MEDISCH BIOLOGISCH LABORATORIUM TNO

Lange Kleiweg 139, Rijswijk ZH.

USE OF A UV ENDONUCLEASE FROM MICROCOCCUS LUTEUS TO MONITOR THE PROGRESS OF DNA REPAIR IN UV-IRRADIATED HUMAN CELLS

M.C. Paterson, P.H.M. Lohman and M.L. Sluyter

Werkgebied 2: Bescherming tegen (ioniserende) straling en chemische mutagentia

USE OF A UV ENDONUCLEASE FROM MICROCOCCUS LUTEUS TO MONITOR THE PROGRESS OF DNA REPAIR IN UV-IRRADIATED HUMAN CELLS

M.C. Paterson, P.H.M. Lohman and M.L. Sluyter

Laboratory for Molecular Genetics, Leiden State University and Medical Biological Laboratory TNO, Rijswijk Z.H., The Netherlands

Motivering en toelichting

Het herstel van schade in het DNA van menselijke cellen die bestraald zijn met ultraviolette straling, werd bestudeerd met behulp van een nieuwe en zeer gevoelige methode. Deze methode maakt gebruik van een enzym (endonuclease), geisoleerd uit Micrococcus luteus, dat specifiek aangrijpt op de door ultraviolette straling geinduceerde pyrimidinedimeren in het DNA. Het resultaat van de behandeling van het bestraalde DNA in vitro met het endonuclease is dat nabij de pyrimidinedimeren enkelstrengbreuken in het DNA worden aangebracht. Het aantal enkelstrengbreuken en dus het aantal pyrimidinedimeren in het DNA kan worden berekend uit de sedimentatiesnelheid van het DNA in alkalische suikergradiënten.

De daling van het aantal gebiedjes in het DNA van bestraalde menselijke cellen (fibroblasten) dat gevoelig is voor het endonuclease als functie van de tijd na de bestraling is een maat voor het herstel van het beschadigde DNA. In normale menselijke fibroblasten in weefselkweek werd bij incubatie van de cellen na bestraling een afname van het aantal voor het endonuclease gevoelige gebiedjes gevonden. De verdwijning van de schade kon reeds worden aangetoond na bestraling met niet meer dan 65 erg/mm^2 ultraviolette straling (254 nm).

In bestraalde cellen afkomstig van Xeroderma patienten was de afname van het aantal voor het endonuclease gevoelige gebiedjes geringer. De geringste afname werd gevonden in cellen afkomstig van patienten met een ernstige vorm van Xeroderma

pigmentosum (klassiek syndroom of het De Sanctis Cacchione syndroom).

De rasultaten met behulp van de nieuwe methode zijn in overeenstemming met eerder uitgevoerde experimenten, waarbij een latere stap in het herstel van schade in DNA ten gevolge van ultraviolette straling werd gemeten, n.l. DNA herstelreplicatie.

USE OF A UV ENDONUCLEASE FROM MICROCOCCUS LUTEUS TO MONITOR THE PROGRESS OF DNA REPAIR IN UV-IRRADIATED HUMAN CELLS

M.C. Paterson, P.H.M. Lohman and M.L. Sluyter

Laboratory for Molecular Genetics, Leiden State University and Medical Biological Laboratory TNO, Rijswijk Z.H., The Netherlands

Summary

A sensitive enzymatic assay has been developed to follow the progress of DNA repair in human cells exposed to ultraviolet (UV) radiation. The assay employs an endonuclease selectively active at sites containing pyrimidine dimers in UV-damaged DNA. Primary fibroblasts are exposed to 254 nm radiation and incubated for specified times, their radioactively labelled DNA is isolated and treated with a UV endonuclease extensively purified from Micrococcus luteus. Endonuclease-susceptible sites remaining in the DNA are subsequently observed as single-strand scissions by sedimentation in alkaline sucrose gradients. In comparison to the situation with excision-proficient normal cells, those derived from patients suffering from either the classical or the De Sanctis-Cacchione clinical form of Xeroderma pigmentosum (XP) exhibit a marked diminution in the rate of disappearance of nuclease-susceptible lesions with time of post-UV incubation.

INTRODUCTION

Human cells in tissue culture are able to circumvent the deleterious effects of ultraviolet (UV) radiation (< 320 nm). This is achieved, at least in part, by the ability of these cells to eliminate or bypass such UV photoproducts as cyclobutyl pyrimidine dimers produced in their DNA (for reviews, see ref. 10, 11, 25). Excision repair, the most extensively studied cellular repair system, involves the physical removal and replacement of the offending photoproducts in the damaged DNA. Based largely upon elaborate studies in bacteria [17, 15, 34], this repair mechanism is believed to proceed by the following chain of enzymatic steps: (i) A UV-specific endonuclease recognizes the lesion and cleaves an adjacent phosphoester bond. (ii) An exonuclease introduces a second single-strand scission so as to excise the lesion as part of an oligonucleotide. The ensuing gap may be widened by additional nucleolytic activity. (iii) The excised genetic information is recovered by "repair replication" in which a DNA polymerase inserts new nucleotides complementary to those on the opposite strand. (iv) Strand continuity is restored when a DNA ligase forms a covalent bond between newly-synthesized and pre-existing DNA.

The importance of a functional DNA repair mechanism to the well-being of man was established by the discovery that cells cultured from individuals having the hereditary skin disease, Xeroderma pigmentosum (XP), were impaired in the elimination of UV-induced damage[6]. Two clinical variants of XP are known: [30, 27] (i) a so-called classical type primarily characterized by abnormalities of the skin including multiple cutaneous carcinomas and (ii) a more severe type, the De Sanctis-Cacchione syndrome, in which various neurological disorders accompany the skin tumors. Many etiological studies on a number of genetically unrelated XP strains have confirmed that with few

exceptions [3,9] both clinical forms are deficient in excision repair [6, 28, 1, 33, 13, 18, 29,35]. Moreover, although apparently blocked at the same operational step, the presumed initial incision [7,33], the two syndromes probably reflect mutations in different genetic complementation groups since binuclear cells harboring a nucleus from each variant exhibit normal levels of UV-induced repair replication [35].

This report describes a sensitive enzymatic assay for quantitating the incidence of pyrimidine dimers in human DNA irradiated in vivo. The in vitro assay depicted in Fig. 1 employs an endonuclease purified from Micrococcus luteus which selectively attacks dimer-containing sites (referred to as nuclease-susceptible sites) in UV-damaged native DNA. The number of endonuclease-induced, single-strand breaks and therefore the number of photoproducts may be measured by subsequent sedimentation of the enzyme-treated DNA through alkaline sucrose gradients. Incubation of UV-irradiated cells for specified periods prior to DNA extraction and analysis enables one to follow the disappearance of nuclease-susceptible sites in vivo. In accord with previous studies [7, 33], results from the application of the assay to normal and XP strains strongly suggest that both De Sanctis-Cacchione and classical XP cells fail to initiate dimer excision.

