.5 ml were collected. The great advantage of this method is that high molecular weight material (> 700) is excluded and that dimers are eluted before thymine.
If some absorption of thymine takes place, it will not interfere with the relatively low radioactivity in the dimer region.

The presence of protein in DNA-hydrolysate and salts in hydrolysate of TCA soluble material cause much less interference in the fractionation on Sephadex G-10.

2.2.6 METABOLISM OF DNA IN VIVO.

2.2.6.1 Labeling of M. luteus DNA.

A single colony was used to inoculate PGY broth that was incubated with aeration until the culture reached mid-exponential phase. Bacteria were collected by centrifugation, washed and resuspended in SMM or SMS (see 2.2.1.3).

To 10 ml of supplemented SMM or SMS, 100 μCi ^{3}H-thymine or thymidine were added and the culture was inoculated with washed bacteria (A_{700} = 0.02) and incubated overnight at 30°C. When DNA bases were added to increase growth, unlabeled thymine was omitted to favour incorporation of radioactive thymine. Deoxyadenosine (200 μg/ml) was sometimes added to increase thymine incorporation because thymine requiring strains of M. luteus were not available. This effect was not observed with ^{3}H-thymidine.

The labeled bacteria were washed twice by centrifugation or by filtration with thymine supplemented minimal medium and were grown for another 2 hours at 30°C to exhaust the radioactive intracellular precursor pool. Finally bacteria were collected, resuspended (A_{700} = 2) and stored in ice untill used in the following experiments. Usually 5-10% of the added radioactivity was incorporated into DNA.

2.2.6.2 Degradation of intracellular DNA.

Bacteria were diluted with SMM or SMS to a A_{700} = 0.2 and irradiated in the cold. After supplementation of the medium (bases, casamino acids, yeast extract and PGY) a sample of 1 ml was transferred directly into 1.2 ml cold 10% TCA. Incubation was at 30°C and samples of control and irradiated cultures were taken at intervals. Growth was monitored by measuring the A_{700}. The division time of the control cultures varied between 120 and 180 min. Thymine (10 μg/ml) was present during incubation to prevent re-incorporation of DNA breakdown products into bacterial DNA.

After standing for 30 min in ice, 200 μg thymus DNA was added to the samples and the radioactivity of the TCA soluble fraction was determined as described in 2.2.5.2. Initially the total radioactivity was determined by lysis of a sample of labeled cells with lysozyme and SDS and counting of the diluted lysate. More recently the tubes with the sediments of the t=0 samples were again brought to 2.4 ml with 5% TCA and subsequently heated for 1.5 hrs at 85°C to render all DNA acid soluble before counting.

2.2.6.3 Sedimentation of DNA on alkaline sucrose gradients.

A technique to measure the amount of single strand breaks in bacterial DNA after X-irradiation has been developed by McGrath and Williams (1966). Their procedure was modified for UV by Rupp and Howard-Flanders (1968). This method was adopted for M. luteus cells.

Gradients.

Gradients were prepared by the method in 2.2.5. Sucrose was dissolved in 1 M NaCl 0.001 M EDTA and the pH was adjusted with concentrated NaOH to 12.1. Three sizes of linear sucrose gradients were used for different ultracentrifuge rotors:

a. SW 50 : Volume 4.8 ml, with a layer of 0.1 ml 0.5 NaOH
b. SW 27 : Volume 16 ml, with a layer of 0.3 ml 0.5 NaOH
c. SW 25.1 : Volume 28 ml, with a layer of 0.5 ml 0.5 NaOH.

The prepared gradients were cooled to 10°C before use.

Spheroplasts

Bacteria were irradiated, incubated and collected by centrifugation in the cold. The interior of the tube was dried with filter paper and spheroplasts were made at $0^{o}C$ with 25-30% of the amount of lysozyme that was used by Rupp and Howard-Flanders (1968) for the preparation of E. coli spheroplasts.

The amount of sucrose buffer in the incubation was decreased to 0.05 ml to avoid that the density of the spheroplast suspension exceeded that of the toplayer of the gradient on which the DNA had to be sedimented.

Lysis.

A spheroplast preparation, that had been checked microscopically was transferred carefully into the alkaline layer on top of the sucrose gradient. The amount of spheroplasts that was used, varied from 10 µl in the SW 50 gradients to 50 µl in the SW 25.1 gradients. Care was taken that no material passed down from the alkaline layer into the sucrose. The spheroplasts were very carefully dispersed in the alkaline layer either by swirling with a small glass rod or by blowing a small stream of air on top of the alkaline layer. Lysis occurred generally within 1 min but tubes were left for another 10 min before the start of centrifugation.

Centrifugation.

The DNA was sedimented in the SW 50 rotor for 1.5 hrs at 40,000 rpm or 2 hours at 35,000 rpm; in the SW 27 rotor for 3 hours at 27,000 rpm and in the SW 25.1 rotor for 3.5 - 4 hours at 24,000 rpm. The temperature of the centrifuge was set at $5^{o}C$; the temperature of the rotor was constant after 1.5 hours.

Fractionation and counting.

Forty to fifty fractions were collected directly into scintillation vials by the method described by Van der Schans et al., (1969) and Lohman (1969). Water (0.35 ml to 0.65 ml depending on the volume of the fractions) was added to decrease the sucrose and NaCl concentrations to the point where complete emulgation with 12-15 ml toluene-TRITON X-100 scintillation mixture could be obtained and a uniform counting efficiency throughout the gradient was ensured.

When lysis was incomplete a considerable amount of radioactivity was recovered at the bottom of the tube.

2.2.6.4 DNA synthesis.

The incorporation of ^{3}H-thymidine into the cells was used to measure the DNA synthesizing capacity of a bacterial culture after irradiation. Bacteria were labeled overnight and grown to the exponential phase as discribed in 2.2.6.1. After irradiation the cells were diluted into SMS supplemented with 0.1% casamino acids, 0.1% yeast extract, 2% PGY and 1 - 3 μCi/ml ^{3}H-thymidine. After standing for 5 min in the cold, cultures were incubated at 30°C and the growth was checked by measuring the A_{700}. One ml samples were taken at intervals, placed in ice, and 1 ml 10% TCA containing 50 μg thymidine was added. After standing for 30 min 0.1 ml of 0.1% BSA was added. The acid insoluble material was collected by filtration on Whatman GF/C glass fibre filters. Filters were washed with 5 ml 5% TCA + thymidine, 5 ml 96% ethanol and 5 ml acetone. The dried filters were transferred to counting vials and counted immersed in 5 ml toluene scintillator.

2.2.7 OTHER METHODS.

2.2.7.1 Counting of radioactive samples.

Scintillators.

In early experiments a dioxane-napthalene scintillation mixture was used. The determination of radioactivity in this solution is not influenced by ammoniumsulphate and ammoniumcarbonate, which are present in chromatography solvents and are eluted together with radioactivity from the paper after chromatography (Setlow et al., 1963). The following compounds were dissolved in freshly distilled p-dioxane (peroxide-free) and the volume was made to 1 litre (Butler, 1961).

Napthalene (chem. pure)	150 grams
2,5-Diphenyloxazole (PPO)	7 grams
2,2-p-Phenylene-bis -(4-methyl-5-phenyloxazolyl)-benzene (diMe-POPOP)	375 mg

After removal of oxygen by bubbling with nitrogen for 15 min, the mixture was kept in completely filled, dark bottles. Stable counting mixtures were obtained with aqueous samples up to 2 ml and the counting efficiency ranged from 15% in the Nuclear Chicago 720 type liquid scintillation counter to 25% in the Mark I type. When samples containing 5% TCA were counted, a sample of 0.4 ml in 15 ml of scintillator was used.

Paper strips from paperchromatograms were either eluted with 1 ml of the eluent (DE 81-paper) or soaked for 1 hour with 0.5 ml of water and shaken occasionally before the scintillator was added.

Vials were always cooled in the dark to about 5^{o}C before counting was started. In most of the experiments another scintillation mixture was employed which made use of the emulgating properties of TRITON X-100 (Patterson and Green, 1965). This method has however its limitations and one must be certain that stable and homogenous solutions are obtained.

Toluene in which 4 grams PPO and 100 mg diMe-POPOP per litre were dissolved, was mixed 4:1 (v/v) with TRITON X-100. This mixture (10-13 ml) was used for both aqueous and TCA samples of 0.4-0.7 ml. For fractions of larger sucrose gradients (SW 25.1 and SW 27) the volume was increased to 12-16 ml. Aqueous samples of 1 ml or more were counted with 12-18 ml of a toluene-TRITON X-100 mixture 3:1 (v/v). The counting efficiency varied from 20 to 35% depending on the type of counter used.

Results of counting are always given in counts/min, but the constancy of counting efficiency (± 2%) was checked by measuring the external standard or internal channel ratios in each experiment. Both low potassium glass vials and polyethylene counting vials were employed.

2.2.7.2 Other methods.

Purified DNA in aqueous solutions was determined by measuring the absorbance at 260 nm and taking $A_{260} = 1$ for 50 μg DNA per ml.

Colorimetric determinations of DNA were done by the diphenylamine method according to Burton (1956).

Protein was measured spectrofotometrically by using the equation:

$$\text{mg/ml protein} = 1.55 \times A_{280} - 0.76 \times A_{260}$$ (Layne, 1957)

For preparations with a high nucleic acid content, more reliable results were obtained by the method of Lowry et al., (1951).

CHAPTER 3

PROPERTIES OF RADIATION SENSITIVE MUTANTS OF M. LUTEUS

3.1 SENSITIVITY TO UV IRRADIATION.

Twelve radiation sensitive mutants were isolated from the wild type strains ML 1 and ML 8 by NTG treatment. Their sensitivity to ultraviolet light was determined according to the methods given in 2.2.1. The survival curves after UV irradiation of the one-step mutants ML 2 to ML 7 are presented in fig 3-1A and 3-1B. The sensitivity to UV irradiation of the double mutants derived from ML 2 and ML 5 are shown in fig 3-2A and 3-2B respectively.

The UV dose which gives 37% survival was calculated from the survival curves and compared to the UV dose giving 37% survival in E. coli wild type and several uvr mutants (table 3-3). The fact that the various M. luteus mutants have survival curves of different shape and with different slopes, was not taken into account, so the values are only an approximation.

From the values given in table 3-3 it can be seen that the wild type strain ML 1 is more radiation resistant than the E. coli wild type strain. The various M. luteus strains have sensitivities to UV light, ranging from intermediate in the case of strain ML 3 to highly sensitive in the case of strain ML 2-1. The latter has a UV sensitivity which is comparable to that of E. coli uvr mutants (Van de Putte et al., 1965). In section 3-3 a preliminary classification of the radiation sensitive M. luteus strains is given.

Several other M. luteus mutants have been isolated, which belong to a different class. The UV sensitivity and several properties of these strains are discussed elsewhere (section 6.3 and 6.4).

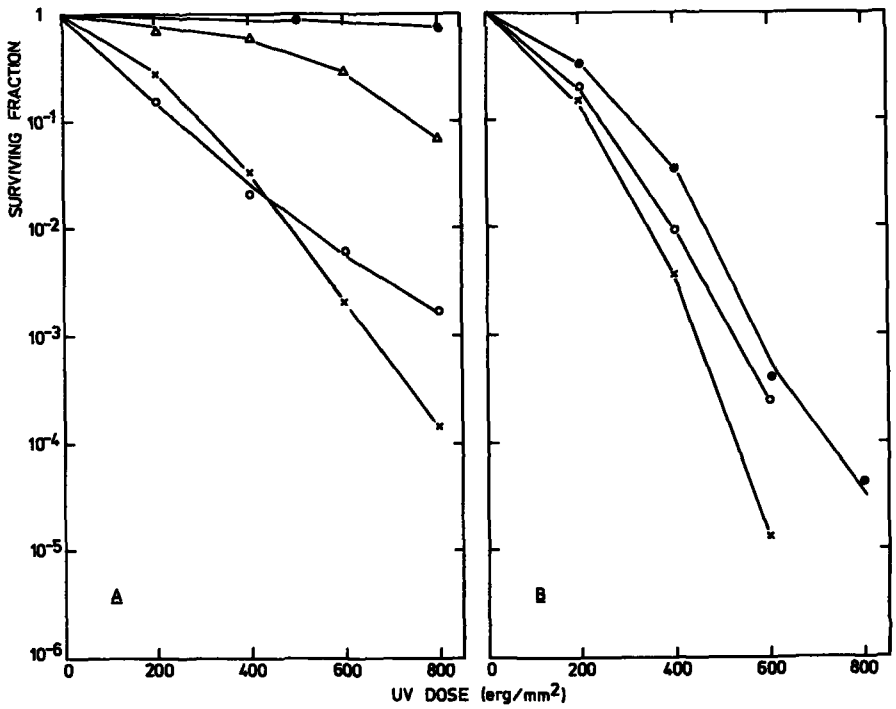

Fig. 3-1 Survival of M. luteus one-step mutants after UV irradiation.

A: ML 1 (•——•); ML 2 (o——o);
ML 3 (Δ——Δ); ML 4 (x——x).
B: ML 5 (•——•); ML 6 (o——o); ML 7 (x——x).

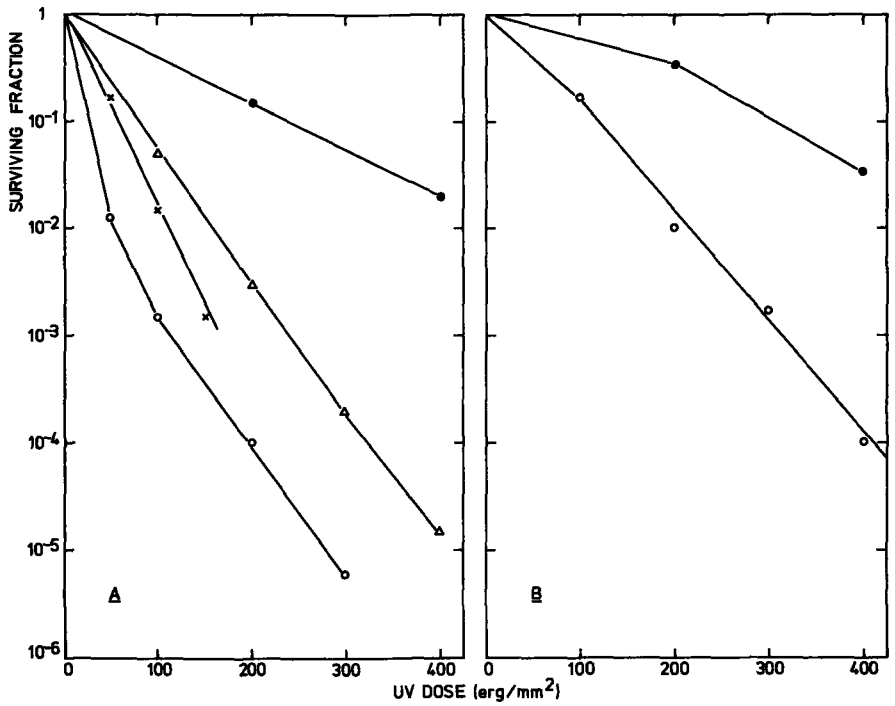

Fig. 3-2 Survival of M. luteus double mutants after UV irradiation.

A: ML 2 (•——•); ML 2-1 (o——o);
ML 2-2 (Δ——Δ); ML 2-4 (x——x).
B: ML 5 (•——•); ML 5-1 (o——o).

TABLE 3-3

UV sensitive mutants of M. luteus.

Strain	Hcr type	D_{37} (erg/mm^2)	Reduction factor of D_{37}
ML 1	+	1525	-
ML 2	+	100	15.2
ML 2-1	-	21	15.2 and 4.8
ML 2-2	-	37	15.2 and 2.7
ML 2-4	-	27	15.2 and 3.7
ML 3	-	530	2.9
ML 4	+	150	10.2
ML 5	-	170	9.0
ML 5-1	-	57	9.0 and 3.0
ML 6	-	127	12.0
ML 7	-	115	13.2
(E. coli)			
KA46 uvr^+	+	950	-
KMBL90 uvrB501	-	17	56
KA173 uvrA6	-	12	78

3.2 SENSITIVITY TO X-IRRADIATION.

Several mutations in E. coli (exr and rec) cause an increase in X-ray sensitivity in addition to the sensitivity to UV light (Van de Putte, Zwenk and Rörsch, 1966). A number of the above mentioned M. luteus mutants were also tested for their survival after X-rays under aerobic conditions. ML 2-1 and ML 3 are slightly more sensitive, but the difference with wild type is not as great as with the Exr^- and the Rec^- mutants (Rörsch et al., 1967) of E. coli. The survival curves after X-irradiation of the strains ML 2-1 and ML 3 have a different shape than the wild type strain. The length of the shoulder is reduced but the exponential part of the curves has the same slope.

Recently it has been observed that radiation sensitive E. coli strains

having a recB or recC mutation, are lacking an ATP dependent exonuclease (Barbour and Clark, 1970). This discovery prompted us to isolate several M. luteus mutants with an increased sensitivity to X-rays (table 3-4). Examination of the extract of these mutants shows that the ATP-exonuclease activity is the same as in the wild type strain.

Because it was also found that the activity of UV-endonuclease (section 4.7.2) was normal in these strains, they have not been characterized further.

TABLE 3-4

Properties of X-ray sensitive M. luteus mutants.

Strain	ML 1 ML 8 (wild type)	ML 12	ML 14	ML 15
X-ray sensitivity	+	±	-	-
UV sensitivity	+	-	±	±
Repair of N5 phage	+	-	±	±
MMS sensitivity	+	+	+	+
UV-endonuclease (chapter 4)	+	+	+	+

Resistent or present (+); sensitive or absent (-); intermediate character (±).

3.3 HOST CELL REACTIVATION OF UV IRRADIATED N5 PHAGE.

Reactivation of extracellularly irradiated phage has been observed in several microorganisms: E. coli, B. subtilis, H. influenzae (for a review see Strauss, 1968).

Micrococcal phages N1, N5 and N6 are temperate phages and are subject to host cell reactivation in M. luteus (Field and Naylor, 1962; Wetmur et al., 1966 and Lee and Davidson, 1970).

Phage N5 was irradiated with UV and the survival was measured on M. luteus mutants. The survival in one-step mutants is given in fig 3-5A and 3-5B; while the inactivation measured in double mutants is shown in fig 3-5C and 3-5D.

According to their UV sensitivity and their ability to repair UV-irradiated phage, the M. luteus mutants can be divided into several phenotypical classes. The mutants ML 2 and ML 4 belong to one class and are very sensitive but still Hcr^+ (compare E. coli uvr-504 and uvrE502 (Van de Putte et al., 1965).

The strain ML 3 has an intermediate sensitivity for UV but has lost the capacity to reactivate phage N5.
In E. coli a similar mutant was isolated:uvrA503 (Van de Putte et al., 1965). The second mutation in the strains ML 2-1, ML 2-2 and ML 2-4 which are derived from ML 2, has the same influence on survival and renders the mutants Hcr^-. The mutant ML 5-1 can probably also be placed in this group if it is assumed that the influence of the mutation on the Hcr^- phenotype is masked by the first mutation in ML 5.

The mutants ML 5, ML 6 and ML 7 belong to a third class with the same general characteristics as the E. coli Uvr^- mutants (Van de Putte et al., 1965; Howard-Flanders et al., 1966).

No genetic support for this classification exists, but since Kloos (1968) developed a method to transform M. luteus, complementation experiments may throw more light on this problem. Okubo and Nakayama (1968) as well as Mahler and Grossman (1968) have shown that transfer of mutations which influence radiation sensitivity is possible.

M. luteus possesses an efficient mechanism to remove UV damage from DNA. It can be calculated that wild type cells are able to survive a UV

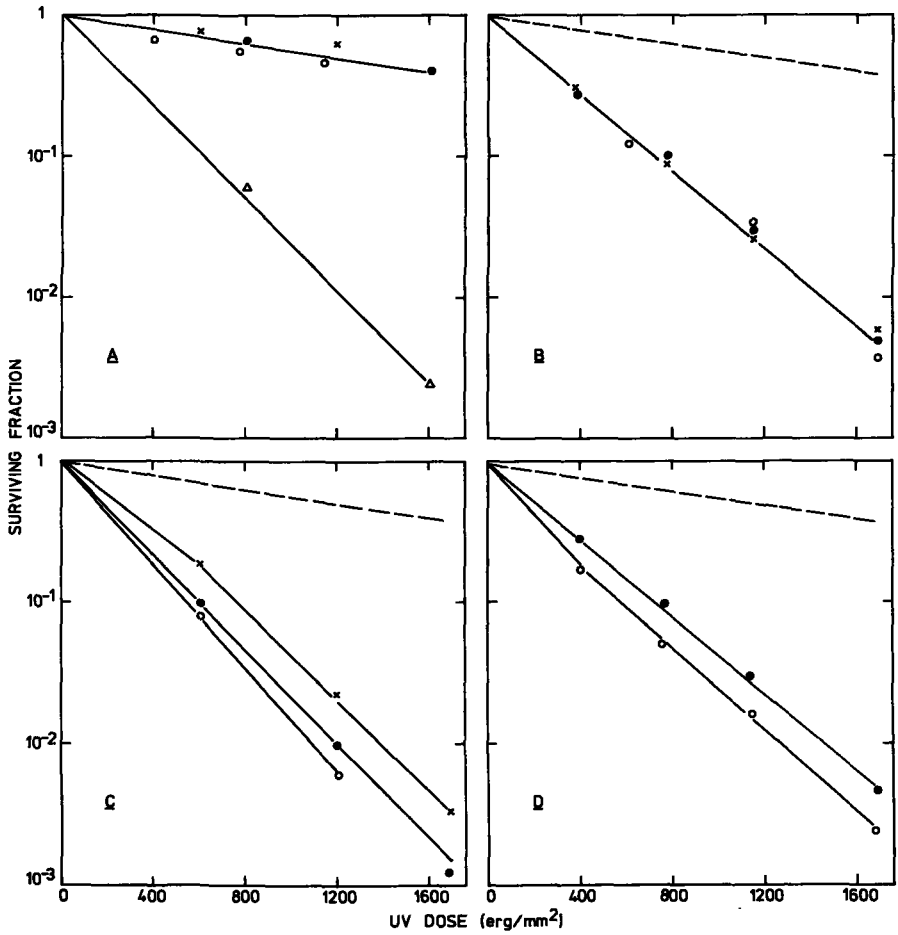

Fig. 3-5 Survival of UV irradiated N5 phage in M. luteus mutants.

A: ML 1 (•——•); ML 2 (o——o); ML 3 (Δ——Δ);
ML 4 (x——x).

B: ML 5 (•——•); ML 6 (o——o); ML 7 (x——x);
The dotted line represents the survival in the wild type strain ML 1.

C: ML 2-1 (•——•); ML 2-2 (o——o); ML 2-4 (x——x);
The dotted line represents the survival in strain ML 2.

D: ML 5 (•——•); ML 5-1 (o——o).
The dotted line represents the survival in strain ML 1.

dose after which pyrimidine dimers in the DNA are separated by only 500 base pairs. The intracellular repair of UV damage in irradiated phage is less efficient than the repair of damage in the host cell. It can be calculated that the number of pyrimidine dimers which can be removed from an infecting bacteriophage genome is about one order of magnitude lower than that for bacterial DNA. The number of dimers, however, that is repairable per unit length of DNA is roughly the same for bacterial and bacteriophage DNA. This is in agreement with the observation for E. coli and bacteriophage λ (Boyle and Setlow, 1970).

Feiner (1967) isolated UV sensitive mutants of M. luteus some of which are of the Hcr^- phenotype. Mahler and Grossman (1968) isolated several types of mutants which could be divided into similar classes as our mutants.

3.4 OTHER PROPERTIES.

During the isolation of the M. luteus mutants, it was observed that all the mutants had acquired additional mutations, which led to slow growth on unsupplemented minimal media. These additional mutations resulted from the heavy mutagenic treatment which was required to induce UV-sensitivity mutations in M. luteus.
In addition to auxotrophic mutations, color mutations were observed frequently (see also Feiner, 1967). The auxotrophic mutations in the radiation sensitive mutants were not characterized further. The minimal media in which the mutants were grown, were supplemented to permit sufficient growth of all strains.

Several agents which are known to interfere with the reactivation process for UV damage in E. coli were investigated. Caffeine and proflavine were tested at concentrations which still permitted growth and phage N5 multiplication. Caffeine (0.2%) causes a slight decrease in survival after irradiation (fig 3-6), although much less than in E. coli (Harm, 1967). Caffeine (0.2%), proflavine (0.5 μg/ml) or iodoacetic acid (1 mM) depress phage N5 production considerably, but no inhibition of repair of irradiated phage is observed. Addition of caffeine increases the survival of phage N5 in the double mutant ML 2-1. As will be shown later in chapter 4 and 6, this strain contains a UV-endonuclease and is probably affected in a late step of the repair process which leads to excessive DNA degradation after irradiation. Caffeine inhibits nuclease activity (Roulland-Dussoix, 1967) and the addition of this substance after irradiation might lead to a lower

DNA degradation resulting in an increased phage repair.

Iodoacetic acid (0.5 mM) also causes an increased sensitivity to UV (fig 3-6). This is in agreement with the experiments of Elder and Beers, (1965), who showed that liquid holding recovery occurs even at low temperature and can be blocked by iodoacetic acid. Their survival data however, are not in agreement with the data presented in fig 3-1A and 3-5A.

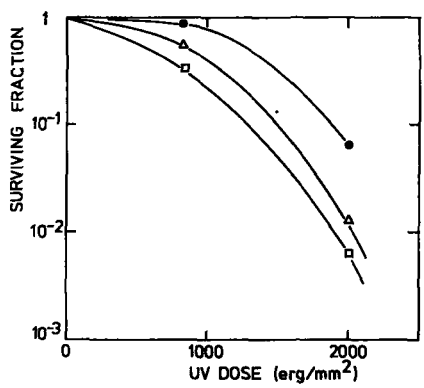

Fig. 3-6 Influence of inhibitors of excision repair on the survival of strain ML 1 after UV irradiation. Control (●——●); caffeine, 0.2% (Δ——Δ); iodoacetic acid, 0.5 mM (□——□).

