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MUTATION AND REPAIR IN AN ULTRAVIOLET-SENSITIVE CHINESE HAMSTER OVARY CELL LINE

Richard D. Wood
(Ph.D. thesis)

November 1981

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MUTATION AND REPAIR
IN AN ULTRAVIOLET-SENSITIVE CHINESE HAMSTER OVARY CELL LINE

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Ph.D. Thesis

November 1981

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Mutation and Repair

in an Ultraviolet-Sensitive Chinese Hamster Ovary Cell Line

By

Richard D. Wood

ABSTRACT

An ultraviolet (UV) light-sensitive mutant of Chinese hamster ovary cells (CHO) has been isolated and characterized with respect to a number of post-irradiation responses. The UV-sensitive mutant, termed 43-3B, was isolated by replica plating of mutagenized CHO cells, followed by a challenge with UV radiation. 43-3B has the same growth rate and chromosome number as the wild-type CHO-9.

43-3B is hypersensitive to the lethal effects of UV light (D_o of 0.3 J/m^2 as compared to 3.2 J/m^2 for the wild-type). A marked UV-hypermutability is observed in 43-3B as compared to the wild-type, as measured with markers for induced resistance to 6-thioguanine, ouabain, and diphtheria toxin. A factor of 38 to 65 more mutations are induced per unit fluence in 43-3B than in CHO-9.

The UV-sensitive mutant is also sensitive to killing by simulated solar light, although the D_o ratio is not as great as for germicidal UV. This is an indication that

the lesions produced by far UV and solar light are partially separable in terms of the types of repair dealing with them.

43-3B exhibits only about 17% of the wild-type level of UV-stimulated DNA repair synthesis, as measured by autoradiography of G_1 phase cells. When UV repair-induced strand breaks are measured by the nucleoid sedimentation method, 43-3B appears to be capable of carrying out only limited incision.

A much reduced ability to recover control rates of semiconservative DNA synthesis after UV irradiation was observed in the repair-deficient 43-3B cell line, suggesting that the removal of UV-induced replication blocks by excision repair is the most important factor in allowing recovery of UV-inhibited DNA synthesis.

Recovery of colony-forming ability between fractionated UV exposures was observed in the wild-type CHO-9, but little recovery was seen in 43-3B. This indicates that excision repair capability can also be important in split-fluence recovery.

The present investigation demonstrates that a sensitive/wild-type pair of CHO cell lines can be used in comparative studies to determine the involvement of repair in a wide range of post-irradiation phenomena.

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This dissertation is dedicated to the memory of Jack Burki, a teacher in every sense of the word.

CHAPTER 1

INTRODUCTION

1.1. Background

Probably the major function of DNA repair systems is the elimination of potentially lethal damage. This is emphasized by the fact that many repair mutants in bacteria (reviewed in Kimball, 1978; 1979), yeast (reviewed in Lemontt, 1980; Cox 1977), and mammalian cells were initially isolated on the basis of their sensitivity to killing by radiation or other agents which damage DNA. However, the ways in which damage to DNA eventually leads to cell death or mutation induction are rather poorly understood and a number of pathways appear to be involved.

Visible changes in the genetic material as seen in radiation-induced chromosome aberrations have long been interpreted as being due to mis-rejoining of broken chromosomes. In this sense, the idea that mutation induction is related to faulty repair is an old one. The explicit statement that mutations can be caused by a repair system that makes errors in fidelity was put forth by Witkin (1967). There is now abundant evidence that mutation induction is dependent on the presence of specific repair pathways in bacteria and yeast. In mammalian cells, most

of our information on the relationship of repair to mutation induction and cell death has come from studies of several inherited human diseases which show hypersensitivity to radiation or other DNA damaging agents (reviewed in Cleaver, 1980).

The following discussion is limited to pathways of repair and mutation induction after ultraviolet irradiation.

The large number of genes known to affect UV sensitivity in various organisms can be divided into several classes on the basis of their effect on UV-induced mutability. Some loci appear to have little effect on UV mutability (such as phr and polA in E. Coli, and a number of rad mutants of S. cerevisiae). Mutants defective in excision repair of UV damage include uvrA and uvrB in E. coli, and at least 10 different loci in S. cerevisiae comprising the rad3 epistatic group (Kimball, 1978; Reynolds and Friedberg, 1981). These excision-defective mutants of E. coli and yeast are all more mutable by UV than the wild-type (Hill, 1965; Witkin, 1967,1969; Eckardt and Haynes, 1977; Lawrence and Christensen, 1976; Lemontt, 1980; Haynes, 1981).

On the other hand, mutants at a number of loci show little or no induced mutation after UV. These loci in E. coli include recA, lexA (exr) and umuC which function in

an inducible repair system (Witkin, 1971; Kato and Shinoura, 1977). The increased mutability is thought to be due to a loss of replicative fidelity in repaired regions of the DNA. Mutants in the rad6 epistatic group of yeast also show absence of mutation induction by ultraviolet irradiation (Prakash, 1977). The loci necessary for mutation induction do not appear to control excision repair.

Cells from human xeroderma pigmentosum (XP) are UV sensitive and deficient in excision repair (Cleaver 1968, 1970b). An increased response to UV mutability is found in these cells. Several UV sensitive lines of Chinese hamster ovary cells also appear to be deficient in excision repair and show a high yield of UV-induced mutation (Busch et al., 1980; Thompson et al., 1980; this work). At least one UV sensitive isolate of CHO is apparently proficient in excision repair and hypomutable by UV, but deficient in a "postreplication recovery" process (Stamato et al., 1981).

The general picture thus emerges that excision defective cells are UV-hypermutable, and that mutations are dependent on another pathway (or pathways) of repair.

What are the primary lesions responsible for UV-induced mutations and lethality? Pyrimidine dimers are an abundant lesion detected in DNA after UV-irradiation, and there are two main types of evidence supporting their

importance in killing and mutation induction.

(1) In completely repair-deficient bacteria and yeast, only 1 to 2 dimers per genome are required to provide 1 lethal "hit" per cell (Howard-Flanders et al., 1969; Cox and Game, 1974).

(2) A large fraction of lethal damage is photoreactivatable in various organisms (reviewed in Harm, 1980). Photoreactivating enzyme is thought to be specific for pyrimidine dimers, but as pointed out by Zelle et al.(1980), this specificity is not definitely proven. Pyrimidine dimers seem to be lethal in completely repair-deficient strains; but their importance is less clear for repair-proficient cells. Either unrepaired dimers or some other unrepaired lesion most likely leads to lethality.

For mutation induction, similar arguments for the involvement of dimers can be given. Excision-repair deficient strains are generally hypermutable, as discussed above. Excision repair is certainly not specific for dimers, so this argument alone does not prove that pyrimidine dimers are the main mutagenic lesion. There is a large photoreactivatable sector (but less than 100%) for induced mutation in bacteria and yeast.

The sequence data of Coulondre et al. (1978) for UV-induced GC to AT transitions to nonsense mutations in the

E. coli lac I gene show that almost all the mutations occur in the 3′ base of a pyrimidine pair. Likewise, the data of Lawrence and Christensen (1979) for UV-induced transitions in the iso-1-cytochrome c gene of yeast show that virtually all of the base substitutions occur in a position which includes the 3′ end of a pair of pyrimidines (thymine or cytosine).

Lesions involving pairs of pyrimidines thus seem to be strongly implicated in UV-induced mutation. It must be emphasized, however, that there are many other types of lesions induced by UV light in DNA besides pyrimidine dimers. For a review of non-dimer lesions in DNA induced by UV, see Rahn (1979).

There is evidence that UV-induced mutation may be "untargeted" as well as "targeted". Mutations that originate as targeted errors are of the type just described, and can be explained as directly arising from mis-pairing at template lesion sites during repair of DNA damage. Untargeted errors do not arise directly at primary lesion sites, and might occur independently of DNA repair activity, or during repair replication in relatively long patches which include undamaged regions. Witkin and Wermundsen (1978) have argued that untargeted errors contribute little to bacterial UV mutagenesis, but may cause a substantial fraction of UV-induced mutations in bac-

teriophages. Lawrence (1981) has proposed that a large fraction of UV-induced mutation in yeast may be untargeted.

DNA repair and the processes leading to mutagenesis appear to be more complicated in yeast (a eucaryote) than in bacteria (a procaryote). These processes may be even more complicated in mammalian cells. In bacteria and yeast, there are a large number of UV-sensitive and DNA repair-deficient mutants which have allowed and will continue to allow a study of mutagenesis in some detail. The situation in mammalian cells is much weaker, with fewer mutants and systems available for the study of mechanisms leading to cell death and mutation induction.

Some understanding of the nature of DNA repair mechanisms in mammalian cells has been provided by the study of inherited diseases with increased sensitivity to DNA damaging agents, such as xeroderma pigmentosum (XP), ataxia telangiectasia, Fanconi´s anemia, Bloom´s syndrome, and Cockayne´s syndrome (reviewed in Cleaver, 1980). In recent years, a number of UV-sensitive mammalian cell lines have been isolated from mouse and hamster cells, some showing DNA repair deficiencies (Thompson et al., 1981; Stamato and Waldren, 1977; Busch et al., 1981; Adair, 1980; Kuroki and Miyashita, 1977; Sato and Hieda, 1979).

It appeared to this investigator that a study of the mechanisms of radiation- or chemical-induced effects on mammalian cells would be facilitated by a study of a repair-deficient cell line of Chinese hamster ovary (CHO) cells.[1]

An examination of DNA damage, repair, replication, and mutagenesis in this new system has a number of advantages over studies in human cell lines:

(1) The cells are permanent cell lines, are relatively easy to grow, and are fast-growing for a mammalian cell line (generation time of about 12 hours). Assays for colony-forming ability can be performed easily.

(2) Conditions have been worked out for a relatively straightforward assay of mutations at several well-studied markers.

(3) Variants can be compared directly with a "wild-type" parental cell line, in a near-isogenic background.

(4) The cells can be synchronized by several methods, allowing examination of the age-responses to a number of agents.

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[1]A summary of the history of the CHO cell line is given in Appendix A.

(5) Very sensitive or unusual variants can be obtained that might not be found in a population of viable humans.

1.2. Assays for Mutation in CHO

There are more than 20 markers developed in CHO alone for somatic cell mutagenesis studies with a few more added each year (reviewed in Lewin, 1980). Utilization of these markers (mostly as assays for acquisition of drug resistance or reversion to prototrophy) provides a measure of specific types of mitotically stable phenotypic variants in a population. At least six of the markers, including 6-thioguanine resistance, ouabain resistance, and diphtheria toxin resistance, have been characterized to an extent which makes them suitable for quantitative studies in CHO cells (reviewed in Gupta and Siminovitch, 1980). This is because for each marker, the existence of an altered protein product in variant cells has been established (see below), allowing identification of these variants as mutants of genetic origin.

Resistance to 6-thioguanine under the selective conditions described arises from a deficiency in hypoxanthine-guanine phophoribosyl transferase (HGPRT), an enzyme in the pyrimidine salvage pathway (Sharp et al., 1973; Aebersold, Ph.D. Thesis U.C. Berkeley, 1975; Beaudet, et al., 1973). These resistant mutants can arise

either by point mutation or by deletions in the HGPRT gene (Chu, 1971; Cox and Masson, 1978). 6-thioguanine resistance has been used as a marker for assaying radiation- and chemical-induced mutations in a number of laboratories (Hsie et al., 1975, 1977, 1978; Thacker et al., 1977; Cox et al., 1977; Burki, 1980; Burki and Aebersold, 1978).

Ouabain resistance arises from an alteration which prevents the drug from its usual action of inhibiting the Na^{+}/K^{+} ATPase, while still allowing the ATPase to function (reviewed by Baker and Ling, 1978). This alteration might be in the ATPase itself or in adjacent membrane components. Ouabain-resistant mutants of CHO were isolated and characterized by Baker et al. (1974). Induction of ouabain resistance has been in use as a selective marker in Chinese hamster cells for a number of years (Baker et al., 1974; Arlett et al., 1975; Burki and Aebersold, 1978; Goth-Goldstein and Burki, 1980; Chang et al., 1978; Thacker et al., 1978).

Resistance to diphtheria toxin is the most recently described marker for quantitative mutagenesis studies in CHO cells. Some of the characteristics of the assay for this mutational change are described in this thesis. Diphtheria-toxin resistance (DT^{r}) has been characterized in CHO cells in several laboratories (Moehring and Moehring, 1979; Gupta and Siminovitch, 1978; Draper et al.,

1979). Virtually all DT^r mutants isolated in CHO cells fall into two major classes (in the terminology of Moehring and Moehring): class I and class II. Class I mutants have a membrane alteration which prevents entry of the toxin into the cell. Class II mutants have an altered protein synthesis factor, EF-2, which is the target for diphtheria toxin in the cell. The altered EF-2 protein in a resistant class II mutant cannot be inactivated by toxin-catalyzed ADP-ribosylation. At concentrations around 1 Lf/ml (see below), virtually all mutants recovered are found to be in class II; that is, they are true protein synthesis mutants.

One way to distinguish between EF-2 mutants and membrane mutants is to test for cross-resistance to _Pseudomonas aeuroginosa_ exotoxin A, a toxin which has the same action as diphtheria toxin once it is inside the cell, but which has a different mode of internalization via receptor-mediated endocytosis (Fitzgerald et al., 1980). Cells isolated in a single-step selection and which are simultaneously resistant to diphtheria toxin and _Pseudomonas_ toxin have been found to be EF-2 mutants (Moehring and Moehring, 1979).

1.3. Sensitivity to Solar Light

Sunlight is the only radiation in the natural environment which has substantially harmful consequences

on biological systems. There is a large body of evidence linking chronic sunlight exposure with skin cancer in humans (Blum, 1959). Patients with the inherited disease xeroderma pigmentosum show a greatly increased induction of skin cancer by sunlight, associated with a defect in repair of lesions induced by ultraviolet irradiation. Most of the far-UV irradiation emitted by the sun is absorbed by stratospheric ozone; so that the shortest wavelengths reaching the earth's surface are around 290 nm. The solar spectrum thus consists mostly of near-ultraviolet, visible light and infrared radiation. Since there is an overlap with the tail of the DNA absorption spectrum between 290 and 320 nm, the adverse effects of sunlight are critically dependent on the fluence in this region.[2]

In the region from 320 to 290 nm, the spectral irradiance of sunlight decreases at the earth's surface by about 5 orders of magnitude (Green et al., 1974). The

[2]Action spectra for various endpoints in mammalian cells show clearly that biological effectiveness falls off rapidly above 300 nm. Action spectra for cell lethality in this region have been reported by Todd et al. (1968) and Rothman and Setlow (1979) for Chinese hamster cells, and by Kantor et al. (1980) for human cells. An action spectrum for mutagenesis in L5178Y mouse cells was reported by Jacobson et al. (1981) and for cell transformation by Doniger et al. (1981). A short review of this subject has been presented by Coohill and Jacobson (1981). In general the action spectra for all of these biological endpoints in mammalian cells show a broad peak between 260 and 280 nm.

fluence in this region is also the most subject to change by factors such as variation in latitude, time of day, altitude, and natural or artificial atmospheric contaminants.

Although sunlight produces pyrimidine dimers (Trosko et al., 1970), and induces DNA repair synthesis (Cleaver, 1970a), there appears to be a spectrum of near UV-induced lesions which is different than that for far UV lesions. For example, Mang and Hariharan (1980) found that the ratio of CT to TT dimers was about 0.3:1 after 254 nm irradiation but that CT dimers predominate after 313 nm irradiation. Hariharan and Cerruti have found (quoted in Webb, 1977) that the ratio of thymine glycols to thymine dimers produced in HeLa S3 cells increases by a factor of 14 from 260 nm to 313 nm. Sites in phage DNA which are sensitive to endonuclease V can be specifically induced by near UV (Childs et al., 1978). Some type of single strand break, rather than dimers, may be critical for the lethal effect of near UV (Elkind et al., 1978; Elkind and Han, 1978; Webb and Brown, 1976).

There is also an oxygen dependence for near-UV-induced DNA breakage, lethality, and mutation induction (Webb, 1977). The oxygen dependence is thought to be due to oxygen-sensitized photodynamic action resulting in the production of damaging free radicals. The oxygen depen-

dence for near UV lethality is not seen for far UV, and has been shown to occur in mammalian cell lines (Danpure and Tyrrell, 1976).

Very few studies have actually used sunlight. The ability of sunlight to inactivate bacteria was reported by Downes and Blunt (1877) who exposed tubes of bacteria cultures to sunlight and sunlight filtered through different colored glasses. Apparently the first survival curve for bacteria exposed to sunlight was produced by Luckiesh (1946) at the G.E. Research Labs in Cleveland. Hollaender and Emmons (1946) first studied the mutagenic effects of sunlight, by scoring morphological variants of Aspergillus terreus. Ashwood-Smith et al. (1967) examined the lethal and mutagenic effects of sunlight on E. coli and found more killing and mutation induction when the cells were exposed at -60° rather than at ambient temperature.

Harm (1969) showed that a UV sensitive strain of E. coli (uvrA recA) was also sensitive to sunlight. Survival decreased to 1% within a minute's exposure. However, for inactivation to the 10^{-3} survival level, E. coli B/r phr requires a 600 times greater fluence of 254 nm radiation than the uvr recA strain, but only a 30 times greater fluence of sunlight. Evidently, sunlight produces some damage which is not repaired by the normal UV repair systems. In fact it has been generally noted (Calkins and Barcelo,

1979), that very UV-sensitive mutants of various organisms are not sensitized equally to near- and far-UV, suggesting that the repair mechanisms do not completely overlap.

Resnick (1970) studied sunlight-induced killing in wild type and rad 2 (uvs 9) strains of yeast. A large fraction of killing in the excision defective strains appeared to be photoreactivatable. In a phr background, a rad 2 strain was sensitive to sunlight and was killed to a 20% survival level after 10 to 20 minutes exposure on a sunny day. The RAD^+ strain required 90 to 120 minutes exposure to reach the same survival level.

Mammalian cells also suffer lethal and mutagenic consequences of sunlight, as shown by Hsie et al. (1975) in CHO cells, and by Krell and Jacobson (1980) in L5178Y mouse cells. Single strand breaks are produced in mammalian cells by sunlight, and can be measured by alkaline elution (Erickson et al. 1980) or by nucleoid sedimentation (Parsons and Goss, 1980). Parsons and Goss were also able to conclude that DNA repair kinetics were different for far-UV and sunlight-induced damage.

1.4. Rationale for the Study

A major purpose of this study was to isolate a UV-sensitive, DNA repair deficient CHO cell line and to determine the effect of this deficiency on mutation induc-

tion by ultraviolet light. As discussed above, current evidence from both microorganisms and mammalian cells suggested that marked effects would be seen. The UV-sensitive cell line which was isolated compares closely to the wild-type in terms of its growth rate and chromosome number. These properties, in combination with the other advantages of CHO cells mentioned above, suggested that the UV-sensitive mutant could be used in parallel with the wild-type CHO to study post-irradiation phenomena which have been proposed to involve DNA repair. The second objective of the study was to examine two of these phenomena:

(1) Recovery of DNA synthesis rates after UV irradiation, which has been proposed to require repair or modification of UV-induced replication template lesions.