MATERIALS AND METHODS

Human strains

The experiments reported here were conducted with the following primary strains: AH[19], derived from a normal individual having no clinical symptoms; XP4[1], XP5[1], established from patients having the classical form of Xeroderma pigmentosum; and XP12[35], XP25, XP26, obtained from De Sanctis-Cacchione patients. XP25 and XP26 were established by D. Bootsma (University of Rotterdam) from biopsies provided by V. Der Kaloustian (American University of Beirut).

Tissue culture conditions

Monolayer cultures of primary fibroblasts were grown routinely in F12 medium supplemented with 15% calf serum. All incubations were at 37°C in air containing 5% CO_2. Stocks were maintained in T flasks while experimental cultures were grown in Falcon plastic petri dishes (Φ, 9 cm).

Cellular DNA was labelled with either ^{3}H-methyl thymidine (2 Ci/mmol) or 2-^{14}C thymidine (53 mCi/mmol). (Both radionuclides were purchased from the Radiochemical Centre, Amersham, Great Britain.) Confluent monolayers of experimental cultures were obtained by adding ~10^{6} cells to growth medium supplemented with either 0.5 μCi ^{3}H-thymidine or 1.0 μCi ^{14}C-thymidine per ml and incubating the inoculum for 24-30 hr.

UV irradiation

Upon removing the radioactive medium, cultures were rinsed once with balanced salt solution. After carefully draining off the rinsing solution, tritium-labelled samples were exposed to specified fluences of chiefly 254 nm radiation emitted from a Philips TUV low pressure mercury tube with an incident dose rate of 8 ergs/(mm^{2}.sec). The ^{14}C-labelled samples served as an unirradiated control. Immediately after irradiation non-radioactive growth medium was added, and the UV-damaged cultures were incubated for various periods of time. Cells were then scraped from the dishes with a rubber policeman, washed twice in ice-chilled physiological saline, collected by centrifugation (4°C) and finally the cell pellets were stored at -70°C.

DNA isolation

Cell pellets of the control samples were resuspended in physiological saline and ~7 x 10^5 were added to ~2 x 10^6 UV-irradiated ones. The combined mixture of cells were centrifuged and resuspended in a 1 ml solution (pH 7.5) of 0.01 M Tris - 0.15 M NaCl - 0.01 M ethylenediaminetetraacetic acid (EDTA) - 0.1% sarkosyl (Geigy). Cell lysis was achieved by incubating the suspension at 60°C for 30 min after which the DNA was extracted with an equal volume of chloroform-isoamyl alcohol (24 : 1, $v/_v$ according to a modified procedure of Marmur[23]. The aqueous phase containing the DNA was withdrawn and dialyzed overnight at 4°C against 1l of the buffer used in the assay for UV endonuclease activity (see below).

Assay for endonucleolytic activity towards UV-damaged DNA

The components of the substrate mixture, in 120µl, usually were: ^{3}H-labelled, UV-irradiated DNA (0.5 - 1.0 µg containing 2 - to 4 x 10^4 dpm), ^{14}C-labelled, unirradiated DNA (0.15 - 0.30 µg containing 2 - to 4 x 10^3 dpm), and 20 µM t-RNA (General Biochemicals, Chagrin Falls, Ohio, U.S.A.) dissolved in a potassium phosphate buffer (10 mM, pH 7.5) containing 10 mM β-mercaptoethanol- 1 mM EDTA. After prewarming the mixture to 37°C, the reaction was initiated by introducing, with thorough mixing, 20 µl of UV endonuclease (in 10 mM potassium phosphate - 1 mM EDTA (pH 7.0) - 10 mM β-mercaptoethanol - 10% ethylene glycol). The endonuclease was purified from M. luteus essentially as described by Nakayama and co-workers[24] (personal communication, R.A. Oosterbaan). As a control, 20 µl of the UV endonuclease buffer was added to a second substrate solution. After incubating at 37°C for 20 min, conditions permitting the UV endonucleolytic reaction to go to completion, each solution was added to an

equal volume of 1 M NaOH.

Sucrose gradient analysis of UV endonuclease-induced strand breaks

The alkaline solution containing the products of the endonucleolytic reaction was directly layered on a 3.6 ml linear gradient of 5 to 20% sucrose (w/v) in 0.5 M NaCl - 0.2 M NaOH - 0.01 M EDTA. Gradients were spun at 40,000 rev/min (21°C) for 3 hr in an SW56 swinging-bucket rotor powered by a Spinco model L ultracentrifuge (Beckman). Approximately 28, 6-drop, fractions were collected, by pumping from the bottom of the gradient, on paper strips according to the method of Carrier and Setlow[5]. Radioactivity profiles were generated after double-label counting each fraction (counting fluid, toluene - 2,5 - diphenyloxazol (4g/l), 2,2' p-phenylene - bis - (4-methyl-5-phenyl-oxazolyl)- benzene (0.1 g/l)) in a Nuclear Chicago Mark II scintillation counter. The total recovery of both ^{3}H and ^{14}C radioactivities was between 85 and 105% of the input activity. Weight average molecular weight values for the sedimenting DNA's were computed essentially as described by Ley and Setlow [22]. Our computer program permitted us to exclude fractions from the top and bottom of each gradient which were clearly separate from the main body of sedimenting radioactivity. This treatment of our data increased greatly both the reproducibility and the sensitivity of the UV endonuclease assay.

Photoreactivation

In certain experiments the UV-irradiated DNA was photoreactivated before treatment with the M. luteus enzyme. This was achieved by incubating the substrate solution with 20 µl of photoreactivating enzyme (in 10 mM NaCl - 1 mM potassium

phosphate (pH 7.4) - 3% glycerol) for 40 min in a 37°C incubator illuminated with two Philips TL20w/08 black-light lamps with an incident intensity of $\sim 10^4$ ergs/ (mm^2.sec). The photoreactivating light (300-400 nm) was filtered with glass 13 mm thick to remove any radiation below 320 nm. The photoreactivating enzyme was purified from Streptomyces griseus by the regimen of Eker [14].

RESULTS

Assay for UV endonuclease activity towards human DNA damaged in vivo

(i) General properties

The consequence of incubating DNA co-isolated from unirradiated and UV-irradiated normal human cells with the UV endonuclease from M. luteus is illustrated in Fig. 2. When the DNA sample is incubated alone, the difference in the weight average molecular weight (Mw) of the two populations of macromolecules corresponds to 0.3 single-strand breaks per 10^7 daltons number average molecular weight (Mn) in the UV-damaged in comparison to the undamaged DNA. (Although, like Regan et al.,[29] we prefer to compute Mw's, the frequency of strand breaks should be calculated from Mn values. It is assumed that the distribution of strand breaks is random (i.e. dimers are induced randomly during irradiation) and thus Mn = Mw/2) . When the same sample is incubated with the purified enzyme however there is a noticeable decrease in the Mw of the damaged DNA to the extend that this DNA has acquired 6.6 breaks per 10^7 daltons. Thus, the UV endonucleolytic activity per se has selectively introduced 6.3 strand incisions per 10^7 daltons (Mn) in the DNA pre-exposed in vivo to a fluence of 250 ergs/mm^2 of germicidal light.