All M. luteus mutants are more sensitive to mitomycin C compared to wild type. The double mutant ML 2-1 is the most sensitive strain. The influence of 4-nitro-quinoline-1-oxide (4NQO) (Felkner and Kadlubar, 1968), which is regarded as a good radiomimetic agent for UV irradiation, was tested in several M. luteus mutants. The wild type strain still forms colonies with 3 μg/ml 4NQO, while strain ML 2-1 tolerates only 0.003 μg/ml.

The effect of methylmethanesulphonate (MMS) on radiation sensitive M. luteus mutants was not significantly different from the wild type. In this character the mutants differ from those described by Mahler and Grossman (1968), who isolated a group of mutants which are UV and MMS sensitive. The recently isolated mutants ML 14 and ML 15 were also insensitive to MMS. The survival of MMS treated N5 phage is the same on all M. luteus strains isolated so far.

CHAPTER 4

IN VITRO REACTIVATION OF UV IRRADIATED RF DNA

4.1 INTRODUCTION.

ØX 174 infection of E. coli C cells results in the intracellular production of replicative form (RF) DNA. The uptake of RF DNA by E. coli spheroplasts can result in the production of new phage (Guthrie and Sinheimer, 1963). RF DNA molecules have a molecular weight of 3.4×10^6 and have a double stranded circular structure. The physical and biological properties of RF RNA have been described by Jansz and Pouwels (1965) and Pouwels et al., (1966).

In contrast to the single-stranded form of ØX 174 DNA (Rörsch, 1962), the irradiated replicative form (RF) is subject to host cell reactivation as described by Jansz and Pouwels (1963) and Yarus and Sinsheimer (1964).

Thus the biological activity of UV-irradiated RF DNA is higher when assayed on spheroplasts prepared from wild type cells than from Hcr^- cells (uvrA, B or C).

The circular RF DNA molecule is not degraded by exonucleases and can be incubated with a bacterial extract without a serious loss of biological activity. A reasonably high biological activity can be maintained, depending of course on the endonuclease level of the extract. Moreover it has been shown by Pouwels et al., (1966) that a circular RF molecule can withstand the introduction of 20 single strand breaks by an endonuclease without loss of biological activity. Double strand breaks, however, destroy the circularity of the molecule and result in the simultaneous loss of biological activity. An experimental system can be devised in which the influence of bacterial extracts in vitro on UV inactivated RF DNA can be investigated by measuring the changes in biological activity as

measured on spheroplasts of E. coli which are Hcr^-.

Strauss (1962) reported an enzymatic activity in extracts of M. luteus, which specifically inactivated transforming B. subtilis DNA, that had been irradiated with UV. Rörsch et al. (1964a) showed that preincubation of UV irradiated RF DNA with extract from M. luteus, increased the biological activity measured on Hcr^- spheroplasts, but not when measured on spheroplasts of E. coli wild type (Hcr^+). The properties of the reaction indicated the presence of a "dark reactivating enzyme" in contrast to the "photoreactivating enzyme" (Rupert, 1962).

A detailed study on the nature of "reactivation in vitro" was undertaken and results have been partially published elsewhere (Rörsch et al., 1967).

The reactivating system consists of two parts:

An in vitro preincubation with M. luteus extract which initiates the repair reaction and the subsequent in vivo completion of the repair process in spheroplasts which take up the pretreated DNA and finally produce new phage.

The in vitro reaction has been studied under a variety of conditions by adding substances during the incubation and by using extract from several UV sensitive M. luteus mutants.

The in vivo completion of repair in E. coli spheroplasts was investigated by adding inhibitors of repair (caffeine and proflavine) to the spheroplasts. When genetic and biochemical details of the excision repair process in E. coli became available (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964; Van de Putte et al., 1965; Howard-Flanders et al., 1966), the influence of several uvr mutations was also studied by using a number of E. coli mutants in the spheroplast assay. Studies presented in this chapter, have to be seen in the light of knowledge concerning excision repair, which was available in 1964-1966.

It is likely that in simple incubation systems, in vitro repair of DNA is restricted to the excision/degradation steps. The completion of repair in vivo (in spheroplasts) consists of the action of DNA polymerase and DNA ligase, which require several cofactors and nucleosidetriphosphates.

4.2 CONTROL EXPERIMENTS.

Before the reactivation of irradiated RF DNA in vitro by M. luteus extract was investigated in more detail (see section 4.4. to 4.7) a number of experiments had to be done to establish that the reaction concerned was an enzyme-catalyzed repair reaction.

The first control experiment was done to see whether the following assumption was legitimate.

If the in vitro reaction replaces an in vivo reaction which is normally carried out in the spheroplasts of wild type cells by the uvr^+ gene products, then repair in vitro using wild type speroplasts cannot lead to a further increase in biological activity. The in vitro reaction - and in particular with a non purified enzyme preparation -can never compete for a well coordinated repair process in a living cell. Several nucleases which are present in the crude extract probably interfere with repair by aspecific DNA degradation. However, in the case of repair in vivo, nucleases are localized in the bacterial cell and are therefore unable to degrade DNA which is repaired at a specific site in the cell.

In table 4-1 results are represented with a uvr^+ and a uvrA strain. As expected the survival of irradiated DNA measured on wild type spheroplasts is not increased by incubation with extract while reactivation is found in the case

TABLE 4-1

Effect of preincubation of RF DNA measured on spheroplasts from wild type cells.

Incubation mixture	Mutation in spheroplast	Survival of RF	FLR
		(900 erg/mm^2)	
Buffer	uvrB501	4.0×10^{-3}	—
Extract	uvrB501	7.5×10^{-2}	0.52
Buffer	uvr^+	1.4×10^{-1}	—
Extract	uvr^+	1.7×10^{-1}	0.05
		(5000 erg/mm^2)	
Buffer	uvr^+	8.5×10^{-4}	—
Purified enzyme	uvr^+	9.2×10^{-4}	0.01

RF DNA was incubated with crude extract of ML 1 (5 μg protein) or with a DEAE-cellulose fraction (0.6 μg protein). Incubation and assay were done as indicated in 2.2.4.

of uvrA spheroplasts. It was also found that, with a partially purified enzyme fraction, repair was not observed when the DNA was irradiated with a high UV dose. The survival in this experiment was comparable to the value that was measured in uvrA spheroplasts after a low UV dose. If it is assumed that comparable survival levels can be explained in terms of the same amount of irreparable damage to the DNA, the following conclusion is justified. The incubation of irradiated DNA with M. luteus extract, leads to repair of the fraction UV lesions in the DNA, which can be repaired in vivo by uvr^+ spheroplasts.

The second control experiment was done to exclude that the observed repair in vitro was an artifact and had to be ascribed to the relative inefficiency of the uptake of bacteriophage DNA in this system. Normally a RF DNA preparation gives rise to the formation of 2-5 x 10^8 phage particles for one μg of RF DNA. Assuming that the burst size of spheroplasts is 100 and that each absorbed DNA molecule can induce the formation of one plaque forming unit, it can be calculated that only one in a thousand of the RF molecules is taken up. After UV irradiation the solution contains a mixture of damaged and undamaged molecules. Because only a small fraction of DNA is absorbed by the spheroplasts it is possible that competition between damaged and undamaged molecules occurs for absorption by spheroplasts. The large excess of damaged molecules prevents undamaged molecules to be taken up and their biological activity is masked.

The reactivation process can -by this assumption- be regarded as a specific degradation of the damaged molecules (Strauss, 1962) by the M. luteus extract, after which undamaged molecules are taken up to a greater extent. To test this possibility, the effect of heavily irradiated DNA was studied in two experiments (table 4-2). Firstly the biological activity of unirradiated DNA was not decreased by the addition of heavily irradiated DNA, which had no remaining biological activity. Secondly the action of pre-incubation with M. luteus extract was still observable when heavily irradiated DNA was added to the mixture after the incubation. Therefore it seems to be justified that repair in vitro is not attributable to specific degradation of UV damaged DNA-molecules.

Rörsch et al.(1964a) showed that the biological activity of unirradiated DNA was not increased by the incubation with M. luteus extract. Assuming that the M. luteus extract acts specifically on UV irradiated DNA and not on unirradiated DNA, it can be expected that incubation of unirradiated DNA prior to its exposure to UV will not alter its UV-sensitivity. A third control experiment was therefore carried out, in which the order of incubation and irradiation was re-

TABLE 4-2

Effect of heavily irradiated DNA on biological activity and repair of RF DNA.

	Addition of RF-UV	Biological activity	Survival of RF (2000 erg/mm^2)	FLR
No preincubation				
control	-	5.9×10^6	-	
irradiated	-	2.4×10^3	4.1×10^{-4}	-
control	+	2.5×10^6	-	
irradiated	+	7.0×10^2	2.7×10^{-4}	-
Preincubation				
control	-	3.2×10^6	-	
irradiated	-	4.8×10^4	1.5×10^{-2}	0.45
control	+	2.5×10^6	-	
irradiated	+	3.5×10^4	1.4×10^{-2}	0.45

RF DNA was incubated with 5 µg of ML 1 extract as indicated in 2.2.4. An equal amount of heavily irradiated RF DNA (10,000 erg/mm^2: RF-UV) without biological activity was added before DNA was absorbed to uvrA516 spheroplasts.

versed. In table 4-3 two experiments of this type are described which differ in the method used to denature the M. luteus extract. From the results it can be concluded that incubation prior to irradiation has no influence on the survival of the biological activity measured on uvr$^+$ as well as in uvrA spheroplasts. It is therefore unlikely that incubation of unirradiated DNA with M. luteus extract altered its conformation in such a way that this change had consequences for the UV inactivation.

Heating at 65^{o}C or treatment of M. luteus extract with trypsine completely destroyed its repair capacity (table 4-4). Both observations made it highly probable that the active component in the M. luteus extract was a protein. The method to terminate the in vitro incubation by heating before spheroplasts were mixed with the incubation mixture (see section 2.2.4) was based on this heat sensitivity.

TABLE 4-3

Influence of pre-incubation on subsequent UV inactivation.

Extract	Incubation terminated by	Surviving fraction uvr$^+$	Surviving fraction uvrA516
-	heat	3.4×10^{-1}	7.0×10^{-3}
4 μg	heat	3.7×10^{-1}	1.4×10^{-2}
-	trypsin	3.0×10^{-1}	8.1×10^{-3}
4 μg	trypsin	3.1×10^{-1}	6.5×10^{-3}

In a volume of 2 ml RF DNA (2 μg) was incubated in 0.01 M K-phosphate buffer (pH 7) for 30 min at 37°C in the presence of 4 μg partially purified extract. The reaction was stopped either by heating (10 min at 65°C) or by 30 min incubation with 20 μg trypsin. The incubation mixture was subsequently diluted into 8 ml buffer and irradiated with 1100 erg/mm^2. The biological activity was measured on spheroplasts as described in 2.2.4. Incubation before irradiation caused a decrease in biological activity to 25-50%.

In the calculation of the fraction of UV lesions restored, given in section 2.2.4, it was said that the reactivation in vitro can be expressed as a dose reduction. To illustrate this apparent reduction of the UV dose, the experiment given in fig 4-5 was performed. DNA was irradiated with several UV doses, incubated with M. luteus extract and repair was measured on E. coli spheroplasts. It can be seen that at UV doses of 1150, 1500 and 2000 erg/mm^2 about two-fold reduction of the UV dose was obtained.

The fraction of UV lesions restored (FLR) were 0.50, 0.46 and 0.50 respectively. This means that the incubation of UV irradiated DNA with extract led to restoration of 50% of the damage originally present. When the repair activity is compared to repair of UV damage in uvr$^+$ spheroplasts, the repair capacity of the extract is even more than 50% of the repair in the uvr$^+$ spheroplasts, because 20% of the UV damage in RF DNA is not repaired in uvr$^+$ spheroplasts.

The extent of repair is dependent on the amount of extract in the in-

TABLE 4-4

Inactivation of restoring capacity of M. luteus extract.

Incubation mixture	Survival of RF (800 erg/mm^2)	FLR
Buffer	7.5×10^{-3}	-
Extract (5 μg protein)	4.7×10^{-2}	0.40
heated extract	7.0×10^{-3}	-
trypsinized extract	9.1×10^{-3}	0.05

Extract of ML 1 (1 mg/ml protein) was heated for 10 min at 65°C or treated with 10 μg/ml trypsin prior to the incubation. After 10-fold dilution in phosphate buffer, incubation and repair were performed according to section 2.2.4.

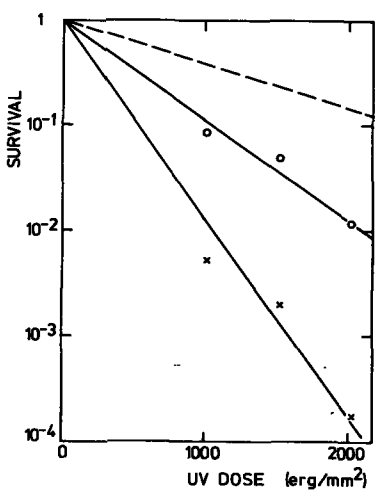

Fig. 4-5 Inactivation of RF DNA.
x——x, biological activity determined on E. coli Hcr$^-$ spheroplasts without preincubation.
o——o, preincubation with M. luteus extract (3 μ g protein) prior to absorption to E. coli Hcr$^-$ spheroplasts.
The dotted line represents the survival measured on E. coli Hcr$^+$ spheroplasts. The preincubation and assay of biological activity were carried out as given in section 2.2.4.

cubation mixture and the duration of the incubation. Repair is also influenced by the presence of other nucleases in the enzyme preparation. With partially purified extract as well as with crude extract the inactivation of unirradiated DNA is small, probably because all nuclease inhibitors are still present. In addition the M. luteus DNA in the extract competes for the nucleases and protects RF DNA against degradation. From fig 4-6 it can be seen that reactivation is linear to approx. 3 μg protein in the incubation mixture. Addition of higher amounts of extract (10-50 μg protein) decreased repair and a generally inhibited the spheroplast assay. The influence of the incubation time is represented in fig 4-7 and 4-8. In most cases longer incubation gives a decrease in repair. The reason for this is probably that in our system early and late repair steps are separated in time. At longer incubation times the probability is increased that partially repaired DNA molecules are destroyed by an aspecific nuclease. In vivo the repair proceeds rapidly and sites which undergo excision repair are only momentarily sensitive to attack by nucleases (Setlow, personal communication, 1966).

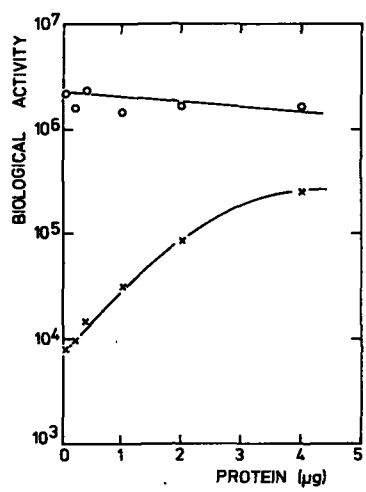

Fig. 4-6 Repair of RF DNA in vitro by M. luteus extract.
The biological activity was measured on E. coli uvrA516 spheroplasts.
o——o, unirradiated DNA; x——x, UV irradiated DNA.

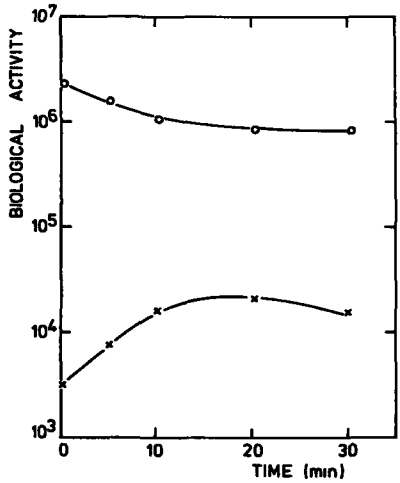

Fig. 4-7 Repair of RF DNA in vitro by M. luteus extract.
Extract of strain ML 2-1 (4 μg protein) was used in the incubation. For explanation of symbols see fig 4-6.

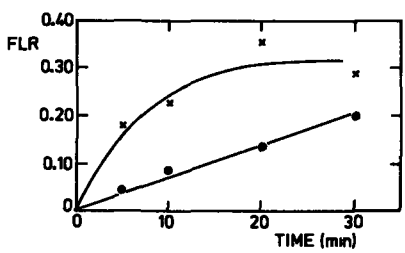

Fig. 4-8 Influence of protein concentration on repair of RF DNA in vitro by M. luteus extract.
x——x, 3 μg protein; o——o, 1 μg protein.
The fraction of restored UV lesions (FLR) was determined according to the method given in section 2.2.4.

4.3 ATTEMPS FOR THE PARTIAL PURIFICATION OF A REACTIVATING ENZYME ACTIVITY

As outlined in chapter 2, nucleic acid can be removed from crude extracts by streptomycin and ammonium sulphate precipation. Further purification on Sephadex served two purposes. Firstly traces of streptomycin were removed

completely and in this way the spheroplast assay, which is very sensitive to this drug, was not affected. Secondly the material could be brought into the buffer required for the next purification step.

Chromatography on Sephadex columns gave no real improvement in specific repair activity compared to the crude extract, but pigments present in high concentrations in crude extract that interfered with the determination of protein content by spectrophotometry, were removed. The stability of the Sephadex eluate was comparable to the crude extract.
Material could be stored at -25°C for at least 3 months without any loss of activity.

When partially purified preparations were used to determine the repair at various protein concentrations (fig 4-9), it was shown that the biological activity of unirradiated DNA was reduced considerably during incubation. Probably the aspecific nucleases were more active on RF DNA due to the removal of M. luteus DNA from the partially purified preparations. If irradiated DNA after preincubation with M. luteus extract contains single stranded regions (as can be expected when excision has taken place in vitro) the DNA is not protected against the action of aspecific nucleases. Every break in a single stranded region destroys the continuity of the DNA molecule. Even if repair replication takes place afterwards in the spheroplast, linear DNA molecules do not

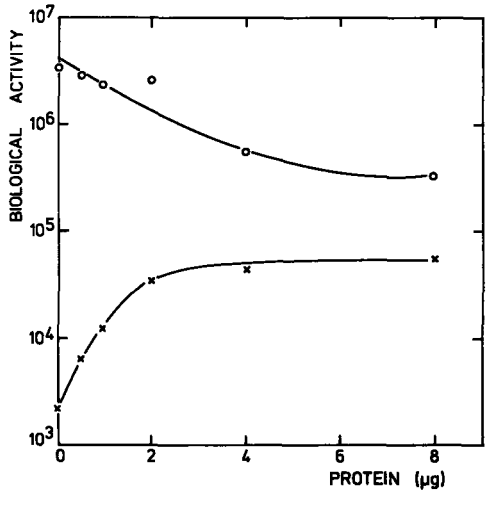

Fig. 4-9 Repair of RF DNA by partially purified M. luteus extract. For explanation of symbols see fig 4-6.

give phage progeny. Because the fraction of UV lesions restored (FLR) is based on the activity of treated irradiated DNA compared with treated unirradiated DNA, correction for aspecific nucleases can only be made if their action on both kinds of DNA is equal (see also 2.2.3.2). If the action is not equal there is no method to detect this and the estimation of repair will be erroneous.

It was attempted to remove aspecific nucleases selectively with streptomycin. The idea was that nucleases could be co-precipitated with the DNA in the extract, while the reactivating activity was not bound to DNA. The streptomycin concentration was varied between 0.5% and 10% but the inactivating action of the extract on the biological activity of unirradiated DNA could not be decreased. The method in which DNA and nucleases were coprecipitated at low streptomycin concentrations, followed by elution of nucleases from the precipitate at higher ionic strength, has been unsuccessful.

Further purification was attempted on DEAE-cellulose in several buffer systems at various pH-values. The reactivating activity was absorbed between pH 7.0 and 8.5 and could be eluted with NaCl. Nevertheless considerable loss of activity was encountered and could not be easily avoided. Changing NaCl for KCl in the elution buffer, led to complete loss of activity. The most suitable method was to remove oxygen from the buffer by nitrogen bubbling and addition of 2-mercaptoethanol (1 mM).

Both stepwise elution and elution with a linear salt gradient resulted in one peak of activity between 0.1 M and 0.2 M NaCl. In phosphate and Tris buffers the activity was eluted at the same ionic strength. In fig 4-10 the results of a 0-0.5 M NaCl linear gradient elution in 0.01 M Tris buffer are presented. The enzyme is eluted just before the main protein peak. Determination of the specific activity was unreliable due to the low optical density of the fractions which had repair activity, but the degree of purification was estimated to be approx. 10-fold (see fig 4-11). Except after dialysis, these preparations could be stored for several weeks at -20°C. This material has been used in a number of experiments which were devised to investigate a difference in the in vitro reaction, catalyzed by crude extract and by an enriched enzyme sample.

Pooled fractions from a DEAE-cellulose column were dialyzed, concentrated by lyophilization and chromatographed on a G-100 Sephadex column. The recovered activity was low. In some cases the activity was eluted in two peaks. The first peak of activity was eluted together with high molecular weight material which was excluded from the Sephadex particles and probably repre-

sents enzyme that was still bound to DNA. The elution volume of the second peak suggested a molecular weight of the free enzyme below 50,000. Further attempts to purify the activity by chromatography on Sephadex were not done, because the results were not reproducible and a considerable loss of activity occurred.

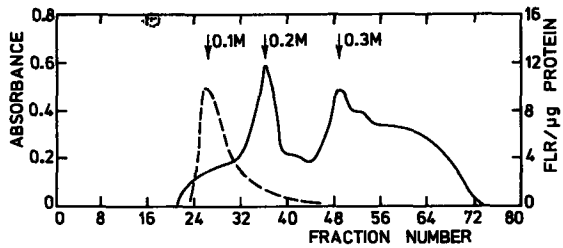

Fig. 4-10 Chromatography of M. luteus extract on DEAE-cellulose.
A preparation from which nucleic acid had been removed, was absorbed on a column, which was washed with 0.05 M Tris buffer (pH 8.0). Subsequently the proteins were eluted with a linear gradient of 0-0.5 M NaCl in Tris buffer.
(———), absorbance at 280 nm; (– – – –) specific repair activity measured by the method given in section 2.2.4.

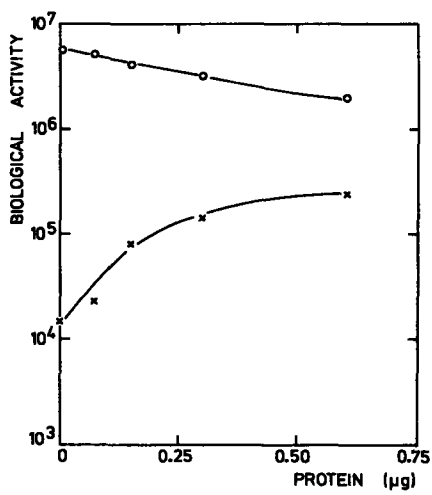

Fig. 4-11 Repair of RF DNA by purified M. luteus extract. A fraction collected after DEAE-cellulose chromatography was used. For explanation of symbols see fig 4-6.

The reactivating activity could be absorbed on phosphocellulose and hydroxyapatite columns at pH 6.5 and subsequently eluted with more concentrated buffer. Although some purification was found, the material was unstable and could not be used further. Absorption to calciumphosphategel and elution with concentrated buffer has been more successful (A. Hout, unpublished). This material has been used in some experiments which are described later.

Naber et al., (1965) described a purification procedure for nucleases on agarose columns with immobilized DNA. When a nucleic acid free M. luteus extract was chromatographed on agarose columns without DNA, nearly all activity could be eluted with a buffer of low ionic strength. When UV irradiated thymus DNA (5000 erg/mm^2) was embedded in the agarose, about 10% of the applied protein was retained by the column and could be eluted with buffer containing 1 M NaCl. The reactivating activity was eluted behind the protein peak (fig 4-12). Based on the very low absorbance at 280 nm of the active fractions, the material must have been purified to a considerable extent. However, the activity was lost rapidly and could not be stabilized by freezing or by addition of 2-mercaptoethanol. Aspecific nucleases which decrease the biological activity of unirradiated DNA were separated from the reactivating activity and were eluted after the main protein peak (fig 4-12; fraction 20-28).

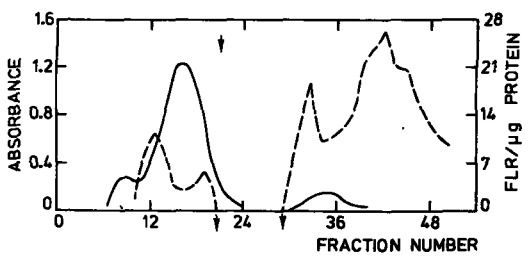

Fig. 4-12 Chromatography of M. luteus extract on agarose containing UV irradiated DNA. A nucleic acid free M. luteus extract was absorbed to an agarose colum which was washed with 0.05 M phosphate buffer (pH 7.5) 0.001 M EDTA 0.001 M 2-mercaptoethanol. After the collection of 20 fractions the protein was eluted with phosphate buffer containing 1 M NaCl (see arrow). The incubation of UV irradiated RF DNA with fraction 20-28 resulted in a further decrease of the survival of the biological activity.

(———), absorbance at 280 nm; specific repair activity (-----).

Nakayama et al., (1967) fractionated M. luteus extract on TEAE-cellulose and obtained a preparation similar to our DEAE-cellulose fraction. The enzyme preparation of Nakayama et al., degrades UV irradiated DNA in combination with other components from the M. luteus extract. This matter will be dealt with in chapter 5.

Several attemps have been made to detect a similar reactivating activity, in extracts of the radiation resistant M. radiodurans. Preparations that were able to repair RF DNA in vitro have not been obtained. M. radiodurans extract contained a high level of aspecific endonuclease which could not be removed by ammoniumsulphate precipitation and DEAE-cellulose chromatography.

Extracts from E. coli have been tested by several methods. Crude extract could not be used in the E. coli spheroplast assay, due to the endonuclease present. In extracts of E. coli endA mutants no repair of RF DNA was found. Fractionation on DNA agarose columns according to Naber et al. (1965) was not conclusive, although some positive results were obtained. Extracts of E. coli endA strains were able to degrade UV irradiated E. coli DNA to a greater extent than unirradiated DNA, indicating that a UV specific nuclease is probably present in the extract (Hout, unpublished and section 5.2).