(2) Recovery of colony-forming ability between fractionated exposures of UV light, which has been ascribed to the action of a repair process for sublethal damage between the fractionated exposures.

Many studies on lethality induced by near UV and visible light have used monochromatic irradiation (reviewed in Webb, 1977). This allows the determination of an action spectrum for a specific effect and possible elucidation of the responsible chromophores. However, strong synergistic effects between light of different

wavelengths are known to occur in a number of systems (Webb, 1977). This means that some effects seen with broad-band sources may not be explained from action spectra obtained with monochromatic light.

As a third objective, it was decided to compare the responses of CHO-9 and 43-3B to broad-band solar wavelengths. As discussed earlier, dosimetry of sunlight is subject to many variations in the biologically relevant range, and reproducible conditions are difficult to obtain. For this reason, the light source used was a commercial solar simulator which closely reproduces both the spectrum and the total fluence from the sun under standard conditions.

On the one hand, the present study illustrates the extreme importance of repair capability in many of the effects of UV on mammalian cells. On the other hand, it is clear that all responses to radiation (UV and otherwise) are not simply dependent on one repair pathway.

CHAPTER 2

MATERIALS AND METHODS

2.1. Isolation of UV-Sensitive Mutants

The exact procedure used for replica plating and mutant isolation is described in Appendix B. The method was adapted from one described by Busch (Busch, Ph.D. Thesis, U.C. Berkeley, 1980; Busch et al., 1980). This method in turn was based on the principle of isolation by colony detachment from a monolayer described by Stamato and Waldren (1977) and a filter paper replica plating method described by Esko and Raetz (1978).

Cultures of CHO-9 were mutagenized by treatment with N-nitroso-N-ethylurea (ENU; CAS registry # 759-73-9). ENU was chosen in the hope that different complementation groups could be obtained with this mutagen than had been obtained from previous isolations using different mutagens (Thompson et al., 1980; Busch et al., 1980).

From a mutagenized population of CHO-9, two UV-sensitive cell lines were isolated from about 8000 colonies examined. In preliminary experiments the two UV-sensitive cell lines showed similar responses; 43-3B was chosen for detailed study so that the results reported here refer to this line and CHO-9.

2.2. Cell Culture

Both of these lines are negative in tests for PPLO and have a modal chromosome number of 21. The mycoplasma tests were performed both by testing for the incorporation of Hoechst 33258 dye into cell cytoplasm (examining by fluoresence microscopy), and by a number of tests performed on the cultures several times during this study by an independent commercial agency. The cells were grown in McCoy´s 5a medium supplemented with 7.5% fetal calf serum, 100 units per ml penicillin, 100 μg/ml steptomycin, and 1mM HEPES buffer. Cultures were kept at 37.5^{o} either in open plastic tissue culture flasks in a CO_2 incubator or in large, closed glass roller bottles in a warm room.

2.3. UV Exposures and Survival Determination

Cells were irradiated with germicidal UV in monolayer after rinsing with clear Puck´s saline A, in 90 mm plastic tissue culture dishes (asynchronous experiments), or $75cm^2$ tissue culture flasks with the tops removed (synchronous experiments). For asynchronous experiments, cells were plated at 2×10^6/dish and irradiated 18 hours later, at which time the cultures were in exponential growth. The ultraviolet light was produced by two germicidal UV lights in a specially constructed irradiation box. These lights produce more than 85% of their output at the 254 nm Mercury line. The box was equipped with a solenoid-operated

shutter and a rotating platform, so that dishes were rotated during the irradiation to help assure an even exposure. The UV fluence rate was determined with a Spectroline DM-254N ultraviolet fluence meter (Spectronics Corporation). The probe of this meter is very selective for the 254 nm line, and so gives 75% of the fluence rate registered by the broad-band YSI meter used in previous studies (Burki et al., 1980). Fluence rates of 0.3 to 1.5 J/m^2 were used.

After exposure to UV at room temperature, the cells were trypsinized (0.03% trypsin, Worthington, in Puck's saline A; 10 minutes at 37°) and a monocellular suspension was made in warm culture medium by pipetting. An aliquot was counted in normal saline in the Coulter counter, and dilutions were made in McCoy's 5a containing 1% fetal calf serum. Some cells were plated in 90 mm dishes to determine the plating efficiency after treatment.

After eight days of growth in normal medium at 37°, the dishes containing the surviving colonies were stained with 1% methylene blue. The media containing the stain was poured off and the plates were dried and then gently rinsed with water. The number of cells surviving irradiation was determined by counting visible colonies of 50 or more cells. This numerical cutoff mainly serves to eliminate from the count those small secondary colonies which

might arise during the final 2 to 3 days of incubation from the large primary colonies containing 1000 or more cells. This can occur if the dish is physically disturbed during the growth period.

2.4. Irradiation with Simulated Solar Light

Cells were irradiated with light by an Oriel 1000 watt Solar Simulator (Oriel Corporation of America, Stamford, Conn.). The characteristics of the light source are described in the results section. A 1000 watt high pressure Xenon lamp is powered by a model 8550-7 power supply. This lamp is situated in an illuminator housing containing collection optics that direct the light through a 12-inch fused silica collimating lens (figure 6). Since the standard xenon lamp produces some ozone, exhaust was led away from the lamp housing through a 4 inch diameter hose with a remote velocity blower connected to the fume hood in the laboratory.[1]

Four 6 cm dishes containing cells in suspension to be irradiated were placed on a metal tray in a 20° circulating water bath. The dishes (lids on) were covered with a

---

[1] Ozone at concentrations around 1 ppm has been shown to have toxic effects on man and on microorganisms, including tissue culture cells. A review of some of the toxicology appears in <u>Ozone and Other Photochemical Oxidants</u>, National Research Council, Committe on Medical and Biologic Effects of Environmental Pollutants, National Academy of Sciences (Washington, D.C., 1977).

quartz-bottom tray containing 50 ml of water at 37°. (see figure 7).

The total fluence rate to the cells through this filtering system (a spectral correction filter installed in the lamp housing, the Falcon lid dish, 3 mm water, and the quartz tray) was 700 $J/m^2/sec$ and was directly measured in each experiment using a YSI Model 65A radiometer (Yellow Springs Instrument Co.) equipped with a thermopile sensor. The probe window has a flat response (near 100% transmittance) from 300 to 3000 nm. In order to obtain this fluence, the Xenon lamp (less than 100 hrs lifetime in all experiments) was run at 40.5 Amps and 24 volts.

Cells were prepared in suspension by trypsinizing a 150 cm^2 flask containing 2-3 X 10^7 cells in exponential growth. An aliquot was removed and counted, and the remainder of the cells were centrifuged, washed once in Hanks' balanced salt solution, recentrifuged, and resuspended in Hanks' to 1.5 x 10^6 cells per ml. Two ml of this suspension was placed in a 6 cm plastic dish. After irradiation, or sham-irradiation in the dark, the cell suspension was decanted into a tube. To remove cells which had attached to the dish during irradiation, 1 ml trypsin was added, the dish was incubated for 3 min. at 37°, and the trypsinate was mixed with the suspension in the tube. Aliquots of the cell suspension were counted

and diluted for survival assay as described earlier in Chapter 2.

A number of early experiments showed that the cells were subject to both acute and delayed effects of heating from the light source. After 30 minutes exposure of cells in monolayer with no water filter many of the cells had disintegrated and detached, while those that remained attached required long trypsinization times to remove them. Similar effects have been described by Parsons and Goss (1980) in their experiments on human cells irradiated by sunlight. The problems were eliminated in the present experiments by irradiating the cells in suspension, using a water layer to filter out heat, and carefully circulating water around the dish bottoms to cool the cells.

2.5. Chromosome Spreads and Karyotyping

Chromosome spreads were made and karotyping performed essentially as described by Worton and Duff (1979).

To obtain mitotic spreads, a roller bottle was set up to contain about 5×10^7 cells in exponential growth on the day of the collection. Colcemid solution (Gibco) was added to fresh medium to give a final concentration of 0.6 µg/ml. Cells were incubated for 90 min at 37° in 20 ml of this medium in the roller bottle. The bottle was then spun at 180 rpm for three minutes on the cell synchronizer

apparatus (Talandic Research Corporation; Klevecz, 1975) to detach mitotic cells. The supernatant was centrifuged in a counter-top centrifuge for 10 minutes (800 rpm). All but 0.5 ml of the supernatant was decanted and the cell pellet was resuspended quickly but gently with a Pasteur pipette. Four ml of 0.075 M KCl was added and mixed well. The suspension was held for 4 minutes at room temperature to swell the chromosomes, then centrifuged for 5 minutes and the supernatant discarded. The pellet was resuspended, and 4 ml of cold, freshly made fixative (methanol: acetic acid, 3:1) was mixed with the cell suspension, which was then centrifuged for 5 minutes. The fixation step was repeated twice, after which 8 drops of fixative were mixed with the pellet. A drop of this suspension was dropped onto the slide from a height of about three inches. The slides were air-dried and then solid stained with Giemsa (2% Gurr's R66 Giemsa in 10mM potassium phosphate (pH 6.8) for 4 minutes, and rinsed in water.

2.6. Photomicrography

Chromosomes were counted at 630 X with bright field illumination on a Zeiss Photomicroscope II. Pictures were taken with Kodak SO-2415 film using the built-in 35 mm camera at ASA 6.3 and 12.5 and near-maximum illumination. Film was developed in Kodak HC-110 dilution D (8 min,

21°), rinsed 15 to 30 seconds in Kodak Stop Bath Sb-1a and fixed with Kodak Fixer for 2 to 4 minutes. The film was rinsed in running water for 15 seconds, bathed in Kodak Hypo Clearing Agent for 30 seconds, washed one minute in running water, and air dried.

Enlargements were made onto Kodabromide single-weight F3 paper which was developed for 2 minutes (Dektol:H_2O 1:2) followed by 10 seconds in Sb-1a stop bath, 10 minutes in Kodak Fixer, and 30 minutes in running H_2O. Some printing was done on a Rapidoprint machine with Agfa-Gevaert chemicals. Printing operations were carried out under sodium lamp illumination. Modal chromosome number was determined by counting 50 spreads from each cell type. The chromosomes from a print of a representative spread were cut out and arranged according to size and morphology in order to establish the karyotype.

2.7. Growth Rate After UV

The cell number in cultures after UV irradiation was determined by plating 1-2 X 10^6 cells in replicate 100 mm dishes, UV irradiating 18 hrs later, and then trypsinizing and counting the cells in one of the replicates at various times afterwards. Lethally irradiated cells lyse and detach from the dish and are not detected by the Coulter counter.

2.8. Selection for Drug and Toxin Resistance

After exposure to UV, the cells were trypsinized, the cell titre determined, and at least 2 X 10^6 cells were inoculated into a large roller bottle. An aliquot of cells was used at this time to measure cell survival. The cells were then grown for eight days; care was taken to prevent the cultures from becoming confluent. This required two subcultures of the bottles in control cultures and one subculture of the cells exposed to the higher UV fluences. After eight days, the cells were trypsinized, the cell number determined, and 10^5 cells were exposed to 6-thioguanine (6TG) at 5µg/ml, or 10^6 cells were exposed to either ouabain (OUA) at 3mM or diphtheria toxin (DT) at 1.0 Lf/ml in McCoy's 5a medium plus 7.5% fetal calf serum. A separate dilution was used to measure the plating efficiency of cells in drug-free medium. Plating efficiencies for unexposed cultures were about 70% for both CHO-9 and 43-3B. Diphtheria toxin was the product of Connaught Laboratories Limited, Willowdale, Ontario, Canada, lot 408.

Diphtheria toxin concentrations are expressed in Lf/ml. One Lf is the amount of toxin which reacts with one standard antitoxin unit in a Ramon-type flocculation test (Chase et al., 1977). The toxin was tested and given an Lf value by Connaught Labs, and this is the value used

in this work. One Lf contains about 2.5 μg protein.[2]

After 9 to 12 days of growth, diphtheria-toxin resistant colonies were stained with methylene blue and counted. Medium that included diphtheria toxin was poured into a plastic vessel containing formalin solution to deactivate any residual toxin activity before final disposal.

Pseudomonas aeuroginosa exotoxin A was a gift from Dr. S.J. Leppla of the U.S. Army Medical Research Institute of Infectious Diseases. It has 5000 MLD/mg of protein, and was used in selection experiments at 0.2 μg per ml. The toxins are stored in concentrated form (sterile aqueous solution) in 1 ml aliquots at -70°.

2.9. Measurement of Inhibition of DNA Synthesis

The UV-induced depression of DNA synthesis was measured by the incorporation of tritiated deoxythymidine into acid-precipitable material. About 10^6 cells in 6 cm plastic tissue culture dishes were rinsed once with clear

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[2] The symbol "Lf" was introduced by Glenny and Okell in 1924, as an *in vitro* unit of toxicity, in their development of the flocculation reaction between DT and antitoxin. Previously, two *in vivo* units of toxicity had been widely used. The symbol L_o stood for "limes nul", or "minimum boundary" in Latin; it was an amount producing minimal edema in a guinea pig when mixed with a unit of antitoxin. The symbol L_+ was given by Erlich ("limes tod") for a lethal amount of toxin mixed with one unit of antitoxin. "Lf" refers to flocculation limit and is thus another step in a Latin/German/English hybridization which is discussed more fully by Wilson and Miles (1964).

Puck's Saline A (PSA) and irradiated. The cells were then overlayed with 2 ml medium, or with 2 ml medium containing tritiated deoxythymidine (10 µCi/ml, 73 Ci/mmol) for 10 minute pulses or 1 µCi/ml 6.7 Ci/mmol for 2 hr periods, as indicated in the figure legends. At the end of the labeling period the medium was decanted, and the dishes were rinsed twice with PSA. One ml trypsin was added followed by one ml of medium; 1/2 ml of this cell suspension was used to determine the cell number and 1-1/2 ml were processed for determination of radioactivity.

The cells were then spun down in glass tubes (7 minutes, 800 RPM) and fixed by resuspending in 5 ml cold trichloroacetic acid (TCA), followed by recentrifugation. The fixation was repeated and the resulting pellet was resuspended in 1 ml TCA and heated at 90° for 30 minutes. The cooled supernatant was counted in 10 ml scintillation cocktail (PCS Phase Combining System, Amersham-Searle). Results were expressed as counts per minute per cell.

2.10. Unscheduled DNA Synthesis (UDS)

Autoradiographic measurement of repair synthesis was made on G_1 populations. About 3 x 10^6 cells were dispensed into replicate 75 cm^2 plastic flasks using the automatic cell cycle analyzer apparatus (Talandic Research Corporation; Klevecz, 1975). After allowing 30 minutes for cell attachment, the flask tops were cut, and the

cells were rinsed and then irradiated or sham-irradiated (controls). The medium was replaced with 5 ml McCoy's 5a medium containing tritiated thymidine (10 μCi/ml, 70 Ci/mmol), 2 mM hydroxyrea, and 2 x 10^{-6} M fluorodeoxyuridine (FUdR). Cultures were incubated at 37° for 2 hours. Hydroxyurea was added to suppress entrance of G_1 cells into S phase which would obscure measurement of repair synthesis; FUdR was added to suppress endogenous pyrimidine synthesis, so that effects due to any differences in nucleotide metabolism or pool size between the two cell types would be minimized (Cleaver, 1974).

After incubation, the radioactive medium was decanted, the cells were rinsed 3 times with 10 ml saline, and then 15 ml of cold, freshly made 3:1 methanol:acetic acid fixative was added. The fixative was poured off after 7 minutes and fresh fix was added for 7 additional minutes; this was followed by rinsing in 70% and 95% cold ethanol. The bottoms of the 75 cm^2 flasks were cut with a band saw into pieces the size of a standard microscope slide, four per flask. Rough edges were smoothed with a knife-edged scraper, taking care not to damage the preparation. These plastic slides were soaked overnight in 5% TCA at 4° to extract soluble radioactive material, and rinsed 10 minutes in cold 70% ethanol followed by 10 minutes in cold 95% ethanol.

Autoradiograms were prepared by methods similar to those described by Baserga and Malamud (1969). The slides were dipped into Kodak NTB-3 emulsion at 44° for 10 seconds followed by air drying for one hour. Dipping was carried out with intermittent illumination from a safelight with a 15 watt bulb and a Wratten #2 filter.

After 8 days exposure at 10° in light-tight boxes containing drying capsules autoradiograms were developed in Kodak D-19 (3 minutes, 18°) followed by a 15 second rinse in water and 10 minutes in Kodak Fixer (18°). Slides were then rinsed in two changes of water for 15 minutes each and air dried.

Giemsa staining through the emulsion was performed by rehydrating in freshly prepared phosphate buffer (0.1 M, pH 6.8) for 30 minutes, air drying, and staining for 5 minutes with 2% Gurr's Giemsa in buffer (6.8 mM citric acid, 18 mM Na_2HPO_4) containing 2.4% methanol. Slides were rinsed in tap and distilled water.

Grains were counted in each cell at 630 X using bright-field illumination with the Zeiss Photomicroscope II.

2.11. Nucleoid Sedimentation

Detection of UV repair-induced incision breaks leading to changes in supercoiling of DNA in nucleoids was

carried out essentially as described by Cook and Brazell (1975; 1976) with modifications suggested by Dr. J.E. Cleaver. Cultures in 35 mm plastic tissue culture dishes were labeled for 24-48 hrs with ^{14}C-deoxythymidine (0.01 µCi/ml, 50 mCi/mmol). The cultures, containing 2-5 x 10^5 cells in exponential growth, were rinsed, irradiated with 2.5 J/m^2 UV (or sham-irradiated) and incubated with non-radioactive medium.

At times between 0 and 150 minutes after irradiation, a culture was rinsed with ice-cold clear Puck's Saline A and 1.0 ml cold trypsin added for 3 min. The trypsin was poured off and a monocellular suspension was made in 300 µL of lysis solution layered on top of a 12 ml, 15-30% sucrose gradient (2 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 8.0). Final concentrations in the lysis mixture were 2M NaCl, 0.10 M EDTA, 0.5% Triton X-100, and 2 mM Tris, pH 8.0.

Lysis on the gradients at room temperature took place for 25 minutes before centrifugation at 15,000 rpm for 60 minutes in a Beckman L5-75 centrifuge with an SW41 rotor. Eighteen to twenty fractions were collected into scintillation vials by pumping out from the bottom. One ml water and 10 ml PCS scintillation fluid were added to each vial and radioactivity determined in a scintillation counter. In this way, the position of the nucleoids along the gra-

dient could be ascertained from the radioactivity in each fraction. The distance sedimented in the gradient was determined as the fraction containing maximum radioactivity. The gradient containing unirradiated nucleoids served as a reference; the distance traveled by nucleoids in other tubes in a rotor is expressed relative to the distance sedimented by nucleoids in the reference tube (Cook and Brazell, 1975).

CHAPTER 3

RESULTS

3.1. Isolation of UV-Sensitive Mutants

The method used to isolate UV-sensitive clones of CHO was based on the principle that colonies of CHO cells lyse and detach from a tissue culture dish after a lethal dose of UV-irradiation. Therefore, one should be able to choose a UV fluence which will leave a high percentage of wild-type cells viable but which will kill a colony of UV-sensitive mutants and cause the colony to fall off the dish. In this method one makes a replica of a dish before the master is irradiated; this way, affected colonies on the master can be picked from the unirradiated replica. The UV-sensitive cell lines obtained for the present study are apparently the first extremely sensitive variants to be obtained in mammalian cells by replica plating. Replica plating has the advantage over some other isolation techniques in that the cells obtained do not need to be exposed to or modified by the selective agent.

