ENZYMES INVOLVED IN THE REPAIR OF DNA, DAMAGED BY ULTRAVIOLET LIGHT

H. L. HEIJNEKER

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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 15 OKTOBER 1975 TE KLOKKE 15.15 UUR

DOOR

HERBERT LOUIS HEIJNEKER
GEBOREN TE AMSTELVEEN IN 1944

Promotor : Prof.Dr.Ir. A.Rörsch

Het onderzoek, dat in dit proefschrift is beschreven,werd uitgevoerd in het Medisch Biologisch Laboratorium TNO te Rijswijk en in het Laboratorium voor Moleculaire Genetica te Leiden en stond onder leiding van Dr. P.H.Pouwels.

STELLINGEN

I

De conclusie van Konrad en Lehman, dat de *E. coli* mutant *polA*ex1 conditioneel lethaal is ten gevolge van de afwezigheid van een functioneel 5'-3'exonuclease, geassocieerd met DNA polymerase I, is voorbarig.

E.B.Konrad en I.R.Lehman, *Proc.Natl.Acad.Sci.U.S.A.*71, 2048 - 2051 (1974).

II

Het is onwaarschijnlijk, dat de 5'-3' exonuclease functie van DNA polymerase I, geïsoleerd uit *E. coli*, alleen verantwoordelijk is voor de excisie van uracil-bevattende oligonucleotiden uit duplex DNA.

M.G.Wovcha en H.R.Warner, *J.Biol.Chem.* 248, 1746 - 1750 (1973).

III

Het moet worden betwijfeld of in menselijke cellen fotoreactiverend enzym aanwezig is.

B.M.Sutherland, *Nature* (London) 248, 109 - 112 (1974).

IV

De aanwezigheid van bacterieel DNA, dat covalent gebonden is aan het linker uiteinde van het bacteriofaag Mu-1 DNA, kan het gevolg zijn van herstelreplicatie tijdens excisie van de Mu-1 profaag.

B.Allet en A.I.Bukhari, *J.Mol.Biol.* 92, 529 - 540 (1975).

V

De conclusie van Robertson en medewerkers, dat het RNA van bacteriofaag f2 geen stabiele secondaire structuur heeft, is niet in overeenstemming met de waarneming, dat f2 RNA hypochromiciteit vertoont.

H.D.Robertson, in *RNA phages*, N.D.Zinder, ed.,Cold Spring Harbor Laboratory (1975), pp.113 - 145.

VI

De aangeslagen toestand, waarin purines en pyrimidines onder invloed van exciterende straling kunnen geraken, zou aanleiding kunnen geven tot mutatie-inductie, zonder dat daarbij enig herstelproces is betrokken.

J.K.Setlow en M.E.Boling, *Mutation Res.* 9, 437 - 442 (1970).

VII

Het niet aangepast zijn van sportaccomodaties aan de behoefte van de gehandicapte sportbeoefenaar werkt frustrerend en belemmert de invalide sport in zijn geheel.

W.Y.Sijtsema, *Huisarts en Wetenschap*, 18, 254 - 256 (1975).

VIII

De "super-winsten", die het staatsbedrijf der PTT sinds enige jaren maakt, konden mede worden behaald door de dienstverlening aan het publiek tot een hinderlijk minimum te beperken.

IX

Het werkelijk aantal wachtenden vóór U, dat telefonisch via 008 inlichtingen wenst, is steeds één minder dan het aantal wachtenden, dat U door de telefoniste wordt voorgespiegeld.

Leiden, 15 oktober 1975 H.L.Heijneker

Aan mijn ouders
Aan Pauline

CONTENTS

INTRODUCTION

In 1960, Beukers and Berends demonstrated that in a frozen solution of thymine, which was irradiated with UV-light of 254 nm dimers are formed between two thymine molecules.
Subsequently it was found by Wacker (1963) that in UV-irradiated DNA of the bacterium *Escherichia coli* the same type of dimers are formed between two adjacent thymine bases. Besides thymine:thymine, also thymine:cytosine and cytosine:cytosine dimers are introduced by UV-irradiation into DNA (Setlow and Carrier, 1966).
The presence of pyrimidine dimers in DNA is the main cause for the inactivation of the organism by ultraviolet light (Setlow, 1966).
The inactivation is the result of the inhibition of DNA replication at the site of the dimer , due to a distortion of the base pairing (Bollum and Setlow, 1963).
The first observations that repair of UV-damage can occur in cells came from Kelner in 1949, who showed that cultures of *Streptomyces griseus*, *Penicillium natatum* and yeast cells survive much better after UV-irradiation when they are exposed to visible light than when they are kept in the dark. This photoreactivation was found to occur in many other microorganisms (Jagger, 1958) and also in eukaryotes.

Goodgall *et al.*,(1957) were the first to demonstrate that photoreactivation is an enzymatic process. A survey on photoreactivation is given by Cook,(1970).
In 1958,Hill isolated a mutant from a radiation resistant strain of *E.coli*, which is 50 to 100 times more sensitive to UV-light than the parent strain. This strain is also defective in its ability to reactivate UV-irradiated phage DNA. This phenomenon is often referred to as host cell reactivation (hcr) (Ellison *et al.*,1960).
The molecular mechanism underlying the defect in hcr^- strains was explained by experiments performed by Setlow and Carrier (1964), who studied DNA synthesis in these strains after UV-irradiation. By labelling the thymine residues in DNA with a radioactive isotope they showed that the thymine dimers are released from DNA in the resistant strain, but not in the sensitive one.
Similar observations were reported by Boyce and Howard-Flanders (1964) for other combinations of UV-resistant and UV-sensitive strains of *E.coli*. On the basis of these results a model for excision-repair was

proposed, which has come to be known at the "cut and patch" mechanism: the damaged regions are cut out from the DNA and new nucleotides are inserted into the resulting gap.
Pettijohn and Hanawalt (1964) obtained evidence that insertion of nucleotides indeed occurs in DNA in UV-irradiated cells which are able to excise dimers, but not in those cells which do not excise the dimers.
Arguments in favour of the hypothesis that the excision repair process is enzymatic in nature have come from experiments performed by Rörsch *et al.*,(1964). They found that incubation of UV-irradiated double stranded DNA of bacteriophage ØX174 (RF DNA) with an extract from *Micrococcus luteus* leads to a marked increase of the biological activity of the DNA when measured on hcr^-, but not when measured on hcr^+ spheroplasts. This result indicates that the reaction carried out *in vitro* replaces of paralells the host cell reactivation capacity of the wild type bacteria. The repair capacity of the extract was destroyed by heating and by treatment with proteolytic enzymes, suggesting that enzyme(s) are involved in the reaction. In more recent experiments (Rörsch *et al.*, 1966) it was demonstrated that upon incubation with a partially purified enzyme preparation from *M.luteus* UV-irradiated RF I DNA is converted into RF II DNA, concomitantly with an enhancement of the biological activity. Therefore it was concluded that excision of dimers is preceded by nicking of the UV-irradiated DNA.
From the available data, the following model for excision-repair was presented (Kelly, 1969)(Fig.1) :

1. Irradiation. Under the influence of UV-irradiation,(pyrimidine) dimers are introduced in the DNA, resulting in stable local distortion of the double helix.
2. Incision. An UV-specific endonuclease detects the distortion in the DNA, and introduces a single strand scission at the 5' site of the lesion.
3. Excision. The defective segment is removed through cleavage by an exonuclease.
4. Repair replication. A DNA polymerase uses the intact complementary strand as template for replication. The 3' end of the nicked strand serves as primer and new nucleotides are inserted in the 5'-3' direction.

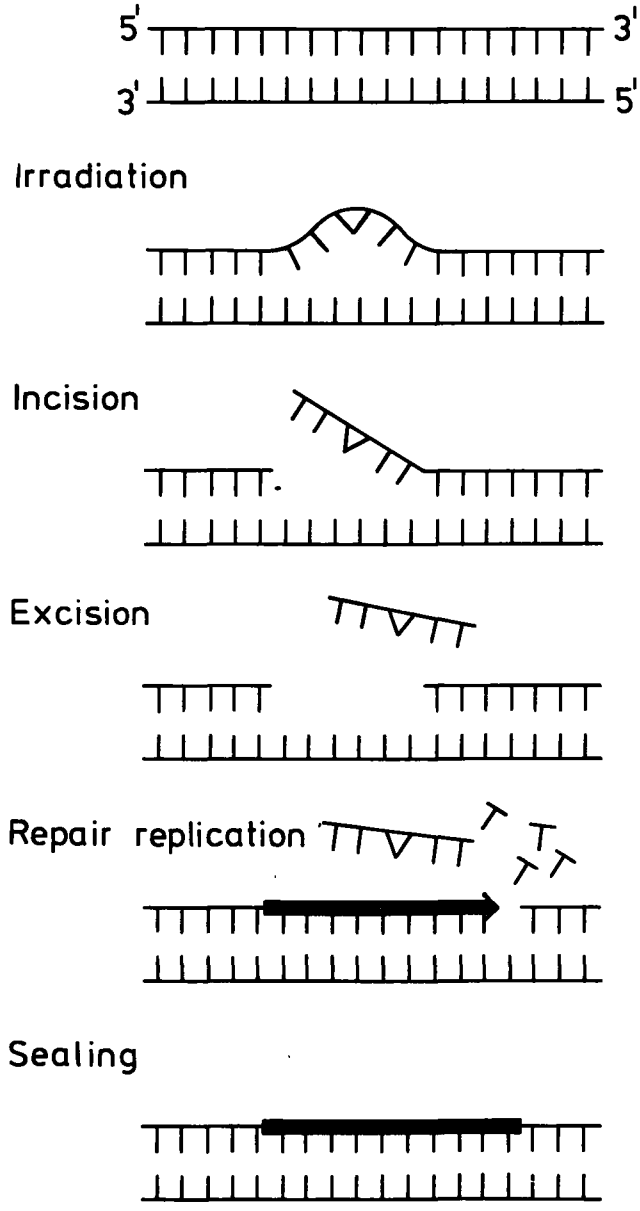

Fig.1, Model for excision-repair.

5. Sealing. By the action of DNA ligase, the newly synthesized DNA is covalently attached to the juxtaposed preserved DNA strand.

The different steps in the enzymatic removal of dimers from UV-irradiated DNA have been studied in some detail with purified enzymes, obtained from various organisms.

Incision

The properties of an UV-specific endonuclease from *M.luteus*, have been studied most extensively (Kaplan *et al.*,1971; Nakayama *et al.*,1971; Carrier and Setlow, 1970).
The enzyme consists of one polypeptide chain of a molecular weight of about 15,000. Unlike most other nucleases, the UV-specific endonuclease does not require divalent cations. The enzyme introduces a single stranded nick 5' to the dimer (Kushner *et al.*,1971) in the DNA strand containing the dimer (Carrier and Setlow, 1970; Paribok and Tomilin, 1971).
An analysis of the type of nick generated by UV-specific endonuclease originally revealed 3'P-5'OH endgroups (Kushner, 1971; Grossmann, 1973). More recently however it was found that 3'OH-5'P termini are generated (Hamilton *et al.*,1974).
The characteristics of the T4-induced UV-specific endonuclease (Sekiguchi *et al.*,1970; Friedberg and King, 1971) and those of the *E.coli* UV-endonuclease (Braun and Grossman,1974) are comparable to those of the *M.luteus* enzyme, with respect to molecular weight, divalent cation specificity, strand selectivity and type of incision made.

Excision

An exonuclease has been isolated from *M.luteus* which acts both on UV-irradiated and non-irradiated DNA (Kaplan *et al.*,1971; Kushner *et al.*, 1971; Nakayama *et al.*,1971). The enzyme (UV-exonuclease) is highly selective for single stranded DNA. Hydrolysis is initiated at either the 3' or the 5' terminus. Normally 5'P mononucleotides are released. However when the single stranded DNA is UV-irradiated small oligonucleotides containing the UV-photoproducts are released as well. The combined activities of UV-specific endonuclease and UV-exonuclease on dimer-containing double stranded DNA result in incision and subsequently a limited release of nucleotides.
It has been found that for each incision made, 6 nucleotides are removed from UV-irradiated *E.coli* DNA (Kaplan *et al.*,1969).

Since the exonuclease acts only on single stranded DNA it has been concluded that degradation of DNA is limited to the distorted region in the strand containing the dimer. UV-exonucleases, have also been isolated from phage T4 infected *E.coli* cells (Friedberg and King, 1971; Onshima and Sekiguchi, 1972) and from *E.coli* (Chase and Richardson, 1974a, and 1974b). These exonucleases have properties which are comparable to those of the *M.luteus* enzyme.

Repair replication.

From *in vivo* and *in vitro* studies it has been established that DNA polymerase I of *E.coli* is involved in excision-repair (de Lucia and Cairns, 1969; Gross and Gross, 1969; Boyle *et al.*,1970; Kelly *et al.*,1969; Cooper and Hanawalt, 1972; Tait *et al.*,1974; Glickman, 1974). For a comprehensive review of the properties of the enzyme see Kornberg, 1974.
DNA polymerase I is a multifunctional enzyme contained on a single polypeptide chain of molecular weight of 109,000 dalton. The various activities of the enzyme are : a DNA polymerizing activity, a 3'-5' exonucleolytic activity and a 5'-3' exonucleolytic activity on double stranded DNA. Limited proteolytic cleavage of the enzyme results in the formation of a large fragment containing the DNA polymerizing and the 3'-5' exonucleolytic activity and s small fragment containing the 5'-3' exonucleolytic activity (Brutlag *et al.*,1969; Klenow and Henningsen, 1970; Setlow and Kornberg, 1972; Setlow *et al.*,1972).
The 5'-3' exonuclease normally hydrolyzes DNA to 5' mononucleotides (Klett *et al.*,1968). However the enzyme can also excise oligonucleotides up to 10 residues in length from DNA containing mismatched or distorted regions (subterminal endonucleolytic activity), (Kelly *et al.*,1969).
When DNA polymerase I is bound to DNA at the site of a single strand nick, the nick serves as a starting point for DNA replication, provided that a 3'OH endgroup is present.
In the presence of the precursors for DNA replication, synthesis will proceed in the 5'-3' direction. At the same time, however, by virtue of its 5'-3' exonucleolytic activity, the enzyme degrades the DNA strand which is lying downstream of the nick. The result of these combined activities is a "translation of the nick" in the 5'-3' direction, which is a unique property of DNA polymerase I.
It has been found that DNA synthesis can stimulate concurrent 5'-3' exonucleolytic activity about 10 fold (Lehman, 1967). Coupling of the polymerizing function and the excising function in a single enzyme, may be

of advantage in excision-repair of damaged regions in DNA. As a thymine dimer is removed by the 5'-3' exonuclease function, the polymerase function concomitantly will fill in the gap caused by excision. Therefore, at no time during excision-repair by DNA polymerase I a single strand DNA region is present: only "nick translation" occurs by the combined action of the two enzymatic functions of DNA polymerase I (Cozzarelli *et al.*,1969). Translation of the nick is arrested when DNA polymerase I is displaced by DNA ligase. Although the 3'-5' exonucleolytic activity of DNA polymerase I is not involved in excision-repair of damaged regions, it is of interest to mention that this activity has a repair function as well. When by mistake a wrong base is incorporated into DNA, causing mismatching, further polymerization is arrested, until the unpaired base is removed by the 3'-5' exonucleolytic activity. This process of "error-correction" is called proofreading (Brutlag and Kornberg, 1972). It assures a high fidelity of replication.
From *M.luteus* a DNA polymerase has been isolated with properties similar to that of DNA polymerase I of *E.coli* (Litman, 1970; Miller and Wells, 1972; Hamilton *et al.*,1974).

Sealing.