4.4 CROSS REACTIVATION EXPERIMENTS.

When UV irradiated RF DNA is reactivated in vitro with M. luteus extract it is changed in such a way, that an E. coli Hcr^- spheroplast can proceed with the repair process. Because repair of UV damage consists of a series of reactions which have to occur in a fixed order, it is likely that some early steps of the overall repair process are preformed in vitro by the M. luteus extract. Late steps are subsequently carried out in the spheroplast which absorbs the pretreated DNA. Incision, excision and degradation are probably catalyzed by the M. luteus extract, while the spheroplast is responsible for the last two steps: resynthesis of the excised parts of the DNA (repair replication) and reestablishment of the continuity of the phosphate ester backbone (ligase action).

Two ways of approach were used to obtain more information about the steps carried out in vivo and in vitro. Firstly extracts were used from several M. luteus mutants (4.4.1), secondly E. coli spheroplasts carrying several other uvr mutations were used to study the completion of repair (4.4.2).

4.4.1 Extracts from M. luteus mutants.

Extracts from M. luteus mutants of different UV phenotypes (table 3-1) were prepared and tested for reactivation in vitro by the standard method as described in 2.2.4, using spheroplasts from an E. coli uvrA strain. This strain is unable to repair bacterial DNA or bacteriophage DNA and does not excise pyrimidine dimers (Harm, 1963; Howard-Flanders et al., 1966). The fact that no excision takes place in E. coli uvrA mutants, can be caused by a mutation in the gene responsible for either the incision or the excision reaction. Experiments of Shimada et al., (1968) suggest that the Uvr^- mutants are unable to perform the first step (incision).

From the experiments summarized in table 4-13 it can be concluded that extracts from all the investigated M. luteus mutants are able to repair RF DNA in vitro. Apparently all these mutants are blocked in a step which

TABLE 4-13

Repair by extracts from radiosensitive M. luteus strains.

Strain	FLR	Strain	FLR
ML 1	0.62	ML 2-1	0.55
ML 2	0.55	ML 2-2	0.44
ML 3	0.55	ML 2-3	0.62
ML 4	0.54	ML 2-4	0.55
ML 5	0.52		
ML 6	0.60	ML 5-1	0.53
ML 7	0.55		

Crude extract (5 μg protein) was used. Incubation and assay of repair in uvrA516 spheroplasts were as indicated in 2.2.4.

is different from the one affected by the uvrA mutation. Extracts from X-ray sensitive M. luteus strains (see table 3-4) were also investigated for repair in vitro with E. coli uvrA516 spheroplasts. Extract from ML 14 and ML 15 showed normal activity; the repair capacity of strain ML 13 was reduced.

It cannot be concluded whether the incision step is sufficient for repair in vitro or whether also excision of dimers is required. It is surprising that all the extracts -although derived from various phenotypical groups- repair phage ØX 174 DNA in vitro.

If it is assumed that the incision and excision step are performed by different enzymes, fractionation of the reactivating activity should result in separation of these enzymes. If both enzymes are required for the in vitro reactivation, this should result in loss of reactivating activity. It was shown by the study of repair with partially purified extract from wild type and several M. luteus mutants, that removal of nucleic acid, ammoniumsulphate precipitation and chromatography on Sephadex columns did not decrease the repair activity (table 4-14).

TABLE 4-14

Reactivation by partially purified extract from several M. luteus mutants.

Mutant	FLR
ML 1	0.36
ML 2-1	0.32
ML 2-2	0.34
ML 5-1	0.53
ML 7	0.32

The crude extract was treated with streptomycin, fractionated with ammoniumsulphate and chromatographed on Sephadex G-50. In all incubations 5 μ g protein was used. Repair in vitro was measured on uvrA516 spheroplasts as described in 2.2.4.

4.4.2 Spheroplasts of several E. coli uvr mutants.

The completion of the repair process was studied, using spheroplasts with mutations of the uvrA, uvrB or uvrC type and with a number of multiple uvr mutations.

In table 4-15 and 4-16 the results of experiments with the extracts of several M. luteus strains and spheroplasts of various E. coli mutants are represented. Results are an average from several experiments and the values have an accurancy of ± 20%. Because extracts were made from cells in mid to late exponential phase, variations in the content of other cellular nucleases might contribute to the variation among the extracts. Moreover not all uvrA, B and C mutations in the strains lead to the same survival of RF DNA measured on the corresponding spheroplasts. The dose reduction compared to E. coli uvr^+ spheroplasts varied from 4 to 5, which can be explained by a residual host cell reactivation activity in the various mutants (Van de Putte, 1967).

TABLE 4-15

Reactivation by extracts from UV sensitive M. luteus strains measured on spheroplasts of several UV sensitive E. coli strains.

Mutation in spheroplast	FLR by extract from				
	ML 2	ML 2-1	ML 5	ML 5-1	ML 7
uvrA503	0.32	0.25	0.38	0.44	0.51
uvrA516	0.52	0.42	0.50	0.44	0.56
uvrB5	0.32	0.42	0.21	0.32	0.44
uvrB501	0.42	0.48	0.51	0.55	0.55
uvrB506	0.18	0.23	0.38	0.51	0.49
uvrC505	0.30	0.35	0.43	0.41	0.34

Extract from the above listed M. luteus strains (5 μg protein) were used as indicated in 2.2.4. The E. coli strains carrying the uvr mutations listed above, are given in tables 2-1 and 2-2.

TABLE 4-16

Reactivation by extracts from M. luteus mutants measured in spheroplasts of several E. coli uvr strains.

Mutant	FLR measured on		
	uvrA516	uvrB5	uvrB506
ML 2	0.58	0.36	0.53
ML 2-1	0.53	0.36	0.47
ML 2-2	0.53	0.47	0.60

Crude extract (3 μg protein) was used and repair was measured as indicated in 2.2.4. The E. coli strains are listed in tables 2-1 and 2-2.

Repair by a purified DEAE-cellulose fraction and by a crude extract were measured in spheroplasts of several single E. coli uvr mutants (table 4-17). No significant differences were found between spheroplasts with different uvr mutations. Because E. coli strains having one uvr mutation are probably not completely inhibited in excision repair, a double mutant (uvrA uvrC) was also used in our test system (table 4-18). Purified enzyme preparations -Ca-phosphate fraction- (section 4-3) were used. Reactivation was somewhat higher compared to reactivation measured on spheroplasts of E. coli mutants with one uvr mutation. This can be explained by the observation that double mutants (uvrA uvrC) are completely inhibited in host cell reactivation (Van de Putte, 1967).

The repair of RF DNA was also measured in spheroplasts with three uvr mutations (uvrA, uvrB and uvrC). Treatment of UV irradiated RF DNA with crude extracts of several M. luteus mutants and with a partially purified preparation still resulted in an increase in biological activity (table 4-19). Moreover it was found that caffeine had no influence on the survival of UV irradiated RF DNA measured with spheroplasts of the triple uvr mutant. The conclusion seems to be justified that repair can still be completed in a spheroplast which is mutated in the three uvr genes which code for an early function in the repair of UV damage.

TABLE 4-17

Effect of uvr mutations in E. coli spheroplasts on the completion of repair.

Mutation in spheroplast	FLR	
	crude extract (2 μg protein)	purified enzyme (0.4 μg protein)
uvrA516	0.31	0.26
uvrA503	0.17	0.19
uvrB501	0.26	0.16
uvrC505	0.37	0.38

Crude extract of ML 1 and an enzyme fraction collected after DEAE-cellulose and G-100 Sephadex gel-filtration were used. Incubation and assay of repair were according to 2.2.4. For the strains carrying the above mentioned mutations see table 2-1 and 2-2.

TABLE 4-18

Repair measured on E. coli spheroplasts with two uvr mutations.

Mutation in spheroplast	FLR by extract from			
	ML 1 4 μg	ML 1 (purified) 1 μg	ML 2-1 3 μg	ML 5-1 3 μg
uvrA516	0.47	0.52	0.52	0.53
uvrB5			0.54	0.45
uvrB506		0.37	0.36	0.41
uvrA6 uvr-504	0.40	0.48		
uvrA6 uvrE502	0.43	0.56		
uvrA503 uvrE502	0.58			
uvrA6 uvrC505	0.75		0.65	0.57

For E. coli strains carrying the above mentioned mutations, refer to tables 2-1 and 2-2. Incubation and assay of repair were as indicated in 2.2.4.

TABLE 4-19

Repair of RF DNA measured on E. coli spheroplasts carrying three uvr mutations.

Preparation	Strain	FLR
Crude extract	ML 1	0.46
(3 μg protein)	ML 2-1	0.40
	ML 5-1	0.17
Purified extract	*ML 1*	*0.57*
(0.5 μg protein)		

Spheroplasts were used from the E. coli mutant MBL 212 uvrA6 uvrB5 uvrC505. Incubation and assay of repair were according to 2.2.4.

E. coli strains which have a uvrE502 or uvr-504 mutation, have other properties than the uvrA, B or C mutants (Van de Putte et al., 1965; Mattern et al., 1965 and Mattern, 1971). The mutations in these strains have only a minor influence on repair of UV irradiated phage and probably result in an alteration in one of the late steps in the excision repair process. Because the survival of the biologival activity of UV irradiated RF DNA measured on spheroplasts of these two E. coli mutants is similar as in the wild type strain, the completion of repair could not be measured directly. Therefore E. coli strains were used with a uvrA mutation and either a uvrE502 or a uvr-504 mutation. If these uvr mutations alter the way in which repair is completed, this will be shown by measuring repair in the double mutant. The experiments done with crude extracts from several M. luteus strains as well as with partially purified enzyme preparations, suggest that the uvrE502 and uvr-504 mutations have no influence on late steps of repair in E. coli spheroplasts (table 4-18).

Repair of UV damaged RF DNA could also be measured in spheroplasts of the E. coli B strain KMBL 2003 uvrC500. Experiments with the E. coli B strain B_{s-1} (exr-11 uvrB513) were not successful due to the low production of phage.

Spheroplasts of other Enterobacteriaceae are also able to produce ØX 174 phage after absorption of ØX RF DNA. S. typhimurium wild type and a Hcr^- strain were used to measure the UV inactivation of RF DNA. The

survival on the Hcr⁻ strain is much lower as on the wild type strain and it seems likely that this organism can be used for the measurement of repair in vitro.

The possibility to develop a more homologous test system has been examined. Several experiments were done to see whether DNA of the micrococcal phage N5 could induce the production of phage in spheroplasts of M. luteus prepared by the method given in section 3.2.4.1 and in competent M. luteus bacteria (Mahler and Grossmann, 1968; Okubo and Nakayama, 1968). However, no production of phage was observed in these experiments.

4.5 INFLUENCE OF SEVERAL IONS AND BUFFERS

In order to establish several features of the in vitro repair reaction the effect of several ions of the pH of several buffer systems was investigated.

The presence of Mg^{++} ions in the incubation mixture resulted in a decrease of the restoring activity of a crude extract. This inhibition is probably caused by the activation of aspecific nucleases by Mg^{++} ions, partially destroying repaired RF DNA molecules (table 4-20). The influence of Mg^{++} ions

TABLE 4-20

Effect of Mg^{++} on repair of RF DNA.

Incubation mixture	Addition	Survival of RF (1000 erg/mm^2)	FLR
Buffer	-	1.1×10^{-2}	-
Extract	-	1.1×10^{-1}	0.50
Buffer	1 mM Mg^{++}	1.0×10^{-2}	-
Extract	1 mM Mg^{++}	4.5×10^{-2}	0.30

Incubation and assay of repair were as indicated in 2.2.4. Extract of ML 1 (5 µg of protein) was used.

using purified M. luteus extracts was variable probably because the aspecific nucleases were not removed. A second possibility is that M. luteus DNA which competes with phage DNA for degradation, is removed. Fractions collected after DEAE-cellulose chromatography were inactive in the repair reaction in the presence of 0.05 M Mg^{++} and caused a 50-fold decrease in the biological activity of unirradiated DNA.

The effect of EDTA was also investigated. Concentrations upto 30mM had no influence on the biological activity of RF DNA. Repair in vitro mediated by M. luteus extract was not affected by EDTA (table 4-21). In experiments with purified enzyme preparations it was observed that the decrease of biological activity of unirradiated RF DNA could be avoided by the addition of EDTA, while repair of UV irradiated RF DNA was similar as in experiments without EDTA.

TABLE 4-21

Effect of EDTA on reactivation of RF DNA.

Incubation mixture	Addition	Survival of RF (1200 erg/mm^2)	FLR
Buffer	-	9.4×10^{-3}	-
Extract	-	1.1×10^{-1}	0.60
Buffer	3 mM EDTA	1.1×10^{-2}	-
Extract	3 mM EDTA	1.5×10^{-1}	0.60
Buffer	30 mM EDTA	4.7×10^{-3}	-
Extract	30 mM EDTA	1.3×10^{-1}	0.55

Incubation and assay of repair were as indicated in 2.2.4. Extract of ML 1 (5 µg protein) was used.

The observation that tRNA has a similar effect as EDTA suggests that an endonuclease interferes with repair (Bernardi, 1964). The introduction of single strand breaks in the double stranded form of ØX DNA, does not lead to a rapid loss of its biological activity (Pouwels, 1965). However, the situation is different when breaks are introduced into a region of a DNA molecule, which is undergoing excision repair. In this case endonucleolytic action leads to lineari-

zation of RF DNA and loss of biological activity in the spheroplast system.

The assumption that the effect of Mg^{++} has to be ascribed to aspecific nucleases is sustained by the experiments presented in table 4-22. An enzyme fraction, prepared by selective elution from Ca-phosphate gel (see section 4.3), was employed. Addition of 0.01 M Mg^{++} to the incubation mixture has no influence on the extent of repair in vitro.

TABLE 4-22

Reactivation of RF DNA by purified extract.

Protein concentration	FLR	
	1 mM EDTA	10 mM Mg^{++}
3 µg	0.40	0.48
1 µg	0.32	0.46
0.3 µg	0.33	0.41

Incubation and assay of repair were as indicated in 2.2.3. Phosphate buffer was used to dilute the incubation mixture 10-fold before spheroplasts were added. An enzyme preparation was used, which was purified 25-fold by ammoniumsulphate fractionation and selective elution from Ca-phosphate gel.

The influence of several buffers was investigated because it was observed that the assay of biological activity was higher in Tris and Tricine buffer as compared to phosphate buffer. Good et al., (1966) reported that several enzyme-catalyzed reactions proceeded more rapidly in Tricine buffer. The results in table 4-23 indicate that the highest level of repair in vitro was obtained in Tricine buffer.

The influence of the pH on repair in vitro has been investigated only in some preliminary experiments. It must be realized that measurement of repair at various pH values using partially purified enzyme preparations is unreliable. Activation of the incision step may be counteracted by a higher activity of aspecific nucleases and vice versa, resulting in faulty conclusions. If ex-

TABLE 4-23

Influence of buffer on repair in vitro.

Enzyme fraction	FLR	
	Tris buffer	Tricine buffer
Crude extract (2.5 μg protein)	0.20	0.66
Partially purified fraction (1 μg protein)	0.20	0.56

Tris and Tricine buffers were used at a concentration of 0.01 M (pH 7.5) and 1 mM EDTA was added during incubation. Subsequently the incubation mixture was diluted 10-fold with 0.01 M phosphate buffer (pH 7.0) 1 mM EDTA. Incubation and determination of repair were carried out as indicated in section 2.2.4.

cision is favoured at high pH values, single stranded DNA regions may be formed which render the RF molecules more sensitive to nucleolytic attack. Experiments in phosphate and Tricine buffer show that repair is maximal at high pH (8-9). Recent results obtained with highly purified UV-endonuclease in the repair of transforming DNA show maximal activity at pH 6-7 (Carrier and Setlow, 1970).

4.6 REPAIR INHIBITORS

Substances which decrease the survival of UV irradiated bacteria, are believed to interfere with the repair processes which take place after irradiation. Addition of these inhibitors to plating media decreases survival. Metzger (1964) and Sauerbier (1964) observed that caffeine inhibits repair of UV irradiated bacteriophage T1 measured on E. coli B. Harm (1966, 1967) investigated the action of several acridines (caffeine, proflavine and acriflavine) on HCR and on cell survival in E. coli and found that the inhibition is most likely in the excision repair process, because the survival of uvrA, B and C mutants could not be decreased further by acridines. The mechanism of action is not known, but is most probably the formation of a complex of acridines and DNA, which

interferes with the binding of nucleases and the DNA substrate. Roulland-Dussoix (1967) reported the inhibition of several nucleases by caffeine and found a decreased degradation of UV irradiated λ phage DNA in Hcr^+ strains of E. coli. Shimada and Tagaki (1967) observed that the introduction of single strand breaks in circular DNA of bacteriophage λ after UV irradiation of phage-infected cells was inhibited in the presence of caffeine. These inhibitors most probably act on the early steps of the excision repair process.

Elder and Beers (1965) showed that survival of UV irradiated M. luteus is increased when the irradiated cells were held in buffer. This liquid holding recovery phenomenon can be abolished by the addition of iodoacetic acid.

The effect of substances which inhibit excision repair in E. coli was not found in M. luteus (section 3.4). Only a small inhibitory effect of caffeine was found on cell survival, but a decrease in repair of N5 phage was not observed.

Several experiments were carried out in which the influence on the in vitro incubation as well as on the in vivo termination of repair in spheroplasts was investigated.

4.6.1 Acriflavine, proflavine

Iodoacetate fails to inhibit host cell reactivation of ØX 174 DNA in spheroplasts of wild type E. coli. A concentration of 0.3 mM present during DNA absorption and phage maturation in speroplasts, depresses phage production to 3% of the control value, but the survival of UV irradiated RF DNA is only decreased by a factor of 2-3. Higher concentrations cannot be investigated due to the toxicity of iodoacetate. The same concentration (0.3 mM) inhibits the in vitro reaction. Incubation of irradiated RF DNA with M. luteus extract which leads normally to 40% repair of UV damage measured in uvr A516 spheroplasts gives only 4% repair in the presence of iodoacetic acid. Iodoacetamide is too toxic in the spheroplast assay system to be investigated.

The effect of proflavine was studied in concentrations varying from 1 to 30 μg/ml. The biological activity of unirradiated RF DNA measured on E. coli Hcr^+ spheroplasts with 30 μg/ml proflavine is depressed to 1% of the control value. The surviving fraction of UV irradiated RF DNA decreases from 10^{-2} at 1 μg/ml to 10^{-3} at 10 μg/ml. Still higher concentrations are probably

required to reduce the survival of biological activity on spheroplasts of the Hcr^+ type to the level of the Hcr^- spheroplasts. These experiments could not be carried out as a consequence of the high toxicity of proflavine.

Low concentrations of proflavine (2 μg/ml) were used to study repair of UV irradiated RF by crude extract and by partially purified enzyme (table 4-24). Addition of proflavine during incubation and subsequent dilution to 0.2 μg/ml during absorption and phage production has no influence on the extent of repair catalyzed by crude extract. Even when proflavine was added during the in vitro incubation as well as during the completion of repair in spheroplasts, repair was observed.

TABLE 4-24

Influence of proflavine on reactivation of RF DNA.

Enzyme preparation	Addition of proflavine: Incubation	Addition of proflavine: Absorption Maturation	FLR
Crude extract	-	-	0.48
(10 μg protein)	+	-	0.51
	-	+	0.33
	+	+	0.35
Purified	-	-	0.45
enzyme	+	-	0.47
(3 μg protein)	-	+	0
	+	+	0

Proflavine was added to a final concentration of 2 μg/ml. After incubation in subdued light, the mixture was diluted 10-fold into phosphate buffer + EDTA and mixed with spheroplasts. Incubation and assay of repair were as given in section 2.2.4.

When a partially purified M. luteus extract is used, the effect of proflavine on repair in vitro is different. Addition during incubation and subsequent dilution before DNA is absorbed to spheroplasts has no influence on re-

pair. The presence of proflavine (2 μg/ml) during completion of repair in spheroplasts, however, inhibits repair completely. This inhibition is independent of the addition of proflavine during in vitro incubation.

The different effect of proflavine on repair by crude extract and by a more purified preparation might be explained by the assumption that the purified preparation is unable to catalyze the excision or degradation step in vitro. Further support for this assumption is given in chapter 5, where it is shown that the excision activity of M. luteus extract decreases with purification of the repair activity. UV damaged RF DNA treated with crude extract would have undergone excision while DNA treated with the purified preparation would only have received incision breaks. This signifies that for a successful repair of UV damage this DNA must undergo excision/degradation in the spheroplast. If it is assumed further that proflavine inhibits the excision reaction in the spheroplast, the influence on the repair process can be understood.

In table 4-25 an experiment is described where acriflavin has been added after the incubation with crude extract. The completion of repair in E. coli spheroplasts was not inhibited by acriflavin and has probably to be ex-

TABLE 4-25

Influence of acriflavine on termination of repair.

Incubation mixture	Addition of acriflavine		Survival of RF ($1600\ erg/mm^2$)	FLR
	Absorption	Maturation		
buffer	-	-	1.3×10^{-3}	
extract	-	-	8.2×10^{-2}	0.63
buffer	+	-	3.4×10^{-3}	
extract	+	-	2.2×10^{-1}	0.74
buffer	+	+	1.0×10^{-3}	
extract	+	+	6.1×10^{-2}	0.60

Acriflavine was added to a final concentration of 5 μg/ml. When maturation of the phage took place without acriflavine the mixture was diluted 10-fold with NBS prior to maturation. Extract of ML 2-1 (4 μg protein) was employed. Incubation and assay of repair were as indicated in section 2.2.4.

plained by the same arguments as given for proflavin.

4.6.2 Caffeine

The advantage of using caffeine as a repair inhibitor is that phage production is not as sensitive to this substance as to acridines. Concentrations to 0.4% can be used and result in 5 to 10-fold decrease in phage-yield. Repair of UV irradiated RF is nearly completely inhibited in Hcr^+ spheroplasts. DNA irradiated with 1500 erg/mm^2 having 20% surviving biological activity, gave only 0.1-0.2% survival in the presence of caffeine (0.2-0.4%). Several wild type E. coli strains were tested and could be made phenotypically Uvr^- by the addition of caffeine.

TABLE 4-26

Influence of caffeine on the completion of repair.

Mutation in spheroplast	Addition of caffeine		FLR	
	Absorption	Maturation	Protein concentr.	
			3 µg	1 µg
uvrA516	-	+	0.65	0.42
uvrA516	+	+	0.53	0.41
uvr^+	+	+	0.34	0.37

Caffeine (0.4%) was added during the absorption period and during the phage maturation phase. When caffeine was to be diluted after the absorption period, the mixture of DNA and spheroplasts was diluted 10-fold with NBS prior to phage maturation. Extract of strain ML 2-1 was used. Incubation and assay of repair were as indicated in 2.2.4. For E. coli strains see tables 2-1 and 2-2.

In table 4-26 several experiments on the effect of caffeine on the repair by M. luteus extract are presented. Caffeine has no influence on the in vitro incubation nor on the in vivo completion of repair on wild type spheroplasts (see 4.2) was observed in the presence of caffeine. This suggests that

the in vitro incubation replaces a step of the in vivo repair process which is sensitive to caffeine. Considering the results of Shimada and Tagaki (1967), it has to be concluded that caffeine inhibits the incision step in Hcr^+ spheroplasts and that incubation with M. luteus extract replaces the in vivo reaction.

Surprisingly caffeine has no influence on the in vitro reaction as indicated in table 4-27. The entire reactivation process could even be carried out in the presence of caffeine. The different action of caffeine on repair in vitro and in vivo is not understood, but it has to be considered that repair in vivo occurs in a well coordinated way by a functional unit of several nucleases. This system is probably more sensitive to inhibitors than nucleases in a buffered solution in vitro.

TABLE 4-27

Effect of caffeine on initiation and completion of repair.

Mutation in spheroplast	Addition of caffeine		FLR
	Incubation	Absorption Maturation	
uvrA516	-	-	0.50
	+	-	0.58
	-	+	0.51
	+	+	0.61
uvr^+	-	+	0.48
	+	+	0.44

Caffeine was used at a concentration of 0.2%. When caffeine was to be diluted after incubation, the incubation mixtures were diluted 10-fold with phosphate buffer before mixing with spheroplasts. Extract of ML 2-1 (2 μg protein) was used. Incubation and assay of repair were as indicated in 2.2.4.

Besides the E. coli strains usually employed (KA15, KA16), several other strains were investigated. Wild type strain KMBL 49 can also be made Hcr^- by 0.2% caffeine. In table 4-28 an experiment is given in which

another type of mutant (uvrE502) is used. This strain is radiation sensitive but is still able to reactivate bacteriophage. Addition of caffeine renders this strain Hcr^- and repair can be measured. Caffeine added during incubation increases repair, probably by inhibition of aspecific nucleases in the extract of ML 2-1 (see chapter 3 and Roulland-Dussoix, 1967).

TABLE 4-28

Effect of caffeine on repair measured in various E. coli strains.

Mutation in spheroplast	Addition of caffeine		FLR
	Incubation	Absorption Maturation	
uvrB501	-	-	0,59
uvrE502	-	+	0,41
	+	+	0,79

Caffeine (0.2%) was added during or after incubation. The surviving biological activity of irradiated DNA measured on E. coli uvrE502 decreased from 1.3×10^{-1} to 1.5×10^{-2} in the presence of caffeine. Extract of ML 2-1 (5 µg protein) was used. Incubation and assay of repair were as indicated in 2.2.4.

The effect of caffeine on repair by partially purified M. luteus extract was investigated using uvrA and uvrB spheroplasts. The presence of caffeine during absorption and maturation of phage has no influence on reactivation (table 4-29). Spheroplasts of an E. coli strain carrying three different mutations (uvrA, uvrB uvrC) were able to complete repair in the presence of caffeine. Repair is still found when caffeine is also added during the incubation of RF DNA with purified M. luteus extract. This suggests once more that incision in vitro is not influenced by caffeine. The activity of the incision enzyme (UV-endonuclease) in a M. luteus extract is probably resistent to caffeine. The small effect of caffeine on the survival of UV irradiated M. luteus cells is in agreement with this supposition. In this property M. luteus behaves differently as E. coli where caffeine has a great influence on the survival after UV irradiation (Harm, 1967).

TABLE 4-29

Influence of caffeine on reactivation assayed on spheroplasts of various E. coli mutants.