A "rescue" method was developed by Thompson et al. (1980) for isolation of UV-sensitive mutants. In this method, cell colonies on a tissue culture dish are irradiated with a dose that will kill all but a very small frac-

tion of a UV-sensitive colony A large number of dishes are scanned with a dissection microscope; those colonies which look especially damaged after irradiation are carefully isolated in the hope that a few survivors of the UV-sensitive subpopulation will grow up. This technique was tried a number of times, unsuccessfully, with our CHO-KK cell line. The CHO strain used by Thompson et al. had been selected for use in suspension culture and attaches relatively weakly to a culture dish. On the other hand, the CHO-KK strain was selected to attach to substrates with some tenacity so that only mitotic cells can be shaken off a rapidly rotating bottle in a synchronization procedure (Klevecz, 1975). Thus, these different attachment properties may be responsible for our failure to isolate mutants by Thompson's method.

The UV-sensitive mutant obtained by replica plating which was used in this study is referred to by its experimental isolation code, 43-3B.

3.2. Sensitivity to Killing by Ultraviolet Light

When a seven day old colony of CHO-9 cells is irradiated with 6 J/m^2 of UV light, and then examined 48 hours later, few obvious morphological changes are observed as compared to an unirradiated colony (figure 1a). However, when a seven day old colony of 43-3B is irradiated with the same fluence, marked effects are seen (figure 1b).

During the two days after irradiation, growth ceases as the cells swell, lyse, and detach from the dish. Only a few rounded and aberrant cells from the colony remain.

Survival curves for colony-forming ability were determined. The CHO mutant 43-3B is hypersensitive to UV-induced reproductive death, as compared to the wild-type CHO-9 (figure 2). The D_0 UV-fluences are 0.3 J/m^2 and 3.2 J/m^2 for CHO-9 and 43-3B, respectively. Thus, in terms of a ratio of D_o fluences, 43-3B is 10.7 times more sensitive than CHO-9. Extrapolation of the linear portion of the survival curves back to the ordinate gives n = 2.5 for both cell lines.

Cultures of 43-3B maintained continuously for up to six months have shown no alteration in UV-sensitivity; this indicates that the phenotype is a stable one.

The extreme sensitivity of 43-3B to UV irradiation is further illustrated when one measures the rate of cell growth in replicate cultures (figure 3). After a low (sublethal) fluence, the cells continue to divide, but are delayed with respect to the control rate. After a high (supralethal) fluence the cells stop dividing and begin to fall off the dish after a day. After an intermediate fluence there is a growth delay after which wild-type cultures resume growth, but cultures of the sensitive mutant begin to deteriorate. This sort of response to UV has

been seen by Cleaver (1970b) for xeroderma pigmentosum versus normal human cells; this type of measurement has also been used to examine the time-course of cell death after X-irradiation (see Okada, 1970).

3.3. Growth Rate

Exponential-phase cultures of both CHO-9 and 43-3B had a doubling time of 11.8 hours as determined by counting replicate cultures (figure 4). Control plating efficiencies were 60% to 80% for both types. Both CHO-9 and 43-3B have similar cell-cycle phases as measured by incorporation of tritiated thymidine in pulses in synchronized cell populations, with a 3.5 - 4 hour G_1 period, a 6 - 6.5 hour S phase, and a G_2 plus M period of 1.5 hours.

3.4. Karyotype

The modal chromosome number for both cell lines was 21. The chromosome complement from five spreads of each cell line was examined in detail. Pictures of the chromosomes were arranged in size order to faciliate comparision (figure 5). No differences between the two cell types were detected in the solid-stained spreads, and the chromosome complement was identical amongst the cells examined.

The chromosome complement exhibited by the two cell types is very similar to that reported for the CHO cell

line by Worton et al. (1977). The short marker chromosome number 2 is present, as is the very small chromosome Z-13 which may be a result of bi-arm deletions from a chromosome 10. There are a few minor but consistent differences from the karyotype given by Worton et al.; one member of the number 6 set is longer than the other. Since there are no obvious differences between the cell types, it is not possible to speculate on the possible map position of a gene controlling UV-sensitivity. If the modification leading to UV sensitivity has resulted from a large deletion (rather than a point mutation), a detailed study of chromosome banding patterns might provide a clue in this regard.

The very similar growth rates and karyotypes exhibited by CHO-9 and 43-3B make them a very good choice for comparative studies on mutation induction and repair.

3.5. Diphtheria Toxin

Some aspects of the assay for diphtheria toxin resistance are described below.

Experiments in this study were performed with toxin at either 0.1 or 1.0 Lf/ml, since the cells had a response similar to that reported by Gupta and Siminovitch (1978) for CHO cells. This is a concentration giving a low spontaneous rate of 0.0 to 1.5 DT^r colonies per 10^6 cells

plated. Medium containing this concentration of diphtheria toxin and incubated at 37° remains highly toxic to cells for at least 8 days, showing loss of activity toward the end of that period.

In order to determine the class of CHO DT^{r} mutants that is selected for under these conditions, experiments were performed to measure induction of resistance to both diphtheria toxin and *Pseudomonas aeuroginosa* exotoxin A. Very similar numbers were obtained when an experiment was performed to measure the induction of mutants resistant either to diphtheria toxin, or to diphtheria toxin and *Pseudomonas* toxin in combination (Table I). Spontaneous variants resistant to *Pseudomonas* exotoxin A at 0.2 µg/ml arise with a frequency of 5×10^{-6} per viable cell plated. UV-induced variants resistant to *Pseudomonas* toxin at this concentration show a linear induction up to at least 9 J/m^2, at which exposure 240 are recovered per viable wild-type cell plated (data not shown). This number is expected to include both EF-2 mutants and membrane-associated variants.

Putative mutant colonies which had been grown up in the absence of toxin and retested for diphtheria toxin resistance always exhibited resistance to diphtheria toxin at 10 or 100 times the selecting concentration of 0.1 or 1.0 Lf/ml although at 10 Lf/ml they grew slower. Indivi-

dual DT^r clones also proved to be cross-resistant to <u>Pseudomonas</u> exotoxin A at 0.2 µg/ml (5/5 tested).

Taken together, these results indicate that under the conditions used, class II DT^r mutants are being selected.

A reconstruction experiment was performed in order to determine if diphtheria-toxin resistant clones could be recovered in the presence of 10^6 wild-type cells. With one of the clones tested (2-DT), virtually all of the resistant mutants plated were recovered 8 days later (Table II). With the other clone (8-DT), which had a low control plating efficiency, the response for number plated vs. number recovered was linear, but only about one-half the number of mutants plated were recovered. The results indicate that in the selective system used, the number of DT^r resistant colonies counted on the dish after a challenge with toxin is, conservatively, proportional to the number present in the population plated.

In these experiments, the cells were routinely challenged with toxin 8 days after mutagen treatment. This allowed ample time for healthy recovery of the cultures, and was also convenient in our system, where 8 days had already been shown to be a suitable expression time for 6-thioguanine resistance and ouabain resistance. However, the results indicate that much shorter expression times can be used with this marker (Table I).

3.6. UV-Induced Mutation in 43-3B

The extreme sensitivity of 43-3B to ultraviolet light is also seen for induction of mutations to 6-thioguanine resistance ($6TG^r$), ouabain resistance (OUA^R), and diphtheria toxin resistance (DT^r) (figure 6).

Mutation frequencies were calculated for a given experimental point according to the formula:

$$\frac{(TM)}{(nXN)(PE)} = MF$$

where

MF = mutation frequency

TM = total mutant colonies observed

n = number of dishes used per point

N = number of cells plated per dish

PE = plating efficiency of cells used in the sample, obtained from 3 plates unexposed to the challenging drug or toxin.

As a rule, 3 dishes were plated per point per drug or toxin (see Materials and Methods), so n = 3. N = 1 to 1.5 X 10^6 for DT^r and OUA^R, and N = 1 to 1.2 X 10^5 for $6TG^r$.

In figures 6, 7, and 8, background mutation rates have been subtracted. The background rates were 0.0 to 2.0 X 10^{-6} per viable cell for ouabain resistance and diphtheria toxin resistance in both CHO-9 and 43-3B. Background rates for 6-thioguanine resistance were 0.0 to

2.0 X 10^{-5} per viable cell for CHO-9 and 5.0 to 10.0 X 10^{-5} for 43-3B, depending on the individual experiment.

For CHO-9, the absolute number of colonies observed on a mutation assay plate varied from 0 to about 40 depending on the UV fluence, giving the mutation frequencies reported in Burki et al. (1980) and Table III.

For 43-3B, the absolute number of resistant colonies was strikingly higher; for the fluences used (figure 6), 50 to 150 colonies were counted on each plate.

3.7. Solar-Induced Lethality

Several experiments were performed to measure reproductive death by solar-simulated light using the arrangement described (figure 7). The UV-sensitive 43-3B is more sensitive than the wild-type CHO-9 to the lethal effects of this light (figure 8). The times required to reach the 10% and 1% survival levels with CHO-9 cells compare very favorably with those obtained for several mammalian cell types irradiated with actual sunlight (Hsie et al., 1977; Parsons and Goss, 1980; Krell and Jacobson, 1980). This serves to support the assertion that the solar simulator produces biologically effective light closely simulating that of the sun (figure 9). The physical characteristics of the simulated solar light are described in Appendix C.

To more directly compare results obtained with solar light to those obtained with germicidal UV, several experiments were performed with 43-3B and CHO-9 in suspension using the same protocol as used for the solar experiments, but with ultraviolet light as the source (figure 8).

Cells irradiated in suspension survive better at a given fluence than cells irradiated in monolayer (figures 8,2; Table IV). This effect has been noticed by other investigators and can be accounted for by increased internal screening associated with rounding up of the cells and the high UV absorbance of cellular structures (Freed, 1980; Collins et al., 1980).

3.8. DNA Repair

Unscheduled DNA Synthesis

43-3B is deficient in unscheduled DNA synthesis in comparision to the wild-type CHO-9, as measured by autoradiography of G_1 populations (Table V). G_1 cells were used for this assay to eliminate confusion from the high proportion of S-phase cells in the relatively short CHO cell cycle. The unirradiated populations had some background incorporation, probably due to the long labeling time (2 hours) and high specific activity used (figure 10). In the irradiated populations, CHO-9 cells showed a very significant amount of incorporation over the control level.

This indicates the presence of repair of UV-damaged DNA in the wild-type. 43-3B showed a much smaller amount of incorporation (an average of 17% of that shown by the wild-type); (figure 11), indicating a deficiency in UV-stimulated DNA repair in the UV-sensitive cells.

Sedimentation of Nucleoids After UV

If cells are gently lysed in 2.0 M NaCl, structures are released which resemble nuclei but are depleted of protein. These structures sediment in neutral gradients as masses of supercoiled DNA and are termed nucleoids (Cook and Brazell, 1975; 1976).

When single-strand breaks are introduced into supercoiled DNA by X-rays, the rate of sedimentation of nucleoids in sucrose gradients decreases as supercoils in DNA are relaxed (Cook and Brazell, 1975). After UV irradiation, breaks can be detected by changes in nucleoid sedimentation because they are introduced enzymatically as excision repair begins (Cook and Brazell, 1976).

After 2.5 J/m^2, a rapid decrease and slower increase of sedimentation distance was seen in CHO-9 as expected, showing incision at damaged sites followed by rejoining; but this effect was not seen in 43-3B (figure 12). This provides further evidence for a DNA repair deficiency in the UV-sensitive mutant.

3.9. Inhibition of DNA Synthesis by UV

The fluence-response for the inhibition of cellular DNA synthesis by UV is very similar for CHO-9 and 43-3B when a 2 hr labeling period is used (figure 13a). Ten minute pulses were used to measure the rates of DNA synthesis at various times after irradiation. After a low fluence, the UV-sensitive mutant was able to recover to control rates of DNA synthesis (figure 13b). However, after a moderate fluence (1.0 to 2.5 J/m^2), 43-3B was unable to return to control rates at times when the wild-type showed almost complete recovery (figure 13c,d).

3.10. Survival Following Fractionated Exposures

Experiments were performed to measure the survival of CHO-9 and 43-3B cells after a single UV fluence, or the same fluence given in two fractions, but separated by 12 hours. Recovery between fractionated exposures has been studied previously in Chinese hamster cells (Humphrey et al., 1970; Todd, 1973) and was observed with CHO-9 (figure 14a). Little recovery was seen for 43-3B. The difference in survival at 1.5 and 2.0 J/m^2 between single and fractionated exposures is not statistically significant (figure 14b). The word "recovery" refers to the observation of increased survival, with no implication of the underlying mechanism (although repair is often assumed). Several experiments to observe the time-course of recovery between

fractionated exposures were performed and again showed that CHO-9 was able to significantly recover within 12 hours, while 43-3B showed only a very small amount of recovery (figure 15). Initial fluences of 0.5 J/m^2 for 43-3B and 6.0 J/m^2 for CHO-9 were chosen to give similar survival levels for the two cell types.

TABLE I

INDUCTION OF DT-RESISTANT CLONES

	Expression time (days)	DT^r	$DTPA^r$
0 J/m^2			
	0	0.5	
	3	0.0	0.0
	7	0.0	0.0
	9	0.3	0.0
8.4 J/m^2			
	0	0.0	
	3	7.5	6.9
	7	6.3	7.5
	9	10.1	11.3
16.8 J/m^2			
	0	0.3	
	3	18.3	11.9
	7	12.7	14.6
	9	13.8	11.6

The frequencies given are for resistant clones recovered per 10^6 cells plated, normalized to control plating efficiency and averaged for three dishes per point. The average absolute number of resistant colonies per dish can be obtained by multiplying the frequency given in the table by the control plating efficiency (generally 0.6 to 0.7). DTPA is an abbreviation for diphtheria toxin and *Pseudomonas* exotoxin A used in combination.

TABLE II

RECONSTRUCTION EXPERIMENT

	DT^r cells plated	DT^r colonies recovered
2DT		
	0	0.4
	22	25
	44	44
	111	124
	221	211
8DT		
	0	1.1
	27	10
	54	24
	109	62
	218	83

Colonies recovered are normalized to the control plating efficiencies which were 0.80 for 2DT and 0.45 for 8DT. 2DT and 8DT were isolated by colony cloning and grown first in DT for purification, then several weeks in the absence of DT before this experiment.

TABLE III

Comparision of UV-induced mutation frequencies in CHO-9 and 43-3B*

(mutations/10^6 viable cells/J m^{-2})

Cell Line	$6TG^r$	OUA^R	DT^r	Average
CHO-9	20	2	2	
43-3B	750	75	130	
Ratio 43-3B/CHO-9	38	38	65	47
Ratio per D_o dose 43-3B/CHO-9	3.5	3.5	6.1	4.4

* Mutation frequencies per J/m^2 were obtained as slopes of the curves in the linear region, from figure 9. For CHO-9, the data are from unpublished experiments and are very similar to the frequencies for CHO-KK reported by Burki et al. (1980). The linear region of mutation induction vs. fluence for CHO-9 is approximately 0 to 12 J/m^2 for all three markers. The ratio of mutation induction per D_o dose was calculated by dividing the ratio of mutation induction per unit fluence (43-3B/CHO-9) by the ratio of D_o fluences for the two cell types (10.7). This is equivalent to taking the ratios of the final linear portions of a plot of survival vs. mutation frequency such as figure 16.

TABLE IV

D_o FLUENCES FOR CHO-9 AND 43-3B ($J\ m^{-2}$)

Irradiation	D_o CHO-9	D_o 43-3B	Ratio CHO-9/43-3B	Ref.
UV (monolayer)	3.2	0.3	10.7	fig. 2
UV (suspension)	5.3	0.5	10.6	fig. 8
Solar (suspension)	2.9E05	4.5E04	6.4	fig. 8

TABLE V

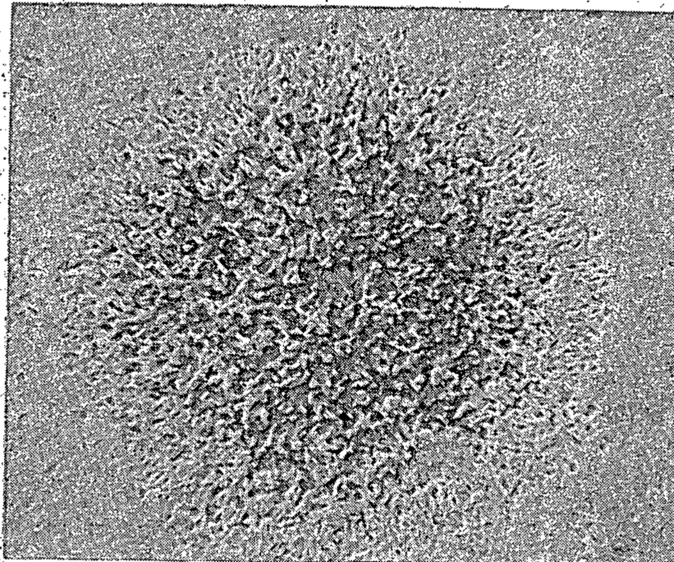
UV-Stimulated Unscheduled DNA Synthesis

Average grains per cell*

	5 J/m^2	10 J/m^2
CHO-9	23.7$\pm$1.6 (100)	24.7$\pm$0.8 (100)
43-3B	4.8$\pm$1.1 (20.1)	3.3$\pm$0.7 (13.3)

* Average backgrounds of 11.7 grains per cell for CHO-9 and 12.9 grains per cell for 43-3B were observed for the controls (0 J/m^2) and subtracted to give the results shown in the table. 95% confidence limits for the average incorporated grains per cell are indicated. Shown in parentheses is the percent of grain (background-corrected) counts in 43-3B compared to CHO-9 at the same fluence. At least 2000 grains were counted at each fluence.

(a)



(b)

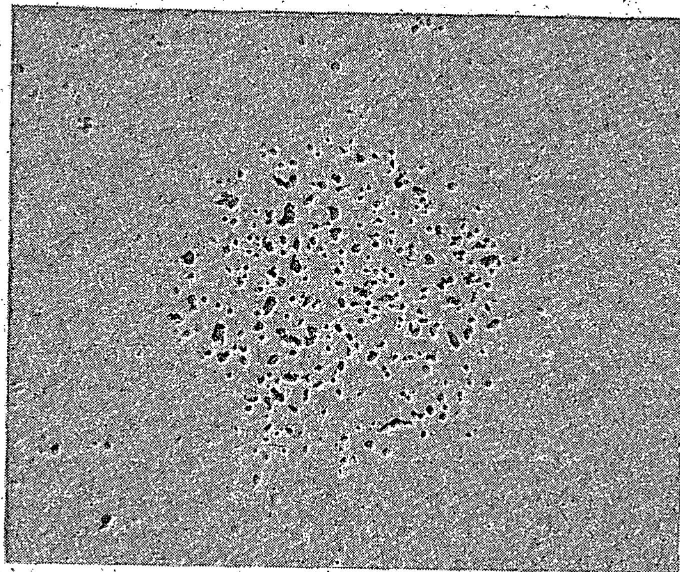


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Figure 1. (a), a 9-day-old CHO-9 (wild-type) cell colony 48 hours after irradiation with 6 J/m^2 ultraviolet light; (b), The remains of a colony of 43-3B at 9 days after the same treatment described for (a). Magnification 700X.