The joining of the remaining nick between the newly replicated DNA chain and the juxtaposed preserved DNA chain is catalysed by DNA ligase (Gellert, 1967; Weiss and Richardson, 1967; Olivera and Lehman, 1967; Gefter *et al.*, 1967; Cozzarelli *et al.*, 1967). In a variety of prokaryotic as well as eukaryotic cells (Lindahl and Edelman, 1968; Howell and Stern, 1971) ligase activity has been demonstrated and the enzyme probably is a normal constituent of all living cells. DNA ligase is an essential enzyme required not only for DNA repair but also for DNA replication in *E.coli* (Pauling and Hamm, 1968; Gellert and Bullock, 1970; Konrad *et al.*, 1973). DNA ligase from *E.coli* (Modrich *et al.*,1973) and DNA ligase induced by phage T4 (Panet *et al.*,1973) have been purified to homogeniety and their catalytic properties have been investigated (Modrich and Lehman, 1973). A recent survey on the structure, the mechanism of action and the function of these enzymes has been presented by Lehman (1974). The formation of a phosphodiester bond between a 3'OH terminus and a adjacent 5'P endgroup by *E.coli* or T4-induced DNA ligase is accomplished in three sequential steps. In the first step, the enzyme is activated

by NAD (*E.coli* DNA ligase) or ATP (T4 induced DNA ligase) leading to the formation of an enzyme-adenylate complex and nicotineamide mononucleotide or pyrophosphate(Little *et al.*,1967; Weiss *et al.*,1968; Gumport and Lehman, 1971). In the second step, the adenylgroup which is attached to the enzym is transferred to DNA to generate a pyrophosphate linkage between adenosine monophosphate (AMP) and the 5'phosphoryl terminus at the nick (Olivera *et al.*, 1968; Wang, 1971). In the last step the 5' pyrophosphate linkage is attacked by the 3' hydroxyl group to form a phophodiester bond and AMP is released.

REPAIR OF OTHER DAMAGE

There is strong evidence that the mechanism of excision-repair is not only restricted to UV-lesions, but might also apply to a variety of lesions induced by physical agents such as ionizing radiation or chemical agents *e.g.* carcinogens (Haynes, 1964; Lawley and Brookes, 1965; Boyce and Howard-Flanders, 1964b; Friedberg and Goldthwait, 1969). An endonuclease acting specifically on γ-irradiated DNA has been isolated from *M.luteus* (Paterson and Setlow, 1972) and an endonuclease specific for alkalyted DNA has been isolated from *E.coli* (Hadi *et al.*,1973).

REPAIR IN EUKARYOTIC CELLS

Using similar techniques as employed in bacteria to study excision-repair, it was found that excision-repair also occurs in a number of mammalian cells, including human cells (Regan *et al.*,1968; Cleaver and Painter, 1968; Regan *et al.*,1971; Mattern *et al.*,1973) and protoplasts of the carrot (Howland, 1975). From mammalian cells specific endonucleases have been isolated as well : Ljungquist and Lindahl (1974) have isolated an endonuclease specific for a-purinic sites in DNA and Bacchetti and Benne (1975) have purified and characterized a γ-specific endonuclease from calf-thymus. The importance of a functional excision-repair system in human cells to remove UV-induced photoproducts became apparent by the discovery of Cleaver (1968) that skin fibroblasts from patients suffering from the hereditary disease *Xeroderma pigmentosum* are unable to repair UV-induced damage from their DNA. Cells from *Xeroderma pigmentosum* patients are unable to carry out one of the early steps of the excision-repair process (Setlow *et al.*,1969; Paterson *et al.*,1973). Also host cell reactivation of UV-irradiated Herpesvirus or

SV40 virus is markedly impaired in these cells (Rabson *et al.*,1969; Aaronson and Lytle, 1970).

A STUDY OF EXCISION-REPAIR IN VITRO

In the previous section we have described a model for excision-repa. This model is based largely on the results of experiments *in vivo* and on the properties of the enzymes, which are thought to be involved in this repair process. At the time our investigation was started, the complete excision-repair process had not been realized *in vitro*, although the invidual steps had been studied in some detail. Therefore we have started an investigation of the repair of the biological activity of transforming DNA from *B.subtilis* after irradiation with UV-light, using purified enzymes. The important property of this system is that the complete repair process has to be carried out *in vitro*, in order to measure recovery of biological activity. The results of this study are presented in the first publication. In the second publication we have described the properties of DNA polymerase I isolated from a strain of *E.coli* which is sensitive to UV-light. From a genetic analysis of this strain it appeared that the mutation (polA107) leading to sensitivity to UV-light, was located on the gene coding for DNA polymerase I. Therefore we have investigated which of the enzymatic functions of DNA polymerase I was altered in this strain.
In the third publication results are presented of a study about the localization of the polA107 mutation in the gene coding for DNA polymerase I.
In the fourth publication a new method to study excision-repair of UV-damaged DNA *in vitro* is described. Repair is carried out on covalently closed double stranded DNA from phage ØX174 instead of transforming DNA from *B.subtilis*. The attraction of this method is that the repair reaction can be followed with biological as well as with physico-chemical techniques. The possible functions of exonuclease III and DNA polymerase I isolated from the *E.coli* polA107 mutant in excision-repair was investigated.

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In Vitro Excision-Repair of Ultraviolet-Irradiated Transforming DNA from *Bacillus subtilis*

(DNA excision/DNA ligase/gene repair/F⁻strain/DNA polymerase)

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ABSTRACT **The biological activity of UV-inactivated *Bacillus subtilis* DNA is partly restored after incubation with a UV-specific endonuclease from *Micrococcus luteus* in conjunction with DNA polymerase and DNA ligase, both isolated from *Escherichia coli*. The restored activity is not further increased by photoreactivation. Pyrimidine dimers are specifically liberated when irradiated DNA is exposed to the three enzymes. None of these effects is observed when pancreatic DNase is used instead of UV-specific endonuclease.**

Since the demonstration by Rörsch *et al.* (1) and by Elder and Beers (2) that dark-repair of UV-irradiated DNA is enzymatic in nature, much effort has been given to elucidate its mechanism. Using UV-irradiated ϕX 174 replicative form type I (RFI) DNA and extracts from *Micrococcus luteus*, Rörsch *et al.* (3) showed that simultaneously with an increase in biological activity (assayed as the ability to form virus particles in spheroplasts of *Escherichia coli*), single-strand breaks are introduced in RFI-DNA, suggesting that an endonuclease is involved in the reactivation process.

Setlow and Carrier (4) and Boyce and Howard-Flanders (5) observed that thymine dimers are excised from UV-irradiated DNA in the *in vivo* dark-repair process. They proposed a repair mechanism in which dimers are removed by the combined action of an endonuclease, which recognizes dimers, and an exonuclease, which excises the UV-damaged region. After repair-replication (6), the physical continuity of the DNA strand is restored by DNA ligase (7).

Results of *in vitro* experiments support this model: Carrier and Setlow (8) were able to demonstrate excision of pyrimidine dimers from UV-irradiated DNA in extracts from *M. luteus*. Both a UV-specific endonuclease and an exonuclease have been purified from this organism (9, 10). The endonuclease specifically recognizes dimers (11, 12). Recently, Kelly *et al.* (13) demonstrated that DNA polymerase from *E. coli* is able to excise pyrimidine dimers and to insert new nucleotides into the DNA chain in the 5′ to 3′ direction, thus 'translating' single-strand nicks.

These results prompted us to investigate whether UV-irradiated DNA can be repaired *in vitro* with UV-specific endonuclease, DNA polymerase, and DNA ligase. To detect repair, we measured the recovery of biological activity of UV-inactivated *Bacillus subtilis* transforming DNA.

The *B. subtilis* transformation system is particularly well suited for this purpose. First, mutants of *B. subtilis* are known in which *in vivo* dark-repair of UV-inactivated transforming DNA is blocked (14)§, so that *in vitro* repair of UV-damage is not obscured by repair occurring *in vivo*. Second, in UV-sensitive mutants of *B. subtilis*, biological activity of UV-inactivated transforming DNA is further decreased after it is nicked with UV-specific endonuclease (14), implying that complete dark-repair would have to occur *in vitro* before an increase in biological activity could be observed. This is in contrast with other biological test systems, such as *Haemophilus influenzae* transformation (15) and the previously mentioned *E. coli* spheroplast assay with ϕX 174 RF DNA, in which nicking of their DNA with UV-specific endonuclease suffices to restore biological activity as measured in host-cell reactivation negative (*hcr*⁻) mutants.

MATERIALS AND METHODS

Bacterial Strains. The following strains were used: *M. luteus* ATCC 4698; *E. coli* KMBL 1067 F⁻ *thy* A 301 *bio*-87 *end* A 101 *pol* A 1; and *E. coli* KMBL 1068 F⁻ *thy* A 301 *bio*-87 *end* A 101. The *E. coli* strains (constructed by B. Glickman and M. Groothuis) were derived from *E. coli* KMBL 1060 F⁻ *thy* A 301 *bio*-87 *end* A 101 *met* E 72 by means of transduction with bacteriophage P_1 grown on *E. coli* W 3110 *pol* A 1 (kindly provided by Dr. J. Cairns). The suppressor-free strain KMBL 1060 was derived from wild-type *E. coli* W 1485 through a series of P_1-mediated transductions.

B. subtilis strain 8G-5 was derived from *B. subtilis* 168 *ind*⁻ Marburg strain[§] and carries an *ade*, *his*, *nic*, *ura*, *rib*, and *met* marker, in addition to the linked markers trp_2 (= ind_{168}) and tyr_1 that show 70% joint transfer in transformation. *B. subtilis* strain 8G-11 is a *hcr*⁻ derivative of strain 8G-5, in which DNA breakdown and recovery of DNA synthesis after UV-exposure is blocked. Repair of UV-inactivated transforming DNA is severely impaired or completely absent in this strain.[§]

DNA Preparations. *B. subtilis* transforming DNA was isolated from the wild-type Marburg strain according to Venema *et al.* (16). [^{3}H]Thymidine-labeled DNA (3 Ci/g of DNA) was

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isolated from a thymine-requiring derivative of the Marburg strain.

Transformation. Cultures were made competent as described by Venema *et al.* (16) and transformed with limiting amounts of DNA (0.04 μg/ml). *Trp*$_3^+$, *tyr*$_1^+$, or *ade*$^+$ single transformants and *trp*$_3^+$-*tyr*$_1^+$ double transformants were scored on appropriately supplemented minimal-agar plates. To prevent *in vivo* photorepair, the procedure was done under dim light and the plates were incubated in the dark.

UV-Irradiation. 2-ml samples of DNA (9 μg/ml) in potassium phosphate buffer (10 mM, pH 7.5, containing 1 mM EDTA and 90 mM KCl) were irradiated with UV on gently agitated watchglasses with a Hanovia low-pressure germicidal lamp (254 nm, dose rate 50 ergs/mm^2 per sec).

Photoreactivation. Photoreactivation (1 hr, 37°C) was performed with a Philips TL 20W/08 black-light lamp (8-cm distance) in the presence of photoreactivating enzyme, obtained from yeast by the method of Wulff and Rupert (17).

Enzymes. UV-specific endonuclease was purified from *M. luteus* according to Nakayama *et al.* (18). The phosphocellulose fraction was free from other endonucleases, as determined by the resistance to endonucleolytic cleavage of φX 174 RFI-DNA. DNA polymerase was purified from *E. coli* KMBL 1068 as described by Jovin *et al.* (19). The DEAE-cellulose fraction contained 60 units/ml and was free of endonuclease activity, but contaminated with exonuclease III activity.

DNA ligase was purified from *E. coli* KMBL 1067 (*pol* A$_1^-$) as described by Gefter *et al.* (20). After treatment with alumina Cγ, the enzyme was further purified by DEAE-cellulose chromatography (21). The preparation was free of polymerase and endonuclease activity, but was contaminated with exonuclease III activity, as measured by the formation of acid-soluble products when φX 174 RFII-DNA was incubated with the enzyme preparation in the presence of nicotinamidemononucleotide (NMN).

Conditions Used for Dark-Repair of UV-Irradiated B. subtilis DNA. 0.1 ml of *B. subtilis* DNA (9 μg/ml) was incubated for 20 min at 34°C with 0.1 ml of UV-specific endonuclease (in 10 mM potassium phosphate–1 mM EDTA, pH 7.5) and 0.1 ml of 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 6 μM t-RNA, and 90 mM KCl. The mixture was subsequently heated for 15 min at 68°C to inactivate the enzyme. 0.1 ml of the reaction mixture was then incubated (30 min, 34°C) with 0.1 ml of enzyme mixture, consisting of 0.03 ml DNA polymerase [diluted 1:10 with 0.05 M potassium phosphate buffer (pH 7.5)], 0.07 ml of DNA ligase, and 0.1 ml of 0.06 M Tris·HCl buffer (pH 8.0) containing 13 mM $MgCl_2$, 3 mM EDTA, 0.16 M KCl, 1 mM dithiothreitol, 1 mM NAD^+, and 20 μM of each of the four deoxyribonucleoside triphosphates. In experiments in which UV-specific endonuclease was replaced by pancreatic DNase, the incubation mixture was additionally supplemented with 10 mM $MgSO_4$. 0.1 ml of the final mixtures were used for transformation of 2.5 ml of competent cells (5×10^8 cells/ml).

Determination of Pyrimidine Dimers. UV-irradiated DNA was hydrolyzed in formic acid and the amount of pyrimidine dimers was determined by two-dimensional paper chromatography according to Setlow *et al.* (22).

RESULTS

Typical results of *in vitro* reactivation of UV-irradiated transforming DNA are shown in Table 1. The data presented in columns I–IV show that none of the enzymes (UV-specific endonuclease, DNA polymerase, and DNA ligase) affects the activity of unirradiated transforming DNA, indicating both the absence of endonuclease activity and the absence of repairable breaks in the DNA preparation. On the other hand, the biological activity of UV-irradiated DNA (V) is reduced by UV-specific endonuclease (VI), showing that the enzyme specifically attacks UV-irradiated DNA. In the absence of prior treatment with UV-specific endonuclease (VII), no change in biological activity is observed after the irradiated

TABLE 1. *Reactivation of UV-irradiated transforming DNA from* B. subtilis

		Unirradiated				UV-irradiated			
		I	II	III	IV	V	VI	VII	VIII
		No enzymes	UV-specific endonuclease	Polymerase and ligase	UV-specific endonuclease, followed by polymerase and ligase	No enzymes	UV-specific endonuclease	Polymerase and ligase	UV-specific endonuclease, followed by polymerase and ligase
Number of transformants per ml; and, in parentheses, residual transforming activity (%)	*trp*$_3^+$	80600 (100)	79900 (99)	83400 (103)	85500 (106)	27320 (34)	11620 (14)	27710 (34)	42420 (53)
	tyr$_1^+$	85200 (100)	93900 (111)	89650 (105)	89000 (104)	27200 (32)	9930 (12)	29730 (35)	39100 (46)
	trp$_3^+$-*tyr*$_1^+$ doubles	49400 (100)	47300 (96)	52300 (106)	48300 (98)	6580 (13)	1330 (2.7)	7190 (15)	15120 (31)

DNA extracted from prototrophic cells was irradiated with 1500 ergs/mm^2. Irradiated and unirradiated samples were incubated (*a*) without enzymes (I and V); (*b*) with UV-specific endonuclease (II and VI); (*c*) with DNA polymerase and DNA ligase (III and VII), and (*d*) with UV-specific endonuclease, followed by DNA polymerase and DNA ligase (IV and VIII). Samples from the incubation mixtures were used for transformation of competent 8G-11 (*hcr*$^-$) cells. Platings were in triplicate. At least 1000 transformed colonies were counted for each value presented.

TABLE 2. *Excision of pyrimidine dimers from UV-irradiated DNA*

	UV-irradiated DNA treated with:		
	UV-specific endonuclease	UV-specific endonuclease, followed by polymerase and ligase	Pancreatic DNase, followed by polymerase and ligase
$\frac{\text{Dimers}}{\text{Monomers}}$, cpm in alcohol-soluble fraction	$\frac{133}{5705} = 0.02$	$\frac{9224}{59798} = 0.15$	$\frac{1613}{95081} = 0.02$
$\frac{\text{Dimers}}{\text{Monomers}}$, cpm in alcohol-precipitable fraction	$\frac{22081}{555014} = 0.04$	$\frac{27615}{1019277} = 0.03$	$\frac{36915}{949930} = 0.04$
Percentage of total DNA made alcohol-soluble	1	6	10
Percentage of dimers made alcohol-soluble	0.5	25	5

B. subtilis DNA (9 μg/ml) labeled with [^{3}H]thymidine (3 μCi/ml) was irradiated with 7000 ergs/mm^2 of UV-light and incubated under conditions used for dark-repair. After incubation, carrier DNA from *B. subtilis* and two volumes of absolute ethanol were added to the fractions. The mixtures were kept at 0°C for 1 hr, before centrifugation for 20 min at 5000 × *g*. The supernates which contained alcohol-soluble nucleotides, and the pellets, which contained DNA, were assayed for the presence of pyrimidine dimers.

