DEFECTIVE DNA REPAIR IN THE HUMAN SKIN DISEASE XERODERMA PIGMENTOSUM

PROEFSCHRIFT

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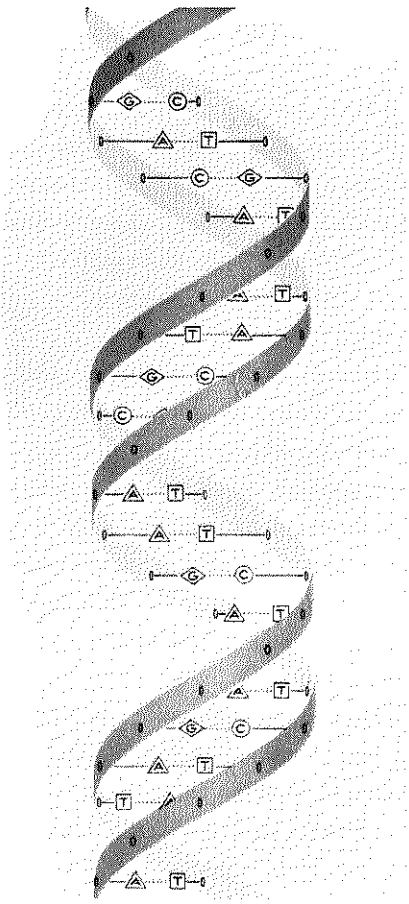
INTRODUCTION

Deoxyribonucleic acid (DNA) forms the genetic code, which is unique for each species and in most cases for each individual. Together with mainly proteins, the DNA is packed in the chromosomes of each cell. The code is determined by the sequence of the four bases - thymine, cytosine, adenine and guanine - which are bound to the deoxyribose groups of the DNA chains (Fig.1).



Alterations in the genetic code, for example by physical or chemical induction of chain breaks or base damages, often lead to the death of the cell or become manifest as changed properties, i.e. mutations. The occurrence of mutations in proliferating somatic cells in tissues of higher organisms results in the formation of groups of deviating cells which are often harmful to the organism and may occasionally be followed by the formation of tumors. If a mutation occurs in the germ cells this mutat-

Fig.1 The double helix structure of DNA. The four bases, thymine (T), cytosine (C), adenine (A) and guanine (G), are bound to deoxyribose-phosphate chains. The two complementary strands are held together by hydrogen bonds between the bases, which always form fixed pairs: A--T and G--C.

ion can be passed on to the following generations. Since most mutations are harmful or even lethal, the induction of mutations is dangerous not only for the individual organism but also for the genetic stability and consequently for the survival of the species.

The genetic code is threatened by various external agents. Such agents are:

(a) Ionizing radiation, e.g. cosmic rays, X-rays used for example for medical purposes and radiation occurring concomittant with the desintegration of radioactive isotopes which occur by nature or are produced for research purposes on behalf of biochemical laboratories, hospitals etc. and which are applied as well as produced in nuclear power plants and in large quantities in tests of nuclear weapons.

(b) Numerous chemical compounds which have in many cases been shown to be mutagenic or carcinogenic or both. The threat of these agents, like that of ionizing radiation, has increased strongly due to the enormous enlargement in recent years of the quantities and the variety of chemicals produced and applied for many purposes.

(c) Ultra-violet (UV) light, occurring in sunlight.

In the UV-spectrum (i.e. wavelengths shorter than 380 nm; visible light ranges from 380 to 780 nm) only light of wavelengths shorter than 310 nm can directly cause damage to the DNA, the main photoproducts being pyrimidine dimers (1.2;Fig.1-1). However, since all UV-irradiation occurring in sunlight of wavelenths shorter than 290 nm is absorbed in the atmosphere, due to the presence of ozon at heights between 15 and 35 km, only the narrow region between 290 and 310 nm is of importance for the induction of DNA damage. It is also this range of wavelengths that causes the highest lethality to micro-organisms and in man sunburn and skin cancer. These observations suggest a causal relationship between on the one hand DNA damage and on the other hand cell death, or in man damage to skin cells and possibly skin carcinogenesis. Nevertheless micro-organisms and

also cultured mammalian cells do survive low doses of UV light and this suggests that cells can repair DNA-damage to a certain extent.

Investigations of UV- and X-irradiated bacteria and of mutant bacterial strains, which are much more radiosensitive than the wild-type strains, have established that repair mechanisms operate in bacteria indeed. The rapid progress made in the past decade in the elucidation of repair mechanisms in bacteria has strongly stimulated the study of repair processes in mammalian cells. Several lines of investigation have provided evidence for the occurrence of DNA repair in mammalian cells. In 1968 it was discovered that a defect in a DNA repair mechanism is associated with the human inherited disease xeroderma pigmentosum (XP). Patients who suffer from this disease are extremely sensitive to UV light and develop severe skin lesions (e.g. skin tumors) after exposure to sunlight. Investigations of the cells cultured from XP patients have demonstrated that DNA repair is a biologically significant process in mammalian cells too.

It seems likely that all living organisms are able to repair DNA that has been damaged by UV light, ionizing radiation, mutagenic and carcinogenic chemical compounds and other agents. Besides being of direct importance for the individual organism, this repair ability will minimize the occurrence of lethal or harmful mutations and chromosome aberrations and thereby help to maintain the genetic stability of the species. On the other hand, from an evolutionary point of view, new mutations should occur to some extent, since evolution is only possible if there is a sufficient degree of genetic variability to select continuously the best adapted properties. Therefore the survival of the species presumably depends on a critical equilibrium between the genetic variability and the genetic stability. DNA repair mechanisms may play a role in establishing this equilibrium by minimizing the mutation frequency but possibly also by introducing mutations by making errors during

repair.

This thesis deals with DNA repair mechanisms in normal human cells and in the repair deficient cells of XP patients. In the first part of this thesis a review and a discussion is given of literature data, including our own contributions, on the damage induced by UV light and ionizing irradiation (chapter 1), DNA repair mechanisms in mammalian cells (chapter 2) and investigations of the defect in various forms of XP (chapter 3). In the second part -the appendix- 5 papers are presented in which our investigations of the above mentioned subjects are described and discussed in more detail.

CHAPTER 1

RADIATION DAMAGE IN LIVING CELLS

1.1 INTRODUCTION

DNA is the most extensively studied target for the induction of radiation damage in micro-organisms as well as higher organisms. Much less knowledge is available about radiation damage to proteins and membranes and the contribution of such damage to cell death. Some evidence exists that damage to structural proteins, involved in the maintenance of the chromosome structure, may contribute significantly to the appearance of chromosome aberrations and consequently to cell death (Zirkle and Uretz, 1963; Chu, 1965). On the other hand Griggs and Bender (1973) have observed that UV-induced chromosome aberrations can be prevented by photoreactivation (see chapter 2.2) and concluded that DNA lesions are the major cause of UV-induced chromosome aberrations. Without any doubt damage to the DNA structure is an important, if not the most important, cause of radiation-induced mutations and cells death.

1.2 DNA DAMAGE INDUCED BY ULTRA-VIOLET LIGHT

In 1960 Beukers and Berends discovered that UV irradiation of a solution of thymine caused the formation of photoproducts with a thymine dimer structure. Subsequently, it was shown that UV light induces also in DNA one of the possible stereoisomers, the *cis-syn* pyrimidine dimers (dithymine, dicytosine and thymine-cytosine) (Fig. 1-1). The efficiency of pyrimidine dimer formation in bacteria and cultured mammalian cells is of the same order: 1 - 5 pyrimidine dimers per DNA molecule of 10^9 dalton at a UV dose of 1 erg/mm^2 (micro-organisms: Howard-Flanders, 1968; mammalian cells: Setlow et al., 1969; Kleijer et al.,1973a:

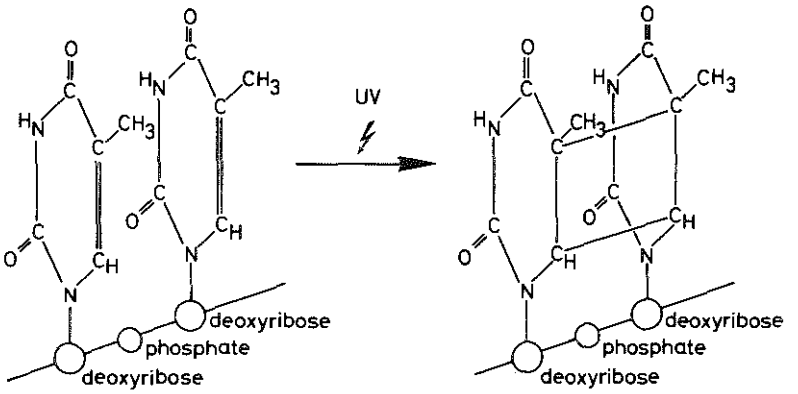


Fig.1-1. Formation of a *cis-syn* cyclobutane thymine dimer by ultra-violet irradiation. Two adjacent thymine bases in the DNA strand are linked by bonds between their respective C-5 and C-6 atoms.

paper II).

In experiments with UV-inactivated transforming bacterial DNA it has been shown that pyrimidine dimers are the main DNA-inactivating lesions. The inactivation could be significantly reduced by specifically splitting the dimers by either irradiation with UV light of a shorter wavelength (the monomer $\rightleftharpoons$ dimer equilibrium is shifted then to the monomer side) or by treatment of the DNA with photoreactivating enzyme and visible light (photoreactivation; see 2.2) (Setlow, 1966). The replication of bacterial DNA *in vitro* (Bollum and Setlow, 1963) and *in vivo* (Rupp and Howard-Flanders, 1968) is inhibited by the presence of pyrimidine dimers in the DNA. Also in mammalian cells the DNA synthesis is inhibited by UV irradiation, but it has not been established whether dimers are the only, or even the most important cause of this effect (Painter, 1970a). It is assumed that the replication of DNA is delayed but not completely blocked by the dimers.

Other DNA damages induced by UV irradiation are: pyrimidine hydrates (Miller and Cerutti, 1968); pyrimidine adducts (Varghese and Day, 1970; Hauswirth and Wang, 1973); single-strand breaks (Marmur et al., 1961; Kleijer et al.,

1973a: paper II) and cross-links between DNA-strands (Marmur and Grossman, 1961) or DNA and the surrounding protein molecules (Smith, 1962). Although these types of damage are less important than pyrimidine dimers in micro-organisms, to our knowledge there is no information available about their importance in mammalian cells.

1.3 DNA DAMAGE INDUCED BY IONIZING IRRADIATION

Ionizing radiation causes ionizations and the formation of reactive radicals in the DNA, either directly or indirectly by radical formation in the neighbourhood of the DNA. The most important result of these reactions is the formation of single- and double-strand breaks in the deoxyribose phosphate chains. Although double-strand breaks occur at a much lower frequency than single-strand breaks, (Freifelder, 1966; v.d. Schans and Blok, 1970) the former are of greater importance for the inactivation of bacteriophages, bacteria and possibly also mammalian cells because it is much more difficult or perhaps not possible at all to repair double-strand breaks (Painter, 1970b and 2.5).

Ionizing radiation can also induce damage to the pyrimidine and purine bases without the consequence of chain breaks (Setlow and Carrier, 1973). Only few data are available on the relative contribution of the different types of DNA damage to the inactivation of micro-organisms, whereas for higher organisms such data are completely lacking.

CHAPTER 2

DNA REPAIR MECHANISMS

2.1 INTRODUCTION

In bacteria several mechanisms involved in DNA repair have been found and are known as: photoreactivation, excision repair, post-replication repair and rejoining of single-strand breaks. These bacterial mechanisms have served as models for repair processes in mammalian cells. For that reason some attention will be paid in this chapter to the findings in bacteria.

The isolation of a large number of radiosensitive bacterial mutants and the genetic and biochemical analysis of these mutants have contributed strongly to the elucidation of repair mechanisms and their significance to the survival of irradiated bacteria. A similar approach for mammalian cells has been hampered by difficulties in the selection of radiosensitive or resistant mutants. The few cases of radiosensitive cell lines reported sofar were isolated from heteroploid mouse (Alexander and Mikulski, 1961), Chinese hamster (Cleaver, 1969a; Humphrey et al., 1970) and human cell lines (Randtke et al., 1972; Isomura et al., 1973). It has not been established whether the radiosensitivity in these cell lines is due to point mutations or to chromosome rearrangements or changes in the chromosome number. This disadvantage is partially compensated by the natural occurrence of UV-sensitive mutants in the human hereditary disease xeroderma pigmentosum (XP). The UV-sensitivity in this disease appeared to be associated with a defect in a DNA repair mechanism, i.e. excision repair. The study of repair mechanisms in mammalian cells, discussed in this chapter, has been stimulated strongly by the investigations on XP, which will be discussed separately in chapter 3.

Very recently it has been reported that another human

disease -the Hutchinson-Gilford progeria syndrome- is associated with a defect in the rejoining of X-ray induced single-strand DNA breaks (see 2.5).

2.2 PHOTOREACTIVATION

Photoreactivation is an enzymatic process in which specifically pyrimidine dimers are monomerized under the influence of light in the range of 310 - 500 nm as an energy source. The DNA is thus repaired in a simple and efficient way without the necessity of DNA chain breakage and replacement of damaged bases. Photoreactivation has been observed in: bacteria, protozoa, some higher plants, vertebrates such as: fish, amphibia, reptilia, birds and in the lower order of mammals: the marsupials (e.g. rat kangaroo) but not in placental mammals (Cook, 1970).

Since photoreactivation acts specifically on pyrimidine dimers it is a valuable tool to study the involvement of pyrimidine dimers (relative to other UV-induced lesions) in the cause of cell death (Regan and Cook, 1967) and chromosome aberrations (Griggs and Bender, 1973) or to prove that dimers are the substrate for other repair processes (e.g. repair replication, Krishnan and Painter, 1973; or UV endonuclease, Bacchetti et al., 1972; Paterson et al., 1973).

2.3 EXCISION REPAIR

(a) *The mechanism in bacteria as a model for higher organisms*

In contrast to photoreactivation, excision repair occurs in the dark; the less accurate term dark-repair has therefore been used for this process. Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964) found that UV-induced thymine dimers were excised from the DNA of wild type *Escherichia coli* cells but not from the DNA of certain UV-sensitive mutants. Pettijohn and Hanawalt (1964) showed that after UV irradiation of *E. coli* small patches of DNA

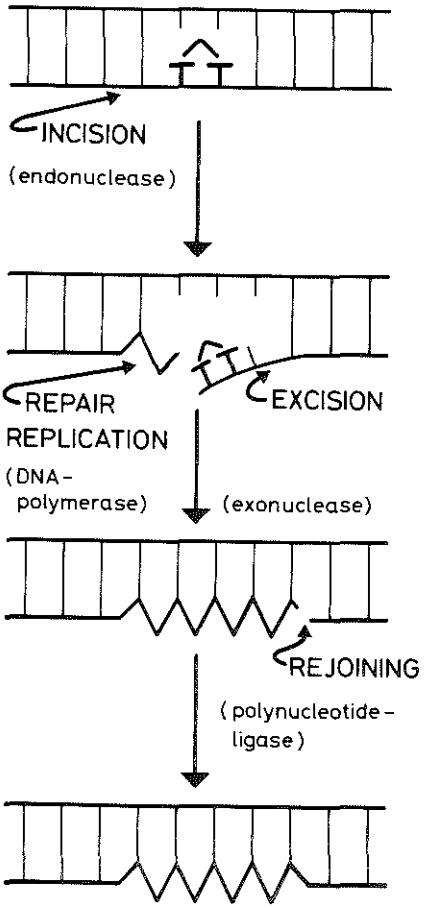


Fig.2-1.Scheme showing the steps and the enzymes involved in the excision repair mechanism for bacteria and higher organisms. The lesion shown is a thymine dimer; other base damages and single-strand breaks might be repaired by the same mechanism except that for the repair of single-strand breaks the first step (incision) can be omitted.

were synthesized and inserted dispersively in the pre-existing DNA-strands. From these and other observations a mechanism was proposed (Fig. 2-1) that repairs UV damaged DNA in four steps by the combined action of: an endonuclease, which incises the DNA strand adjacent to a pyrimidine dimer; an exonuclease, which excises the dimer; a DNA polymerase, which inserts complementary nucleotides using the opposite strand as a template; and a polynucleotide ligase, which closes the DNA strand (Setlow, 1967). Enzymes for each step have been isolated from bacteria and characterized (Grossman, 1973), whereas Heijneker et al. (1971) have demonstrated that the biological activity of UV-irradiated

Bacillus subtilis DNA can be restored by the combined action of purified preparations of UV-endonuclease (i.e. an endonuclease that acts specifically on UV-damaged DNA),DNA-polymerase (which has an exonuclease function as well) and a DNA-ligase.

Evidence that mammalian cells can repair their DNA by a similar excision repair mechanism (see Fig.2-1) has come from the demonstration of unscheduled DNA synthesis, repair replication and the removal of pyrimidine dimers.

(b) DNA repair synthesis

Unscheduled DNA synthesis and repair replication, first demonstrated in human (HeLa) cells by Rasmussen and Painter (1964, 1966), are assayed in quite different ways, but both phenomena reflect the same fundamental process (Painter and Cleaver, 1969). The incorporation of ^{3}H-thymidine, shown by autoradiography, in UV-irradiated cells which are not in S-phase, has been called unscheduled DNA synthesis (Djordjevic and Tolmach, 1967) because under normal conditions non-S-phase cells do not incorporate any ^{3}H-thymidine. Repair replication on the other hand is the synthesis and integration of short single-strand regions in the pre-existing DNA, measured after centrifugation of the DNA in density gradients. Semiconservatively synthesized DNA is separated from repaired DNA on the basis of the higher density it acquires because of the extensive incorporation of bromodeoxyuridine (BUdR given before and after irradiation. The procedure for this assay, developed by Pettijohn and Hanawalt (1964), is illustrated in Fig. 2-2, whereas a modified technique is described in paper III of the appendix (Lohman et al., 1973).

The necessity to separate semiconservatively synthesized DNA and repaired DNA can be avoided by using lymphocytes and an inhibitor (e.g. hydroxyurea) to suppress the semiconservative replication in the low fraction of cells in S-phase (about 1%) to a negligible level. It is possible then to determine repair synthesis by direct measurement of UV-

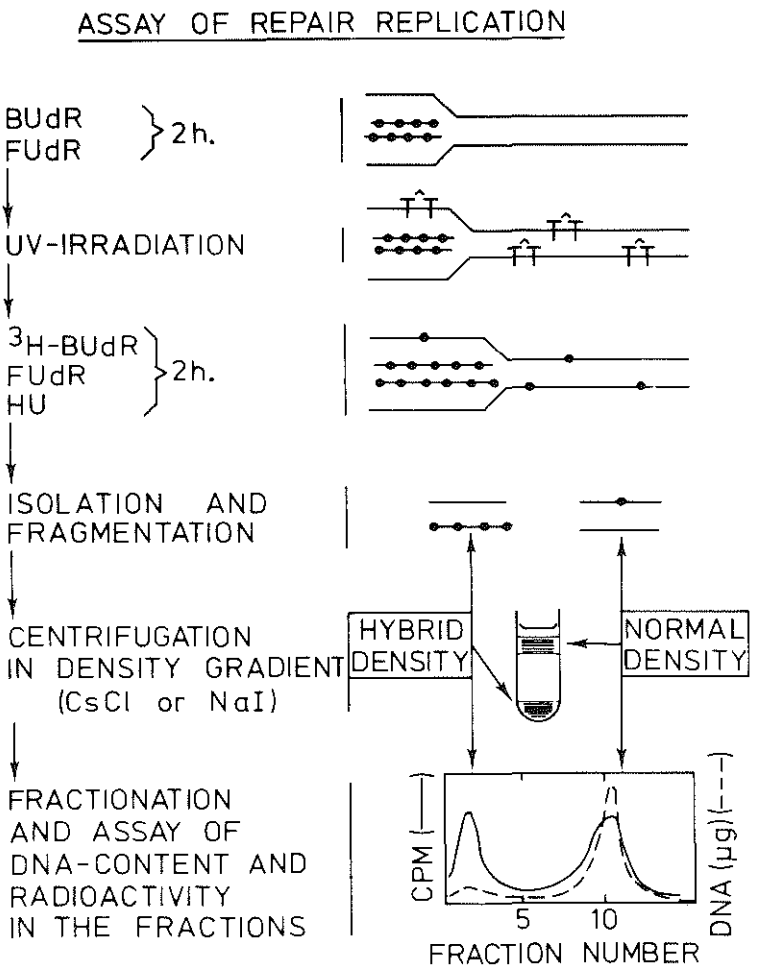


Fig.2-2. Protocol of the repair replication assay. Repair replication and semiconservative replication are measured by the incorporation of radioactive bromodeoxyuridine (^{3}H-BUdR). Both forms of DNA synthesis are distinguished by the different densities which repaired parental DNA and newly synthesized DNA acquire as a result of the different extents of BUdR incorporation(●). Fluorodeoxyuridine promotes the BUdR incorporation by preventing the *de novo* synthesis of thymidine phosphates. Hydroxyurea minimizes semiconservative replication.

stimulated ^{3}H-thymidine incorporation into the DNA (Evans and Norman, 1968; Burk et al., 1971). The use of this simple and rapid method for cultured fibroblasts would require almost perfect measures to prevent S-phase DNA replication, which are not yet available.

A fourth method to determine DNA repair synthesis, introduced by Regan et al. (1971a, b) utilizes the photolysis of BUdR. In this method repaired regions in the DNA, which have incorporated BUdR, are selectively broken by 313 nm radiation. The molecular weight of the resulting DNA molecules can be determined by alkaline sucrose gradient centrifugation and give a measure for the number of repaired regions.

After the first demonstration by Rasmussen and Painter (1964, 1966) the occurrence of DNA repair synthesis in mammalian cells has been confirmed in many reports. The amounts of repair synthesis performed in human cells are larger than in bovine cells (Cleaver et al., 1972) and in some Chinese hamster and mouse cell lines (Painter and Cleaver, 1969; Cleaver, 1973a). These variations are probably due to differences in the number of repaired regions rather than differences in the size of the patches inserted into the DNA (Setlow et al., 1972).

It has been estimated by several methods that the size of the patches inserted by excision repair in human cells is of the order of 30 - 100 nucleotides (Regan et al.,1971b; Edenberg and Hanawalt, 1972; Cleaver, 1973a). The synthesis of patches of this length requires the insertion of purines as well as pyrimidines. Failures to demonstrate repair synthesis by using ^{3}H-deoxyadenosine as a precursor (Smets, 1969; Lieberman et al., 1971), were probably caused by the conversion of this precursor to adenine and the predominant use of adenine for biochemical pathways other than DNA repair synthesis (e.g. RNA synthesis). Recently Cleaver (1973b) showed that purines (e.g. hypoxanthine, adenine) are used for repair synthesis.

After UV-irradiation the repair process probably starts

immediately, as is suggested by the time courses of repair synthesis which shows no time lag (Cleaver et al., 1972; Kleijer et al., 1973b: paper V) and can be measured after short incubation periods (15-30 min),(Kleijer, De Weerd-Kastelein, unpublished results). Gautschi et al. (1973) and Gianelli et al. (1973) have shown normal repair synthesis activities under conditions in which protein synthesis was inhibited. It seems likely therefore that the repair enzymes are continuously present in the cell and have long life times.

The rate of repair replication is dose dependent up to a UV-dose of 100-200 erg/mm^2 in human cells (Cleaver et al., 1972; Lohman et al., 1973: paper III; Kleijer et al., 1973b: paper V); at higher doses the repair system seems to be saturated. Although after doses up to 100 erg/mm^2 most of the repair synthesis occurs during the first few hours (2-4h) (Cleaver, 1973a) this is not true for higher doses; after 200 and 500 erg/mm^2 repair synthesis continues at an almost constant rate for 8 and more than 16 h respectively (Kleijer et al., 1973b: paper V).

(c) Excision of photoproducts

The second phenomenon which reflects the operation of an excision repair mechanism is the excision of pyrimidine dimers from the DNA of UV-irradiated human cells. Using chromatographic procedures to measure the dimer content of the DNA,it was found that after UV-doses of 100-200 erg/mm^2 about 50% of the induced dimers are removed within 24 h (Regan et al., 1968; Setlow et al., 1969; Cleaver and Trosko, 1970; Isomura et al., 1973; Kleijer et al., 1973b: paper V). Although the data are scarce, the time courses of excision suggest a similarity with those obtained for repair synthesis, which is consistent with the hypothesis that both phenomena are manifestations of steps in the same repair process (Cleaver et al., 1972; Kleijer et al.,1973b: paper V).

Two other methods have recently been developed with

which the presence of pyrimidine dimers in mammalian cell DNA and their excision has been demonstrated. The first is an immunological technique in which specific antibodies are used against dimers in UV-irradiated DNA (Seaman et al., 1972; Lucas, 1972). The second technique makes use of a purified bacterial UV-endonuclease *Micrococcus luteus* which produces breaks specifically at dimer sites in the DNA isolated from the mammalian cells (Paterson et al., 1973). With both techniques it was observed that the susceptibility of human cell DNA to the binding of antibodies or to the action of bacterial UV-endonuclease, decreased during post-irradiation incubation. The technique using bacterial UV-endonuclease and possibly also the immunological technique seem promising in giving more accurate results than the chromatographic method.

Although these studies have clearly demonstrated the occurrence of excision in human and in bovine cells there are two problems to be taken into consideration: the first being the high fraction of the dimers which remains in the DNA at a time when repair replication no longer occurs and the second being the fact that excision of dimers was not observed in mouse and Chinese hamster cells (Klimek, 1965; Trosko and Kasschau, 1967) although the UV-sensitivity of these cells is not significantly different from that in human and bovine cells. It has been suggested that dimers can be excised as oligonucleotides which would coprecipitate with the high molecular weight DNA in the usual chromatographic technique. However a similar remaining dimer fraction was found by Paterson et al. (1973) who used a UV-endonuclease from *Micrococcus luteus* to detect the dimers. The results obtained with this technique indicate that the remaining dimers are in the DNA and not in oligonucleotides. The imcompleteness of dimer excision can also not be attributed to an early inactivation of the repair system, or even of the whole cell, by the UV-doses usually applied (100-200 erg/mm^2), since it has been shown that even at higher doses (e.g. 500 erg/mm^2) the repair process

(i.e. repair replication) can continue for longer times (Kleijer et al., 1973b: paper V). Therefore it is suggested that part of the dimer sites is less accessible to the excision repair system. The cells might bypass the remaining dimers by the post-replication repair process described in section 2.4. Excision repair in rodent cells occurs at a slower rate than in human cells as is also shown by the repair replication levels, which are in most rodent cell lines significantly lower but not absent. Because of the relative insensitivity of the method a slow excision rate could not be excluded and recently Setlow et al. (1972) have reported a slight rate of excision of dimers in Chinese hamster and mouse cells after low UV-doses. The relative contributions of excision repair and post-replication repair to the survival of cells of various organisms is still obscure and has to be established in future experimental work.

(d) Incision and rejoining

The excision repair process involves the formation of incisions in the DNA molecules -preceding repair synthesis and excision- and the rejoining of the interrupted single strands afterwards (Fig. 2-1). Experiments have been carried out to demonstrate these steps directly by the detection of breaks in the DNA using sedimentation analysis in alkaline sucrose gradients. Before centrifugation whole cells are lysed and the DNA is extracted in a layer on top of the gradient in order to minimize shearing of the DNA (Mc Grath and Williams, 1966). The lysis layer consists of NaOH (pH > 12), usually EDTA and NaCl and occasionally a detergent or sucrose. A quantitative determination of molecular weights from the sedimentation profiles requires freely sedimenting single-stranded DNA molecules of a size which may probably not exceed 2.10^8 - 5.10^8 daltons (120 - 180 S particles). The release of such molecules from the original fast-sedimenting complex material takes relatively long lysis periods in alkali (1- 20 hours, depending on the temperature) but is strongly accelerated by the use of detergents in the

lysis layer or by prior irradiation with low doses of X-rays or UV light. More details about the alkaline sucrose gradient method, including the problems associated with the interpretation of the release of freely sedimenting single-stranded DNA from a complex DNA structure will be discussed in section 2.5.