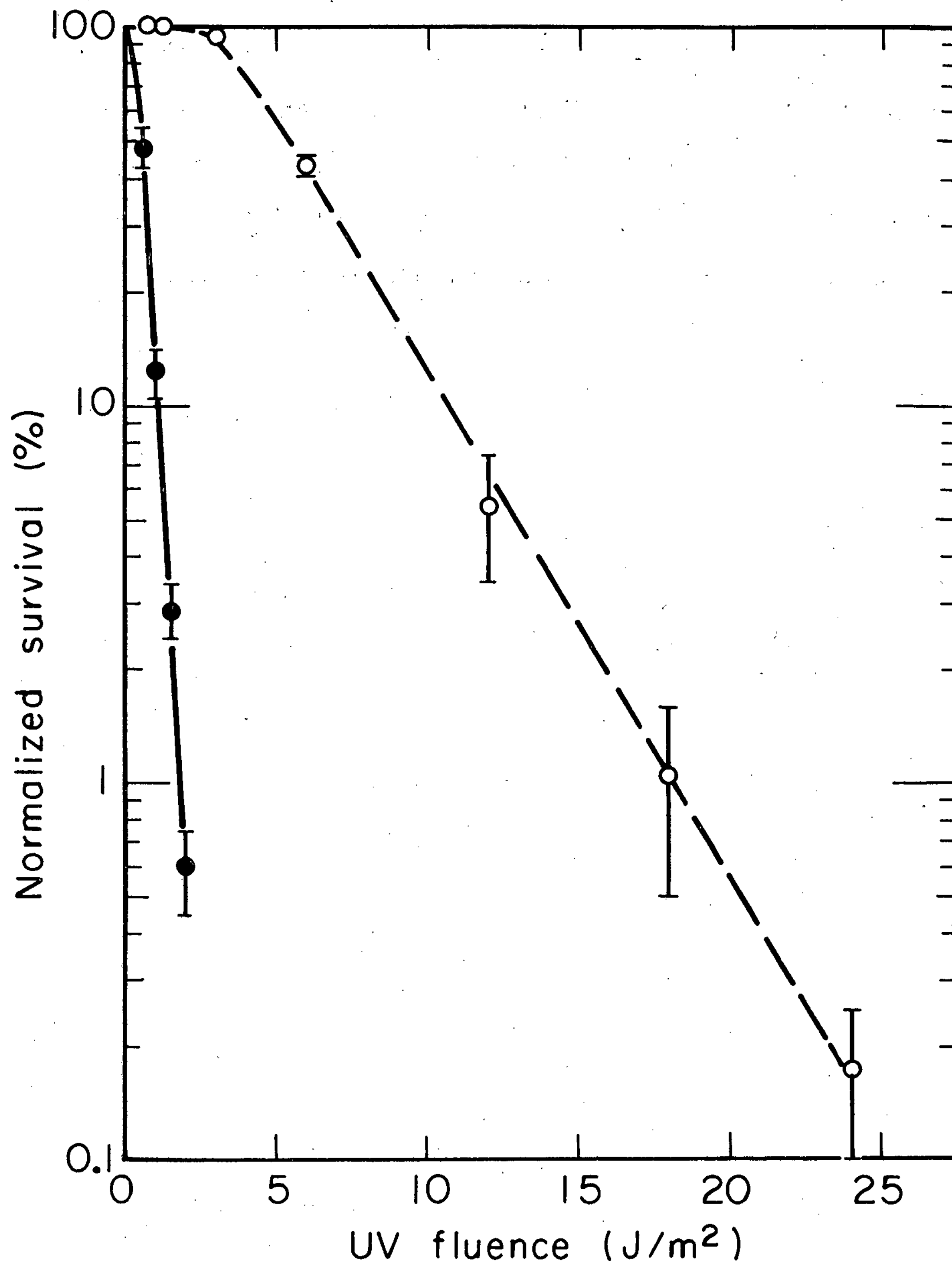


Figure 2. Percent survival (normalized to control plating efficiency) of CHO-9 (open circles) and 43-3B (solid circles) exposed to UV. The error bars represent standard errors of the mean for 2 to 4 experiments per point.

Figure 3 (overleaf). The detachment of cells from a 90 mm petri dish after UV irradiation. Cells were irradiated 18 hours after plating (2 to 4 X 10^6 per dish) and the cell number remaining in the dish was measured at various times thereafter. The solid line in the top panel is a growth curve for unirradiated replica cultures of either CHO-9 or 43-3B.

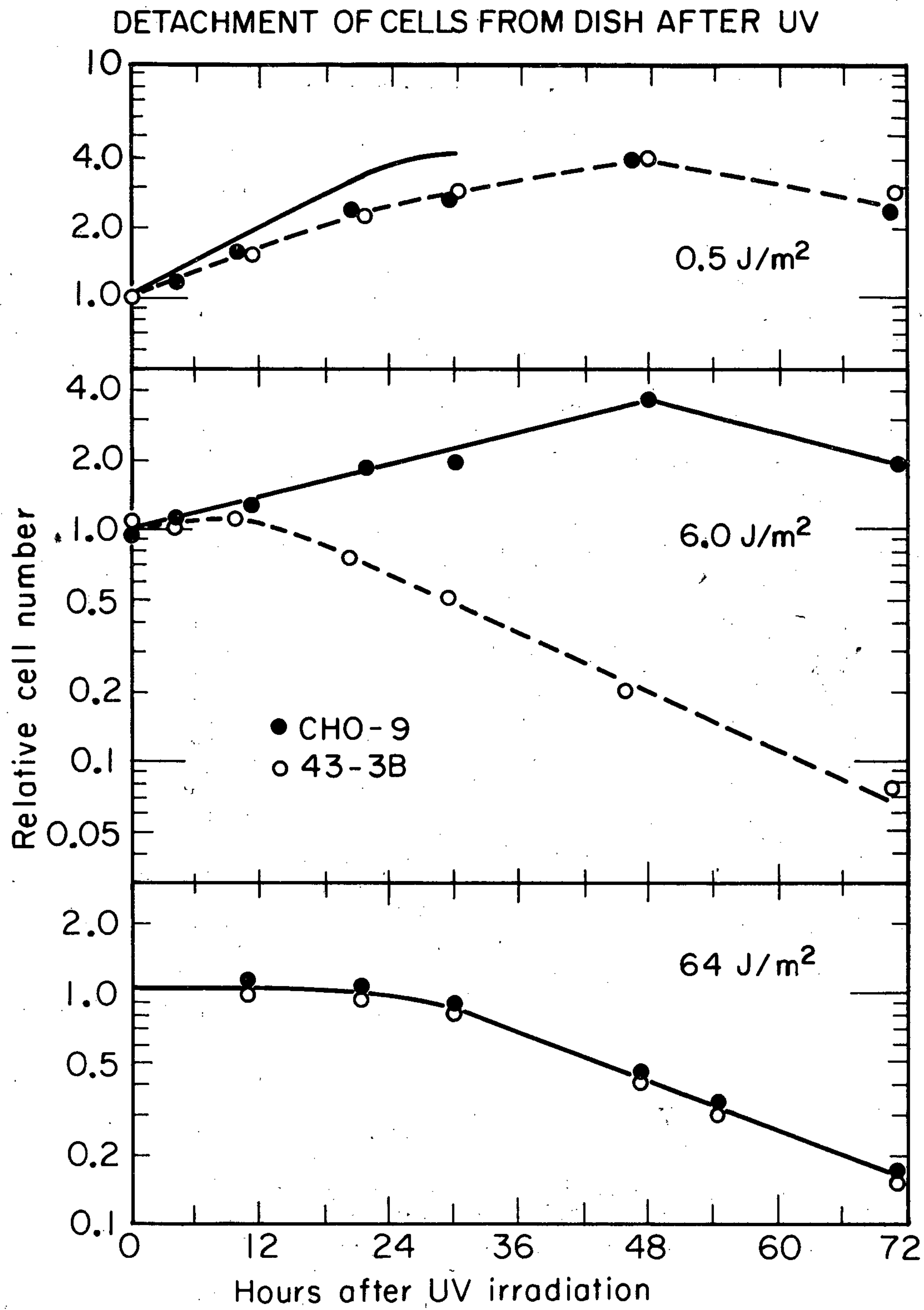


Figure 3.

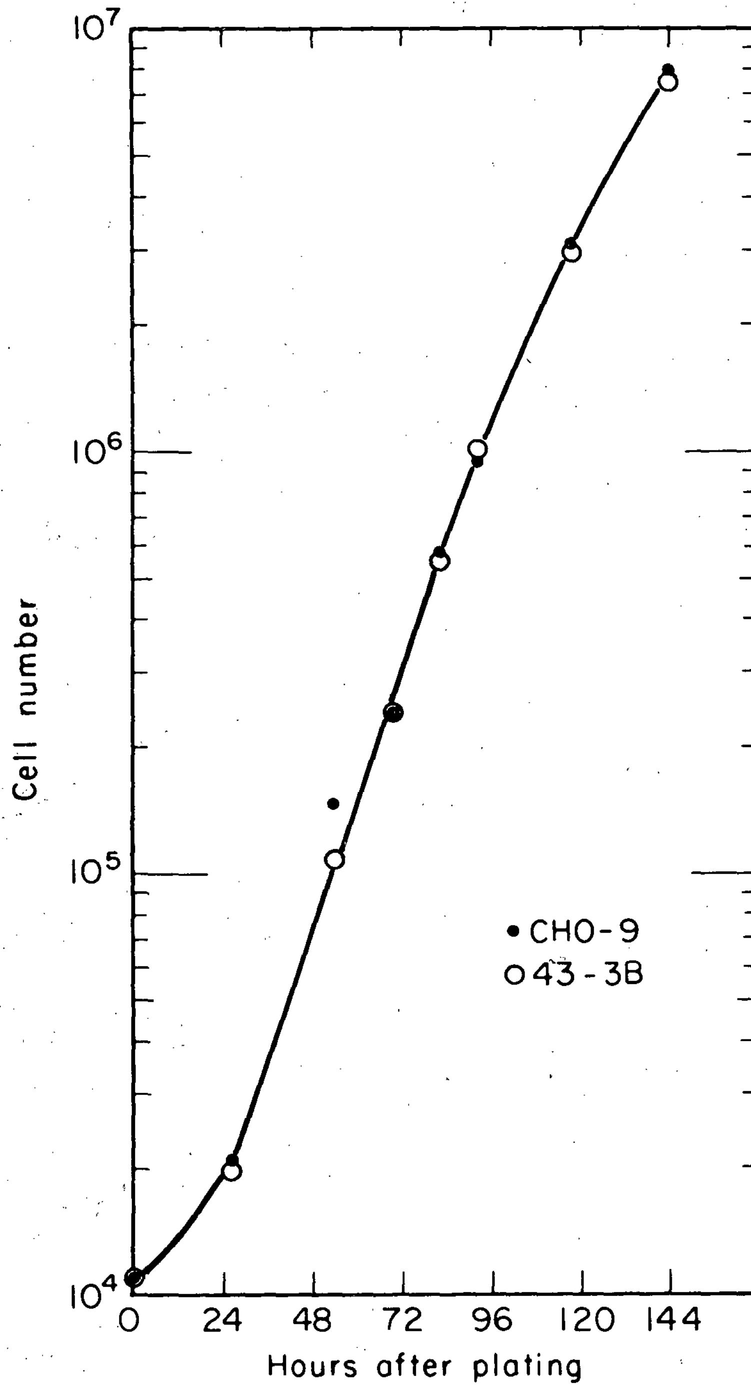
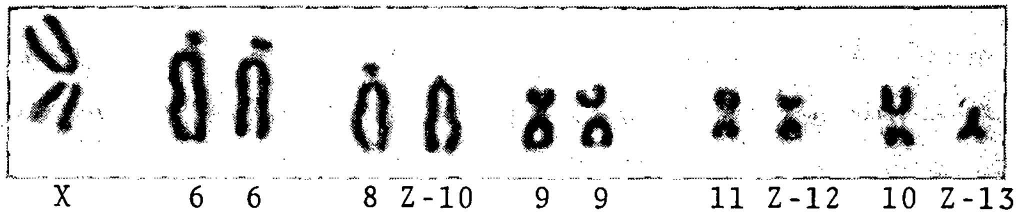
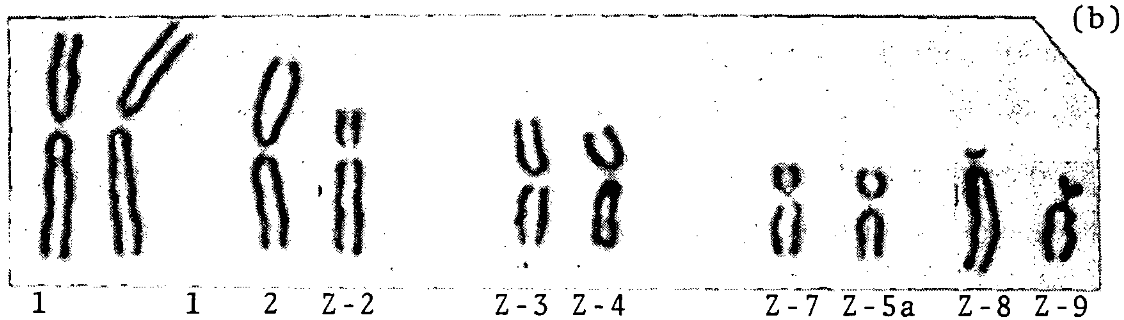


Figure 4. Growth curves for CHO cells, determined by seeding 1.1 X 10^4 cells into replicate dishes and then trypsinizing and counting a dish at various times thereafter. Same symbols as figure 3.





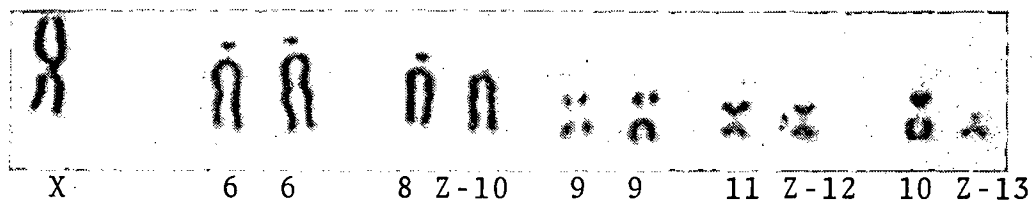


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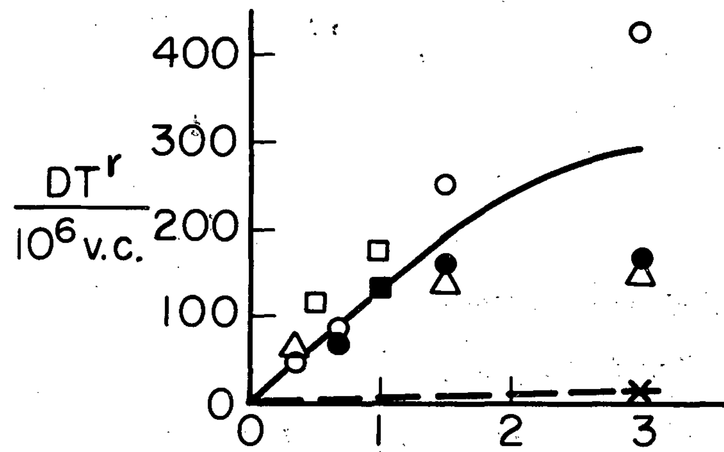
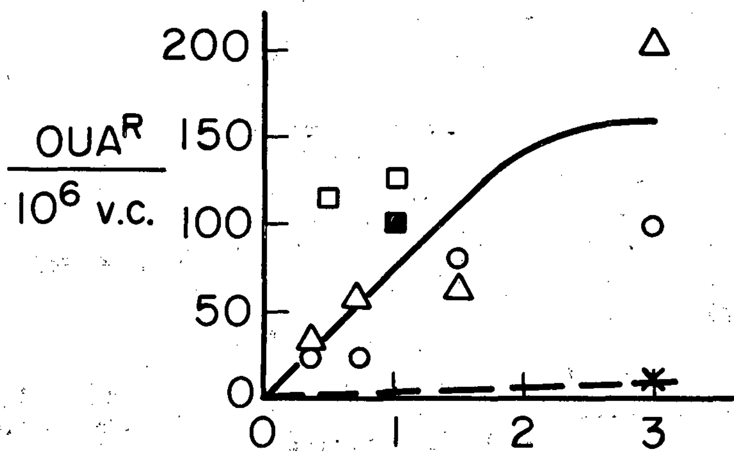
Figure 5. Mitotic chromosomes of CHO-9 (a) and 43-3B (b) from representative spreads, magnified approximately 3000 times. The chromosomes are arranged according to size; the nomenclature is that of Worton et al. (1977) with the modifications mentioned in the text.

Figure 6 (overleaf). Induction of mutations by UV light in 43-3B (solid line) and CHO-9 (dotted line). Top panel, cells resistant to 1.0 Lf/ml diphtheria toxin (per 10^6 viable cells). Middle panel, cells resistant to 3mM ouabain (per 10^6 viable cells). Bottom panel, cells resistant to 5μg/ml 6-thioguanine (per 10^5 viable cells). Background mutation rates have been subtracted as described in the text. Different symbols indicate separate experiments.





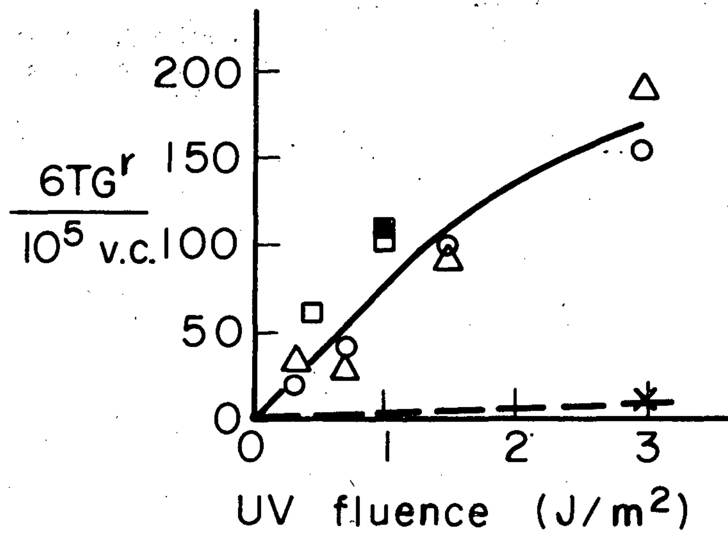


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Figure 6.