DNA is incubated with polymerase and ligase. However, when DNA is first incubated with UV-specific endonuclease and subsequently with polymerase and ligase (VIII), biological activity is increased appreciably, as compared to the UV-induced extent of inactivation (V). This demonstrates that after nicks have been introduced by the UV-specific endonuclease, UV-damage is repaired by the joint action of DNA polymerase and DNA ligase. The biological activity of the single markers trp_2 and tyr_1 recovers from 33% (after irradiation) to 50% (after repair). The unlinked *ade* marker behaves similarly (results not shown). Cotransformation of the linked trp_2-tyr_1 markers is an even more sensitive assay for recovery of biological activity (from 13 to 32%). This is due to the fact that joint transformation of linked markers is more sensitive to UV than transformation of single markers[4](23); conversely, removal of UV-damage will restore joint transformation more efficiently than single-marker transformation.

The type of experiment presented in Table 1 was repeated 10 times. Single-marker activities increased by a factor 1.5 ± 0.2 and the joint activity of the trp_2-tyr_1 marker pair by a factor 2.2 ± 0.3. The results of Table 1 allow us to compute the fraction of inactivating lesions repaired. Based on the theory of inactivation of transforming DNA, Bresler and coworkers (24) derived the relationship: $Z = 2(\sqrt{1/\beta} - 1)$, where Z stands for the number of inactivating lesions per genetic unit of length, and β for residual transforming activity. If it is assumed that the genetic unit of length for a particular marker is independent of the amount of damage present, the fraction of inactivating lesions repaired is equal to $(1 - Z_2/Z_1)$, where Z_1 and Z_2 stand for the number of inactivating lesions per unit length before and after repair, respectively. The mean value $(1 - Z_2/Z_1)$ calculated from our data is 0.50 for the trp_2 marker, 0.39 for the tyr_1 marker, and 0.56 for the trp_2-tyr_1 linked marker pair. This means that about 50% of all inactivating lesions were repaired successfully.

When the hcr^+ strain 8G-5 was used as a recipient in transformation with irradiated DNA, no, or only a slight, increase in biological activity was observed after repair by UV-specific endonuclease, DNA polymerase, and DNA ligase. This is expected, since the 8G-5 strain is able to perform dark-repair of irradiated transforming DNA, thus obscuring the effect of *in vitro* repair.

TABLE 3. *Photoreactivation after incubation of UV-irradiated DNA with various enzymes*

			Unirradiated DNA	UV-irradiated DNA				
			I	II	III	IV	V	VI
		Photo-reactivation	No enzymes	No enzymes	UV-specific endonuclease	Pancreatic DNase	UV-specific endonuclease, followed by polymerase and ligase	Pancreatic DNase, followed by polymerase and ligase
Residual transforming activity (%)	trp_2^+	−	100	15	3.3	5.9	27	15
		+	91	32	2.9	11	34	33
	(trp_2^+-tyr_1^+) doubles	−	100	5.6	0.4	1.4	15	6.5
		+	84	23	0.4	3.6	19	16

Donor DNA (20 μg/ml) irradiated with 2000 ergs/mm^2, was incubated with UV-specific endonuclease (III) or pancreatic DNase (IV). After they were heated for 15 min at 68°C, both samples were incubated with DNA polymerase and DNA ligase (V and VI). The reaction was stopped by the addition of sodium citrate to 0.15 M. Photoreactivation was subsequently performed in solutions consisting of equal volumes of treated sample, SSC (0.15 M NaCl + 0.015 M sodium citrate), and photoreactivating enzyme. Controls were treated similarly, except that they were kept in the dark. Treated samples were used to transform 8G-11 (hcr^-) cells. Platings were in triplicate. At least 1000 transformed colonies were counted for each value presented, except for values less than 4%. (0.4% represents 100 colonies counted.)

In order to investigate whether the *in vitro* restoration of biological activity is due to dimer excision, we measured the release of dimers from irradiated [^{3}H]DNA. The data (Table 2) show that incubation of irradiated *B. subtilis* DNA with UV-specific endonuclease, followed by DNA polymerase and DNA ligase, results in the specific liberation of pyrimidine dimers as alcohol-soluble products. The specificity of the reaction is demonstrated by the observation that no specific excision occurs when UV-specific endonuclease is replaced by pancreatic DNase with comparable endonucleolytic activity. Furthermore, incubation of the irradiated DNA with UV-specific endonuclease alone does not result in excision of dimers, indicating that the enzyme preparation is not contaminated with exonucleases capable of excising dimers. In contrast to the calculation from Table 1 that 50% of the inactivating lesions are repaired, this experiment shows that only 25% of the dimers were removed from the DNA. Since the UV doses in the two experiments were different (1500 and 7000 ergs/mm^2, respectively), the results are not directly comparable.

The effect of photoreactivation on irradiated DNA treated with the various enzymes (Table 3) gives additional information about the repair mechanism studied. As can be seen from the data in column III, photorepair does not increase biological activity of irradiated DNA treated with UV-specific endonuclease, presumably because the presence of a single-strand nick very close to a pyrimidine dimer obscures the effect of photoenzymatic monomerization. However, when single-strand nicks are introduced randomly by pancreatic DNase, one may expect to find partial restoration of biological activity after photorepair, since the nicks will then only occasionally be located close to dimers. This expectation is borne out by the experiment (column IV).

The observation that the biological activity of DNA treated with UV-specific endonuclease, DNA polymerase, and DNA ligase is only slightly increased upon photoreactivation (V), whereas the biological activity of irradiated DNA incubated with pancreatic DNase, followed by repair with DNA polymerase and DNA ligase, is substantially increased (VI), justifies the conclusion that in the repaired DNA a large fraction of pyrimidine dimers is replaced by the normal constituents.

DISCUSSION

Our results demonstrate that UV-damage in DNA is repaired by the combined action of a UV-specific endonuclease, DNA polymerase, and DNA ligase. The three enzymes restore partial biological activity of UV-irradiated transforming DNA and specifically excise pyrimidine dimers. If UV-specific endonuclease is replaced by pancreatic DNase, restoration of biological activity is not observed, nor could excision of pyrimidine dimers be found. The susceptibility to photorepair is greatly diminished after incubation of UV-irradiated DNA with UV-specific endonuclease, DNA polymerase, and DNA ligase, but substantial photorepair is still observed when pancreatic DNase replaces UV-specific endonuclease. These results lend further support to the conclusion that the restoration of biological activity is due to the specific removal of pyrimidine dimers. The number of nucleotides excised for each dimer can be computed from the ratio of radioactivity in the monomer and dimer fraction of the alcohol-soluble material. Based on the thymine content of *B. subtilis* DNA (28%), and an assumed relative yield of 50:40:10 for thymine–thymine, thymine–cytosine, and cytosine–cytosine dimers after UV-irradiation (25), values of 29 and 38 nucleotides excised per dimer were calculated from two independent experiments. These values are close to those obtained by Setlow and Carrier (4), and Pettijohn and Hanawalt (6) from *in vivo* experiments (20 and 30 nucleotides excised per dimer, respectively).

As has been mentioned, both the DNA polymerase and DNA ligase preparations are contaminated with exonuclease III activity. Since exonuclease III operates in the 3′ to 5′ direction (26), and UV-specific endonuclease from *M. luteus* introduces nicks at the 5′ side of pyrimidine dimers (27), exonuclease III cannot be held responsible for the excision of dimers. For this reason, the excision must have been done by the 5′ to 3′ exonucleolytic activity of DNA polymerase (13). However, the possibility that exonuclease III is involved in the repair system has been suggested by Kelly *et al.* (13) in that the phosphatase activity of the enzyme may remove the 3′-phosphate end-groups generated by the UV-specific endonuclease (27) to provide the 3′-OH end-groups required for polymerization in the 5′ to 3′ direction.

Although the components of our *in vitro* repair system have been obtained from different organisms, we believe that the three enzymes used are, at least partly, responsible for the excision of pyrimidine dimers *in vivo* and act in a way as suggested by Kelly *et al.* (13). A possible role of DNA polymerase and DNA ligase in the process of repair of UV-damage was already suggested by the increased sensitivity to UV-light of mutants of *E. coli* lacking active DNA polymerase (28, 29) or DNA ligase (30, 31). Nevertheless, it cannot be excluded that excision of pyrimidine dimers can also occur through an alternative enzymatic pathway. The moderate UV-sensitivity of *E. coli pol* A 1 as compared to *uvr*A, *uvr*B or *uvr*C mutants (32), and the observation (33) that the rate and final extent of dimer excision are normal in the *pol* A 1 mutant, support the view that other enzyme(s) can replace DNA polymerase in the repair process. Recently, Monk *et al.* (34) suggested that the product of the *rec* A gene in *E. coli* can replace DNA polymerase in the repair-replication step.

The function of UV-specific endonuclease in *in vivo* repair of UV-damage is not clear. A number of UV-sensitive strains from *E. coli* and from *M. luteus* are unable to excise dimers after UV-irradiation and show severely restricted reactivation of UV-damaged bacteriophage. When assayed *in vitro* for the presence of UV-specific endonuclease, these strains have normal levels of enzyme activity (35, 36), while a mutant from *M. luteus* lacking this UV-specific endonuclease is only slightly UV-sensitive (36, 37). The discrepancy between *in vivo* and *in vitro* experiments could be resolved if excision-repair of UV-damaged DNA occurs *in vivo* by two different mechanisms localized in different parts of the cell. Pyrimidine dimers might normally be excised by membrane-bound enzymes controlled by *uvr*-genes. A second mechanism of dimer excision that acts by UV-specific endonuclease and DNA polymerase might be localized in the cytoplasm.

Apart from functioning in the removal of pyrimidine dimers, DNA polymerase in conjunction with DNA ligase might also play a role in the repair of single-strand breaks and gaps in DNA. The high sensitivity of *E. coli pol* A 1 to x-rays and methyl methane sulfonate, in comparison to its sensitivity to UV-light (28), and the occurrence of single-strand breaks in newly synthesized DNA in this mutant (38), support this view.

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A Mutant of *Escherichia coli* K12 Deficient in the 5′—3′ Exonucleolytic Activity of DNA Polymerase I

II. Purification and Properties of the Mutant Enzyme

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Summary. The enzymatic properties of purified DNA polymerase I from a strain of *Escherichia coli* K12 with a mutation in the *polA* gene have been studied. The polymerizing activity of the mutant enzyme is similar to that of the enzyme from isogenic wild-type cells, when the activity is measured on exonuclease III treated calf-thymus DNA. Also the 3′—5′ exonucleolytic activity is not significantly different for both enzyme preparations. The 5′—3′ exonucleolytic activity of DNA polymerase I isolated from the mutant strain, however, is much lower than that of wild-type DNA polymerase I. The products formed by the action of the wild-type and the mutant enzyme on nicked circular double-stranded DNA of phage ϕX174′(RFII DNA) were analysed by sucrose-gradient sedimentation and electron-microscopy. When RFII DNA was incubated with wild-type enzyme 80% of the molecules were converted into linear molecules. All linear molecules were shorter than one phage genome. Only 25% of the molecules were branched. After incubation of RFII DNA with the mutant enzyme 62% of the molecules have become linear. More than 90% of these linear molecules were branched and the majority of them was longer than one phage genome.

Introduction

In the preceding paper (Glickman *et al.*, 1973) it was shown that the mutation responsible for the sensitivity of *Escherichia coli polA′107* to irradiation with ultraviolet light or X-rays and to methyl methanesulfonate is located in the gene coding for DNA polymerase I. Since DNA polymerizing activity was present in crude extracts prepared from this strain (Glickman *et al.*, 1972), we were interested to see if one of the exonucleolytic activities, which are normally displayed by the enzyme had been affected by the mutation (Lehman and Richardson, 1964; Klett *et al.*, 1968; Deutscher and Kornberg, 1969; Kornberg, 1969). Therefore we have purified DNA polymerase I from the mutant strain and compared its enzymatic properties with purified DNA polymerase I from an isogenic wild-type strain.

Materials and Methods

Bacteria. Wild-type DNA polymerase I was purified from *E. coli* strain KMBL 1790 and mutant DNA polymerase I was purified from *E. coli* strain KMBL 1789. The characteristics of these strains were described by Glickman *et al.* in the preceding paper.

Bacteria were cultivated in 5 litre batches in minimal medium (Vogel and Bonner, 1956) supplemented with 0.25% casamino acid, 0.2 μg biotine per ml, 20 μg thymine per ml and 0.4% glucose. Bacteria, which were in the exponential phase of growth, were harvested by centrifugation, washed once with 50 mM Tris-HCl buffer pH 7.6, containing 0.2 M KCl, 1 mM MSH, 1 mM EDTA, 1 mM NaN_3 and 5% glycerol and stored at −20° until use.

Enzymatic Assays. DNA polymerase I activity was assayed according to Richardson *et al.* (1964a). 3′--5′ exonucleolytic activity (Lehman and Richardson, 1964) was assayed by measuring the degradation at 37° of single-stranded T7 DNA to acid-soluble products in 0.25 ml containing: 17 μmoles potassium-phosphate (pH 7.4), 1.7 μmoles $MgCl_2$, 17 μg BSA, 0.6 μg heat-denatured ^{32}P-labelled T7 DNA and DNA polymerase I (0.5–5 units) (Setlow *et al.*, 1972). The reaction was stopped by the addition of 0.1 ml carrier DNA (2 mg/ml) in 0.1 M EDTA and 0.35 ml ice-cold 10% TCA. After leaving the material for 20 min in ice, the precipitate which was formed was removed by centrifugation (1 min at 12000 rpm in an Eppendorf 3200 centrifuge). Of the supernatant solution 0.5 ml was pipetted onto planchets and the radioactivity which was present was measured with a Geiger-Müller counter. Exonuclease III activity was determined according to Richardson *et al.* (1964b). A combined assay for the 5′—3′ exonucleolytic activity and DNA polymerase I activity was carried out as follows: the incubation-mixture (0.3 ml) contained 21 μmoles glycine-KOH buffer (pH 9.2), 1.8 μmoles $MgCl_2$, 0.3 μmole DTT, 5.1 nmoles of each of the four deoxyribonucleoside triphosphates (dTTP was labelled with ^{3}H; 500 mC per mmole) and 0.3 μg [^{32}P]-RFII DNA. The reaction which was carried out at 37° C was started by the addition of DNA polymerase I and was terminated by the addition of 0.2 ml of a mixture of sodium-pyrophosphate (33 mM), EDTA (33 mM), and carrier DNA (2 mg per ml). The DNA was precipitated by the addition of 0.5 ml ice-cold 10% TCA in 10 mM sodium-pyrophosphate, collected on glass filters, washed 3 times with 3 ml 5% TCA in 10 mM sodium-pyrophosphate and once with 10 ml ice-cold ethanol. After drying the filters at 100°, the radioactivity of the filters (^{3}H label), which is a measure for the activity of DNA polymerase I, was counted in a liquid scintillation counter. The filtrate was collected directly in scintillation vials and the radioactivity (^{32}P label), representing 5′—3′ exonucleolytic activity, was measured as Čerenkov radiation in the ^{3}H-channel of a liquid scintillation counter.

Enzyme Units. One polymerizing unit of DNA polymerase I is defined as the amount of enzyme catalyzing the incorporation of 10 nmoles of nucleotide into acid-insoluble product in 30 min at 37° on calf-thymus DNA "activated" with exonuclease III (Richardson *et al.*, 1964a). One unit of exonuclease III activity is defined as the amount of enzyme rendering 1 nmole of phosphate acid soluble in 30 min at 37° C (Richardson *et al.*, 1964b).

Purification of DNA Polymerase I and Exonuclease III. All procedures were carried out at 0–4°. The buffers used contained 1 mM EDTA, 1 mM MSH, 1 mM NaN_3 and 5% glycerol.