FLR

Enzyme preparation	Control		Caffeine	
	uvrA516	uvrB506	uvrA516	uvrB506
Crude extract (1 μg protein)	0.37	0.48	0.46	0.50
Purified enzyme (0.3 μg protein)	0.52	0.36	0.43	0.45

Caffeine (0.3%) was added after incubation with enzyme. Incubation and assay of repair were as indicated in 2.2.4.

4.7 CONVERSION OF ØX 174 RF I DURING IN VITRO REPAIR

Examining the results in the preceeding sections, the conclusion can be drawn that the reaction which is responsible for the reappearance of biological activity in UV irradiated RF DNA in vitro is probably the incision/excision step of the excision repair process. The RF DNA used in the repair system has some physical properties which makes it suitable to test this hypothesis. ØX 174 RF DNA isolated from phage infected E. coli cells has a closed double stranded circular form (RF I), which has a sedimentation coefficient of 21 S, measured on linear sucrose gradients. As soon as one single strand interruption is introduced in one of the two DNA strands, the molecule changes its conformation into a less compact one (RF II), resulting in a sedimentation value of 17 S. If the assumption that in vitro repair requires at least the incision step, is valid, molecules which have regained biological activity must have received single strand breaks next to each UV lesion present and will therefore sediment as RF II DNA.

In section 4.7.1 experiments are described in which the action of partially purified M. luteus extract on ØX 174 RF I was investigated by neutral sucrose gradient sedimentation. The biological activity of fractions containing RF I or RF II was also determined. In addition a test for UV specific-endonuclease (incision enzyme) was developed (section 2.2.5.1), which was used to investigate the presence of the enzyme in crude M. luteus extracts.

4.7.1 Sedimentation on sucrose gradients

^{32}P-labeled ØX 174 RF DNA was used to detect the introduction of breaks during incubation with M. luteus extract. After incubation samples were taken for biological activity and the remainder was subjected to centrifugation as described in 2.2.5.1.

Initial difficulties with the preparation of RF DNA were solved by the method of Jansz et al., (1966). However, the isolation of pure (> 95%) RF I DNA was still tedious because the decaying radioactive phosphorus atoms in the RF DNA gradually cause a conversion from RF I into RF II. Therefore in

TABLE 4-30

Reactivation of ^{32}P-RF DNA.

	High dose (2000 erg/mm^2)	Low dose (900 erg/mm^2)
Survival without incubation.	1.2×10^{-3}	6×10^{-2}
Survival after incubation.	3.9×10^{-2}	1.4×10^{-1}
FLR	0.51	0.40
Figure	4-31	4-32

A DEAE-cellulose fraction (3 μg and 1.5 μg protein respectively) was used in the incubation. Biological activity and repair were determined as indicated in 2.2.4.

most experiments a mixture of RF I and RF II was used.

The introduction of nucleases during manipulation was not always prevented and occasionally disturbed the experiments. The specific introduction of single stranded breaks in UV irradiated DNA by the micrococcal extract is shown with a DEAE-cellulose preparation (table 4-30; figs 4-31 and 4-32). The sedimentation profile of the non-incubated unirradiated sample is not represented, because the pattern is equal to the irradiated control. Irradiation as well as incubation with buffer alone causes no change in the sedimentation pattern or the biological activity of RF DNA.

Incubation of irradiated DNA with extract leading to 51% and 40% repair of UV damage is accompanied by a complete conversion of RF I into RF II in the high dose experiment (fig 4-31) and by a partial conversion in the low dose experiment (fig 4-32). The biological activity measured on uvrA516 spheroplasts is only found in the RF II peak in the high dose experiment. It is therefore concluded that completion of repair of UV damaged RF DNA on Hcr^- spheroplasts, is only possible after the introduction of single strand breaks. Although all RF I DNA is converted into RF II DNA, this does not signify that all molecules which are converted into RF II can be repaired in Hcr^- spheroplasts. Only those molecules where incision has taken place at all UV lesions will become biologically active. Others which are only partially "repaired" will be scored as converted into RF II but will not be biologically active on Hcr^- spheroplasts. As can be seen from the sedimentation profile the degradation of DNA is low at the conditions employed. Excision and degradation probably take place in the Uvr^- spheroplast.

In the low dose experiment a considerable fraction of the biological activity remains after irradiation (fig 4-32). After incubation the biological activity in the RF I peak remains constant and a fraction of the RF I material is not converted into RF II. This fraction probably represents undamaged RF molecules which are not incised by the UV specific endonuclease.

Before definite conclusions from the above mentioned results were drawn, several control experiments were performed. The conversion of RF I and the concomitant recovery of biological activity does not automatically imply that the processes are related. It can be imagined that the RF II DNA initially present in the incubation mixture is more easily repaired and that the RF II formed by conversion of RF I is not biologically active. Unirradiated RF I and RF II have the same biological activity (Jansz and Pouwels, 1965). To test

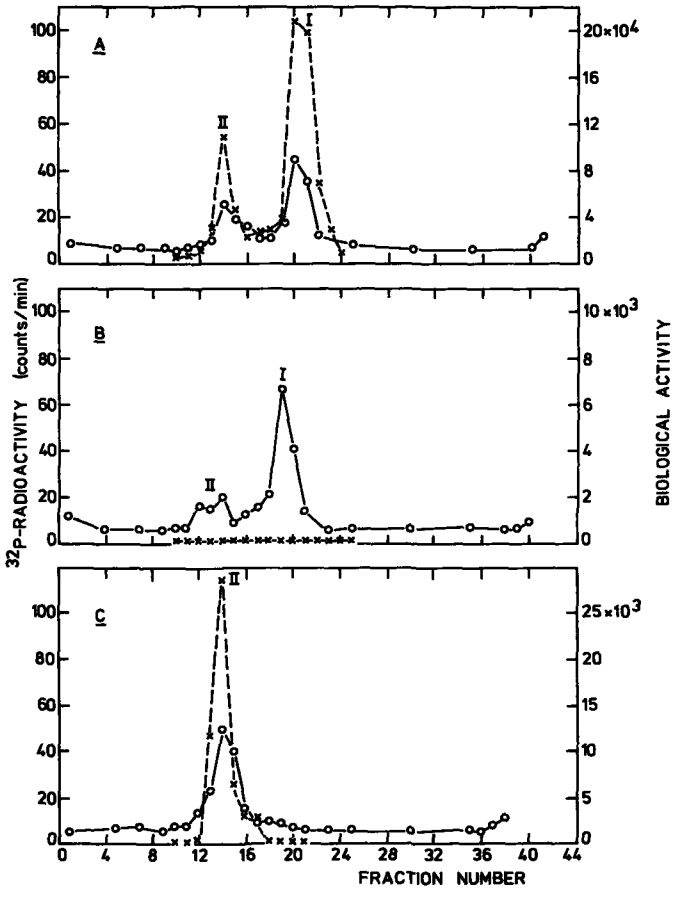

Fig. 4-31 Sedimentation of RF DNA on sucrose gradients.

^{32}P-RF DNA was irradiated with 2000 erg/mm^2 and incubated with an enzyme fraction collected after DEAE-cellulose chromatography.

(o——o), ^{32}P-radioactivity; (x——x), biological activity measured on E. coli uvrA516 spheroplasts according to section 2.2.4.

A: unirradiated DNA incubated with purified M. luteus extract.

B: irradiated DNA, no incubation.

C: irradiated DNA, incubated with purified M. luteus extract.

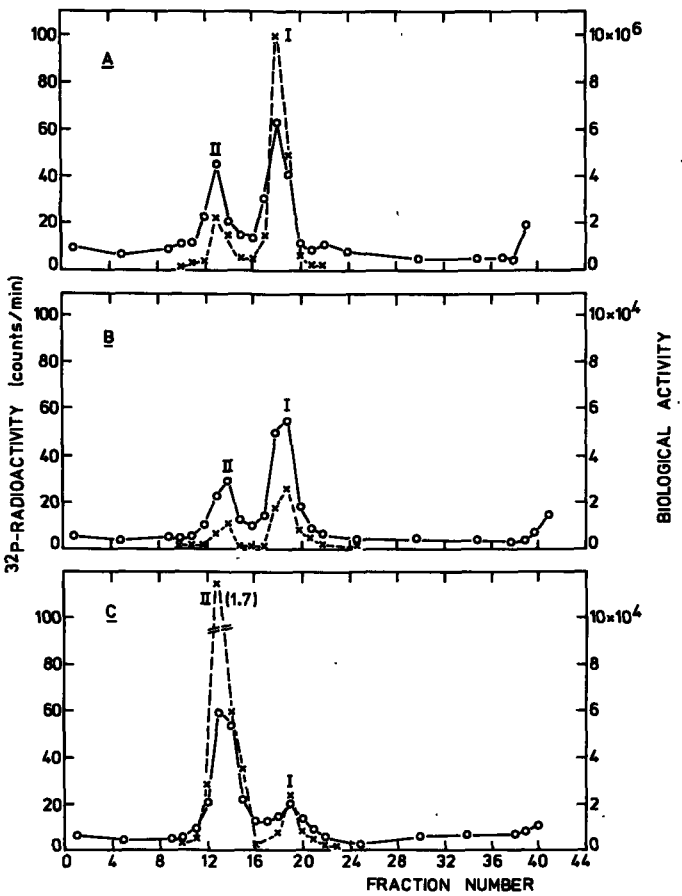

Fig. 4-32 Sedimentation of RF DNA on sucrose gradients. The experimental details were the same as those given in fig 4-31, except that the UV dose was 900 erg/mm^2.

this possibility RF I and RF II DNA were prepared by sucrose gradient sedimentation and the inactivation by UV was determined (table 4-33). Repair by M. luteus extract was equal for RF I and RF II. The presence of several single stranded breaks in RF II had no influence on the repair of UV damage.

TABLE 4-33

Comparison of RF I and RF II.

Survival after irradiation.		
UV dose (erg/mm^2)	RF I	RF II
500	1.3×10^{-1}	1.2×10^{-1}
1000	3.0×10^{-2}	1.9×10^{-2}
1500	5.7×10^{-3}	4.7×10^{-3}
Repair in vitro (FLR)		
Enzyme		
2 µg	0.53	0.54
0.5 µg	0.35	0.29

Extract from ML 1 was partially purified by ammoniumsulphate precipitation and chromatography on Sephadex G-75. Spheroplasts of E. coli uvrA516 were used to measure the surviving biological activity and repair according to section 2.2.4.

Single stranded ØX 174 DNA has a 10 to 20-fold higher specific biological activity than the double stranded RF I and RF II and the sedimentation coefficient is only slightly different from RF II. It can be imagined that "reactivation" takes place by the formation of undamaged single stranded DNA molecules from the small fraction of irradiated RF molecules which have one strand left with no damage. Incision in the damaged strand and subsequent removal of this strand by other nucleases liberates undamaged single stranded circular DNA molecules which have a much higher biological activity in the spheroplast assay as the double stranded DNA. This possibility was tested by several experiments. Firstly the RF DNA was treated with M. luteus extract and subsequently heated for 10 min at 95°C and not at 65°C. At this temperature double stranded RF DNA with single stranded regions is denatured to fragments while single stranded circular DNA remains unimpaired and retains its biological ac-

tivity. It was observed that heating at 95°C destroyed the increase in biological activity of partially repaired RF DNA.

RF II containing fractions from sucrose gradients, in which reappearance of biological activity after incubation with M. luteus extract was observed, were combined and dialyzed. The effect of UV irradiation on the biological activity of this RF II preparation was measured on spheroplasts of uvr$^+$ and uvrA516 bacteria. It was found that the inactivation was higher with uvrA spheroplasts. The collected RF II material is therefore double stranded DNA and not single stranded DNA, which would have given the same survival with both strains (Jansz et al., 1963).

The hypothesis that undamaged single stranded circular DNA molecules may contribute to the increase in biological activity after repair in vitro was tested further after a UV dose which resulted in 25-50% survival on Uvr$^-$ spheroplasts. After this low UV dose, RF DNA contains only 2-3 pyrimidine dimers per molecule and it is expected that a considerable fraction of the DNA molecules has strands without dimers. Incubation with a partially purified M. luteus fraction and subsequent heating at 95°C did not result in an increase in biological activity of the DNA. It is therefore unlikely that single stranded DNA molecules contribute to the observed increase in biological activity.

The conclusion seems to be justified that the in vitro repair of UV irradiated RF DNA by micrococcal extract is the incision step of the excision repair process at pyrimidine dimers in RF DNA. This is supported by experiments of Strauss et al., (1966) who found that the number of breaks in UV irradiated DNA treated with M. luteus extract is equal to the number of dimers. Setlow and Carrier (1970) observed that purified UV-endonuclease from M. luteus only introduced breaks in the DNA strand which contained pyrimidine dimers.

The influence of Mg^{++} and EDTA on the conversion of RF I DNA was also studied with sucrose gradient sedimentation. A mixture of 90% RF I DNA and 10% RF II DNA was incubated with a partially purified enzyme fraction under various conditions (table 4-34). Incubation of DNA in buffer with 0.01 M Mg^{++} did not result in conversion of RF I DNA. This suggests that the employed DNA preparation contained a negligible amount of endonuclease. Incubation of unirradiated DNA with enzyme showed that the enzyme preparation had an endonucleolytic activity, which resulted in the complete conversion of

RF I DNA. The biological activity did not change in agreement with the observation that only RF II DNA was formed. However the incubation of irradiated DNA under similar conditions, resulted in a degradation of the DNA and no increase of the biological activity was observed. It was concluded from the seimentation on a sucrose gradient that the DNA was degraded into small fragments. This observation supports the assumption made in section 4.5 that the activation of aspecific nucleases, causes the destruction of the DNA molecules and is initiated in the regions which are under repair.

In the presence of EDTA the aspecific endonucleolytic activity of the employed enzyme preparation was inhibited considerably. Irradiated DNA could be repaired under these conditions. RF I DNA was converted into RF II DNA and no degradation was observed.

TABLE 4-34

Conversion of RF I DNA with purified enzyme.

Enzyme	Addition	Biol. activity on uvrA516 spheroplasts	% RF I	% RF II
Control DNA				
-	10 mM Mg^{++}	4.7×10^6	90	10
+	10 mM Mg^{++}	4.3×10^6	-	(100)
+	1 mM EDTA	2.0×10^6	70	30
Irradiated DNA (1500 erg/mm^2)				
-	10 mM Mg^{++}	$< 10^4$	90	10
+	10 mM Mg^{++}	$< 10^4$	-	fragments
+	1 mM EDTA	1.3×10^5	-	100

Purified enzyme (Ca-phosphate fraction, 25-fold purified) was used. Incubation was in 0.05 M phosphate buffer (pH 7.5). Assay for repair and sedimentation on sucrose gradients were as indicated in 2.2.4 and 2.2.5.

The addition of tRNA (1 μM) to the incubation mixture had an effect similar to the presence of EDTA. The conclusion which can be drawn from the effect of EDTA and tRNA is that partially purified M. luteus extracts still contain nucleases which interfere with the in vitro repair reaction. Firstly an endonuclease is present which is activated by Mg^{++} and can be inhibited by tRNA, this obscures the action of the UV specific endonuclease. Secondly another nucleolytic activity is present, acting on irradiated DNA which has been incised by the UV-endonuclease and probably has single-stranded regions. These single-stranded regions are the vulnerable places in the DNA. Exonucleases probably enlarge the single-stranded regions to the point that these regions overlap each other in the same DNA molecule and the DNA looses its biological activity. Endonucleases which are active on the opposite undamaged strand have also a deteriorating effect on repair in vitro.

The observation that repair in vitro can be measured in the presence of EDTA and tRNA, is further sustained by experiments which are described in the following section.

4.7.2 Determination of UV endonuclease activity

Several methods have recently been developed to measure the activity of UV specific endonuclease in bacterial extracts. Kaplan et al., (1969) determined the release of inorganic phosphate from UV irradiated DNA by incubation with alkaline phosphatase after pretreatment of the DNA with M. luteus extract. The observed solubilization of inorganic phosphate is a measure for the number of incisions made in the DNA by UV endonuclease.
Carrier and Setlow (1970) denatured the UV irradiated DNA after incubation with enzyme and fractionated the DNA fragments on Sepharose columns. By this method large fragments (derived from intact DNA) could be separated from small fragments (derived from incised DNA) and the amount of the latter was taken as a measure of UV endonucleolytic activity.

Both methods have the disadvantage that they use DNA which is not biologically active. The method which has been employed in the following experiments is based on the rapid denaturation of covalently closed circular RF DNA (see also section 4.7, first paragraph). The technique proved to be specific for UV irradiated DNA. Incubation of unirradiated RF DNA with crude M. luteus extract gave no conversion of RF I into a denaturable form. DNA irradiated with 300 erg/mm^2 was rapidly converted (fig 4-35). Experiments

under various conditions revealed that a fraction of the RF I DNA molecules in the incubation mixture was resistent to UV-endonuclease. This is explained by the average number of pyrimidine dimers which is induced after 300 erg/mm^2 UV in this DNA. It can be calculated that at this UV dose approximately 10% of the DNA molecules had no dimers.

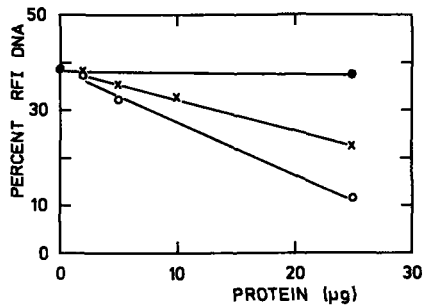

Fig. 4-35 Conversion of RF I DNA by M. luteus extract.
A mixture of RF I and RF II DNA (1 µg) was incubated at 37°C as indicated in section 2.2.5.1.
Unirradiated DNA, 30 min incubation (•——•); UV irradiated DNA (300 erg/mm^2), 10 min incubation (x——x); UV irradiated DNA, 30 min incubation (o——o).

An experiment with nucleic acid free M. luteus extract is represented in table 4-36. When the amount of converted RF I DNA in this experiment is compared to the repair activity of M. luteus extract (section 4.2), it is observed that the maximal conversion of RF I DNA is reached at lower protein concentrations. A possible explanation is that the introduction of the first single strand break in irradiated RF I DNA results already in the conversion into RF II DNA. As has been pointed out earlier, repair in vitro is only observed when UV endonuclease causes incision at all damaged sites in the irradiated RF DNA.

From heat stability measurements of UV-endonuclease it was concluded that the enzyme is inactivated in 10 min at 70°C, but remains unimpaired by heating at 60°C. This value is in accordance with the observation of Setlow and Carrier (1970) who found that the activity was reduced to 50% by incubation during 10 min at 63°C.

The denaturation-filtration technique to measure UV-endonuclease is more sensitive and less time consuming than the in vitro repair spheroplast system. Because repair in vitro can be measured under the same conditions as

those which are used for the determination of UV-endonuclease, the two methods are comparable. If a considerable DNA degradation occurs during the incubation of ^{32}P-RF DNA with M. luteus extracts, the breakdown products could be determined by measuring the acid soluble radioactivity in the filtrate. Denatured DNA of high molecular weight is retained by nitrocellulose filters, while the DNA fragments are not absorbed and are found in the filtrate.

TABLE 4-36

Conversion of ØX RF I by partially purified M. luteus extract.

Extract concentration	DNA	Irradiated DNA (700 erg/mm^2)
-	1.6%	2.2%
0.6 µg	3.2%	64.2%
2 µg	6.0%	67.1%
6 µg	4.9%	73.9%

Micrococcal extract (ML 1) was chromatographed on Sephadex G-25 and subsequently fractionated by ammoniumsulphate precipitation. Incubation and determination of RF I in the reaction mixture was as indicated in 2.2.5.1.

CHAPTER 5

DEGRADATION OF IRRADIATED DNA IN VITRO

5.1 INTRODUCTION

In vivo restoration of UV radiation damage (excision repair) is always accompanied by partial degradation of cellular DNA. Mutations in bacterial genes which are responsible for the excision repair process may lead to alterations in the level of bacterial DNA degradation after irradiation. The degradation of E. coli DNA by extracts from M. luteus strains was used to investigate DNA degradation.

Increased degradation of UV irradiated DNA compared to unirradiated DNA by a bacterial extract implies that (i) the extract must contain an enzymatic activity which recognizes UV lesions and initiates the degradation and (ii) enzymes must be present which excise pyrimidine dimers and degrade the DNA.

An UV-specific endonuclease, which recognizes the damage and is responsible for the incision step in vitro has been discussed in section 4.7. It remains however, unclear whether specific excision/degradation nucleases are present in extracts of M. luteus or whether aspecific enzymes are able to degrade DNA which has incision breaks. An exonuclease, which attacks irradiated native DNA only after treatment with UV-endonuclease has been purified (Kaplan et al., 1969). It has also been observed that a phosphodiesterase is active on UV irradiated DNA, which has been pretreated with UV-endonuclease purified from T4-infected E. coli cells (Yashuda and Sekiguchi, 1970a).

5.2 SPECIFIC REMOVAL OF DIMERS

5.2.1 Experiments with crude extracts

In table 5-1 results of an experiment with extract of the wild type strain ML 8 are given. E. coli DNA irradiated with relatively high doses of UV (20,000-80,000 erg/mm^2) is degraded to a greater extent than unirradiated DNA. At low protein concentrations the difference in degradation of unirradiated and irradiated DNA is more pronounced. At higher protein concentrations, the difference decreases, probably due to substrate dilution by M. luteus DNA, present in the extract (see also section 4.2).

TABLE 5-1

Degradation of E. coli DNA by ML 8 extract.

Extract concentration (μg protein)	Percent degraded DNA	$\hat{TT}$/T ratio in acid soluble material
DNA		
1000	15.5	< 0.010
2000	17.7	< 0.020
DNA (80,000 erg/mm^2)		
250	6.7	0.218
500	19.8	0.162
1000	22.4	0.165 corr. 0.490
2000	21.8	0.160 corr. 0.620

DNA was incubated for 2 hr at 37°C in 0.05 M phosphate buffer (pH 7.5) 0.01 M $MgCl_2$. Experimental details are given in section 2.2.5.

Analysis of the acid soluble degradation products by hydrolysis and paperchromatography, reveals that oligonucleotides have a significantly higher content of pyrimidine dimers compared with the unincubated DNA.

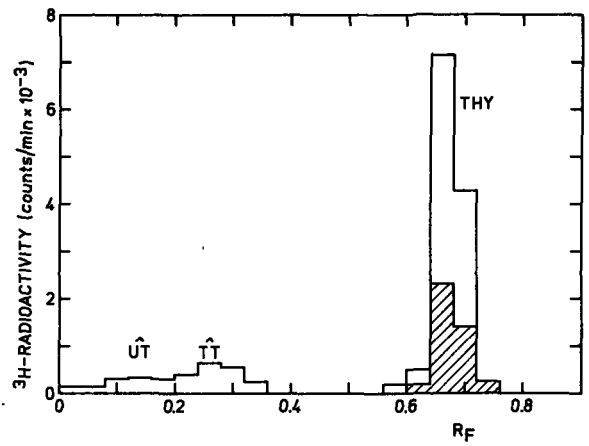

Fig. 5-2 Fractionation of hydrolyzed DNA degradation products.

E. coli DNA was incubated for 2 hrs at 37°C with partially purified extract from strain ML 8 (1 mg protein) in 0.05 M phosphate buffer (pH 7.5) 0.01 M $MgCl_2$ according to section 2.2.5. The acid soluble fraction was concentrated, hydrolyzed by heating with formic acid and subjected to paperchromatography.

Hatched area: unirradiated DNA; open area: irradiated DNA (20,000 erg/mm^2)

In fig 5-2 the radioactivity profile of a paperchromatogram of hydrolyzed oligonucleotides is represented. From R_F values published in the literature it was concluded that the radioactivity at $R_F = 0.20$ and $R_F = 0.30$ represent $\widehat{CT}$ and $\widehat{TT}$ dimers respectively. The amount of dimers in the acid insoluble DNA could not be measured in this experiment because hydrolysis and chromatography were disturbed by protein. In later experiments protein was removed from the incubation mixture by phenol extraction. When the observed degradation was corrected for degradation of unirradiated DNA, it was found that 50% of the increase could be ascribed to thymine containing dimers. The action of M. luteus extracts on irradiated E. coli DNA is thus a preferential removal of pyrimidine dimers. From the amount of degradation and the fraction of dimers removed, it can be calculated that about 5-10 nucleotides are degraded for each excised pyrimidine dimer. This value is somewhat lower than the value that has been determined in vivo in UV irradiated E. coli bacteria. At a UV dose, which results in 75% cell survival in E. coli, 30-50 nucleotides are removed for each excision event (Setlow and Carrier, 1964).

The in vivo situation could be different because enzymes which play a role in repair and degradation of DNA in vivo, might be inactive in the in vitro incubation (ATP exonuclease; Barbour and Clark, 1970; DNA polymerase ; Kelly et al., 1969).

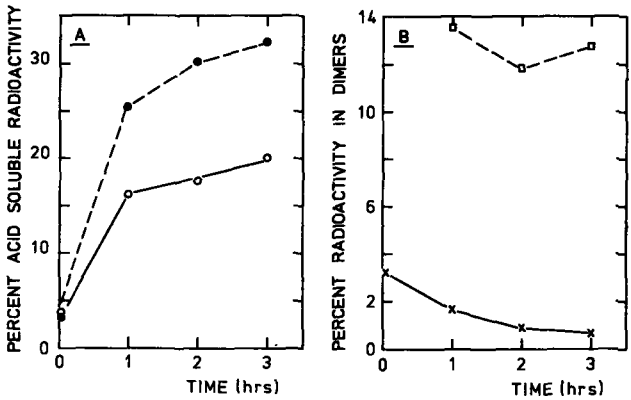

Fig. 5-3 A and B

Excision on vitro by extract of ML 8

E. coli DNA was incubated with M. luteus extract (1 mg protein) in 0.05 M phosphate buffer (pH 7.0) 0.01 M $MgCl_2$ according to the method in section 2.2.5.

A. o——o, unirradiated DNA; •----•, irradiated DNA (40,000 erg/mm^2).

B. x——x, TCA insoluble fraction (DNA); □----□, TCA soluble fraction (degradation products.).

Degradation as a function of incubation time is represented in fig 5-3A. The rate of breakdown decrases at longer incubation times. In fig 5-3B the excision of pyrimidine dimers from UV irradiated DNA is illustrated. Rapid removal of UV damage from the DNA is probably the reason that degradation levels off as excision of dimers from DNA leads to a decrease in the number of sites where degradation can start. The influence of Mg^{++} was investigated in an experiment given in table 5-4. Omission of Mg^{++} from the incubation mixture decreases degradation by a factor of 4-5 for both unirradiated and UV-irradiated DNA. If it is assumed that the degradation of irradiated DNA is mainly carried out by the same aspecific nucleases which degrade unirradiated DNA, it can be expected that the effect of Mg^{++} will be of the same order of magnitude with both DNA substrates.