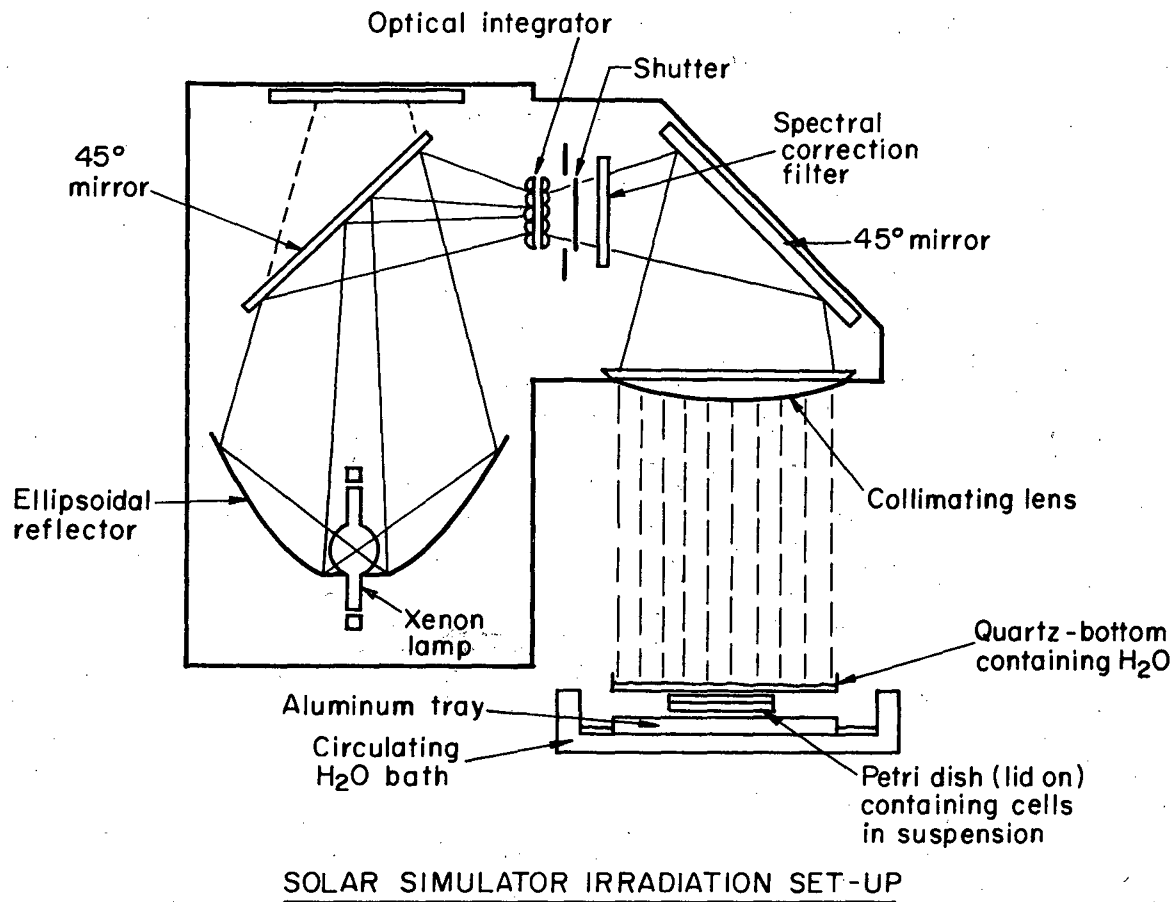


Figure 7.

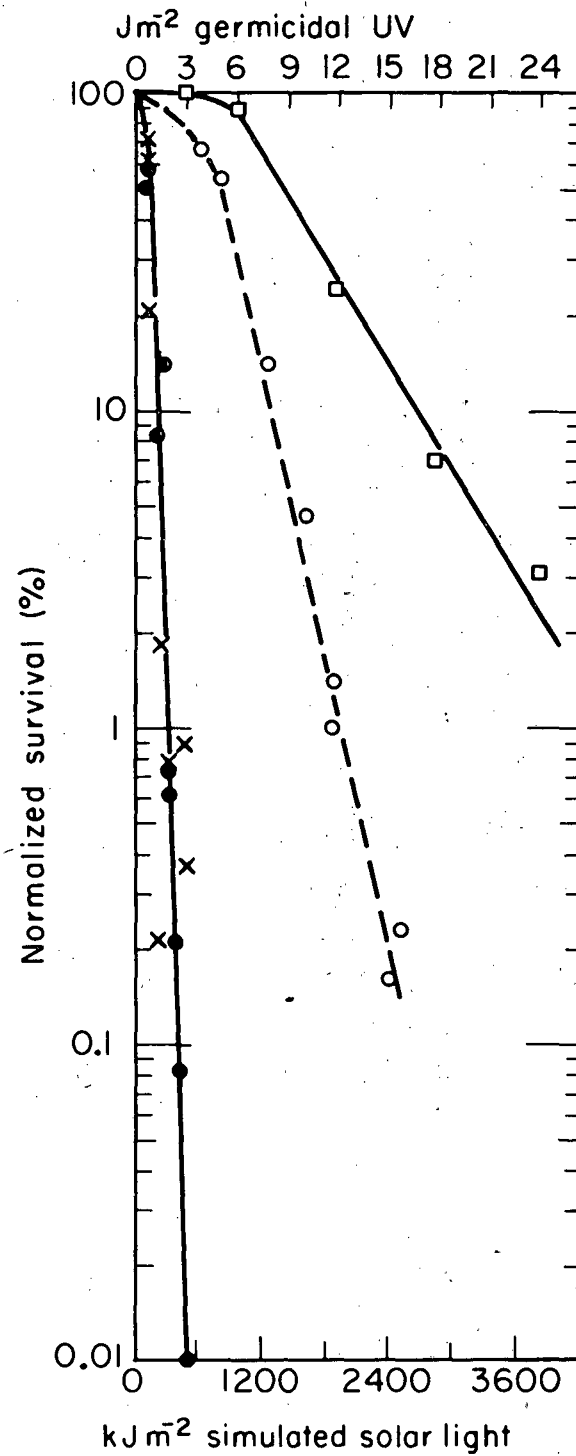


Figure 8. Survival curves for cells exposed to simulated solar light (solid circles, 43-3B; open circles, CHO-9) or to germicidal UV light in suspension (crosses, 43-3B; squares, CHO-9).

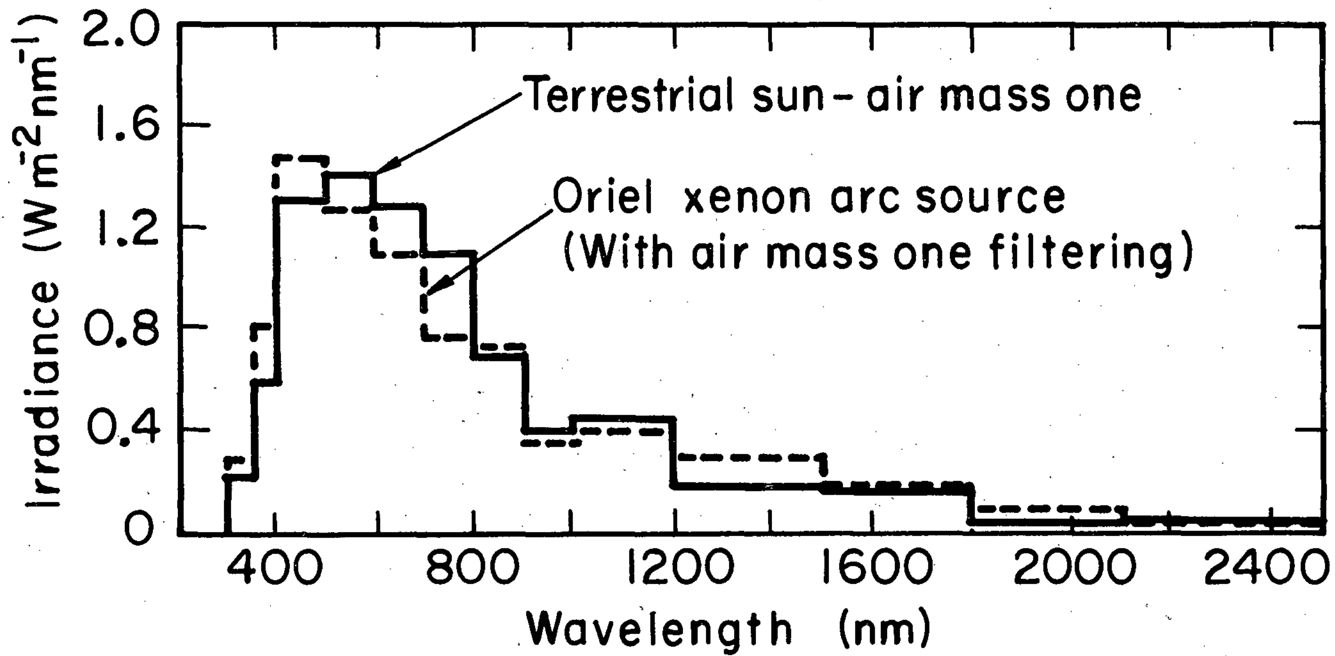
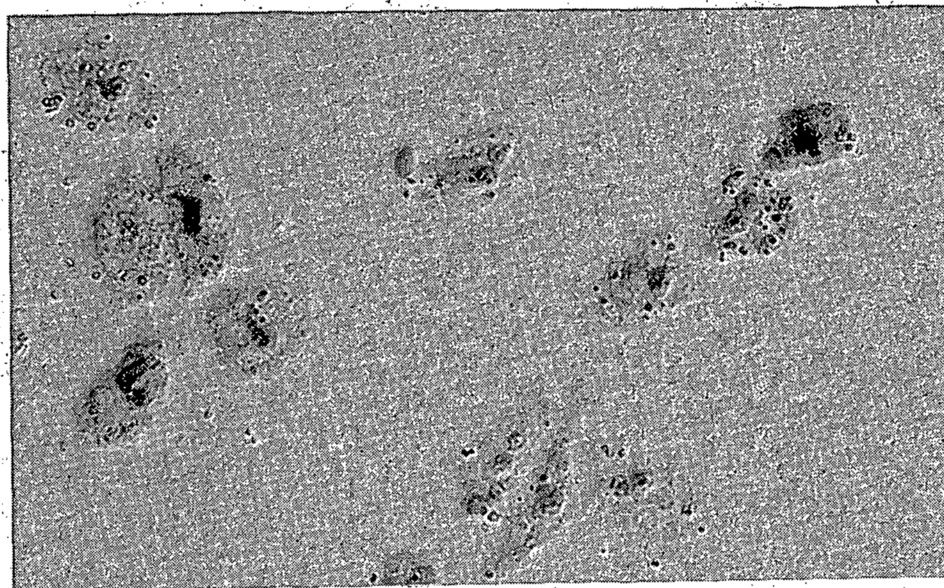


Figure 9. Spectral irradiance from the sun and solar simulator, normalized to one solar constant. The measured fluence rate of 700 W m^{-2} from the solar simulator compares closely to the solar fluence rate on a clear Berkeley day.



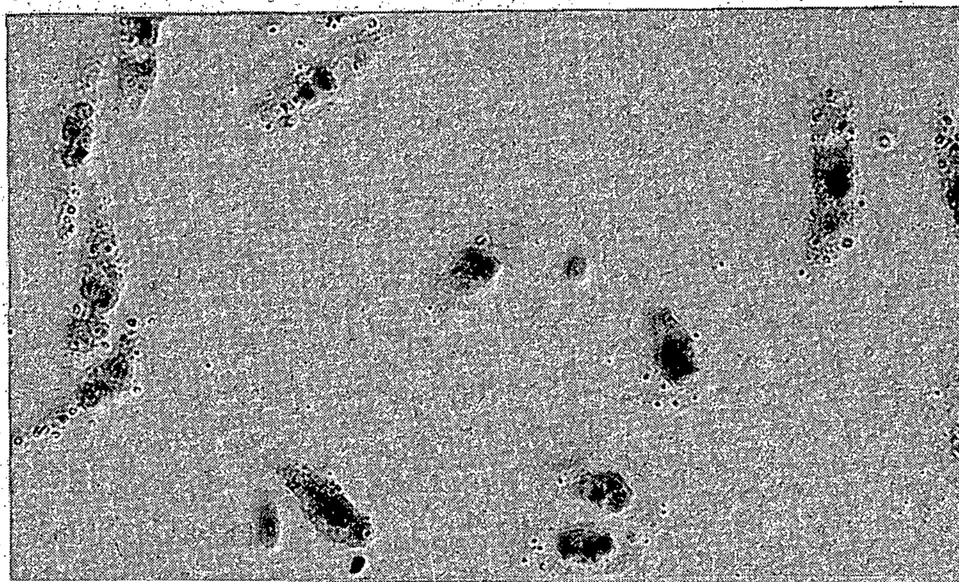


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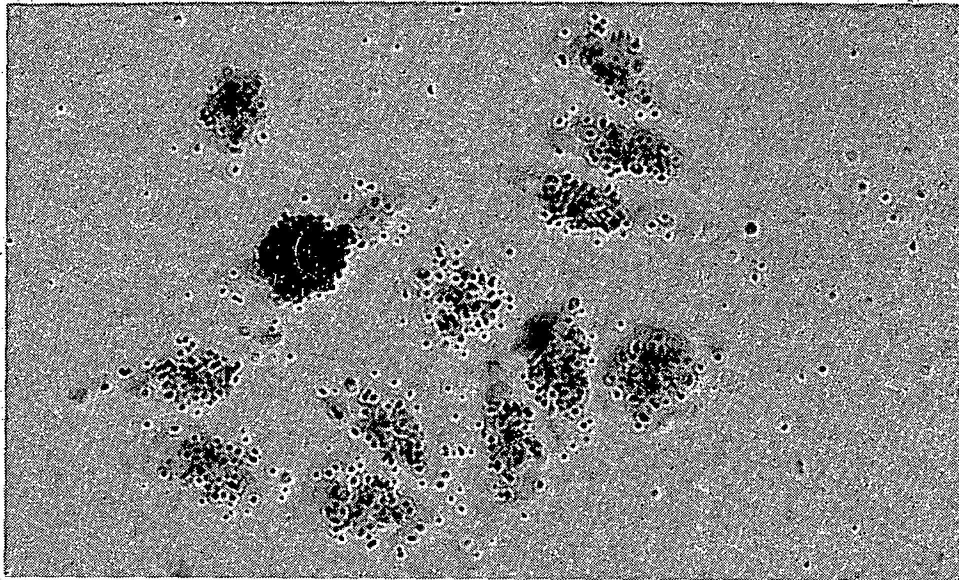
Figure 10. Unirradiated CHO-9 cells (top) and 43-3B cells (bottom) labeled for 2 hours with tritiated thymidine as described in the text. Magnified approximately 1000 times.



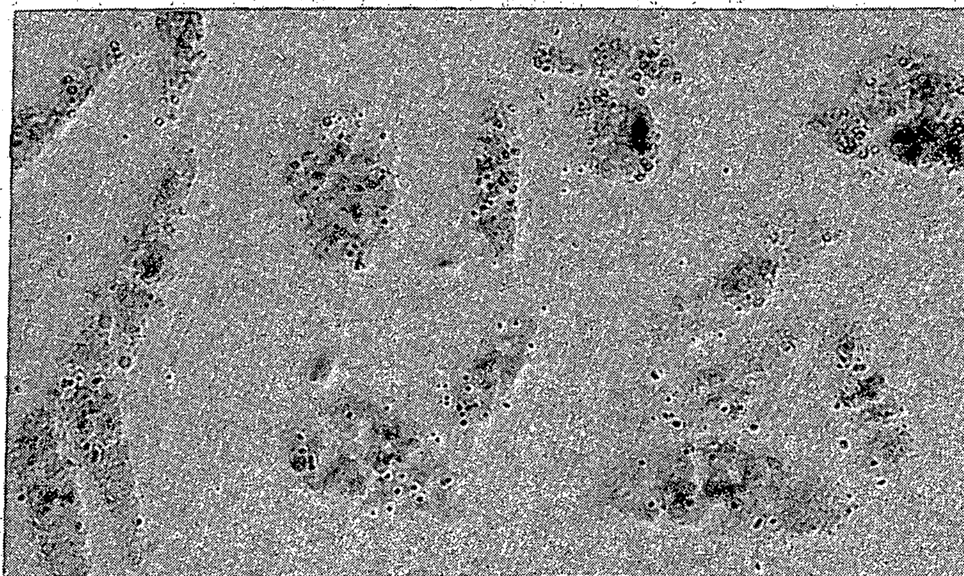


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Figure 11. Unscheduled DNA repair synthesis in G_1 cells. CHO-9 cells (top) and 43-3B cells (bottom) irradiated with 10 J/m^2 UV light and labeled with tritiated thymidine as described in the text. A heavily labeled S-phase cell is present in the field for comparision.

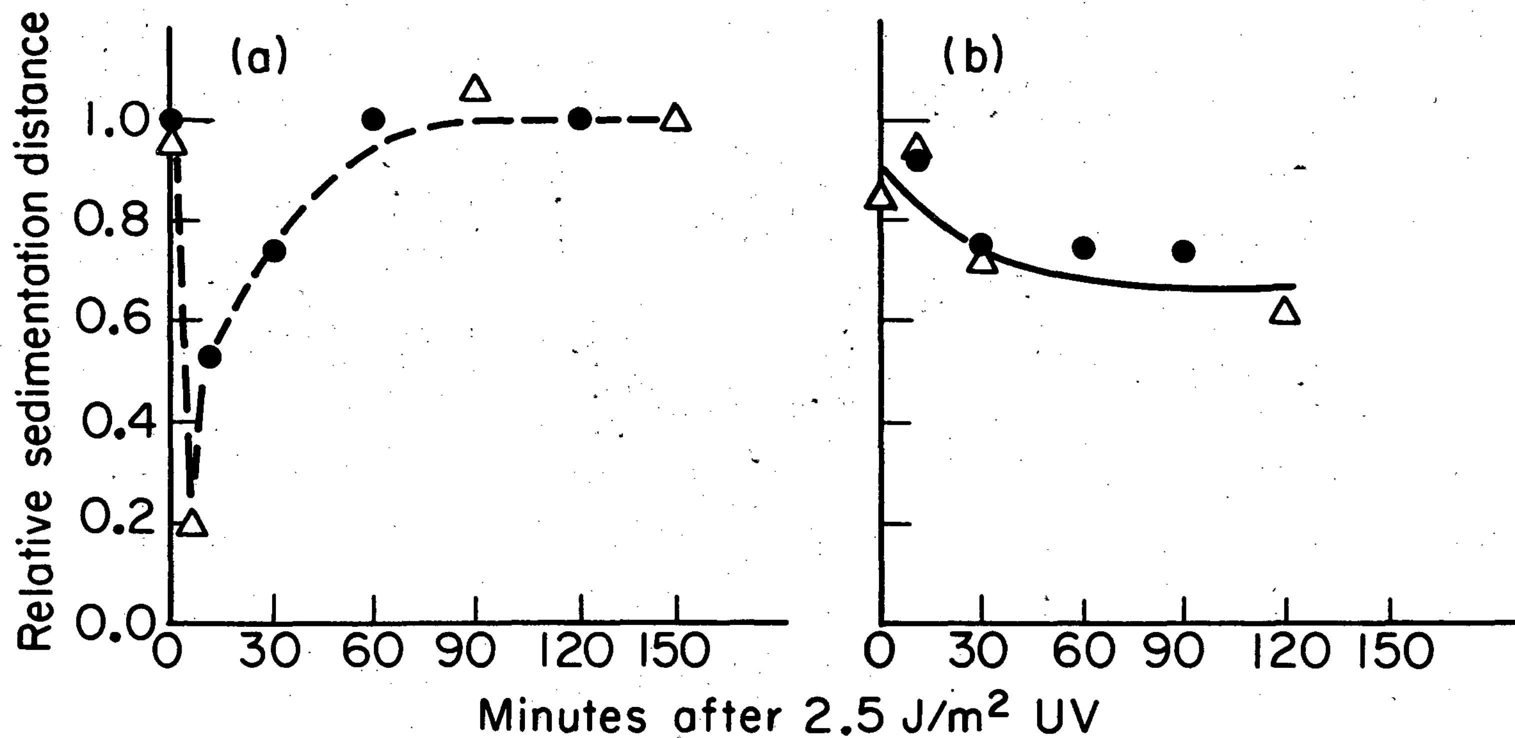

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Figure 12. Relative sedimentation distance in sucrose gradients of nucleoids from CHO-9 (a) and 43-3B (b) at various times after irradiation with 2.5 J/m^2 light. Different symbols represent separate experiments.