A bacterial extract was prepared by grinding a suspension of 50 g bacteria (*E. coli* strain KMBL 1790 *pol*$^+$ or strain KMBL 1789 *polA'107*) together with 150 g glass-beads in 80 ml 50 mM Tris-HCl buffer pH 7.6, 0.2 M KCl in a Sorvall omnimixer (speed-control in position 8) twice for 4 min. The cellular debris and glass-beads were removed by centrifugation (10 min at 5000 × g) and the sediment was extracted once with 20 ml of the same buffer. After combining the supernatant solutions KCl was added to a final concentration of 1.7 M and the solution was centrifuged for 3 hours at 140000 × g. To the supernatant solution solid polyethylene glycol 6000 was added to a final concentration of 10% (w/v) and the solution was kept in ice during 5 h. The precipitate which was formed and which contained the bulk of the DNA was removed by centrifugation (10 min at 10000 × g) (Alberts and Herrick, 1971). The KCl concentration of the supernatant solution, which contained the enzymatic activity, was reduced to 0.1 M KCl by dialysis against 10 mM Tris-HCl (pH 7.6).

A column (16 cm^2 × 8 cm) filled with DNA-agarose was washed with 10 mM Tris-HCl buffer pH 7.6, 0.1 M KCl and the extract (~400 ml) was passed through the column in upwards direction. More than 90% of the total protein did not adsorb to DNA-agarose (Schaller *et al.*, 1972) and was removed by washing the agarose with 150 ml of the same buffer. When a salt gradient was applied (0.1 → 1.0 M KCl in the same buffer; total volume 500 ml), exonuclease III eluted at 0.3 M KCl and DNA polymerase I at 0.5 M KCl. The speed of elution was approximately one half column-volume per hour. The fractions containing exonuclease III

activity (75 ml; 350 units/ml) were pooled and stored at 4°. This enzyme preparation was used without further purification to prepare "activated" calf-thymus DNA. The fractions containing DNA polymerase I activity were pooled, dialyzed against 20 mM potassium-phosphate buffer (pH 6.5) and chromatographed on phospho-cellulose as described by Jovin *et al.* (1969). The enzymatic activity of the pooled fractions (20 ml) eluted from phospho-cellulose was about 250 units/ml. The specific activity of both preparations was >20000 units/mg. When purified preparations of DNA polymerase I from wild-type bacteria or mutant bacteria were subjected to electrophoresis in polyacrylamide gels in the presence of SDS only one protein band was visible after staining with amido black. Purified preparations of DNA polymerase I from wild-type bacteria or mutant bacteria were essentially free from exonuclease III and endonuclease activity as measured by the conversion of RFI DNA to RFII DNA (Center *et al.*, 1970).

DNA Preparations. Covalently closed, circular, double-stranded ϕX174 DNA (RFI DNA) was prepared according to Jansz *et al.* (1966). Nicked, circular, double-stranded ϕX174 DNA (RFII DNA) was prepared from RFI DNA (5 μg/ml) by incubation with pancreatic DNAase (Worthington, 2× crystallized; 40 ng/ml) in 10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ during 15 min at 25°C. The reaction was stopped by the addition of excess EDTA. To inactivate the pancreatic DNAase, the solution was heated for 10 min at 65°C. The RFII DNA, which was formed, contained on the average 3 to 4 breaks per molecule as was calculated from the amount of radioactive material sedimenting at the position of RFI DNA in a neutral sucrose gradient. After dialysis against 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, the DNA was stored at −20°C. T7 DNA was prepared as described by van Dorp *et al.*, 1973.

Activated Calf-Thymus DNA. A limited number of single-stranded breaks was introduced into calf-thymus DNA (highly polymerized; EGA-chemie, Germany) by incubating 50 mg DNA with 0.5 μg pancreatic DNAase in 10 ml 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM $MgCl_2$ and 5 mg BSA (crystalline; Nutritional Biochemicals Corporation, Cleveland, USA) for 10 min at 37°. The reaction was terminated by the addition of excess EDTA and the enzyme was inactivated by heating for 10 min at 65°. After dialysis against 10 mM Tris-buffer (pH 7.6) this DNA was incubated subsequently with exonuclease III as described by Richardson *et al.* (1964b), until about 10% of the DNA had become acid-soluble. The reaction was stopped by the addition of excess EDTA and the enzyme was inactivated by heating the solution for 10 min at 65°. Finally the "activated" calf-thymus DNA was dialysed against 10 mM Tris-HCl buffer pH 7.6, 1 mM EDTA, 1 mM NaN_3 and stored at 4°C.

Electron-Microscopy. The DNA samples were prepared for electron-microscopy by the modified protein film technique described by Chattoraj and Inman (1972). 20 μl of a solution containing DNA was mixed with 10 μl of a solution containing 68 mM Na_2CO_3, 10.7 mM EDTA and 34% HCHO. Before spreading 30 μl formamide and 6 μl cytochrome-c (filtered through a 0.1 μ Sartorius membrane filter) were added. 20 μl of the mixture were spread on a 1.2 ml drop of water. Copper grids (400 mesh) covered with a thin carbon film, were touched to the surface. The samples were stained for 30 sec with 50 μM uranyl-acetate in 90% ethanol (Davis *et al.*, 1971), immersed in isopentane for 10 sec and dried. Some of the grids were rotary shadowed with platinium for bright field electron-microscopic observation. Dark field electron-micrographs were obtained in a Philips EM 200 electron-microscope, at a magnification of about 10000, which had been placed in the dark field mode by tilting the electron beam. Contrast was inverted by printing the electron-exposed film (Kodalith LR 2572) onto 24 × 30 cm^2 Kodalith ortho III followed by printing onto a normal contrast grade paper. Length of the molecules was determined with a map measure.

Miscellaneous. Electrophoresis in polyacrylamide gels in the presence of SDS was carried out as described by Laemmli (1970).

Agarose, prepared for electrophoresis, was from the British Drug Houses.

dATP, dCTP, dGTP and dTTP were purchased from Sigma, St. Louis, USA. ^{3}H-labelled dTTP and ^{32}P-labelled inorganic phosphate, carrier free, were obtained from the Radiochemical Centre, Amersham, England. DNA-agarose was prepared according to Schaller *et al.* (1972). 100 ml of a 4% agarose solution at 100° was mixed with 100 ml of a solution of calf thymus DNA in 20 mM NaOH (15 mg DNA per ml) which was heated to 50°. The mixture was cooled by pouring on a cold surface and was stored overnight in a refrigerator.

The agarose which contained the denatured DNA was pressed through a sieve of 90 mesh, washed with 1 M KCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM NaN_3 and stored at 4° C. The DNA-agarose contained 750 μg DNA per ml bed volume as measured by the method of Bendich and Bolton (1968).

Results

Purification of the Enzyme. DNA polymerase I was isolated and purified from *E. coli* KMBL 1789 *polA'107* and from the isogenic strain *E. coli* KMBL 1790 *pol*$^+$. Starting with the same amount of bacteria the yield of purified enzyme was comparable for the two strains, when the DNA polymerizing activity was assayed with exonuclease III treated calf-thymus DNA, but the polymerizing activity of the mutant enzyme was approximately 50% lower when it was assayed on RF II DNA (see below). The chromatographic behaviour of wild-type enzyme and mutant enzyme on DNA-agarose columns or phospho-cellulose columns was similar. Also the rate of migration of the two enzyme preparations in polyacrylamide gels, in the presence of SDS, was not significantly different: a mixture of the two enzymes showed only one protein band. These results suggest that the mutation in the *polA* gene does not affect the physical properties of the enzyme to a great extent.

Enzymatic Properties. We have next compared the enzymatic properties of the two enzyme preparations. DNA polymerase I consists of one polypeptide chain of MW 109000 (Jovin *et al.*, 1969); besides polymerizing activity, 3′—5′ and 5′—3′ exonucleolytic activities are present in DNA polymerase I. 3′—5′ exonucleolytic activity was found in purified preparations of wild-type DNA polymerase I as well as mutant DNA polymerase I. For the same amount of enzyme (an equal number of polymerizing units) we have repeatedly observed that the exonucleolytic degradation of denatured T7 DNA was 1.3 times faster with the mutant enzyme than with the wild-type enzyme (Fig. 1).

Under appropriate conditions the 5′—3′ exonucleolytic activity can be assayed concomitantly with the determination of DNA polymerizing activity. For this purpose ^{32}P-labelled RF II DNA, containing on the average 3 to 4 nicks per molecule, was incubated together with the enzyme in the presence of the four deoxyribonucleoside triphosphates (dTTP was labelled with ^{3}H). The incorporation of ^{3}H-labelled material into acid-insoluble product (a measure for the DNA polymerizing activity) and the release of ^{32}P-labelled material as acid-soluble product (a measure for the 5′—3′ exonucleolytic activity) was followed as a function of the duration of incubation. Under these conditions the 3′—5′ exonucleolytic activity is completely suppressed (Brutlag and Kornberg, 1972). The results have been presented in Fig. 2. Incubation of [^{32}P]-RF II DNA with wild-type enzyme resulted in the release of ^{32}P-labelled material, reaching a plateau after 15 to 30 min, when 130 pmoles of nucleotides or approximately 40% of the total radioactivity had become acid soluble. Under these conditions little ^{32}P-labelled material was rendered acid soluble with an equivalent amount of mutant enzyme. This result suggests that the capacity of the mutant enzyme to catalyse the hydrolysis of DNA in the 5′—3′ direction is greatly reduced. With the wild-type enzyme the incorporation of ^{3}H-labelled material into DNA kept pace with the release of ^{32}P-labelled material from DNA and reached a plateau after 15 to 30 min of synthesis, when 130 pmoles nucleotides had been incorporated into DNA.

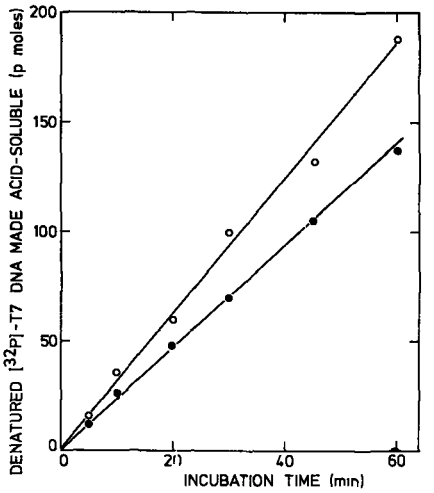

Fig. 1. 3′—5′ exonucleolytic activity of wild-type and mutant DNA polymerase I as a function of the duration of incubation. The assay was carried out as described in Materials and Methods. For each assay 1.5 polymerizing units of enzyme were used. • wild-type enzyme, ∘ mutant enzyme

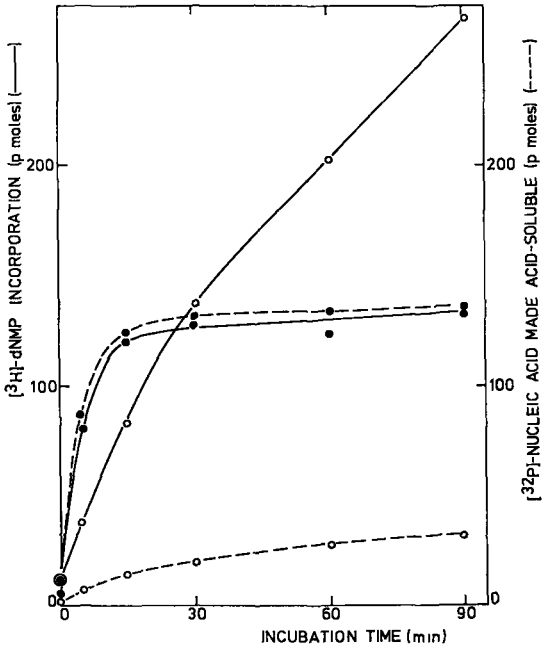

Fig. 2. Determination of 5′—3′ exonucleolytic activity and polymerizing activity of wild-type and mutant DNA polymerase I. ^{32}P-labelled RFII DNA was incubated as described in the experimental part with wild-type DNA polymerase I (closed symbols) or mutant DNA polymerase I (open symbols). The incorporation of [^{3}H]-dTTP into acid-insoluble material and the formation of acid-soluble ^{32}P-labelled product was followed as a function of the duration of incubation. Solid lines represent incorporation of [^{3}H]-dTMP into acid-precipitable material; dotted lines represent breakdown of ^{32}P-labelled RFII DNA into acid-soluble product

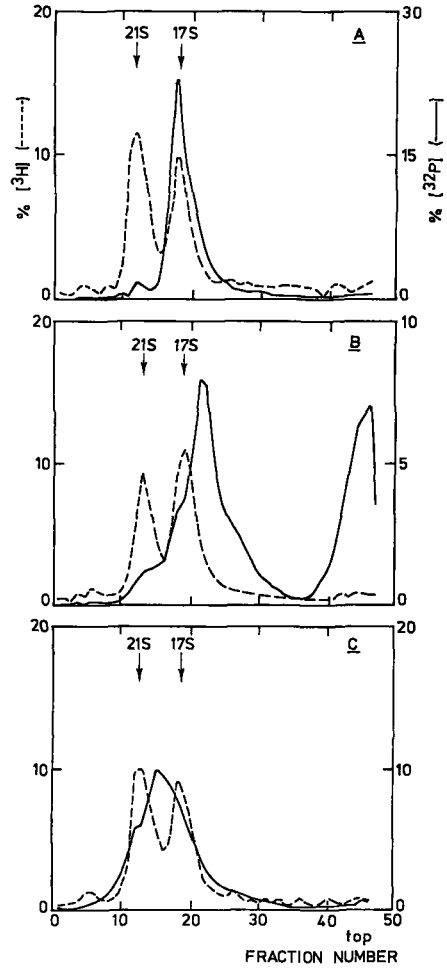

Fig. 3A—C. Analysis of RFII DNA after incubation with wild-type or mutant DNA polymerase I on sucrose-gradients. ^{32}P-labelled RFII DNA was incubated for 0 and 60 min with wild-type or mutant DNA polymerase I under conditions used for the combined assay for the 5′—3′ exonucleolytic activity and DNA polymerizing activity, except that ^{3}H-dTTP was replaced by unlabelled dTTP. The reaction was stopped by the addition of excess EDTA and the products were analysed by electron-microscopy and by sedimentation on neutral sucrose-gradients (5—20% sucrose in 1 MNaCl, 10 mM EDTA (pH 7.5); centrifugation was for 15 h at 33 000 rpm in a Spinco L3, rotor SW 41). The dotted lines represent ^{3}H-labelled RFI DNA and RFII DNA used as internal markers. The solid lines represent the reaction products: A after 0 min incubation with wild-type DNA polymerase I. (The pattern for mutant enzyme after 0 min incubation, which was not significantly different from that obtained with wild-type enzyme, has been left out from the figure.) B after 60 min incubation with wild-type DNA polymerase I. C after 60 min incubation with mutant DNA polymerase I

The initial rate of incorporation of ^{3}H-labelled material into DNA with the mutant enzyme was approximately half of that of wild-type enzyme, but DNA synthesis continued for a much longer period of time than with the wild-type enzyme.