Setlow et al. (1969) first described the demonstration of DNA breaks as a result of incision in UV-irradiated human cells. However the variable sedimentation profiles presented in their paper do probably not justify a definite conclusion. Experiments of Ben-Hur and Ben-Ishai (1971) have suggested that during post-irradiation incubation of HeLa cells breaks appear and subsequently disappear again. The profiles in some of their gradients showed fast-sedimenting DNA and did not enable the determination of the number of breaks. However the low molecular weight (M_n = 0.32 x 10^8D) observed 1.5 h after UV-irradiation (250 erg/mm^2) suggested a relatively large number of breaks. In contrast Kleijer et al. (1971, 1973a: paper II), using a detergent for lysis, found no change in the molecular weight (M_n = 10^8D) of DNA from human cells after UV-doses of 100 - 500 erg/mm^2 and various incubation intervals. Taking into account the sensitivity limitation of the technique, this indicated that the number of UV-induced breaks, if present, did not exceed 0.2 breaks/10^8D. Cleaver et al. (1972) found in bovine cells a UV-induced acceleration of the degradation of complex DNA comparable to earlier observations after low X-ray doses (see section 2.5). After lysis times long enough to obtain relatively stable DNA, Cleaver et al. observed a UV-induced shift of the sedimentation profile corresponding to a change of the molecular weight (M_n) of 4 x 10^8 to 2 x 10^8D, or to the induction of 0.25 breaks/10^8D. This number of breaks was present immediately after irradiation even when the cells were kept at 0°C and, in contrast to the results of Ben-Hur and Ben-Ishai (1971) with HeLa, cells, no further degradation occurred during post-irradiation incubation at 37°C. Therefore it might be questioned

whether the accelerated degradation of complex DNA and the presence of breaks were caused by an enzymatic incision process. A direct effect of UV-irradiation, resulting in the formation of breaks or alkali-labile lesions (Kleijer et al. 1973a: paper II) might also account for an accelerated degradation of complex DNA, although it is probably too small to cause a number of breaks as estimated by Cleaver et al. (1972). This type of investigations has recently been extended to normal human and xeroderma pigmentosum fibroblasts (Cleaver, 1973c). For the conversion of double-stranded DNA from unirradiated human cells a lysis period of at least 1h appeared necessary, whereas this process required only 5 - 10 min., or less, if normal human cells were irradiated with UV-doses of 13 erg/mm^2 and higher immediately before lysis and centrifugation. In contrast such a rapid conversion during brief lysis periods was not observed for DNA from UV-exposed xeroderma cells. The latter observations, which will be discussed further in chapter 3, are in agreement with the hypothesis that xeroderma cells are unable to make incisions in the DNA at pyrimidine dimer sites. Moreover these observations support Cleaver's interpretation that the accelerated release of single-stranded DNA from complex DNA after UV-irradiation is caused by single-strand breaks occurring during excision repair rather than by direct physical induction of breaks or alkali-labile bonds in the DNA.

It can be concluded from the reported data, discussed above, that soon after irradiation a steady state between incision and rejoining is established with a relatively small number of breaks present. In this concept the application of specific inhibitors of one or more of the later steps in the repair mechanism would lead to an accumulation of breaks. Therefore experiments have been performed to increase the number of breaks by blocking their rejoining by the use of compounds which were shown to inhibit the rejoining of X-ray induced breaks (Kleijer et al., 1971, 1973a: paper II). However the inhibitors of the rejoining process

used in these experiments (e.g. potassium cyanide, dinitrophenol, ethylenediaminotetracetate, iodoacetate and crystal violet) could not effect an accumulation of breaks after UV-irradiation. Similar results were obtained when some inhibitors of semiconservative DNA replication were tested, e.g. hydroxyurea, fluorodeoxyuridine, and dideoxythymidine (Kleijer and Bootsma, 1971). In contrast Ben-Hur and Ben-Ishai (1971) found an inhibiting effect of hydroxyurea on the return of the DNA to fast sedimenting, complex DNA if this compound was present during incubation after UV-irradiation. These results of Ben-Hur and Ben-Ishai were not confirmed in comparable experiments with bovine cells by Cleaver et al. (1972). Thus it seems to be impossible to accumulate breaks by changing the steady state equilibrium between incision and rejoining. Although it cannot be ruled out that the inhibitors used are not adequate for this purpose, there might be a more fundamental reason for the absence of break accumulation; this will be discussed in the next section.

(e) The excision repair mechanism and its biological significance

The investigations with living cells, discussed above, support the repair model consisting of the four steps shown in Fig. 2-1: incision, excision, repair replication and strand rejoining. The enzymes involved in this mechanism have not yet been characterized. A UV-specific endonuclease activity (Bacchetti et al., 1972; Brent, 1972), exonucleases (Lindahl, 1971), DNA-polymerases (Weissbach et al., 1971)and a polynucleotide ligase (Lindahl and Edelman,1968; Spadari et al., 1971) have been isolated from mammalian cells but it has not yet been established whether they play a role in DNA repair.

Although the steps in excision repair are known, at least two questions about the mechanism remain: First, what is the order of excision and repair replication? There is at present no evidence available to choose between a "cut

and patch mechanism" (excision followed by repair replication) and a "patch and cut mechanism" (repair replication followed by excision). Secondly, do the repair enzymes operate independently or does the repair of each lesion take place by a concerted action of the enzymes such as has been proposed for bacteria by Hanawalt and Haynes (1967)? Our observation in mammalian cells that incision breaks were not accumulated under conditions which exclude the rejoining might indicate a concerted mechanism. It was postulated (Kleijer et al. 1971, 1973a: paper II) that repair enzyme complexes systematically scan the DNA strands and repair the DNA at each lesion before moving further along the DNA to the next lesion. Such a mechanism would restrict the number of breaks at any time to at most the number of enzyme complexes involved in the repair process even under conditions where repair replication and rejoining are inhibited. Similarly Cleaver et al. (1972) suggested that the repair might occur by enzyme complexes because of the close correlation of the time courses of various repair phenomena in bovine cells. Taking into consideration the complexity of the chromosome structure in mammalian cells, a systematic scanning of the DNA and a co-ordinated repair of lesions by enzyme complexes seems an attractive model to guarantee an efficient repair process. Moreover the accumulation of dangerous intermediate phases during repair, such as breaks or gaps, will be avoided.

The biological significance of the excision repair process became clear after the discovery that excision repair is deficient in the disease xeroderma pigmentosum (XP) (Cleaver, 1968). This repair deficiency appeared to be associated with an increased UV-sensitivity of XP cells and a reduced ability to reactivate UV-inactivated viruses, indicating that excision repair is a functional process in human cells (3.1).

2.4 POST-REPLICATION REPAIR

A third repair mechanism, called recombination- or post-replication repair, was first demonstrated in 1968 by Rupp and Howard-Flanders in an *Escherichia coli* mutant which was unable to excise thymine dimers but could nevertheless survive low UV-doses. These authors as well as others observed that during DNA replication in UV-irradiated *E. coli* cells, gaps (of 10^3 nucleotides long; Iyer and Rupp, 1971) were left in the newly synthesized strands opposite to dimers in the parental strands. These gaps were filled in upon continued incubation by a process involving recombination with parental strands (Rupp et al., 1971) but probably also by some exogenous base insertion (Ley and Setlow, 1972).

For mammalian cells similar observations, i.e. synthesis of relatively short segments of DNA during semiconservative replication after UV-irradiation and subsequent gap-filling have been described (Cleaver and Thomas, 1969; Meyn and Humphrey, 1971; Lehmann, 1972a, b; Fujiwara, 1972 and Buhl et al., 1972a, b). The gap size was estimated to be, like in bacteria, about 1000 nucleotides (Lehmann, 1972a; Buhl et al., 1972a).

Despite the similarities between the observations in bacteria and in mammalian cells the processes involved may differ in at least one important respect. Recombinational events, involving exchanges of parental DNA-regions, such as found in *E. coli*, have not been observed in mammalian cells. The gaps appeared to be filled in completely (Lehmann, 1972a) or at least partially (Buhl et al., 1972a) by *de novo* DNA synthesis.

The biological function of the gap-filling process is still obscure. The inhibitory action of caffeine on the recovery of UV-irradiated cells, especially during S-phase (Domon and Rauth, 1969) and its inhibitory effect on gap-filling (Cleaver and Thomas, 1969; Lehmann, 1972c) may be correlated and considered as an indirect indication for a functional role of this mechanism. Mutant cells, which can-

not perform post-replication repair, have as yet not been found. Nevertheless it is tempting to speculate that post-replication repair is an important mechanism that can operate in concert with excision repair. Since excision repair requires an undamaged complementary strand, any gap left in the newly synthesized strand opposite a dimer site, would make this site irreparable. Apparently the function of post-replication repair is to overcome the gaps in the newly synthesized DNA and to keep the dimer sites in the old strands susceptible to excision repair.

2.5 REPAIR OF DAMAGE INDUCED BY IONIZING RADIATION

The induction and rejoining of single-strand breaks in cellular DNA has been studied mainly with the alkaline sucrose gradient technique devised by McGrath and Williams (1966). This technique is based on the gentle lysis of cells in an alkaline layer on top of the gradient to obtain single-stranded DNA with a minimal number of breaks introduced during isolation (see also 2.3.d). The molecular weights of the DNA molecules can be derived from the determination of sedimentation coefficients (Svedberg and Pederson, 1940; Burgi and Hershey, 1963) and the use of the relation of Studier (1965) between the sedimentation coefficient (S) and the molecular weight (M) in the sucrose gradients:

$$S = 0.0528\ M^{0.40}$$

After the determination of the average molecular weights of unirradiated (M_o) and irradiated DNA (M_r), the number of induced breaks (P) is calculated from the equation:

$$P = \frac{M_o}{M_r} - 1$$

(Lohman, 1968,1969; v.d. Schans, 1969; Ehmann and Lett,1973)

Mc Grath and Williams (1966) demonstrated that the molecular weight of the DNA in bacteria was lower immediately after X-irradiation but increased during incubation. They

attributed these effects to the induction and subsequent rejoining of breaks. Similar observations have since been made for mammalian cells, although some modifications of the lysis procedure were necessary to obtain reproducible sedimentation profiles from unirradiated cells. The induction of single-strand breaks after doses of 5 - 30 krad, and the rejoining of breaks, was shown in mouse cells (Lett et al., 1967), Chinese hamster cells (Humphrey et al., 1968), human cells (Lohman, 1968) and has later been confirmed in many other reports.

Most of the breaks are rejoined very rapidly during post-irradiation incubation at 37° (e.g. within 30 min. after 20 krad, Kleijer et al., 1970, : paper I) but the process is inhibited strongly at lower temperatures suggesting the enzymatic nature of the process (Kleijer et al., 1971, 1973a: paper II; Ormerod and Stevens, 1971; Donlon and Norman, 1971). The rejoining can also be inhibited by several chemical compounds, such as potassium cyanide, ethylenediaminotetracetate, iodoacetate, crystal violet (Kleijer et al., 1971, 1973a: paper II), and dinitrophenol (Ormerod and Stevens, 1971; Moss et al., 1971; Kleijer et al., 1971, 1973a: paper II).

The biological relevance of strand rejoining has remained questionable because of the supralethal X-ray doses (5 - 30 krad). At lower doses (1 - 5 krad) the lower limit of detection of breaks is reached. A higher sensitivity would be achieved if molecular weights could be measured higher than $2 - 5 \times 10^8$ daltons, which is the range of values usually found when lysis procedures are used which give rise to stable single-stranded DNA molecules. In the last few years modified conditions of lysis, gradient composition and centrifugation have been described with which much higher sedimentation coefficients were measured (Lett et al., 1970; Elkind and Kamper, 1970; Moroson and Furlan, 1970; Mc Burney et al., 1971, 1972; Elkind, 1971; Mc Burney and Whitmore, 1972). Using such methods several groups have observed significant changes in the sedimentation profiles

after low doses of X-rays (100 - 1500 krad) (Lett and Sun, 1970; Moroson and Furlan, 1970). After post-irradiation incubation of the cells, the profiles returned to the original position (Elkind and Kamper, 1970; Elkind and Chang-Liu, 1972; Mc Burney et al., 1972). However it is difficult to interprete unequivocally the varying sedimentation profiles obtained by the different groups. In some reports bimodal sedimentation patterns were shown, suggesting the gradual release of slower sedimenting DNA molecules at the expense of fast sedimenting material (Elkind and Kamper, 1970; Lett et al., 1970; Elkind and Chang-Liu, 1972). Other reports showed shifts of very narrow DNA bands occurring after irradiation (Mc Burney et al., 1972). Such observations are not consistent with the profiles which would be expected after random fragmentation of freely sedimenting single-stranded DNA molecules. The changes in the profiles at these high sedimentation coefficients ($>$200S) observed after X-irradiation rather seem to reflect the release of single-strands from a complex DNA structure or changes in the conformation of a DNA complex (Elkind and Kamper, 1970; Lett et al., 1970; Mc Burney et al., 1972).

Several suggestions about the possible composition of the fast sedimenting, probably complex, material have been put forward: association of proteins and lipids to DNA (Elkind and Chang-Liu, 1972); binding of DNA to membranes (Ormerod and Lehmann, 1971a, b); composition of long DNA strands of subunits linked by labile components (Lett et al., 1970); and incomplete denaturation of double-stranded DNA (Mc Burney et al., 1972).

Elkind and Kamper (1970) suggested that there might be two types of damage and also two distinct repair processes: first the induction and the rejoining of strand breaks at high doses and secondly the resolution and subsequent reassociation of a functional DNA/lipid/protein complex at lower doses. However it seems possible that the accelerated resolution of the complex results exclusively from strand breakage and that therefore the two repair processes are

not fundamentally different.

DNA repair synthesis has been observed as a second repair phenomenon after ionizing irradiation in addition to strand rejoining. Unscheduled DNA synthesis and repair replication (see 2.3.b) have been demonstrated in cells of various origins after supralethal doses of 5 - 30 krad by Rasmussen and Painter (1966), Painter and Cleaver (1967), Spiegler and Norman (1969), Kleijer et al. (1970: paper I), Schaeffer and Merz (1971) and Painter and Young (1972). The detection of repair synthesis is difficult to achieve at lower doses (500 - 5000 rad) but seems possible in lymphocytes (Spiegler and Norman, 1970) or in synchronous G_1 - phase cells (Brent and Wheatley, 1971) where repair synthesis is not masked by semiconservative DNA replication.

Spiegler and Norman (1970) and Painter and Young (1972) have estimated that an average of 1 - 3 nucleotides is inserted per DNA lesion. Apparently much less nucleotides are inserted per X-ray lesion than per UV-lesion (30 - 100 nucleotides: see 2.3.b) but it remains to be established whether this difference is determined by the probably larger DNA distortion at a pyrimidine dimer site or by the involvement of different enzyme systems in the repair of X-ray and UV-lesions.

Owing to the high, supralethal doses required to demonstrate strand rejoining and repair synthesis, their contribution to the survival of the cell is still obscure. Double-strand DNA breaks might contribute much more to the killing effect of ionizing radiation, although they occur in a much lower frequency than single-strand breaks (see 1.3). Very little information is available on the repair of double-strand breaks. Sedimentation experiments, using neutral sucrose gradients, have sofar not indicated the repair of double-strand breaks (Veatch and Okada, 1969; Lehmann and Omerod, 1970), but because of the high X-ray doses ($>$20 krad) used, these experiments did not rule out the possibility that double strand breaks can be rejoined at lower doses.

Some of the questions concerning the mechanism of the

repair of ionizing radiation damage and the significance to cell survival might be answered if mammalian mutant cells were available. Very recently such a mutant has probably been found in a human genetic disorder, the Hutchinson-Gilford progeria syndrome which is characterized by precocious aging of the patients. Epstein et al. (1973) observed that cultured skin fibroblasts of a progeria patient failed to show normal DNA strand rejoining after exposure to γ-irradiation. It was shown earlier that progeria cells perform a normal amount of repair synthesis after UV-irradiation (Cleaver, 1970b). Further investigations are necessary to demonstrate the general occurrence of an association between the DNA repair deficiency and precocious aging in the progeria syndrome. If so, then this discovery will undoubtedly be of great value for future studies on DNA repair mechanisms.

CHAPTER 3

XERODERMA PIGMENTOSUM

3.1 INTRODUCTION

Xeroderma pigmentosum (XP) is a rare disease, which is characterized by sensitivity to sunlight and an autosomal recessive inheritance. Two clinically distinct forms have been recognized: the classic form of XP, which was first described by Hebra and Kaposi (1874), and the De Sanctis-Cacchione syndrome (DSC) (De Sanctis and Cacchione, 1932). Patients of both types show an extreme skin sensitivity to UV-light, probably with wavelengths of 290 - 310 nm, occurring in sunlight. In sun-exposed regions of the body, the skin undergoes pigmentary changes with freckling, a thickening of the epidermis by hyperkeratosis and acanthosis and often, already starting in the first years of life, a continuous development of skin cancers of ectodermal as well as mesodermal origin (Reed et al., 1969). These malignancies often lead to the early death of the patients. In addition to the skin lesions the DSC-syndrome is characterized by neurological complications such as microcephaly with mental deficiency, premature closure of epiphyses and cranial sutures, retarded growth and sexual development, choreoathetosis and cerebellar ataxia (Reed et al., 1969). The two forms of the disease have never been observed within the same family.

Since the first report of Hebra and Kaposi in 1874 many studies on XP have appeared but the basic cause of the disorder -a defect in the DNA-excision repair mechanism- was discovered only in 1968 by Cleaver (1968). Later, the relationship between the defect at the molecular level and cell survival was demonstrated by the increased UV-sensitivity of XP cells (Cleaver, 1970a; Goldstein, 1971). Also the reduced ability of XP cells, in comparison to normal

cells, to reactivate UV-inactivated herpes simplex virus (host-cell reactivation), indicated that the excision repair mechanism, which is deficient in XP-cells, plays a functional role in normal human cells (Rabson et al., 1969; Aaronson and Lytle, 1970; Lytle et al., 1972; Bootsma et al., 1972).

The study of XP does not only contribute to a better understanding of DNA repair mechanisms in mammalian cells, but might also deepen our insight in the mechanisms leading to cancer. XP is unique in sofar that in this condition a relatively well defined biochemical defect is associated with the development of tumor cells. Recently Cleaver (1973 d) has speculated about the possible relationship between defective DNA repair and carcinogenis (e.g. via an increased mutation rate or the involvement of oncogenic viruses).

3.2 DEFECTIVE EXCISION REPAIR IN XERODERMA PIGMENTOSUM

(a) DNA repair synthesis and rejoining of breaks

The first indication for a defect in excision repair in XP was obtained by Cleaver (1968) who found that DNA repair synthesis (chapter 2.3.b) was strongly reduced in the cells cultured from XP patients. This observation has since been confirmed by several groups, using different techniques, in cultured skin fibroblasts (Cleaver, 1969b, 1970a, 1972; Bootsma et al., 1970; Regan et al., 1971a, b, Kleijer et al., 1973b: paper V), in lymphocytes (Burk et al., 1971; Robbins and Kraemer, 1972), in excised skin fragments (Jung, 1971) and *in vivo* in the skin of patients (Epstein et al., 1970).

In the previous chapter (2.5) it has been described that the repair of X-ray damage involves repair replication and rejoining of single-strand breaks. Both phenomena occurred at a normal rate in XP cells of the classic form as well as the DSC-syndrome (Cleaver, 1969b; Kleijer et al., 1970, 1973b: papers I and V). These results indicate that in XP cells, the capacities to perform repair replication and rejoining of breaks are not deficient. Therefore it was concluded that in XP cells an initial step of the excision

of pyrimidine dimers, probably the incision step, is defective. The hypothesis of a defective initial step in XP is supported by studies on the repair of damage induced by a number of chemical compounds which are carcinogenic in many cases.

Two groups of compounds can now be recognized:
1. Compounds which, like UV-irradiation, induce repair replication in normal human cells but not in XP cells. Examples of such compounds are: 4-nitroquinoline-1-oxide (Stich and San, 1971), N-acetoxy-2-acetyl-aminofluorene (Setlow and Regan, 1972; Stich et al., 1972), 8-methoxypsoralen + irradiation with 360 nm UV-light (Baden et al., 1972), 3 - methyl-4-nitropyridine-1-oxide (Stich et al., 1973), benz (a) anthracene epoxide (Stich and San, 1973), 7-bromobenzyl (a) anthracene (Slor, 1973) and 1, 3-bis (2-chloroethyl)-1-nitrosourea (Cleaver, 1973b). These compounds bind to the pyrimidine or purine bases, whereas some of them can form intra- and inter-strand cross-links. These damages do not lead to breaks in the DNA-strands, unless a specific endonuclease is present to initiate excision repair.
2. Compounds which, like ionizing irradiation, induce a normal amount of repair replication in XP cells. Examples of such compounds are: Bromodeoxyuridine, which is incorporated into the DNA and causes strand-breakage upon irradiation with visible or UV-light (Cleaver, 1969b) and the alkylating agents: methyl methanesulphonate and N-methyl-N'-nitro-N-nitrosoguanidine (Cleaver, 1971). The DNA is alkylated by these compounds and subsequently depurination and strand breakage can occur without the interference of a specific repair-endonuclease. Excision repair might therefore be performed without the endonucleolytic function which might be defective in XP cells.

However two remarks should be made here: first, the spontaneous depurination and strand-breakage after alkylation damage are rather slow processes under physiological conditions (Craddock, 1973) and secondly ionizing irradiation produces base damages in addition to strand-breaks.

Therefore it cannot be excluded than an endonuclease, perhaps specific for base damages of the type produced by X-rays, is still required. In bacteria such endonuclease activities, specific for X-ray damage (Setlow and Carrier, 1973) or alkylation damage (Strauss and Robbins, 1968) have been reported. Another question which has not yet been answered is whether the large difference between the number of new bases which are inserted during the repair of UV-damage (30 - 100 bases; see 2.3.b) and X-ray damage (1 - 3 bases; see 2.5) is caused only by the different nature of the lesions (and distortions of the double helix) or by the involvement of different exonucleases and repair polymerases.

The conclusion, mentioned above, that the incision step in excision repair is deficient in XP was, and is still, based mainly on the observations that XP cells are able to perform repair replication and rejoining of breaks after X-irradiation. It is obvious that this conclusion would not be justified if different UV-specific and X-ray specific exonucleases and polymerases would exist.

(b) Incision and Excision

Since excision repair at a DNA-lesion is thought to be initiated by incision, and the rejoining of the strand must be the last step, breaks should be present transiently during the repair process. If the incision step is deficient in XP cells the number of breaks should be lower than in normal cells. On the other hand a higher number might be expected if one of the steps following incision is deficient. Therefore attempts have been made to measure the production of breaks after UV-irradiation of normal and XP-cells by sedimentation analysis of the DNA in alkaline sucrose gradients.

In one report (Setlow et al., 1969) it was claimed that incision breaks could be observed indeed in normal cells but not in XP cells. However the reported sedimentation profiles showed large variations and did probably not support

this conclusion (see 2.3.d).

The results obtained by Kleijer et al. (1971, 1973a: paper II) indicated that incision-breaks did not accumulate to a detectable level in UV-irradiated normal as well as XP cells. Because of the absence of accumulated breaks in XP cells, the negative conclusion might be drawn that these cells are not deficient in one of the steps following incision. However this interpretation is not valid if the repair process proceeds by a sequence of concerted steps as was suggested before (2.3.e).

Very recently Cleaver (1973c) has used a modified method, already mentioned in the previous chapter (2.3.d), which permits the qualitative detection of probably a very low number of breaks. Changes of the sedimentation rate of DNA, observed after UV-irradiation, were attributed to the occurrence of small numbers of breaks which act as sites for strand separation during brief lysis in alkali (conversion of double-stranded into single-stranded DNA). Large differences were revealed between the sedimentation properties of DNA from UV-irradiated normal and XP cells. The results suggested that immediately after UV-irradiation of normal cells, breaks appeared and disappeared after a certain incubation interval depending on the UV-dose used. In contrast, the results for XP-cells (a DSC-cell strain) suggested that breaks did not occur after a low dose (13 erg/mm^2) whereas after higher doses breaks seemed to accumulate slowly, but did not disappear. Despite these clear differences between normal and XP (DSC) - cells, an unequivocal conclusion with respect to the defect in XP can as yet not be drawn. The accumulation of breaks in the DSC-cells might indicate, as Cleaver suggested, that a UV-specific exonuclease is defective, but other interpretations are possible; particularly the slow appearance of breaks in DSC-cells relative to normal cells might rather indicate a defect in the incision step.

Studies on the excision of pyrimidine dimers, by using the chromatographic procedure to determine the dimer content

of DNA, have shown that classic XP and DSC cells do not excise dimers (Setlow et al., 1969; Cleaver and Trosko, 1971; Kleijer and Bootsma 1971).

Paterson et al. (1973) (see 2.3.c) have demonstrated, by using a UV-specific endonuclease isolated from *Micrococcus luteus*, that the endonuclease susceptible sites, induced by UV light do not disappear from the DNA of classic XP and DSC cells. This implies that incision at dimer sites did not occur in these cells. Nevertheless these observations do not prove that the incision function is defective, because if the repair process is co-ordinated all steps might be blocked by a defect in any other step.

The preferential way to determine which of the proposed repair enzymes is defective in XP would be a direct enzyme assay. Following this approach Bacchetti et al. (1972) have demonstrated a UV-specific endonucleolytic activity in extracts of normal as well as XP cells. However this enzyme shows a rather peculiar substrate specificity, since it also acts on UV-irradiated and subsequently photoreactivated DNA. This suggests that the enzyme did not recognize pyrimidine dimers but either other UV-lesions or possibly DNA distortions which might remain in the DNA after the dimer is monomerized by the *in vitro* photoreactivation procedure.

Although some of the data discussed in this section are tempting to assume that an endonuclease involved in the initiation of excision repair is defective in XP cells (Cleaver, 1969b; Setlow et al., 1969; Kleijer et al., 1970: paper I), there is as yet no direct proof for this hypothesis. A defect in a UV-specific exonuclease, suggested by Cleaver (1973c) for DSC-cells, remains possible and even a defect in the polymerase cannot be excluded.

3.3 GENETIC HETEROGENEITY IN XERODERMA PIGMENTOSUM

The distinct clinical symptoms of the classic form of XP and the DSC-syndrome and also the various genetically determined levels of repair activity in a series of classic

XP patients suggests that different mutations may be involved in XP (Bootsma et al., 1970; Kleijer et al., 1973b: paper V).

The existence of different forms of XP has been examined by the study of repair synthesis which would occur as a result of complementation in heterokaryons obtained by fusion of cells originating from different repair deficient XP-patients. Three groups of cell strains which complement each other have been demonstrated by De Weerd-Kastelein et al. (1972, 1973a, b,: paper IV) and Kleijer et al (1973c) using autoradiographic as well as density gradient methods to determine repair synthesis in the fused cell populations. These three complementation groups represented also three distinct classes of cell strains with respect to their repair capacities. The first group (6 DSC strains) had no repair capacity at all whereas the second group of cell strains (from 7 severe classic XP patients) showed a repair rate of 5 - 15% of that in normal cells and the third group (from 2 genetically related, less severe classic XP patients) showed intermediate and dose-dependent relative repair rates (30% at 100 erg/mm^2 to 80% at 1000 erg/mm^2) (Kleijer et al. 1973b: paper V). Similarly Kraemer et al. (1973) have recently reported briefly the presence of at least three complementation groups in a series of 7 patients, each group representing a distinct class of repair rates.

The demonstration of several complementation groups implies that different mutations are responsible for the repair deficiency in different forms of XP. The distinct classes of repair capacities which seem to coincide with the complementation groups, probably reflect the different enzyme defects in each group.

As discussed in the previous section the defective step in excision repair has not yet been established precisely. Several possible causes of the decreased catalytic function have to be considered, such as defects in the reaction catalyzing function, a lowered substrate-affinity or a lower amount of any of the enzymes involved. In a co-ordinated

repair model, as discussed before, one can moreover envisage a defect that affects the rate at which the repair complexes move along the DNA from one lesion to the next. The precise location of the defect may be difficult to reveal with the present techniques, especially if the repair process is co-ordinated indeed, since in that case any possible defect will block or delay all repair steps. Further evidence to support some of the possible defects or to reject others may be achieved by careful kinetic studies of repair phenomena such as repair synthesis (Kleijer et al., 1973b: paper V) and excision as well as incision by using the sensitive techniques which have recently been developed to detect in the DNA small numbers of dimers (Paterson et al., 1973, Lucas, 1972) and single-strand breaks (Cleaver, 1973c).

The definite and precise elucidation of the defect in the different forms of XP will probably require the development of methods to purify and characterize the excision repair enzymes *in vitro*.

A variant form of XP has been found in patients who were clinically diagnosed as classic XP cases, but who exhibit normal cellular DNA repair capacities. In some cases the clinical symptoms were similar to those seen in the common form with respect to the severity and the age of onset (Burk et al., 1971; Cleaver, 1972; Kleijer et al., 1973 b: paper V), whereas in other cases the patterns of skin lesions observed were less severe or arose at a later age than in the common form of XP (Jung, 1971; Bootsma et al., 1970; Kleijer et al., 1973b: paper V). The involvement of defects in a post-replication mechanism (Jung, 1971) or of defects leading to errors in the genetic code (misrepair) cannot *a priori* be excluded for all cases. However, because of the observations of a normal UV-sensitivity (Cleaver, 1972) and a normal gap-filling capacity (Buhl et al., 1973 b), it seems more likely that there is no defect associated with any DNA-repair mechanism in these patients.

3.4 BIOCHEMICAL DIAGNOSIS OF XERODERMA PIGMENTOSUM

Several methods, already mentioned before, are now available to establish a biochemical diagnosis of XP. Such a diagnosis by analysis of skin fibroblasts or lymphocytes, makes adequate measures possible either for patients who have been diagnosed clinically or in the case of birth of a child from presumed heterozygous parents. Prenatal diagnosis can probably succesfully be performed in pregnancies at risk by examination of foetal cells obtained by amniocentesis, preferably in the 12th - 16th week of pregnancy. Especially for prenatal diagnosis the method of choice will be the autoradiographic technique to measure UV-induced DNA repair synthesis because of the low number of cells needed. The assay conditions, such as UV-doses and length of labelling periods, should be chosen with some care because the cells of some XP patients have shown relatively high residual repair activities which may at high doses approach the normal level (Kleijer et al., 1973b: paper V), whereas also after long labelling periods normal amounts of repair synthesis can be found (Robbins and Kraemer, 1972; Kleijer et al., 1973b: paper V). It is evident that the variant form of XP with a normal DNA repair capacity cannot be detected.