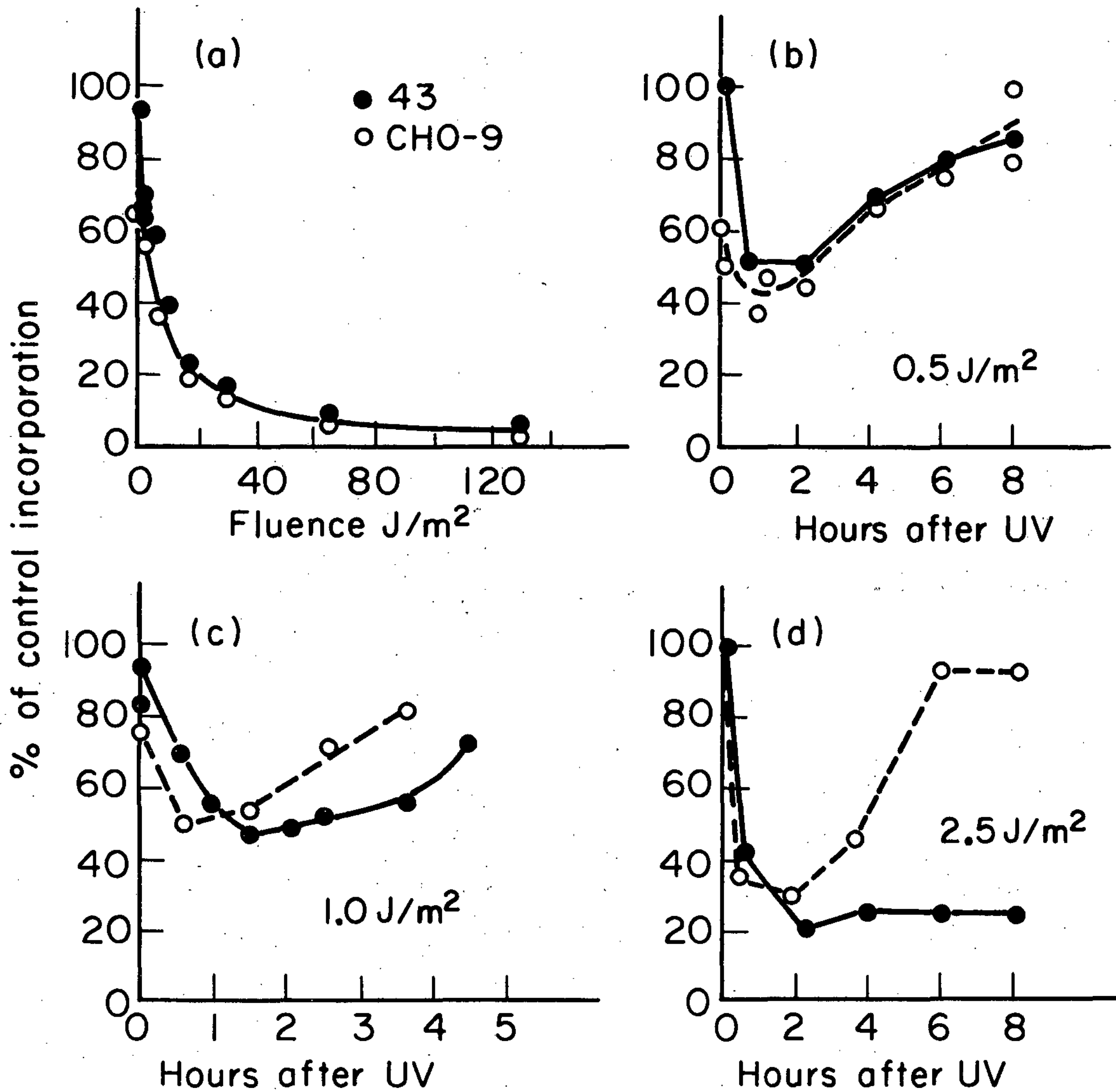


Figure 13. Fluence-response for the amount of DNA synthesized within two hours after exposure to UV light (a). Inhibition and recovery of DNA synthesis rates (measured in 10 minute pulses) after UV irradiation with 0.5 J/m^2 (b), 1.0 J/m^2 (c), or 2.5 J/m^2 (d), in 43-3B and CHO-9.

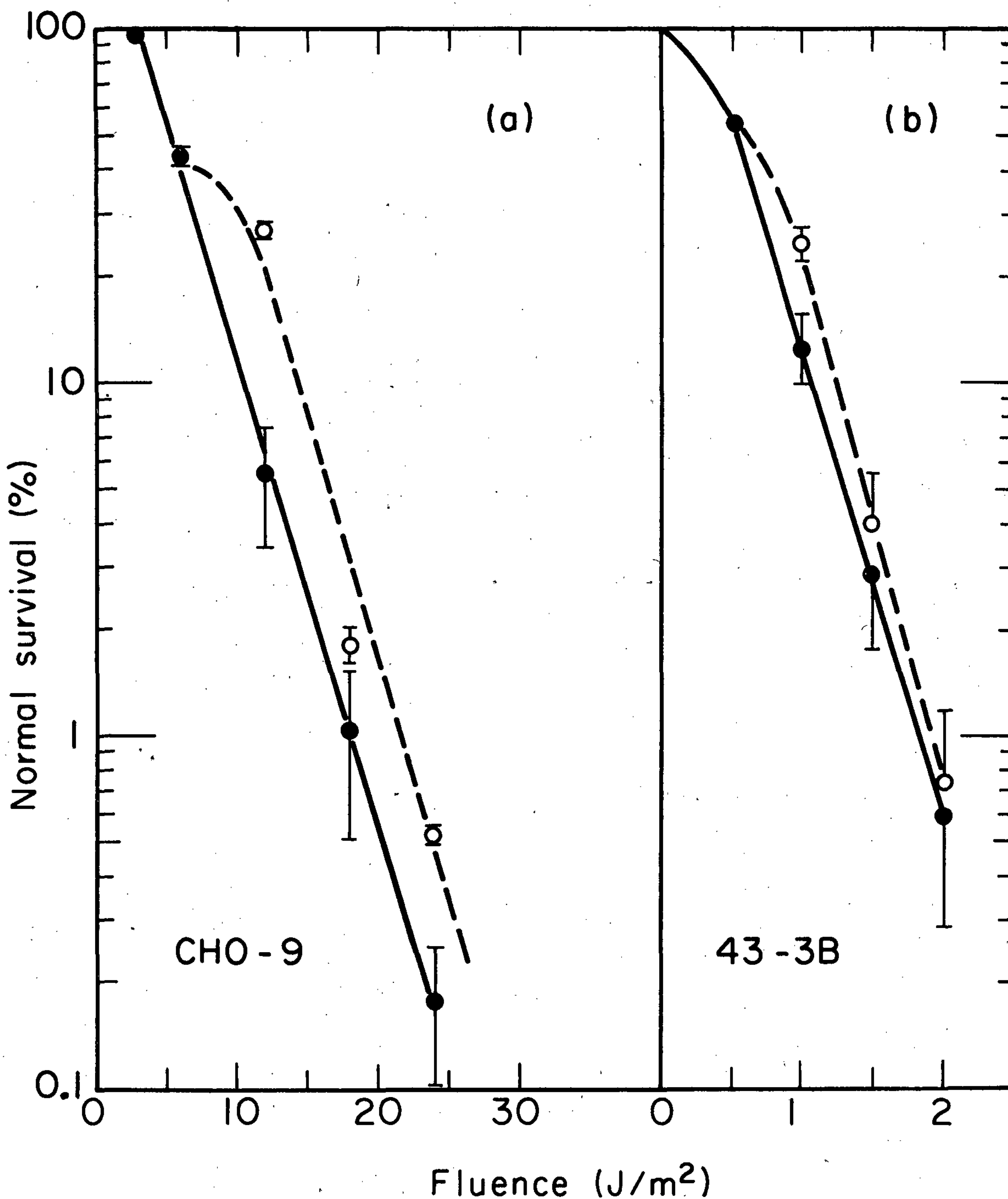


Figure 14. Recovery between fractionated UV fluences in (a) CHO-9 and (b) 43-3B. Each point represents the mean of two to four experiemnts. Exponential protions of the curves were fitted by least-squares analysis. CHO-9 cells received 6 J/m^2 followed by the remainder of the dose 12 hours later. 43-3B cells received 0.5 J/m^2 followed by the remainder of the dose 12 hours later. Solid line, single exposures; dotted line, fractionated exposures.

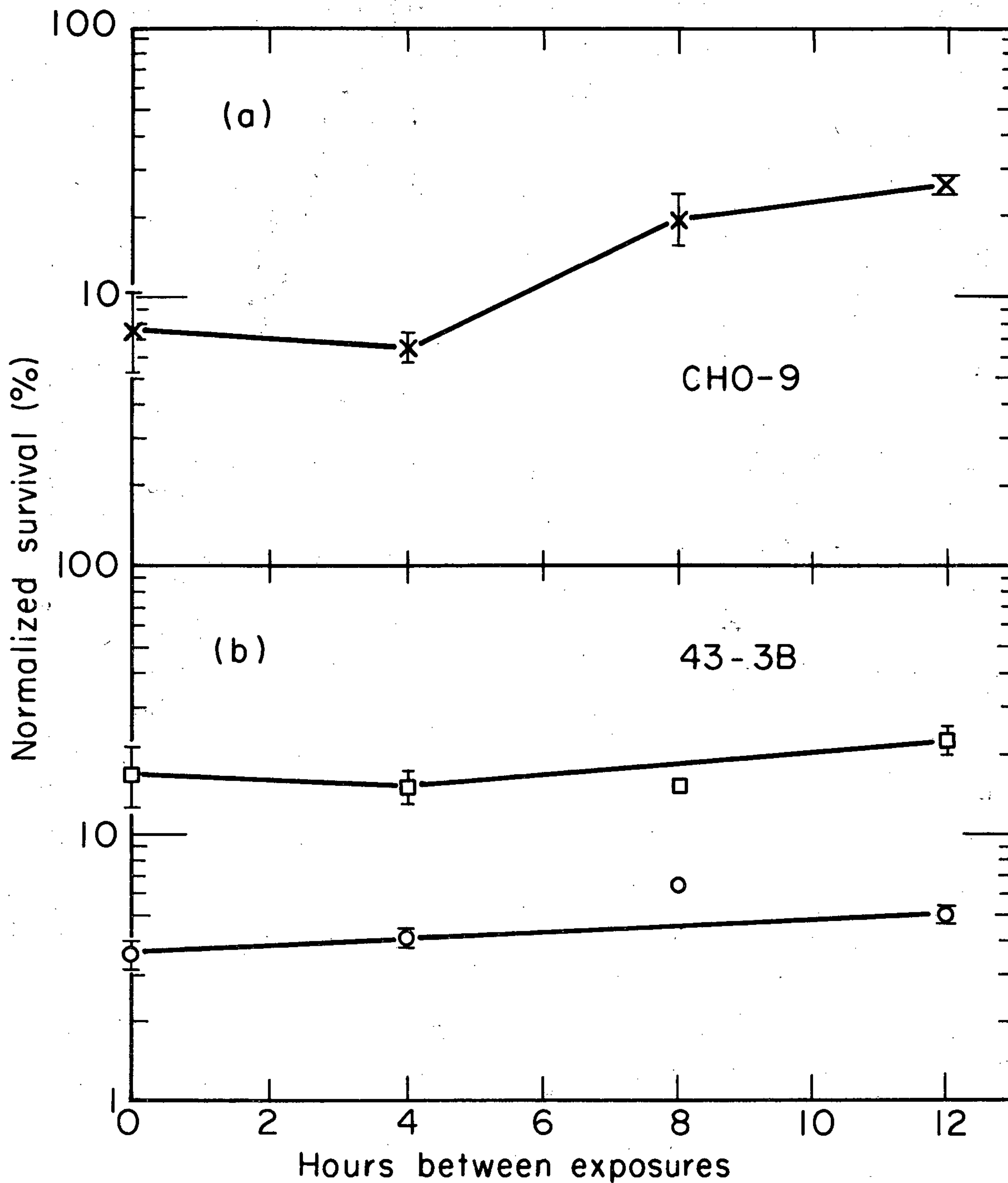


Figure 15. Time course of recovery between fractionated UV exposures. (a) CHO-9, 6 J/m^2 + recovery time + 6 J/m^2. (b) 43-3B, 0.5 J/m^2 + recovery time + 1.0 J/m^2 (squares); 0.5 J/m^2 + recovery time + 1.0 J/m^2 (circles). The bars represent standard errors of the mean for two experiments per point.

CHAPTER 4

DISCUSSION

4.1. Sensitivity to UV-Induced Lethality

In terms of D_0 fluence, 43-3B is 10.7 times more sensitive than CHO-9 to UV-induced reproductive death (figure 2). This ratio of D_0 values (wild-type/sensitive mutant) is greater than that reported for previously isolated UV sensitive mutants of CHO (Busch et al.,1980; Thompson et al.,1980; Stamato and Waldren, 1977). The ratio is similar to that reported for normal human fibroblasts as compared to xeroderma pigmentosum group A or D cells (Andrews et al., 1978; Maher et al. 1975). The extreme sensitivity resembles that of excision-deficient strains of bacteria (*uvr*) or yeast (the *rad 1 to 4* epistatic group).

4.2. DNA Repair Deficiency in 43-3B

Excision repair can be monitored by a number of assays. Repair patching, detected autoradiographically as unscheduled DNA synthesis has been shown to occur in Chinese hamster cells (Rasmussen and Painter, 1966; Painter and Cleaver, 1969; Zelle et al. 1980) including CHO cells (Cleaver, 1974). Insertion of bases into parental DNA during excision repair patching has also been detected as repair replication in wild-type CHO cells

(Gautschi et al., 1973; Thompson et al. 1980; Busch et al., 1980). Excision of pyrimidine dimers in CHO cells has been directly observed by chromatography (Meyn et al., 1974; Cleaver and Park, in press, Photochem. Photobiol.), and by removal of UV-endonuclease sensitive sites (Zelle et al., 1980).

A number of the UV-sensitive clones of CHO isolated by Busch et al.(1980) and Thompson et al. (1980) have been assigned to complementation classes on the basis of somatic cell hybridization studies (Thompson et al.,1981; Adair, 1980). The UV-sensitive mutant 43-3B appears to fall into complementation group 2 on the basis of a hybridization test (Thompson, personal communication). Several representatives of this class have been shown to be deficient in repair replication after irradiation with UV light (Thompson et al.,1980; Busch et al., 1980). This result, coupled with the observation of greatly reduced DNA repair patching after UV (unscheduled DNA synthesis; figures 10,11; Table V) shows that 43-3B is a DNA repair-deficient isolate with similarities to some of those described by the above investigators.

Excision repair of UV-induced damage involves incision of a DNA strand, removal of photoproducts, resynthesis of DNA and ligation to restore the DNA duplex (for reviews see Cleaver, 1974; 1978). Endonucleolytic inci-

sion at the onset of repair of UV damage appears to relax supercoiling and cause nuclear structures isolated in 2M salt to sediment slower than controls in sucrose gradients (Cook and Brazell, 1976; Yew and Johnson, 1978; Cook et al., 1978). Repair during incubation after UV allows the DNA to regain its normal supercoiled configuration within several hours.

Studies with intercalated ethidium bromide support the view that excision breaks decrease the sedimentation distance by decreasing the amount of supercoiling in nucleoids (Cook and Brazell, 1975). A biphasic response in sedimentation distance with increasing concentration of ethidium bromide is seen as negative supercoils are relaxed in chromatin and positive supercoils are introduced.

Repair differences between cell types can be demonstrated by the nucleoid sedimentation technique. Xeroderma pigmentosum cells have been shown by this method to be defective in incision at damaged sites (Cook et al., 1978). Human T lymphocytes, which are more sensitive to killing by UV than B lymphocytes, have a slower recovery to normal supercoiling after UV as measured by sedimentation of nucleoids (Yew and Johnson, 1978).

When the method was applied to CHO-9 and 43-3B, the normally observed biphasic response was seen for CHO-9,

but not for the mutant 43-3B. A smaller but significant decrease in sedimentation rate was seen in 43-3B; it was not accompanied by an increase to control values during the times examined. These results suggest that 43-3B may be able to incise at UV lesions with a much reduced efficiency. However, eventual repair with restoration of supercoiling apparently does not take place. This interpretation is in agreement with preliminary results obtained using controlled alkaline denaturation of isolated DNA to measure UV incision (Sakai and Burki, unpublished observations).

4.3. Sensitivity to Solar Light

43-3B is clearly more sensitive than CHO-9 to the lethal effects of solar wavelengths. However, the relative sensitivity as measured by a ratio of D_0 values for the two cell types (6.4) is less than the ratio obtained for germicidal UV.

As pointed out by Calkins and Barcelo (1979), this appears to be a general phenomenon for UV repair deficient mutants of a number of organisms as compared to the wild type. Very UV-sensitive mutants are not sensitized equally for near and far UV; they are always less sensitive to near-UV relative to the wild-type than to far-UV. All of the mutants, of course, were originally selected for sensitivity to far-UV. These results imply that

near-UV produces some types of damage that are not handled by exactly the same repair pathways used for far-UV-type damage.

It is possible to calculate the survival expected if killing from the solar light is due only to far-UV type damage arising from the radiation in the 280-320 nm region.

For radiation of a given wavelength, a fluence rate in photons m^{-2} sec^{-1} can be calculated according to:

$$f = F/E$$

where

$$E = \frac{hc}{\lambda}$$

and

f = fluence rate, photons m^{-2} sec^{-1}

F = fluence rate, J m^{-2} sec^{-1}

E = energy of one photon, J

h = Planck's constant, 6.624×10^{-34} J sec

c = speed of light, 3×10^{8} m^{-2} sec-1

λ = wavelength in meters.

Substituting,

$$f = \lambda F(5.03 \times 10^{15}) \qquad (1)$$

The integrated biological effectiveness from 280 to 320 nm, in units of equivalent 254 nm quanta per m^2 per

sec, was calculated from Table VI as 4.0×10^{15}. This represents the measured total (energy) fluence rate F from the solar simulator of 700 J m^{-2} sec^{-1}.