The difference between the Mw values of the UV endonuclease-treated and untreated DNA extracted from undamaged cells is well within the gradient-to-gradient reproducibility of the velocity sedimentation method. Furthermore, in many instances the Mw of the enzyme-treated DNA actually exceeds that of the untreated DNA. This absolute dependence on UV damage for endonucleolytic activity indicates that the purified enzyme is free of contaminating non-specific endonucleases.

Data in Fig. 3 show that a linear relationship exists between production of strand breaks by the UV endonuclease in vitro and UV dose incident on the DNA in vivo. Such a direct dependence of endonucleolytic activity on UV dose is expected if the M. luteus enzyme quantitatively introduces a single incision near each photoproduct.

The standard conditions (20 min, 37°C) for incubation with the UV endonuclease permit the endonucleolytic reaction to proceed to completion as further treatment with a second aliquot of the enzyme results in relatively little additional strand breakage (see Fig. 4).

(ii) Substrate specificity

To establish the substrate specificity of the M. luteus UV endonuclease DNA from UV-irradiated human cells was first subjected to conditions permitting photoreactivation. It can be seen in Fig. 4 that pre-incubation with purified photoreactivating enzyme in the presence of near UV light essentially converts the radition-damaged DNA into an unsuitable substrate for the UV endonuclease. Since pyrimidine dimers are the only known substrate for photoenzymatic repair of UV-irradiated DNA[32] it is concluded that the endonucleolytic activity present in the M. luteus enzyme is highly selective for

stretches in UV-damaged DNA containing these photoproducts. This conclusion is substantiated further by the observation that the UV endonuclease treatment induces ~ 6 strand scissions per 10^7 daltons in DNA from cells exposed to a UV fluence of 250 ergs/mm^2 (Fig. 3); a value in excellent agreement with the number of dimers, ~ 5 per 10^7 daltons, expected in the UV-damaged DNA[33].

Time course for the *in vivo* disappearance of nuclease-susceptible sites

(i) in normal cells

Fig. 5 depicts the results of several experiments in which normal AH cells were exposed to various UV doses and subsequently incubated for specified time intervals prior to treating the extracted DNA with the UV endonuclease. The disappearance of nuclease-susceptible sites with time of post - UV incubation of the cells appears to follow first-order kinetics. It should also be noted that the fraction of sites affected decreases with increasing UV dose to the cells. After exposure to 65 ergs/mm^2, about 70 percent of the sites originally present were attacked by 9 hours. With a dose of 500 ergs/mm^2 a considerably reduced proportion (~ 10 - 15%) was converted during the same incubation period.

The data in Fig. 5 can be expressed in terms of rate of substrate turnover; that is, absolute numbers of dimer-containing sites in the UV-damaged DNA per cell affected during a 9 hr incubation period. This treatment of the data permits us to estimate the incident UV dose necessary to saturate the cellular enzyme system(s) catalyzing dimer attack. Table 1 demonstrates that at low UV doses (65-125 ergs/mm^2) the rate of disappearance of nuclease - susceptible sites increases with UV dose. At fluences of 250 - 500 ergs/mm^2, however, the absolute number of sites affected is independent of the original number present in the cell. Hence, between 65 to 125 ergs/mm^2 the concentration of enzyme(s) mediating the removal of nuclease-susceptible sites becomes limiting.

At fluences of 65 and 125 ergs/mm^2, both curves in Fig. 5 exhibit a marked break

after approximately 70% of the nuclease-susceptible sites in the DNA have been acted upon during post-UV incubation of the cells. The fact that we observe this break repeatedly suggests that it is not an artefactual effect of the *in vitro* assay. Extrapolation of the second slope to the ordinate indicates that during repair incubation *in vivo* about 40% of the sites are affected at a slower rate.

(ii) in XP cells

In sharp contrast to the proficiency exhibited by normal cells, XP cells derived from both forms of the skin disease are able to remove only a limited number of nuclease-susceptible sites during 30 hours of post-radiation incubation (see Fig. 6). In XP5, for example, about 65% of the sites initially induced by 250 ergs/mm^2 of 254 nm radiation is still susceptible to attack by the UV endonuclease from *M. luteus*. The level is even higher ($\sim$ 85%) in the remaining XP cell lines. In post-UV incubated normal cells, however, only 35% of the original number of sites is attacked by the purified enzyme. The simplest interpretation of these data is that XP cells possess a reduced capacity to overcome dimers in their DNA.

DISCUSSION

In agreement with earlier investigations [16, 4, 24, 26], we have found that the UV endonuclease purified from *M. luteus* attacks UV-irradiated DNA at specific sites distorted by the formation of pyrimidine dimers. Hence, the enzymatic assay outlined in Fig. 1 provides a simple and highly selective method for monitoring the disappearance of dimer-containing sites from DNA during incubation of UV-irradiated human cells. The assay also exhibits a high degree of sensitivity as it is possible to detect easily one

dimer in native DNA having a Mn of 10^7 daltons.

Our results strongly suggest that normal human cells, but not those derived from patients having either the classical or the De Sanctis-Cacchione clinical form of Xeroderma pigmentosum, possess efficient metabolic machinery to act on pyrimidine dimers induced in their DNA by ultraviolet radiation. This is inferred from the observation that during post-UV incubation, a much larger fraction of the dimer-containing sites in normal cells than in XP cells become insensitive to subsequent in vitro treatment with the UV endonuclease from M. luteus. Although the in vitro assay provides no information on the actual enzymatic process involved, extensive complementary studies (reviewed in references 10, 11) leave little doubt that nuclease-susceptible sites disappear in vivo primarily as a consequence of excision repair. Our data are consistent with those measuring the excision and repair replication steps in the scheme for excision repair. The time course of the disappearance of nuclease-susceptible sites in normal AH cells reported here is strikingly similar to that of dimer excision in other normal human strains [28, 33]. Various XP strains have been shown to be defective in the excision of dimers [28, 33, 13, 19]; we find that analogous XP strains are deficient in the removal of nuclease-susceptible sites. The relative kinetics for the disappearance of these sites during a 9-hour period following exposure to 250 ergs/mm^2 of 254 nm radiation in XP cells in comparison with control AH cells (taken as 100%) are: XP5~35%; XP4, XP12, XP25, XP26~15%. These relative values are in agreement with the relative extent of UV-induced repair replication in the same strains [1, 20].

At low incident UV fluences (65 and 125 ergs/mm^2) to normal cells there is a distinct break in the curve depicting the elimination of nuclease-susceptible sites with time of incubation (see Fig. 5). We do not know the nature of the cellular events

responsible for the slower process which occurs after about 70% of the dimers have been removed by excision repair. It may be, for example, that the remaining sites contain pyrimidine dimers in a stretch of DNA less accessible to excision repair such that, although the same repair enzymes are involved, the process occurs at a reduced rate. Alternately, the residual process may stem from postreplication repair. Although little is known about this DNA repair mechanism in mammalian cell systems [12, 31, 21, 2], de novo synthesis is thought to skip past a dimer resulting in a single-strand region containing the photoproduct with a gap in the complementary strand. These gaps remain open for substantial times (~ 1 hr in mouse lymphoma cells[21]) before being closed by a second polymerisation process. It is reasonable to expect that the intermediate single-strand structure, like denatured UV-irradiated bacterial DNA [16, 4], constitutes an unsuitable substrate for the UV endonuclease from M. luteus. Hence, though still present in the DNA, the dimers are undetected by our in vitro assay. Such a possibility could well account for at least a fraction of the nuclease-susceptible sites which disappear from the DNA of UV-damaged XP cells during post-radiation incubation.