TABLE 5-4

Influence of Mg^{++} on DNA degradation.

	Percent degraded DNA	
	1 mM EDTA	10 mM Mg^{++}
Unirradiated DNA	0.5	2.3
Irradiated DNA (40,000 erg/mm^2)	2.5	11.6
	Fraction of total radioactivity in dimers	
Acid soluble fraction	0.220	0.206
Corrected for degradation of unirradiated DNA	0,248	0.282

DNA was incubated for 2 hrs at 37°C with M. luteus extract (200 µg protein) in 0.05 M phosphate buffer (pH 7.5). The method mentioned in section 2.2.5. was employed, except that TCA was replaced by perchloric acid. The UV irradiated DNA had 4% of the total activity in dimers.

The excision process itself i.e.the incision event followed by the removal of a small oligonucleotide, has to be independent of Mg^{++} ions. Therefore an experiment was carried out in which the fraction of radioactivity present in pyrimidine dimers was determined (table 5-5).

The values were corrected for the solublilization of radioactivity from unirradiated DNA, giving the net increase in breakdown caused by UV irradiation. The values given, show that the supposition is valid. The dimer content of the acid soluble fraction is not altered by the addition of Mg^{++}. The incision reaction does not require Mg^{++} as has been shown by the experiments given in section 4.7.2. The excision in vitro by M. luteus extracts is somewhat decreased in the presence of EDTA (Carrier and Setlow, 1966). In their experiments the number of oligonucleotides removed at each dimer, was not determined.

TABLE 5-5

Effect of purification on DNA degradation by M. luteus extract.

	Percent degraded DNA	
	Crude extract (1 mg protein)	Purified extract (20 μg protein)
Unirradiated DNA	2.5	0.4
Irradiated DNA ($80,000$ erg/mm^2)	4.4	0.3

DNA was incubated for 2 hrs at 37°C in 0.02 M phosphate buffer (pH 7.5) 0.002 M EDTA. The UV irradiated DNA had 7-8% of the total radioactivity in dimers; the acid soluble fraction of the incubation with crude extract 25-30%.

The size of oligonucleotides produced by the in vitro degradation of irradiated DNA was studied by chromatography on Sephadex and on DEAE-cellulose. The elution of oligonucleotides from Sephadex G-15 and G-25 columns reveals that in the presence of Mg^{++}, degradation products have a length of 2 to 5 nucleotides. Elution patterns from Sephadex columns, which were loaded with the entire incubated mixture show that the amount of low molecular weight material is lower in the presence of EDTA. Probably less mononucleotides are formed due to inhibition of aspecific exonucleases.

Fractionation of oligonucleotides on DAEA-cellulose columns was also used to determine the maximal size of oligonucleotides which were soluble in TCA. In a separate experiment, TCA soluble material prepared by partial degradation of E. coli DNA by pancreatic DNAse was fractionated according to Haberman, (1962). It was found that molecules longer than 10-12 nucleotides were insoluble in 5% TCA.

5.2.2 Experiments with purified extracts

From experiments, described in chapter 4, it has been concluded that reactivation of biologically active DNA in vitro in our test system, only requires the incision step and that subsequent steps are performed in sphero-

plasts of the Uvr⁻ mutants. However, in the in vitro degradation of E. coli DNA by M. luteus extract, several enzymes are required for the excision step and for subsequent degradation. It can be expected that purification based on repair activity (incision enzyme) will change the degrading activity of the preparation.

Treatment of M. luteus extract with streptomycin and chromatography on DEAE cellulose does not alter the excision/degradation activity. The increased degradation of UV irradiated DNA and the removal of pyrimidine dimers from DNA are comparable to those observed in experiments with crude extracts. Although aspecific nucleases will be partially removed during chromatography on DEAE-cellulose, the remaining nucleolytic activity is not inhibited by M. luteus DNA which is also removed in this purification step. This probably explains why the aspecific nuclease activity in this preparation is comparable with crude M. luteus extract (see also section 4.3).

When Mg^{++} is omitted from the reaction mixture the degrading activity of partially purified preparations is lower than that of crude extract. In particular, when purification is done with Ca-phosphate gel, the degrading activity is low (table 5-5), while the same preparation shows a 50-fold increase in specific activity for repair of biologically active DNA. No dimers are excised by this enzyme preparation; the dimer content of the DNA degradation products is equal to that of the undegraded irradiated DNA.

The observed results can be explained as follows:
Extracts of M. luteus contain several aspecific exonucleases. Some of these enzymes are not active in vivo, but are probably active in the in vitro system. Their action on UV irradiated DNA which has undergone incision, leads to excision and subsequent degradation. Depending on the purification methods which are employed and on the degree of purification which is attained, the aspecific nucleases are removed to various levels. This will not only be observable as a decrease in the excision/degradation activity of the preparation but also as a change in response to several cations.

In table 5-6 the excision by a crude extract and by a 10-fold purified Ca-phosphate fraction is compared. The effect on the biological activity of unirradiated DNA was less than with crude extract. The low degradation and excision capacity of the Ca-phosphate preparation indicates that exonucleases which are responsible for the excision/degradation step are present at a low level. Although only a small number of dimers is removed, the number

TABLE 5-6

Influence of purification on excision in vitro

Enzyme fraction	Percent degraded DNA	Fraction of total radioactivity in dimers	
		DNA	Acid soluble fraction
Ca-phosphate eluate (1 mg protein)	7.8	0.043	0.360
Crude extract (1 mg protein)	15.3	0.011	0.390
control	-	0.069	-

DNA (60,000 erg/mm^2) was incubated for 2 hrs at 37°C in 0.05 M phosphate buffer (pH 7.5) 0.01 M $MgCl_2$ according to section 2.2.5.

TABLE 5-7

Balance sheet of radioactivity in dimers.

Incubation	Percent of total activity in DNA	Fraction of total activity in dimers	Activity in dimers (dpm)
Control	100	0.069	102,155
Crude extract			
acid insoluble	84.7	0.011	13,927
acid soluble	15.3	0.390	89,505
			103,432
Ca-phosphate-eluate			
acid insoluble	92.2	0.043	59,271
acid soluble	7.8	0.360	39,120
			98,391

Total radioactivity in irradiated DNA 1.5 x 10^6 dpm. For experimental details see table 5-6.

of nucleotides solubilized at each excision event is comparable to that with crude extract.

A calculation was made to see whether the total number of pyrimidine dimers is unchanged during in vitro incubation. The results in table 5-7 show that pyrimidine dimers are not lost during in vitro incubation (see also Carrier and Setlow, 1966).

5.2.3 Experiments with crude extracts from M. luteus mutants

The excision/degradation capacity of extracts from a number of UV sensitive mutants was investigated (table 5-8). Extracts from the M. luteus mutants ML 2-1 and ML 5 degrade UV irradiated E. coli DNA more rapidly than unirradiated DNA. The dimer content of the acid soluble oligonucleotides, corrected for the degradation of unirradiated DNA, was lower with the mutant extracts. For the same number of excised dimers a higher amount of DNA was degraded.

TABLE 5-8

Degradation by extract from M. luteus mutants

Extract	Percent DNA degraded	Fraction of total activity in dimers in acid soluble fraction	
Unirradiated DNA			
ML 8	3.0	< 0.050	
ML 2-1	2.1	< 0.020	
ML 5	2.4	< 0.010	
Irradiated DNA ($80,000$ erg/mm^2)			Corrected
ML 8	8.7	0.225	0.335
ML 2-1	6.4	0.185	0.271
ML 5	8.0	0.091	0.157

DNA was incubated with extract (500 μg protein) for 1.5 hr at 37°C in 0.02 M phosphate buffer (pH 7.5) 0.002 M EDTA.

The extract from another mutant (ML 4) has an excision/degradation capacity comparable to that in wild type extract. In a particular experiment 60% of the dimers were removed. About 10% of the total radioactivity in the degradation products was found in dimers in the degradation products while the UV-irradiated DNA only had 2.7% of the total activity in dimers.

TABLE 5-9

DNA degradation by several extracts in the presence of Mg^{++}.

Strain	Percent degraded DNA	
	Unirradiated DNA	Irradiated DNA ($50,000$ erg/mm^2)
ML 1	0.4	2.5
ML 2-1	0.4	3.2
ML 3	0.3	5.2

DNA was incubated for 1.5 hrs at 37°C with extract (500 µg protein) in 0.1 M Tris buffer (pH 8.0) 0.02 M $MgCl_2$ according to the method in section 2.2.5.

In table 5-9 the results of an experiment in which Mg^{++} was added, are represented. Even during short incubation times, the extract from strain ML 3 degraded more DNA than wild type extract. In another experiment the excision of dimers with an extract from ML 1 wild type and the mutant ML 2-1 is compared. The extract from ML 1 removes 40% of the dimers while the extract from ML 2-1 removes 22% of the dimers during the same incubation period.

Several suppositions can be made about the nature of the differences between extracts of wild type and mutant strains: Firstly, the increased degradation of UV irradiated DNA indicates that extracts from UV-sensitive mutants contain an enzyme specific for UV irradiated DNA. This is in agreement with results on repair of biologically active DNA in vitro. (section 4.4). Secondly, the higher number of nucleotides removed during excision of dimers when extracts from several UV-sensitive M. luteus strains are used, can be explained in various ways. (i) Assuming that the level of the incision enzyme is not altered in the mutants, the lower amount of excision at the same level of degradation must be ascribed to changes in enzymes responsible for

late steps. The high degradation of UV irradiated DNA is then caused by increased levels of aspecific nucleases, which act after dimers have been excised. In this case the number of nucleotides removed with each dimer is apparently higher, but the excision event itself may be as effective as with wild type extract. (ii) Alterations in the level or in the specificity of a certain nuclease may lead to uncontrolled degradation at sites in the DNA where incision has taken place. Degradation can be in the wrong direction in the dimer-containing strand or in the opposite undamaged strand.

It has to be realized that the degradation in vitro can be different from the situation in vivo. Extrapolation of the in vitro results to the in vivo situation suggests that after the same UV dose, more DNA is degraded in the mutants.. These experiments will be described in chapter 6.

The excision/degradation activity was also investigated with extracts of other organisms. Extracts of Micrococcus radiodurans which is known for its extreme radiation resistance (Boling and Setlow, 1966) also shows increased degradation of UV irradiated DNA. The excision of dimers however is obscured by very high aspecific degradation. Attempts to reactivate RF DNA in vitro (see chapter 4) were unsuccessful for the same reason, even if EDTA and tRNA were added to the incubation mixture.

Incision and excision are blocked in E. coli mutants, carrying a uvrA, B or C mutation (for review see Strauss, 1968). It was therefore of considerable interest to measure the excision/degradation activity in E. coli strains. Moreover experiments done by Riklis (1965) with lysates of cells with labeled DNA, showed that degradation of DNA could be increased by irradiation of cells prior to lysis. No excision could be detected, which was ascribed to high intracellular nuclease levels.

Extracts were prepared from E. coli endA strains and the degradation of unirradiated and UV irradiated E. coli DNA was measured. Irradiated DNA was degraded to a greater extent than unirradiated DNA, Whether dimers are excised is not known (fig 5-10). However the extracts must contain UV-specific nuclease activity. Experiments with extracts from E. coli mutants to study the influence of uvr mutations have not yet been conclusive.

Similar observations were reported in a study of the introduction of single strand breaks in UV irradiated E. coli DNA after incubation with extract (Tagaki et al., 1968). When purified T4-induced UV-endonuclease (coded for by the v-gene) is added to extracts of uninfected E. coli bacteria, specific

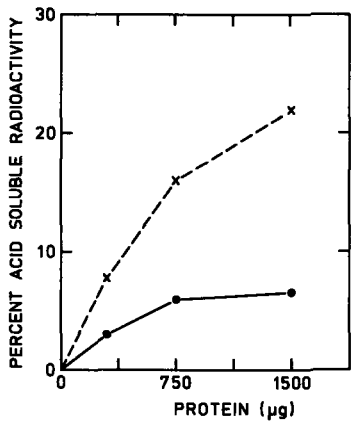

Fig.5-10 DNA degradation by E. coli extract.

DNA was incubated for 2 hr at 37°C with extract of strain KA 89 endA in 0,05 M Tris buffer (pH 7.0) 0,01 M $MgCl_2$. Unirradiated DNA, ●——●; irradiated DNA (50,000 erg/mm^2), x——x.

removal of pyrimidine dimers has been found (Sekiguchi et al., 1970).

5.3 OTHER NUCLEASES IN M. LUTEUS

5.3.1 Nuclease specific for denatured DNA

Moriguchi and Suzuki (1966) have shown that M. luteus extracts contain a nuclease which degrades heat denatured DNA more rapidly as native double stranded DNA. A nuclease with this substrate specificity could play a role in the repair of UV damage in several ways.

Firstly, the dimer containing strand is thought to denature partially after incision, leaving a nucleotide with single stranded properties. The action of an exonuclease which is able to degrade this type of DNA irrespective of the presence of dimers, could result in the excision of dimers.

The exonuclease, which was purified by Kaplan et al., (1969) was able to perform this reaction. Another nuclease specific for single stranded DNA probably degrades the excised oligonucleotides and decreases their length so far that they can be excreted from the cell.

From a regulation point of view, the level of this type of nucleases can be important for the stability of the opposite undamaged DNA strand, which is formed after excision and is the template for subsequent repair repli-

cation. Nucleolytic attack of these DNA regions will lead to double strand breaks and loss of biological activity.

The results of experiments on the degradation of denatured E. coli DNA by extracts from M. luteus wild type and mutant strains are given in table 5-11. More than 50% of the DNA was degraded during 30 min incubation, while degradation of unirradiated double stranded DNA was negligible under

TABLE 5-11

Degradation of single stranded DNA by M. luteus extracts.

Extract	Percent degraded DNA	
	Unirradiated DNA	Irradiated DNA ($50,000$ erg/mm^2)
ML 1	30	24
ML 2-1	86	46
ML 3	97	44

DNA was denatured by heating for 10 min at 100°C followed by rapid cooling in ice. Incubation with M. luteus extract was during 30 min at 37°C. For experimental details see table 5-9.

the same conditions (table 5-9). UV-irradiation of denatured DNA decreases the extent of degradation, in contrast to the situation with double stranded DNA (section 5.2). Our results are not in agreement with those of Kaplan et al., (1969) who reported that the purified exonuclease is equally active on denatured DNA with and without dimers. The difference might be the result of another nuclease present in crude M. luteus extract, which is active on single stranded DNA.

5.3.2 ATP-dependent nuclease

E. coli mutants having a recB or recC mutation are UV and X-ray sensitive and are affected in their recombination ability. These mutants lack an ATP-dependent exonuclease (Barbour and Clark, 1970). A similar enzyme has been detected in M. luteus (Tsuda and Strauss, 1964) and has been

purified (Hout et al., 1970). Because no genetic methods are available to measure recombination in M. luteus, the activity of ATP-exonuclease was determined in extracts of several M. luteus strains.

In table 5-12 the results of degradation of E. coli DNA by M. luteus

TABLE 5-12

ATP-stimulated DNA degradation by M. luteus extracts.

	Percent degraded DNA	
Incubation time	30 min	120 min
Extract		
ML 1	34	96
ML 2-1	36	97

DNA was incubated at 37°C with M. luteus extract (100 μg protein) in 0.1 M Tris buffer (pH 8.0) 0.02 M $MgCl_2$ 0.002 M ATP. Degradation without ATP did not exceed 2%.

extracts in the presence of ATP are represented. The extract of ML 2-1 contains a normal level of this nuclease. Irradiation decreases the degradation by ATP-exonuclease, probably because pyrimidine dimers block the enzyme (table 5-13).

TABLE 5-13

Influence of UV irradiation on ATP-dependent DNA degradation.

	Percent degraded DNA	
ATP concentration (mM)	Unirradiated DNA	Irradiated DNA (50,000 erg/mm^2)
0	1.3	4.4
0.1	62	42
0.3	73	63

DNA was incubated for 4 hr at 37°C with extract from strain ML 1 (400 μg protein). For experimental details see table 5-9.

The activity of the other nucleases present in the M. luteus extract is insufficient to remove pyrimidine dimers so rapidly that the ATP-exonuclease is not inhibited.

The ATP-exonuclease is unable to remove pyrimidine dimers. Incubation of UV irradiated RF DNA which was treated previously with purified UV-endonuclease did not lead to further degradation. (A. Hout and B. van Dorp, unpublished). The supposition that ATP-exonuclease does not play a role in excision of pyrimidine dimers was sustained further by the observation that the enzyme is less active on single stranded DNA than on double stranded DNA and that the activity is reduced by UV irradiation of the substrate.

5.3.3 Influence of several repair inhibitors on degradation

A number of substances which interfere with the excision repair process in vivo have been described (Strauss, 1968). Some of these repair inhibitors have also been tested on the activity of nucleases in vitro (Roulland-Dussoix, 1967). In experiments, discussed in section 4.6 and 4.7, it has been shown that the in vitro repair of biologically active DNA by micrococcal extract is not influenced. It was therefore of interest to know whether these substances inhibited degradation of UV irradiated E. coli DNA by M. luteus extract. The influence of tRNA, caffeine and proflavine was investigated. Transfer RNA, which selectively inhibits E. coli endonuclease I, was added during incubation with extract of wild type ML 1. The results given in table 5-14 indicate that the increase in degradation by UV-irradiation is not altered by the addition of tRNA. Degradation of unirradiated DNA is lower, probably due to the inhibition of aspecific endonucleases. As has already been mentioned in section 4.5 and 4.7.2, the UV-endonuclease which is responsible for the incision step, is not inhibited by tRNA. The conclusion seems to be valid that the nucleases which perform the reactions following incision, are also insensitive to tRNA.
The influence of caffeine and proflavine on degradation is shown in table 5-15. Although degradation of irradiated DNA is slightly inhibited, it is still more rapid than that of unirradiated DNA. Similar results were obtained when EDTA instead of Mg^{++} was present in the incubation mixture. The results given here are similar to those described in section 4.6 where it was shown that in vitro repair of phage DNA was not inhibited by caffeine or proflavine. As these compounds do inhibit excision of pyrimidine dimers in vivo, the structural organization of the enzymes involved in a membrane-bound complex might be of importanc

TABLE 5-14

Influence of tRNA on DNA degradation.

	Percent degraded DNA			
	Double stranded DNA		Single stranded DNA	
Concentration of tRNA (μg/ml)	Unirradiated	Irradiated (50,000 erg/mm^2)	Unirradiated	Irradiated (50,000 erg/mm^2)
0	0.3	4.4	10.2	5.9
1	1.1	4.8	11.2	4.8
5	0.1	4.8	9.3	4.0

DNA was incubated at 37°C with extract from ML 1 (400 μg protein). The incubation times were 4 hr for double stranded DNA and 30 min for single stranded DNA respectively. For experimental details see table 5-9.

TABLE 5-15

Influence of inhibitors of excision repair on DNA degradation.

	Percent degraded DNA	
Additions	Unirradiated DNA	Irradiated DNA (50,000 erg/mm^2)
Control	0.4	8.4
Caffeine (0.15%)	0.5	6.6
Proflavine (3 μg/ml)	1.0	6.9

DNA was incubated for 3 hr at 37°C with extract of ML 1 (750 μg protein). For experimental details see table 5-9.

CHAPTER 6

METABOLISM OF IRRADIATED DNA IN M. LUTEUS MUTANTS

6.1 INTRODUCTION

If one assumes that the repair of UV damage in M. luteus involves the same type of excision repair as occurs in E. coli, it is then possible to indicate some critical phases which must be overcome by an irradiated cell if it is to survive.

Firstly pyrimidine dimers must be recognized as potential damage and DNA replication must be retarded until this damage has been removed. If DNA replication proceeds beyond the dimers their repair can only be achieved by a complicated recombinational repair mechanism which is based upon the selective recombination of the two daughter chromosomes within one cell (Rupp and Howard-Flanders, 1968).

When dimers are recognized, they must be removed by the excision of small pieces of DNA by an excision/degradation process which requires fine regulation. Degradation must be limited to prevent single stranded regions in the DNA strand from overlapping and resulting in a double strand break. Furthermore end groups at the sides of the "excision gap" have to be such that new DNA can be formed by a repair polymerization reaction.

It can be easily understood that degradation and repair synthesis must be carried out simultaneously to keep the length and the number of single stranded regions in the cellular DNA to a minimum. By this localized repair the cell minimizes the risk of breakage or nucleolytic attack of the exposed single stranded repair regions.

Finally a rejoining must be achieved between the repaired re-

gions and the non-damaged part of the bacterial chromosome. It is not yet clear whether replication in M. luteus proceeds at the point where it was inhibited at the time of irradiation or whether a new replication cycle starts at the origin. In irradiated E. coli replication starts from both points (Hewitt and Billen, 1965).

In this chapter several techniques are described which use intracellularly labeled DNA to study the fate of the M. luteus chromosome after irradiation. Radiation sensitive mutants are compared with a wild type strain in order to obtain information about the step where repair in the mutants deviates from the repair in the wild type strain.

Secondly a new class of mutants will be described, which lack a UV-specific endonuclease, but are nevertheless only slightly radiation sensitive.

6.2 EXPERIMENTS WITH RADIATION SENSITIVE MUTANTS

6.2.1 Degradation of DNA after UV irradiation

The DNA of the M. luteus ML 1, ML 2 and ML 3 was labeled with ^{3}H-thymidine, as described in section 2.2.6; breakdown was investigated during post-irradiation incubation after various UV doses.

Firstly, the stability of labeled DNA in irradiated cells was studied (fig 6-1).

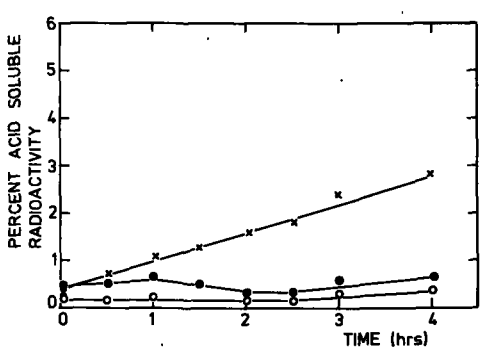

Fig. 6-1 Stability of DNA in unirradiated M. luteus strains. ML 1 (•——•); ML 2-1 (×——×) and ML 3 (○——○).

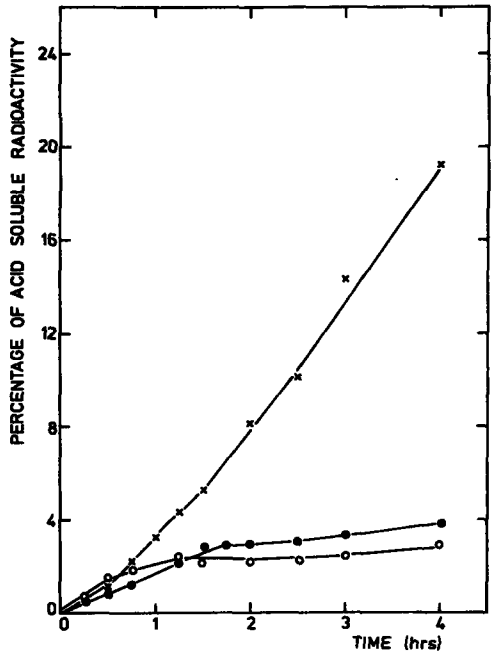

Fig. 6-2 Degradation of intracellular DNA in M. luteus strains after 250 erg/mm^2. For explanation of symbols see fig 6-1.

Fig 6-2 shows that after 250 erg/mm^2 the M. luteus wild type ML 1 and the mutants ML 2-1 and ML 3 degrade their DNA. In ML 1 and ML 3 which both have more than 50% survival at this UV dose, DNA degradation levels off after 90-120 min. In strain ML 2-1, however, survival is less than 10^{-4} and DNA degradation continued for at least 4 hours. Apparently the regulation mechanism which terminates degradation after excision does not operate properly in this strain.

The differences between the wild type and the two mutant strains are clearer after a UV dose of 1000 erg/mm^2 (fig 6-3). During the first 60 min the DNA degradation in the three strains is comparable. In ML 1 and ML 3, DNA degradation reaches a maximum between 4 and 6 hours after irradiation. Strain ML 2-1 fails to stop degradation and after 6 hours and 70-80% of the cellular DNA is degraded. Whether this high degradation is the primary reason of cell death or whether it is only caused by a faulty regulation of another step in the repair process cannot be deduced from these results.

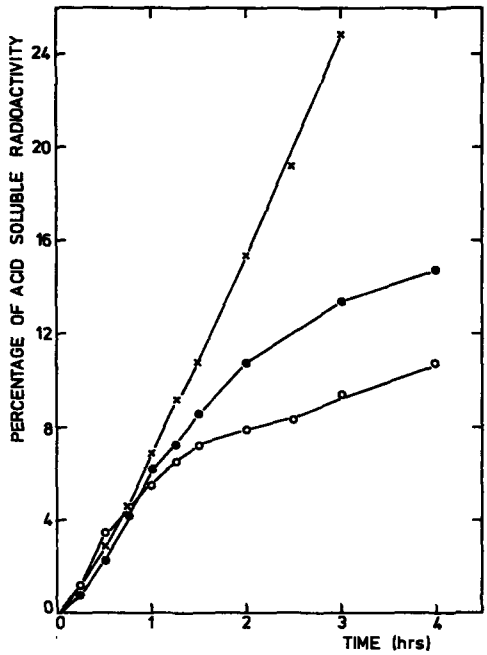

Fig. 6-3 Degradation of intracellular DNA in M. luteus strains after 1000 erg/mm^2. For explanation of symbols see fig 6-1.