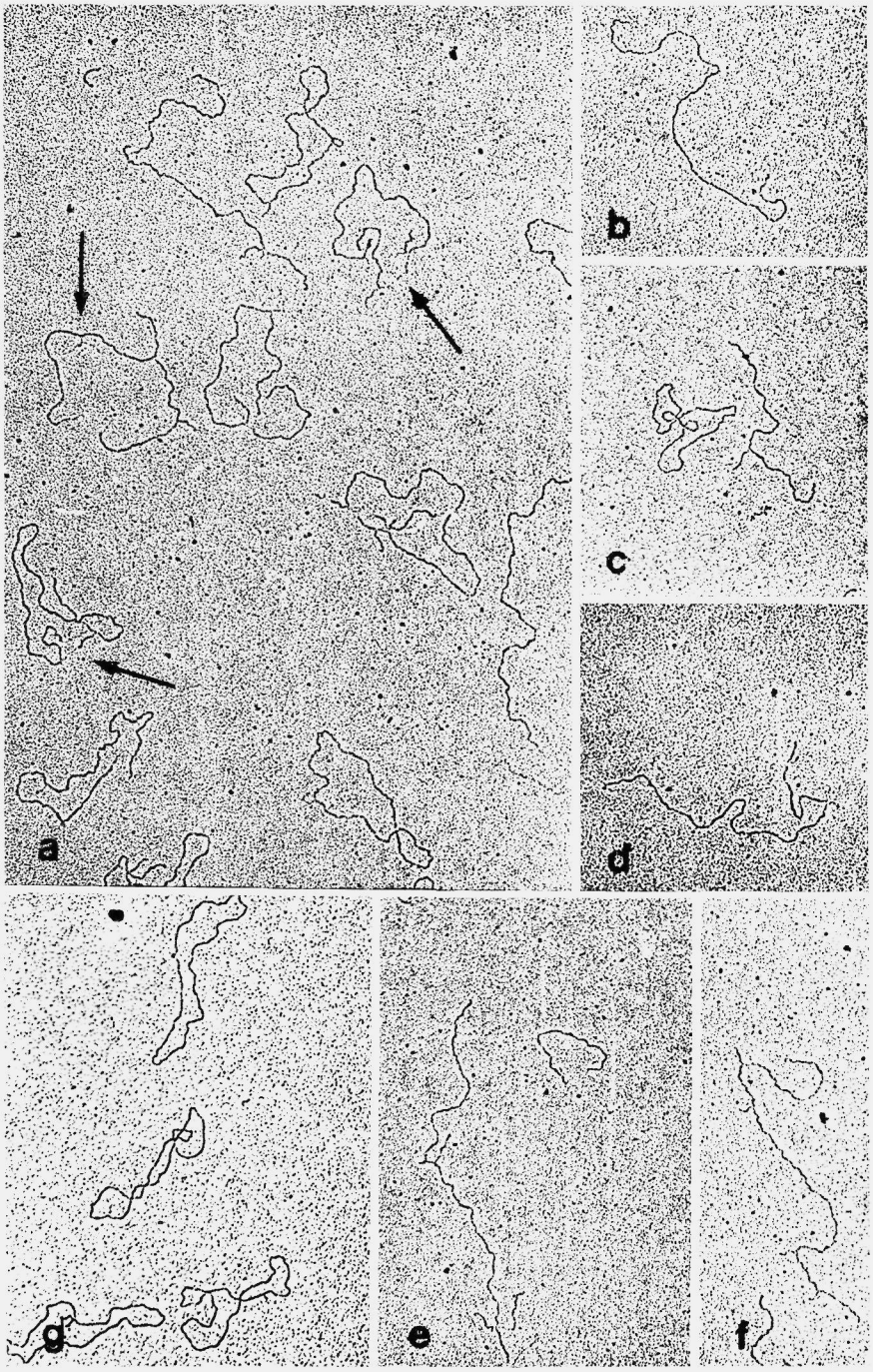

Fig. 4a—g. Bright-field electron micrographs of RFII DNA. a Incubated for 60 min with mutant DNA polymerase I, b—f incubated for 60 min with wild-type DNA polymerase I, g untreated. Arrows indicate the presence of single-stranded DNA. Magnification × 36000

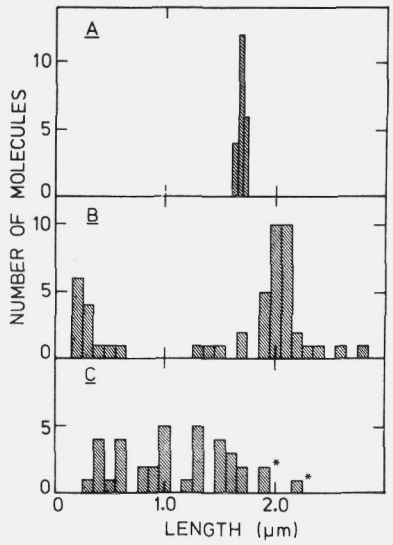

Fig. 5A—C. Length distribution of RFII DNA; A untreated, B after 60 min incubation with mutant DNA polymerase I, C after 60 min with wild-type DNA polymerase I. Conditions of the experiment are described in the legend to Fig. 3. The total length of the molecules, including the "offshoots", was measured. The columns marked with an asterisk represent branched circular molecules

Analysis of the Reaction Products on Sucrose-Gradients. When ^{32}P-labelled RFII DNA was incubated with DNA polymerase I and the products of the reaction were analysed on neutral sucrose-gradients the following results were obtained (Fig. 3). After 60 min of incubation with wild-type enzyme a significant proportion of the radioactivity was found at the top of the gradient, while the remainder was found predominantly at the position of linear double-stranded ϕX174 DNA (15S). With the mutant enzyme, however, radioactivity was not found at the top of the gradient; signifying the absence of low-molecular weight products; all the radioactivity was recovered in a rather broad peak, sedimenting significantly faster than RFII DNA.

Electron-Microscopy. Samples of RFII DNA which had been treated for 60 min at 37° with wild-type or mutant enzyme under conditions favouring polymerization were also examined with an electron-microscope. After incubation with wild-type enzyme 65% of the DNA molecules were converted into unbranched linear molecules and 15% into branched linear molecules (Table 1; Fig. 4). The remaining 20% of the molecules had a circular configuration approximately half of which showed the presence of "offshoots". DNA molecules which were partially or completely single-stranded were not observed in these preparations. The length of the linear molecules varied considerably, ranging from 0.2 to 1.7 μ (Fig. 5). When RFII was incubated with the mutant enzyme, only 4% was present as unbranched linear molecules, while 58% was present as branched linear molecules. Of the remaining 38% of the circular molecules a high percentage (90%) showed the presence of "offshoots" (Table 1; Figs. 4 and 6). In a number of cases, especially for branched molecules, single-stranded DNA regions were observed

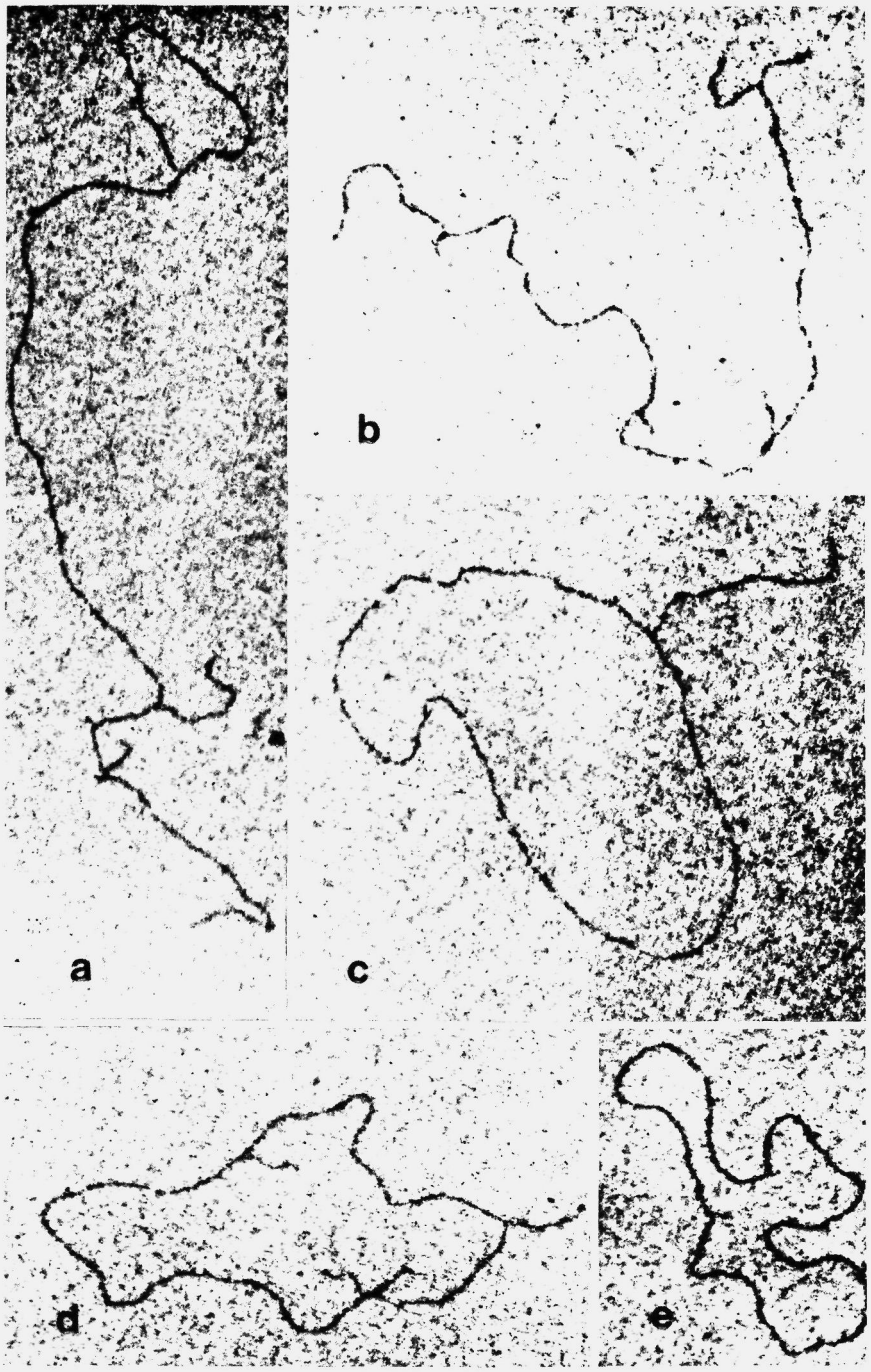

Fig. 6a—e. Dark-field electron micrographs of RFII DNA. a—d Incubated for 60 min with mutant DNA polymerase I, e untreated. Magnification × 103000

Table 1. Reaction products formed after incubation of RF II DNA with wild-type or mutant DNA polymerase I

	0 min incubation wild-type DNA polymerase I	60 min incubation wild-type DNA polymerase I	60 min incubation mutant DNA polymerase I
Linear DNA	10%	65%	4%
Branched linear DNA	0%	15%	58%
Ciruclar DNA	90%	9%	4%
Branched circular DNA	0%	11%	34%
Number of molecules inspected	173	111	348

(Fig. 4a). The length of the molecules showed a rather narrow distribution: for the majority of the molecules the length varied between 1.8 and 2.2 μ ("off-shoots" included). Besides these long molecules very short molecules (0.2–0.6 μ) were also observed.

Discussion

The DNA polymerizing and 3′—5′ exonucleolytic activity of purified DNA polymerase I from *E. coli* KMBL 1789 *polA'107* are comparable with those of the wild-type enzyme, but the mutant strain lacks the 5′—3′ exonucleolytic activity which is present in wild-type enzyme, as is shown in this paper. When DNA synthesis was measured on RFII DNA with equivalent amounts of mutant or wild-type enzyme (estimated from the polymerizing activities on exonuclease III treated calf-thymus DNA) the initial rate of synthesis was approximately twice as high with the wild-type enzyme as with the mutant enzyme. A possible explanation for the lower rate of DNA synthesis, might be that synthesis of DNA, which requires the displacement of a preexisting strand, is energetically or sterically less favourable than DNA synthesis accompanied by DNA degradation. In keeping with this is our observation that at 15° no DNA synthesis takes place with mutant enzyme on RFII DNA, while the initial rate of DNA synthesis with wild-type enzyme at this temperature is still 20% of the rate found at 37°. Our conclusion that the 5′—3′ exonucleolytic activity facilitates the DNA polymerization reaction is at variance with the results found by Masamune and Richardson (1971), who observed no difference in the rate of DNA synthesis on RFII DNA from phage PM2, comparing DNA polymerase I and the large fragment of the enzyme, possessing DNA polymerizing activity, but lacking the 5′—3′ exonucleolytic activity (Brutlag *et al.*, 1969; Klenow and Overgaard-Hansen, 1970).

The 3′—5′ exonucleolytic activity is present in both DNA polymerase I preparations. The 1.3 times faster rate of degradation of denatured T7 DNA with the mutant enzyme can be accounted for by a slightly lower rate of synthesis on "activated" calf-thymus DNA with the mutant enzyme than with wild-type DNA polymerase I. In that case a polymerizing unit of the wild-type enzyme will represent approximately 1.3 times less protein molecules than a polymerizing unit of the mutant enzyme.

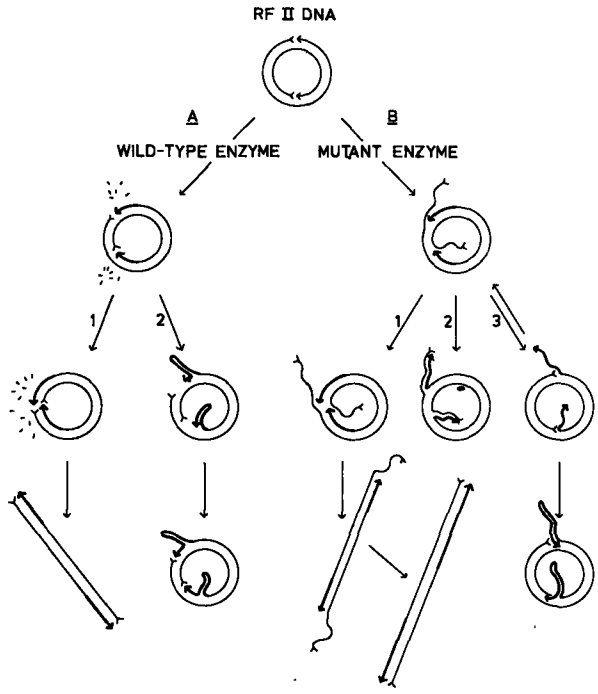

Fig. 7. Schematic presentation of the reaction products formed upon incubation of RF II DNA with wild-type or mutant DNA polymerase I, as modified from Masamune *et al.* (1972). Newly synthesized DNA has been drawn in heavy lines; template DNA has been drawn in thin lines

When circular double-stranded ΦX174 DNA containing 3 to 4 nicks per molecule was incubated with wild-type or mutant enzyme a large percentage of the molecules was converted into linear molecules, which were maximally one genome in length (1.7 μ), in the case of the wild-type enzyme but longer than one genome, in the case of the mutant enzyme. In accordance with this is our observation that molecules generated by the action of the wild-type enzyme sediment at 15 S, a rate expected for molecules of that size (Studier *et al.*, 1965), while the molecules formed by the action of the mutant enzyme sediment much faster (19 S). This result may be explained as follows (Fig. 7).

With the wild-type enzyme DNA synthesis starts at a nick on either strand and proceeds in the 5′—3′ direction, while the 5′—3′ exonucleolytic activity simultaneously releases nucleotides from the primer strand ahead of the nick. Consequently the nicks are translated in opposite directions. Finally, when the nicks have approached each other, a linear molecule will be formed (Fig. 7, A, 1). These linear molecules will be maximally one genome in length depending on the number and the relative position of the nicks. Since linear double-stranded DNA molecules do not serve as a template for DNA polymerase I, both incorporation of DNA precursors and release of nucleotides from DNA will stop (Fig. 3). In a number of cases branched molecules are found, probably because DNA poly-

merase I switches from the DNA template strand to the newly synthesized strand and continues synthesis with the primer strand as template (Schildkraut *et al.*, 1964) (Fig. 7, A, 2).

With the mutant enzyme DNA will be synthesized starting at the nicks in the 5′—3′ direction along both strands, by displacement of the DNA strand ahead of the nick. When the nicks have approached each other a linear molecule will be formed with single-stranded protruding ends, which the enzyme can convert into double-stranded DNA. The product of the reaction is a linear DNA molecule, which may be longer than one phage genome (Fig. 7, B, 1). When DNA synthesis involves strand displacement, as is the case with mutant DNA polymerase I, the enzyme may also switch to the displaced single strand and use that strand as a template (Fig. 7, B, 2). As proposed by Masamune *et al.* (1971), there will be an equilibrium between the original displaced strand and the newly synthesized DNA after incubation with DNA polymerase I (Fig. 7, B, 3) (see also Lee *et al.*, 1970). The 3′OH endgroup of the single-stranded tail might then loop back on itself and serve as its own primer template (Schildkraut *et al.*, 1964). Since the percentage of branched molecules is much higher after incubation with the mutant enzyme (92%) than with the wild-type enzyme (26%) it appears that strand switching is mainly due to the presence of free single-stranded ends.

When wild-type enzyme is used, the amount of nucleotides incorporated into RFII DNA is not significantly different from that released from DNA, at any time of the reaction. This means that for every nucleotide which is incorporated into DNA also a nucleotide is removed. This result is in agreement with those of Kelly *et al.* (1970) and Setlow *et al.* (1972b), but is at variance with the results of Masamune and Richardson (1971), who observed net synthesis of DNA on PM2 DNA preceeded by nick translation. From our data we conclude that the action of DNA polymerase I in repair of DNA from *E. coli* is to "wipe out" damaged regions by "nick translation".