For 700 J m^{-2} sec^{-1} of 254 nm germicidal UV radiation (from equation 1):

$$(254)(700)(5.03 \times 10^{15}) = 8.94 \times 10^{20} \text{photons } m^{-2}\ sec^{-1}$$

This means that if solar killing can be ascribed <u>only</u> to far-UV-type damage from the region 280-320 nm then 1 J m^{-2} of solar radiation should be

$$\frac{4.0 \times 10^{15}}{8.94 \times 10^{20}} = 4.47 \times 10^{-6}$$

times as effective as 1 J m^{-2} of 254 nm radiation.

For instance, a solar simulator fluence of 1.2×10^{6} J m^{-2} is equivalent to a 254 nm fluence of

$$(1.2 \times 10^{6})(4.47 \times 10^{-6}) = 5.4 \text{ J } m^{-2}$$

if only the 280-320 nm region is relevant for cell killing.

However, for CHO-9, the survival after 1.2 J m^{-2} solar-simulated light is 0.13; for 5.4 J m^{-2} germicidal UV the survival is 0.9 (figure 8). Thus, solar-induced killing is much greater than that expected if only the UV components 280-320 nm were involved. This, of course, simply means that the other wavelengths are also involved in killing and is confirmed, for instance, by action

spectra which show a relative biological effectiveness per quantum of 10^{-5} to 10^{-4} for visible wavelengths as compared to germicidal UV (Smith, 1977).

Tuveson (1980) has provided evidence that near UV sensitivity is under separate genetic control from far UV sensitivity in _E. coli_. He found that mutants at the _nur_ locus are very sensitive to near UV irradiation but show a normal response to far UV. This is to be expected if the types of lesions produced by the two types of radiation are at least partially separable in terms of the types of repair which deal with them.

Based on the D_0 fluences for killing of CHO-9 and 43-3B by germicidal UV and by simulated solar light, a calculation can be made to compute the relative contributions of short-wavelength and long-wavelength damage to solar-induced killing. The details of this calculation are given in Appendix D. The results show that the short-wavelength and long-wavelength contributions to solar sensitivity are about equal in the wild-type. On the other other hand, for the UV-sensitive mutant, the short wavelength component is much more important; about 97% of the lethality can be attributed to the short-wavelength component (far-UV-type damage), because this is the type of damage which is not repaired efficiently in the UV-sensitive mutant.

4.4. UV-Induced Mutation

A marked hypersensitivity to UV-induced mutagenesis was observed. For all three loci studied, more mutations are induced at a given fluence in the UV-sensitive 43-3B than in the wild-type CHO-9. When induced mutations are compared at a given survival level, 43-3B is still clearly hypermutable (figure 16). Similar curves are obtained when data for the other two loci are plotted. 43-3B is on the average 47 times more mutable than CHO-9 on a per fluence basis and an average of 4.4 times more mutable on a per D_0 fluence basis (Table III). Thus, the sensitivity of these cells in comparision to the wild-type is similar to that reported for excision defective XP cells, while the hypermutability (as a function of dose and survival) is similar to that reported for the XP variants, although quantitatively much larger (Maher et al., 1976; Myhr et al., 1979). Excision defective XP cells apparently are hypermutable by UV only on a per unit fluence basis, and not when compared on a per D_0 fluence (survival) basis (McCormick and Maher, 1978; Glover et al. 1979). The level of unscheduled DNA synthesis observed as compared to the wild type (13 to 20%) is more characteristic of XP group C cells (Bootsma, 1979; Andrews, 1978). These UV sensitive CHO cells therefore have a phenotype that does not compare in detail to any of the XP complementation

groups.

The excision-defective strains of bacteria and yeast are also UV-hypermutable in comparision to the wild-type per unit fluence, but not at the same survival level. Eckardt and Haynes (1977) made a plot of mutation frequency vs. survival similar to figure 16 for wild-type and *rad 2* strains of yeast and found that the frequencies for both cell types fell along the same line, indicating that similar mutation frequencies are obtained in the two strains at the same survival level. Hill (1965) came to a similar conclusion for wild-type and UV sensitive *hcr* strains of *E. coli*.

Another way to plot mutation frequencies is as mutant yield i.e., (mutants recovered per cell exposed). This can be important, for instance, in considerations of using a particular strain in an environmental test system. The mutant yield Y is obtained by multiplying the mutation frequency at a given fluence by the survival at that fluence. The plots for Y in figure 17 were derived by taking the mutation frequencies at 0, 0.5, 1.0, 1.5, 2.0, and 3.0 J/m^2 for 43-3B, and for 0, 3.0, 6.0, 12.0, 18.0, and 24.0 J/m^2 for CHO-9 and multiplying by the survival at each fluence according to the survival curve (figure 2). The units of Y are mutants obtained per 10^6 cells exposed, and so the number represents the number of independently aris-

ing viable mutant colonies arising from 10^6 exposed cells. The plot of yield vs. fluence has approximately the same integral for both cell types, indicating that neither has a greater overall detection sensitivity. At very low doses of a UV-like mutagen, 43-3B would be a better detector, but at higher doses, mutagens might not be detected at all by the sensitive mutant because all cells would be killed (figure 17).

The CHO 43-3B cell line thus appears to be deficient in a DNA repair process, leading to both increased cell death and mutation induction. Apparently, this repair process is essentially error-free, because the mutation frequencies are increased in the deficient mutant, rather than decreased.

4.5. Inhibition and Recovery of DNA Synthesis

Inhibition of DNA synthesis by UV shows a biphasic, fluence-dependent response (Rasmussen and Painter, 1964; Cleaver, 1970b). This type of response was observed in this study and was almost identical for CHO-9 and 43-3B (figure 13a). When XP and normal human cells are compared in this way they also show the same fluence response for total DNA synthesis within short times after UV (Cleaver, 1970b; Kaufmann and Cleaver, 1981).

Exposure to low UV fluences produces a transient reduction in the rate of DNA synthesis, followed by a return to control values (Rudé and Friedberg, 1977; Dahle et al., 1980). One current hypothesis is that lesions act as blocks to DNA chain growth and that recovery involves bypass of blocks, or growth from adjacent unblocked replication forks, or removal of the blocks by repair (Cleaver, 1978; Park and Cleaver, 1979b). The similar inhibition of DNA synthesis in the UV-sensitive mutant and wild-type within the first 2 hours post-UV indicates that this inhibition is a reflection of the initial damage leading to replication blocks.

After a low UV fluence, both CHO-9 and 43-3B were able to recover normal rates of DNA synthesis (figure 13b). However, after higher fluences, 43-3B is deficient in recovery. In fact, at 2.5 J/m^2, CHO-9 had recovered to near control values 8 hours after irradiation while 43-3B had not recovered at all. This suggests that continued DNA synthesis depression results in large part from the excision repair deficiency which allows UV-induced lesions to act as replication blocks for a longer period of time. These results are in accord with those reported for normal human vs. XP cells (Rudé and Friedberg, 1977; Park and Cleaver, 1979a).

4.6. Recovery Between Fractionated Exposures

The ability of Chinese hamster cells (V-79, M3-1, and CHO) to recover between fractionated UV exposures has been observed a number of times (Todd, 1973; Humphrey and Meyn, 1970; Lam, Ph.D. Thesis, U.C. Berkeley, 1981). Todd (1973) observed that a slightly UV-sensitive variant of V-79 cells (2 or 3 times more sensitive than the wild-type on a D_o basis) was still able to show split-fluence recovery, and suggested that recovery capability may be independent of the ability of a cell to repair lesions. On the other hand, excision deficient XP cells have been shown to lack the ability to recover from potentially lethal damage after UV (Maher et al., 1979).

Little or no recovery between fractionated exposures was observed in 43-3B. However, although the survival levels for CHO-9 and 43-3B in these experiments are similar, the actual fluences used in the experiments with 43-3B are much smaller (1.0-2.0 J/m^2) than those used with CHO-9 (12-24 J/m^2).

Therefore, the following two hypotheses are suggested for the lack of split fluence recovery after UV in 43-3B:

(1) Recovery is repair-dependent; the repair deficiency in 43-3B prevents recovery, as measured by increased survival with fractionated exposures, from taking place.

(2) Since UV damage recovery is thought to be S-phase dependent (Humphrey and Meyn, 1970), it is possible that a delay in S-phase precipitated by UV is necessary in order for the recovery to take place. At the lower fluences received by 43-3B, DNA synthesis is inhibited much less than for an equivalent survival fluence for CHO-9; recovery may not be able to take place because S-phase is not sufficiently prolonged.

For example, little growth delay is seen after 0.5 J/m^2 in 43-3B, but significant immediate growth delay in CHO-9 is seen after 6 J/m^2, probably due to S retention.

The reason that a delayed S phase may allow recovery in the wild-type is that the delay allows time for repair to take place; if repair deficient, a cell may not be able to recover regardless of whether S phase is prolonged. The two hypotheses cannot be resolved without further experiments.

TABLE VI

RELATIVE SHORT-WAVELENGTH EFFECTIVENESS OF THE SOLAR SIMULATOR AND THE SUN

λ, (nm)	fluence sol. sim.	fluence from sun	RBS per quantum	E sol. sim.	E sun
280	0	5.3E-01	.65	0	3.4E-01
285	1.4E13	5.9E07	.35	4.9E12	2.1E07
290	1.7E14	1.6E12	.20	3.4E13	3.2E11
295	5.2E15	5.3E14	.10	5.2E14	5.3E13
300	1.6E16	1.4E16	.015	2.4E14	2.1E14
305	4.3E16	8.3E16	.003	1.3E14	2.5E14
310	2.1E16	2.3E17	.0005	1.1E13	1.2E14
315	2.5E16	4.1E17	.0001	2.5E12	4.1E13
320	2.9E16	5.9E17	.0001	2.9E12	5.9E13

Total irradiance, 280-320 nm (W m^{-2}):
3.19 (sun) 0.320 (solar simulator)

Integrated effectiveness
(equivalent 254 nm quanta m^{-2} sec^{-1}):

3.5×10^{15} (sun) 4.0×10^{15} (solar simulator)

---

† Fluences in the table are given in quanta m^{-2} sec^{-1} nm^{-1}, computed from the energy fluences in Table VII. The fluences are thus normalized to one A.M. 1 solar constant, 925 W m^{-2}. The integrated values for relative biological effectiveness (E) given above for the solar simulator have been adjusted to the output of the simulator (700 W m^{-2}) by multiplying the integral by the factor 700/925. The relative biological sensitivity (RBS) at a given wavelength is taken from the action spectra given by Rothman and Setlow (1979).

Figure 16 (overleaf). Induction of mutation to diphtheria toxin resistance vs. surviving fraction for CHO-9 (open circles) and 43-3B (solid circles). Each point shown for 43-3B corresponds to one indicated in figure 9; the mutation frequencies for each fluence are plotted as a function of the survival level at that fluence, read from the survival curve in figure 2. The points for CHO-9 were plotted in the same manner, using data from experiments of Burki and Wood (see footnote to Table III).

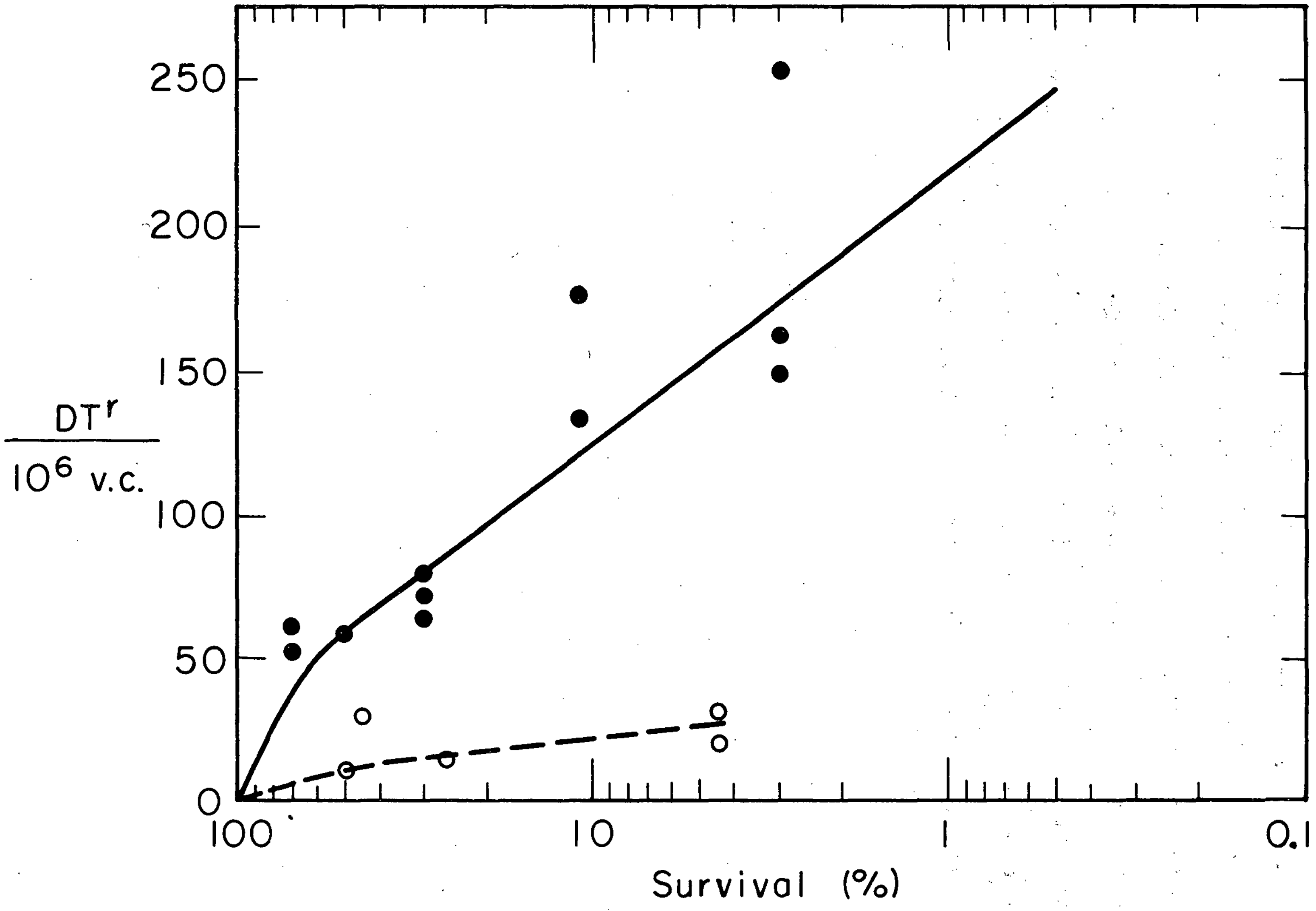
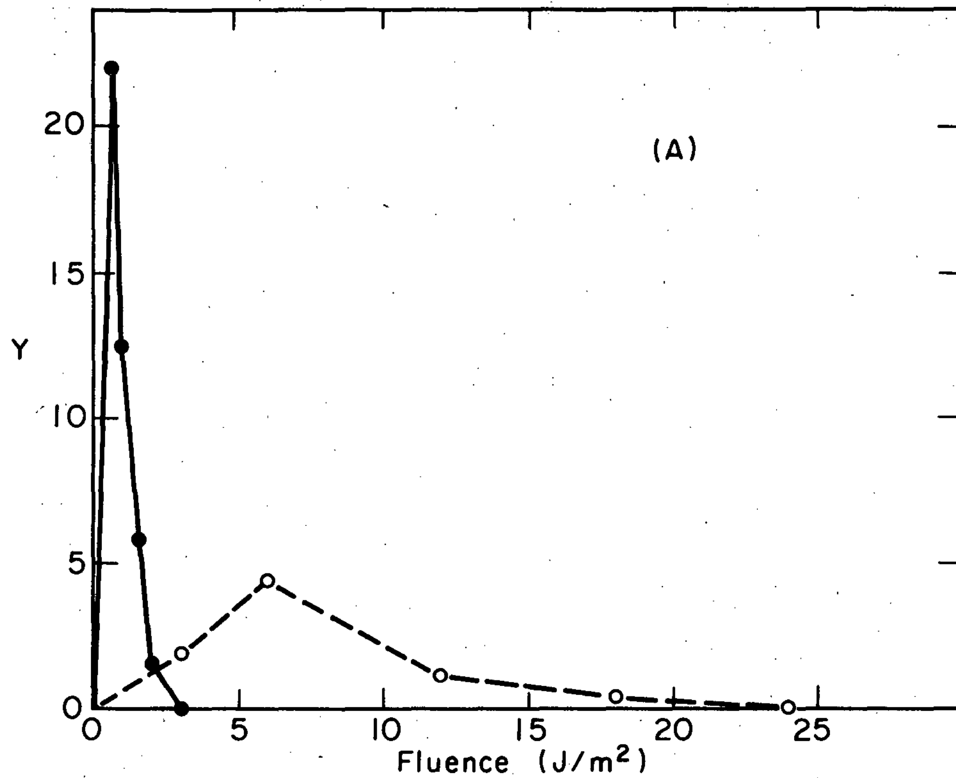


Figure 16.



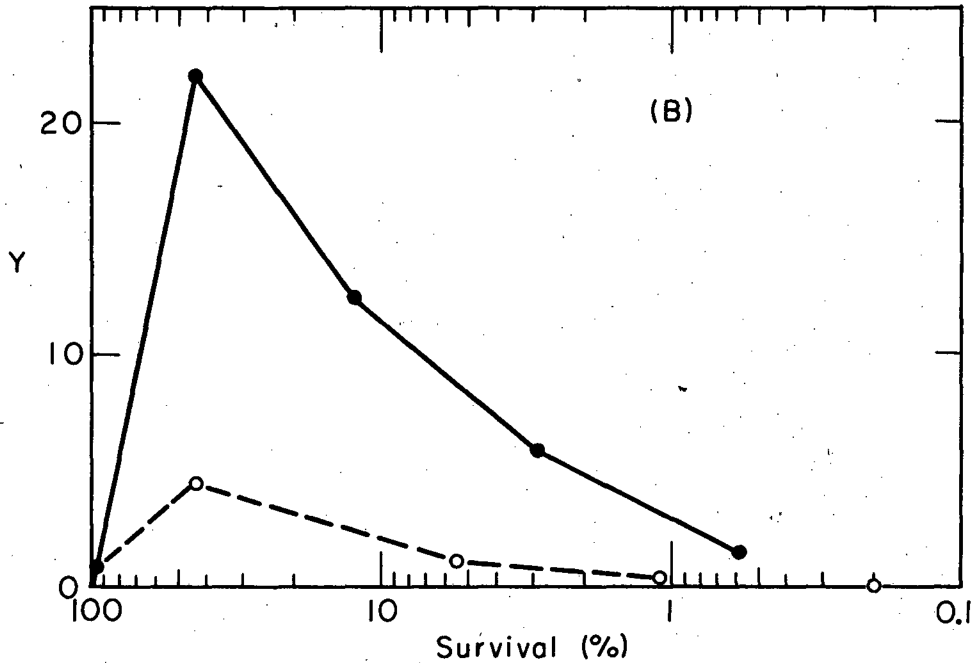


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Figure 17. Mutant yield Y (= mutants per viable cell X survival) for induced diphtheria toxin resistance, plotted vs. fluence (a) and survival (b). The units of Y are mutants per 10^6 cells irradiated. Solid lines, 43-3B; dotted lines, CHO-9. See text.