We have observed consistently that regardless of the strain, DNA from UV-irradiated cells has a slightly lower sedimentation constant (corresponding to 0.2 to 0.6 breaks per 10^7 daltons Mn after 250 ergs/mm^2)than DNA co-extracted from un-irradiated cells. One potential explanation for this phenomenon is that the observed molecular weight diminution in the DNA containing UV photoproducts simply reflects the instantaneous number of excision repair events between the incision and ligation steps at the time of cell lysis. The failure to detect an increase in the number of single-strand breaks in UV-damaged DNA extracted from incubated as compared to non-incubated repair-proficient AH cells argues against this possibility. Although the cause of the

difference in molecular weight between DNA's from damaged and undamaged cells has not been extensively studied, the phenomenon appears to be dependent upon the UV dose administered to the cells; the higher the initial dose, the greater the difference in molecular weight between the two populations of polynucleotide.

The precise step in excision repair monitored by the in vitro enzymatic assay is the one that renders the site containing a dimer no longer susceptible to attack by the purified UV endonuclease. The operational step affecting this conversion is most likely an incision event in which the presumed human analogue of the Micrococcus UV endonuclease breaks a phosphoester bond near the dimer. The normal AH strain is distinctly more proficient at performing this step than are the XP strains investigated here. Hence, it seems reasonable to conclude that in both classical XP and De Sanctis-Cacchione syndromes the functional form of the enzymatic machinery mediating strand incision is either present at a deficient level or in a conformation that severely retards its activity. The same conclusion has been reached in earlier independent studies [7, 33]. It should be pointed out that our data do not exclude other less direct explanations for the basic defect in XP cells. For example, as proposed elsewhere [19], the various steps in excision repair appear to be co-ordinated to the extent that the enzymes involved in mediating these steps may exist as one multi-enzyme complex. In such a complex, the failure to perform a later step in excision repair may in effect block the initiation of the repair process.

Evidence presented above strongly suggests that the in vitro assay described here follows the kinetics of initiation of dimer excision from the DNA of UV-damaged human cells. As such, the method complements two other well-established techniques used to quantitate dimer excision and repair replication (presumably later operational steps in excision repair) and is therefore useful for the characterisation of XP strains

which may be defective beyond the incision step. In principle, the procedure may be employed to follow any number of metabolic processes acting on a variety of chemically distinct lesions; the only limitation being the substrate specificity of the purified test endonuclease.

ACKNOWLEDGEMENTS

We are deeply indebted to R.A. Oosterbaan and H.L. Heijneker for their generous supply of the UV endonuclease. We are also grateful to A.P.M. Eker for providing the photoreactivating enzyme and to V. Der Kaloustian for supplying biopsies of human strains XP25 and XP26. We thank J.E. Donnellan, Jr., for assistance with the computer programming.

One of us (M.C.P.) is a Postdoctoral Fellow of the Medical Research Council of Canada.

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 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.

3 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.

4 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.

5 CARRIER, W.L., AND R.B. SETLOW, A paper strip method for assaying gradients containing radioactive macromolecules, Anal. Biochem., 43 (1971) 427-432.

6 CLEAVER, J.E., Defective repair replication of DNA in Xeroderma pigmentosum, Nature, 218 (1968) 652-656.

7 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.

8 CLEAVER, J.E., DNA damage and repair in light-sensitive human skin disease, J. Invest. Derm., 54 (1970) 181-195.

9 CLEAVER, J.E., Xeroderma pigmentosum: variants with normal DNA repair and normal sensitivity to ultraviolet light, J. Invest. Derm., 58 (1972) 124-128.

10 CLEAVER, J.E., Repair of damaged DNA in human and other eukaryotic cells, in D.W. RIBBONS, J.F. WOESSNER AND J. SCHULTZ (eds), Nucleic Acid-Protein

Interactions - Nucleic Acid synthesis in viral infection, North-Holland, Publishing Company, Amsterdam, London, (1971) 87-111.

11 CLEAVER, J.E., Excision repair: Our current knowledge based on human (Xeroderma pigmentosum) and cattle cells, in R.F.BEERS, R.M.HERRIOTT AND R.C.TILGHMAN (eds), Molecular and Cellular Repair Processes, Fifth International Symposium on Molecular Biology, Baltimore, June 3-4, Supplement Number 1 of the Johns Hopkins Medical Journal, The Johns Hopkins University Press, (1972) 195-211.

12 CLEAVER, J.E., AND G.H.THOMAS, Single strand interruptions in DNA and the effects of caffeine in chinese hamster cells irradiated with ultraviolet light, Biochem.Biophys.Res.Commun., 36 (1969) 203-208.

13 CLEAVER, J.E., AND J.E.TROSKO, Absence of excision of ultraviolet-induced cyclobutane dimers in Xeroderma pigmentosum, Photochem. Photobiol., 11 (1970) 547-550.

14 EKER, A.P.M., DNA-photoreactivating enzyme from Streptomyces griseus, Proc. VI Intern. Congr. Photobiol., Bochum, (1972) abstr. no. 118.

15 HEIJNEKER, H.L., H.PANNEKOEK, R.A.OOSTERBAAN, P.H.POUWELS, S.BRON, F.ARWERT AND G.VENEMA, *In vitro* excision-repair of ultraviolet-irradiated transforming DNA from Bacillus subtilis, Proc.Natl.Acad.Sci.(U.S.), 68 (1971) 2967-2971.

16 KAPLAN, J.C., S.R.KUSHNER AND L.GROSSMAN, Enzymatic repair of DNA. I. Purification of two enzymes involved in the excision of thymine dimers from ultraviolet-irradiated DNA, Proc.Natl.Acad.Sci.(U.S.), 63 (1969) 144-151.

17 KELLY, R.B., M.R.ATKINSON, J.A.HUBERMAN AND A.KORNBERG, Excision of thymine dimers and other mismatched sequences by DNA polymerase of Escherichia coli, Nature, 224 (1969) 495-501.

18 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.

19 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.

20 KLEIJER,W.J., E.A. DE WEERD-KASTELEIN, P.H.M.LOHMAN, M.L.SLUYTER, W. KEIJZER, J.DE WIT AND D.BOOTSMA, The kinetics of UV-induced repair DNA synthesis in different forms of Xeroderma pigmentosum patients and their heterozygote parents, To be submitted to Mutation Res.

21 LEHMANN,A.R., Postreplication repair of DNA in ultraviolet-irradiated mammalian cells, J.Mol.Biol., 66 (1972) 319-337.

22 LEY,R.D., AND R.B.SETLOW, Rapid repair of lesions induced by 313 nm light in bromouracil-substituted DNA of Escherichia coli, Biochem.Biophys.Res.Commun., 46 (1972) 1089-1094.

23 MARMUR,J., A procedure for the isolation of deoxyribonucleic acid from microorganisms, J.Mol.Biol., 3 (1961) 208-218.

24 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.