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INVOLVEMENT OF *ESCHERICHIA COLI* DNA POLYMERASE-I-ASSOCIATED 5'-3' EXONUCLEASE IN EXCISION-REPAIR OF UV-DAMAGED DNA

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ABSTRACT

From comparative studies between *Escherichia coli* PolA107 cells (lacking 5'-3'exonucleolytic activity associated with DNA polymerase I) and the isogenic wild-type strain, and between the purified DNA polymerase I preparations isolated from these strains, it can be concluded that the 5'-3' exonuclease is involved in excision of pyrimidine dimers in *E.coli*. Evidence is presented that the polA107 mutation is located on that part of the DNA polymerase I gene coding for the small fragment on which 5'-3' exonucleolytic activity is found.

The discovery by de Lucia and Cairns (1969) of a mutant of *Escherichia coli* (polA1) lacking DNA polymerase I initiated many studies on the role of this enzyme in dark repair of UV-irradiated DNA both *in vivo* (Gross and Gross, 1969; Boyle *et al.*,1970; Cooper and Hanawalt, 1972; Tait *et al.*, 1974; Glickman, 1974) and *in vitro* (Kelly *et al.*, 1969; Heyneker *et al.*, 1971). DNA polymerase I consists of one polypeptide chain of molecular weight 109,000 (Jovin *et al.*, 1969), which by mild proteolytic treatment (Brutlag *et al.*, 1969; Klenow and Overgaard-Hansen, 1970) can be split into two fragments : a large fragment of

molecular weight 76,000 containing DNA polymerizing and 3'-5' exonucleolytic activities, and a small fragment of molecular weight 36,000 containing 5'-3' exonucleolytic activity.

The elegant experiments performed by Brutlag and Kornberg (1972) and Setlow and Kornberg (1972) demonstrated that the multiple functions of DNA polymerase I give the enzyme the necessary characteristics to catalyze repair reactions on damaged DNA. DNA polymerase I binds at nicks introduced by a UV-specific endonuclease in UV-irradiated DNA (Braun *et al.*, this volume). Polymerization then proceeds in the 5'-3' direction, while the 5'-3' exonucleolytic activity associated with DNA polymerase I simultaneously releases nucleotides, including pyrimidine dimers, from the primer strand ahead of the nick. This reaction is called "nick translation".

In addition to PolA1, other PolA mutants have been isolated such as the PolA12 mutant with a thermosensitive DNA polymerase I (Monk and Kinross, 1972) and the ResA mutant (Kato and Kondo, 1970). All of these mutants retain the 5'-3' exonucleolytic activity unaffected by the mutation (Lehman and Chien, 1973; Lehman, personal communication). Glickman *et al.*,(1973) described the phenotypic characteristics of a PolA mutant with normal levels of DNA polymerizing activity but deficient in the 5'-3' exonucleolytic activity (Heyneker *et al.*,1973). This mutant (PolA 107) is moderately UV-sensitive and excises pyrimidine dimers from UV-irradiated DNA more slowly than isogenic wild-type or PolA1 strains (Glickman, 1974). It has been shown that DNA polymerase I purified from *E.coli* cells carrying the polA107 mutation is able to perform "strand-displacement synthesis" instead of "nick translation" (Heyneker *et al.*, 1973). Since the PolA107 mutant is only moderately sensitive to UV-irradiation, one possibility is that DNA polymerase I binds to nicks introduced by a UV-specific endonuclease into irradiated DNA, then catalyzes polymerization in the 5'-3' direction by displacement of the strand ahead of the nick without the excision of dimers. The displaced strands may be subsequently digested by exonuclease VII, which degrades single-stranded DNA in the 5'-3' direction (Chase and Richardson, this volume). Other possibilities are that some residual 5'-3' exonucleolytic activity is associated with the mutant DNA polymerase I, or that the displaced strand is nicked by a single-strand-specific endonuclease and further degraded by exonuclease I. Recently,Konrad and Lehman(1974) (communicated by Uyemura *et al.*, this volume) isolated another mutant (PolAex1) also lacking 5'-3' exonucleolytic activity. Unlike the polA107

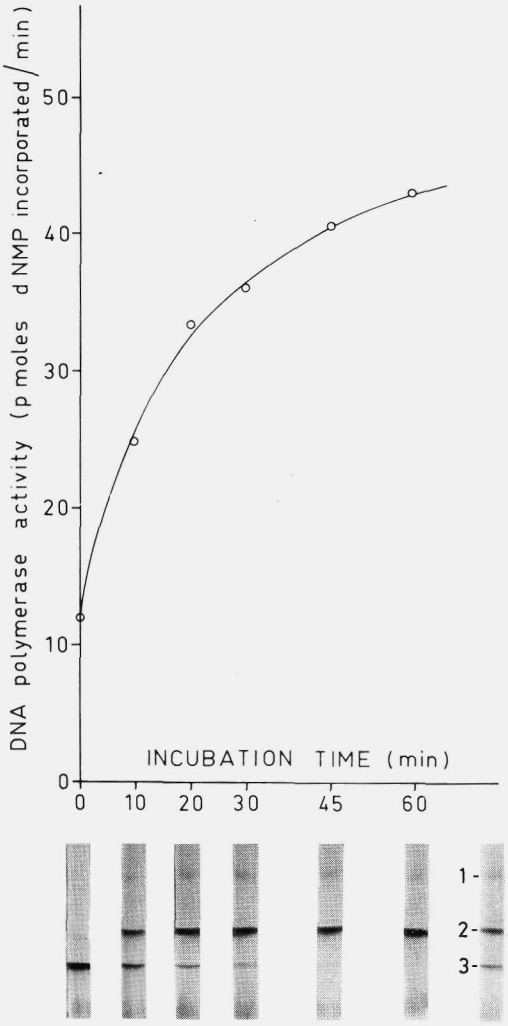

Fig.1. The effect of subtilisin treatment on the rate of polymerization by DNA polymerase I.
Treatment with subtilisin was carried out at 37°C in 0.13 M potassium phosphate buffer, pH 6.5, containing 40 µg/ml calf thymus DNA (Sigma, type V), approximately 7 µg/ml DNA polymerase I (spec.act. > 20,000 U/mg, and 75 ng/ml subtilisin (type Carlsberg). At times 0, 10, 20, 30, 45 and 60 min, aliquots of 2.5 ml were withdrawn from the reaction mixture and the reaction was terminated by the addition of 0.3 ml 50% TCA. After 30 min on ice, the precipitated protein was collected by centrifugation (5 min, 20,000 rpm, 0°C, Spinco rotor 40) and dissolved in 0.1 ml 0.1 M tris-acetate buffer, pH 7.5, containing 1% SDS, 0.02 M dithiothreitol, and 25% sucrose. The mixture was incubated for 1 hr at 37°C and subjected to electrophoresis in polyacrylamide gels in the presence of SDS for 16 hr at 1.5 mA per gel at 22°C as described by Klenow *et al.* (1971). The gels are shown below their respective sampling times except for the reference gel (right frame), which represents the electrophoretic pattern of a mixture of small fragment (1), large fragment (2), and intact DNA polymerase I (3), purified from wild-type *E.coli* cells. At the indicated times, aliquots of 25 µl were removed from the reaction mixture and mixed with 25 µl 1% albumin (Armour Pharmaceutical Co.) containing 25 µg/ml phenylmethane sulfonylfluoride. DNA polymerase I activity was subsequently measured in 0.05 M potassium phosphate buffer, pH 7.4, supplemented with the four deoxyribonucleoside triphosphates: 17 µM each (TTP was tritium-labeled (NEN), 100 mCi/mmole); calf thymus DNA activated with pancreatic DNase, 300 µg/ml; 3 mM $MgCl_2$; and 0.3 mM dithiothreitol. Samples were taken at 5, 10, 15 and 20 min and assayed for acid-precipitable radioactivity, and the results were used to calculate the reaction rate shown in the figure.

mutation, the polAex1 mutation is reported to result in temperature-dependent lethality, suggesting that the 5'-3' exonuclease has multiple functions in the repair and synthesis of DNA.
To determine whether the polA107 mutation is due either to a mutation in the large fragment and influencing the structure of the enzyme in such a way that the 5'-3' exonucleolytic activity is affected or to a mutation in the small fragment affecting the active site of the 5'-3' exonuclease or influencing the tertiary structure of the small fragment, the mutant enzyme was treated with subtilisin. Klenow *et al.*, (1971) have found that the action of subtilisin on DNA polymerase I, resulting in the splitting of the enzyme into a small and a large fragment, can be monitored by the concomitant 3- to 4-fold increase in the reaction rate of DNA polymerizing activity. When PolA107 DNA polymerase I is split with subtilisin, an almost 4-fold stimulation of polymerizing activity is obtained under the same conditions which are optimal for stimulation of wild-type polymerizing activity (fig.1). Furthermore, the size of the fragments obtained by splitting the mutant enzyme is indistinguishable from the size of the fragments of wild-type DNA polymerase I (fig.1). The 5'-3' exonucleolytic activity of the polA107-DNA polymerase I, measured by the formation of acid-soluble material from tritium-labeled poly d(A.T), however, was less than 5% of the wild-type 5'-3' exonuclease both before and after the subtilisin treatment. From these experiments we conclude that the polA107 mutation is located in the region of the polA gene coding for the small fragment of DNA polymerase I and is propably a point mutation.
It might be of interest to investigate which difference in amino acid composition is responsible for the inactivation of the 5'-3' exonuclease associated with DNA polymerase I.

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PHYSICO-CHEMICAL AND BIOLOGICAL STUDY OF EXCISION-REPAIR OF UV - IRRADIATED ØX174 RF DNA *IN VITRO*.

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SUMMARY

We have studied excision-repair of UV-irradiated ØX174 RFI DNA *in vitro* with UV-specific endonuclease from *Micrococcus luteus* (UV-endo), DNA polymerase I from *Escherichia coli* and DNA ligase from phage T4 infected *E.coli*.
Excision-repair was measured a) by physico-chemical methods, *i.e.* by determination of the conversion of RF I DNA intc RF II DNA by UV-endo and by the subsequent conversion of RF II DNA into RF I DNA by the joint action of DNA polymerase I and DNA ligase, b) by biological methods *i.e.* by measuring the ability of the reaction product to form phages upon incubation with spheroplasts from the appropriate strains of *E.coli*.
Using the first method, we have shown, that more than 90% of the pyrimidine dimers can be repaired *in vitro*; with the latter method we have shown, that the molecules which are repaired as defined by method a) have regained full biological activity.
Exonuclease III was found to be not essential for excision-repair *in vitro* and also did not stimulate repair. From this result we conclude that UV-endo generates 3'OH endgroups, in agreement with results obtained by Hamilton *et al.*(1974).
The usefulness of the method presented in this paper with regard to the study of excision-repair is discussed.

INTRODUCTION

The ability of cells to survive from the lethal and mutagenic effects of UV-irradiation can be largely attributed to enzymatic repair processes. The main part of DNA damage induced by UV-irradiation consists of pyrimidine dimers (Setlow, 1966).
It was discovered in 1964 by Setlow and Carrier and by Boyce and Howard-Flanders that UV-irradiated *E.coli* cells were able to "excise" pyrimidine dimers from their DNA. The excision-repair process has been studied in detail both *in vivo* as well as *in vitro*. For recent reviews of this subject see : Howard-Flanders (1973), Berndt (1973), Grossman (1973), Kornberg (1974). The excision-repair process can be devided into 4 steps :

1. Incision of UV-irradiated DNA by an UV-specific endonuclease.
2. Excision of the dimer by a 5'-3' exonuclease.
3. Reinsertion of new nucleotides by DNA polymerase I.
4. Sealing of the single stranded nick by DNA ligase.

Excision-repair has been carried out *in vitro* by measuring the restoration of the biological activity of *B.subtilis* transforming DNA (Heyneker *et al.*, 1971), Hamilton *et al.*, 1974). In this system the biological activity is restored only when all the steps of the excision-repair process are carried out *in vitro* (Strauss, 1966). However a disadvantage of the *B.subtilis* transformation system lies in the difficulty to monitor the different steps of the reaction by physical methods, as well as in the rather insensitive determination of biological activity.
The main purpose of this investigation is to develop a sensitive excision-repair assay *in vitro*, where repair of UV-irradiated DNA can be followed both with physico-chemical and with biological methods. For our assay we have used UV-damaged RF I DNA of ØX174. Physico-chemically the repair reaction can be studied by determination of the conformational changes in DNA. Biologically the repair reaction can be followed by measuring the ability to form virus particles after incubation of RF DNA with spheroplasts.
With these test systems we examined the incision and excision reactions in more detail. It has been shown, that incision takes place at the 5'-side of a dimer (Setlow, 1970; Paribok, 1971), but there has been conflicting data about the nature of the endgroups generated by the UV-specific endonuclease from *M.luteus*. Initially Kushner *et al.*,(1971) reported

that 3'P and 5'OH termini were generated by the enzyme, thus implying, that the 3'P endgroup had to be converted to a free 3'OH endgroup, which is essential for binding of DNA polymerase I, to start with reinsertion of new nucleotides. More recently Hamilton *et al.*,(1974) concluded from enzymatic studies, that the incision made by the *M.luteus* UV-specific endonuclease carries a 3'OH terminal group. The results described in this paper support the latter finding.

The role of DNA polymerase I in repair has been well established, both *in vivo* and *in vitro*. Previous work from our laboratory pointed out that *E.coli polA107* (Glickman, 1973), which is lacking 5'-3' exonucleolytic activity associated with DNA polymerase I (Heyneker *et al.*, 1973; Heyneker and Klenow, 1975) excises dimers at a reduced rate *in vivo* (Glickman, 1974). In this study we have investigated the role of the mutant DNA polymerase I in excision-repair *in vitro*; our results support the conclusion that the 5'-3' exonucleolytic activity of DNA polymerase I plays a role in the excision-repair process.

MATERIALS AND METHODS

ENZYMES

DNA ligase

DNA ligase was prepared from phage T4 amN82 infected *E.coli* B cells according to the procedure of Weiss *et al.*,(1968). Fraction VI was further purified by hydroxylapatite chromatography according to Panet *et al.*, (1973). The enzyme eluted from the column at 3.7% ammonium sulfate saturation. Subsequently the enzyme was dialysed against 10 mM potassium phosphate buffer pH 7.6 in 50% glycerol, supplemented with 1 mM dithiotreitol and 50 mM KCl. The final enzyme preparation had a concentration of 250 Units/ml. Analysis of 50 µl of the ligase solution by the SDS-polyarylamidegel-electrophoresis showed the presence of only one band.

DNA polymerase

The isolation of DNA polymerase I and polA107-DNA polymerase I was described before (Heijneker *et al.*,1973). The specific activity of both preparations was > 20,000 U/mg. The enzyme concentration for both enzymes was adjusted to 100 Units/ml in 20 mM potassium phosphate buffer (pH 6.5), containing 10 mM 2-mercaptoethanol, 1 mM NaN_3, 1 mM EDTA and 5% glycerol.

Exonuclease III

Exonuclease III was purified according to Heyneker *et al.*,(1973) with additional purification steps of the DNA agarose fraction by phosphocellulose columnchromatography (Whatman P 11) and Sephadex G 100 gelfiltration according to Jovin *et al.*,(1969). The final preparation was dialyzed against o.1 M potassium phosphate buffer (pH 6.5), containing 10 mM 2-mercaptoethanol, 1 mM NaN_3, 1 mM EDTA and 5% glycerol. The activity of the preparation used was 2200 Units/ml.

UV-specific endonuclease

The preparation of a crude extract from 50 grams of *M.luteus* AATC 4698 cells and the separation of UV-endonuclease from nucleic acids by phase-partition was carried out as described by Nakayama *et al.*,(1971).
The enzyme-fraction 2 of Nakayana (250 ml) was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM NaCl and 10% ethylene glycol. The solution containing the enzyme was passed through a DEAE-cellulose column (Whatman DE 52), which was equilibrated in the same buffer. UV-endo does not bind under these conditions. The DEAE fraction containing UV-endo activity was adsorbed directly to a single stranded DNA agarose column (4.5 cm Ø x 10 cm), which was equilibrated with 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl,1 mM EDTA, 1 mM NaN_3, 1 mM 2-mercaptoethanol and 10% ethylene glycol. A linear gradient from 0.1 M NaCl to 1.2 M NaCl in the same buffer was applied (total volume 600 ml). UV-endo is eluted from the column at 0.8 M NaCl. The active fractions were pooled, dialyzed extensively against 10 mM potassium phosphate buffer pH 7.0, containing 5 mM 2-mercaptoethanol and 5% glycerol. A column (2 cm Ø x 5 cm) of hydroxylapatite (prepared according to Mazin *et al.*,(1974) was poured and equilibrated in dialysis buffer. The diluted enzyme was adsorbed to hydroxylapatite and eluted with 50 mM potassium phosphate buffer (pH 7.0), containing 5 mM 2-mercaptoethanol and 5% glycerol. The active fractions were pooled (10 ml) and dialyzed against 10 mM potassium phosphate buffer pH 7.0 in 50% glycerol, 5 mM 2-mercaptoethanol and 1 mM NaN_3. The purified enzyme was stored at $-20^{o}C$ without loss of activity.