Studies of cell strains from the heterozygous parents of XP patients have shown a significantly reduced repair activity in only a few cases, whereas in most cases a normal rate of repair was found (Bootsma et al., 1970; Cleaver, 1972; Kleijer et al., 1973b: paper V). The latter observations are in agreement with the recessive character of the disease and suggest that the enzyme or enzyme complex which is defective in XP cells is present in excessive amounts in normal and heterozygous cells. The direct determination of repair synthesis levels is therefore not a reliable method to detect the heterozygous condition. Theoretically it seems possible to solve this problem by "dilution" of the unaffected enzyme by fusion of either

heterozygous or normal cells with XP cells to produce multikaryotic cells (Gianelli et al., 1973). At certain ratios of the numbers of parental nuclei in the multikaryons (heterozygous/XP or normal/XP) the amount of unaffected enzyme might no longer be excessive and a difference between the repair levels in the heterozygous/XP and the normal/XP fusions might be revealed.

REFERENCES

AARONSON, S.A., and C.D. LYTLE, (1970), Nature, 228, 359.

ALEXANDER, P., and Z.B. MIKULSKI, (1961), Nature, 192, 572.

BACCHETTI, S., A. v.d.PLAS, and G. VELDHUISEN, (1972), Biochem. Biophys. Res. Comm., 48, 662.

BADEN, H.P., J.M. PARRINGTON, J.D.A. DELHANTY, and M.A. PATHAK, (1972), Biochim. Biophys. Acta, 262, 247.

BEN-HUR, E., and R. BEN-ISHAI, (1971), Photochem. Photobiol., 13, 337.

BEUKERS, R., and W. BERENDS, (1960), Biochim. Biophys. Acta, 41, 550.

BOLLUM, F.J. and R.B. SETLOW, (1963), Biochim. Biophys. Acta, 68, 599.

BOOTSMA, D., M.P. MULDER, F. POT, and J.A. COHEN, (1970), Mutation Res., 9, 507.

BOOTSMA, D., E.A.DE WEERD-KASTELEIN, G. VELDHUISEN and W. KEIJZER, (1972), in: DNA repair mechanisms; Symposia Medica Hoechst, F.K. Schattauer Verlag, Stuttgart - New York, 1972, p.87.

BOYCE, R.B., and P. HOWARD-FLANDERS, (1964), Proc. Natl. Acad. Sci. (US), 68, 2967.

BRENT, T.P., (1972), Nature New Biol., 239, 172.

BRENT, T.P., and G.A. WHEATLEY, (1971), Int. J. Radiat. Biol., 19, 339.

BUHL, S.N., SETLOW, R.B., and J.D. REGAN, (1972a), Int. J. Radiat. Biol., 22, 417.

BUHL, S.N., R.M. STILLMAN, R.B. SETLOW, and J.D. REGAN, (1972b) Biophys. J., 12, 1183.

BURGI, E., and A.D. HERSHEY, (1963), Biophys. J., 3, 309.

BURK, P.G., M.A. LUTZNER, D.D. CLARKE, and J.H. Robbins, (1971), J. Lab. Clin. Med., 77, 759.

CHU, E.H.Y., (1965), Mutation Res., 2, 75.

CLEAVER, J.E., (1968), Nature, 218, 652.

CLEAVER, J.E., (1969a), Int. J. Radiat. Biol., 16, 277.

CLEAVER, J.E., (1969b), Proc. Natl. Acad. Sci. (US), 63, 428.

CLEAVER, J.E., (1970a), Int. J. Radiat. Biol., 18, 557.

CLEAVER, J.E., (1970b), J. Invest. Dermatol., 54, 181.

CLEAVER, J.E., (1971), Mutation Res., 12, 453.

CLEAVER, J.E., (1972), J. Invest. Dermatol., 58, 124.

CLEAVER, J.E., (1973a), in: Advances in Radiation Biology, J.T. Lett, M.R. Zelle and H. Adler (eds.), Academic Press, New York, (in press).

CLEAVER, J.E., (1973b), Cancer Res., 33, 362.

CLEAVER, J.E., (1973c), Radiation Res., in press.

CLEAVER, J.E., (1973d), Cancer Genetics, in press.

CLEAVER, J.E., and G.H. THOMAS, (1969), Biochem. Biophys. Res. Comm., 36, 203.

CLEAVER, J.E., G.H. THOMAS, J.E. TROSKO, and J.T. LETT, (1972), Exptl. Cell Res., 74, 67.

CLEAVER, J.E., and J.E. TROSKO, (1970), Photochem. Photobiol., 11, 547.

COOK, J.S., (1970), in: A.C. Giese (ed.), Photophysiology, Current Topics in Photobiology and Photochemistry, Vol. V, p.191, Academic Press, New York and London.

CRADDOCK, V.M.,(1973), Biochim. Biophys. Acta, 312, 202.

DE SANCTIS, C., and A. CACCHIONE, (1932), Riv. Sper. Freniat., 56, 269.

DJORDJEVIC, B., and L.J. TOLMACH, (1967), Radiation Res., 32, 327.

DOMON, M., and A.M. RAUTH, (1969), Radiation Res., 40, 414.

DONLON, T., and A. NORMAN, (1971), Mutation Res., 13, 97.

EDENBERG, H., and P. HANAWALT, (1972), Biochim. Biophys. Acta, 272, 361.

EHMANN, U.K., and J.T. LETT, (1973), Radiation Res., 54, 152.

ELKIND, M.M., (1971), Biophys. J., 11, 502.

ELKIND, M.M., and C.M. CHIANG-LIU, (1972), Int. J. Radiat. Biol., 22, 75.

ELKIND, M.M., and C. KAMPER, (1970), Biophys. J., 10, 237.

EPSTEIN, J.H., K. FUKUYAMA, W.B. REED, W.L. EPSTEIN, (1970), Science, 168, 1477.

EPSTEIN, J., J.R. WILLIAMS, and J.B. LITTLE, (1973), Proc. Natl. Acad. Sci. (US), 70, 977.

EVANS, R.G., and A. NORMAN, (1968), Radiation Res., 36, 287.

FREIFELDER, D., (1966), Radiation Res., 29, 239.

FUJIWARA, Y., (1972), Exptl. Cell Res., 75, 483.

GAUTSCHI, J.R., B.R. YOUNG, and J.E. CLEAVER, (1973), Exptl. Cell Res., 76, 87.

GIANELLI, F., P.M. CROLL, and S.A. LEWIN, (1973), Exptl. Cell Res., 78, 175.

GOLDSTEIN, S., (1971), Proc. Soc. Exp. Biol. Med., 137, 730.

GRIGGS, H.G., and M.A. BENDER, (1973), Science, 179, 86.

GROSSMAN, L., (1973), in: Advances in Radiation Biology, (J.T. Lett, M.R. Zelle, and H. Adler, eds.), Academic Press, New York, in press.

HANAWALT, P.C., and R.H. HAYNES, (1967), Scientific American, 216, 36.

HAUSWIRTH, W., and S.Y. WANG, (1973), Biochem. Biophys. Res. Comm., 51, 819.

HEBRA, F., and M. KAPOSI, (1874), The New Sydenham Soc. London, 3, 252.

HEIJNEKER, H.L., H. PANNEKOEK, R.A. OOSTERBAAN, P.H. POUWELS, S. BRON, F. ARWERT, and G. VENEMA, (1971), Proc. Natl. Acad. Sci. (US), 68, 2967.

HOWARD-FLANDERS, P., (1968), in: Ann. Rev. Biochem., 37, 175.

HUMPHREY, R.M., B. A. SEDITA, and R.E. MEYN, (1970), Int. J. Radiat. Biol., 18, 61.

HUMPHREY, R.M., D.L. STEWARD, and B.A. SEDITA, (1968), Mutation Res., 6, 459.

ISOMURA, K., O. NIKAIDO, M. HORIKAWA, and T. SUGAHARA, (1973), Radiation Res., 53, 143.

IYER, V.N., and W.D. RUPP, (1971), Biochim. Biophys. Acta, 228, 117.

JUNG, E.G., (1971), Arch. Derm. Forsch., 241, 33.

KLEIJER, W.J., P.H.M. LOHMAN, M.P. MULDER, and D. BOOTSMA, (1970), Mutation Res., 9, 517.

KLEIJER, W.J., and D. BOOTSMA, (1971), Proc. 1st. European Biophysics Congress, Baden, Vol. II, p. 129.

KLEIJER, W.J., H.L. HOEKSEMA, M.L. SLUYTER and D. BOOTSMA, (1973a), Mutation Res., 17, 385.

KLEIJER, W.J., E.A. DE WEERD-KASTELEIN, W. KEIJZER, M.L. SLUYTER, J. DE WIT and D. BOOTSMA, (1973b), Mutation Res., submitted for publication.

KLEIJER, W.J., E.A. DE WEERD-KASTELEIN, P.H.M. LOHMAN, and D. BOOTSMA, (1973c), Studia Biophys., 36/37, 375.

KLIMEK, M., (1966), Photochem. Photobiol., 5, 603.

KRAEMER, K.H., J.H. ROBBINS, and H.G. COON, (1973), Clinical Res., 21, 480.

KRISHNAN, D., and R.B. PAINTER, (1973), Mutation Res., 17, 213.

LEHMANN, A.R., (1972a), J. Mol. Biol., 66, 319.

LEHMANN, A.R., (1972b), European J. Biochem., 31, 438.

LEHMANN, A.R., (1972c), Biophys. J., 12, 1316.

LEHMANN, A.R., and M.G. ORMEROD, (1970), Biochim. Biophys. Acta, 217, 268.

LETT, J.T., I. CALDWELL, C.J. DEAN and P. ALEXANDER, (1967), Nature, 214, 790.

LETT, J.T., E.S. KLUCIS, and C. SUN, (1970), Biophys. J. 10, 277.

LETT, J.T., and C. SUN, (1970), Radiation Res., 44, 771.

LEY, R.D., and R.B. SETLOW, (1972), Abstr. Biophys. Soc. 12th annual Meeting, p. 151a.

LIEBERMAN, M.W., S. SELL, and E. FARBER, (1971), Cancer Res., 31, 1307.

LINDAHL, T., (1971), European J. Biochem., 18, 407.

LINDAHL, T., and G.M. EDELMAN, (1968), Proc. Natl. Acad. Sci. (US), 61, 680.

LOHMAN, P.H.M., (1968), Mutation Res., 6, 449.

LOHMAN, P.H.M., (1969), thesis, Leiden.

LOHMAN, P.H.M., M.L. SLUYTER, I.A.A. MATTHIJS and W.J. KLEIJER, (1973), Anal. Biochem., 54 (1973) 178-187.

LUCAS, C.J., (1972), Exptl. Cell Res., 74, 480.

LYTLE, C.D., S.A. AARONSON, and E. HARVEY, (1972), Int. J. Radiat. Biol., 22, 159.

MARMUR, J., W.F. ANDERSON, L. MATTHEWS, K. BERNS, E. GAJEWSKA, D. LANE and P. DOTY, (1961), J. Cell. Comp. Physiol., suppl. 58, 33.

MARMUR, J., and L. GROSSMAN, (1961), Proc. Natl. Acad. Sci. (US), 47, 778.

MCBURNEY, M.W., F.L. GRAHAM, and G.F. WHITMORE, (1971), Biochem. Biophys. Res. Comm., 44, 171.

MCBURNEY, M.W., F.L. GRAHAM, and G.F. WHITMORE, (1972), Biophys. J., 12, 369.

MCBURNEY, M.W., and G.F. WHITMORE, (1972), Biochem. Biophys. Res. Comm., 46, 898.

MCGRATH, R.A., and R.W. WILLIAMS, (1966), Nature, 212, 534.

MEYN, R.E., and R.M. HUMPHREY, (1971), Biophys. J., 11, 295.

MILLER, N., and P. CERUTTI, (1968), Proc. Natl. Acad. Sci. (US), 59, 34.

MOROSON, H., and M. FURLAN, (1970), Radiation Res., 44, 713.

MOSS, A.J., G.V. DALRYMPLE, J.L. SANDERS, K.P. WILKINSON, and J.C. NASH, (1971), Biophys. J. 11, 158.

ORMEROD, M.G., and A.R. LEHMANN,(1971a), Biochim. Biophys. Acta, 228, 331.

ORMEROD, M.G., and A.R. LEHMANN,(1971b), Biochim. Biophys. Acta, 247, 369.

ORMEROD, M.G., and U. STEVENS, (1971), Biochim. Biophys. Acta, 232, 72.

PAINTER, R.B., (1970a), in: Photophysiology, A.C. Giese (ed.), Vol. V, p.169, Academic Press, New York, London.

PAINTER, R.B., (1970b), in: Current Topics in Radiation Research, M. Ebert and A. Howard (eds.), Vol.7, p.45, North Holland, Amsterdam.

PAINTER, R.B., and J.E. CLEAVER, (1967), Nature, 216, 369.

PAINTER, R.B., and J.E. CLEAVER, (1969), Radiation Res., 37, 451.

PAINTER, R.B., and B.R. YOUNG, (1972), Mutation Res., 14, 225.

PATERSON, M.C., P.H.M. LOHMAN, and M.L. SLUYTER, (1973), Mutation Res., in press.

PETTIJOHN, D., and P. HANAWALT, (1964), J. Mol. Biol., 9, 395.

RABSON, A.S., S.A. TYRRELL, and F.Y. LEGALLAIS,(1969), Proc. Soc. Exptl. Biol. Med., 132, 802.

RANDTKE, A.S., J.R. WILLIAMS, and J.B. LITTLE (1972), Exptl. Cell. Res., 70, 360.

RASMUSSEN, R.E., and R.B. PAINTER, (1964), Nature, 203, 1360.

RASMUSSEN, R.E., and R.B. PAINTER, (1966), J. Cell Biol., 29, 11.

REED, W.B., B. LANDING, G. SUGARMAN, J.E. CLEAVER, and J. MELNYK, (1969), J. Am. Med. Ass., 207, 2073.

REGAN, J.D., and J.S. COOK (1967), Proc. Natl, Acad. Sci. (US), 58, 2274.

REGAN, J.D., R.B. SETLOW, M.M. KABACK, R.R. HOWELL, E. KLEIN, and G. BURGESS, (1971a), Science, 174, 147.

REGAN, J.D., R.B. SETLOW, and R.D. LEY, (1971b), Proc. Natl. Acad. Sci. (US), 68, 708.

REGAN, J.D., J.E. TROSKO, and W.L. CARRIER, (1968), Biophys. J., 8, 319.

ROBBINS, J.H., and K.H. KRAEMER, (1972), Mutation Res., 15, 92.

RUPP, W.D., and P. HOWARD-FLANDERS, (1968), J. Mol. Biol., 31, 291.

RUPP, W.D., C.E. WILDE, D.L. RENO, and P. HOWARD-FLANDERS, (1971), J. Mol. Biol., 61, 25.

SCHANS, G.P. v.d., (1969), thesis, VU - Amsterdam.

SCHANS, G.P. v.d., and J. BLOK, (1970), Int. J. Radiat. Biol., 17, 25.
SEAMAN, E., H. VAN VUNAKIS and L. LEVINE, (1972), J. Biol. Chem., 247, 5709.
SETLOW, J.K., (1966), in: Current Topics in Radiation Res., 2, 197, M. Ebert, and A. Howard (eds.), North Holland, Amsterdam.
SETLOW, R.B., (1967), in: V.V. Koningsberger and L. Bosch (eds.), Regulation of Nucleic Acid and Protein Biosynthesis, BBA Library, Vol. 10 Elsevier, Amsterdam, p. 51.
SETLOW, R.B., and W.L. CARRIER, (1964), Proc. Natl. Acad. Sci. (US), 51, 226.
SETLOW, R.B., and W.L. CARRIER, (1973), Nature New Biol., 241, 170.
SETLOW, R.B., and J.D. REGAN, (1972), Biochem. Biophys. Res. Comm., 46, 1019.
SETLOW, R.B., J.D. REGAN, and W.L. CARRIER, (1972), Biophys. J., 12, 19a.
SETLOW, R.B., J.D. REGAN, J. GERMAN, and W.L. CARRIER, (1969), Proc. Natl. Acad. Sci. (US), 64, 1035.
SHAEFFER, J., and T. MERZ, (1971), Radiation Res., 47, 426.
SLOR, H., (1973), Mutation Res., in press.
SMETS, L.A., (1969), Int. J. Radiat. Biol., 16, 407.
SMITH, K.C., (1962), Biochem. Biophys. Res. Comm., 8, 157.
SPADARI, S., G. CIARROCCHI, and A. FALASCHI, (1971), European J. Biochem., 22, 75.
SPIEGLER, P., and A. NORMAN, (1969), Radiation Res., 39, 400.
SPIEGLER, P., and A. NORMAN, (1970), Mutation Res., 10, 379.
STICH, H.F., and R.H.C. SAN, (1971), Mutation Res., 13, 279.
STICH, H.F., and R.H.C. SAN, (1973), Proc. Soc. Exptl. Biol. Med., 143, 155.
STICH, H.F., R.H.C. SAN, and Y. KAWAZOE, (1973), Mutation Res., 17, 127.
STICH, H.F., R.H.C. SAN, J.A. MILLER, and E.C. MILLER, (1972), Nature New Biol. 238, 9.
STRAUSS, B., and M. ROBBINS, (1968), Biochim. Biophys. Acta, 161, 68.
STUDIER, F.W., (1965), J. Mol. Biol., 11, 373.
SVEDBERG, T., and K.O. PEDERSON, (1940), Die Ultrazentrifuge, Handbuch der Kolloid Wissenschaft, VIII, Th. Steinkoff, Dresden.
TROSKO, J.E., and M.R. KASSCHAU, (1967), Photochem. Photobiol., 6, 215.
VARGHESE, A.J., and R.S. DAY, (1970), Photochem. Photobiol., 11, 511.
VEATCH, W., and S. OKADA, (1969), Biophys. J., 9, 330.
WEERD-KASTELEIN, E.A. DE.,W. KEIJZER, and D. BOOTSMA, (1972), Nature New Biol., 238, 80.
WEERD-KASTELEIN, E.A. DE.,W. KEIJZER, and D. BOOTSMA, (1973a) manuscript in preparation.
WEERD-KASTELEIN, E.A. DE., W.J. KLEIJER, M.L. SLUYTER, and W. KEIJZER, (1973b), Mutation Res., 19, 237.
WEISSBACH, A., A. SCHLABACH, B. FRIDLENDER, and A. BOLDEN, (1971), Nature New Biol., 231, 167.
ZIRKLE, R.E., and R.B. URETZ, (1963), Proc. Natl. Acad. Sci. (US), 49, 45.

SUMMARY

Most organisms, including man, are capable of repairing their DNA when it has been damaged by either ultra-violet light (UV), ionizing radiation or several other, physical or chemical, agents. In one of the repair mechanisms -excision repair- the damage (e.g. UV-induced pyrimidine dimers; Fig. 1-1, p.14) is removed from the DNA by an incision and an excision step, and the DNA strand is repaired by the insertion of new nucleotides, in which the opposite complementary strand serves as a template (repair replication) and finally by the rejoining of the strand (Fig. 2-1; p.19).

The biological significance of excision repair to man is probably demonstrated in the inherited disease xeroderma pigmentosum (XP). In this disease a hypersensitivity of the skin to sunlight, leading to severe skin lesions (e.g. skin tumors) is associated with a deficient excision repair of UV-damaged DNA.

In the first part of this thesis a review is given of the data and the conclusions obtained from experiments described in the literature and from our own investigations on the types of DNA-damage induced by UV light and ionizing radiation (chapter 1); on the DNA repair mechanisms occurring in micro-organisms and higher organisms (chapter 2); and on DNA repair in XP (chapter 3).

In the second part -the appendix- five papers are presented describing our investigations which were designed:

a) to obtain more insight into the excision repair mechanism;
b) to locate the defective step in the repair process in XP; and
c) to examine whether a different genetic and biochemical basis of the deficiency exists in different forms of the disease such as in the two clinically distinct forms

:classic XP and the De Sanctis-Cacchione syndrome (DSC).

In paper I of the appendix the repair of X-ray induced DNA damage is studied by using the technique of centrifugation in alkaline sucrose gradients to detect single-strand breaks in the DNA and the autoradiographic technique to measure repair replication. Cultured cells from several classic XP patients (and DSC patients; paper V) were able to rejoin single-strand breaks at the same rate as cells from healthy individuals. Moreover these XP strains, which exhibit a strongly reduced capability to perform repair replication after UV-irradiation, showed normal amounts of repair replication after X-irradiation. These observations suggest that in XP cells the deficient excision repair of UV-damage is not due to a defect in either the repair replication step or in the rejoining of breaks but rather in an earlier step, e.g. the incision. This hypothesis was supported by experiments which showed that pyrimidine dimers are excised from the DNA in normal cells (paper V) but not in XP cells (see 3.2.b).

The experiments described in paper II were designed to obtain direct evidence for a defective incision step in XP cells by comparing the number of breaks appearing after UV-exposure of normal and XP cells. Although breaks were demonstrated in normal as well as XP cells after high UV-doses ($>$ 1000 erg/mm^2) these breaks were probably caused by a direct induction rather than by the enzymatic incision action. After lower doses (100 - 500 erg/mm^2) no significant changes were observed in the sedimentation patterns in alkaline sucrose gradients of DNA from normal and XP cells. The number of transiently present breaks did not exceed the minimum number which was required for their detection (0.2 breaks/10^8 daltons) possibly because of the rapid rejoining of the breaks. Therefore it was not possible to demonstrate directly a deficient incision function in XP.

In further investigations of the rejoining of X-ray induced breaks it was found that this process was inhibited by lowered temperatures ($<22^{\circ}$) and by chemical compounds

such as: potassium cyanide, 2,4-dinitro-phenol, ethylene-diaminotetracetate, iodoacetate and crystal violet. These inhibitors of rejoining were applied to UV-exposed normal cells but an accumulation of breaks was not observed. The hypothesis was postulated that excision repair at each DNA lesion takes place by the concerted action of the repair enzymes which may operate as an enzyme complex.

In paper IV and V the possible existence of a genetic and biochemical heterogeneity in XP is examined. Two distinct techniques to measure DNA repair synthesis have been used: the autoradiographic method and the method of isopycnic centrifugation of DNA in density gradients (2.3.b and Fig. 2-2: p.21). For the latter method a modified procedure, in which NaI is used for the density gradients and a fluorimetric method to measure the amount of DNA in the gradient fractions, is introduced in paper III.

In paper IV repair replication is studied in the UV-irradiated cell population obtained after fusion of classic XP and DSC cells. In the multinucleate heterokaryons, which occurred at a high frequency, the capacity to perform repair replication appeared to be restored completely. The occurrence of complementation between the classic XP and the DSC cells indicates that the defect in the two forms of the disease is caused by different mutations.

In paper V investigations are described on the DNA repair capacities of cell strains from a large number of XP patients. On the basis of the kinetics of DNA repair synthesis after UV-irradiation four distinct groups of XP patients can be recognized: the DSC patients with a complete deficiency of the repair capacity in all cases which we investigated, and three groups of classic XP patients with a low, an intermediate and a normal repair capacity respectively. In the latter group the disease is presumably not caused by a repair defect. In the other two groups of classic XP patients a correlation was indicated between the degree of repair deficiency and the severity of the clinical symptoms. Genetically related patients always be-

longed to the same group. Complementation studies reported in paper IV and elsewhere (see 3.3) have shown that at least three different mutations are responsible for the repair deficiency in the DSC patients and the two groups of classic XP patients. These observations suggest that the different rates of repair synthesis in cells of XP patients are genetically determined and are based on different mutations.

Most of the cell strains derived from heterozygotes have shown normal repair activities; however some cases with a significantly reduced level of repair synthesis have been found.

SAMENVATTING

DNA, de chemische struktuur die de erfelijke informatie bevat, kan door verscheidene fysische en chemische agentia worden beschadigd. Tot de fysische agentia behoren ultraviolette (UV) straling die pyrimidinedimeren in de DNA-streng veroorzaakt (Fig. 1-1; blz.14) en röntgenstraling die enkelstrengbreuken induceert. Een overzicht van de types DNA-schade veroorzaakt door UV-licht en röntgenstraling wordt gegeven in hoofdstuk 1.

Evenals mikro-organismen beschikken hogere organismen over het vermogen beschadigd DNA te herstellen. In hoofdstuk 2 worden de verschillende DNA-herstelmechanismen besproken gebruik makend van gegevens uit de literatuur en uit het eigen onderzoek dat is beschreven in de appendix van dit proefschrift. In het voor zoogdiercellen meest onderzochte mechanisme -excisie herstel- wordt de schade door middel van een incisie- en een excisiestap uit de DNA streng verwijderd. De DNA-streng wordt hersteld door de inbouw van nieuwe nucleotiden, waarbij de tegenoverliggende komplementaire streng als matrijs wordt gebruikt (herstelsynthese) en tenslotte sluiting van de DNA-keten (herstel van breuken) (zie Fig. 2-1; blz.19).

Een aanwijzing voor de biologische betekenis van dit herstelmechanisme is de overgevoeligheid voor UV-straling bij patiënten die lijden aan de erfelijke ziekte xeroderma pigmentosum (XP). De ernstige huidaandoeningen, waaronder frekwent optredende huidtumoren, bij blootstelling van deze patiënten aan zonlicht zijn waarschijnlijk het gevolg van een defekt in het mechanisme van excisieherstel. In hoofdstuk 3 wordt een beschouwing gegeven van de resultaten en konklusies uit onderzoekingen over het DNA-herstelvermogen van XP-cellen die in de literatuur en in de appendix zijn beschreven.

Het in de appendix beschreven onderzoek was gericht op:

a) het verkrijgen van nader inzicht in het mechanisme van excisieherstel;
b) de vraag welke stap in het herstelproces is gestoord in XP; en
c) de vraag of de genetische en biochemische achtergrond van de deficiëntie verschilt in verschillende vormen van de ziekte, zoals de twee klinisch onderscheiden vormen: -klassieke XP en het De Sanctis-Cacchione syndroom (DSC).

In artikel I van de appendix worden experimenten beschreven waarin het herstel werd bestudeerd van DNA-schade die door röntgenstraling wordt veroorzaakt. Enkelstrengbreuken die het gevolg zijn van deze straling werden aangetoond door de bepaling van de sedimentatiesnelheid van het DNA in alkalische sucrosegradiënten. Gekweekte cellen die werden verkregen uit huidbiopsieën van verscheidene klassieke XP-patiënten (en DSC-patiënten; artikel V) bleken breuken even snel te herstellen als de cellen van gezonde personen. Bovendien waren de XP-cellen, die een sterk gereduceerd vermogen tot herstelsynthese vertonen na UV-bestraling (artikel V), in staat een normale hoeveelheid herstelsynthese uit te voeren na röntgenbestraling. Deze waarnemingen suggereren dat in XP cellen het defekt in het herstelproces na UV-bestraling niet ligt in de herstelsynthese stap of het herstel van breuken maar in een eerdere stap, bijvoorbeeld de incisie. Deze veronderstelling wordt gesteund door experimenten die hebben aangetoond dat pyrimidinedimeren wel uit het DNA van normale cellen kunnen worden verwijderd (artikel V) maar niet uit het DNA van XP-cellen (3.2.b).

Met de in artikel II beschreven experimenten werd beoogd op direkte wijze het optreden van de incisiestap in het herstelproces in normale en XP-cellen te vergelijken. Bij een storing van de incisie in XP-cellen zou men na UV-bestraling een geringer aantal tijdelijk aanwezige DNA-breuken mogen verwachten in XP-cellen dan in normale cellen. Hoewel na hoge UV-doses ($>$1000 erg/mm^2) breuken konden

worden aangetoond in zowel normale als XP-cellen, waren deze breuken waarschijnlijk het gevolg van een direkte induktie en niet van de aktie van een incisieenzym. Na lagere doses werden in de alkalische sucrosegradiënten geen veranderingen in het sedimentatiepatroon van DNA van zowel normale als XP-cellen waargenomen. Het aantal breuken dat tegelijkertijd aanwezig is blijft beneden het niveau dat voor hun detektie vereist is (0.2 breuken/10^8 dalton), mogelijk doordat de breuken zeer snel na hun ontstaan weer worden hersteld. Hierdoor was het niet mogelijk om op direkte wijze een deficiënte incisiefunktie in XP aan te tonen.