25 PAINTER,R.B., The importance of repair replication for mammalian cells, in R.F.BEERS, R.M.HERRIOT AND R.C.TILGHMAN (eds), Molecular and Cellular Repair processes, Fifth International Symposium on Molecular Biology, Baltimore, June 3-4, Supplement Number 1 of The Johns Hopkins Medical Journal, The Johns Hopkins University Press, Baltimore, 1972, 140-146.

26 PARIBOK,V.P., AND N.V.TOMILIN, Recognition of pyrimidine dimers in DNA by the incision enzyme from Micrococcus lysodeikticus, Nature New Biol. 230 (1971) 210-211.

27 REED,W.B.,B.LANDING,G.SUGARMAN,J.E.CLEAVER AND J.MELNYK, Xeroderma pigmentosum:Clinical and laboratory investigation of its basic defect, J.Amer.Med.Ass., 207 (1969) 2073-2079.

28 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.

29 REGAN,J.D., R.B.SETLOW AND R.D.LEY, Normal and defective repair of damaged DNA in human cells: A sensitive assay utilizing the photolysis of bromodeoxyuridine, Proc.Natl.Acad.Sci.(U.S.), 68 (1971) 708-712.

30 ROOK,A.,D.S.WILKINSON AND F.J.G.EBLING, Textbook of Dermatology, Vol. I, Blackwell, Oxford and Edinburgh, 1968, p. 62.

31 RUPP,W.D.,E.ZIPSER,C.VON ESSEN,D.RENO,L.PROSNITZ AND P.HOWARD-FLANDERS, Repair and reconstruction of chromosomal DNA after replication, in Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy,Brookhaven Natl.Lab. Publication BNL 50s03 (C-57), 1970,pp. 1-13.

32 SETLOW,J.K.,M.E.BOLING AND F.J.BOLLUM, The chemical nature of photoreactivable lesions in DNA, Proc.Natl.Acad.Sci. (U.S.), 53 (1965) 1430-1436.

33 SETLOW,R.B.,J.D.REGAN,J.GERMAN AND W.L.CARRIER, Evidence that Xeroderma pigmentosum cells do not perform the first step in repair of ultraviolet damage to their DNA, Proc.Natl.Acad.Sci.(U.S.), 64 (1969) 1035-1041.

34 SETLOW,R.B., AND J.K.SETLOW, Effects of radiation on polynucleotides, Ann.

Rev. Biophys. Bioeng., 1 (1972) 293-346.

35 WEERD-KASTELEIN, E.A. DE, W. KEIJZER AND D. BOOTSMA, Genetic heterogeneity of Xeroderma pigmentosum demonstrated by somatic cell hybridization, Nature New Biol., 238 (1972) 80-83.

Table 1.

Estimation of the absolute number of UV endonuclease-susceptible sites disappearing from UV-damaged DNA per normal human cell during 9 hours of post-radiation incubation.

UV dose (ergs/mm^2)	Number of dimers initially induced ($\times 10^{-4}$) a)	% Sites disappearing b)	Number of sites disappearing ($\times 10^{-4}$)
65	65	69	45
125	125	54	71
250	250	28	70
500	500	14	70

a) Calculation based on data in reference 10. Although the actual values may be in error by a factor of two, our conclusion would remain the same.

b) From fig. 5.

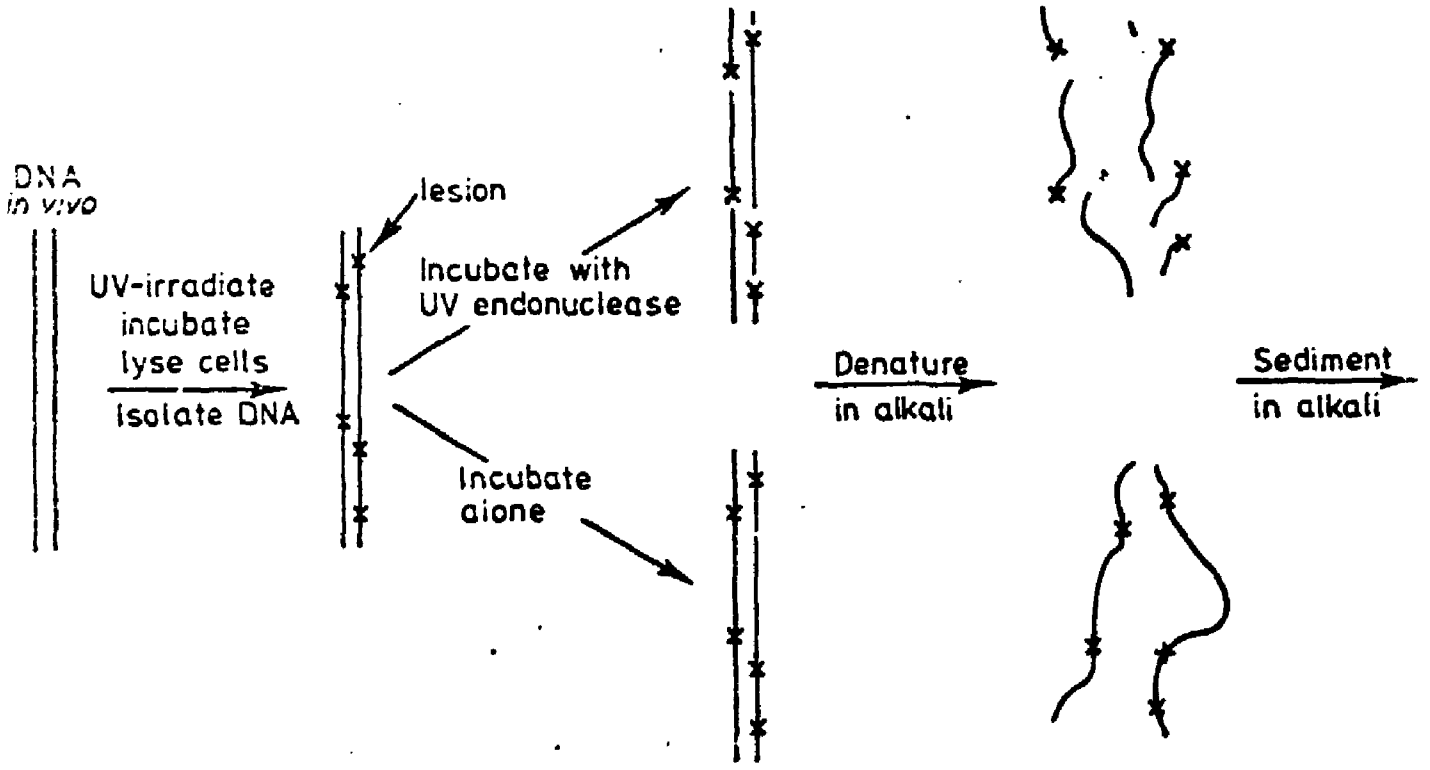

Fig. 1.