The enzymatic apparatus responsible for DNA degradation is present in M. luteus at the time of irradiation. Incubation in buffer or the addition of chloramphenicol lead to the same amount of degradation. It seemed however that an energy source is required to stop degradation as incubation in medium without glucose leads to a higher level of DNA breakdown. 1969)

Addition of an inhibitor of excision repair (caffeine 0.2%) has only a minor effect on degradation. This is in accordance with the effect of caffeine on bacterial survival (fig 3-6). In evaluating these results, one must realize that no distinction can be made between cells which are able to form colonies and cells which stop division. If only a small fraction of the bacterial population is lethally hit and this fraction contributes strongly to the overall DNA degradation, the final result will be the same as when all cells have an identical level of DNA degradation. As long as techniques to separate cells which are moribund from the surviving ones are not available, a differentiation between the two alternatives cannot be made. Recently Hildebrand and Pollard (1969) showed that a difference in density between both types of cells is

present 1 hour after X-irradiation in E. coli. Attempts to apply this method on UV-irradiated M. luteus cells have not been successful sofar.

6. 2. 2 SEDIMENTATION ANALYSIS OF DNA

McGrath and Williams (1966) developed a technique to determine the average molecular weight of bacterial DNA. During the excision repair process the DNA contains breaks which result in a lower sedimentation value in alkaline sucrose gradients. This technique was used to study the introduction of breaks in M. luteus DNA after irradiation (section 2. 2. 6. 3).

In some early experiments M. luteus cells were converted into spheroplasts by incubation with lysozyme at 25°C. The introduction of single strand breaks could not be observed, which was probably due to rapid repair of breaks at this temperature. Modification of the technique as indicated by Rupp and Howard-Flanders (1968) gave satisfactory results.

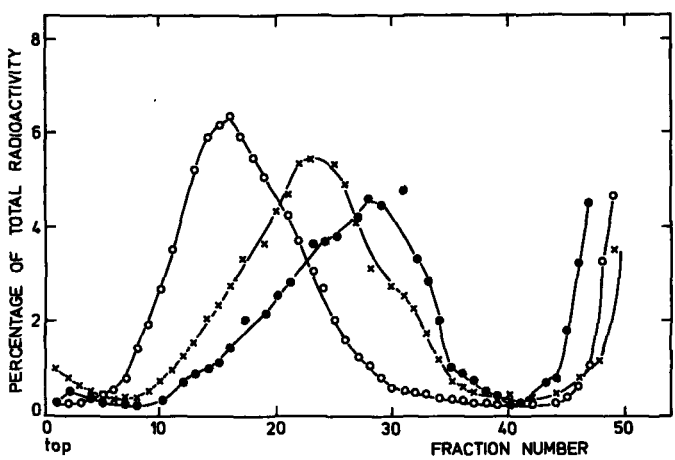

Fig. 6-4 Alkaline sucrose gradient sedimentation of DNA from M. luteus wild type strain ML 1. Unirradiated control (•——•); irradiated with 1000 erg/mm^2 (o——o); irradiated and incubated for 60 min at 30°C (x——x).

In fig 6-4 an experiment with the wild type strain ML 1 is presented. Irradiation of cells with 1000 erg/mm^2 in the cold and subsequent incubation for 10 min at 0°C with lysozyme causes a decrease of the molecular weight. Post-irradiation incubation in enriched minimal medium gives repair

of breaks. It is surprising that the initiation of the repair process (incision step) takes place even at low temperature.

A preliminary calculation of the number of single strand breaks revealed that 3-5 breaks are introduced per 10^8 dalton. Assuming the molecular weight of the M. luteus chromosome 2×10^9 dalton it can be calculated that the number of single strand breaks per chromosome is 80-120. This is considerably less than the number of pyrimidine dimers introduced by 1000 erg/mm^2 UV (1500-2000). Similar results have been reported for E. coli by Setlow, (1967). Upon further incubation the number of breaks increases during the first 15 min after irradiation after which it decreases. It seems therefore that the number of sites which are simultaneously under repair is kept at a low level. Most sites are probably repaired before incision takes place at other pyrimidine dimers.

Repair of DNA could be observed after 60 min incubation. In the experiment given in fig 6-4, 60% of the number of breaks present immediately after irradiation were repaired. The actual number of breaks introduced and repaired during the 60 min incubation period is higher than can be calculated from the sedimentation experiments.

Caffeine inhibits excision repair in E. coli and in bacteriophages (Harm, 1967 and Shimada and Tagaki, 1967), but has only a minor effect on the survival of M. luteus (fig 3-6). The addition of 0.25% caffeine to irradiated cells, results in a lower amount of breaks (fig 6-5). Postirradiation incubation for as

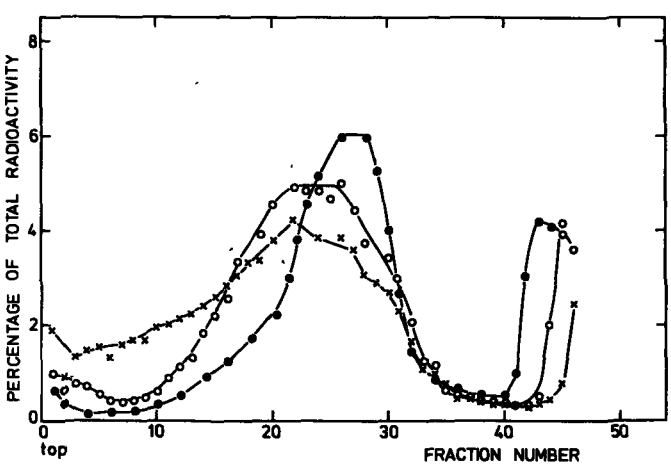

Fig 6-5
Influence of caffeine on sedimentation of DNA from strain ML 1. Unirradiated control (●—●); irradiated with 1000 erg/mm^2 and 0.25% caffeine added (o—o); irradiated and 60 min at 30°C incubated in the presence of caffeine (x—x).

much as 90 min shows that repair of breaks still occurs under these conditions.

When irradiated cells are incubated in buffer or in medium without an energy source, repair of breaks is slower, This explains why DNA degradation reaches a higher level under these conditions. The delayed repair-replication and rejoining of DNA fragments probably permit nucleases to degrade DNA for a longer period after irradiation.

The introduction of breaks is also observed in the radiation sensitive mutant ML 2-1. This is in agreement with the UV-endonuclease activity in bacterial extracts as reported in chapter 4 and 5. The number of breaks present immediately after irradiation is of the same order of magnitude as in the wild type strain (fig 6-6). Upon further incubation however, no

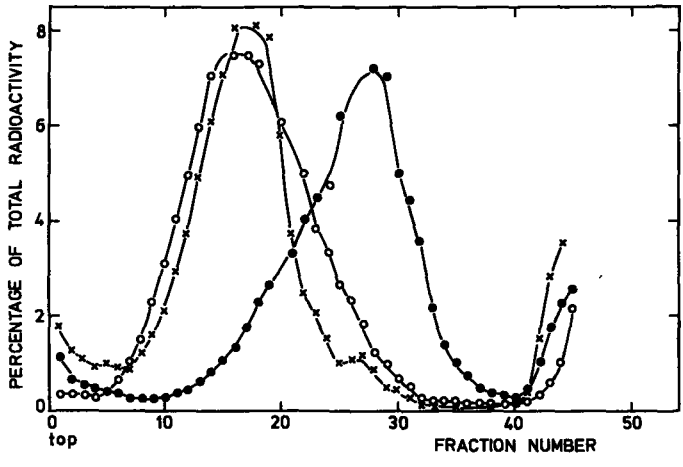

Fig. 6-6 Alkaline sucrose gradient sedimentation of DNA from strain ML 2-1. For explanation of the symbols see fig 6-4.

repair of breaks occurs within 60 min. After prolonged incubation the molecular weight decreased further. As has been pointed out, it cannot be decided whether the high level of degradation (see section 6. 2. 1) made repair of breaks in this strain impossible or whether absence of rejoining of breaks caused the high degradation of DNA.

In strain ML 3, which is slightly UV sensitive, incision occurs as in the wild type strain. The repair of breaks during postirradiation incubation is slower: only 15-20% of the breaks present after irradiation were repaired within 60 min.

6.2.3 DNA synthesis after UV irradiation

The incorporation of labeled nucleotides into cellular DNA after irradiation in E. coli is inhibited (Setlow et al., 1963; Swenson and Setlow 1966; Smith, 1969). The length of the inhibition period and the degree of inhibition are dose dependent. Although it was thought earlier that pyrimidine dimers are a complete block for DNA replication, Rupp and Howard-Flanders (1968) and Rupp et al., (1971), showed that DNA replication is able to circumvent pyrimidine dimers in excisionless E. coli mutants.

Different opinions exist about the experimental set-up for measuring DNA synthesis after irradiation and about what conclusions can be drawn from the results (Smith and O'Leary, 1968; Setlow and Setlow, 1970). In the experiments reported here, unlabeled M. luteus cells were irradiated and subsequently incubated in radioactive growth medium (section 2. 2. 6. 4):

The incorporation of radioactive thymidine into strain ML 1 was investigated after a UV dose of 200 and 800 erg/mm^2 (fig 6-7). At a dose of

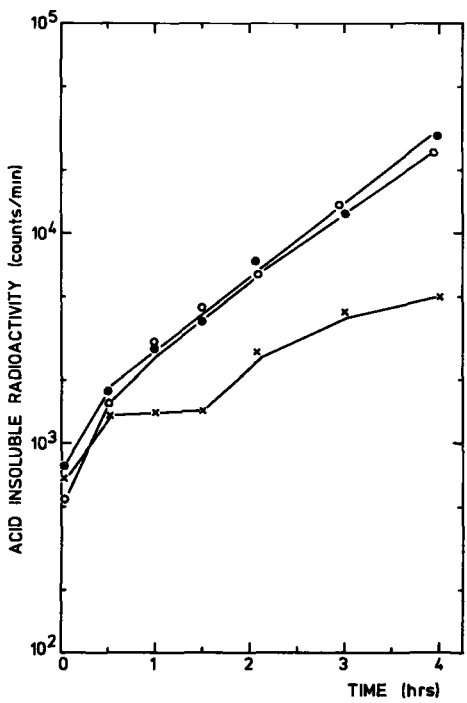

Fig. 6-7 Incorporation of radioactive thymidine in wild type strain ML 1 after irradiation. Unirradiated control (•——•); irradiated with 200 erg/mm^2 (o——o); irradiated with 800 erg/mm^2 (x——x).

800 erg/mm^2 DNA synthesis is delayed for 60-75 min. Despite this long delay 60% of the cells are able to form colonies. Apparently the removal of pyrimidine dimers and the restoration of the M. luteus chromosome requires 60 min to be completed.

Results on the rate of repair from other investigators are inconclusive. Mahler et al., (1971) observed 70% excision and nearly complete repair of breaks in 30 min after irradiation, while Okubo et al., (1971) found that only 25% of pyrimidine dimers were removed in the same period. The discrepancy is probably caused by differences in the growth medium. In our experiments supplemented minimal medium was used and this is probably the reason that the delay observed in our experiments is somewhat longer than reported by Mahler et al., (1971).

At a low UV dose (200 erg/mm^2) no delay in DNA synthesis is observed. This can be explained in various ways: (i) repair of a small number of pyrimidine dimers occurs so rapidly that the delay is too short to be measured. (ii) DNA synthesis will not be delayed if DNA replication in M. luteus is able to circumvent a small number of dimers. There are some indications that the recombinational repair mechanism as reported by Howard-Flanders et al., (1968), can play a role in M. luteus (see also section 6.3).

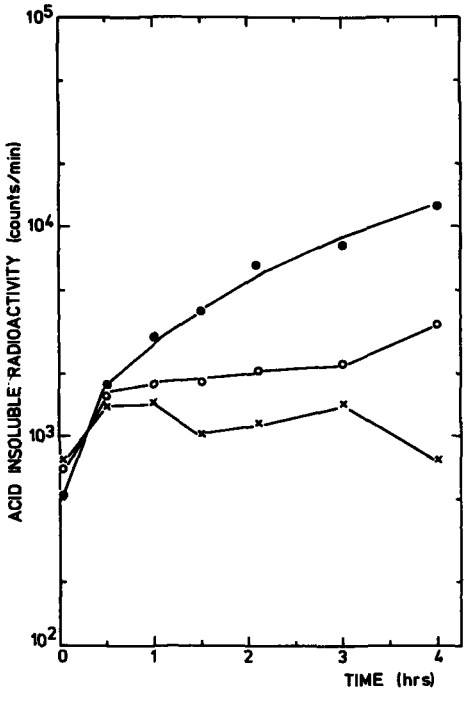

Fig. 6-8 Incorporation of radioactive thymidine in mutant strain ML 2-1 after irradiation. For explanation of the symbols see fig. 6-7.

The effect of irradiation on DNA synthesis in mutant ML 2-1 is as expected for a sensitive mutant (fig 6-8). After 200 erg/mm^2 (survival 10^{-4}) incorporation of radioactivity is delayed for at least 3 hours. After a dose of 800 erg/mm^2, DNA synthesis is blocked permanently.

Experiments to study repair replication in irradiated M. luteus have not been done for several reasons. Firstly, the UV dose required to inhibit the DNA replication is large and secondly techniques for the efficient incorporation of bromodeoxyuridine into M. luteus DNA are not available. Furthermore the difference in buoyant density between normal and bromouridine labeled DNA fragments is expected to be small due to the high GC content of M. luteus DNA.

6. 3 MUTANTS LACKING UV-ENDONUCLEASE ACTIVITY

From the experiments which were described in the preceding parts of this chapter, the conclusion was drawn that the UV sensitive mutants ML 2-1 and ML 3 are able to introduce single strand breaks into their UV irradiated DNA. An active UV-endonuclease is thus present in these strains. This is in agreement with the experiments reported in chapter 4 and 5 on the reactivation of RF DNA and on the degradation of UV irradiated E. coli DNA. The mutants ML 2-1 and ML 3, as well as the other UV sensitive mutants studied, are altered in a late step in the excision repair process, probably after the removal of the pyrimidine dimers. It was of interest to know whether mutants lacking UV-endonuclease could be isolated and to study their radiation sensitivity.

The first mutant of this type has been isolated by Okubo et al., (1967) and shown to be radiation resistent (strain 1312; included in our strain collection as ML 22). Two mutants with similar properties (ML 9 and ML 10) have been isolated from the wild type strains ML 1 and ML 8 respectively. This was done by the selection of colonies, of which the extract did not degrade UV irradiated DNA (section 2. 2. 5). In this section the in vivo properties will be discussed, while in the following one the properties of their extracts are investigated (section 6. 4).

6. 3. 1 Cellular survival and reactivation of phage N5

In preliminary experiments it was observed that the mutant strain ML 9 had a UV sensitivity which differs only slightly from the wild type

strain. To obtain more reliable results it was assured that the irradiated bacterial suspensions contained only a small number of aggregates. The inactivation curves which are given in fig 6-9 are averaged from at least 4 experiments. From fig 6-9A it can be seen that strain ML 9 is slightly more UV sensitive as the wild type strain. At a dose of 3200 erg/mm^2 the difference with the wild type is only a factor of two.

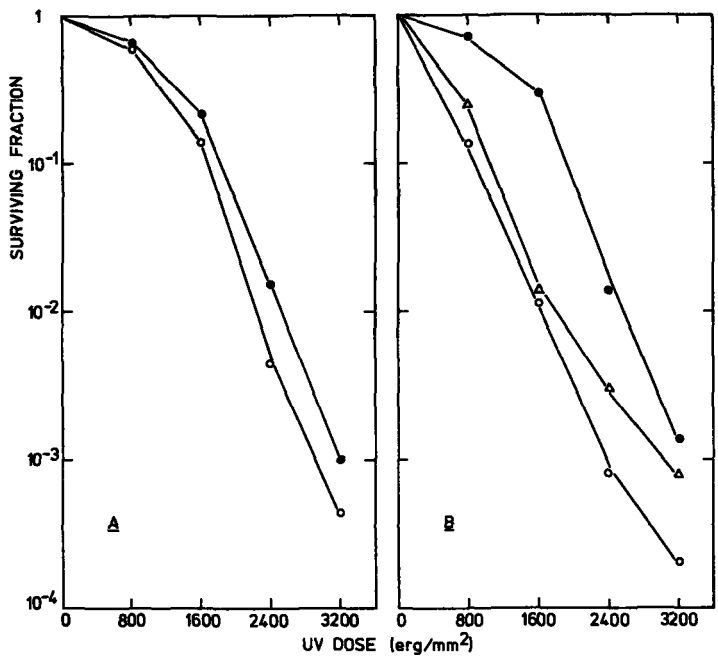

Fig. 6-9 Survival of various M. luteus strains after irradiation.
A. ML 1 (•——•); ML 9 (o——o).
B. ML 8 (•——•); ML 10 (o——o); ML 22 (Δ——Δ).

Strain ML 22, which was isolated by Okubo et al., (1967) is probably derived from the wild type strain ML 8 (ATCC 4698). The survival curve of this mutant is therefore included in fig 6-9B together with the results for strain ML 10. Both mutants are more UV sensitive than the parent strain, but the slope of the exponential part of the survival curve is comparable to that of the wild type strain. The three mutants isolated so far have survival curves intermediate between the wild type and mutant ML 3 which is the most resistant strain listed in table 3-3.

The sensitivity of the mutants ML 9, ML 10 and ML 22 to X-rays and some chemical compounds has also been determined. The strains are resistant to X-rays and MMS and are slightly sensitive to mitomycin C and 4NQO. In comparison with the radiation sensitive mutants (ML 2 to ML 7), however, they must be regarded as radiation resistant. Caffeine decreases the survival of strain ML 9 to the same extent as the survival of the wild type strain (fig 3-6). All three mutants form colonies of a different colour than the wild type strain. ML 9 forms pale yellow colonies. ML 10 gives a mixture of white and yellow colonies, which both have the same UV characteristics. ML 22 excretes a brownish substance and gives pale yellow colonies. Whether these features are related to the loss of UV-endonuclease activity can only be determined by the transfer of the mutations into other M. luteus strains.

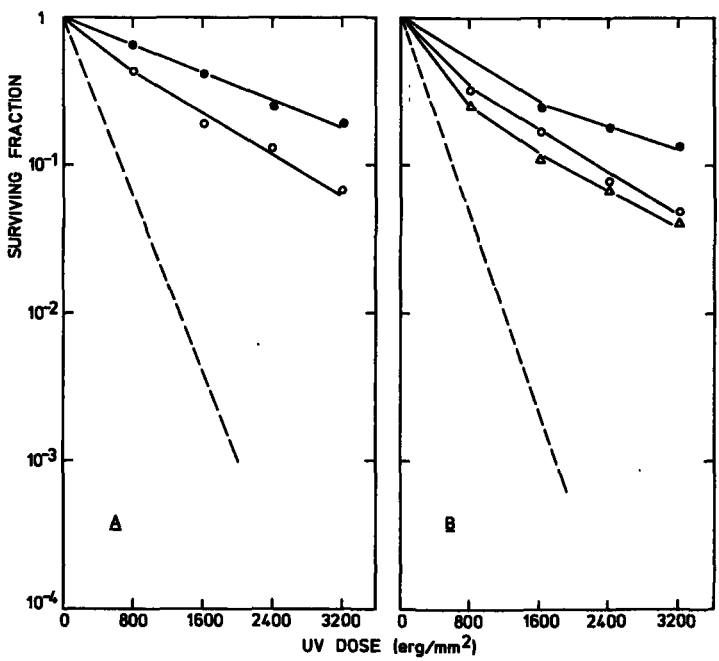

Fig. 6-10 Survival of phage N5 after irradiation measured on various M. luteus strains. The dotted line (– – –) represents the survival on an Hcr$^-$ mutant. For the explanation of the symbols see fig 6-9 A and 6-9 B respectively.

The survival of UV irradiated phage N5 was determined in the three mutants. From fig 6-10A it can be seen that repair of UV damaged phage in ML 9 is less efficient than in the wild type, but is more efficient than in an Hcr$^-$ mutant (e.g. ML 2-1).

Phage repair is also reduced in the two other mutants (fig 6-10B) The addition of caffeine has no influence on the survival of irradiated phage in any of these mutants.

6.3.2 DNA degradation

Cells were labeled with ^{3}H-thymidine and the release of radioactivity into the acid soluble fraction was investigated after a UV dose of 1000 erg/mm^2. The DNA degradation in strain ML 9 remains low for the first 30 to 45 min after irradiation and subsequently increases to the same level as in the wild type (fig 6-11). This delay in degradation of DNA in a relatively resistant mutant is in striking contrast with the behaviour of the radiosensitive strains

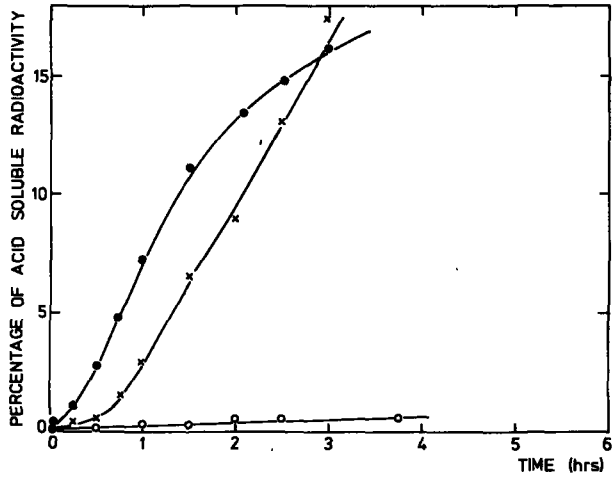

Fig. 6-11 Degradation of intracellular DNA in strain ML 1 and ML 9 after irradiation. Unirradiated controls (o——o); ML 1, 1000 erg/mm^2 (•——•); ML 9, 1000 erg/mm^2 (x——x).

ML 2-1 and ML 3 (section 6.2.2). DNA degradation in these strains starts immediately after irradiation and single strand breaks are introduced even at low temperature (0^{o}C).

Obviously DNA degradation in this new class of mutants is delayed but occuring later with the same kinetics as in the wild type strain.

Similar results were obtained with the strains ML 10 and ML 22. The level of DNA degradation in ML 22 remains lower than in the parent strain ML 8 for at least 6 hours after irradiation (fig 6-12 and 6-13).

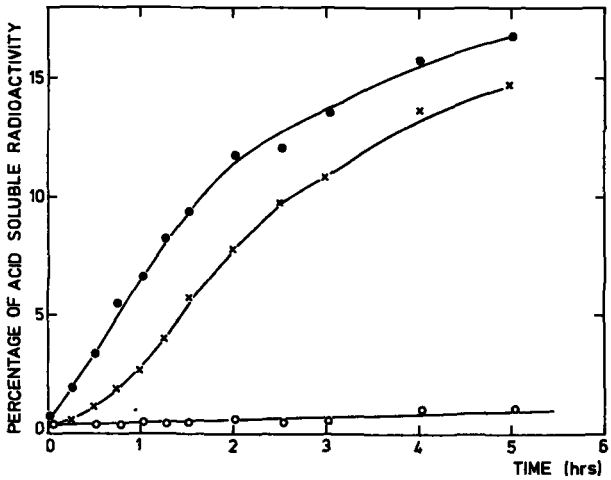

Fig. 6-12 Degradation of intracellular DNA in strain ML 8 and ML 10 after irradiation. Unirradiated controls (o——o); ML 8, 1000 erg/mm^2 (•——•); ML 10, 1000 erg/mm^2 (x——x).

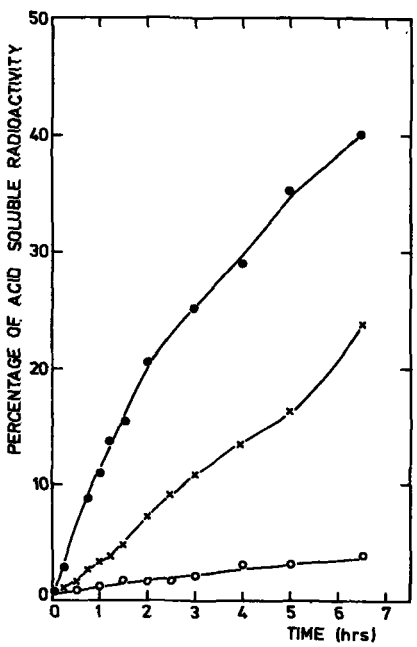

Fig. 6-13 Degradation of intracellular DNA in strain ML 8 and ML 22 after irradiation. Unirradiated controls (o——o); ML 8, 1000 erg/mm^2 (•——•); ML 22, 1000 erg/mm^2 (x——x).

Several explanations for the delayed degradation of DNA in these mutants can be given. Firstly, the physiological state of the cells after irradiation can be different for the wild type and the mutant. The lower the metabolic activity of a particular strain, the more reduced repair and DNA degradation will be. This possibility was excluded by repeating the degradation experiment in medium without amino acids or an energy source. Under these circumstances DNA degradation starts immediately in the wild type strain and the length of the delay in ML 9 is 45-60 min.

Secondly the formation of a UV-endonuclease might be induced by UV irradiation. To test this possibility the effect of chloramphenicol, which inhibits protein synthesis, was investigated. The addition of cloramphenicol has no influence on the onset of DNA degradation in the wild type or in the mutant ML 9. However the final level of degradation is higher in both strains. This could be ascribed to an inhibition of repair replication or rejoining. Also the fact that chloramphenicol does not lengthen the delay period makes it unlikely that the formation of UV-endonuclease is induced by UV.

Thirdly, the delayed appearance of acid soluble material may be inherent to another type of repair e.g. the recombinational repair process (Howard-Flanders, 1968). Preliminary experiments show that DNA degradation products are temporarily accumulated in the cellular cytoplasm of ML 9, while with wild type they are mainly found in the medium.

Attempts to measure the rate of excision of pyrimidine dimers have not been successful. Recently excision has been studied by Mahler et al., (1971) in the UV-endonuclease negative mutant G7. In this strain excision was delayed. Okubo et al., (1971) measured excision and degradation in the strains G7 and 1312 (ML 22). Their data on removal of dimers are quantitatively different from the results of Mahler et al., (1971), but show the same tendency. Our DNA degradation experiments with the mutants ML 9, ML 10 and ML 22 also suggest a slow repair of UV damage. Whether the same mechanism of repair occurs in wild type and mutant cannot yet be established.