In een nader onderzoek van het herstel van door röntgenstraling geïnduceerde enkelstrengbreuken werd gevonden dat dit herstel geheel of gedeeltelijk werd geremd door een verlaagde temperatuur en door verscheidene verbindingen zoals kaliumcyanide, 2,4-dinitrophenol, ethyleendiaminotetraacetaat, joodacetaat en kristalviolet. Deze remmers werden vervolgens toegepast na UV-bestraling om het evenwicht tussen de vorming van breuken (incisie) en het herstel van breuken te wijzigen ten gunste van de incisie. Echter ook onder deze omstandigheden werd geen akkumulatie van breuken in normale UV-bestraalde cellen gevonden. Op grond van deze resultaten werd de hypothese opgesteld dat de stappen in het herstelproces zodanig gekoördineerd zijn dat het gehele proces tot stilstand komt zodra één van de stappen is geblokkeerd. Een gekoördineerd herstelmechanisme zou tot stand kunnen komen doordat de herstelenzymen, eventueel als één enzymkomplex, zich systematisch langs het DNA bewegen en het herstel van elke afzonderlijke DNA-schade volledig uitvoeren alvorens verder te gaan naar een volgende schade.

In de artikelen IV en V werd de aandacht vooral gericht op een mogelijke genetische en biochemische heterogeniteit in XP (zie bovengenoemd punt c). Voor de bepaling van DNA-herstelsynthese werden twee verschillende methoden toegepast: de autoradiografische methode en de methode van evenwichtssedimentatie van DNA in dichtheidsgradiënten (2.3.b

en Fig. 2-2; blz.21). In laatstgenoemde methode werden enkele wijzigingen aangebracht (met name het gebruik van natriumjodide voor de dichtheidsgradiënten en een fluorimetrische bepaling van DNA in de gradiëntfrakties), waarvan de voordelen ten opzichte van de tot dusverre gebruikelijke procedure in artikel III worden beschreven.

In artikel IV werd onderzocht of komplementatie kan optreden tussen de cellen van patiënten met de twee verschillende vormen van XP -de klassieke vorm en het DSC-syndroom. De twee types hersteldeficiënte cellen werden gefuseerd onder zodanige omstandigheden dat meerkernige cellen in een hoge frekwentie werden gevormd. Het vermogen tot het verrichten van herstelsynthese na UV-bestraling bleek in de cellen, die kernen van beide types oudercellen bevatten, volledig te zijn teruggekeerd tot het niveau van normale cellen. Het optreden van komplementatie tussen de klassieke XP-cellen en de DSC-cellen toont aan dat de hersteldeficiëntie in de twee vormen van de ziekte het gevolg zijn van verschillende mutaties.

In artikel V wordt een onderzoek naar het DNA-herstelvermogen in de cellen van een groot aantal XP-patiënten beschreven. Op grond van de kinetiek van DNA-herstelsynthese na UV-bestraling kunnen vier groepen XP-patiënten worden onderscheiden nl.: de DSC-patiënten die in alle door ons onderzochte gevallen het herstelvermogen geheel missen en drie groepen klassieke XP-patiënten met resp. een lage, een intermediaire en een normale herstelaktiviteit. In de laatstgenoemde groep is de ziekte vermoedelijk niet het gevolg van een gestoord herstelvermogen. In de andere twee groepen klassieke XP-patiënten is een korrelatie aanwijsbaar tussen de mate van deficiëntie van het DNA-herstelvermogen en de ernst van de klinische symptomen. Patiënten uit dezelfde families behoorden steeds tot dezelfde groep. In komplementatiestudies werd aangetoond dat tenminste drie verschillende mutaties betrokken zijn bij de hersteldeficiëntie in resp. de groep DSC-patienten en de twee eerstgenoemde groepen klassieke XP-patiënten (artikel IV en el-

ders; zie 3.3). Het lijkt daarom waarschijnlijk dat de verschillende herstelsyntheseniveaus in de cellen van XP-patiënten genetisch bepaald zijn en berusten op verschillende mutaties.

Cellen van de heterozygote ouders van XP-patiënten vertonen in de meeste gevallen een normale aktiviteit van DNA-herstelsynthese; slechts in enkele gevallen werd een aanmerkelijk verlaagd herstelsyntheseniveau gevonden.

NAWOORD

Op verzoek van de faculteit der Geneeskunde volgen hier enkele persoonlijke gegevens.

Na het afleggen van het eindexamen h.b.s.B aan de Kon. h.b.s. te Apeldoorn begon ik in 1960 de studie in de scheikunde aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen (g) legde ik af in 1964; het doctoraalexamen, met het hoofdvak biochemie en het bijvak microbiologie, in 1967. In 1964 en 1965 was ik als student-assistent verbonden aan het Analytisch Chemisch Laboratorium van de Rijksuniversiteit te Utrecht. Voor het vervullen van de militaire dienstplicht werd ik gedetacheerd in het Medisch Biologisch Laboratorium TNO te Rijswijk.

In dit laboratorium werd vanaf 1969 het beschreven onderzoek grotendeels verricht. In mei 1969 trad ik in dienst van de Medische Faculteit te Rotterdam als wetenschappelijk medewerker aan de afdeling Celbiologie II.

Graag wil ik mijn dank betuigen aan allen die op enigerlei wijze hebben bijgedragen aan het tot stand komen van dit proefschrift. In het bijzonder dank ik mijn promotor Prof. Dr. D. Bootsma voor de wijze waarop hij heeft bijgedragen tot mijn wetenschappelijke vorming; zijn stimulerende begeleiding tijdens het onderzoek en bij het schrijven van het manuskript zijn voor mij van grote waarde geweest. De belangstelling van Prof. Dr. O. Vos gedurende het onderzoek en de wijze waarop hij en Prof. Dr. W.C. Hülsmann dit proefschrift als co-referenten hebben willen beoordelen heb ik zeer op prijs gesteld.

Van vele medewerkers van het MBL en van de afdeling Celbiologie en Genetica heb ik steun ondervonden. Met name ben ik dank verschuldigd aan Dr. R.A. Oosterbaan en Dr. G. Veldhuisen voor hun kritische opmerkingen bij het schrijven

van de publikaties waarop dit proefschrift berust; aan Dr. P.H.M. Lohman van wiens deskundigheid ik veel heb mogen profiteren bij de toepassing van biochemische technieken en bij de verwerking van de experimentele gegevens met de computer en voorts aan hem evenals aan Drs. E.A. de Weerd-Kastelein voor hun samenwerking bij de voorbereiding van enkele gezamenlijke publikaties die in dit proefschrift zijn opgenomen; aan Mej. M.L. Sluyter en Mej. H.L. Hoeksema voor het belangrijke aandeel dat zij bij de uitvoering van de experimenten met enthousiasme en kundigheid hebben geleverd en evenzo aan Mevr. E.C. Mulier-Groos voor haar hulp in de beginfase van het onderzoek; aan Mej. M. van Duuren, de heren T.M. van Os en J.G.H. Fengler van de afdeling Celbiologie en de heren H.E. Groot Bramel en M.J. Boermans van het MBL voor de verzorging van de figuren en aan de medewerkers van de sekretariaten van beide instituten waarvan vooral Mevr. R.J. van den Hoek-Boucke veel voortreffelijk typewerk heeft verricht voor het definitieve manuskript.

De direktie van het Medisch Biologisch Laboratorium en het hoofd van de afdeling Celbiologie II ben ik zeer erkentelijk voor de gelegenheid die zij mij hebben geboden het onderzoek in het MBL te voltooien.

Op deze plaats wil ik eveneens mijn dank betuigen aan de betrokken patiënten, hun ouders en hun artsen, evenals aan de gezonde donoren van huidbiopsieën, die door hun bereidwillige medewerking het onderzoek mogelijk hebben gemaakt.

Tenslotte dank ik mijn ouders voor hun steun en belangstelling, die mij in staat hebben gesteld deze weg te volgen en mijn vrouw voor haar vele initiatieven om mijn belangstelling voor andere hoofd- en zijwegen levend te houden.

APPENDIX

PAPER I

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REPAIR OF X-RAY DAMAGE IN DNA OF CULTIVATED CELLS FROM PATIENTS HAVING XERODERMA PIGMENTOSUM

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SUMMARY

Repair replication and rejoining of single-strand breaks after X-irradiation in human-skin fibroblasts from normal donors and several patients with xeroderma pigmentosum have been compared. The xeroderma strains showed different levels of repair replication following UV exposure. Repair replication and rejoining of breaks, which are considered to be part of the repair mechanism after damage due to X-irradiation and UV-irradiation, appeared to be performed in all xeroderma pigmentosum strains tested to the same level as in control strains.

These results, and the observation that in the same xeroderma pigmentosum strains repair replication after UV irradiation was considerably reduced, suggest that the xeroderma strains investigated were deficient in the enzyme(s) involved in the excision of pyrimidine dimers from the DNA.

INTRODUCTION

In the preceding paper[1] experiments are described that show a defective repair replication after UV irradiation in cultured cells from patients having xeroderma pigmentosum. The results are in agreement with CLEAVER's[2] suggestion that xeroderma pigmentosum cells lack the ability to repair radiation damage. It cannot yet be concluded that the repair-replication process itself has been disturbed, because defects in any step of the repair process may be responsible for the failure of repair replication.

The major biological damage in bacteria after UV irradiation is attributed to pyrimidine dimer formation in the DNA[13]. The excision-repair model which is based on experiments with different radiosensitive bacterial mutants[12] involves at least the following four steps[14]: incision in the DNA chain adjacent to a dimer, excision of the dimer and further degradation of the DNA in the dimer region, repair replication and finally rejoining of the single-strand break.

In mammalian cells, evidence for this kind of repair has also been found. Repair replication following UV irradiation occurs in various mammalian cell types[10], and dimer excision has possibly been shown in some human cell lines[11]. X-Rays induce

single-strand breaks in the DNA which can be repaired in mammalian cells. The repair process following X-irradiation involves repair replication[9] and rejoining of the broken DNA strand[5–7]. It seems reasonable to assume that after UV and after X-irradiation the repair replication and the rejoining step are catalyzed by the same two enzymes, a DNA polymerase and a polynucleotide ligase respectively.

The present communication deals with studies on the repair replication and the rejoining of single-strand breaks in the DNA of the normal human and xeroderma pigmentosum skin fibroblasts after exposure to X-rays. Repair replication was shown by autoradiographic methods following irradiation and pulse labeling of cells with tritiated thymidine. Rejoining of single-strand breaks was studied by alkaline sucrose-gradient sedimentation of the labeled DNA.

The results show that xeroderma pigmentosum cells can perform repair replication and rejoining of single-strand breaks after X-irradiation. This is in agreement with similar observations on repair replication in xeroderma pigmentosum cells that were published by CLEAVER[3] during the preparation of this manuscript.

MATERIALS AND METHODS

Cells and culture techniques

The cell strains used in the experiments were XP2, XP4 and XP9 primary fibroblasts originating from xeroderma pigmentosum patients, XP4SV40 an XP4 strain transformed by SV40 virus, and two strains that were considered to be control cells (R and LN). These strains are described in more detail in the preceding paper[1].

For the experiments 150 000 cells were seeded in culture dishes (4 cm diameter) with a Mylar bottom in 4 ml F12 medium supplemented with 5% fetal calf serum and 5% newborn calf serum. These cultures were incubated at 37° in air containing 5% CO_2. For the autoradiographic experiments the dishes were provided with a coverslip before the cell suspension was added.

Irradiation

Monolayers of the cells were irradiated in medium by a 250-kV X-ray machine at room temperature. Radiation constants were 250 kV (constant potential), 15 mA, no filtration, distance to target 22.5 cm, dose rate 1000 R/min.

Autoradiographic experiments

After 2–4 days growth the cultures were irradiated at 37°. Immediately after irradiation the medium was replaced by fresh medium containing 10 μCi/ml tritiated thymidine (spec. act. 2 Ci/mmole, The Radiochemical Centre, Amersham, Great Britain). After 3 h, cells were fixed in acetic acid–ethanol (1:3, v/v). Autoradiography was performed with Kodak AR-10 stripping film after treatment with 2% perchloric acid to remove acid-soluble radioactive precursors. The preparations were then stained with hematoxylin–eosin. The exposure time was 21 days. The average number of grains per cell was obtained by counting 50 labeled cells per slide.

Sucrose-gradient experiments

Two days after seeding, the medium was replaced by fresh medium containing tritiated thymidine (2.5 μCi/ml, spec.act. 2 Ci/mmole), and the cultures were grown

for 18 h at 37° and irradiated. After various intervals (0–90 min) the cells were suspended in the medium by scraping with a rubber policeman. The suspension was cooled and centrifuged at 300 *g* for 10 min at 0–3°, and the pellet washed in 0.15 *M* NaCl. Finally a suspension of $2 \cdot 10^5$ cells/ml in cold 0.15 *M* NaCl was prepared. Isokinetic sucrose gradients were made in polypropylene centrifuge tubes as will be described elsewhere[8]. On top of each gradient was pipetted successively: 1.5 ml 0.5 *M* NaOH, 0.5 ml of an aqueous solution containing 6% butan-2-ol (by volume) and 2% 4-aminosalicylic acid and 2% triisopropylnaphthalenesulfonic acid (both by weight) and 0.5 ml of the cell suspension (10^5 cells). The gradients were centrifuged at 22 500 rev./min for 3.5 h at 20° in a SW-27 swinging bucket rotor of a Spinco model L centrifuge.

Fractionation of the gradients and radioactivity assay were performed as described elsewhere[7].

RESULTS

Repair replication studied by autoradiography

Fibroblast cultures were irradiated with 1–20 krad doses of X-rays followed by incubation in tritiated-thymidine-containing medium for 3 h. Autoradiographs of such cultures showed that all cells were radioactive. Cells in the S-phase could be recognized by their heavy labeling which resulted from continued semi-conservative DNA replication. Cells in the G_1 or in the G_2 phase were lightly labeled as a result of repair replication.

The results of grain countings from an experiment with a xeroderma pigmentosum cell strain (XP4) and a control cell strain (R) are shown in Fig. 1. Both cell strains showed the same amount of [^{3}H]thymidine incorporation after X-irradiation. Identical results were obtained with all other xeroderma and control cell strains (XP2, XP9, LN and XP5, XP6, XP7, DB, MM, mentioned in the preceding paper[1]).

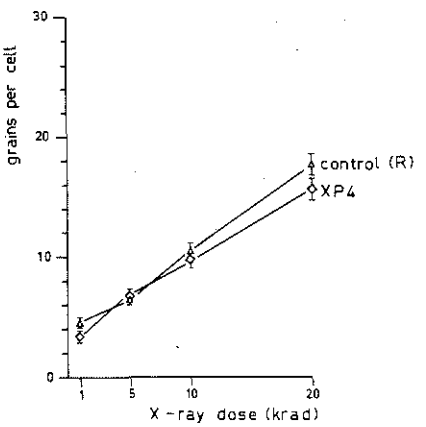


Fig. 1. Average grain number over lightly labeled cells in xeroderma pigmentosum and control cultures after X-irradiation. Autoradiographic exposure time, 21 days.

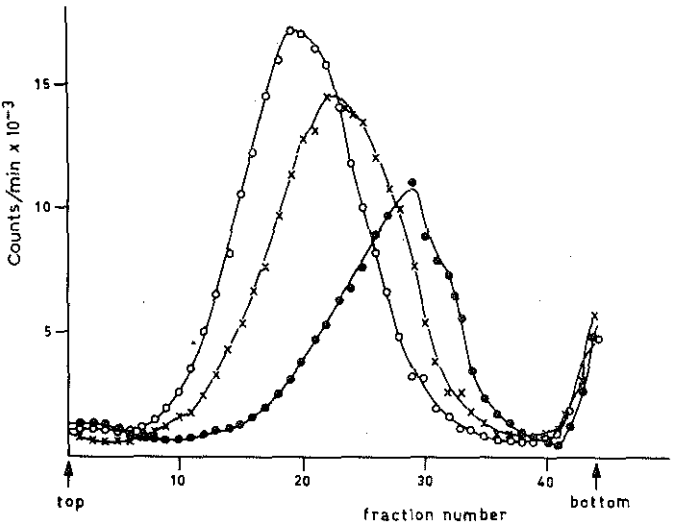


Fig. 2. Sedimentation patterns in alkaline sucrose gradients of DNA from xeroderma pigmentosum (XP2) cells. Unirradiated (●); 20 krad (○); 20 krad incubated at 37° for 1 h (×).

Rejoining of breaks studied by alkaline sucrose gradients

Xeroderma pigmentosum cell cultures (XP2, XP4, XP9, XP4-SV40) and control cultures (LN and R) were irradiated with X-rays at a dose of 20 krad. The cells were collected immediately or 1 h after irradiation. Fig. 2 shows the sedimentation patterns obtained with XP2 cells. Similar patterns were obtained with the other cell strains tested. The induction of breaks is indicated by the shift of the curve to lower fraction numbers, corresponding to lower molecular weights. After 1 h incubation following irradiation the bulk of the DNA bands at higher fraction numbers indicating repair of breaks. This was found in control as well as in xeroderma cells. In Table I the weight average molecular weights, calculated by use of the relation of STUDIER[15] for DNA on alkaline gradients and the number of breaks (for calculations see refs. 4, 7, 8) have been summarized. In all cell types an appreciable number of breaks appears to have been rejoined after 1 h incubation. Although there is some variation in the percentages of rejoined breaks, these results suggest that there is no significant difference in rejoining capacity between xeroderma and control cell strains.

TABLE I

REJOINING OF X-RAY-INDUCED SINGLE-STRAND BREAKS IN THE DNA OF SEVERAL XERODERMA PIGMENTOSUM AND CONTROL CELL STRAINS

Cell strain	*Mol. wt. ($\times 10^{-6}$ dalton) 0 krad*	*Mol. wt ($\times 10^{-6}$ dalton) 20 krad*	*Mol. wt. ($\times 10^{-6}$ krad) 20 krad and 60 min at 37°*	*Breaks per 10^8 dalton 20 krad*	*Breaks per 10^8 dalton 20 krad and 60 min at 37°*	*Rejoining (%)*
R	132	67	97	1.44	0.55	62
LN	135	70	92	1.36	0.68	50
XP2	117	65	86	1.36	0.62	54
XP4	147	83	105	1.03	0.53	49
XP4-SV40	221	96	168	1.17	0.28	76
XP9	184	66	119	1.95	0.59	70

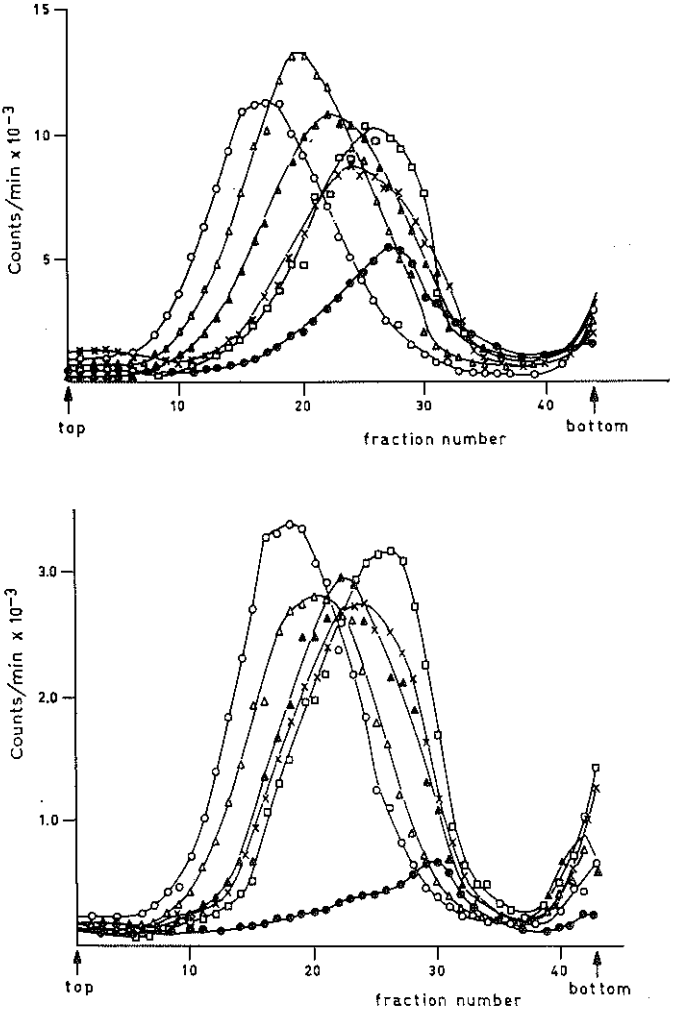
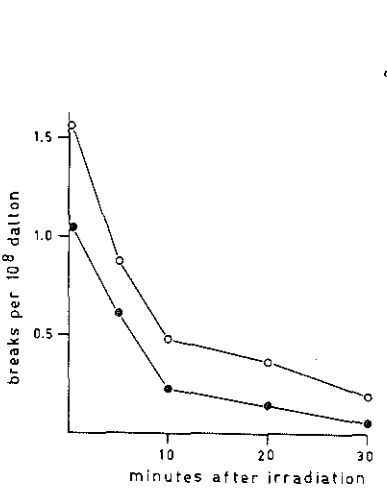


Fig. 3. Sedimentation patterns in alkaline sucrose gradients of DNA from cells incubated for various times (0–30 min) after irradiation (20 krad): 0 min (○); 5 min (△); 10 min (▲); 20 min (×) and 30 min (□); unirradiated control (●). (a) Xeroderma pigmentosum cells (XP4). (b) Control cells (R).

To obtain information about the time course of the rejoining process, cultures of XP4 and cells R were incubated after X-irradiation for different times before the cells were collected. In Figs. 3a and 3b the sedimentation patterns from these experiments are shown, and the numbers of breaks per 10^8 mol. wt. are given in Fig. 4. For both cell strains the rejoining process appears to start rapidly, and after 10 min most of the breaks have been rejoined.

Usually the molecular weight of DNA from unirradiated cells varied in independent experiments. Since these control values are used for the calculations of the number of breaks[4] after irradiation, these variations will lead to a systematic difference when the data of two experiments are compared (Fig. 4). Extension of the incu-



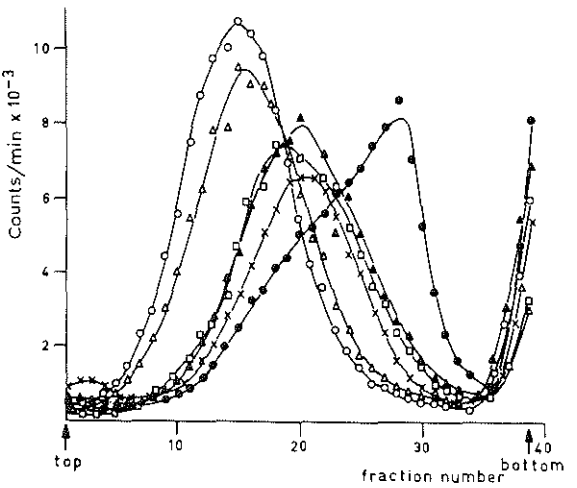


Fig. 4. The time course of the rejoining of single-strand DNA breaks during incubation at 37° after a 20 krad X-ray dose. ○, xeroderma pigmentosum cells (XP4); ●, control cells (R).

Fig. 5. Sedimentation patterns in alkaline sucrose gradients of DNA from R cells incubated for various times (0–90 min) after irradiation (20 krad): 0 min (○); 15 min (△); 30 min (▲); 60 min (×); and 90 min (□); unirradiated control (●).

bation period to more than 30 min after irradiation did not result in a further increase of molecular weight; this is shown in Fig. 5 for R cells; the same result was obtained with XP4 cells.

DISCUSSION

The sucrose-gradient experiments described here show that cells from different patients having xeroderma pigmentosum are able to rejoin single-strand breaks in DNA caused by X-irradiation at the same rate and to the same level as normal human fibroblasts. In the autoradiographic experiments we found that the cells of six patients who suffered in different degrees from xeroderma pigmentosum and four, in this respect normal persons, showed equal levels of repair replication after X-irradiation. This is in contrast with our previous finding[1] that after UV irradiation each of these xeroderma pigmentosum strains showed a significant and characteristic reduction in repair replication when compared with normal cells. From these results it can be concluded that the six different xeroderma cell strains should all be defective in the exision of pyrimidine dimers or even in the first step of the process, the incision adjacent to a dimer by an endonuclease.

Our results on the rejoining of breaks in xeroderma cells present additional evidence for this conclusion. Therefore, our cell strains seem not to be different from the strain studied by CLEAVER[3] who also found a defective early step of the repair process. However, these results do not yet explain the characteristic differences in repair-replication levels among the xeroderma strains after UV exposure.

These differences could originate from mutations in different genes (*e.g.* genes coding for enzymes involved in different early steps, or regulatory genes) or also from different mutations in a single gene coding for the same enzyme in all cases.

At the present time we are studying the reduction of dimer excision[11] in SV40-transformed xeroderma cells. The study of break induction after UV-irradiation might offer further evidence for the incision deficiency in xeroderma pigmentosum cells. However, we found no breaks in the DNA of T cells (an established normal human-kidney cell line) or in SV40-transformed xeroderma pigmentosum cells after a UV dose of 100 erg/mm^2 and incubation up to 4 h at 37° or at 0–4°. These results can be explained by supposing that incision is the rate-limiting step. In order to accumulate incision breaks in the DNA of UV irradiated cells various agents are being tested that might inhibit the repair replication or the rejoining.

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REFERENCES

1 Bootsma, D., M. P. Mulder, F. Pot and J. A. Cohen, Different inherited levels of DNA repair replication in xeroderma pigmentosum cell strains after exposure to ultraviolet light, *Mutation Res.*, 9 (1970) 507–516
2 Cleaver, J. E., Defective repair replication of DNA in xeroderma pigmentosum, *Nature*, 218 (1968) 652–656.
3 Cleaver, J. E., Xeroderma pigmentosum: a human disease in which an initial stage of DNA-repair is defective, *Proc. Natl. Acad. Sci. (U.S.)*, 63 (1969) 428–435.
4 Corry, P. M., and A. Cole, Radiation-induced double-strand scission of the DNA of mammalian metaphase chromosomes, *Radiation Res.*, 36 (1968) 528–543.
5 Humphrey, R. M., D. L. Steward and B. A. Sedita, DNA-strand breaks and rejoining following exposure of synchronized Chinese hamster cells to ionizing radiation, *Mutation Res.*, 6 (1968) 459–465.
6 Lett, J. T., I. Caldwell, C. J. Dean and P. Alexander, Rejoining of X-ray induced breaks in the DNA of leukaemia cells, *Nature*, 214 (1967) 790–792.
7 Lohman, P. H. M., Induction and rejoining of breaks in the deoxyribonucleic acid of human cells irradiated at various phases of the cell cycle, *Mutation Res.*, 6 (1968) 449–458.
8 Lohman, P. H. M., O. Vos, C. A. v. Sluis and J. A. Cohen, in preparation.
9 Painter, R. B., and J. E. Cleaver, Repair replication in HeLa cells after large doses of X-irradiation, *Nature*, 216 (1967) 369–370.
10 Rasmussen, R. E., and R. B. Painter, Radiation-stimulated DNA synthesis in cultured mammalian cells, *J. Cell Biol.*, 29 (1966) 11–19.
11 Regan, J. D., J. E. Trosko and W. J. Carrier, Evidence for excision of ultraviolet-induced pyrimidine dimers from the DNA of human cells *in vitro*, *Biophys. J.*, 8 (1968) 319–325.
12 Rörsch, A., P. v. d. Putte, I. E. Mattern, H. Zwenk and C. A. v. Sluis, Bacterial genes and enzymes involved in the recovery from lethal radiation damage, in G. Silini (Ed.), *Radiation Research, Proc. 3rd Intern. Congr. Radiation Res., Italy, 1966*, North-Holland, Amsterdam, 1967, pp. 771–789.
13 Setlow, R. B., and J. K. Setlow, Evidence that ultraviolet-induced thymine dimers in DNA cause biological damage, *Proc. Natl. Acad. Sci. (U.S.)*, 48 (1962) 1250–1257.
14 Setlow, R. B., Repair of DNA, in V. V. Koningsberger and L. Bosch (Eds.), *Regulation of Nucleic Acid and Protein Biosynthesis, (BBA Library, Vol. 10)*, Elsevier, Amsterdam, 1967, pp. 51–62.
15 Studier F. W., Sedimentation studies of the size and shape of DNA, *J. Mol. Biol.*, 11 (1965) 373–390

PAPER II

Mutation Research, 17 (1973) 385–394

EFFECTS OF INHIBITORS ON REPAIR OF DNA IN NORMAL HUMAN AND XERODERMA PIGMENTOSUM CELLS AFTER EXPOSURE TO X-RAYS AND ULTRAVIOLET IRRADIATION

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SUMMARY

Experiments were carried out to obtain direct evidence for the hypothesis that in human cells the repair of UV-damaged DNA is initiated by an incision step, and that this step is defective in cells from patients having Xeroderma pigmentosum (XP). The alkaline sucrose gradient centrifugation technique was used to detect breaks in the DNA.

A decreased sedimentation velocity of the DNA was found after exposure of normal and XP cells to high doses of UV (5000 erg/mm^2). Breaks were induced in the DNA by the UV irradiation without the action of an enzyme. After exposure of both types of cell to UV doses of 100–500 erg/mm^2, breaks that might occur by enzymic incision were not observed, possibly because of immediate rejoining.

After single-strand breaks had been induced by X-rays, rejoining did not occur at temperatures lower than 22°. Rejoining was inhibited by KCN, 2,4-dinitrophenol, EDTA, iodoacetate and crystal violet. Actinomycin D, acriflavine and phleomycin, also tested as potential inhibitors of the repair process, induced breaks or conformational changes in the DNA of unirradiated normal and XP cells.