General protocol illustrating the use of a UV endonuclease from *M. luteus* to measure the number of UV photoproducts in ^{3}H-labelled human DNA damaged *in vivo*. Although not depicted here, ^{14}C-labelled DNA from undamaged cells was co-extracted with damaged DNA and thus served as an internal control to detect the number of non-specific strand breaks occurring during the nuclease treatment.

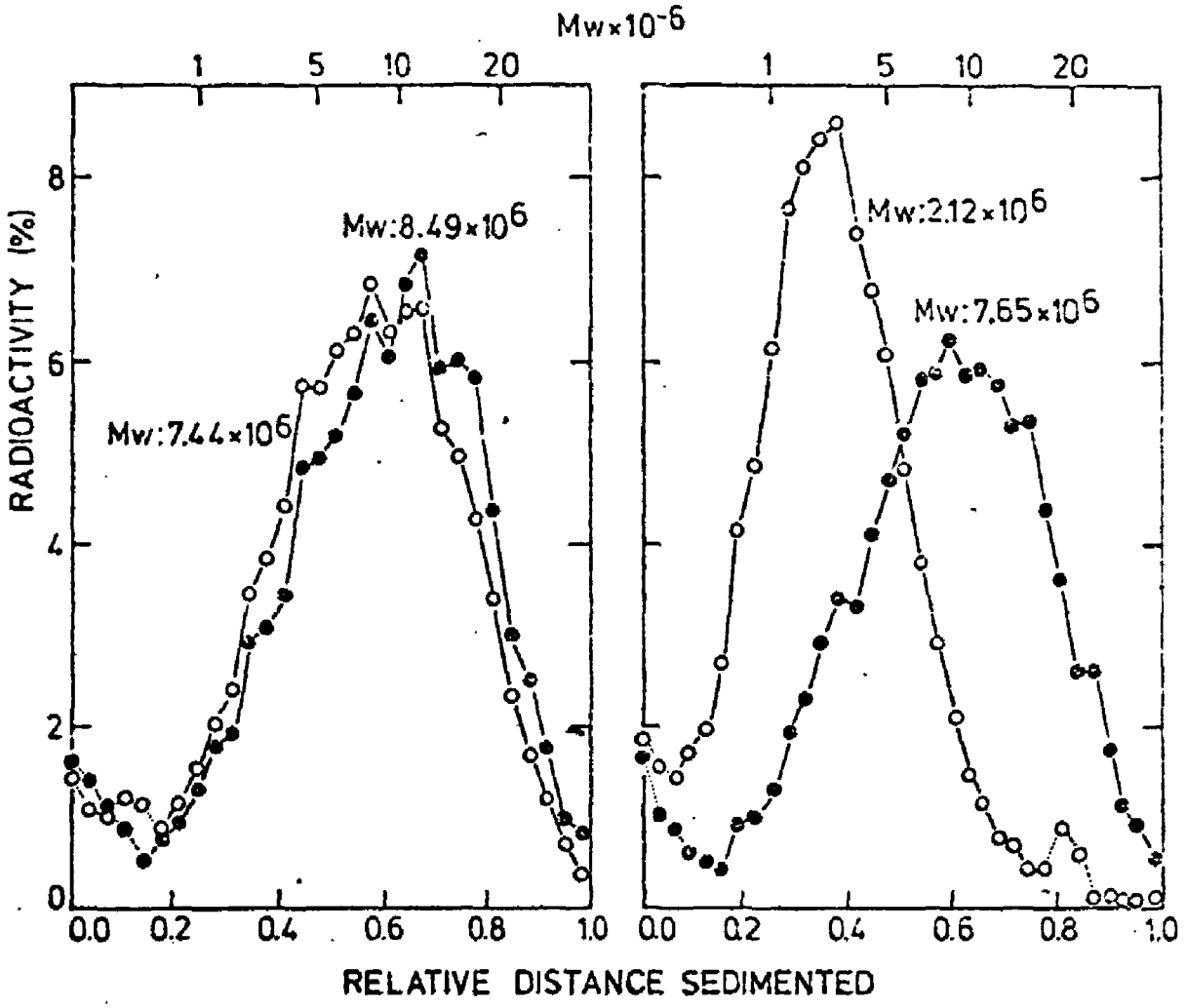

Fig. 2

Sedimentation profiles in alkaline sucrose gradients of radioactive DNA co-extracted from ^{3}H-labelled, UV-irradiated (250 ergs/mm^{2}) (o) and ^{14}C-labelled, unirradiated (•) AH human cells and incubated in the absence (left panel) or presence (right panel) of an endonuclease from M. luteus selectively active on UV-irradiated DNA. The radioactivity patterns were reproduced from tracings plotted by a computer. The portions of each pattern traced with a dotted line correspond to those gradient fractions excluded in the calculation of the weight average molecular weight of the sedimenting DNA.