6.3.3 Sedimentation analysis of DNA

The excision repair process is initiated by the introduction of single strand breaks. To investigate the incision step in the mutants ML 9 and ML 10 and ML 22, sedimentation in alkaline sucrose gradients was performed according to section 2.2.6. Cells were irradiated and kept in buffer to prevent re-

joining of breaks (see section 6. 3. 2). From fig 6-14 it can be concluded that single strand breaks are introduced into the DNA in the wild type strain ML 1

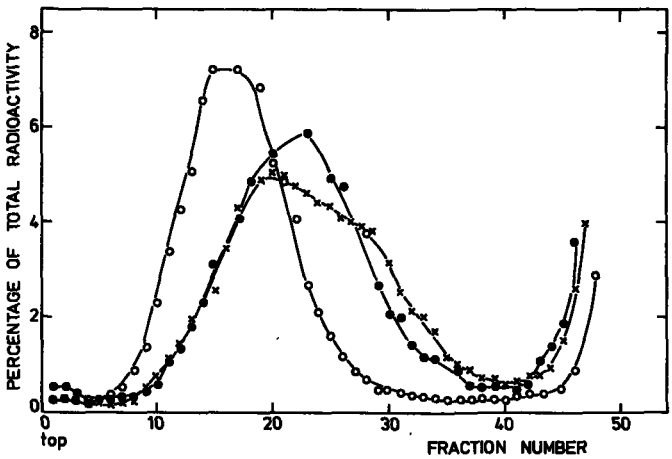

Fig. 6-14 Alkaline sucrose gradient sedimentation of DNA from strains ML 1 and ML 9. Unirradiated controls, ML 1 (•——•); ML 1 irradiated with 1000 erg/mm^2 (o——o); ML 9 irradiated with 1000 erg/mm^2 (x——x).

after irradiation but not into the DNA of mutant ML 9. After incubation for 60 min under non-growth conditions, breaks can also be observed in the DNA of ML 9. No repair of breaks occurs in strain ML 1 in the same period, when the cells are kept in buffer (fig 6-15). In growth medium the repair of breaks is observed in both strains.

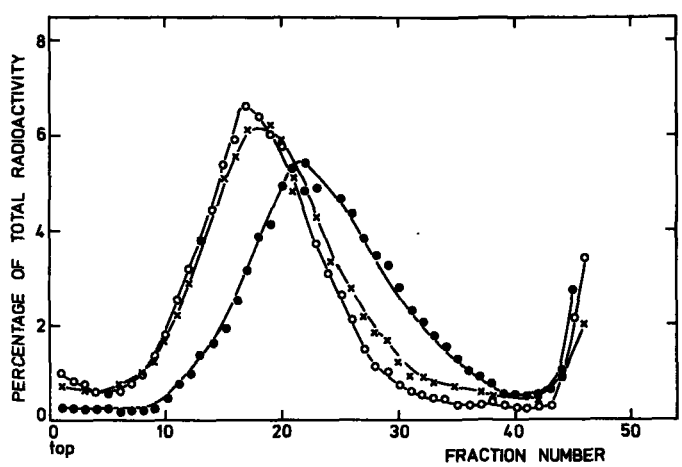

Fig. 6-15 Alkaline sucrose gradient sedimentation of DNA from strains ML 1 and ML 9. All samples were incubated for 60 min in medium without amino acids and a carbon source. For explanation of the symbols see fig 6-14.

From experiments with cells that were incubated for various intervals following irradiation it could be concluded that the repair of breaks in ML 9 occurs later than in the wild type strain. This difference has also been reported by Mahler et al., (1971). The results obtained with strain ML 10 and ML 22 are similar to those obtained with ML 9. In fig 6-16 and 6-17 the sedimentation patterns of DNA from ML 8 and ML 22 are presented.

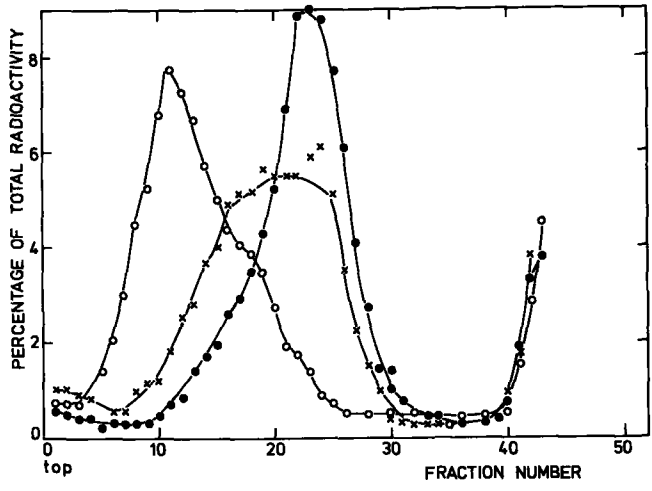

Fig. 6-16 Alkaline sucrose gradient sedimentation of DNA from wild type strain ML 8. For explanation of the symbols see fig. 6-4.

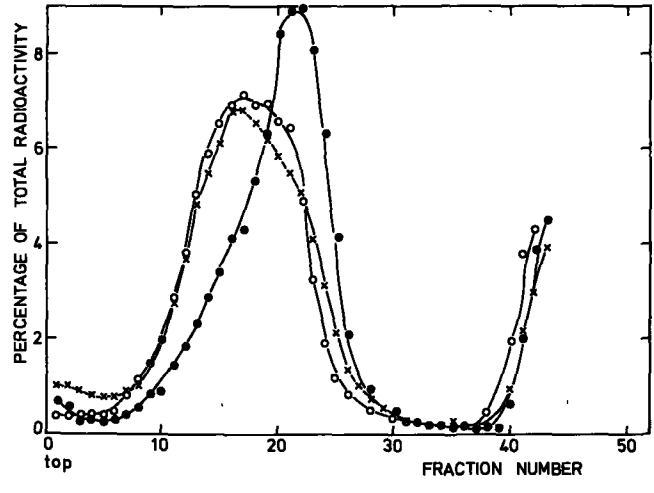

Fig. 6-17 Alkaline sucrose gradient sedimentation of DNA from mutant strain ML 22. For explanation of symbols see fig 6-4.

In ML 8 the introduction of breaks and their repair during incubation in broth is evident. The number of breaks in ML 22, however remains low. It cannot be decided whether this result for the mutant after 60 min incubation has to be explained as due to DNA in which little incision has occurred or due to DNA in which repair is nearly completed. Okubo et al., (1971) conclude from their results that strain ML 22 is a "leaky" mutant and probably contains some UV-endonuclease activity in vivo.

However, the conclusion from experiments, both in our and in other laboratories, is that the slow repair of UV damage in this class of mutants is caused by the fact that incision does not occur immediately after irradiation.

6.3.4 DNA synthesis

If the repair of UV damaged DNA proceeds slower in the UV-endonuclease mutants, it is expected that this slow repair will also be observable as a late resumption of DNA replication. Therefore the incorporation of ^{3}H-thymidine into the acid insoluble fraction after irradiation was investigated as described in section 2.2.6. After a UV dose of 200 erg/mm^2, which allowed more than 95% survival, DNA synthesis in ML 9 is inhibited for about 60 min, while DNA replication in the wild type strain ML 1 is not inhibited (fig 6-18).

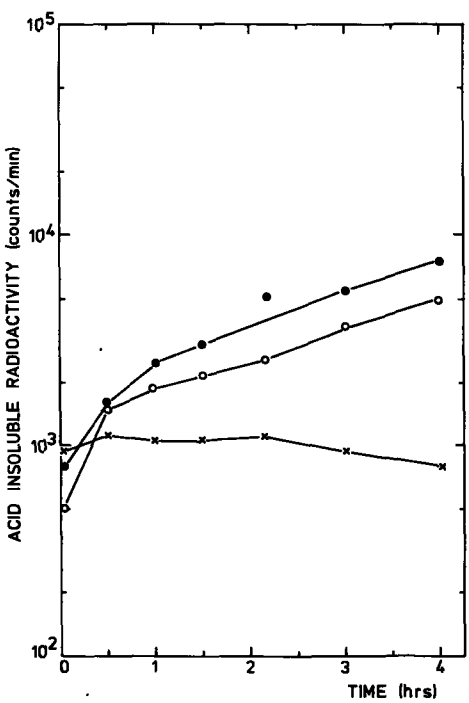

Fig. 6-18 Incorporation of radioactive thymidine in mutant strain ML 9 after UV irradiation. For explanation of symbols see fig. 6-7.

TABLE 6-19

Reativation of biological activity of RF DNA by various extracts.

Mutant	FLR
ML 1	0.34
ML 9	0.02
ML 8	0.56
ML 10	0.05
ML 22	0.00

Restoration of biologically active ØX RF DNA was measured by the method described in section 2.2.4. Incubation was with 3 μg protein for 30 min at 37°C in 0.01 M phosphate buffer (pH 7) 0.001 M EDTA. Spheroplasts of strain KA16 uvrA516 were used.

After a UV dose of 800 erg/mm^2, DNA synthesis in ML 9 is postponed for a much longer period than in the wild type strain. During the first 4 hours after irradiation no incorporation of radioactivity can be detected, while DNA replication was resumed after 60-90 min in the wild type strain. The fact that the survival of mutant ML 9 is only slightly lower than of the wild type (fig 6-9A), suggests that DNA synthesis is not blocked permanently at this UV dose.

6.4 BIOCHEMICAL PROPERTIES OF EXTRACTS

6.4.1 UV-endonuclease activity

In chapter 4 (section 4.4 and 4.7) experiments have been described which establish the presence of a UV-endonuclease in M. luteus. The activity of this enzyme can be measured by either the restoration of biologically active RF DNA or by the specific introduction of single strand breaks.

Extracts were prepared from strains ML 9, ML 10 and ML 22 and the repair of irradiated phage DNA was measured. The results in table 6-19 indicate that extracts of these strains did not increase the biological activity of RF DNA when assayed in spheroplasts of E. coli uvrA516. The possibility that the three mutants lack the UV-endonuclease activity coded for by the

TABLE 6-20

Reactivation of biological activity by extracts from irradiated M. luteus cells.

Mutant	Postirradiation incubation time	Survival of biological activity	FLR
no extract	-	3.7×10^{-4}	0
ML 1	-	2.1×10^{-2}	0.53
ML 1	120 min	1.1×10^{-2}	0.45
ML 9	-	2.2×10^{-4}	0
ML 9	60 min	3.3×10^{-3}	0.06
ML 9	120 min	1.3×10^{-4}	0
ML 9	180 min	2.9×10^{-4}	0.02

Extracts were prepared from irradiated cells (1000 erg/mm^2) which were reincubated in PGY broth for 60-180 min. The experimental details for the measurement of the fraction of UV lesions restored (FLR) were similar to those in table 6-19 except that 5 μg protein was used in the incubation.

uvrA gene only in E. coli was excluded as repair was also not found when spheroplasts with a uvrB or uvrC mutation were used. The conclusion can be drawn that these M. luteus mutants lack one or more UV-specific endonucleases which can replace the in vivo repair reaction which is affected in E. coli uvrA, uvrB and uvrC mutants.

Combinations of extracts from ML 9, ML 10 and ML 22 were not active in the in vitro repair of RF DNA. It is therefore unlikely that the mutations in these strains are in different genes. Otherwise complementation is likely to have been found. It thus appears that the mutants are missing the same enzyme. The results of Kaplan et al., (1969), Setlow and Carrier, (1970) and Nakayama et al., (1971) are in accordance with this supposition.

The possibility remains that the absence of UV-endonuclease activity in extracts of this class of mutants is due to either the presence of an inhibitor or the absence of an activator. The activity in the extract of the wild type strain ML 1 is not reduced by the addition of extract from strain ML 9. It can therefore be concluded that an inhibiting substance is not present. The

possible role of an activator was investigated by dialysis and ultrafiltration experiments. The activity of extracts from the wild type strain is not reduced by dialysis or by ultrafiltration. The diffusate of wild type extract has no UV-endonuclease activity and does not restore the UV-endonuclease activity in an extract of strain ML 9.

Induction of UV-endonuclease by UV irradiation as proposed in 6. 3. 2 was also investigated. Bacterial cells were harvested in exponential phase, washed in phosphate buffer (pH 7) and irradiated with 1000 erg/mm^2. Growth in broth was than continued and at various postirradiation incubation times, samples were withdrawn and extracts were prepared. No activity is found in mutant ML 9, while the activity in the wild type strain remains constant after irradiation (table 6-20).

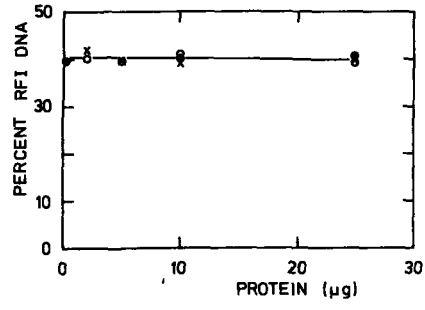

Fig. 6-21 Conversion of ØX RF I DNA into RF II DNA by extract from mutant ML 9. Unirradiated DNA, 30 min incubation (•——•); irradiated DNA, 300 erg/mm^2, 10 min incubation (x——x); irradiated DNA, 30 min incubation (o——o).

The UV-endonuclease was also measured by the conversion of irradiated ØX 174 RF I DNA (see section 4. 7. 2). During 30 min incubation with ML 9 extract in the presence of EDTA and tRNA no breaks are introduced into the RF DNA (fig 6-21). The conversion of RF DNA was also measured with extracts from irradiated cells of ML 1 and ML 9 (table 6-22). The UV-endonuclease activity is the same in wild type cells immediately after irradiation as well as after 120 min. It is concluded that UV irradiation of ML 9 does not induce the formation of UV-endonuclease.

TABLE 6-22

Endonuclease activity in extracts from irradiated M. luteus cells.

Mutant	Postirradiation incubation time	DNA	UV-DNA (300 erg/mm^2)
Control	-	1.0	1.8
ML 1	0	5.5	17.2
ML 1	120	7.4	12.2
ML 9	60	6.2	3.9
ML 9	120	6.4	5.8
ML 9	180	6.6	6.3

The endonucleolytic activity is expressed as the percentage of ØX RFI DNA in the incubation mixture converted into RFII DNA as measured by the technique given in section 2.2.5.1. The same extracts were used as in table 6-20. RF DNA was incubated for 30 min with 100 µg protein.

6.4.2 Excision and degradation

As has been shown in chapter 5, extracts from M. luteus strains which contain a UV-endonuclease degrade UV irradiated E. coli DNA to a greater extent than unirradiated DNA. This degradation is accompanied by a specific removal of pyrimidine dimers from the acid insoluble fraction (see table 5-6). Our results and those of Nakayama et al., (1971) and Kushner et al., (1971), suggest that UV-endonuclease together with a second enzyme (UV-exonuclease; Kaplan et al., 1971) is responsible for the in vitro excision of dimers. Absence of UV-endonuclease in a micrococcal extract should then result in an equal degradation of unirradiated and UV irradiated DNA and an incapacity to excise pyrimidine dimers. In the experiment given in table 6-23, E. coli DNA was incubated with extracts from M. luteus wild type ML 1; mutant ML 2-1 (UV-sensitive) and ML 9. Extracts of ML 1 and ML 2-1 excise dimers as judged from the decrease of dimers in the DNA and the increase in the acid soluble fraction. ML 9 extracts show no excision activity as the dimer content of the acid insoluble and acid soluble fraction are not different.

TABLE 6-23

Excision of pyrimidine dimers by extracts from various M. luteus strains.

Strain	DNA	UV irradiated DNA		
	fraction acid soluble	fraction acid soluble	fraction of total radioactivity in dimers.	
			acid soluble	acid insoluble
no extract	0.014	0.011	0.095	-
ML 1	0.079	0.155	0.055	0.249
ML 2-1	0.115	0.198	0.071	0.232
ML 9	0.118	0.110	0.092	0.10
no extract	0.020	0.022	0.069	-
ML 10	0.084	0.092	0.064	0.06

Incubation with extract and the determination of pyrimidine dimers in the acid insoluble fraction (DNA) and in the acid soluble excision products were performed as described in section 2.2.5. DNA was irradiated with 50,000 erg/mm^2 in the first experiment and with 30,000 erg/mm^2 in the experiment with ML 10. Incubation was for 3 hr with 400 µg protein.

In an experiment with ML 10 it was also found that dimers are not removed during incubation. An extract from the corresponding wild type is able to excise dimers efficiently (table 5-8). Shimada et al., (1967) obtained similar results with extracts from ML 22. Combinations of extracts from ML 9, ML 10 and ML 22 show no increased degradation of UV irradiated DNA.

To test the conclusion that UV-endonuclease is absent in the extracts of ML 9, ML 10 and ML 22, the effect of addition of purified UV-endonuclease (E_1) and of UV-exonuclease (E_2) on DNA degradation was investigated. From the results, presented in table 6-24, it can be seen that the addition of E_1, stimulates the degradation of UV irradiated DNA while the addition of

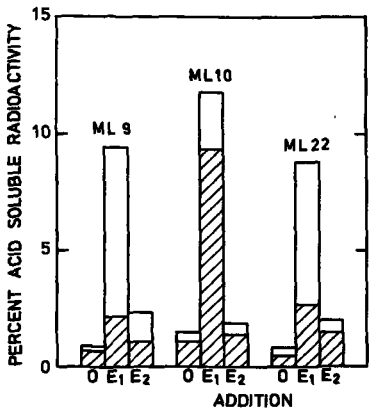

Fig. 6-24

Complementation with purified UV-endonuclease (E_1) and UV-exonuclease (E_2). The degradation of unirradiated DNA and irradiated DNA by the E_1 or E_2 fraction alone was maximally 1%. The DNA was irradiated with 50,000 erg/mm^2. Incubation was for 3 hours as indicated in section 2.2.5. The hatched area of the vertical bars represents the degradation of unirradiated DNA; the open area illustrates the increase in degradation caused by irradiation of the DNA.

E_2 has only a minor influence. The conclusion is thus valid that extracts of all three mutants lack UV- endonuclease. Furthermore the observation that the addition of E_1 alone stimulates degradation of UV-irradiated DNA, suggests that the extracts have UV-exonuclease activity (see also section 6.4.3). Experiments done by Okubo et al., (1967) with extract of mutant ML 22 are in accordance with our results.

6.4.3 Other nucleolytic activities

Two other nucleolytic activities were measured in the extract of UV-endonuclease deficient mutants. As has been concluded in the preceding section, extracts of these mutants probably contain a UV-exonuclease, which is required for excision and degradation of irradiated DNA. This enzyme, which has been purified and investigated in detail by Kaplan et al., (1971) and Kushner et al., (1971), is also active on single-stranded DNA irrespective of the presence or absence of dimers. In table 6-25 an experiment is described in which single stranded DNA is incubated with extract of the three M. luteus mutants. The results indicate that the extracts from strains ML 9, ML 10 and ML 22 contain an activity toward single stranded DNA. Irradiation of this DNA de-

TABLE 6-25

Degradation of denatured DNA by extracts from various M. luteus strains.

Strain	Fraction acid soluble (%)	
	DNA	UV-DNA
ML 1	71	37
ML 9	86	42
ML 8	42	12
ML 10	88	36
ML 22	56	21

DNA was irradiated with 50,000 erg/mm^2 and subsequently denatured. Incubation was for 30 min at 37°C with 400-700 µg of protein in 0.01 M Tris buffer (pH 8.0) 0.01 M $MgCl_2$. The method described in section 2.2.5.2 was used.

creases degradation, in contrast to the results of Kushner et al., (1971).

ATP-exonuclease activity was also measured in extracts of strain ML 9, ML 10 and ML 22. The level of this enzyme, which is involved in bacterial recombination in E. coli (Barbour and Clark, 1970), is the same in the mutants and in the corresponding wild type strains. The conclusion is that UV-endonuclease mutants have an active RecBC recombination system.

Although alterations in the activity of other nucleases cannot be fully excluded, the absence of UV-endonuclease activity in ML 9, ML 10 and ML 22 is the best explanation for the radiation properties of this new class of M. luteus mutants. The possibility that several repair processes operate simultaneously, will be discussed in more detail in the following chapter.

CHAPTER 7

DISCUSSION AND SUMMARY

The purpose of the experiments with M. luteus described in this thesis was the study of the relationship between the in vivo response to radiation and the biochemical reactions occurring with extracts from M. luteus mutants with DNA in vitro. A number of radiation sensitive M. luteus mutants was isolated and their sensitivity to UV and X-rays was determined (chapter 3). Several mutants are unable to propagate UV irradiated micrococcal phage and display various sensitivities towards radiomimetic agents such as methyl methanesulphonate and mitomycin C. According to their radiation phenotype, the mutants were classified into several groups, similar to the classification given by Rörsch et al., (1967) for E. coli. This classification could not be further substantiated by genetical evidence because reliable techniques for the genetic examination of M. luteus are not available.

In the first part of chapter 4, evidence is presented that extracts from M. luteus contain an enzymatic activity which initiates the repair of UV damage in phage DNA. From the observed features of the in vitro reaction it was concluded that this reaction is the incision of UV irradiated DNA by a UV-endonuclease. Our results are in agreement with the properties of the UV-endonuclease which has been purified by Kaplan et al., (1969); Carrier and Setlow, (1970) and Nakayama et al., (1971).

The effect of mutations leading to UV sensitivity was investigated by two types of experiments. Firstly, the influence of uvr mutations was studied, using extracts from UV sensitive M. luteus strains in the in vitro incubation. From the results it could be concluded that the extract of all M. luteus strains which were investigated, contain UV-endonuclease.

This observation suggested that the genetic alteration in these M. luteus mutants did not concern the first step of the excision repair process but rather the later steps.

Secondly the completion of repair of phage DNA in spheroplasts was measured in E. coli mutants, carrying mutations with different chromosomal locations (uvrA, B, C, E). It was found that the presence of a uvr mutation leading to an Hcr^- phenotype of the E. coli bacteria, is a prerequisite for the observation of repair in vitro. Combination of several uvr-mutations in the E. coli spheroplast has no effect on the completion of repair. It can be concluded that the repair of UV damage in phage DNA is not altered in these E. coli mutants in a step beyond the incision reaction. Alterations in the excision step cannot be fully excluded, because a number of experiments were carried out with enzyme preparations still showing excision activity in vitro.

The influence of inhibitors of excision repair on the in vitro system reveals several inconsistencies between the repair reaction in vitro and the repair process in vivo, Acridines and caffeine added at low concentrations have no effect on the in vitro reaction, while the same concentrations inhibit the incision reaction in E. coli in vivo. Setlow et al., (1970) and Nakayama et al., (1971) report similar observations. This difference can probably be ascribed to different properties of UV-endonuclease in E. coli and in M. luteus since excision repair in M. luteus cells in much less sensitive to repair inhibitors than excision repair in E. coli.

The observation that UV-endonuclease is present in a number of UV-sensitive M. luteus strains force the conclusion that these mutants are affected in later steps in repair. This was further investigated by measuring other nuclease activities in the extracts and by the study of intracellular DNA metabolism after UV irradiation. From in vitro studies on the degradation of UV irradiated DNA, it was concluded that extracts of all strains contain exonuclease activity which removes pyrimidine dimers. However, the number of nucleotides solubilized, concomitantly with the excision of one pyrimidine dimer is different for the wild type strain and several mutants. Because it was thought that the level of UV-exonuclease (Kaplan et al., 1969; Kaplan et al., 1971 and Kushner et al., 1971) might vary in the mutants, the level of this enzyme was determined by measuring the nuclease activity for single stranded DNA. No differences were found between the wild type and the mutant strains and it was concluded that variations in the effectiveness of pyrimidine dimer

removal have to be ascribed to changes in the level of other nucleases which act upon single stranded regions in the DNA present after excision. This supposition is tentative as the incubation of DNA in vitro was done under conditions which differ from those in a living cell. It should be noted that changes in the level of DNA-polymerase and polynucleotide ligase are not expressed in vitro but probably have influence in vivo (De Lucia and Cairns, 1969; Boyle et al., 1970 and Gellert and Bullock, 1970). Recently it was shown by Heijneker et al., (1971), that UV irradiated transforming DNA could be repaired with UV-endonuclease, exonuclease III, DNA-polymerase and DNA-ligase. In this case the entire excision repair reaction is required while in our test system the occurence of the first step is sufficient to complete the repair reaction in uvr-spheroplasts,

Harwood et al., (1970) showed that M. luteus DNA polymerase I is associated with a triphosphate dependent exonuclease activity, which probably plays a role in excision. Recent experiments show that DNA polymerase activity is present in the extracts of UV sensitive M. luteus mutants. This observation does not exclude the possibility that the DNA polymerase I is changed because the 5'-3'-exonuclease activity of the enzyme might be affected similar to the E. coli DNA polymerase (Kelly et al., 1969 and Setlow and Kornberg, 1972). It was found in our laboratory that alterations in the functioning of a mutant DNA polymerase I in E. coli may lead to other repair kinetics and an increased DNA degradation after irradiation (B.W. Glickman and H. L. Heijneker, unpublished). A detailed study of DNA polymerase and other nuclease activities in M. luteus should be done to elucidate the cause of radiation sensitivity in these mutants.

Additional evidence that in UV sensitive M. luteus mutants the excision repair process starts normally, was obtained from the investigation of incision and DNA degradation in M. luteus cells. The introduction of single-strand breaks in DNA in strains ML 2-1 and ML 3 occurs immediately after irradiation suggesting that the UV-endonuclease activity is present in these mutants. In the wild type strain these breaks are repaired during post-irradiation incubation. In strain ML 2-1, single strand breaks are not repaired and DNA degradation continues even after the first 60 min. DNA degradation in the wild type and in strain ML 3 stops gradually after 60 min. The results however, do not permit a conclusion about the primary lesion in strain ML 2-1. High DNA degradation might be the reason that no breaks are repaired or vice versa. The extensive delay of DNA replication after irradiation is another indication that repair of UV damage is blocked in strain ML 2-1. Strain ML 3

shows a decrease in DNA degradation and partial repair of breaks after irradiation. The intermediate radiation sensitivity of this strain is in accordance with its DNA metabolism.

The slightly increased X-ray sensitivity of strains ML 2-1 and ML 3 prompted us to study another nuclease activity in M. luteus mutants. RecB and C type mutants of E. coli are UV and X-ray sensitive, show a decreased intracellular DNA degradation and lack an ATP-stimulated exonuclease (Clark, 1971). The determination of ATP-exonuclease activity in M. luteus was carried out to see whether any mutant belonged to the RecBC-type. No significant differences were found in the level of ATP-exonuclease in extracts of M. luteus wild type and mutant strains. It was therefore concluded that the isolated M. luteus strains do not have RecBC-type mutations and that their phenotype should be ascribed to other types of mutation. The more direct approach: the measurement of recombination ability in bacterial conjugation experiments was not done because of technical complications.