Application to UV-exposed cells of conditions that inhibit the rejoining of breaks did not cause accumulation of breaks in the DNA. The results suggest a coordinated and sequential performance of the steps in the repair of each UV lesion by repair enzymes which may act as a complex.

INTRODUCTION

In human cells the DNA that has been damaged by exposure of the cells to UV or X-irradiation can be effectively restored by enzymic repair processes. Cells from patients suffering from XP show an increased sensitivity to UV irradiation[13], and appear to be defective in the excision of pyrimidine dimers[12,20,31] and in repair replication after exposure to UV[6,9]. After X-irradiation, repair replication[10,21] and rejoining

Abbreviations: DNP, 2,4-dinitrophenol; XP, Xeroderma pigmentosum.

of single-strand breaks[21] were normal in these cells. These observations suggest that the first step in the repair process after UV irradiation, the incision next to a pyrimidine dimer in the DNA, is defective in XP cells[10,21,31]. In normal cells one would expect to find single-strand breaks after exposure to UV as a result of the incision of the damaged strand by an endonuclease. Evidence for the formation of DNA breaks after exposure to UV has been presented by SETLOW *et al.*[31] and by BEN-HUR AND BEN-ISHAI[2]. However, in both reports and also in the present study it is suggested that most of the breaks produced escape detection owing to their rapid rejoining in the subsequent steps in the repair process. One would expect to observe an accumulation of breaks under conditions in which rejoining is inhibited.

In the present study the effects of two groups of compounds on the rejoining of breaks were investigated after the induction of breaks by X-irradiation: (*I*) compounds that might inhibit the action of the enzyme(s) involved in the repair either directly (iodoacetate) or by depletion of the cell from necessary cofactors (DNP, KCN, EDTA); and (*II*) compounds that bind to the DNA (actinomycin D[4], acriflavine[3], crystal violet, phleomycin[17] and caffeine[15]). CLEAVER[11] showed that iodoacetate, actinomycin D, acriflavine and crystal violet inhibit repair replication after irradiation by UV. Recently, the inhibition of the rejoining of X-ray-induced breaks in mammalian cells has been reported by MOSS *et al.*[25] and ORMEROD AND STEVENS[28] for DNP and by TSUBOI AND TERASIMA[32] for proflavine.

The results obtained when inhibitors of the rejoining of breaks were applied after UV irradiation of human cells will be described.

MATERIALS AND METHODS

(a) Cells and culture techniques

The cell lines used in the experiments were: two heteroploid human cell lines, the T-cell line (originating from human kidney[5]) and the HeLa cell line; a diploid fibroblast strain from a normal donor (R)[6]; a diploid Xeroderma strain from a patient having severe symptoms of the disease (XP4)[6]; and four strains[6] transformed with SV40 virus, namely RSV40, XP10SV40 (XP10, heterozygous for XP), XP4SV40 and XP9SV40 (XP9, severe XP case).

The T cells were grown in Dulbecco's salt solution containing 0.5% lactalbumin hydrolysate, penicillin (100 I.U./ml), streptomycin (0.1 mg/ml) and 6% newborn calf serum. F12 medium was supplemented with penicillin, streptomycin and 15% foetal calf serum for primary fibroblast cultures or with 10% newborn calf serum for the SV40-transformed strains and for the HeLa cells. For each experiment 150000–200000 cells were seeded in plastic culture dishes (5 cm diameter). The cultures were incubated at 37° in a controlled atmosphere of 95% air and 5% CO_2.

(b) Labelling of the DNA

1 or 2 days after seeding of the cells, when the cultures were in logarithmic growth, the medium in the dishes was replaced by medium containing (methyl) tritiated thymidine (2.5 μCi/ml, 2 Ci/mmole from the Radiochemical Centre, Amersham). The cultures were grown for at least 20 h at 37°. Fresh unlabelled medium was supplied to the cells 30 min before irradiation.

(c) Irradiation

For UV-irradiation the medium was removed, and the cultures were washed with a balanced salt solution and drained. Relatively low does of UV irradiation (100–500 erg/mm²) were generated from a Philips TUV lamp (15 W) at a rate of 8 erg/mm²/sec predominantly at 254 nm). Exposure to 5000 erg/mm² was obtained with a Philips TUV lamp (30 W) at a rate of 56 erg/mm²/sec.

X-Irradiation was performed at room temperature with a 250-kV X-ray machine. Radiation constants were: 250 kV (constant potential), 15 mA, distance to target 22.5 cm, dose rate 1000 rad/min. Immediately after irradiation with X-rays or UV the cultures were either incubated in medium at 37° or scraped from the dishes in medium at 0°.

(d) Alkaline sucrose gradient sedimentation

Immediately after irradiation or after the incubation period the cells were suspended in cold medium by scraping with a rubber policeman. The suspension was centrifuged at 3000 *g* for 10 min at 0–3° and the pellet washed in 0.15 *M* NaCl. The cells were finally suspended in 0.15 *M* NaCl at a concentration of $2 \cdot 10^5$ cells/ml. 10^5 cells were layered gently over a layer of 0.5 ml of an aqueous solution containing 12% v/v butanol-2 and sodium 4-aminosalicylate (2% w/w), sodium triisopropylnaphthalenesulphonate (2% w/w) and 1.5 ml 0.5 *M* NaOH on top of a 5–23% alkaline (pH 12.2) sucrose gradient of constant velocity type[27]. 20 min after layering the cells, the gradients were centrifuged in a SW27 rotor in a Spinco Model L2 ultracentrifuge for 3.5 h at 22500 rev./min at 20°. Fractionation, radioactivity assay and calculation of molecular weights and number of breaks were carried out as described by LOHMAN (ref. 24).

RESULTS

(a) Sedimentation of DNA after exposure to UV

A UV dose of 500 erg/mm² or lower did not change the sedimentation properties of the DNA immediately after exposure. Fig. 1A shows that, after exposure of primary human skin fibroblasts (R cells) to 150 erg/mm² and incubation at 37° for 0, 20 and

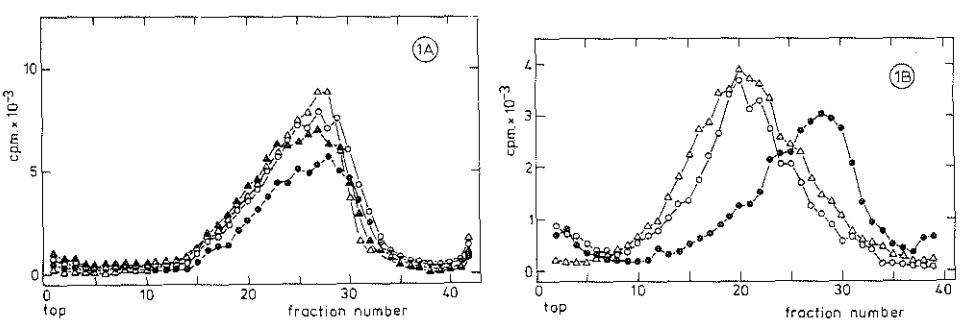

Fig. 1A. Sedimentation patterns of DNA from primary human fibroblasts (R). ●, unirradiated. Irradiated with 150 erg/mm² and incubated at 37° during: ○, 0 min; ▲, 20 min; △, 120 min.

Fig. 1B. Sedimentation patterns of DNA from primary human fibroblasts (R). ●, unirradiated; ○, 5000 erg/mm² and no incubation; △, 5000 erg/mm² and 1 h at 37°.

120 min, no shift of the DNA peak in the gradient occurred. The molecular weight of the DNA calculated for each profile was $2 \cdot 10^8$ dalton. Similar experiments were performed with heteroploid T cells (100 erg/mm^2 and 0, 10, 30, 90, 120 and 240 min incubation; 500 erg/mm^2 and 0, 15, 60 and 120 min incubation), HeLa cells (250 erg/mm^2 and 0, 15, 90, 180 and 360 min incubation) and XP4SV40 cells (100 erg/mm^2 and 0, 120 and 240 min incubation). In these experiments no clear shift or change in the shape of the profile was observed when compared with the profile of the unirradiated control.

Immediately after exposure of primary human cells to a high dose of UV (5000 erg/mm^2) at 0°, a decrease in sedimentation velocity was observed (Fig. 1B). The shift and the skewing of the DNA peak did not change when the cells were incubated in medium at 37° for 1 h. The same change in sedimentation properties of the DNA, as shown for normal cells in Fig. 1B, was found in Xeroderma cells (XP4-cell strain).

(b) Inhibitors of rejoining of X-ray-induced breaks in the DNA

In this series of experiments conditions were tested that might inhibit the rejoining of breaks after X-irradiation. Irradiation with a dose of 20 krad caused a decrease of the (weight-average) molecular weight of the DNA in human cells (primary fibroblasts[21]) and T cells[24] from $2.0 \cdot 10^8$ to $0.7 \cdot 10^8$ dalton. As described previously, in primary fibroblasts[21] and heteroploid T cells[23], 50–75% of the breaks were repaired during an incubation period of 60 min at 37° after irradiation.

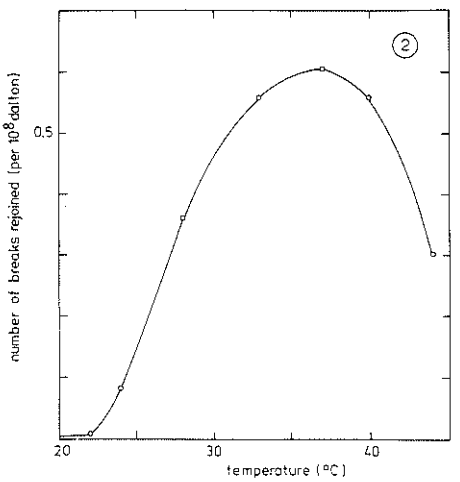

Fig. 2. Numbers of breaks rejoined after 20 krad X-rays and incubation during 45 min at various temperatures.

First, the influence of temperature on the rejoining activity was investigated. Fig. 2 shows the temperature dependency of the rejoining activity in T cells. The number of rejoined breaks per 10^8 dalton was calculated from the sedimentation profiles of the DNA and plotted against temperature. The rejoining activity appears to be almost absent below 22° and optimal at about 37°.

Among the chemical agents tested for inhibition of rejoining, four compounds

—iodoacetate, DNP, KCN and EDTA—were chosen because they might inhibit the enzyme(s) involved, either directly or by depleting the cell of ATP or Mg^{2+} ions. Control experiments showed that treatment with DNP, KCN or EDTA had no effect on the sedimentation of DNA from unirradiated cells (Table I), whereas iodoacetate induced a small number of breaks in the DNA of T cells. DNP and KCN were added 30 min before irradiation; EDTA was added just before, and iodoacetate immediately after, irradiation. The presence of DNP, KCN or EDTA during irradiation did not influence the number of breaks present immediately after irradiation. As an example, Fig. 3 shows the comparable sedimentation profiles after irradiation in the presence or absence of DNP. The profile of the irradiated DNA shifted towards the position of

TABLE I

THE EFFECTS OF INHIBITORS ON REPAIR OF X-RAY- OR UV-DAMAGED DNA

Compound	*Concentration (M)*	*Breaks induced by the compound*[a]	*Relative rejoining*[b]	*Breaks after UV + compound*[a]
EDTA	10^{-1}	<0.2	3	<0.2
KCN	10^{-3}		12	
DNP	10^{-4}		10	
	10^{-2}		0	
Iodoacetate	10^{-2}	0.2–0.4	5	0.5

[a] Numbers of breaks per 10^8 dalton in the DNA of inhibitor-treated or UV-irradiated and inhibitor-treated cells relative to DNA from unirradiated and untreated cells.
[b] The figures give the number of breaks rejoined after 20 krad and 60 min in the presence of the inhibitor as a percentage of the number of breaks rejoined in medium without the inhibitor.

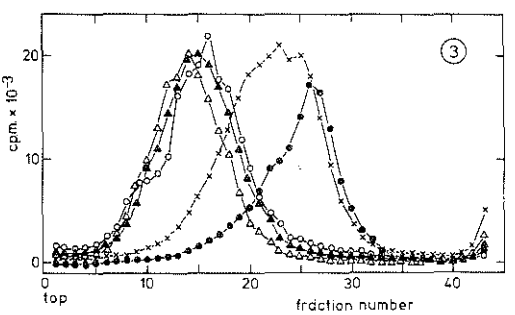

Fig. 3. Effect of DNP on induction and rejoining of single-strand breaks. Sedimentation patterns of DNA from T cells. ●, unirradiated; ▲, irradiated (20 krad X-rays) and no incubation; △, irradiated, no incubation, DNP (10^{-2} *M*) present 20 min before and during irradiation; ×, irradiated, incubated at 37° during 60 min; ○, irradiated, incubated at 37° during 60 min in 10^{-4} *M* DNP.

unirradiated DNA when the cells after irradiation had been incubated in the absence of DNP. However, such a shift was not observed when the cells had been incubated in the presence of DNP. This indicates that DNP abolishes the repair activity in the cells. The same result was obtained with KCN, EDTA and iodoacetate (Table I).

The second group of compounds—actinomycin D, acriflavine, crystal violet, phleomycin and caffeine—were chosen because of their binding to DNA, which might impair the rejoining of single-strand breaks. The influence of incubation for 1 h in the presence of these drugs on the sedimentation properties of the DNA from unirradiated cells is presented in the second column of Table II. Crystal violet and caffeine at the

TABLE II

THE EFFECTS OF DNA-BINDING COMPOUNDS ON REPAIR OF X-RAY- OR UV-DAMAGED DNA, AND ON DNA IN UNIRRADIATED CELLS

Compound	*Concentration (μg/ml)*	*Breaks induced by the compound*[a]	*Relative rejoining*[b]	*Breaks after UV + compound*[a]
Caffeine	400	<0.2	105	—
Crystal violet	40	0.2	0	0.2
Actinomycin D	2	<0.2	98	—
	20	2.0	0	—
Acriflavine	10	1.4	0	—
Phleomycin	10	non-random degradation	95	—

[a] and [b], see Table I.

concentrations used and actinomycin D at the lower concentration (2 μg/ml) had no effect on the sedimentation of DNA from unirradiated cells. Both actinomycin D and acriflavine, at 20 and 10 μg/ml respectively, strongly influenced the sedimentation of the DNA from the unirradiated cells. This effect, which suggests the induction of breaks by these drugs, was similar in normal human and XP cells, as is shown for acriflavine in Fig. 4A. Phleomycin reacted in a more peculiar way: treatment of T cells with this compound at 20 μg/ml for 15 min resulted in the formation of material with a lower molecular weight, leaving part of the original DNA unchanged; this indicates a nonrandom breakdown of the DNA. From the profiles in Fig. 4 B it appears

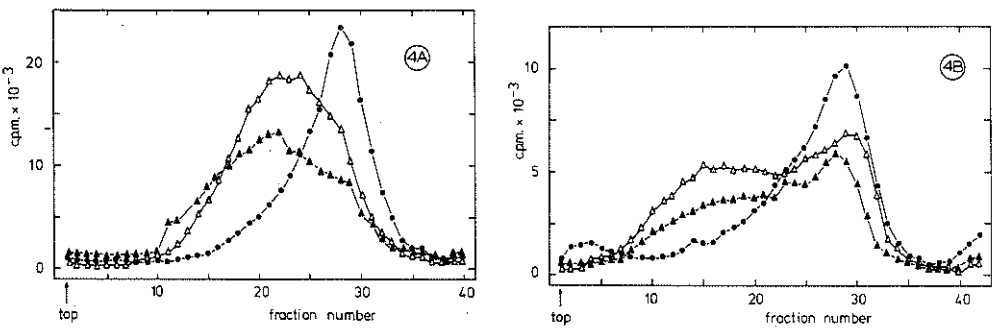

Fig. 4A. Effect of acriflavine on the sedimentation of DNA from (SV40-transformed) human fibroblasts: ●, untreated RSV40 and in the same position XP4SV40. Cells incubated in medium containing acriflavine (10 μg/ml) during 15 min: △, RSV40; ▲, XPSV40.

Fig. 4B. Effect of phleomycin on the sedimentation of DNA from (SV40-transformed) human fibroblasts: ○, untreated XP9SV40 (XP patient) and in the same position XP10SV40 (not affected, heterozygote for XP). Cells incubated in medium containing phleomycin (20 μg/ml) during 15 min: ▲, XP9SV40; △, XP10SV40.

that similar reactions occurred in (SV40-transformed) normal and XP cells. The data on the influence of the drugs on the rejoining activity of the cells are tabulated in the third column of Table II. Caffeine (0.4 mg/ml), actinomycin D (2 μg/ml) and phleomycin (20 μg/ml) did not inhibit the rejoining of breaks when added after X-irradiation. Rejoining of breaks was inhibited completely by crystal violet (40 μg/ml). In the presence of actinomycin D (20 μg/ml) or acriflavine (10 μg/ml) repair of breaks after a 20-krad dose was not seen, but this observation was complicated by the additional effect of the drug alone.

(c) Inhibitors of rejoining used after irradiation by UV

In the previous section several conditions are described that inhibited the rejoining of X-ray-induced breaks and that had no or only slight influence on the sedimentation of DNA from unirradiated cells. These conditions were applied after UV irradiation in order to accumulate breaks which might occur as a result of incision at pyrimidine dimers in the DNA. After incubation of UV-irradiated T cells for 45 min at temperatures that were inhibitory for rejoining, the same DNA sedimentation profiles as in Fig. 1A were found, showing no shift to lower molecular weight values. Similar results were obtained when T cells, after exposure to 100–200 erg/mm^2, were incubated in the presence of KCN (10^{-3} *M*), DNP (10^{-2} and 10^{-4} *M*), EDTA (10^{-2} *M*), Table I, and crystal violet (40 μg/ml), Table II. Fig. 5 shows the sedimentation pro-

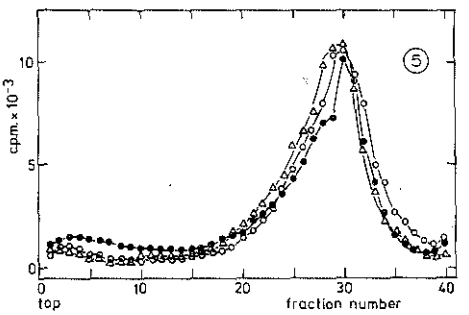

Fig. 5. Effects of EDTA and KCN on the sedimentation of DNA from T cells after exposure to UV: ●, unirradiated, untreated cells; irradiated (200 erg/mm^2) and incubated during 90 min at 37° in: ○, 10^{-1} *M* EDTA; △, 10^{-3} *M* KCN.

files obtained after incubation of UV-exposed cells with EDTA and KCN. Iodoacetate treatment caused DNA breaks in UV-irradiated T cells, but, as mentioned in the previous section, also in unirradiated T cells. However, in similar experiments with primary and SV40-transformed normal and XP cells, iodoacetate did not effect breaks in the DNA of either UV-exposed or unirradiated cells of these strains.

DISCUSSION

The experiments were designed to find direct evidence for incision as the first step in the repair of UV-damaged DNA in human cells. In the cell lines R, T and HeLa, used in our experiments, unscheduled DNA synthesis[6] and excision of thymine dimers (ref. 20) occurs whereas these activities were absent[20] from the two strains from severe XP patients (XP4 and XP9). It has been suggested[10,21,31] that the incision step is defective in XP cells; DNA breaks are therefore not expected after UV irradiation of these cells. In the present experiments breaks were only found after extremely high doses of UV (5000 erg/mm^2), but then even at 0° and in both normal and XP cells. The results suggest that these breaks are not caused by an enzymic action but probably by the direct induction of breaks or alkali-labile lesions by the UV irradiation. This hypothesis is supported by studies on the effect of high doses of UV on isolated double-stranded, circular DNA from bacteriophage PM2 performed in our laboratory[33]. These studies have shown that UV irradiation induces single-strand breaks and alkali-labile lesions in the DNA; the sum of both types of lesion was about the same as the

number of breaks we found in the DNA of human cells (1 break/10^8 dalton after 5000 erg/mm^2). After exposure to relatively low doses (100–500 erg/mm^2) the number of breaks did not exceed 0.2 per 10^8 dalton, which is the lower limit of detection in our studies on the induction of breaks by X-irradiation using the same technique. These results seem to disagree with earlier papers of SETLOW *et al.*[31] and of BEN-HUR AND BEN-ISHAI[2] in which the production of breaks after low doses of UV is reported. In HeLa cells, after a dose of 250 erg/mm^2 and 1.5 h incubation, BEN-HUR AND BEN-ISHAI[2] found a molecular weight of the DNA of $0.32 \cdot 10^8$ dalton, indicating a large, but unknown, number of breaks. However, in our experiments using the same cell line and identical irradiation and incubation conditions the results of BEN-HUR AND BEN-ISHAI[2] could not be reproduced. It cannot be excluded that the discrepancy between our results and those of BEN-HUR AND BEN-ISHAI is caused by the differences in lysing and DNA denaturation procedures. The apparent variability in the profiles shown in SETLOW's paper, possibly due to the low amount of radioactivity in the gradients, makes the interpretation of their results difficult.

From experiments on the excision of thymine dimers[12,20,29,31] the number of incisions, which have to be made during the repair process, can be calculated. After a UV dose of 200 erg/mm^2 we found[20] a dimer percentage ($\hat{X}T/T\%$) of 0.060, which is equivalent to at least 30 dimers per 10^8-dalton DNA[31]. Since, in the cells used (T cells and primary fibroblasts), about 25% of the dimers is excised within the first 8 h (unpublished results), we estimate that about 1 dimer/10^8 dalton is excised per h. If incision were to proceed while the repair of breaks is blocked completely during incubation for 1 h after a dose of 200 erg/mm^2, a clear shift of the DNA peak in the gradient would be expected. To find conditions that might inhibit one of the steps in the repair process after incision, we studied the effects of various agents on the rejoining of breaks induced in T cells by X-irradiation.

The enzymic nature of the rejoining process following X-irradiation is indicated by the temperature dependency found in the present experiments and in those of ORMEROD AND STEVENS[28] and DONLON AND NORMAN[16]. It is a reasonable assumption that the same enzyme, a polynucleotide ligase, is involved in the rejoining of breaks in the repair of both X-ray and UV damage. The first group of compounds tested for inhibition of rejoining inhibits enzymes either directly (iodoacetate) or by an effect on the supply of necessary cofactors (KCN, DNP and EDTA). Iodoacetate inhibits repair replication after UV irradiation[11]. It might be expected that the ATP- and Mg^{2+}-dependent ligase[22] is inhibited by KCN, DNP and EDTA. Inhibition of rejoining has been reported earlier for EDTA in *M. radiodurans*[14] and for DNP in mammalian cells by MOSS *et al.*[25] and ORMEROD AND STEVENS[28].

In the second group of compounds that bind to DNA, actinomycin D (20 μg/ml) and acriflavine (10 μg/ml) were completely inhibitory although the rejoining of a small number of breaks could have been masked by the simultaneous induction of breaks or alternatively by conformational changes in the DNA induced by these compounds. The effects on DNA observed after treatment of unirradiated cells with phleomycin, actinomycin D and acriflavine are still unexplained. GRIGG[18] has postulated that in *E. coli* the UV-specific endonuclease may be responsible for the initiation of the degradation of DNA that he observed in phleomycin-treated UV-resistant, but not in UV-sensitive, *E. coli* mutants. Normal human and XP cells, however, responded similarly to exposure to phleomycin and also to actinomycin D and acriflavine. If in

XP cells a UV-specific endonuclease is defective, this enzyme seems not to be involved in the changes found in the DNA structure in human cells after application of these drugs. Crystal violet inhibited the rejoining without influencing the sedimentation of DNA from unirradiated cells, but like the compounds in the first group (KCN, DNP, EDTA and iodoacetate) it did not effect accumulation of breaks after UV irradiation. The absence of breaks after exposure to UV under conditions where rejoining of breaks was inhibited indicates that incision did not occur. These findings do not necessarily argue against the hypothesis that an incision step is involved in the repair process, because at least two explanations may fit the results in the excision-repair model. (*1*) The endonuclease function *per se* might be inhibited by each of the conditions used in our studies. (*2*) The different steps in the repair process might be performed in a sequential and coordinated way, which does not permit the functioning of the endonuclease when one or more of the other repair enzymes are blocked.

In the microorganisms *M. lysodeikticus*[26] and *M. luteus*[8,19] a UV-specific endonuclease is present that does not require ATP and Mg^{2+} ions and is not inhibited by EDTA; on the other hand the enzyme is inhibited *in vivo* and *in vitro* by KCN[8]. In mammalian cells a UV-specific endonuclease has not been characterized. Recently BACCHETTI *et al.*[1] and BRENT[7] have demonstrated in human cell extracts an endonuclease activity that specifically attacks UV-irradiated DNA; its possible role in the proposed excision repair process, however, has not yet been established. The endonucleolytic activity did not require the addition of ATP or divalent ions and was not inhibited by EDTA. These data do not support the first-mentioned interpretation of our results. The coordinated action of the repair enzymes, as postulated in the second explanation, will be achieved if (*a*) the endonuclease is part of a complex also containing the enzymes necessary for the subsequent repair steps, or (*b*) the endonuclease systematically screens the DNA making an incision as soon as a pyrimidine dimer is recognized but is blocked in its progress until the dimer is excised and the gap is closed. In this model not more than one break per incision enzyme molecule will be present per cell at the same time. A similar model for the action of the excision system in *E. coli* has been proposed by SETLOW AND CARRIER[30]. Confirmation of the coordinated repair model requires more specific inhibitors of repair replication or rejoining of breaks that do not inhibit the proposed endonuclease function *per se*.

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REFERENCES

1 BACCHETTI, S., A. VAN DER PLAS AND G. VELDHUISEN, A UV-specific endonucleolytic activity present in human cell extracts, *Biochem. Biophys. Res. Commun.*, 48 (1972) 662–669.
2 BEN-HUR, E., AND R. BEN-ISHAI, DNA repair in ultraviolet light irradiated HeLa cells and its reversible inhibition by hydroxyurea, *Photochem. Photobiol.*, 13 (1971) 337–345.
3 BLAKE, A., AND A. R. PEACOCKE, The interaction of aminoacridines with nucleic acids, *Biopolymers*, 6 (1968) 1225–1253.
4 BOLAND, L., Actinomycin D binding to isolated deoxyribonucleoprotein and intact cells, *Exptl. Cell Res.*, 63 (1970) 171–188.
5 BOOTSMA, D., Changes induced in the first post-irradiation generation cycle of human cells studied by double labelling, *Exptl. Cell Res.*, 38 (1965) 429–431.