CONDITIONS FOR EXCISION-REPAIR

To 10 µl solution of UV-damaged RF I DNA (5.5 µg/ml) was added : 10 µl 10 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA, 50 mM KCl; 8 µl H_2O and 2 µl UV-endonuclease solution. Incubation was for 10 min. at 35°C (reaction 1).
To the contents of reaction-mixture 1 was added a mixture consisting of 20 µl 0.33 M Tris-HCl buffer (pH 7.6), containing 33 mM $MgCl_2$, 50 mM dithiotreitol; 2 µl 6.6 mM ATP; 4 µl dNTP's (0.5 mM each); 6 µl DNA polymerase I; 2 µl DNA ligase and 36 µl H_2O (reaction 2). The time of incubation is dependent on the temperature of the reaction-mixture. The reaction is terminated by the addition of 10 µl o.1 M EDTA.

SPHEROPLAST-ASSAY

Spheroplasts were prepared from *E.coli* AB 2437 $uvrA_6$, his_{A66} obtained from Dr.Howard-Flanders or from *E.coli* K12S originating from Dr.Weigle. 40 ml of bacteria was grown in LC medium at 37°C to a density of 6 to 8.10^8 cells/ml. LC medium (pH 7) contains per litre : 10 g trypton (Difco), 5 g yeastextract (Difco) and 8 g NaCl. Cells were collected by centrifugation and resuspended in 0.5 ml 0.1 M Tris-HCl buffer (pH 9.0) containing 40% sucrose and 6% Bovine Serum Albumine (Serva, Heidelberg). Further steps, leading to the formation of spheroplasts were carried out at 22°C. The suspension was incubated with 20 µl of a fresh solution of lysozyme (Calbichem, A grade; 5 mg/ml in 0.25 M Tris-HCl pH 8.0). After 5 min. 40 µl 0.15 M EDTA pH 8.0 was added and 4 min. later 5 ml PA medium (pH 7.0) containing per litre 10 g Casaminoacids (Difco, vitamine free), 10 g Nutrient Broth, 1 g Glucose and 100 g Sucrose. After 14 min. the formed spheroplasts were mixed with 0.2 ml 1 M $MgSO_4$. Finally 0.4 ml protamine sulfate (1.2 mg/ml) (Serva) was slowly added, while shaking, to prevent clogging of the spheroplasts. The formation of spheroplasts was checked with the light-microscope; at least 99% of the cells should be converted to spheroplasts. Spheroplasts (80 µl) were preincubated with 100 µl of RF DNA at 35°C in test-tubes made of red glass to prevent repair of dimers by photoreactivation (Cook, 1970). After 10 min., 0.8 ml PA, containing 10 mM $MgSO_4$, was added and incubation was continued under mild shaking for 2 hours. The incubation was terminated with 10 µl chloroform and the concentration of viable phage particles was determined by convential techniques.

SEPARATION OF RF I DNA FROM RF II DNA

Separation of RF I DNA from RF II DNA was performed by centrifugation on a linear alkaline sucrose gradient (5 to 22% sucrose in 1 M NaCl pH 12.8) at 55.000 rpm for 90 min. at 10°C in a Beckman SW56 rotor. Before centrifugation Triton X100 was added to the reaction mixture to a final concentration of 0.5%.
Alternatively RF I DNA was separated from RF II DNA after heat denaturation by filtration on nitrocellulose filters (Schleicher and Schull, Selectron Ba85) as described by Center *et al.*,(1970) : The reaction mixture was heated in thin-walled glass tubes for 2.5 min. on a boiling water-bath and was subsequently quenched in ice-water. Half a ml of cold 10 mM Tris-HCl buffer pH 8.0 containing 1 M NaCl (high-salt buffer) was added to the sample. Nitrocellulose filters were immersed in high-salt buffer before use. The sample was filtered slowly and the filter was washed with three portions of 0.5 ml high-salt buffer. Single stranded DNA, originated from RF II DNA, was retained on the filter. The eluate, containing RF I DNA as well as possible low molecular weight material descended from degraded DNA was collected, supplemented with carrier DNA to a final concentration of 50 µg/ml and the DNA was precipitated by the addition of an equal volume cold 10% perchloric acid. After 30 min. on ice, the precipitate (containing RF I DNA) was collected on a glassfilter (Whatman GF/C). The filter was washed with 5% perchloric acid, containing 10 mM sodium pyrophosphate, and subsequently with ethanol. The filters were dried and the amount of RF I DNA and RF II DNA was determined from the radioactivity present on the filters.
The average number of nicks per RF DNA molecule was calculated from the ratio of RF I DNA to RF II DNA applying Poisson distribution : $N/N_o = e^{-b}$, where N/N_o represents the fraction of RF I DNA molecules in the total population of RF DNA molecules, and b stands for the average number of nicks.

RF DNA

Tritium-labelled RF DNA was prepared according to the method of Godson and Boyer,(1974). The preparation (70% RF I DNA, 30% RF II DNA) was dialyzed against 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA and 50 mM NaCl. The concentration used was 5.5 µg/ml with a specific radioactivity of 0.2 µCi/µg.

UV-IRRADIATION

UV-irradiations were performed with a Philips 30W germicidal lamp at a dose-rate of 30 ergs/mm^2.sec. Dose-rate was determined biologically, from the inactivation of phage ØX174 with increasing doses of UV-light (Rauth, 1965).

ABBREVATIONS USED

UV	Ultraviolet
UV-endo	UV-specific endonuclease from *Micrococcus luteus*
polA107-DNA polymerase I	DNA polymerase I, lacking 5'-3' exonucleolytic activity, isolated from *E.coli* KMBL1789 *polA107*
dimer	cyclobutane-type of dimer formed between two adjacent pyrimidine bases in DNA
dNTP's	equimolar amounts of dATP, dCTP, dGTP and dTTP
RF DNA	Replicative form DNA of phage ØX174
RF I DNA	RF DNA form I, consists of covalently closed circular bi-helical DNA molecules
RF II DNA	RF DNA form II, circular double-stranded DNA molecules, containing one or more nicks

RESULTS

PHYSICO-CHEMICAL DETERMINATION OF THE EXCISION-REPAIR PROCES

ØX174 RF DNA was UV-irradiated and treated with UV-endo, which converts RF I DNA into RF II DNA (reaction 1). The ratio between RF I and RF II DNA molecules was determined by separating the two forms by centrifugation on alkaline sucrose gradients. In reaction 2 of the excision-repair assay, the UV-endo treated DNA was repaired by the combined action of DNA polymerase I and DNA ligase. The progress of the reaction was followed by the reformation of RF I DNA. UV-endo was not inactivated prior to the addition of the components of the second reaction, because we wished to mimick the *in vivo* conditions as closely as possible. Moreover the presence of UV-endo during the excision-repair reaction might prevent premature ligation of nicks introduced by UV-endo, before excision of dimers had taken place.

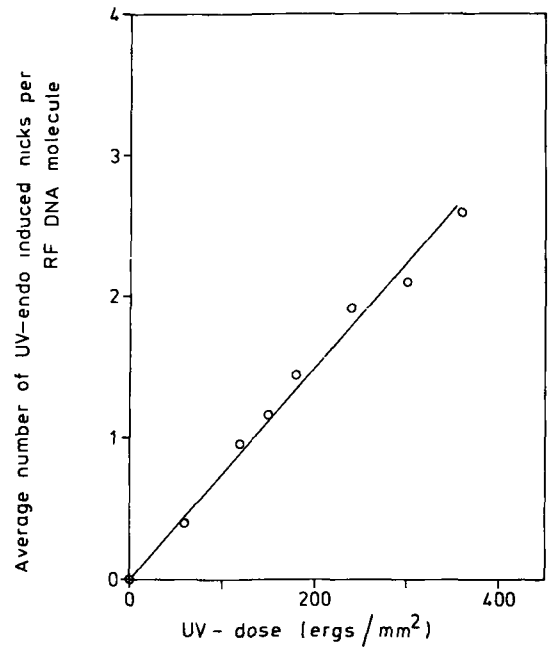

Fig.1. Conversion of RF I DNA into RF II DNA by UV-endo with increasing doses of UV-light.

Conditions for the reaction of UV-endo with RF DNA have been described in Materials and Methods under conditions for excision-repair, reaction I.

Preparations of RF I DNA were irradiated with increasing doses of UV-light and incubated with UV-endo. Subsequently, the ratio of RF I DNA to RF II DNA was determined with the filter-assay, and the data were used to calculate the average number of scissions per RF DNA molecule (fig.1). The amount of UV-endo present in the reaction-mixture was sufficient to attack at least 5 times more dimers than present in our DNA preparation during the time of incubation. Assuming that incisions were made next to all dimers, the average number of nicks equals the average number of dimers per RF DNA molecule. From the results presented in fig. 1 we have computed that an UV-dose of 137 ergs/mm^2 is sufficient to introduce on the average 1 dimer per RF DNA molecule.

Table 1. Conditions for excision-repair *in vitro*.

	RF I DNA (%)
A. Specificity of UV-endo on RF DNA	
Starting material	70
Incubated with UV-endo	63
UV-irradiated with 450 ergs/mm^2	69
UV-irradiated with 450 ergs/mm^2; incubated with UV-endo	> 5
B. Conditions for complete excision-repair of RF DNA	
Optimal conditions	49
Without UV-irradiation	66
Without DNA polymerase I	> 5
Without dNTP's	> 5
With polA107-DNA polymerase I instead of DNA polymerase I	> 5
Supplemented with 2 µl exonuclease III	43
Without DNA ligase	> 5

The conditions for excision-repair have been described in Materials and Methods. Reaction 2 was carried out at 35°C for 20 min.

The specificity of the UV-endo preparation was studied by incubating UV-irradiated and non-irradiated RF DNA with the enzyme (table Ia). Incubation of irradiated RF I DNA with UV-endo led to conversion to RF II DNA. When non-irradiated RF DNA was treated with the same amount of enzyme, only a small amount of RF I DNA was converted to RF II DNA, thus indicating the formation of 1 nick per 10 molecules of RF DNA. Whether the UV-endo introduces occasionally a break in unirradiated supercoiled RF I DNA or is slightly contaminated with an a-specific endonuclease is not known. In table 1b, conditions for excision, reinsertion and ligation are summarized. When excision-repair was carried out under optimal conditions 49% of the DNA was converted back to the RF I form, indicating that more than 90% of the dimers were removed. Unirradiated RF II DNA (the starting material consisted of 70% RF I DNA and 30% RF II DNA) was not converted to RF I DNA molecules after treatment with the repair enzymes. A possible explanation is, that the RF II DNA molecules do not have internal 3'OH termini required for the binding of DNA polymerase I and DNA ligase. The excision-repair reaction was completely dependent on the activity of DNA polymerase I. Omission of the dNTP's resulted in 20% breakdown of the RF DNA to acid-soluble product, with no demonstrable conversion to RF I DNA. PolA107-DNA polymerase I could not replace DNA polymerase I in the excision-repair reaction. In the presence of nicked bi-helical DNA, polA107-DNA polymerase I promotes "strand displacement" synthesis (Heyneker *et al.*, 1973) instead of "nick translation" synthesis, catalysed by DNApolI (Kelly *et al.*,1970). By the mechanism of nick translation, which is a combination of 5'-3' exonucleolytic activity and DNA polymerizing activity, dimers are effectively removed from UV-damaged DNA (Kelly *et al.*, 1969), (Setlow and Kornberg, 1972), (Friedberg and Lehman, 1974). When strand displacement occurs, the newly synthesized strand will displace the strand containing the dimer. Because the displaced strand it not broken down, there will be no free 5'P termini generated and consequently rejoining with DNA ligase cannot occur. It is clear from table 1 that DNA polymerase I and DNA ligase efficiently convert RF II DNA molecules, which were formed by UV-endo, to RF I DNA molecules. Apparently an additional phosphatase treatment is not essential to provide 3'OH endgroups for DNA polymerase I to bind, suggesting that 3'OH termini are generated by UV-endo; in accordance with results obtained by Hamilton *et al.*,(1974). To test whether the addition of a phosphatase improves the efficiency of the *in vitro* repair-system, exonuclease III was supple-

mented to the reaction-mixture. As can be seen from table I, the phosphatase addition was without effect. Other ratio's of DNA polymerase I, DNA ligase and exonuclease III did not improve the reaction either (results not shown). DNA ligase is essential for excision-repair: in the absence of this enzyme no RF I DNA was reformed and about 20% of the DNA was degraded to acid-soluble product, probably due to uncontrolled nick translation by DNA polymerase I (result not shown).

EFFICIENCY OF THE EXCISION-REPAIR PROCESS

The influence of the UV-dose on the efficiency of the excision-repair process *in vitro* was investigated. The results are summarized in table 2. The percentage of nicks remaining after repair in RF I DNA versus increasing doses of UV-light is presented in column VI. This percentage appears to be independent of the doses used, indicating that even at a dose exceeding 2000 ergs/mm^2 more than 90% of the dimers were removed successfully.

Table 2. Efficiency of the *in vitro* excision-repair process.

I UV dose in ergs/mm^2	II % RFI DNA without repair	III % RFI DNA after repair	IV Average number of nicks per RF DNA molecule introduced by UV-endo	V Average number of nicks per RF DNA molecule remaining after repair	VI Percentage of nicks in RF DNA, remaining after repair
0	67	76	0	0	
450	65	53	3.3	0.3	9
900	65	45	6.6	0.5	8
1350	63	43	9.9	0.6	6
1800	61	27	13.2	1.0	8
2250	61	20	16.5	1.3	8

RF DNA was irradiated with increasing doses of UV-light. Samples were split in two equal portions. One half was not treated with repair enzymes (column II), the other half was repaired under optimal conditions (column III). Incubations were for 90 min. at 20°C. The samples were subjected to centrifugation on alkaline sucrose gradients and the percentage of RF I DNA was determined (column II and III). The average number of nicks per RF DNA molecule, remaining after repair, (column V) was calculated from the ratio of RF I to RF II DNA. Since non-irradiated RF I DNA contained on the average 0.3 nicks per molecule, which could not be repaired under the conditions used, this figure was substracted. The average number of nicks per RF DNA molecule introduced by UV-endo (column IV) was computed from the data given in fig.1. The percentage of nicks, remaining in the RF DNA after repair (column VI) was calculated from the date presented in column IV and V.

BIOLOGICAL DETERMINATION OF THE EXCISION-REPAIR PROCESS

The recovery of RF I DNA after UV-irradiation and subsequent treatment with repair enzymes, suggests that complete repair *in vitro* has taken place. However, this result cannot be taken as proof, that the repaired RF I DNA does not contain residual dimers. We have therefore examined the ability of UV-irradiated RF I DNA to form ØX174 phage particles when incubated with uvr^+ spheroplasts or uvrA spheroplasts, which are impaired in excision-repair due to the absence of UV-specific endonuclease (Braun and Grossman, 1974). It was shown by Jansz *et al.*, (1963) that the biological inactivation, caused by increasing doses of UV-radiation, of RF I DNA, which was not subjected to repair *in vitro*, was about 6 times greater, when measured on uvrA spheroplasts than on uvr^+ spheroplasts. Restoration of biological activity takes place when UV-irradiated RF I DNA is converted to RF II DNA by treatment with UV-endo (Rörsch *et al.*, 1964),(Rörsch *et al.*, 1966). The remaining steps of the repair reaction are then carried out by the spheroplasts. In order to demonstrate complete repair *in vitro* it is necessary to separate the completely repaired molecules (RF I DNA) from partially repaired molecules(RF II DNA).This was achieved by sedimentation on alkaline sucrose gradients of the two forms of RF DNA. After neutralization of the alkali, the specific biological activity of RF I DNA was determined. We have found that the specific biological activity of UV-irradiated RF I DNA after excision-repair *in vitro*, was the same when measured on uvrA spheroplasts or on uvr^+ spheroplasts (fig.2). From this result we conclude that dimers are removed effectively *in vitro*. Despite the removal of dimers, there is still a decrease in biological activity of RF I DNA with increasing doses of UV-light. About the same decrease is observed, with RF I DNA, which is not repaired *in vitro* but *in vivo* (on uvr^+ spheroplasts). This decrease in biological activity is due to another type of damage, which is neither repaired *in vitro* nor *in vivo*. It has been reported by Grossman (1973),(unpublished observation H.Ono and L.Grossman) that cytosine:cytosine dimers introduced in polydC.polydG by UV-irradiation are not recognized by UV-endo of *M.luteus*. This would imply that cytosine-cytosine dimers are not excised from UV-damaged DNA *in vitro* and perhaps neither *in vivo*.