It is obvious that the investigations, with the UV-sensitive M. luteus strains described sofar, do not lead to a full explanation of the relationship between radiation phenotype and intracellular nuclease activities. Another experimental approach was therefore attempted based upon the following assumption. If a certain nuclease plays an important role in the repair of UV damage in M. luteus, a strain which is deficient in this particular enzyme must be more radiation sensitive than the wild type strain. To test this hypothesis, mutants were isolated lacking the UV-endonuclease. As has been discussed previously, this enzyme is active on irradiated DNA and catalyzes an in vitro repair reaction. Two mutant strains isolated by us and one strain, which was isolated by Okubo et al., (1967), were investigated.

Surprisingly this class of mutants is only moderately UV sensitive and shows only a slightly lowered repair of micrococcal phage DNA. Investigation of the DNA metabolism in UV irradiated cells showed that repair of UV damage, as observed by the introduction of single strand breaks and by partial DNA degradation, is delayed by 30-45 min. Several explanations can be given:

1. The delayed incision and degradation found in UV-endonuclease deficient strains can be ascribed to a low intracellular enzyme activity. The process would then still occur but at a lowered rate. It is also

possible that several UV-endonuclease enzymes are present in M. luteus and that one component is still active. An endonuclease which is active on γ-ray damage in DNA as well as on UV irradiated DNA has recently been detected (W. L. Carrier, unpublished). Nakayama et al., (1971), found two UV-endonuclease activities after isoelectric focusing. They reported however, that both activities were absent in the UV-endonuclease mutant G7. On the other hand the absence of UV-endonuclease activity in vitro, does not exclude that an unstable mutant enzyme, allowing slow repair, is present in vivo.

2. It is also possible that UV-endonuclease is of minor importance in M. luteus. The recombinational repair process as described by Rupp and Howard-Flanders, (1968) and Rupp et al., (1971) should then play a mayor role in repair in M. luteus. In E. coli this repair process operates with a lower efficiency than the excision repair process: 30-50 dimers can be removed by the latter mechanism compared with about 2000 by excision repair. The importance of recombination repair can be established by measuring the UV sensitivity of Rec⁻ strains. These mutants have not yet been isolated in M. luteus. On the other hand the very radiation sensitive mutant ML 2-1 contains the ATP-exonuclease activity, suggesting that the RecBC-function is operative in this strain. Lysogenic derivatives of ML 2-1 were inducible by UV irradiation, indicating that this mutant is not equivalent to an E. coli recA strain. This experimental evidence is not sufficient to decide whether recombination repair is an important repair process in M. luteus.

An interesting feature of UV endonuclease deficiency was observed during UV induction studies. The lysogenic derivative of ML 9 is much more UV sensitive than the lysogenic wild type strain. Generally the higher UV sensitivity of lysogenic strains is explained by the fact that UV irradiation causes induction in a fraction of the cells which are then killed by the initiation of a lytic cycle. If it is assumed that recombinational repair is a mayor repair process in M. luteus strains which show a delayed excision of dimers, it is likely that the high level of UV-induced recombination results in an increased phage induction. Before it can be decided whether recombination plays an important role in the recovery from UV damage in M. luteus, detailed studies on DNA replication and sister strand exchange in UV-endoclease mutants are required.

3. Another mechanism, in which replication of DNA precedes the removal of pyrimidine dimers can be devised (see fig 7-1).

a. Dimerization of pyrimidines leads to localized denaturation of DNA. These regions may be stabilized by denaturating proteins. Oligonucleotides bind with the dimer containing strand at a position 5' to the dimer and with the undamaged strand at a similar place.

b. Undamaged strands are then replicated, starting from the oligonucleotides which serve as an initiator. The other strand with the dimer is not replicated.

c. The denatured DNA regions become fused as the asymmetric DNA replication proceeds.

d. When two regions are fused in which the polarity of replication is the same, a single strand containing the dimers is formed. In the case

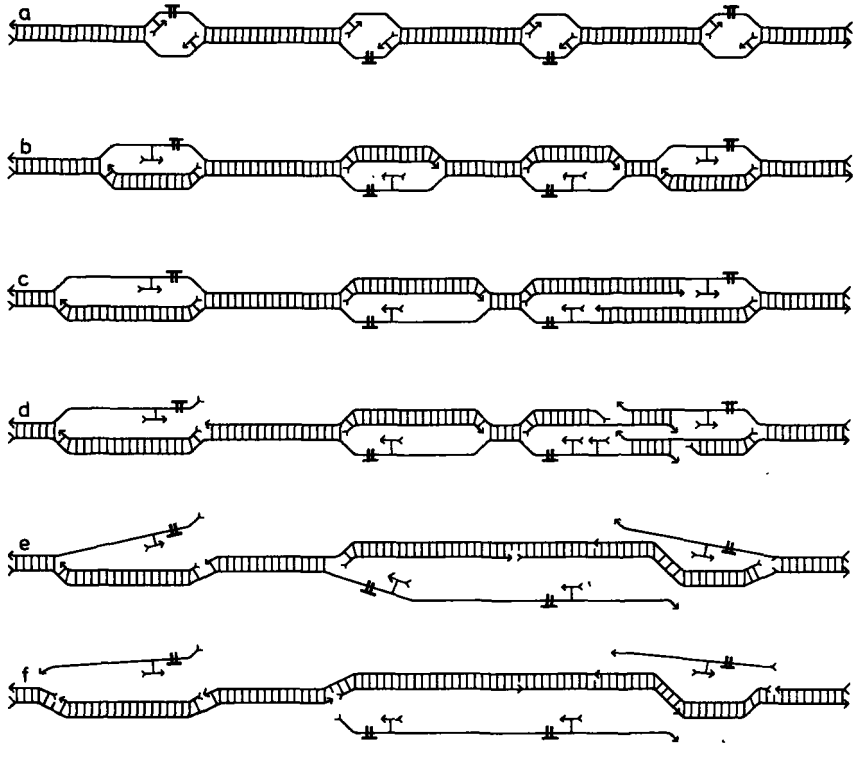

Fig. 7-1 An alternative repair mechanism for UV damage. Recombination of asymmetrically replicated DNA strands. For explanation see the text.

of fusion of two regions with a different polarity of replication, a quadruple stranded structure is formed. This structure may serve as the initiation site for recombination.

e. After recombination, the DNA has a double stranded structure with single strand breaks and single stranded side chains which contain dimers. The DNA consists of parental DNA and hybrid DNA having one parent and one daughter strand.

f. Finally the single stranded side chains are removed by exonucleases and single strand breaks are repaired by polynucleotide ligase.

This proposed reaction scheme has some resemblance to that of Filippov (1970) in which excision of pyrimidine dimers in B. subtilis lacking UV-endonuclease, is thought to occur by a copy choice replication mechanism.

None of the forwarded explanations is completely satisfying. Our results as well as those found by other investigators (Okubo et al., 1971 and Mahler et al., 1971) suggest that the UV-endonuclease has a function in repair of UV damage in vivo. The enzyme might facilitate the removal of dimers but apparently does not play a decisive role in excision repair.

It is also possible that the UV-endonuclease present in M. luteus belongs to a groups of distortion-specific enzymes which are responsible for the recoguition of all kinds of base pairing errors in DNA. These base pairing errors can be of different origin e. g. UV and X-ray damage, spontaneous and induced base alterations (pre-mutations) and intermediate structures in recombination processes. A number of these enzymes has recently been detected for γ-irradiated DNA and bromouridine containing DNA (W. L. Carrier, unpublished) as well as for alkylated DNA (Strauss and Robbins, 1968).

The intracellular localization of repair, replication and recombination of DNA, is just beginning to be explored. Although there are enough indications at present that replication of DNA is located at the cellular membrane, only little is known about the localization of excision repair and post replication/recombination repair. Paterson et al., (1972) reported that incision does not occur in minicells of E. coli. It can be imagined that UV-endonuclease is active in the cellular cytoplasm and that another membrane-bound excision repair process, governed by the E. coli uvrA, B and C genes, is of mayor importance. The observation by Tagaki et al., (1968) that E. coli uvr

mutants have intracellular UV-endonuclease activity also emphasizes that the problem of correlating radiation phenotype with the intracellular nuclease pattern is not yet solved. Repair in mammalian cells after UV irradiation does not show a rapid excision of dimers and other mechanisms may be involved (Painter, 1970).

Continued research on M. luteus will have the disadvantage that appropriate techniques for the genetic analysis of this organism have not yet been developed. Although transformation can be done in M. luteus, the understanding of this process is insufficient to use it for detailed genetic study. It is preferable to make use of the experience and knowledge of bacterial genetics which is available in E. coli. A number of enzymes which influence repair of radiation damage in E. coli, have been purified and characterized. Properties of E. coli strains with several mutations has led to the conclusion that either the uvr-genes, governing excision repair, or the rec-genes responsible for recombinational repair, must be present for repair of radiation damage. Combination of radiation sensitivity mutantions with mutations in DNA-polymerase and in polynucleotide ligase will contribute to further elucidation of the bacterial repair process in the near future.

The elegant selection technique for nuclease-deficient mutants in E. coli recently given by Milcarek and Weiss (1972) will facilitate the isolation of new types of mutants. The isolation of a UV-endonuclease negative E. coli mutant and the introduction of other mutations into this strain will provide an important clue to the complete understanding of repair.

NOTE ADDED IN PROOF

Very recently more details about the excision repair process in E. coli have become available. They emphasize the fact that information about E. coli cannot be extrapolated to M. luteus and vice versa.

Experiments similar to those described in chapter 4 and 5 have been reported in which irradiated phage DNA was incubated with T4-endonuclease V and subsequently absorbed to various E. coli uvr spheroplasts (A. Taketo, S. Yashuda and M. Sekiguchi. J. Molec. Biol. 70, 1 (1972)). It was concluded that E. coli uvrA, B and C mutants are affected in the incision reaction which can be replaced by an incubation in vitro with T4-endonuclease V.

Moreover unpublished results of Kaplan et al., (Brandeis University, Waltham, U. S. A.), have been communicated to us, which strongly indicate that E. coli uvrA, B and C mutants lack a UV-endonuclease activity. In this respect these mutants are different from both the M. luteus mutants of the Hcr^- type which contain a similar enzyme and the UV-endonuclease deficient M. luteus strains which are nevertheless radiation resistant.

SAMENVATTING EN CONCLUSIES

Het doel van de beschreven experimenten was het nagaan van de relatie tussen het gedrag van M. luteus mutanten in vivo na UV bestraling en de reactie van het extract van deze mutanten met DNA in vitro.

Een aantal UV-gevoelige M. luteus mutanten werd geisoleerd en de gevoeligheid voor UV-licht en ioniserende straling werd bepaald (hoofdstuk 3). Enkele mutanten zijn niet in staat UV-geinactiveerde faag N5 te herstellen en zijn in verschillende mate gevoelig voor de radiomimetische agentia MMS en mitomycine C. De mutanten werden op grond van hun gedrag na UV bestraling ingedeeld in verschillende groepen, overeenkomstig de classificatie van E. coli mutanten (Rörsch c. s., 1966). Deze classificatie kon niet worden ondersteund door genetische gegevens, omdat betrouwbare technieken voor genetisch onderzoek in M. luteus niet bekend zijn.

In het eerste gedeelte van hoofdstuk 4 wordt aangetoond dat M. luteus extracten een enzymatisch activiteit bevatten, die verantwoordelijk is voor de initiatie van het herstel van UV bestraald faag DNA in vitro. Uit de eigenschappen van deze in vitro reactie is afgeleid dat deze reactie de incisie van UV bestraald DNA door UV-endonuclease is. Onze resultaten komen overeen met de eigenschappen van het UV-endonuclease dat is geisoleerd en gezuiverd uit M. luteus (Kaplan c. s., 1969: Setlow c. s., 1970 en Nakayama c. s., 1971).

De invloed op de in vitro herstel reactie van mutaties die leiden tot UV gevoeligheid is nagegaan door twee typen experimenten: (a) het effect van mutaties in M. luteus werd bestudeerd door extract van verschillende stralingsgevoelige mutanten te gebruiken. Uit de resultaten kon worden afgeleid, dat alle onderzochte M. luteus stammen UV-endonuclease bevatten. Het is daarom aannemelijk, dat de genetische verandering in deze mutanten niet in de eerste maar in latere stappen van het excisie-herstel proces of in een ander herstel proces is gelegen. (b) het voltooien van het herstel van UV-bestraald faag DNA werd gemeten in sferoplasten van E. coli stammen met uvr mutaties op verschillende plaatsen op het chromosoom (uvrA, B, C, E). De voorwaarde voor het kunnen waarnemen van herstel van UV schade in vitro is, dat de sferoplasten waarin dit herstel wordt gemeten, een uvr mutatie bezitten, die leidt tot een Hcr^--phenotype in de betreffende E. coli stam. Het aanwezig zijn van meerdere uvr mutaties in de E. coli sferoplasten heeft geen invloed op het voltooien van het herstelproces. De onderzochte E. coli mutanten zijn ver-

moedelijk niet gestoord in een stap van het excisie-herstel volgend op de incisie reactie. Veranderingen in de excisie stap zelf kunnen niet geheel worden uitgesloten, omdat in een aantal experimenten enzympreparaten zijn gebruikt die nog een lage in vitro excisie-activiteit vertoonden.

De invloed van remmers op het in vitro systeem, komt niet overeen met het effect van deze stoffen in vivo in de intacte E. coli cel. Toevoegen van acridines en caffeine tijdens de in vitro incubatie heeft geen invloed, terwijl in E. coli de incisie reactie wordt geremd. De resultaten van Setlow c. s., (1970) en Nakayama c. s., (1971) met UV-endonuclease zijn hiermee in overeenstemming. Het verschil in gevoeligheid t. o. v. deze remmers kan misschien worden verklaard door aan te nemen, dat de UV-endonucleases uit E. coli en uit M. luteus andere eigenschappen bezitten. Het geringe effect van deze remmers op de overleving van M. luteus na bestraling -in tegenstelling met E. coli- wijst hierop.

De veronderstelling, dat stralingsgevoelige mutanten niet in de incisie reactie waren gestoord, werd verder onderzocht door het meten van de activiteiten van enkele andere nucleases en door het bepalen van de afbraak van intracellular DNA na UV bestraling. Uit in vitro studies werd geconcludeerd, dat alle extracten een exonuclease bevatten, dat een rol speelt bij de excisie van pyrimidine dimeren (Kaplan c. s., 1971; Kushner c. s., 1971). Het aantal nucleotiden dat wordt verwijderd bij de excisie van één dimeer, is echter verschillend voor het wilde type en de mutanten. Dit verschil in de excisie-efficiëntie is vermoedelijk toe te schrijven aan veranderingen in het niveau van andere nucleases die inwerken op de enkelstreng gedeelten in het DNA, gevormd na de excisie van dimeren.

Bij de extrapolatie van conclusies verkregen uit in vitro experimenten naar de in vivo situatie, moet worden gerealiseerd dat de condities van de in vitro herstel reactie zodanig zijn dat veranderingen in DNA polymerase en polynucleotide ligase activiteit niet worden gemeten. Veranderingen in deze enzymen beinvloeden de stralingsgevoeligheid in E. coli (DeLucia en Cairns, 1969; Boyle c. s., 1970; Gellert en Bullock 1970). In het extract van alle M. luteus mutanten is DNA polymerase I activiteit aangetoond. Het is echter niet uitgesloten, dat de 5'-3'-exonucleolytische functie van dit enzym - waarvan wordt aangenomen, dat het in E. coli bij excisie een rol speelt; Kelly c. s., 1969 - door mutatie is veranderd. Recent onderzoek in ons laboratorium van een E. coli mutant met een veranderd DNA polymerase I bevestigt de rol van 5'-3'-exonuclease in de excisie van dimeren (B. W. Glickman en H. L. Heijneker, in voorbereiding).

Verdere aanwijzingen dat de eerste stap van het excisieherstel proces normaal verloopt in UV-gevoelige M. luteus mutanten, zijn verkregen door het onderzoek van incisie en afbraak in vivo. In de stammen ML 2-1 en ML 3 worden direct na bestraling enkelstreng breuken in het DNA gevormd, wat in overeenstemming is met de aanwezigheid van UV-endonuclease in het extract. In het wilde type worden deze breuken hersteld tijdens verdere incubatie. In stam ML 2-1 treedt geen herstel van breuken op en duurt de DNA afbraak voort na 1 uur incubatie. In het wilde type (ML 1) en de mutant ML 3 neemt de afbraak van DNA geleidelijk af en bereikt 60-90 min na bestraling een maximum. Uit deze resultaten kan niet worden geconcludeerd wat de primaire oorzaak van de hoge UV gevoeligheid in stam ML 2-1 is. De hoge afbraak van DNA kan het herstel van breuken remmen of omgekeerd. Tevens is in deze mutant de DNA synthese na bestraling gedurende zeer lange tijd geremd. In mutant ML 3 worden de enkelstreng breuken langzaam hersteld wat in overeenstemming is met de intermediaire UV-gevoeligheid van deze stam.

De verhoogde röntgengevoeligheid van de stammen ML 2-1 en ML 3 was aanleiding om in deze mutanten de activiteit van het ATP-afhankelijke exonuclease te bepalen (Tsuda en Strauss, 1964). In E. coli zijn namelijk de Rec^- mutanten bekend, die röntgen- en UV-gevoelig zijn en een dergelijk enzym missen (Clark, 1971). Deze methode werd gebruikt, omdat het -door het ontbreken van goede genetische technieken- op een andere wijze niet mogelijk is om vast te stellen of een M. luteus stam tot het RecBC-type behoort. In de stammen ML 2-1 en ML 3 en in de andere M. luteus mutanten was de activiteit van het ATP-exonuclease hetzelfde als in het wilde type.

De resultaten van het onderzoek beschreven in de hoofdstukken 3 t/m 5 en in het eerste gedeelte van hoofdstuk 6, geven wel aanwijzingen waar de storing in het herstel proces in de verschillende mutanten is gelegen, maar definitieve conclusies zijn niet mogelijk. Een tweede mogelijkheid is om het probleem van een principieel andere zijde te benaderen, n.l. door de isolatie van mutanten, die een enzym missen, dat belangrijk is voor het excisieherstel proces. Als UV-endonuclease (beschreven in hoofdstuk 4) het incisieenzym is, dan zullen mutanten die UV-endonuclease deficient zijn, een sterk verhoogde stralingsgevoeligheid moeten vertonen. Twee van deze mutanten (ML 9 en ML 10) en een stam, geisoleerd door Okubo c. s., 1967, (ML 22), werden onderzocht.

De UV-gevoeligheid van dit type mutanten is echter nauwelijks verschillend van het wilde type en het herstel van UV-bestraalde N5 faag is hoog. Na bestraling worden pas na 30 tot 45 min enkelstreng breuken gevormd, waarna het DNA gedeeltelijk wordt afgebroken. Het excisie-herstel proces verloopt langzamer dan in het wilde type. Het gedrag van deze mutanten kan op verschillende manieren worden verklaard:

1. Het uitstel van incisie en DNA afbraak in ML 9, ML 10 en ML 22 kan worden veroorzaakt door een instabiel UV-endonuclease dat in vivo nog enige activiteit bezit maar dat in vitro niet meer aantoonbaar is. Een andere mogelijkheid is, dat in M. luteus meerdere UV-endonucleases aanwezig zijn en dat in vivo één daarvan nog actief is. Nakayama c. s., (1971) toonden bij electroforese van M. luteus extract, twee UV-endonuclease activiteiten aan. In de door deze onderzoekers gebruikte mutant G7, waren beide activiteiten echter afwezig. Zeer recent is een endonuclease aangetoond in M. luteus dat breuken geeft in γ-bestraald DNA en dat ook werkt op UV-bestraald DNA (W. L. Carrier, persoonlijke mededeling).

2. UV-endonuclease speelt geen belangrijke rol bij herstel van UV schade in DNA in M. luteus: verwijdering van pyrimidine dimeren geschiedt volgens een ander mechanisme dan excisie-herstel. In E. coli is een dergelijk proces beschreven (Howard-Flanders c. s., 1968 en Rupp c. s., 1971). Dit zgn. recombinatie-herstel proces kan in E. coli slechts $\pm$ 2% van de UV-schade herstellen die door excisie-herstel kan worden gerepareerd. Het is echter mogelijk dat in M. luteus een dergelijk mechanisme veel belangrijker is. Dit alternatief kan worden getest door de invloed van Rec-mutaties op de stralingsgevoeligheid te bepalen. Een probleem is echter, dat recombinatie in M. luteus niet eenvoudig kan worden gemeten. In alle stralingsgevoelige mutanten werd ATP-exonuclease activiteit aangetoond. Als dit enzym - naar anologie met E. coli - een rol in recombinatie heeft, mag hieruit worden geconcludeerd, dat in deze mutanten het RecBC recombinatie mechanisme functioneel is. De RecA-functie werd onderzocht door de inductie van lysogene derivaten met UV; in E. coli is deze inductie gestoord in $RecA^-$ mutanten. De sterk stralingsgevoelige mutant ML 2-1 vertoont een normale UV-inductie, zodat het niet waarschijnlijk is, dat in deze

stam recombinatie gestoord is.

Interessant is in dit verband de waarneming, dat de UV-endonuclease deficiente mutant ML 9, lysogeen voor faag N5, veel stralingsgevoeliger is dan stam ML 9 zelf. Het ligt voor de hand te concluderen, dat door UV een sterke stimulering van de intracellulaire interchromosomale recombinatie optreedt en dat de cellen worden gedood door de initiatie van een lytische cyclus.

3. Een variant op het zojuist beschreven recombinatie-herstel is een herstel proces, waarbij DNA replicatie van de onbeschadigde DNA keten plaats vindt vanuit de locale denaturatiegebieden rond de aanwezige pyrimidine dimeren (zie fig 7-1). Deze replicatie is asymmetrisch en verloopt in de beide DNA ketens in de 5'-3' richting. De niet gerepliceerde en dimeer bevattende DNA fragmenten worden door exonuclease werking en door recombinatie verwijderd en de resterende enkelstreng breuken worden hersteld door polynucleotide ligase. Door Filippov (1970) is een soortgelijke mechanisme voorgesteld bij het herstel van UV-schade in B. subtilis, in dit organisme is geen UV-endonuclease activiteit aangetoond (Strauss c.s., 1966).

Geen van deze mogelijke verklaringen voor de eigenschappen van UV-endonuclease deficiente M. luteus mutanten is geheel bevredigend. De conclusie is, dat UV-endonuclease een functie heeft bij het herstel van UV-schade in vivo maar dat het geen beslissende rol vervult. De mogelijkheid bestaat, dat dit enzym geen specifiek stralingsherstelenzym is maar behoort tot een groep van enzymen, die afwijkingen van de "double helix" structuur in DNA herkennen. Deze structuurveranderingen kunnen een gevolg zijn van bestraling of van baseveranderingen of kunnen optreden tijdens recombinatie. De endonucleases die specifiek zijn voor Y-schade (W. L. Carrier, persoonlijke mededeling) en voor gealkyleerd DNA (Strauss en Robbins, 1968) zouden tot deze klasse van enzymen kunnen behoren.

De studie van de intracellulaire localisatie van het DNA metabolisme is in ontwikkeling. Replicatie vindt vermoedelijk aan de celmembraan plaats; over herstel is vrijwel niets bekend. In E. coli is door Paterson c.s., (1972) gevonden dat incisie niet plaats vindt in DNA-loze minicellen. Een cytoplasmatisch en een membraan gebonden herstelproces zouden naast elkaar kunnen voorkomen en verschillende nucleasen kunnen hierbij een rol spelen. In Uvr

mutanten van E. coli, is UV-endonuclease activiteit aangetoond (Tagaki c. s., 1968). De correlatie van het stralingsfenotype met het intracellulaire nuclease-patroon is thans nog niet mogelijk.

Het verdere onderzoek dat zal moeten worden uitgevoerd voor het verkrijgen van een beter inzicht in de relatie tussen nucleases en stralingsgevoeligheid, kan beter worden voortgezet in E. coli, daar genetische technieken in dit organisme veel verder zijn ontwikkeld dan in M. luteus. Bovendien zijn E. coli stammen geisoleerd waarin nuclease genen zijn gemuteerd, die een rol spelen bij het herstel van stralingsschade. Het invoeren van uvr en rec mutaties in deze mutanten zal hopelijk in de nabije toekomst leiden tot een beter begrip van het herstel van stralingsschade in bacteriën.

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CURRICULUM VITAE

Na het behalen van het diploma HBS-b aan het Prof. Zeeman lyceum te Zierikzee in 1956, ving de auteur van dit proefschrift zijn studie aan in de scheikundige technologie aan de Technische Hogeschool te Delft. Het kandidaatsexamen richting IV - biologisch-chemische richting - werd in juni 1961 afgelegd. Onder leiding van Prof. W. Berends werd een afstudeeronderzoek uitgevoerd naar het mechanisme van energieoverdracht bij kleurstoffen via triplettoestanden. Gedurende de maanden juli en augustus 1962 was hij tijdelijk werkzaam bij het Natuurkundig Laboratorium van de N.V. Philips' Gloeilampenfabrieken te Eindhoven, waar onder leiding van Dr. J.H. Stuy een onderzoek aan bacterietransformatie werd verricht. Het ingenieursexamen werd in oktober 1962 afgelegd.

Tijdens de vervulling van zijn militaire dienstplicht werd hij door de Koninklijke Luchtmacht in februari 1963 gedetacheerd bij het Medisch Biologisch Laboratorium TNO, waar in de afdeling Genetica van Micro-organismen onder leiding van Prof. dr. A. Rörsch het onderzoek naar het herstel van stralingsschade in E. coli en M. luteus werd aangevangen, dat in dit proefschrift wordt beschreven. In juli 1964 trad hij in dienst bij het Laboratorium voor Fysiologische Scheikunde van de Rijksuniversiteit te Leiden, als medewerker van het J.A. Cohen Instituut voor Radiopathologie en Stralenbescherming. In 1969 volgde zijn benoeming tot wetenschappelijk medewerker bij het Laboratorium voor Moleculaire Genetica van de Rijksuniversiteit te Leiden.

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