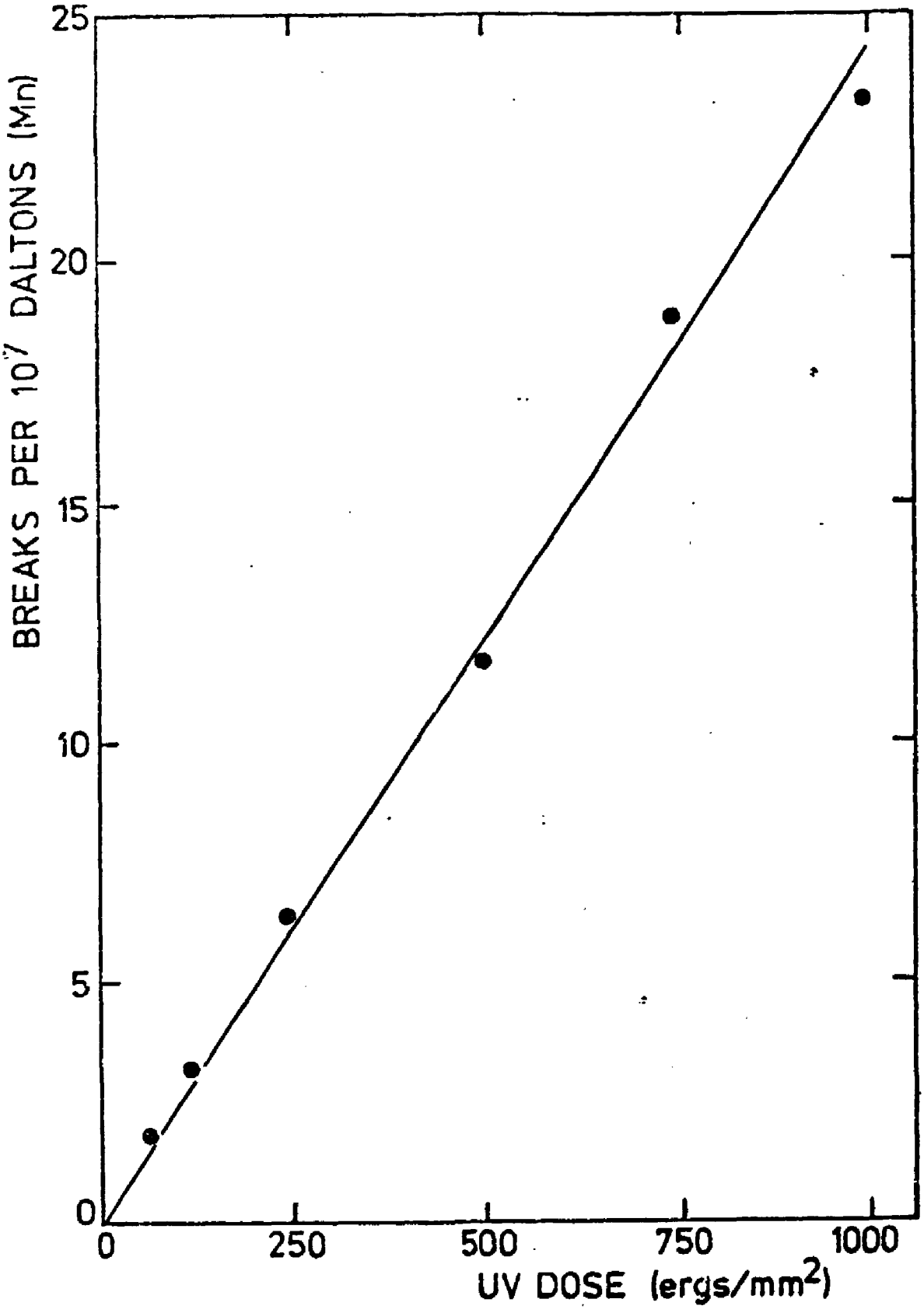

Fig. 3
Relationship between frequency of single-strand incisions selectively introduced by the purified UV endonuclease *in vitro* and UV fluence incident upon the assayed human DNA (extracted from control AH cells) *in vivo*. Each point on the curve was determined by performing an experiment like that depicted in Fig. 1 to generate sedimentation profiles as in Fig. 2. The extent of strand breakage by the purified enzyme was calculated for each irradiated DNA sample as described in the text. Each point is the arithmetic mean of 3 or more independent determinations.

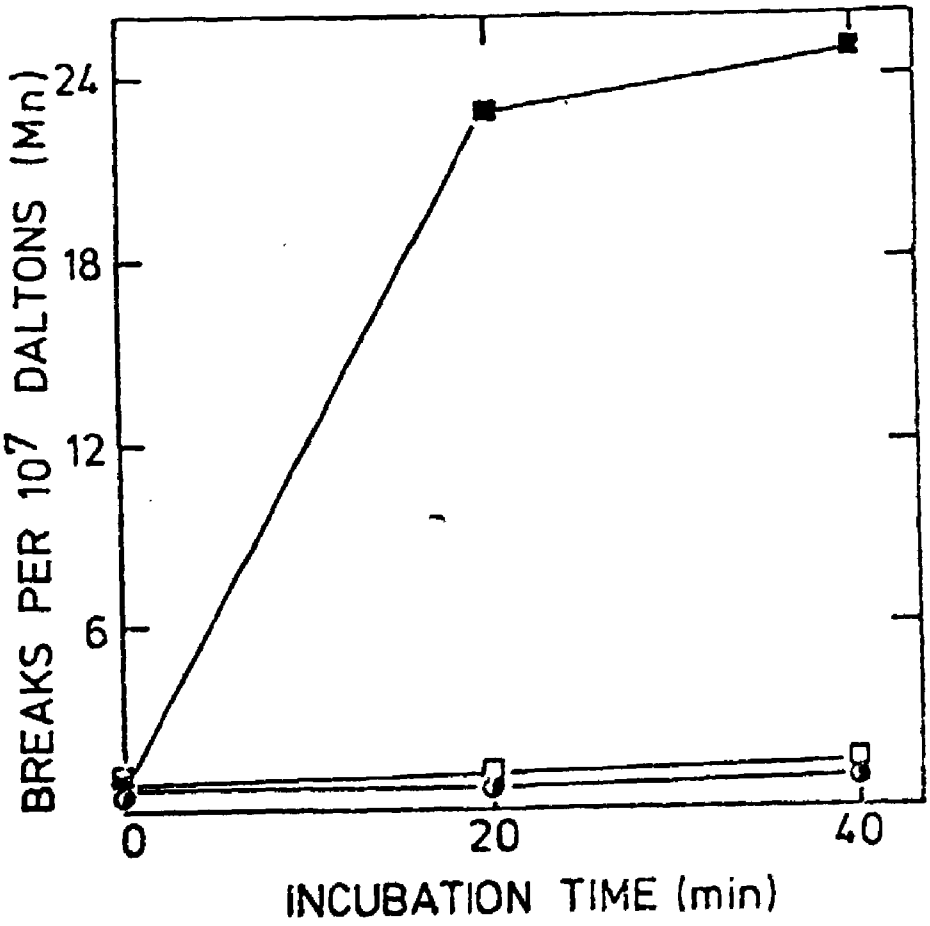

Fig. 4.
Enzymatic photoreactivation of UV endonuclease-susceptible lesions (detected as single strand scissions) in human DNA irradiated in vivo. The DNA of UV-exposed (1000 ergs/mm^2) AH cells was extracted and treated with photoreactivating enzyme under visible light (open symbols) or in the dark (closed symbols). The samples were then incubated for 20 min with (squares) or without (circles) the UV endonuclease from M. luteus. Half of each sample was removed while the remainder was incubated a further 20 min with (squares) or without (circles) an additional aliquot of the purified endonuclease. Finally, the products of the reactions were analysed by sedimentation in alkaline sucrose.

