To the Graduate Council:

I am submitting herewith a dissertation written by Richard Jennings Reynolds entitled "Excision Repair in Saccharomyces cerevisiae." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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EXCISION REPAIR IN SACCHAROMYCES CEREVISIAE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Richard Jennings Reynolds

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ABSTRACT

Two approaches, the first to detect endogenous single-strand (ss) breaks in cellular DNA and the second to detect dimers after low fluences, were used to investigate events during excision repair in wild-type and radiation-sensitive strains of S. cerevisiae. With a modification of the alkaline sucrose gradient sedimentation procedure of McGrath and Williams (Nature 212:534-535, 1966), ss-breaks were found to appear in yeast nuclear DNA after the UV irradiation of cells. In the haploid, wild-type strain S288C the number of ss-breaks was found first to increase to a maximum value proportional to the incident fluence and then to decrease during post-irradiation incubation in potassium phosphate buffer (pH 7.0) at 28°C. Both the maximum number observed and the length of incubation required before the number of ss-breaks again reached a minimum increased with fluence, and were decreased by photoreactivating treatment in the wild-type strain. Single-strand breaks were also detected in haploid, radiation-sensitive strains carrying mutations at rad1, rad2, rad3, and rad4, but the number of breaks was not found to decrease again as in the wild-type strain.

A sensitive and quantitative technique was also developed for the detection of dimers in yeast nuclear DNA. This assay employed a dimer-specific endonuclease found in extracts of Micrococcus luteus and the sensitivity of alkaline sucrose gradient sedimentation to small numbers of ss-breaks. Single-strand breaks induced by the endonuclease

were found to be proportional to fluence and equal to the number of dimers induced. The extract used was found to be totally inactive against unirradiated DNA. UV endonuclease-sensitive sites in the wild-type strain S288C were removed after 5 J m^{-2} in less than 5 min at 28°C. Site removal was less complete and required longer post-irradiation incubations after higher fluences. In the radiation-sensitive strain rad4-3 the number of sites remained constant for 6 h after irradiation with 5 J m^{-2}. The retention of sites indicates this strain is defective in excision repair. Endonuclease-sensitive sites were photoreactivable in both S288C and rad4-3.

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CHAPTER I

INTRODUCTION

Since its first discovery in E. coli (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964) excision repair of UV-induced lesions in DNA has been an intensively studied phenomenon. (For recent reviews in this area see Setlow and Setlow, 1972; Cleaver, 1974a; Grosman et al., 1975; Kondo, 1975; Swenson, in press.) The original discoveries were made during investigations into the fate of cyclobutyl pyrimidine dimers in E. coli. Dimers have since been shown to be the predominant photoproduct produced in hydrated DNA by UV radiation (Setlow and Carrier, 1966) and have also been shown to be biologically important (Setlow, 1968).

During early investigations into the fate of dimers it was found that dimers disappeared from acid precipitable DNA during postirradiation incubations but remained recoverable from acid soluble cell fractions (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). It was concluded that dimers were physically removed from the DNA of E. coli by a dark repair mechanism after UV irradiation. The process by which this was accomplished became known as excision repair and it is now thought to occur through the following enzymatic steps: (1) a specific endonuclease recognizes a lesion in the DNA and makes a single-strand incision near to or at the 5' terminus of the lesion, (2) an exonuclease removes the UV-induced lesion probably along with a few additional

undamaged bases adjacent to the damaged region, (3) a DNA polymerase using the complementary strand as a template replaces the excised region through the polymerization of new DNA in the resulting gap, (4) after the gap is filled the two adjacent, single-strand ends are joined by polynucleotide ligase.

The simple eukaryote Saccharomyces cerevisiae has a number of attributes which make it an interesting organism in which to continue investigations into the response of living systems to UV radiation. Foremost among these attributes is the large number of mutants sensitive to UV radiation which have been isolated. (For a recent review see Game and Cox, 1971). These mutants, now designated "rad" mutants, have been shown by complementation tests to belong to more than 20 distinct complementation groups (Game and Cox, 1971). In addition to the large number of radiation-sensitive mutants S. cerevisiae is readily cultured and manipulated genetically, and largely for these latter reasons considerable progress has been made in the areas of yeast genetics (Mortimer and Hawthorne, 1973; Sherman and Lawrence, 1974) and molecular biology (Hartwell, 1970; 1974).

Investigations into molecular events after UV irradiation in yeast have been hampered by the absence of thymidine kinase (Grivell and Jackson, 1968). The absence of this enzyme prevents the use of thymine and thymidine for the selective labeling of DNA, a process which has been a considerable aid in the elucidation of molecular events during DNA repair in other organisms. The deficiency in yeast precludes the use of the same procedures used for most other organisms and has slowed investigations into DNA repair.