6 BOOTSMA, D., M. P. MULDER, F. POT AND J. A. COHEN, Different inherited levels of DNA repair replication in Xeroderma pigmentosum cell strains after exposure to UV light, *Mutation Res.*, 9 (1970) 507–516.
7 BRENT, T. P., A mammalian endonuclease activity which shows specificity towards UV-irradiated DNA, A possible repair enzyme, *Nature New Biology*, 239 (1972) 172–173.
8 CARRIER, W. L., AND R. B. SETLOW, Endonuclease from *Micrococcus luteus* which has activity toward ultraviolet-irradiated deoxyribonucleic acid; Purification and properties, *J. Bacteriol.*, 102 (1970) 178–186.
9 CLEAVER, J. E., Defective repair replication of DNA in Xeroderma pigmentosum, *Nature*, 218 (1968) 652–656.
10 CLEAVER, J. E., Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective, *Proc. Natl. Acad. Sci. (U.S.)*, 63 (1969) 428–435.
11 CLEAVER, J. E., Repair replication of mammalian cell DNA; Effects of compounds that inhibit DNA synthesis or dark repair, *Radiation Res.*, 37 (1969) 334–348.
12 CLEAVER, J. E., Absence of excision of UV-induced cyclobutane dimers in Xeroderma pigmentosum, *Photochem. Photobiol.*, 11 (1970) 547–550.
13 CLEAVER, J. E., DNA repair and radiation sensitivity in human (Xeroderma pigmentosum) cells, *Intern. J. Radiation Biol.*, 18 (1970) 557–565.
14 DEAN, C. J., M. G. ORMEROD, R. W. SERIANNI AND P. ALEXANDER, DNA-strand breakage in cells irradiated with X-rays, *Nature*, 222 (1969) 1042–1044.
15 DOMON, M., AND A. M. RAUTH, Effects of caffeine on ultraviolet-irradiated mouse cells, *Radiation Res.*, 39 (1969) 207–221.
16 DONLON, T., AND A. NORMAN, Kinetics of rejoining of single-strand breaks induced by ionizing radiation in DNA of human lymphocytes, *Mutation Res.*, 13 (1971) 97–107.
17 FALASCHI, A., AND A. KORNBERG, Phleomycin, an inhibitor of DNA polymerase, *Federation Proc.*, 23 (1964) 940–945.
18 GRIGG, G. W., Induction of DNA breakdown and death in *Escherichia coli* by phleomycin, *Mol. Gen. Genet.*, 104 (1969) 1–11.
19 KAPLAN, J. C., S. R. KUSHNER AND L. GROSSMAN, Enzymatic repair of DNA, III. Properties of the UV-endonuclease and UV-exonuclease, *Biochemistry*, 10 (1971) 3315–3324.
20 KLEIJER, W. J., AND D. BOOTSMA, Repair of DNA damaged by UV- and X-irradiation in cultivated normal human, Xeroderma pigmentosum and Bloom cells, *Proc. 1st European Biophys. Congr., Baden, 1971*, Vol. II, pp. 129–133.
21 KLEIJER, W. J., P. H. M. LOHMAN, M. P. MULDER AND D. BOOTSMA, Repair of X-ray damage of cultivated cells from patients having Xeroderma pigmentosum, *Mutation Res.*, 9 (1970) 517–523.
22 LINDAHL, T., AND G. M. EDELMAN, Polynucleotide ligase from myeloid and lymphoid tissues, *Proc. Natl. Acad. Sci. (U.S.)*, 61 (1968) 680–687.
23 LOHMAN, P. H. M., Induction and rejoining of breaks in the deoxyribonucleic acid of human cells irradiated at various phases of the cell cycle, *Mutation Res.*, 6 (1968) 449–458.
24 LOHMAN, P. H. M., O. VOS, C. A. VAN SLUIS AND J. A. COHEN, Chemical protection against breaks in DNA of human and bacterial cells by X-irradiation, *Biochim. Biophys. Acta*, 224 (1970) 339–352.
25 MOSS JR, A. J., G. V. DALRYMPLE, J. L. SANDERS, K. P. WILKINSON AND J. C. NASH, Dinitrophenol inhibits the rejoining of radiation-induced DNA breaks by L-cells, *Biophys. J.*, 11 (1971) 158–174.
26 NAKAYAMA, H., S. OKUBO AND Y. TAKAGI, Repair of ultraviolet-damaged DNA in *Micrococcus lysodeikticus*, I. An endonuclease specific for ultraviolet-irradiated DNA, *Biochim. Biophys. Acta*, 228 (1971) 67–82.
27 NOLL, H., Characterization of macromolecules by constant velocity sedimentation, *Nature*, 215 (1967) 360–363.
28 ORMEROD, M. G., AND U. STEVENS, The rejoining of X-ray-induced strand breaks in the DNA of a murine lymphoma cell (L 5178 Y), *Biochim. Biophys. Acta*, 232 (1971) 72–82.
29 REGAN, J. D., J. E. TROSKO AND W. L. CARRIER, Evidence for excision of ultraviolet-induced pyrimidine dimers from the DNA of human cells *in vitro*, *Biophys. J.*, 8 (1968) 319–325.
30 SETLOW, R. B., AND W. L. CARRIER, The excision of pyrimidine dimers *in vivo* and *in vitro*, in W. J. PEACOCK AND R. B. BROCK, *Replication and Recombination of Genetic Material*, Australian Academy of Science, Canberra, 1968, pp. 134–141.
31 SETLOW, R. B., J. D. REGAN, J. GERMAN AND W. L. CARRIER, Evidence that Xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA, *Proc. Natl. Acad. Sci. (U.S.)*, 64 (1969) 1035–1041.
32 TSUBOI, A., AND T. TERASIMA, Rejoining of single-strand breaks of DNA induced by X-rays in mammalian cells; Effects of metabolic inhibitors, *Mol. Gen. Genet.*, 108 (1970) 117–128.
33 VAN DER SCHANS, G. P., AND A. C. M. VAN DER DRIFT, personal communication.

PAPER III

REPAIR REPLICATION IN HUMAN CELLS STUDIED BY SODIUM IODIDE ISOPYCNIC CENTRIFUGATION OF DNA IN A FIXED ANGLE ROTOR*

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SUMMARY

A sensitive and quantitative method is described for direct measurement of repair replication in UV-irradiated human cells in tissue culture. The method is based on isopycnic centrifugation of repaired DNA in sodium iodide gradients in fixed angle rotors and determination of the amount of DNA by the fluorescence of a DNA-ethydium bromide complex.

INTRODUCTION

Repair replication is considered to be a step in the repair of damaged DNA. This involves the insertion of a small number of new nucleotides into gaps in single strands resulting from the excision of DNA-regions containing the damage.

Sofar equilibrium centrifugation in linear density gradients of cesium chloride has proven to be a suitable method for studying repair replication in DNA. This method, devised by Pettijohn and Hanawalt[9,10] for bacteria was

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modified by Cleaver [2,3,4,5] for use in mammalian cells.

According to Cleaver [3] accurate quantitative analysis of repair replication with CsCl density gradients is less than satisfactory. The relatively small separation between the radioactivity peaks of semiconservatively-synthesized DNA and repaired DNA makes it often necessary to reband the repaired DNA in a separate CsCl gradient. To calculate the specific radioactivity of repaired DNA, the DNA concentration in the gradient has to be measured. However, the relatively low UV absorbancy in the gradient seems to be not proportional to the DNA concentration [4].

Anet *et al.*[1] and De Kloet *et al.*[7] have shown that the separation power for DNA molecules is enhanced with density gradients containing sodium iodide in comparison to CsCl gradients at the same centrifugal field. Furthermore it has been shown that under suitable conditions in high salt solutions the increase in fluorescence arising from the addition of a given concentration of ethydium bromide[a)] (EB) is proportional to the concentration of DNA[7,8].

In view of the above considerations we have attempted to develop a simple and sensitive method for detecting repair replication in the DNA of mammalian cells by means of NaI density gradients supplemented with an optimal concentration of ethydium bromide.

MATERIALS AND METHODS

Cells and culture techniques

T-cells, heteroploid cells of human origin with a generation time of 24-26 h, were cultivated routinely in F12 medium without thymidine. The medium was supplemented with 6% inactivated newborn calf serum and 100 IU penicillin and 0.1 mg streptomycin per ml.

To study repair replication of DNA, 10^6 cells were

a) (ethydium bromide = 2,7-diamino, 9-phenylphenanthridinium 10-ethyl bromide)

inoculated in 10 ml of medium in plastic petri dishes (inner diameter:90 mm) and incubated at 37°. Experiments were conducted with cells in the exponential growth phase.

Labelling of DNA and Irradiation

Cultures were grown in F12 medium containing 1.5 µg/ml 5-bromodeoxyuridine (BUdR) and 10^{-6} M 5-fluorodeoxyuridine (FUdR) for 2 h. Just before exposure to ultraviolet light, the medium was drained carefully from the cultures and the cells were irradiated with ultraviolet light (254 nm). The light source was a Philips TUV lamp (15 Watt), which emitted 9 erg/mm^2/sec at the exposure distance.

After irradiation the cultures were incubated for 3 h in medium containing ^{3}H-BUdR (10 µCi/ml; specific activity: 15.3 Ci/mmol), 10^{-6} M FUdR and 10^{-3} M hydroxyurea (HU). HU was added to suppress semiconservative replication of DNA.

Then the cells were removed from the petri dishes by trypsinisation, washed twice with cold saline, centrifuged and stored as a cell pellet at -90°.

DNA extraction

The frozen cells (app. 2.10^6) were resuspended in 1 ml buffer (0.15 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 9.5, 0.1% sarkosyl (Geigy)) and incubated for 30 min at 60°.

The cell lysate was deproteinized by mixing with an equal volume of chloroform-isoamylalcohol (24:1).

The extraction mixture was centrifuged at 10,000 x g for 10 min at 5° in a Spinco J21 centrifuge and the aqueous layer containing nucleic acids was recovered.

Isopycnic Centrifugation

(a) Sodium Iodide gradients

The nucleic acid extract was made 0.01 M to sodium bisulfite and ethydiumbromide was added to a final concentration of 20 µg/ml. When equilibrium density centrifugation was performed in the Spinco fixed angle rotor 40,

5 ml of the nucleic acid solution was added to 4.650 g NaI (final density, 1.5300 g/cm^3).

Equilibrium density centrifugation in a swinging bucket rotor was performed in the Spinco 50.1 rotor. Each tube contained 2.5 ml nucleic acid solution and 2.325 g NaI (final density, 1.5300 g/cm^3),

Before centrifugation the tubes were filled to the top with mineral oil. The gradients were spun for 60 h at 33,000 rpm and 20° in rotor 40 and 36 h at 38,000 rpm and 20° in the SW 50.1 rotor.

(b) Cesium Chloride gradients

Five ml nucleic acid extract, without sodium bisulfite and ethidiumbromide, was added to 6.426 g CsCl (final density 1.7000 g/cm^3) when centrifugation was performed in the Spinco rotor 40. For centrifugation in the Spinco SW 50.1 rotor at the same final density 2.5 ml of the nucleic acid extract was added to 3.213 g CsCl. Centrifugation was performed as mentioned above for NaI gradients.

Gradient fractionation and radioactivity assay

After centrifugation the tubes were covered with bored-through Lucite caps. The tube inside each cap was connected to a 20 ml syringe filled with mineral oil. The tubes were punctured at the bottom and equal size fractions were collected by pushing the syringe at a constant speed (1 ml/min) with a Braun infusion pump. Immediately after fractionation the refractive index was measured at room temperature by means of an Abbe refractometer. For NaI gradients, densities were calculated from these data according to the empirical computerized relationship: ρ_{20}= 0.473487E+01 x n_{20} - 0.525000E+01 between the density ρ_{20} and the refractive index n_{20}.

The amount of DNA in each fraction was determined by measuring the fluorescence in a Baird-Atomic Spectrofluorometer. The wavelength used for excitation was 520 nm and the fluorescence was measured at 590 nm. Fractions from NaI gradients could be measured directly. However, fractions

from CsCl gradients first have to be supplemented with ethydium bromide (final concentration, 20 µg/ml).

After the fluorescence assay 5 ml 10% trichloroacetic acid (TCA) (0^{o}) and 0.01 M sodium pyrophosphate were added to each fraction. The samples were collected on Whatman GF/C glass fibre paper (diameter:24 mm), washed thoroughly with cold 5% TCA (0^{o}) containing 0.01 M sodium pyrophosphate and then 96% alcohol; the filters were dried for 30 min at 90^{o}. Liquid scintillation fluor (3 ml; 6 g 2,5-diphenyloxazol (PPO) and 0.1 g 2,2' p-phenyleen-bis-(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl POPOP) per liter toluene) was added to the dried filters and the radioactivity in the samples was measured in a Nuclear Chicago mark1 or mark2 liquid scintillation counter.

RESULTS

Determination of the DNA concentration

When solutions of DNA and EB are mixed, a complex is formed with a very marked increase in fluorescence. Both the width and the maximum of the fluorescence emission spectra (λ_{em} = 590 nm) are identical for the free and the bound dye and EB binding occurs at any salt concentration without a change in the number of binding sites [8]. To test the usefullness of the fluorescence of the DNA-EB complex for measuring a given DNA concentration in sodium iodide solutions different amounts of calf thymus DNA were dissolved in either buffer solutions, used for the extraction of T-cell DNA (0.15 M NaCl, 0.01 M EDTA, 0.01 M Tris - pH 9.5), or in the same buffer solution supplemented with 1 g/ml NaI. After excitation at 520 nm the fluorescence intensity at 590 nm of the pure EB solution (I_0; 20 µg/ml) and of the same EB solution supplemented with DNA (I_m) was measured and plotted against the DNA concentration.(Fig. 1). Nearly linear dependence of the fluorescence on DNA concentration was found. The presence of the high concentration of NaI did not influence the fluorescence measurements.

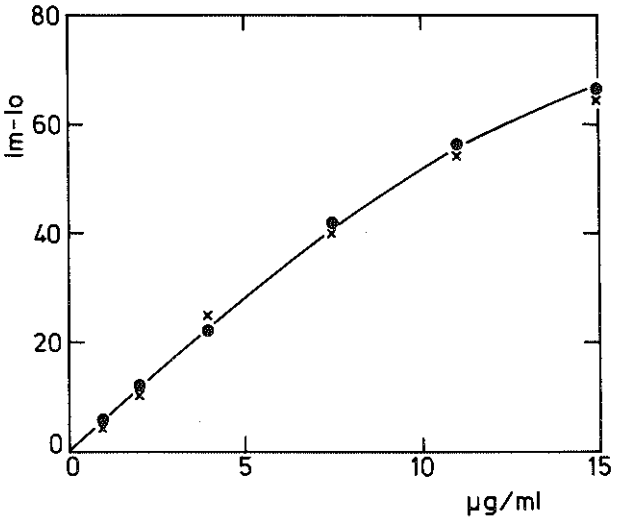

Fig. 1. The fluorescence intensity at 590 nm of the DNA-ethydiumbromide complex as a function of the DNA concentration. Excitation wavelength is 520 nm. (●), DNA dissolved in extraction buffer; (x), DNA dissolved in extraction buffer supplemented with 1 g NaI/ml.

When DNA concentrations in gradient fractions were determined, fluorescence measurements of both gradient and standard DNA solutions were made under identical conditions. The concentration in each gradient fraction was calculated by interpolating a standard curve as shown in Fig.1.

Repair replication after irradiation with ultraviolet light

To study repair replication T-cells were labelled and irradiated with ultraviolet light as described in Materials and Methods. After incubation the cells were harvested and the DNA was extracted. The DNA solution was analysed on NaI density gradients. After fractionation, density, fluorescence and radioactivity measurements were performed.

As shown in Fig. 2. the gradients have an almost linear density over most of the working range [6]. The density of normal DNA in NaI gradients is indicated by the position of the fluorescence peak at approximately 1.525 g/cm^3. T-cell DNA with one strand substituted with BUdR has a

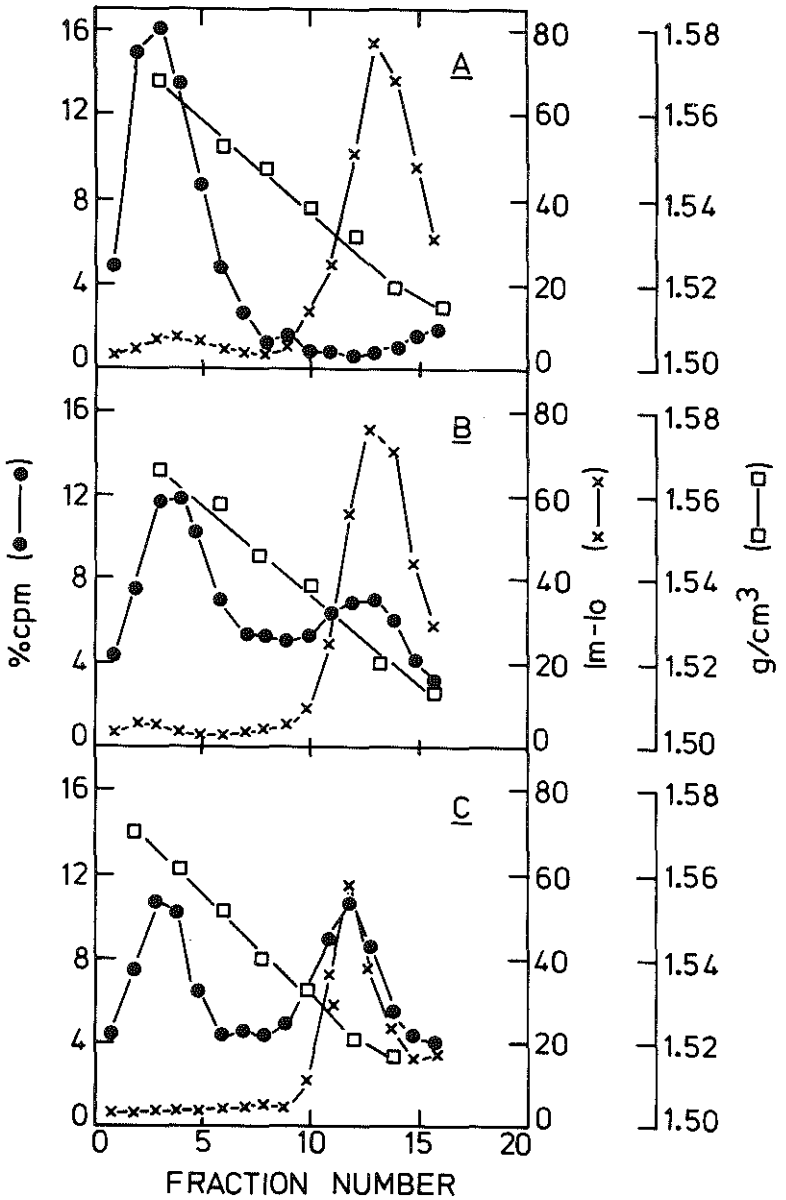

Fig. 2. Relative distribution of the radioactivity and the DNA content after fractionation of T-cell-DNA in NaI isopycnic gradients. Centrifugation 33,000 rpm, 60 h, 20° - Spinco rotor 40.
A: 0 erg/mm^2; B: 100 erg/mm^2, C: 500 erg/mm^2.

density of approximately 1.565 g/cm^3.

The radioactivity and fluorescence profiles in Fig. 2 show the relative distribution of the radioactivity and the DNA content. In samples of unirradiated T-cells practically no radioactivity is found under the fluorescence peak (Fig. 2A). Most of the radioactivity is found in a heavy DNA peak representing DNA formed during semiconservative replication of DNA in the presence of ^{3}H-BUdR, despite the presence of 10^{-3} M HU. Irradiation with 100 erg/mm^2 (Fig. 2B) and 500 erg/mm^2 (Fig. 2C) gives rise to an increase in the amount of radioactivity in a position coinciding with the fluorescence peak. This increased amount of radioactivity in DNA of normal density can be attributed to the occurrence of repair replication in UV-irradiated human cells.

Fractionation of DNA using sodium iodide and cesium chloride gradients

The separation of two types of T-cell DNA, one representing semiconservatively replicated DNA and the other repaired DNA, in NaI gradients was compared with the separation of the same DNA's in CsCl gradients. Also gradients generated in a fixed angle rotor were compared with those formed in a swinging bucket rotor. The NaI gradients and CsCl gradients were collected from the same centrifugation experiment.

The amount of DNA in fractions of both types of gradients was determined by measurement of the fluorescence of the DNA-EB oomplex (see Materials and Methods). The fluorescence, radioactivity and density profiles of the DNA of T-cells irradiated with 200 erg/mm^2 are shown in Fig. 3A and Fig. 3B for NaI and in Fig. 3C and Fig. 3 D for CsCl gradients. The density of repaired DNA, found in the same position as the fluorescence peak, was 1.525 g/cm^3 in NaI gradients and 1.700 g/cm^3 in CsCl gradients.
The DNA labelled by semiconservative replication is identified by the radioactivity peak which does not coincide

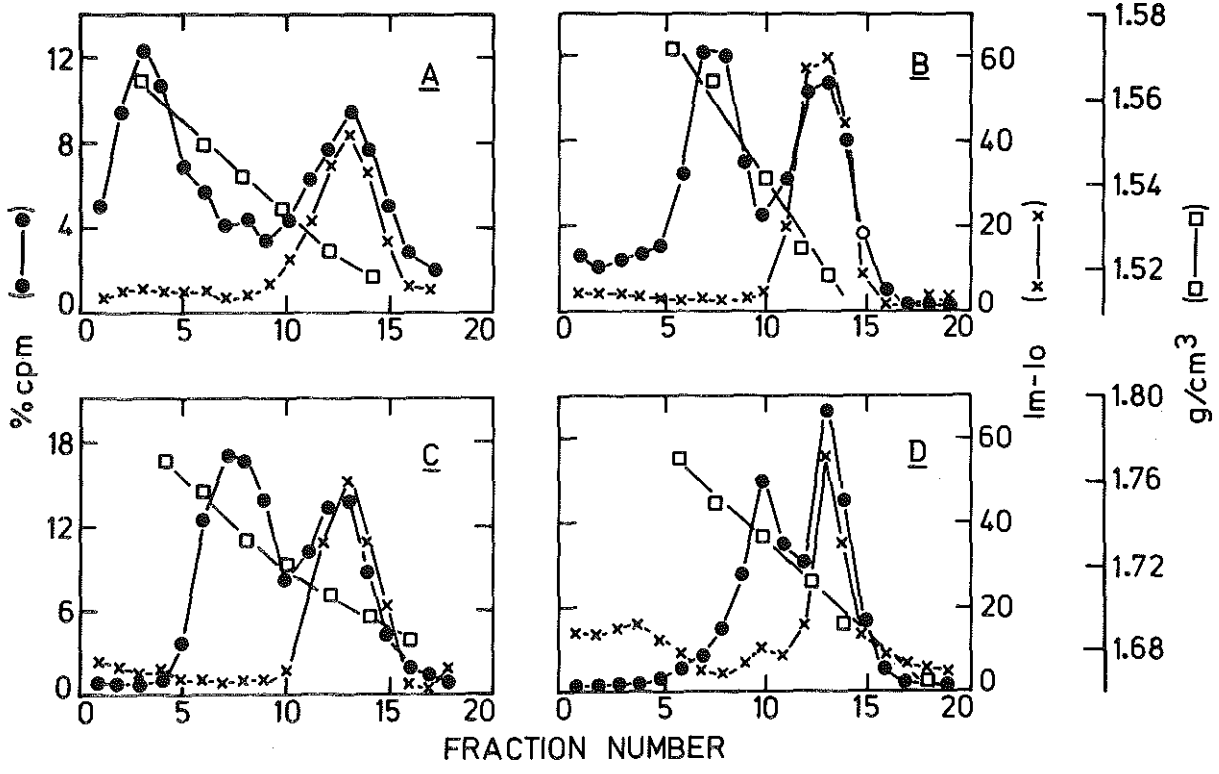

Fig. 3. Fluorescence, radioactivity and density profiles of DNA from T-cells irradiated with 200 erg/mm^2 and fractionated in isopycnic gradients. A: NaI gradient, Spinco rotor 40. B: NaI gradient, Spinco SW 50.1. C: CsCl gradient, Spinco rotor 40. D: CsCl gradient, Spinco 50.1. Centrifugation Spinco rotor 40: 33,000 rpm, 60 h, 20°. Centrifugation Spinco SW 50.1: 38,000 rpm, 36 h, 20°.

with the fluorescence peak. The density of this DNA was 1.565 g/cm^3 in NaI and 1.740 g/cm^3 in CsCl gradients. From the results it can be concluded that for both NaI and CsCl gradients the resolution is better in the gradients centrifuged in the fixed angle rotor (Fig. 3A and 3C). The best resolution was found in the NaI gradient centrifuged in the fixed angle rotor (Fig. 3A). In this gradient the contribution of the DNA peak, labelled by semiconservative replication, to the radioactivity coinciding with the fluorescence peak is minimal.

Specific radioactivity measurements

In Fig. 2 and Fig. 3A, 3B, the distribution of radioactive DNA and of the fluorescent DNA-EB complexes over the NaI gradients is given in arbitrary units. As a quantitative measure of the amount of repair replication in T-cells the specific radioactivity of the DNA in the fluorescence peak was used. The amount of DNA in each fraction was found by interpolating the relative fluorescence to a standard curve as shown in Fig. 1. The radioactivity of each fraction was determined as described in Materials and Methods. According to Cleaver[4,5] in CsCl gradients the specific activity of repaired DNA cannot be determined directly from the radioactivity and transmittance profiles (see Introduction). To demonstrate that the determination of the specific activity of repaired DNA is independent of the amount of DNA present in the NaI gradient, several gradients containing different concentrations of the same DNA preparations were centrifuged. This DNA was extracted from T-cells which have been irradiated with 200 erg/mm^2 and incubated as described in Materials and Methods. The total amount of DNA in the solution was determined by fluorometry. Four gradients with different amounts of DNA were centrifuged in a fixed angle rotor.After fractionation the amount of DNA and the radioactivity in each fraction was measured. The specific radioactivity of the three fractions of the fluorescence peak with maximum radioactivity was calculated. The mean specific

TABLE I

SPECIFIC RADIOACTIVITY OF REPAIRED DNA FROM UV-IRRADIATED T-CELLS AS A FUNCTION OF THE DNA CONCENTRATION IN SODIUM IODIDE DENSITY GRADIENTS

TOTAL AMOUNT OF DNA PER GRADIENT (μg)	*NUMBER OF FRACTIONS*	*MEAN SPECIFIC[b] RADIOACTIVITY cpm/μg DNA*	*SEM[a]*
18.4	3	6251	330
12.3	3	6346	308
5.2	3	6417	271
1.8	3	6379	216

a) Standard error of the mean

b) The correlation coefficient between the total amount of DNA and the specific radioactivity of three major fractions in the fluorescent peak does not differ from zero significantly.

activity as a function of the total DNA content in each gradient is shown in Table 1.

From the results it can be concluded that the specific radioactivity is independent of the DNA concentration; therefore it is possible to calculate directly the specific radioactivity of repaired DNA from the radioactivity and fluorescence profiles in NaI gradients.

Repair replication after various UV-doses

Measurements of the amount of repair replication in UV-irradiated T-cells in relation to radiation dose were made by calculating the specific radioactivity of the DNA in the fluorescence peak in density gradients centrifuged in the fixed angle rotor 40. In Fig. 4 the results of two separate experiments with UV-irradiated T-cells are shown. For comparison in one of the experiments DNA preparations

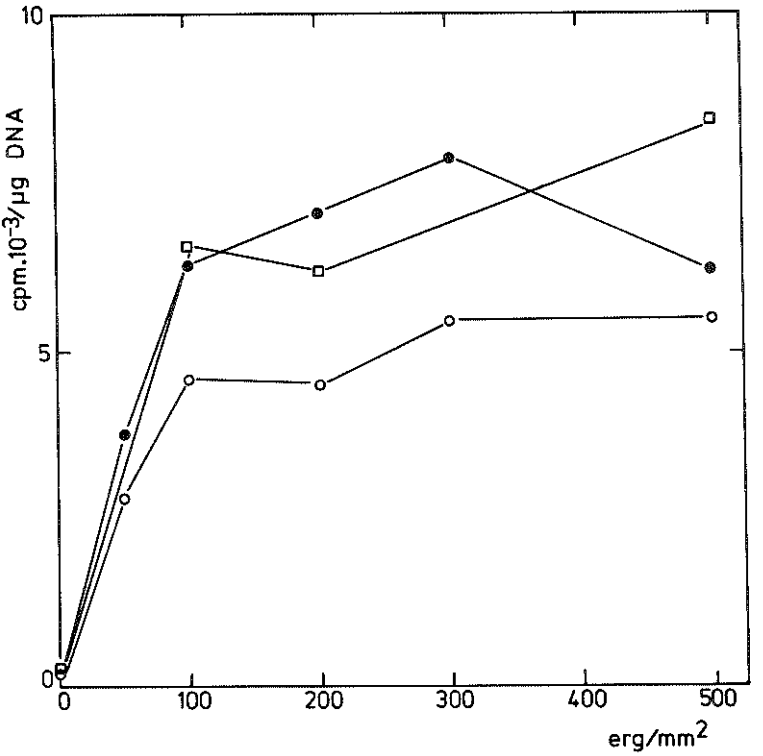

Fig. 4. Specific radioactivity of T-cell-DNA in the fluorescent peak in NaI and CsCl isopycnic gradients as a function of the ultraviolet light dose.
(●), (□) NaI gradients; (○) CsCl gradients. Centrifugation: Spinco rotor 40, 33,000 rpm, 60 h, 20°.

were centrifuged simultaneously in NaI and in CsCl gradients. The data in Fig.4 are the mean specific radioactivity of the three fractions of the fluorescence peak with maximum radioactivity.

The dose-response curves derived from the NaI gradients and the CsCl gradients have the same shape and demonstrate that under the conditions of the experiment the repair system operates at its maximal capacity after irradiation with about 100 erg/mm^2.

DISCUSSION

Anet *et al.*[1] first used NaI density gradients to separate DNA molecules of different densities and showed that the resolution was better than in CsCl gradients. The experiments described in this paper show that the use of NaI density gradients for the study of repair replication in UV-irradiated human cells provides several advantages over the use of CsCl gradients. The separation of semiconservatively replicated DNA, of hybrid density, and repaired DNA of normal density was much better in NaI than in CsCl gradients. In agreement with Falm *et al.*[6] we found the resolution in the fixed-angle rotor superior to that in the swinging-bucket rotor. The higher resolution led to a marked reduction of the contribution of radioactivity in the peak, representing semiconservatively replicated DNA, to the radioactivity present in the peak of repaired DNA. Advantages additional to the optimal resolution in NaI gradients centrifuged in fixed-angle rotors are the low cost of NaI and the larger tube capacities of these rotors in comparison to swinging-bucket rotors. The latter advantage compensates for the longer centrifugation time necessary to attain equilibrium in the fixed-angle rotor.

Another improvement for the quantitative determination of repair replication was the direct measurement of the amount of DNA in the fractions of the gradient, based on the fluorescence properties of DNA-ethidium bromide complex.

LePecq *et al.*[8] have shown that the fluorescence increase, caused by the specific binding of EB to double-stranded regions of DNA, is proportional to the concentration of native DNA. This method to determine DNA concentrations could be applied directly to the fractions of NaI gradients, because the fluorescence was not affected by the high NaI concentration. The presence of EB in the NaI gradient (in contrast to CsCl gradients) does not influence the boyant density of the DNA[1]; therefore EB could be added

to the NaI solution before centrifugation. It was demonstrated that the quantitative measurement of fluorescence provided an adequate and sensitive method to determine the amount of DNA in each fraction. The specific activity of repaired DNA, calculated from fluorescence and radioactivity data, appeared to be independent of the total amount of DNA present in the gradient.