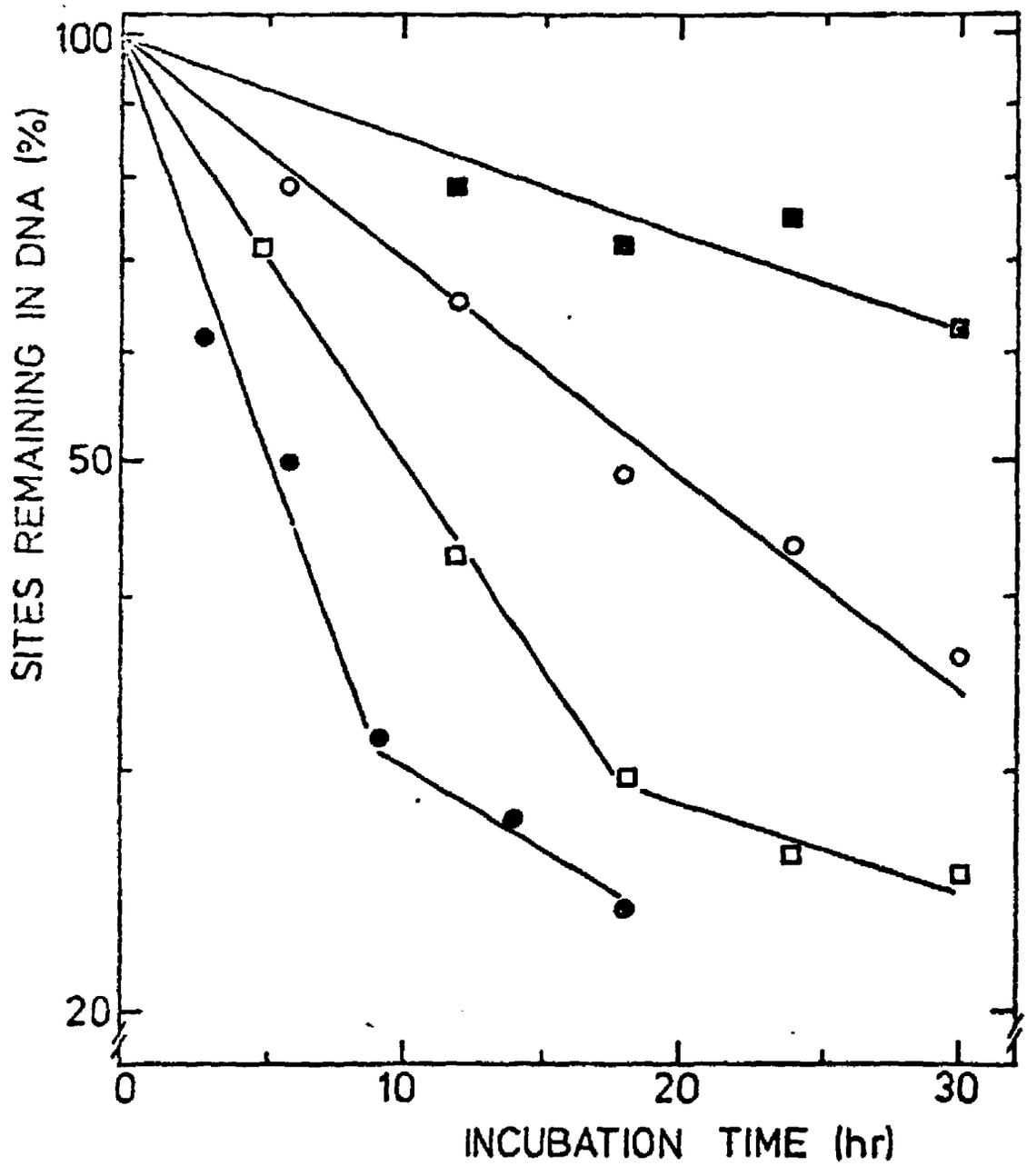

Fig. 5.
Time course of disappearance of UV endonuclease-susceptible sites from the DNA of normal AH cells irradiated with measured fluences of 254 nm radiation. After specified periods of post-UV incubation, cell samples were assayed as depicted in Fig. 1 to determine the number of nuclease-susceptible sites remaining in the extracted DNA. The percentages shown for the incubated samples are relative to that found for the parallel non-incubated ones. The actual number of sites initially induced by each UV dose (i.e. those detected in the non-incubated samples and expressed as 100%) may be determined from Fig. 3. Each point is the arithmetic mean of 2 or more independent determinations. Incident UV dose (ergs/mm^2): (●) 65 - (□) 125, (○) 250, (■) 500.

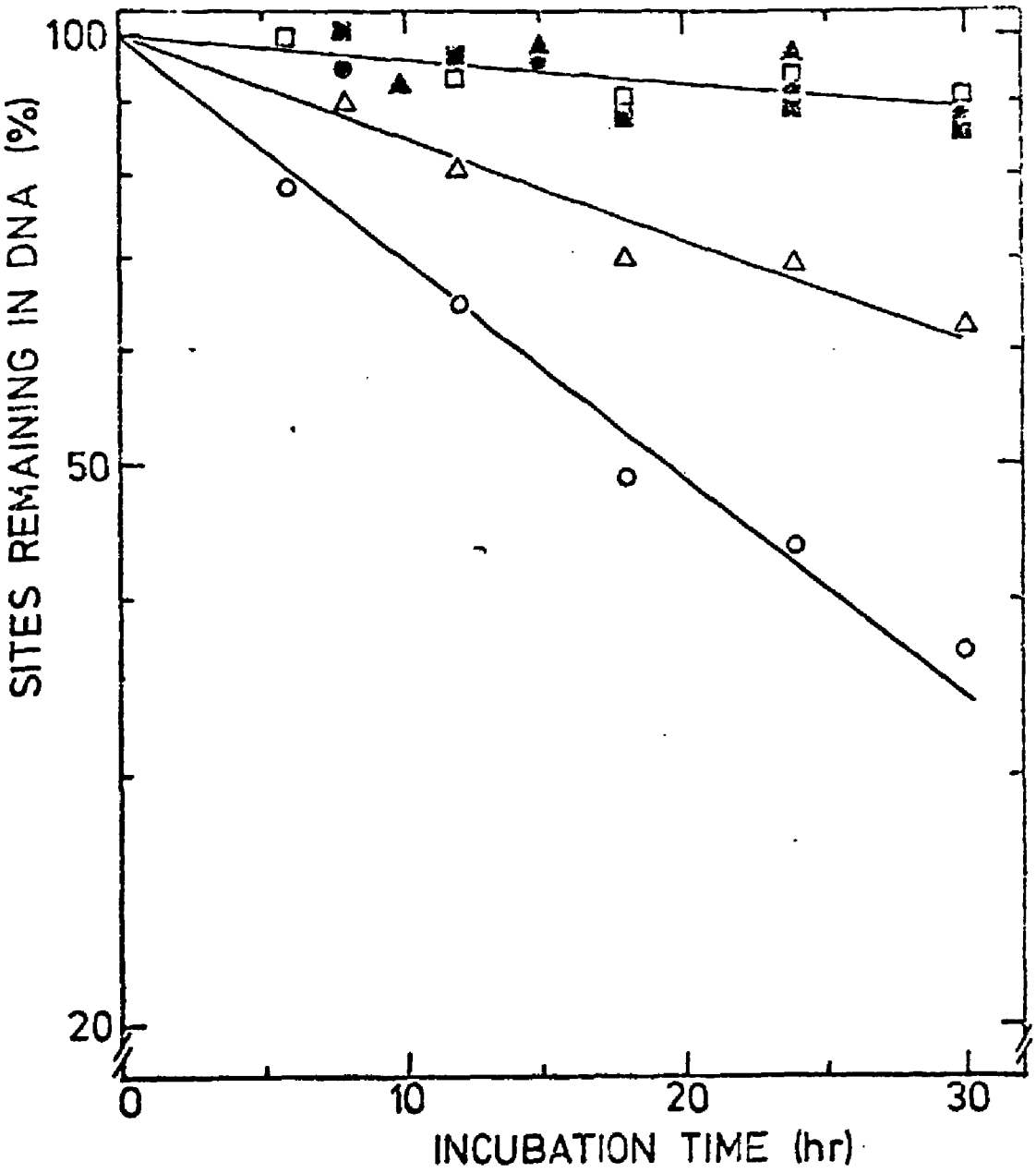

Fig. 6

The relative time course of disappearance of UV endonuclease-susceptible sites from the DNA of several XP strains exposed to 250 ergs/mm^2 of 254 nm radiation. For comparision, corresponding data for normal AH cells, taken from Fig. 5, are re-plotted here. Data were generated as in Fig. 5. Each point is the arithmetic mean of 2 independent determinations. (□), XP4; (△), XP5; (○), XP12; (■), XP25; (▲), XP26; (●), AH.