4.7. General Conclusions and Implications

The use of sensitive, repair-deficient variants to study post-irradiation endpoints in mammalian cells has been expanded to include not only naturally occurring human diseases and syndromes, but also permanent cell lines. There are great advantages in the construction of hypersensitive variants from permanent lines; an obvious one is the ease of handling of the cultures, but more important is the ability to select for a certain phenotype with a definite experimental aim, rather than having to study naturally occurring variations which probably have a much more limited range.

The phenotype of the UV-sensitive mutant 43-3B does not correspond with any of the known human disease syndromes. The closest is xeroderma pigmentosum, but the different groups of this disease seem to have either extreme sensitivity to killing (the excision defective groups) or extreme sensitivity to mutation (the variants), but not both in combination. Despite a number of distressing afflictions, an XP patient is a viable and relatively normal human being. It is conceivable that a repair deficiency which leads to extreme sensitivity both to UV-induced lethality and mutation is not compatible with the proper development of a human being to full term.

The present study demonstrates the importance of DNA excision repair in limiting the frequency of induced mutations in mammalian cells. This repair system seems to control a fairly wide mutational spectrum. Ouabain resistant and diphtheria toxin resistant mutants arise by point mutation, whereas 6-thioguanine resistant mutants can arise by both point mutation and deletion. The similar increase in mutation frequency for all three markers in 43-3B indicates that both types of mutations are affected. Ishii and Kondo (1975) found that excision-defective strains of bacteria also show an increase in both point mutation and deletion after UV-irradiation and exposure to other agents.

Recovery from inhibition of DNA synthesis by UV is strongly dependent on the existence of a repair system for UV-induced damage. Recovery from sublethal damage also seems to be connected with repair capability.

The observations reported here show that two near-isogenic cell types, very similar in most respects except for repair efficiency, can be used to separate post-irradiation phenomena which are repair-related from those which are not affected by repair. By using repair-deficient mutants, rather than by doing studies on the wild-type alone in which conditions are manipulated in the hope of affecting repair, much more direct and convincing

answers can be obtained to questions which center around the involvement of repair in various phenomena.

Only one allele of one gene controlling UV sensitivity has been studied in this work. This limitation has had the advantage of allowing an examination of a number of properties in the sensitive variant. However, as pointed out by Lawrence and Christensen (1976), different alleles of the same gene can show quite variable responses, especially to mutation induction, so that conclusions drawn from results with one strain only might be misleading.

At this time in the yeast Saccharomyces cerevesiae there are at least 9 loci controlling dimer excision, at least 9 loci involved in double strand break repair, at least 11 loci involved in error-prone repair, and probably several other loci involved in minor pathways (Lemontt, 1980; Haynes and Kunz, 1981). The situation in mammalian cells is expected to be at least as complicated. UV sensitive mutants of CHO representing at least 5 complementation classes which seem to be involved in excision repair have been isolated (Adair, 1980; Thompson et al., 1981). Studies with xeroderma pigmentosum cells indicate that there are at least 7 genes associated with the incision step of excision repair in human cells (Bootsma, 1979; Andrews et al., 1978; Cleaver, 1980). The similar numbers

of genes which seem to be involved in excision repair in yeast and mammalian cells indicate that the processes in the two organisms may have features in common. Both organisms are eucaryotes; basic similarities in DNA structure and replication may be accompanied by similarities in the DNA repair processes.

There is expected to be a group of mammalian cell mutants which exhibit hypomutability, analogous to the rad 6 epistatic group of yeast, or to recA, lexA, and umuC in E. coli. Two such mutants in CHO have been found to date (Stamato et al., 1981; Carver et al., 1981). Using mutants such as these, one can study the role of repair not only in preventing mutations, but in causing mutations.

4.8. Directions for Future Research

In a sense, this section serves as an outline of the limitations of this study. There are a number of projects which could be pursued in the study of 43-3B alone. For instance, a direct measurement of pyrimidine dimer excision capacity would be worthwhile. It has been hypothesized that the initial inhibition of DNA synthesis by UV is due to the introduction of breaks in the DNA at damaged sites by enzymes, providing a signal for a temporary halt to DNA replicon initiation in the vicinity of the damage (possibly to allow time for repair to take

place before potentially injurious lesions are "fixed" by replication). In an incision-defective mutant (which 43-3B seems to be, from the results with nucleoid sedimentation) this induced inhibition of replicon initiation would also be predicted to be defective. At least two methods exist to test this hypothesis:

(1) Measurement of DNA size distributions on sucrose gradients, which can distinguish initiating regions from maturing regions (Kaufmann and Cleaver, 1981).

(2) Direct examination of replicon initiation and maturation by DNA fiber autoradiography (Dahle et al.,1980).

The relation of a repair defect to recovery ability between fractionated exposures will certainly be of interest to some. Suitable experiments probably can be designed to determine if 43-3B has any significant recovery capability under a variety of conditions (for instance, conditions which inhibit DNA synthesis) in order to more closely specify the role of repair in recovery phenomena.

The repair defect itself could be more closely studied. One way would involve cloning the gene involved (possibly by plasmid rescue using a plasmid library derived from the wild type). Once cloned, the gene could be mapped to a chromosome by hybridization to specific

chromosome fragments (Gusella et al., 1979). If a system could be developed to obtain the functional gene protein product, the incision protein could be analyzed and compared with the defective protein in the hypersensitive mutant.

The same approach used in this thesis should also be used to isolate mutants sensitive to types of DNA damage other than those produced by UV. Several obvious choices are X-rays, near-UV, and chemical alkylating agents. There are currently no mutants of a permanent cell line which are extremely sensitive to killing by ionizing radiation. Such mutants, expected to be at least partially analogous to the <u>rad 52</u> epistatic group in yeast, would be a great advantage for research on the effects of ionizing radiation on mammalian cells. The relation of repair to mutation induction by X-rays, and to split-dose recovery, are two major potential areas.

The future in this line of research offers ample opportunity for the further development of our ideas on the role that alterations to the genetic material (and their repair) play in the induction of killing, mutation, and other endpoints in mammalian cells.

APPENDIX A

The CHO Cell Line

The CHO cell line was first established in 1957 in T. Puck's laboratory in Denver from a primary culture of macerated hamster ovary (Puck et al., 1958). The cells are fibroblastic in appearance and therefore are probably derived from connective tissue in the organ as opposed to being cells from a functional unit of the ovary. The hamster itself was obtained from Yerganian, who apparently obtained it in turn from T.C. Hsu (Hsu, 1979), who started several other lines from the cells of Chinese hamsters.

The Chinese hamster (Cricetulus griseus) has a diploid chromosome number of 22. In the years since the establishment of the line, CHO cells have undergone some evolution in karyotype in several labs, yet still remain near-diploid (Worton, 1978). The two strains in common use at this time are the CHO-K1 strain, with a modal chromosome number of 18, and the CHO strains with a modal chromosome number of 20 or 21.

The sub-strain of CHO used in our lab was obtained from Klevecz and Kapp at the City of Hope Medical Center, Duarte, California, by H.J. Burki and was designated "CHO-KK". It had been selected for use on the Cell-Cycle

Controller automatic shakeoff machine for use in experiments with synchronous cells (Klevecz, 1975).

APPENDIX B

Procedure for Isolation of UV-Sensitive Mutants

Cultures of CHO-9 were mutagenized by treatment with N-nitroso-N-ethylurea (ENU; CAS registry # 759-73-9). Exponentially growing cells were treated with ENU (Fluka, West Germany) which had been dissolved at 5 mg/ml in phophate-citrate buffer (pH 6). ENU was added to cells at 400 μg/ml, and the cells were incubated at 37° for 30 minutes, followed by 2 rinses with Puck's saline A. About 5 X 10^{6} cells were initially treated, and then grown in a glass roller bottle for four days before harvesting. These cells were frozen in plastic vials (1ml medium plus 5% DMSO, 10^{6} cells per vial), and used as the starting population in the mutant isolation.

The protocol for the mutant isolation was as follows:

Day 0: A vial of mutagenized cells is thawed (approximately 10^{6}) and seeded into a large glass roller bottle.

Day 4: Two hundred fifty cells were plated into each of 100 90 mm dishes containing 10 ml medium and incubated at 37°. Three dishes were seeded with 1.36 x 10^{6} cells in medium containing 3 mM ouabain, as a check that the cells, having grown for 8 days after ENU treatment, had indeed been mutagenized. After 8 days these ouabain plates were

stained and gave 54 OUA^{r} clones per 10^{6} cells plated (value corrected for plating efficiency).

Day 5: Filter paper circles were applied to each plate. The paper circles had been cut using a cardboard template from large circles of Whatman #50 filter paper, and a small notch was made in the edge of each as a reference mark. These disks were autoclaved in groups of ten in glass petri dishes. The filter paper was applied to the dish containing media and cells with a sterile pair of forceps in such a way as to minimize contact with the cells ("floating it down"). Glass beads (washed in 70% ethanol and then repeatedly in H_2O) were poured onto the top of the paper. About 2 liters of sterile #4, 5 and 6 glass beads were required for 100 dishes.

Day 10: Replica plating. This was accomplished according to the protocol given below:

(1) Ten master plates and 10 corresponding "replica" dishes containing 10 ml medium were taken from the incubator. The position of the filter paper notch on the bottom of the master plate was marked.

(2) The media and beads from the masters were poured into a large sterile Buchner funnel.

(3) The filters were removed from the masters without sliding action, rinsed with serum-free medium from a

sterile wash bottle by spraying the cell side, and transferred to the replica dish, cell side down.

(4) Five ml saline (clear Puck's saline A supplemented with 200 mg/liter $CaCl_2$ and 275 mg/liter $MgCl_2$ $6H_2O$) was put on the master plates.

(5) The glass beads from the funnel were poured onto the replica plates and the replicas incubated.

(6) The position of each colony on the master plate was marked by placing a dot on the bottom of the dish with a red felt-tipped marker. This took about one to two minutes per dish, and was best done by transmitted light through the top of the dish.

(7) The saline was aspirated from the marked dishes.

(8) The marked master plates were UV irradiated. 5 sec of UV light inside a tissue culture hood were used for a total fluence of 7.5 J/m^2. Several plates were irradiated with 35 J/m^2 so that the appearance of UV-killed colonies could be positively recognized during the mutant hunt.

(9) Ten ml fresh medium was put onto the masters which were then reincubated. Eighty dishes were put through the above procedure.

Day 12: The master plates were examined for putative mutants, recognized as a red dot on the plate (where a

colony had been on Day 10) where no blue staining colony now appeared, or only a very faint one.

Six colonies were identified as putative sensitive mutants, located on 6 separate dishes. The identification was made on the basis of absent or very faint staining at the sight of the pen mark, coupled with an observation under the tissue-culture microscope of a very sparse group of rounded and lysed cells (figure 1). Their healthy counterparts were removed from the corresponding replicas using metal cloning rings (Ham 1972). Red dots with no visible colony at all also proved to have no colony on the replica. The overall replica plating efficiency was around 83%.

In about a week, 4 of the putative mutants had grown up and were tested for UV sensitivity by irradiating 10^6 cells in a dish with 7.5 J/m^2 (using wild type cells for a comparison). Two days later, 2 of the clones, from dishes 27 and 43, appeared to have a large fraction of UV-sensitive cells, as shown by the moribund appearance of the culture when compared to the relatively unaffected wild type. These two populations were recloned (by replica plating), retested, and proved to be positive for UV sensitivity. Individual subclones were grown up and frozen in liquid nitrogen with a controlled-rate freezing apparatus.

APPENDIX C

Characteristics of the Simulated Solar Light

The high pressure Xenon lamp is a very bright but very small source which allows the Oriel optical system to produce an intense collimated beam. The spectrum approximates a 6000 K black body with superimposed Xenon lines. The equivalent color temperature of the sun is near 5755 K (Henderson, 1977).

A solar spectrum with the sun at the zenith (90° altitude) is referred to as an air mass 1 (A.M. 1) spectrum (see figure 9). For air mass 1 solar simulation the Xenon light is filtered to reduce the excessive power from Xenon lines in the infrared, 800 to 10,000 nm. Excess power is also filter-attentuated in the 250 to 350 nm regions. Oriel supplies two filters to be used in combination in order to simulate the air mass 1 spectrum: an "air mass 1 + 2" filter and an "air mass 1" filter. However, direct measurement of the transmittance of the "air mass 1" filter showed that there was a sharp cutoff at 310 nm with no transmittance below 300 nm. This does not realistically fit the A.M. 1 solar spectrum (Green et al., 1974) and effectively eliminates much of the region which is the most biologically efficient (Table VI).

To provide a filter that more closely simulates air mass 1, the "air mass 1" filter supplied with the device was removed and the plastic lid of a tissue culture dish was used instead. This is also a cutoff filter but with a cutoff at 290 nm (Table VII). All lids and dishes used in these experiments with solar light were from the same lot, Falcon #71361408.

Using data for the output of a standard Xenon lamp, plus absorbance measurements of the filters used in the solar simulator, the output in the range 280 to 320 nm was calculated (Table VII). Total fluence (W m^{-2}) in a given spectral region is obtained by integrating the spectral irradiance (in W m^{-2} nm^{-1}) over the wavelengths in that region. Integrated biological effectiveness is a concept originally developed by Luckiesh (1946). It is obtained by integrating over wavelength the product of the spectral irradiance at a given wavelength and the relative biological effectiveness of a photon of that wavelength in producing a given endpoint. Thus, it is an integral of the output spectrum multiplied by an action spectrum. The total fluence of the solar simulator in the region 280-320 nm is about one-tenth that of the sun in the same region; however, since the fluence in the region 285-295 nm is greater, the integrated biological effectiveness 280-315 nm is very similar for the sun and solar simulator (Table

VI). The net result is that the effectiveness spectrum for the solar simulator is shifted to lower wavelengths relative to that of the sun.

Claims by Oriel (Oriel Catalog and Solar Simulator Technical Bulletin, 1980) that the solar simulator closely simulates the solar output in the visible region (figure 9) have been substantiated by Gay and Vitko (1979) who used direct spectroradiometric measurements.

I also calculated the output of the solar simulator with the commercial filtering (A.M. 1 filter instead of the lid filter) in the 280-320 nm region. Transmittance of the A.M. 1 filter is so low below 300 nm that the biological effectiveness is less than 0.22 that of the sun (results not shown). Some initial experiments with the commercial filtering setup showed that much of the measurable biological effects resulted from prolonged heating and fixation by the light.

TABLE VII
SOLAR SIMULATOR OUTPUT*

λ, nm	relative lamp output	1+2 filter trans.	lid filter trans.	norm. to A.M.1	Sun, A.M. 1
280	7.0	.02	0.0	0.0	3.78E-19
285	7.5	.03	5.6E-04	1.00E-05	4.14E-11
290	8.2	.04	4.5E-03	1.18E-04	1.10E-06
295	8.7	.05	1.0E-01	3.48E-03	3.60E-04
300	9.0	.06	.25	1.08E-02	9.2E-03
305	9.1	.12	.32	2.79E-02	5.4E-02
310	9.5	.05	.40	1.33E-02	.145
315	9.7	.05	.40	1.55E-02	.256
320	10	.05	.45	1.80E-02	.365
350	13	.70	.75	.55	.50
400	17	.83	.87	.98	.60
450	20	.91	.90	1.31	1.10
500	21	.93	.90	1.40	1.40
550	20	.97	.91	1.41	1.40
600	20	.93	.91	1.35	1.35
650	22	.97	.92	1.57	1.35
700	24	.92	.92	1.62	1.30
750	25	.80	.92	1.47	1.15
800	24	.64	.92	1.13	1.05
850	20	.15	.92	.22	.10
900	40	.14	.92	.41	.45

* The values in column two are for the unfiltered fluence from a 1000 W Xenon lamp at 50 cm, taken from a standard spectrum (which can be found in Smith 1977, or the Oriel catalog), and expressed in μwatts cm^{-1} nm^{-1}. To obtain the solar simulator output given in column five, the Xenon lamp fluences are multiplied by the filter factors given in columns three and four, plus a factor of 0.93 for the transmission of the quartz and water filter. Transmittance measurements were made on a scanning spectrophotometer. The value thus obtained is normalized to one solar constant by normalizing to the peak value (1.40 W/m2 at 500 nm). The fluences in columns 5 and 6 are in Watts m^{-2} nm^{-1}. The values for the global solar spectral irradiance are taken from Green et al. (1974).

APPENDIX D

Calculation of Far-UV Component of Solar Killing

A simple model allows a calculation of the relative contributions of short-wavelength and long-wavelength damage to solar-induced killing. Let the exponential portions of the survival curves for solar-simulated light (figure 8, Table IV) be represented by:

$$S = n_1 e^{-(\alpha_1^s + \alpha_2^s) D} \qquad (2)$$

for the UV-sensitive line 43-3B, and by

$$S = n_2 e^{-(\alpha_1^r + \alpha_2^r) D} \qquad (3)$$

for the wild-type CHO-9, where:

S = survival

D = Fluence to cells

α_1^s = inactivation constant, short wavelength component of killing for 43-3B

α_2^s = inactivation constant, long wavelength component of killing for 43-3B

α_1^r = inactivation constant, short wavelength component of killing for CHO-9

α_2^r = inactivation constant, long wavelength component of killing for CHO-9

Equations 2 and 3 are representations of the single hit multitarget model for cell killing, for the

exponential regions of the survival curves. The curves intersect the S axis on a semilog plot at the "extrapolation number" n (n_1 or n_2).

The quantity

$$\frac{1}{\alpha_1+\alpha_2}$$

is the D_o value for the solar simulator survival curves.

From Table IV we know that

$$\frac{1}{\alpha_1^r+\alpha_2^r} = 2.9X10^5$$

and

$$\frac{1}{\alpha_1^s+\alpha_2^s} = 4.5X10^4$$

Also, the ratio of D_o values for the UV survival curves of the two cell types gives $\frac{\alpha_1^s}{\alpha_1^r}=10.6$. Under the assumption that long wavelength and short wavelength sensitivities are independent (handled by separate repair pathways, for instance), then $\alpha_2^s=\alpha_2^r$.

Solving the above equations for the various α's,

$$\alpha_1^r = 1.95X10^{-6}$$

$$\alpha_2^r = \alpha_2^s=1.55X10^{-6}$$

$$\alpha_1^s = 2.16X10^{-5}$$

The results show that with this simple model, the short-

wavelength and long-wavelength contributions to solar sensitivity are about equal in the wild type, with

$$\frac{\alpha_1^r}{\alpha_1^r+\alpha_2^r} = 0.57$$

For the UV-sensitive mutant, the short wavelength component is much more important in solar killing, with

$$\frac{\alpha_1^s}{\alpha_1^s+\alpha_2^s} = 0.97$$

Futher refinement of this type of model requires a direct measurement of α_2^s and α_2^r which could be done by constructing survival curves for solar radiation with the wavelengths < 320 nm filtered out of the light.

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