The increased resolution in NaI gradients in combination with the sensitive fluorimetric DNA determination provided an accurate method to quantitate repair replication in human cells, without the need of rebanding the repaired DNA in an additional gradient, as is often necessary with CsCl gradients.

REFERENCES

1. ANET, R., D.R. STRAYER, Sodium iodide density gradients for the preparative buoyant density separations of DNA mixtures, *Biochem. Biophys. Res. Comm.*, 37 (1969) 52-58.
2. CLEAVER, J.E., Defective repair replication of DNA in Xeroderma pigmentosum, *Nature*, 218 (1968) 652-656.
3. CLEAVER, J.E., Repair replication of mammalian cell DNA; Effects of compounds that inhibit DNA synthesis or dark repair, *Radiat. Res.*, 37 (1969) 334-348.
4. CLEAVER, J.E., DNA repair and radiation sensitivity in human (Xeroderma pigmentosum) cells, *Int. J. Radiat. Biol.*, 18 (1970) 557-565.
5. CLEAVER, J,E., Repair replication in Chinesehamster cells after damage from ultraviolet light, *Photochem. Photobiol.*, 12 (1970) 17-28.
6. FLAMM, W.G., H.E. BOND, H.E. BURR, Density-gradient centrifugation of DNA in a fixed-angle rotor. A higher order of resolution, *Biochim. Biophys. Acta*, 129 (1966) 310-319.
7. KLOET, S.R. de, B.A.G. ANDREAN, Buoyant density gradient centrifugation of RNA and DNA in alkali iodide solutions, *Biochim. Biophys. Acta*, 247 (1971) 519-527.
8. LEPECQ, J.B., C. PAOLETTI, A new fluorometric method for RNA and DNA determination, *Anal. Biochem.*, 17 (1966) 100-107.
9. PETTIJOHN, D.E., P.C. HANAWALT, Deoxyribonucleic acid replication in bacteria following ultraviolet irradiation, *Biochim. Biophys. Acta*, 72 (1963) 127-129.
10. PETTIJOHN, D.E., P.C. HANAWALT, Evidence for repair replication of ultraviolet damaged DNA in bacteria, *J. Mol. Biol.*, 9 (1964) 395-410.

PAPER IV

REPAIR REPLICATION IN HETEROKARYONS DERIVED FROM DIFFERENT REPAIR-DEFICIENT XERODERMA PIGMENTOSUM STRAINS*

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SUMMARY

Repair replication was studied in UV-irradiated cell populations obtained after fusion of cell strains originating from different xeroderma pigmentosum (XP) patients. The capacity to perform repair replication appeared to be restored completely in multinucleate heterokaryons resulting from fusion between a classic XP-strain and a de Sanctis-Cacchione (DSC) strain. In cell populations obtained by fusion of either two different classic XP-strains or two different DSC-strains no repair replication was observed.

These results, obtained with the technique of density labelling and isopycnic centrifugation of DNA, confirm our previously reported results of autoradiographic studies of unscheduled DNA synthesis. The occurrence of complementation between a classic XP-strain and a DSC-strain indicates that the defect in the two forms of the disease is caused by different mutations.

Abbreviations: BUdR, 5-bromodeoxyuridine; DSC, De Sanctis-Cacchione; FUdR, 5-fluorodeoxyuridine; TCA, trichloroacetic acid; XP, xeroderma pigmentosum; HAU, hemagglutinating units.

* Mutation Research, 19 (1973) 237-243.

INTRODUCTION

Two clinically different forms of the hereditary disease xeroderma pigmentosum (XP) have been recognized: the classic form of XP and the De Sanctis-Cacchione (DSC) syndrome[9]. Both forms of XP are characterized by hypersensitivity of the skin to sunlight, leading to the development of severe skin lesions. DSC-patients are distinguished from patients with the classic form of XP by the involvement of neurological complications.

Studies on skin fibroblasts[1,3] and lymphocytes[2] from patients of each XP-type have indicated an impaired ability of these cells to perform repair DNA synthesis after UV-irradiation, probably due to a defective initial step in excision repair [4,7,10]. In cell strains from different patients various levels of repair DNA synthesis ranging from 0-100% of that in normal human cells were found[1]. Cell strains from genetically related patients performed similar amounts of repair DNA synthesis[1]. These findings demonstrated that the degree of reduction of the repair activity is genetically determined.

The different characteristic levels of repair activity in the XP-strains and the occurrence of two clinically distinct forms of XP suggest a genetic heterogeneity in this disease. This hypothesis was supported by autoradiographic studies of repair DNA synthesis in heterokaryons obtained by fusion of different XP-strains, described in a previous paper[11]. In binucleate heterokaryons derived from two repair-deficient XP-strains, one of the classic type and the other of the DSC type, the capacity to perform unscheduled DNA synthesis appeared to be restored. In this report the complementation between a classic XP- and a DSC-strain is confirmed by the demonstration of repair replication in the fused cells, using the technique of BUdR density labelling and isopycnic centrifugation of DNA[8].

MATERIALS AND METHODS

Cell strains and culture techniques

The cell strains used were the following primary fibroblast strains: AH, originating from a normal individual; XP4[I] and XP16[II], from two severe classic XP-patients; and two strains from DSC-patients, XP24 (provided by Dr. Cleaver and designed by him XP17) and XP25 (the biopsy was provided by Dr. Der Kaloustian). Cultures were grown in F12 medium supplemented with penicillin (100 I.U./ml), streptomycin (0.1 mg/ml) and 15% foetal calf serum.

Fusion procedure

The fusions were performed according to the procedure of Harris et al.[5]. Cells of two different XP-strains were mixed in a ratio of 1:1 at a final concentration of 2×10^6 cells per ml. β-Propiolactone inactivated Sendai virus[6] was added (final titer 500 HAU per ml). This mixture was kept during 4 min at 4° and after shaking at room temperature incubated for 20 min at 37°. After fusion the cells were diluted and plated in plastic petri dishes (Greiner, diameter 9 or 14 cm for density gradient experiments or Falcon, diameter 3.5 cm, containing a coverslip for autoradiography).

Irradiation and labelling of the DNA

UV-irradiation (254 nm,) of washed and drained cultures was performed with a Philips TUV lamp (15 W) at 7.5 erg/mm^2/sec, two days after fusion.

The cultures to be analysed in density gradients were incubated in medium containing BUdR (1.5 µg/ml) and FUdR (10^{-6}M) for 2 h before irradiation, and ^{3}H-BUdR (10 µCi/ml; spec.act. 15.3 Ci/mmole), FUdR (10^{-6}M) and hydroxyurea (10^{-3}M) for 2 h after irradiation. The cultures to be processed for autoradiography were incubated during 1 h before and 2 h after irradiation in medium containing ^{3}H-thymidine (10 µCi/ml;spec.act. 2 Ci/mmole).

Extraction and isopycnic centrifugation of DNA

Approximately $2x10^6$ control cells and cells of the fused XP-populations (a number equivalent to $2x10^6$ cells in the mononucleate situation before fusion) were collected by scraping them from the dishes, washed twice in cold saline, centrifuged and stored as cell pellets at -70°. The frozen cells were resuspended in 1 ml buffer (0.15 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 9.5, 0.1% sarkosyl (Geigy)) and incubated for 30 min at 60°. The DNA was extracted by shaking with an equal volume of chloroform/isoamylalcohol (24:1).

Five ml of a solution containing the DNA extract, 0.01 M sodiumbisulfite and 20 µg/ml ethidiumbromide was added to 4.650 g NaI (final density 1.5300 g/cm^3) in tubes which were then filled to the top with mineral oil. The tubes were centrifuged in a Spinco fixed angle rotor (40) for 60 h at 33,000 rpm and 20°.

The technique of isopycnic centrifugation of DNA in NaI gradients has been described and discussed in more detail in a separate paper[8].

Fractionation, DNA concentration and radioactivity assays

The gradients were sampled from the bottom in 15-17 fractions of 0.3 ml. Fluorescence at 590 nm (excitation wavelength : 520 nm) was measured in each fraction using a Baird Atomic spectrofluorometer. The amount of DNA was determined by comparison with a calibration curve made with a solution containing NaI, sodiumbisulfite and ethidiumbromide in the same concentrations as in the gradients and with various known concentrations of DNA[8]. The DNA in each fraction was precipitated with 10% TCA/0.01 M sodium pyrophosphate (0°), collected on Whatman GF/C glass fibre paper and washed with respectively 5% TCA/0.01 M sodium pyrophosphate and 96% ethyl alcohol. The radioactivity in the dried filters was counted in toluene/PPO (6g/l)/POPOP (0.1 g/l) in a liquid scintillation counter (Nuclear Chicago, mark 2).

Autoradiography

Autoradiographs of the cultures grown on coverslips were made as described earlier[1].

RESULTS AND DISCUSSION

Repair replication was studied in cell populations resulting from fusions between a classic XP-strain and a DSC-strain (XP4/XP25), between two genetically unrelated classic XP-strains (XP4/XP16) and between two unrelated DSC-strains (XP24/XP25).

In the population of cells obtained after fusion of two different strains three main classes of cells may be expected, namely: unfused parental cells, fused cells containing nuclei of only one of the parental strains (homokaryons) and fused cells with nuclei of both parental strains (heterokaryons). Complementation and therefore also repair replication may be expected only in heterokaryons. In order to promote a maximal amount of repair replication, fusion conditions were applied that gave a high percentage of multinucleate cells and consequently also a high percentage of heterokaryons.

Fig.1 shows a detail of an autoradiographic preparation of an XP4/XP25 population after UV-irradiation and labelling with ^{3}H-thymidine. Part of the binucleate cells and most of the multinucleate cells were weakly labelled, whereas a very small number of grains was found over all mononucleate cells (except for heavily labelled S-phase cells). In three experiments 50-70% of all nucleic were weakly labelled (i.e. 8-50 grains per nucleus).

Cell populations from the same fusions as used for autoradiography were analysed for repair replication. The profiles in Fig. 2 show the distribution in the NaI gradient of DNA from unirradiated and irradiated cell populations after fusion (XP4/XP25). The distribution of DNA, as measured by the fluorescence of the ethidiumbromide-DNA

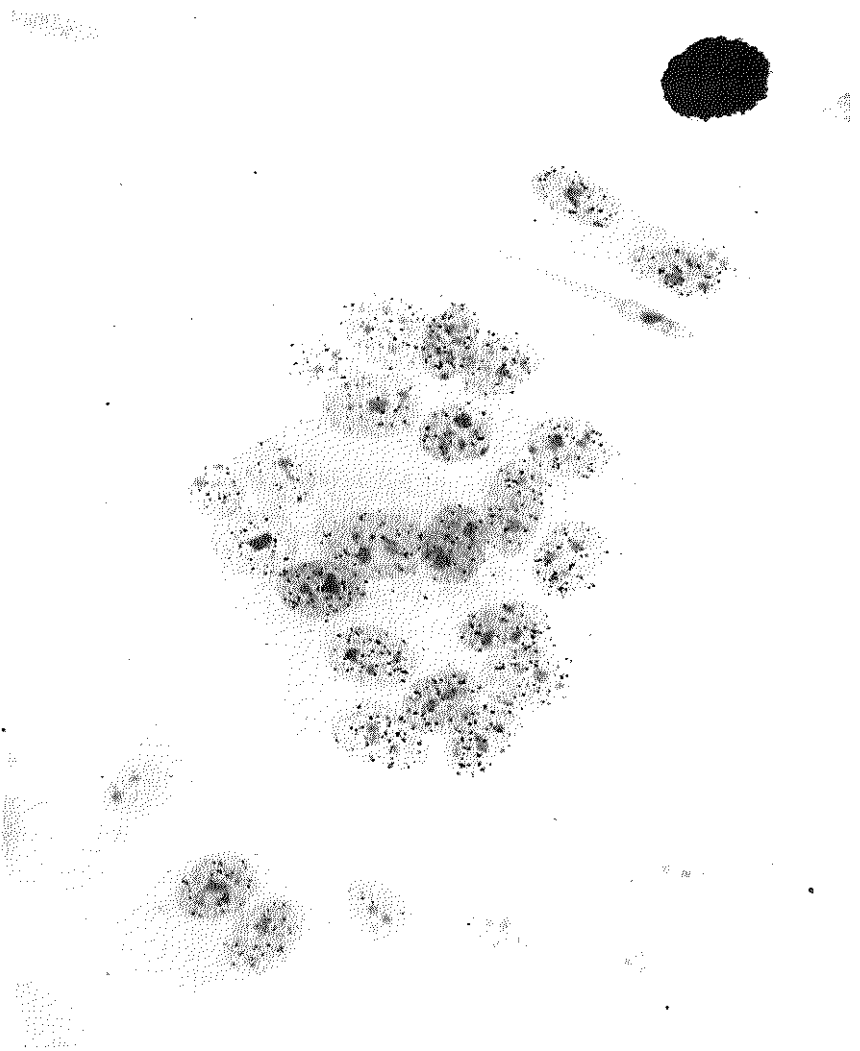

Fig. 1. Autoradiograph of a cell population after fusion (XP4/XP25). Two days after fusion the cells were irradiated with 100 erg/mm^2 UV-light. ^{3}H-thymidine labelling was performed 1 h before and 2 h after UV-irradiation. Autoradiographic exposure time: 1 week. The large multinucleate cell, the trinucleate and the binucleate cell shown are labelled. The unfused XP-cells are not labelled except one heavily labelled S-phase cell.

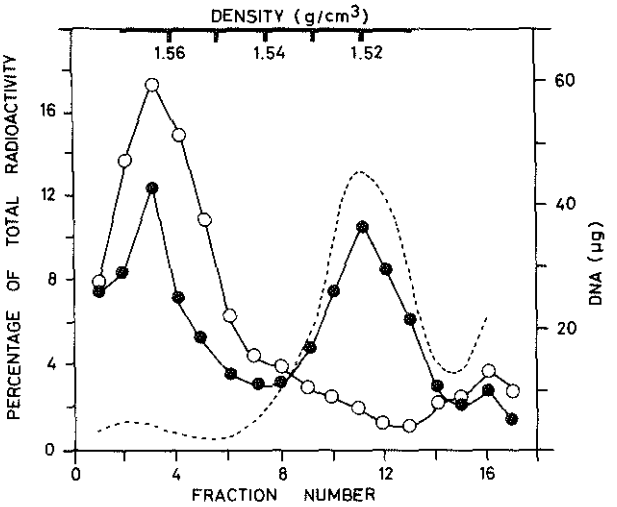

Fig. 2. NaI isopycnic gradients of DNA from cells of an XP4/XP25 population. Two days after fusion the cells were grown for 2 h in BUdR, irradiated with 100 erg/mm^2 UV-light (●) or not irradiated (O), and labelled with ^{3}H-BUdR. The DNA-distribution as measured by fluorescence (- - -) is shown only for irradiated cells because the profile for unirradiated cells was similar. The total amount of radioactivity in the gradient for irradiated cells was 44,000 cpm.

complex shows one peak at the normal density for DNA (app. 1.522 g/cm^3 in NaI). The radioactivity profiles obtained for both unirradiated and irradiated cells show a peak at a higher density (1.565 g/cm^3) representing semiconservatively replicated DNA that has acquired a hybrid density by BUdR substitution in one of the strands. In the case of irradiated cells there is an additional peak coinciding with the peak in the distribution of the total amount of DNA. The radioactivity in this peak at the normal density of DNA demonstrates that ^{3}H-BUdR has been incorporated in the DNA dispersively and indicates repair replication. A quantitative measure for repair replication is given by the specific radioactivity (cpm/µg DNA) of the DNA of normal density. The average specific radioactivity was calculated from the three top fractions of the peak of repaired DNA. The standard deviation of the specific radioactivity was 10% in cont-

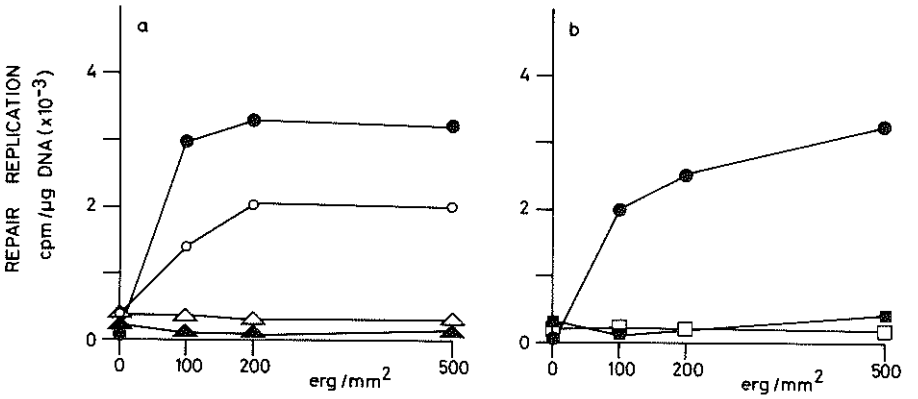

Fig. 3. Repair replication as a function of UV-dose, expressed as specific radioactivity (cpm/µg DNA) of a normal human cell strain (AH) (●) and cell populations obtained after fusion; (a) XP4/XP25 (○), XP4/XP4 (△) and XP25/XP25 (▲); (b) XP4/XP16 (□) and XP24/XP25 (■).

rol experiments in which separate normal human cell cultures were irradiated (100 erg/mm^2), labelled and analysed simultaneously and under identical conditions.

Fig. 3a presents the results of an experiment in which repair replication in the XP4/XP25 population was compared with that in a normal human strain (AH) and two populations of fused parental cells (XP4/XP4 and XP25/XP25) which served as controls. The level of repair replication in the XP4/XP 25 population appeared to be 61% of the level in normal cells, whereas in the XP4/XP4 and XP25/XP25 populations only very low levels were found. In the populations obtained after fusion of either two classic XP-strains (XP4/XP16) or two DSC-strains (XP24/XP25) no repair activity was detected (Fig. 3b).

It is evident from these results that the repair capacity has been restored by complementation in the XP4/XP25 population. Since only a fraction of all nuclei, namely those in heterokaryons XP4/XP25, will have contributed to the measured amount of repair replication, the repair activity in these nuclei relative to normal (AH) nuclei will be higher than the 61% found for the whole population. The

fraction of repair-positive nuclei in the present experiment was obtained by addition of two distinct fractions of nuclei, counted in the autoradiographic preparation: a) a large fraction (61 ± 2%) consisting of the repair-positive nuclei in G_1 or G_2 - phase, recognized as weakly labelled nuclei, and b) a small fraction (2%) consisting of the probably repair-positive S-phase nuclei, situated in multinucleate cells (12% of all nuclei were in S-phase; 18% of these heavily labelled nuclei were found in multinucleate cells). The level of repair replication in repair-positive nuclei relative to that in normal cells (AH) is obtained by dividing the level found for the whole population (61%) by the fraction of repair-positive nuclei (a+b), i.e. 61/63 x 100% = 97%. It is suggested therefore that in those cells where complementation occurs, the repair capacity is restored to the normal human cell level.

The restoration of the repair replication capacity after fusion of a classic XP- and a DSC-cell strain indicates that in the two forms of XP, different mutations are responsible for the defect. These results are in agreement with our earlier findings on UV-induced ^{3}H-thymidine incorporation occurring in classic XP/DSC heterokaryons[11]. The dispersive character of the UV-induced DNA synthesis in these heterokaryons, shown in the present report, confirms that the incorporation of ^{3}H-thymidine represents repair DNA synthesis.

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REFERENCES

1. BOOTSMA, D., M.P. MULDER, F. POT AND J.A. COHEN, Different inherited levels of DNA repair replication in xeroderma pigmentosum cell strains after exposure to ultraviolet irradiation, *Mutation Res.*, 9 (1970) 507-516.
2. BURK, P.G., M.A. LUTZNER, D.D. CLARKE AND J.H. ROBBINS, Ultraviolet-stimulated thymidine incorporation in xeroderma pigmentosum lymphocytes, *J. Lab. Clin. Med.*, 77 (1971) 759-767.
3. CLEAVER, J.E., Defective repair replication of DNA in xeroderma pigmentosum, *Nature*, 218 (1968) 652-656.
4. CLEAVER, J.E., Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective, *Proc. Natl. Acad. Sci. (U.S.)*, 63 (1969) 428-435.
5. HARRIS, H. AND J.F. WATKINS, Hybrid cells derived from mouse and man. Artificial heterokaryons of mammalian cells from different species, *Nature*, 205 (1965) 640-646.
6. KLEBE, R.J., T.R.CHEN AND F.H. RUDDLE, Controlled production of proliferating somatic cell hybrids, *J. Cell Biol.*, 45 (1970) 74-82.
7. KLEIJER, W.J., P.H.M. LOHMAN, M.P. MULDER AND D. BOOTSMA, Repair of X-ray damage in the DNA of cultivated cells from patients having xeroderma pigmentosum, *Mutation Res.*, 9 (1970) 517-523.
8. LOHMAN, P.H.M., W.J. KLEIJER, M.L. SLUYTER AND I.A.A. MATTHIJS, Repair replication in human cells studied by sodium iodide isopycnic centrifugation of DNA in a fixed angle rotor, *Anal. Biochem.*, in press.
9. REED, W.B., B. LANDING, G. SUGARMAN, J.E. CLEAVER AND J. MELNYK, Xeroderma pigmentosum: clinical and laboratory investigations of its basic defect, *J. Am. Med. Ass.*, 207, (1969) 2073-2079.
10. SETLOW, R.B., J.D. REGAN, J. GERMAN AND W.L. CARRIER, Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA, *Proc. Natl. Acad. Sci. (U.S.)*, 64 (1969) 1035-1041.
11. DE WEERD-KASTELEIN, E.A., W. KEIJZER AND D. BOOTSMA, Genetic heterogeneity of xeroderma pigmentosum demonstrated by somatic cell hybridization, *Nature New Biol.*, 238 (1972) 80-83.

PAPER V

UV-INDUCED DNA REPAIR SYNTHESIS IN CELLS OF PATIENTS WITH DIFFERENT FORMS OF XERODERMA PIGMENTOSUM AND OF HETEROZYGOTES*

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SUMMARY

UV-induced DNA repair synthesis, as measured by autoradiography as well as by isopycnic centrifugation methods, was studied in a large number of cell strains from patients with the classic form of xeroderma pigmentosum (XP) or the De Sanctis-Cacchione syndrome (DSC) and several of their heterozygous parents. On the basis of the kinetics of repair synthesis in the cultured skin fibroblasts we can recognize four distinct groups of XP patients: (1) Classic XP patients with low residual repair capacities, (2) Classic XP patients with intermediate, but dose-dependent, levels of repair synthesis relative to the normal level, (3) Patients, diagnosed as having classic XP, with a normal or only slightly reduced repair capacity, (4) DSC patients with a complete deficiency of repair synthesis. Complementation studies reported elsewhere have shown that different mutations are responsible for the defect in at least three of these groups. Cell strains of each of the four XP types were able to rejoin single-strand DNA-breaks induced by

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X-rays. Most of the cell strains derived from heterozygotes showed normal repair activities; however in some cases (the parents of DSC patients) a significant reduction of the level of repair synthesis was found.

INTRODUCTION

The biological significance of an excision repair process in mammalian cells has been demonstrated in studies of DNA repair in cells from patients having the autosomal recessive disease xeroderma pigmentosum (XP). The UV-sensitive cells [7,12], originating from XP patients have a reduced capacity to perform DNA repair synthesis after UV-irradiation [5,1,4] and are unable to excise pyrimidine dimers from their DNA [26,10,15].

Several observations have indicated that the genetic basis of XP is not similar in all patients. Two clinically distinct forms of this disease are known: the classic type of XP and the De Sanctis-Cacchione syndrome (DSC)[23]. Both forms are characterized by hypersensitivity of the skin of the patients to UV-light, leading to severe skin lesions in sun-exposed regions of the body; DSC patients in addition suffer from neurological complications. In previous autoradiographic studies Bootsma et al.[1] found various levels of unscheduled DNA synthesis (UDS) in a number of classic XP strains but similar levels in genetically related patients. Genetic evidence for the involvement of different mutations was obtained from complementation studies by De Weerd-Kastelein et al. [27,28,18]. Complementation, leading to the restoration of the capacity to perform DNA repair synthesis, was demonstrated first in heterokaryons obtained after fusion of classic XP and DSC cells. Similarly we have recently found evidence for the presence of two

Abbreviations: XP, xeroderma pigmentosum; DSC, De Sanctis-Cacchione; UDS, unscheduled DNA synthesis; BUdR, 5-bromodeoxyuridine; FUdR, 5-fluorodeoxyuridine.

complementation groups in classic XP (De Weerd-Kastelein, unpublished results).

From biochemical data it has been suggested that classic XP as well as DSC cells do not perform an initial step of the repair process [6,26,17]. A further biochemical characterization of XP cell strains is required to reveal the defective enzyme function in each of the distinct XP forms. For that purpose we have compared the kinetics of UDS (by autoradiography) and repair replication (by density gradient analysis) in normal human cells and in cell strains from a large number of XP patients and several of their heterozygous parents. The group of patients includes cases of classic XP with clinical symptoms varying from mild to severe and cases of the DSC syndrome.

MATERIALS

(a) Cell strains and culture techniques

Fibroblast cultures were started from skin biopsies in Carrel flasks using F12 medium supplemented with 20% foetal calf serum. Before subculturing in tissue culture bottles, the serum concentration was changed to 15% foetal calf serum. HeLa cells and T cells (established human kidney cell line) used in the excision experiments were grown in F12 medium with 10% newborn calf serum. For UDS experiments cells were seeded in small petri dishes (Falcon, diameter: 3.5 cm) containing a coverslip, whereas for repair replication and excision experiments large petri dishes (Greiner, diameter: 9 cm) were used.

Details on the XP cell strains are presented in Table I, II and III. The designations used in this paper for XP cell strains anticipate on a proposal by Cleaver and Bootsma for the standardization of the nomenclature for XP cell strains. All strains from individuals who are clinically diagnosed as XP patients are called XP (including classic XP, DSC and variant patients with a normal repair ability). The cell strains from heterozygotes are called

XPH. The strains are further characterized by a serial number or by two letters (e.g. the initials of the patient) given in the institute where the cells have been brought into culture and by two letters which denote the city where this institute is situated (e.g. RO for Rotterdam, SF for San Francisco, etc.).

(b) Irradiation

UV-irradiation (254 nm) of the washed and drained cultures was performed with a Philips TUV lamp (15 W) at either 7.5 or 9 erg/mm^2/sec. X-irradiation conditions were as described previously [17].

(c) Unscheduled DNA synthesis

The cultures were labelled with ^{3}H-thymidine (10 µCi/ml; 2.0 Ci/mmole) for 1 h before UV-irradiation in order to label cells in S-phase and for 2 h after irradiation. Fixation of the coverslip cultures, autoradiography, staining and the analysis of the autoradiograms were performed as described previously [1].

(d) Repair replication

The cells were incubated in medium containing BUdR (1.5 µg/ml) and FUdR (10^{-6}M) for 2 h before UV-irradiation. After irradiation incubation was continued in medium containing ^{3}H-thymidine (10 µCi/ml; 20 Ci/mmole, BUdR (1.5 µg/ml), FUdR (10^{-6}M) and hydroxyurea (10^{-3}M) for 2 h (or other periods indicated under Results). After the cells were harvested, (1.10^6-2.10^6 for each experimental point), the DNA was isolated and analysed by sodium iodide density gradient centrifugation as previously described [20].

(e) Excision of pyrimidine dimers

Cells, grown in petri dishes, were labelled for 20 h with ^{3}H-thymidine (2.5 µCi/ml; 26 Ci/mmole). After post-UV-incubation periods of 0,8 and 24 h the cells were collected and washed. DNA preparations, free of protein and

containing relatively low amounts of RNA, were obtained by the phenol extraction procedure described by Kirby and Cook [14]. The DNA was hydrolysed in formic acid and the radioactive products, thymine and thymine-containing pyrimidine dimers were separated by two-dimensional paper chromatography as described by Setlow and Carrier [25]. The pyrimidine dimer content of the DNA was determined by counting the radioactivity in the thymine and the dimer regions of the chromatogram, and was expressed as the per cent radioactivity in dimers of the total radioactivity ($\hat{X}T/T\%$). The amount of radioactivity recovered from the dimer regions of each chromatogram was 400-1200 cpm.

(f) Rejoining of X-ray induced single-strand breaks

The numbers of single-strand breaks in the DNA of X-irradiated cells were calculated from the molecular weights of the DNA as determined by sedimentation in alkaline sucrose gradients. The conditions used for cell-lysis and centrifugation of the DNA were as described earlier [17].

RESULTS

(a) The time course of repair replication in UV-irradiated normal human cells

Fig. 1 shows an example of the separation in NaI-density gradients of repaired DNA with a normal density (Fig. 1b; fractions 10-15) from semi-conservatively synthesized DNA with a higher density (fractions 1-5). The peak representing repaired DNA is found after UV-irradiation and labelling of normal cells as described in Materials and Methods, but is absent if the cells used are not irradiated (Fig. 1a). A quantitative measure for the amount of repair replication is given by the specific radioactivity of repaired DNA (cpm/µg DNA); this value was calculated from the radioactivity and the amount of DNA (as measured by fluorescence of the ethidiumbromide-DNA complex [20]) in the three top fractions of the peak containing the repaired DNA.