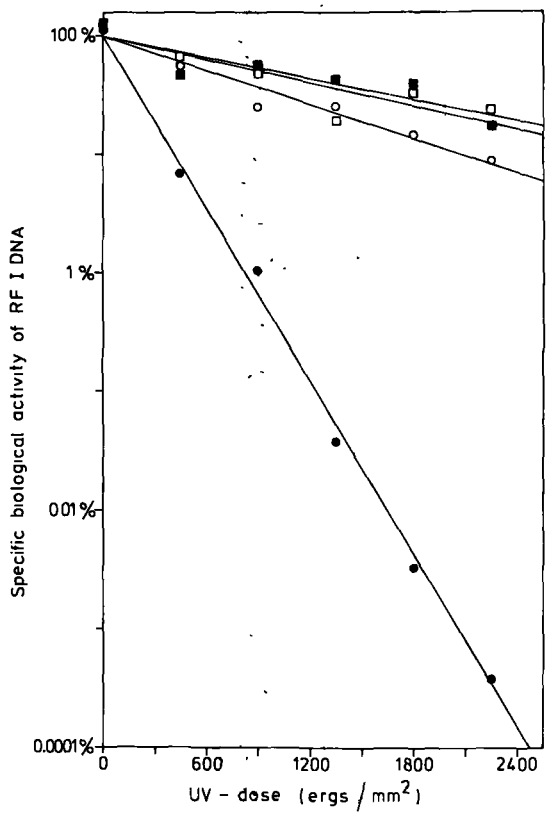

fig.2. Recovery of biological activity of UV-damaged RF I DNA after complete excision-repair.
RF DNA was irradiated with increasing doses of UV-light. Samples were split in two equal portions. One half was not treated with repair enzymes, the other half was repaired under optimal conditions. Incubations were for 90 min. at 20°C. The samples were subjected to centrifugation on alkaline sucrose gradients.
The peak RF I DNA fraction of each gradient was neutralized with 40 mM phosphoric acid and the biological activity determined on uvr^+ and uvrA spheroplasts. The concentration of the RF I DNA in these peak fractions was calculated from the radioactivity. The specific biological activity (no.of viable phage particles per µg RF I DNA) of unirradiated RF I DNA was 2.10^{10} for uvr^+ spheroplasts and 10^{11} for uvrA spheroplasts. In order to compare the results of different experiments more easily, the specific biological activity of unirradiated RF I DNA on both types of spheroplasts was arbitrarily set at 100%. Closed symbols represent specific biological activity of RF I DNA determined on uvrA spheroplasts, circles : without repair; squares : after repair. Open symbols represent specific biological activity of RF I DNA determined on uvr^+ spheroplasts, circles : without repair; squares : after repair.

DISCUSSION

The results presented in this paper are an extention of our previous studies on the repair of UV-irradiated DNA. We have shown before that dimers can be removed from UV-damaged transforming DNA of *B.subtilis* by excision-repair *in vitro*, which was demonstrated by the recovery of transforming activity (Heyneker *et al.*,1971).
The main advantage of using the experimental approach described in this paper is that the complete excision-repair process can be monitored accurately with both physico-chemical and biological methods.
The kinetics of the excision-repair reaction as well as the individual steps can be conveniently studied. The fidelity of DNA-restoration can be tested by determining the specific biological activity of UV-inactivated RF I DNA on uvrA spheroplasts before and after repair. We have shown that only three enzymes are needed for correct repair : UV-specif endonuclease, DNA polymerase I and DNA ligase. The 5'-3'exonucleolytic activity of DNA polymerase I appears to be essential for repair, as polA107-DNA polymerase I can not substitute for DNA polymerase I in excision-repair *in vitro*. This opens the possibility to look *in vitro* for complementation of the 5'-3' exonucleolytic activity by other enzymes of *E.coli*. It is likely that such enzymes exist, because the *E.coli* polA107 mutant is only moderately UV-sensitive (Glickman *et al.*, 1973) suggesting that *in vivo* other nucleases substitute for the defective DNA polymerase I. Recently, Chase and Richardson (1974a) characterized a new exonuclease from *E.coli*. The enzyme, which was identified as exonuclease VII, degrades single stranded DNA in the 3'-5' as well as in the 5'-3' direction. According to Chase and Richardson (1974b), exonuclase VII is not inhibited by the presence of dimers in the DNA. This enzyme is therefore an appropriate candidate to complement in excision-repair for the 5'-3' exonucleolytic activity, which is lacking in polA1 DNA polymerase I.

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SUMMARY

The main lesions introduced in the DNA of living cells by UV-light consist of pyrimidine dimers. They account for a large part for the deleterious effects of UV-radiation on biological systems. Many types of cells are able to repair the UV-damaged DNA by excision of the dimers. The excision-repair process is thought to consist of four consecutive steps : 1. an UV-specific endonuclease recognizes the dimer and introduces a nick near the dimer, 2. an UV-exonuclease excises the photoproduct, 3. new nucleotides are inserted by a DNA polymerase and 4. the remaining nick is sealed by DNA ligase. In this thesis the results are described of an investigation on excision-repair *in vitro* with purified repair enzymes of DNA's from different organisms. In the first publication results have been presented on the repair of UV-irradiated transforming DNA from *Bacillus subtilis* with UV-specific endonuclease from *Micrococcus luteus* and DNA polymerase I and DNA ligase from *Escherichia coli*. We have found that the biological activity of the transforming DNA could be partially restored by treatment with these enzymes. Incubation of partially repaired DNA with photoreactivating enzyme from yeast did not further increase the biological activity, indicating that the restoration of the biological activity is due to the removal of pyrimidine dimers. This conclusion is supported by our finding that a considerable fraction of the dimers are removed by treatment with the repair enzymes.
In the second and third publication experiments have been described on the properties of a defective DNA polymerase I, which was isolated from the *E.coli* mutant polA107. The mutant DNA polymerase I was purified to apparent homogeniety and its properties were compared to those of DNA polymerase I isolated from an isogenic wild-type *E.coli* strain. It was shown that the mutant enzyme lacks the 5'-3' exonucleolytic activity. The products formed by the action of mutant DNA polymerase I on nicked circular double stranded DNA of phage ØX174 have been analyzed by sucrose gradient sedimentation. It was found that "strand displacement" synthesis occurs instead of "nick translation". Visualization of the reaction products by electron-microscopy revealed that significantly more DNA molecules were branched than was seen upon incubation of the DNA with wild-type enzyme. This is due to the fact that strand displacement synthesis gives rise to template switching, yielding branched DNA

structures. DNA polymerase I can be cleaved by mild proteolytic treatment in a large fragment on which DNA polymerizing as well as 3'-5' exonucleolytic activity are located, and a small fragment with 5'-3' exonucleolytic activity. The mobility of the mutant DNA polymerase I and its large and small fragment in polyacrylamide gels during electrophoresis in the presence of SDS are indistinguishable from the mobility of the same components of wild-type DNA polymerase I, suggesting that a point mutation is responsible for the alteration of the enzyme. From enzyme studies evidence has been obtained indicating that the polA107 mutation affects the 5'-3' exonucleolytic activity directly.
In the fourth publication an alternative and sensitive assay has been described to study excision-repair *in vitro*. UV-damaged ØX174 RF I DNA was repaired by the combined action of UV-specific endonuclease from *M.luteus*, DNA polymerase I from *E.coli* and DNA ligase from phage T4 infected *E.coli*. The different steps of the reaction were followed with physico-chemical methods. By measuring the restoration of the specific biological activity of RF I DNA after completion of the excision-repair process it was proven that the dimers were effectively removed from the DNA. It was found that exonuclease III did not influence the excision-repair process indicating that 3'OH and 5'P termini are generated by the action of the UV-specific endonuclease. DNA polymerase I isolated from the polA107 mutant of *E.coli* could not replace wild-type DNA polymerase I in the repair reaction supporting the view that dimers are excised by the 5'-3' exonucleolytic activity associated with DNA polymerase I.

Although the components of the *in vitro* repair system were obtained from different organisms, it seems likely that *in vivo* excision-repair of UV-damage takes place in a similar way as *in vitro*, because 1. the corresponding repair enzymes from *M.luteus E.coli* and phage T4 infected *E.coli* have similar properties, 2. mutations in *E.coli* which affect the structural genes of the enzymes used in our *in vitro* studies, render the bacteria more sensitive to UV-irradiation.

SAMENVATTING

De belangrijkste schade, die wordt veroorzaakt door ultraviolet (UV) licht in het DNA van levende cellen, zijn pyrimidine dimeren. Deze dimeren zijn voor een groot deel verantwoordelijk voor de schadelijke effecten van UV-straling op biologische systemen. Verschillende soorten cellen zijn in staat de dimeren door middel van excisie te verwijderen uit het DNA. Er zijn sterke aanwijzingen dat excisie-herstel een enzymatisch proces is, dat uit vier achtereenvolgende stappen bestaat: 1) *incisie*, een UV-specifiek endonuclease introduceert een breuk in het DNA vlak bij de dimeer, 2) *excisie*, een UV-exonuclease verwijdert de dimeer uit het DNA, 3) *herstel-replicatie*, een DNA polymerase bouwt nieuwe nucleotiden in op de opengevallen plaats, 4) *sluiting*, DNA ligase herstelt de resterende breuk.
In dit proefschrift worden de resultaten beschreven van een onderzoek naar excisie-herstel *in vitro* met gezuiverde enzymen en DNA's afkomstig van verschillende organismen. In de eerste publicatie zijn de resultaten vermeld van herstel van UV-bestraald transformerend DNA van *Bacillis subtilis* met UV-specifiek endonuclease uit *Micricoccus luteus* en DNA polymerase I en DNA ligase uit *Escherichia coli*. Wij hebben gevonden dat de biologische activiteit van het transformerende DNA gedeeltelijk kon worden hersteld door behandeling met deze enzymen. De biologische activiteit van het herstelde DNA nam niet verder toe na incubatie met fotoreactiverend enzym uit gist, hetgeen er op wijst, dat de dimeren uit het DNA zijn verwijderd. In het tweede en derde artikel worden experimenten beschreven betreffende de eigenschappen van een veranderd DNA polymerase I dat uit de *E.coli* mutant PolA107 werd geïsoleerd. De eigenschappen van het gezuiverde mutant DNA polymerase I werden vergeleken met die van DNA polymerase I, dat gezuiverd was uit een isogene wild-type *E.coli* stam. Het bleek, dat het mutant enzym de 5'-3' exonucleolytische activiteit mist. De reactie producten, die werden gevormd na incubatie van wild-type DNA polymerase I of mutant DNA polymerase I met circulair DNA van bacteriofaag ØX174, waarin enkelstrengige breuken waren aangebracht (RF II DNA), werden geanalyseerd door middel van sedimentatie in sucrose gradienten. Het ontbreken van de 5'-3' exonucleolytische functie had tot gevolg, dat synthese van nieuw DNA niet gepaard ging met gelijktijdige afbraak van het stroomafwaarts gelegen ouderlijk DNA ("nick translation"),

maar dat het nieuw gesynthetiseerde DNA de ouderlijke DNA-streng van de matrijs verdrong ("strand displacement"). De reactie producten werden ook met behulp van een electronenmicroscoop bekeken : aanzienlijk meer DNA moleculen vertoonden vertakkingen, na incubatie van het RF II DNA met mutant DNA polymerase I, dan na incubatie met wild-type DNA polymerase I. Dit is het gevolg van het feit, dat bij "strand displacement" synthese het DNA polymerase de verdrongen DNA-streng als matrijs voor DNA synthese kan gebruiken, hetgeen vertakte DNA structuren oplevert. DNA polymerase I kan door een voorzichtige behandeling met proteolytische enzymen worden gesplitst in twee fragmenten: een groot fragment met DNA polymeriserende en 3'-5' exonucleolytische eigenschappen en een klein fragment met 5'-3' exonucleolytische activiteit. De loopsnelheid van het mutant DNA polymerase I en van het grote en kleine fragment in polyacrylamide gels in aanwezigheid van SDS waren niet te onderscheiden van de loopsnelheid van de overeenkomstige componenten geïsoleerd uit wild-type DNA polymerase I. Dit suggereert, dat een puntmutatie verantwoordelijk is voor de verandering van het enzym. Het werd aannemelijk gemaakt, dat de PolA107 mutatie direct aangrijpt op de 5'-3' exonucleolytische activiteit. In de vierde publicatie is een alternatieve en gevoelige bepalingsmethode beschreven om excisie-herstel *in vitro* te bestuderen. UV-beschadigd ØX174 RF I DNA werd hersteld door de gezamenlijke werking van UV-specifiek endonuclease uit *M.luteus*, DNA polymerase I uit *E.coli* en DNA ligase uit faag T4 geïnfecteerde *E.coli*. De verschillende stappen van de reactie konden met physico-chemische methoden worden gevolgd. Door het herstel van de specifieke biologische activiteit van RF I DNA te bepalen na voltooiing van het excisie-herstel proces kon worden bewezen, dat de dimeren daadwerkelijk uit het DNA waren verwijderd. Exonuclease III beïnvloedde het excisie-herstel proces niet hetgeen er op wijst, dat het UV-specifieke endonuclease breuken maakt met 3'OH en 5'P eindgroepen. DNA polymerase I, dat werd geïsoleerd uit de PolA107 mutant van *E.coli* kon wild-type DNA polymerase I niet vervangen in de excisie-herstel reactie. Dit betekent, dat dimeren door de 5'-3' exonucleolytische activiteit van DNA polymerase I worden geëxciseerd.

CURRICULUM VITAE

Op verzoek van de Faculteit der Wiskunde en Natuurwetenschappen volgen hier enkele persoonlijke gegevens.

Na in 1961 het eindexamen HBS-B aan het Eerste Vrijzinnig Christelijk Lyceum te 's-Gravenhage te hebben behaald, liet ik mij in hetzelfde jaar inschrijven aan de Rijksuniversiteit te Leiden.
In oktober 1965 werd het candidaatsexamen in de studierichting G afgelegd (schei- en natuurkunde met biologie), gevolgd door het doctoraalexamen in april 1968. Het hoofdvak biochemie, het bijvak organische chemie en de derde richting pharmacologie stonden onder leiding van Prof.Dr. H.Veldstra, Prof.Dr. E.Havinga en Prof.Dr. E.M.Cohen. Tijdens mijn doctoraalstudie heb ik gedurende twee maanden een stage gelopen bij de Pharmaceuticals Division van de ICI te Alderley Park, Engeland. Voor de vervulling van de militaire dienstplicht werd ik gedetacheerd op het Medisch Biologisch Laboratorium TNO te Rijswijk. Hier werd met het onderzoek begonnen naar herstel van stralingsschade bij micro-organismen, waarvan de resultaten in dit proefschrift zijn beschreven. In november 1969 trad ik in dienst van het J.A.Cohen Instituut voor Radiopathologie en Stralenbescherming, dat mij als wetenschappelijk medewerkers detacheerde bij het Laboratorium voor Moleculaire Genetica van de Rijksuniversiteit te Leiden. Tot september 1973 bleef ik als gast-medewerker werkzaam op het Medisch Biologisch Laboratorium TNO, daarna kreeg het Laboratorium voor Moleculaire Genetica een eigen behuizing in Leiden, waar ik sindsdien werkzaam ben.

NAWOORD

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De medewerkers van de instrumentmakerij en van de afdeling fotografie zowel in het Medisch Biologisch Laboratorium TNO als in het Laboratorium voor Biochemie te Leiden hebben mij op voortreffelijke wijze geholpen.
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