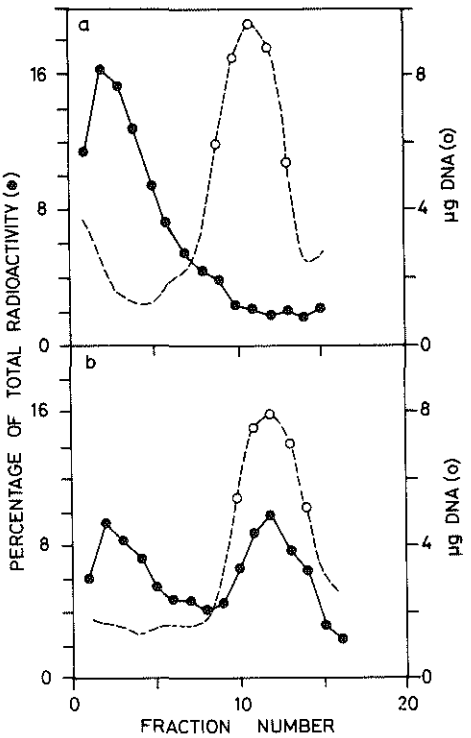

Fig. 1. Repair replication in human cells demonstrated with the NaI isopycnic centrifugation technique. The cells were grown for 2 h in medium containing BUdR and FUdR before UV-irradiation. After irradiation incubation was continued for 2 h in medium containing ^{3}H-thymidine, BUdR, FUdR and hydroxyurea. Distributions in NaI isopycnic gradients of the total amount of DNA (O) and of tritium-labelled DNA (●) from normal human cells (AH). Total amount of radioactivity per gradient: 2 - 3 x 10^{4}cpm. (a) 0 erg/mm^{2}; (b) 100 erg/mm^{2}.

The standard deviation of specific radioactivity measurements was 10% in control experiments in which different cell-samples were irradiated, labelled and analysed simultaneously under identical conditions.

The amounts of repair replication occurring in normal cells following UV-doses of 100, 200 and 500 erg/mm^{2} are presented in Fig. 2. The repair process seems to be completed at about 8 h following a dose of 100 erg/mm^{2}, whereas

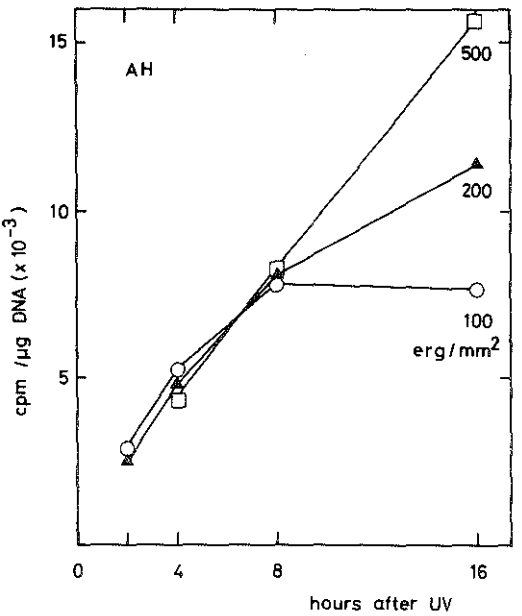

Fig. 2. The amount of repair replication as a function of time after UV-irradiation in normal human cells (AH) determined by NaI isopycnic gradient analysis.

after 200 erg/mm^2 a considerable amount of repair takes place between 8 and 16 h. After 500 erg/mm^2 the amount of repair replication increases almost linearly with time and suggests that the repair replication rate remains constant for at least 16 h.

(b) Excision of pyrimidine dimers

The excision of pyrimidine dimers from the DNA was studied by the determination of the radioactivity in dimers ($\widehat{TT}$ and $\widehat{CT}$) as a percentage of the total radioactivity in the DNA of cells at various time-intervals after UV-irradiation (200 erg/mm^2). A decrease in the dimer content ($\widehat{XT}$/T%) of DNA of about 25% after 8 h and 50% after 24 h post-irradiation incubation was found in experiments with T cells, HeLa cells and normal human skin fibroblasts (Fig.3).

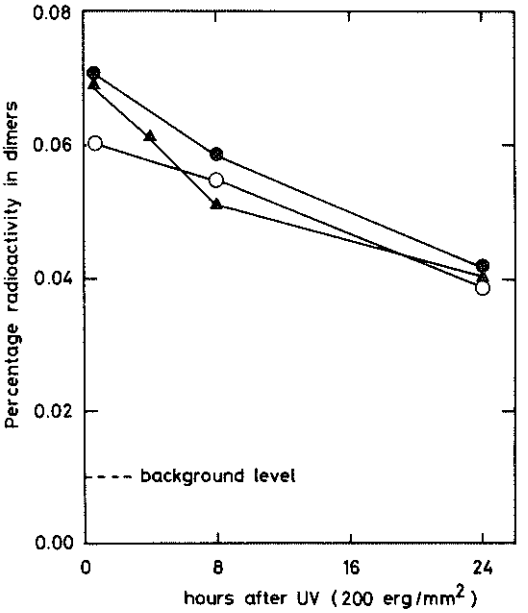

Fig. 3. Excision of pyrimidine dimers in normal human primary fibroblasts (AH)(O), T-cells (●) and HeLa-cells (▲) during incubation after UV-irradiation (200 erg/mm^2). The pyrimidine dimer content ($\widehat{XT}$/T%) of the DNA was determined by measuring the radioactivity in the dimers ($\widehat{TT}$ + $\widehat{CT}$) and thymine (T) after chromatography.

(c) Unscheduled DNA synthesis and repair replication in XP cells

The amounts of UDS and repair replication performed during 2 h following various UV-doses have been measured in a series of classic XP and DSC cell strains. In Table I UDS and repair replication (at 100 erg/mm^2) in the classic XP strains are presented as a percentage of the levels in normal human cells, which were measured simultaneously as controls in each experiment. In a group of 7 cell strains, all originating from severe cases of classic XP (the first group in Table I), low residual repair activities were observed. Cell strains from patients in 2 unrelated families (the second group in Table I) showed intermediate repair

TABLE I

DNA REPAIR CAPACITIES OF CELLS FROM CLASSIC XERODERMA PIGMENTOSUM PATIENTS

CELL STRAIN [a]		SEX	CASE AGE	CLIN.SYMP. (SEVERITY)	UNSCHEDULED DNA-SYNTHESIS [b]	REPAIR REPLIC-ATION [b]	REJOINING OF DNA-BREAKS [c]
XP4RO	d	FEMALE	16	SEVERE	10-15	5-10	n
XP9RO		MALE	9	SEVERE	10-15		n
XP16RO		MALE	9	SEVERE	5-10	5-10	
XP19HO	e	FEMALE	14	SEVERE	10-15		
XP20RO		MALE	3	SEVERE	10-15		
XP21RO	d	MALE	15	SEVERE	10-15		
XP12SF	f	FEMALE	12	SEVERE	10-15		
XP2RO	g	FEMALE	34	MODERATE	40	35	n
XP3RO	g	FEMALE	29	LIGHT/MOD.	45		
XP5RO	h	FEMALE	25	LIGHT/MOD.	25	22	n
XP6RO	h	FEMALE	18	LIGHT	31		
XP7RO	h	FEMALE	18	LIGHT	28		
XP1RO		MALE	48	VERY LIGHT	75	70-100	n
XP11RO		FEMALE	45	VERY LIGHT	100		
XP7TA	i	FEMALE	20	SEVERE	99		
XP30RO	j	MALE	30	LIGHT/MOD.	88	110	n

[a] A new nomenclature for XP-cell strains is used, see under Materials and Methods.

[b] Expressed as a percentage of the levels found in normal cells after a UV-dose of 100 erg/mm^2.

[c] n (normal), means that more than 70 % of the breaks were rejoined within 20 min after X-irradiation (20 krad), which was also found in normal cells.

[d] XP4RO and XP21RO are siblings.

[e] XP19HO provided via Dr. R.M. Humphrey, Houston.

[f] XP12SF, provided by Dr. J.E. Cleaver, San Francisco.

[g] XP2RO and XP3RO are second cousins.

[h] XP5RO, XP6RO and XP7RO are siblings.

[i] XP7TA, provided by Dr. H. Slor, Tel Aviv.

[j] XP30RO, skin biopsy provided by Dr. V. Der Kaloustian, Beirut.

activities. In 4 cases the levels of DNA repair synthesis were normal or only slightly reduced (the third group in Table I). Data on UDS obtained for the strains XP (1-7)RO and XP9RO have been described earlier [1].

Autoradiography and density gradient data obtained with the strains XP2RO and XP4RO, are presented in more detail in Fig. 4. The amount of repair replication observed in XP2RO cells was strongly reduced at low UV-doses, but increased with the dose up to at least 1000 erg/mm^2 and approached the level in normal cells, which reached a maximum already at 100-200 erg/mm^2 (Fig. 4b). A corresponding increase in the relative repair level was apparent from the dose response curves for UDS in XP2RO (Fig. 4a) and XP3RO cells (see ref. 1) and possibly also for XP5RO cells. In other cases (e.g. XP4RO, Fig. 4) the level of repair synthesis relative to that in normal cells was not dose-dependent.

In order to investigate whether XP cells with appreci-

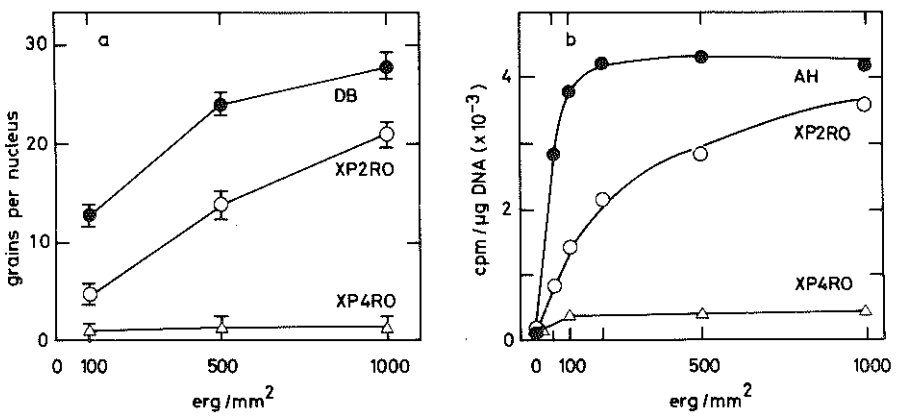

Fig. 4. DNA repair synthesis as a function of UV-dose in normal human fibroblasts (DB and AH), cells from a moderately severe classic XP-patient (XP2RO) and cells from a severe classic XP-patient (XP4RO).
(a) Unscheduled DNA synthesis; ^{3}H-thymidine labelling for 3 h after UV; autoradiographic exposure time: 3 days. (b) repair replication; ^{3}H-thymidine labelling (in the presence of BUdR, FUdR and hydroxyurea for 2 h after UV; NaI isopycnic gradient analysis.

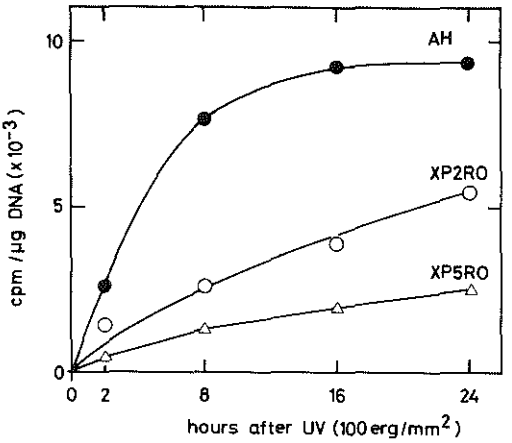

Fig. 5. The amount of repair replication as a function of time after UV-irradiation (100 erg/mm^2) in normal cells (AH) and two cell strains from moderately severe classic XP patients (XP2RO and XP5RO), determined by NaI isopycnic gradient analysis.

able residual repair capacities might be able to perform ultimately as much repair as normal cells, the repair process was followed for extended periods in XP2RO and XP5RO cells. Fig. 5 shows that the repair process in these cells after a dose of 100 erg/mm^2 continued for at least 24 h, whereas in normal cells the repair was almost completed after 8 h.

Table II summarizes the results of experiments on UDS in 6 DSC strains and repair replication in 2 of these strains. In all cases the amounts of repair synthesis at various UV-doses up to 1000 erg/mm^2 did not exceed significantly the background level observed in unirradiated cells.

(d) DNA repair synthesis in cells from XP-heterozygotes

Repair synthesis was studied in 7 cell strains derived from the parents of classic XP and DSC patients. In 2 heterozygous strains (XPH14RO and XPH15RO) from both parents of a DSC patient (XP12RO) a significant reduction of the repair synthesis level was found with the autoradiographic

TABLE II

DNA REPAIR CAPACITIES OF CELLS FROM DE SANCTIS-CACCHIONE PATIENTS

CELL STRAIN [a]	SEX	AGE	CASE CLIN.SYMP.	UNSCHEDULED DNA-SYNTHESIS [b]	REPAIR REPLIC-ATION [b]	REJOINING OF DNA-BREAKS [c]
XP12RO [d]	MALE	13	SEVERE SKIN LESIONS AND NEUROLOGICAL COMPLICATIONS	<5	<5	n
XP4LO [e]	MALE	6		<5		
XP17SF [f]	FEMALE	9		<5		
XP25RO [d]	MALE	1		<5	<5	n
XP26RO [d]	FEMALE	7		<5	<5	
XPPKSF [f]	FEMALE	9		<5		

[a,b,c] See footnotes a, b, c TABLE I.

[d] XP12RO is genetically related to the siblings XP25RO and XP26RO, skin biopsies provided by Dr. V. Kaloustian, Beirut.

[e] XP4LO, provided by Dr. J.M. Parrington, London.

[f] XP17SF and XPPKSF, provided by Dr. J.E. Cleaver, San Francisco.

technique (Table III and ref. 2) as well as with the density gradient technique (Table III). The same mutation is probably present in the heterozygotes XPH27RO and XPH28RO because of family relationships. However the cells of these individuals did not show a consistent decrease of the repair level, although a slight decrease is indicated in the UDS data. The cell strains derived from parents of classic XP patients performed normal amounts of repair after doses up to 1000 erg/mm^2 (Table III).

(e) Rejoining of X-ray induced single-strand DNA breaks

Following X-irradiation (20 krad) normal and XP cells were incubated for 0, 10, 20, 40 and 60 min. The molecular weight of the DNA at the various post-irradiation periods was determined by sedimentation in alkaline sucrose gradients as described previously [17] and the number of breaks relative to DNA from unirradiated cells was calculated. The time courses of rejoining for all cell strains studied

TABLE III

DNA REPAIR CAPACITIES OF CELLS FROM HETEROZYGOTE PARENTS OF CLASSIC XERODERMA PIGMENTOSUM AND DE SANCTIS-CACCHIONE PATIENTS

CELL STRAIN [a]	CASE	UNSCHEDULED DNA SYNTHESIS [b]			REPAIR REPLICATION [b]		
		100	500	1000	100	500	1000
		-erg/mm^2 UV-light -					
XPH8RO	MOTHER OF XP5RO XP6RO, XP7RO (CLASSIC XP)	100	100	100			
XPH10RO	MOTHER OF XP9RO (CLASSIC XP)	100	100	100			
XPH14RO [c]	MOTHER OF XP12RO (DSC)	81	71	83	51	60	56
XPH15RO [c]	FATHER OF XP12RO (DSC)	74	67	72	76	73	61
XPH17RO	MOTHER OF XP16RO (CLASSIC XP)	100	100	100	127	98	78
XPH27RO[c]	MOTHER OF XP25RO XP26RO (DSC)	86	83	81			
XPH28RO	FATHER OF XP25RO XP26RO (DSC)	95	88	94	128	88	97

[a,b] See footnotes a, b TABLE I.
[c] Skin biopsies were provided by Dr. V. Der Kaloustian, Beirut.

(normal and XP, Table I and II) were similar to those published earlier for XP4RO and a normal strain [17].

DISCUSSION

Repair replication (Fig. 2) and excision of pyrimidine dimers (Fig. 3 and refs. 22,26) in UV-irradiated human cells follow time courses which are consistent with the hypothesis that the two phenomena represent steps in the same repair process. A considerable fraction of the dimers is still found in the DNA at a time when the cell ceases

repair replication. Similar observations of Paterson et al. [21], who used UV-endonuclease purified from *Micrococcus luteus* to detect dimer sites, show that the remaining dimers are situated in the DNA indeed and not in large, excised oligonucleotides, as might be supposed in the chromatographic method which we used. The incompleteness of dimer excision can also not be attributed to an early inactivation of the repair system, or even of the whole cell, by the UV-dose used (200 erg/mm^2), because at higher doses (e.g. 500 erg/mm^2) the repair process appeared to continue for longer times. Therefore it is suggested that part of the dimer sites is less accessible to the excision repair system; the cell might cope with these dimers by a post-replication repair mechanism [19,3].

The amount of repair replication, performed in normal cells during incubation for 2 h as a function of the UV-dose, reached a maximum between 100 and 200 erg/mm^2 (Fig. 4b). This can be ascribed to saturation of the repair system in this dose range, rather than to completion of the repair at the lower doses, because the rate of repair replication after 100 erg/mm^2 remains constant for a longer time than the 2 h labelling period (Fig. 2). For the same reason the amount of repair synthesis measured in UV-irradiated normal and XP cells after labelling periods of 2 h are proportional to the initial rates of repair synthesis.

The XP patients involved in this study belong to two main groups according to their clinical symptoms: classic XP and the DSC syndrome. Among the classic XP patients there are differences in the severity of the clinical symptoms. As described by Bootsma et al.[1] and confirmed by the present extended investigations, the severity of the clinical symptoms of the patients and the levels of repair synthesis in the cells seemed to be inversely correlated. The various patients can be divided into four groups according to the different kinetics of the UV-induced DNA repair synthesis in their cells:

1. Cases of classic XP with low, but detectable,

residual repair capacities (Table I, first group; 5-15% of the control).

2. Cases of classic XP with intermediate levels of repair synthesis. (Table I, the second cousins XP2RO and XP3 RO: 35-50%; the siblings XP5RO, XP6RO and XP7RO: 20-30%). However, in XP2RO cells we observed a remarkable increase with dose in the relative repair replication rate from 29% of the control rate at 100 erg/mm^2 to 85% at 1000 erg/mm^2 (Fig. 4b). Like the XP lymphocytes investigated by Burk et al. [4] and Robbins and Kraemer [24], XP2RO and XP5RO cells continued the repair process for a longer time than normal cells did (Fig. 5); so eventually these XP cells may perform as much repair as normal cells.

3. Cases of classic XP with normal or only slightly reduced repair capacities (Table I, third group). Patients having symptoms comparable to those of XP but with normal DNA repair synthesis have also been reported by Jung [13], Burk et al. [4] and Cleaver [8]. The defect involved in these patients is probably not associated with any DNA repair system, including post-replication repair [3], as was suggested by Cleaver [8] because of his finding that cells of such patients have a normal UV-sensitivity.

4. DSC patients with a complete deficiency of the repair capacity (Table II, 6 cases from 4 unrelated families). This complete deficiency is probably not characteristic for the DSC syndrome since DSC patients with residual repair capacities have been reported [7,12,24].

In the study of cell strains from the heterozygous parents of XP patients a reduced repair activity is indicated in the parents of the DSC patients in our series. In contrast, the only two cases of heterozygotes having a reduced repair level reported by Cleaver [8] were the parents of a patient with the classic form of XP. In most cases so-far investigated normal repair levels have been observed, which is consistent with the recessive nature of the disease. Apparently the enzyme which is defective in the XP cells, is present in excessive rather than in rate-limiting

amounts in normal cells. These results indicate that the detection of heterozygotes by means of the present techniques is not reliable.

We have previously demonstrated that classic XP and DSC cells can complement each other [27,28,18]. Recently we have found complementation between the XP2RO cells (group 2) and either DSC cells or classic XP cells of group 1 (unpublished results). These observations indicate that different mutations are involved in the three forms of the disease. As discussed above three of the four groups of XP patients are defective in excision repair. These three groups (1, 2 and 4), which were distinguished by their distinct kinetics of repair synthesis, coincide with the three complementation groups. The data do not indicate whether the mutations are located within one gene or in different genes coding for polypeptides of either the same enzyme or different enzymes.

Thusfar four arguments have been presented for the hypothesis that classic XP as well as DSC cells are defective in an initial step of excision repair, probably incision:

(1) The normal repair replication [6,17] and rejoining of single-strand breaks [17] (Table I and II) after X-irradiation in XP cells.

(2) The inability of XP cells to excise pyrimidine dimers [26,10,15].

(3) The demonstration that dimer sites in the DNA, which are susceptible to attack by UV-endonuclease from *Micrococcus luteus*, remain in XP cells, whereas they disappear from normal cells [21].

(4) The absence of accumulated breaks, which might be expected in the DNA of UV-irradiated XP cells as a result of incision if one of the steps following incision would be defective [16].

However neither of these data is conclusive for the hypothesis of a defective incision function in XP cells. The first argument only holds if the same enzymes are involved in the repair replication and rejoining steps in the

repair of UV and X-ray damage. The other three arguments are no longer valid if the repair enzymes operate in a co-ordinated way [15,9,16] (e.g. by a systematic scanning of the DNA by an enzyme complex), since in that case a defect in any step would block the whole repair process. If the co-ordinated repair hypothesis is correct it cannot be excluded that other steps in excision repair than the incision step can be defective in XP. Moreover in a complex model one can also envisage a mutation that affects the rate at which the complex advances along the DNA-strands from one lesion to the next; such a defect might cause a deviating dose-response curve for repair replication similar to that found for the XP2RO-strain (Fig. 4b).

As described earlier, normal levels of repair synthesis per nucleus were found in most of the heterozygous (XP) strains and also in the heterokaryons obtained by fusion of normal cells with XP cells [11] or by fusion of different XP strains [27,28]. These observations can be explained in the complex model if we assume that repair complexes can start the scanning and repair process only at a limited number of sites on the DNA. Only one complex would then operate effectively in a DNA region belonging to one "initiation" site; subsequent complexes entering the same region would be redundant because the DNA has already been repaired. A reduction of the normal concentration of repair complexes would not affect the repair capacity as long as the concentration remains high enough to bind one complex to each initiation site. A co-ordinated repair mechanism, as proposed here, seems to permit an efficient repair of the DNA in the complicated chromosome structure and would avoid the presence of single-strand DNA regions or breaks for relatively long periods during the repair process.

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REFERENCES

1. BOOTSMA, D., M.P. MULDER, F. POT AND J.A. COHEN, Different inherited levels of DNA repair replication in xeroderma pigmentosum cell strains after exposure to ultraviolet irradiation, *Mutation Res.*, 9 (1970) 507-516.
2. BOOTSMA, D., E.A. de WEERD-KASTELEIN, G. VELDHUISEN AND W. KEIJZER, Genetic studies on repair in xeroderma pigmentosum cells, in: DNA repair mechanisms, *Symposia Medica Hoechst, F.K. Schattauer Verlag, Stuttgart-New York*, 1972, pp 87-97.
3. BUHL, S.N., R.M. STILLMAN, R.B. SETLOW AND J.D. REGAN, DNA chain elongation and joining in normal human and xeroderma pigmentosum cells after ultraviolet irradiation, *Biophys.J.*, 12 (1972) 1183-1191.
4. BURK, P.G., M.A. LUTZNER, D.D. CLARKE AND J.H. ROBBINS, Ultraviolet-stimulated thymidine incorporation in xeroderma pigmentosum lymphocytes, *J. Clin. Med.*, 77 (1971) 759-767.
5. CLEAVER, J.E., Defective repair replication of DNA in xeroderma pigmentosum, *Nature*, 218 (1968) 652-656.
6. CLEAVER, J.E., Xeroderma pigmentosum: a human disease in which an initial stage of DNA-repair is defective, *Proc. Natl. Acad. Sci. (U.S.)*,63 (1969) 428-435.
7. CLEAVER, J.E., DNA repair and radiation sensitivity in human (xeroderma pigmentosum) cells, *Int. J. Radiat. Biol.*, 18 (1970) 557-565.
8. CLEAVER, J.E., Xeroderma pigmentosum: variants with normal DNA repair and normal sensitivity to ultraviolet light, *J. Invest. Dermatol.*, 58 (1972) 124-128.
9. CLEAVER, J.E.,G.H. THOMAS, J.E. TROSKO AND J.T. LETT, Excision repair (dimer excision, strand-breakage and repair replication) in primary cultures of eukaryotic (bovine) cells, *Exptl. Cell Res.*, 74 (1972) 67-80
10. CLEAVER, J.E.AND J.E. TROSKO, Absence of excision of ultraviolet-induced cyclobutane dimers in xeroderma pigmentosum, *Photochem. Photobiol.*, 11 (1970) 547-550.

11. GIANELLI, F., P.M. CROLL AND S.A. LEWIN, DNA repair synthesis in human heterokaryons formed by normal and UV-sensitive fibroblasts, *Exptl. Cell Res.*, 78 (1973) 175-185.

12. GOLDSTEIN, S., The role of DNA repair in aging of cultured fibroblasts from xeroderma pigmentosum and normals, *Proc. Soc. Exp. Biol. Med.*, 137 (1971) 730-734.

13. JUNG, E.G., Das pigmentierte Xerodermoid. Ein Defekt der Rekombinations-Erholung von UV-Schäden, *Arch.Derm.Forsch.*, 241 (1971) 33-43.

14. KIRBY, K.S. AND E.A. COOK, Isolation of deoxyribonucleic acid from mammalian tissues, *Biochem. J.*,104 (1967) 254-257.

15. KLEIJER, W.J. AND D. BOOTSMA, Repair of DNA damaged by UV- and X-irradiation in cultivated normal human xeroderma pigmentosum and Bloom cells, *Proc. 1st European Biophys. Congr., Baden 1971*, Vol.II, pp 129-133.

16. KLEIJER, W.J., H.L. HOEKSEMA, M.L. SLUYTER AND D. BOOTSMA, Effects of inhibitors on repair of DNA in normal human and xeroderma pigmentosum cells after exposure to X-rays and ultraviolet irradiation, *Mutation Res.*,17 (1973) 385-394.

17. KLEIJER, W.J., P.H.M. LOHMAN, M.P. MULDER AND D. BOOTSMA, Repair of X-ray damage in DNA of cultivated cells from patients having xeroderma pigmentosum, *Mutation Res.*, 9 (1970) 517-523.

18 KLEIJER, W.J., E.A.de WEERD-KASTELEIN, P.H.M. LOHMAN AND D. BOOTSMA, Repair of UV-damaged DNA in normal human and xeroderma pigmentosum cells, *Studia Biophys.* 36/37 (1973) 375-386.

19. LEHMANN, A.R., Post-replication repair of DNA in ultraviolet-irradiated mammalian cells, *J. Mol. Biol.*,66 (1972) 319-337.

20. LOHMAN, P.H.M., M.L. SLUYTER, I.A.A. MATTHIJS AND W.J. KLEIJER, Repair replication in human cells studied by sodium iodide isopycnic centrifugation of DNA in a fixed angle rotor, *Anal. Biochem.*, 54 (1973) 178-187.

21. PATERSON, M.C., P.H.M. LOHMAN AND M.L. SLUYTER, Use of a UV-endonuclease from Micrococcus luteus to monitor the progress of DNA repair in UV-irradiated human cells, *Mutation Res.*, in press.

22. REGAN, J.D., J.E. TROSKO AND W.L. CARRIER, Evidence for excision of ultraviolet-induced pyrimidine dimers from the DNA of human cells in vitro, *Biophys. J.*, 8 (1968) 319-325.

23. REED, W.B., B.LANDING, G. SUGARMAN, J.E. CLEAVER AND J. MELNYK, Xeroderma pigmentosum: clinical and laboratory investigations of its basic defect, *J. Am. Med. Ass.*,207 (1969) 2073-2079.

24. ROBBINS, J.H. AND K.H. KRAEMER, Abnormal rate and duration of ultraviolet-induced thymidine incorporation into lymphocytes from patients with xeroderma pigmentosum and associated neurological complications, *Mutation Res.*, 15 (1972) 92-97.
25. SETLOW, R.B. AND W.L. CARRIER, The disappearance of thymine dimers from the DNA: an error-correcting mechanism, *Proc. Natl. Acad. Sci. (U.S.)*, 51 (1964) 226-231.
26. SETLOW, R.B., J.D. REGAN, J. GERMAN AND W.L. CARRIER, Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA, *Proc. Natl. Acad. Sci. (U.S.)*, 64 (1969) 1035-1041.
27. de WEERD-KASTELEIN, E.A., W. KEIJZER AND D. BOOTSMA, Genetic heterogeneity of xeroderma pigmentosum demonstrated by somatic cell hybridization, *Nature*, 238 (1972) 80-83.
28. de WEERD-KASTELEIN, E.A., W.J. KLEIJER, M.L. SLUYTER AND W. KEIJZER, Repair replication in heterokaryons derived from different repair-deficient xeroderma pigmentosum strains, *Mutation Res.*, 19 (1973) 237-243.