Despite the technical difficulties in working with yeast, considerable progress has been made by investigations into excision repair in S. cerevisiae. Patrick and Haynes (1964) examined dark recovery in diploid yeast under nongrowth conditions and established optimum conditions of pH and temperature for recovery. Evidence relating dark recovery processes after UV irradiation to excision repair in yeast was then reported by Kilbey and Smith (1969) who examined the effect of photoreactivating treatment on the viability of wild-type and radiation-sensitive strains of yeast after UV irradiation and liquid holding. Enzymatic photoreactivation involves the in situ monomerization of dimers and increases the surviving fraction of cells in UV-irradiated yeast cultures. (For recent reviews see Harm et al., 1971; Setlow, 1972.) Silbey and Smith noted that the viability was maximally photoreactivable in wild-type, haploid yeast immediately after UV irradiation and that after incubation in potassium phosphate buffer at 32°C increases in viability resulting from photoreactivation treatment were smaller. By contrast, the viability of a radiation-sensitive strain carrying a mutation at radl was equally photoreactivable before and after incubation in potassium phosphate buffer. Similar results have been found with wild-type and radiation-sensitive strains of E. coli (Harm, 1968), where the retention of photoreactivability was attributed to the absence of excision repair in the radiation-sensitive strain. By analogy Kilbey and Smith (1969) suggested that mutations at radl in yeast result in defective excision repair.

Unrau et al. (1972) have developed a chromatographic procedure that enables the separation of pyrimidine dimers from labeled pyrimidine bases when radioactive uracil is used to label cellular nucleic acids. With this procedure pyrimidine dimers were found to be removed from acid precipitable DNA in wild-type, stationary-phase yeast during incubation of irradiated cells in growth medium (Unrau et al., 1971). They also demonstrated that a strain carrying the allele rad1-1 did not lose dimers from acid precipitable DNA under conditions identical to those used for the wild-type strain. This confirmed the earlier conclusion by Kilbey and Smith (1969) that mutations at rad1 confer radiation sensitivity through defective excision repair.

Resnick and Setlow (1972) have developed an assay for dimers based on the ability of DNA from UV-irradiated yeast cells to compete in vitro with UV-irradiated Haemophilus influenzae DNA for photoreactivating enzyme. They found excision of dimers in wild-type, log-phase yeast cells but were unable to confirm the excision of dimers in stationary-phase cells reported by Unrau et al. (1971). Resnick and Setlow also identified a second locus, rad2, mutations at which prevented excision of dimers under conditions which allowed dimer excision in wild-type cells.

Waters and Moustacchi (1974a) separated nuclear (n-) and mitochondrial (mt-) DNA on CsCl buoyant density gradients to permit separate investigations into events in the two DNA's. They found dimer excision from n-DNA in both log-phase and stationary-phase cultures during incubations in both saline and growth medium. But no dimer

excision from mt-DNA could be detected under any of the conditions used and nonspecific degradation of mt-DNA was reported after the relatively large fluence of 500 J m^{-2}. Most recently Prakash (1975) has used the specificity of UV endonuclease from bacteriophage T4 infected E. coli and the sensitivity of DNA sedimentation through alkaline sucrose gradients to small numbers of ss-breaks to detect dimers in yeast DNA. With this procedure Prakash was able to confirm the inability of strains carrying mutations at rad1 to excise dimers but at much lower fluences than those previously used.

In this thesis two basic approaches have been developed to investigate excision repair in S. cerevisiae. In the first approach the presence of endogenous ss-breaks which are predicted by current models for excision repair to be made by cellular enzymes was investigated in UV-irradiated, wild-type and radiation-sensitive strains of yeast. To determine the number of endogenous ss-breaks, spheroplasts formed by a procedure modified from that of Duell et al. (1964) were lysed on top of calibrated alkaline sucrose gradients (McGrath and Williams, 1966). Single-strand breaks were found in the DNA of rad1, rad2, rad3, rad4, and wild-type strains. In the wild-type strain the number of ss-breaks was found to be linearly related to fluence and to be photoreactivable.

The second approach was directed at determining the fate of dimers in yeast cells after low fluences of UV radiation. Most previous studies on excision repair in yeast have used fluences greater than 100 J m^{-2}. In this study events after fluences as low as 5 J m^{-2} have been investigated in wild-type and a radiation-sensitive strain of

S. cerevisiae. To accomplish this, DNA released from yeast spheroplasts was partially purified and then treated with an extract from M. luteus which contained UV endonuclease activity (Carrier and Setlow, 1970). This activity appears to be specific for pyrimidne dimers in UV-irradiated DNA (Carrier and Setlow, 1970; Patrick and Harm, 1973). Single-strand breaks resulting from incisions by the UV endonuclease at or near pyrimidine dimers were quantitated by sedimentation of DNA through calibrated alkaline sucrose gradients. Similar procedures have been developed for bacteria (Wilkins, 1973), yeast (Prakash, 1975), and human cells (Paterson et al., 1973).

CHAPTER II

MATERIALS AND METHODS

Strains

Pertinent information about strains used in this study is presented in Table 1. All strains used were haploids of α mating type. The wild-type strain S288C and the UV radiation-sensitive strains carrying the mutant alleles rad1-2, rad4-2, and rad4-3 are closely related genetically (Snow, personal communication). These four strains were kindly provided by Dr. Dorma Gottlieb. The radiation-sensitive strains carrying the mutant alleles rad2-5 and rad3-2 were isolated from strain 197/2d (α ade2-1) (Cox and Parry, 1968) which has a normal, wild-type sensitivity to UV radiation. UV sensitivity was determined for each strain and in each case was found to be similar to published results. Survival curves for S288C and rad4-3 are presented in Fig. 1.

The strain S288C EB1 was derived from S288C by ethidium bromide mutagenesis after the method of Goldring et al. (1970). A stationary phase culture of S288C was diluted 100 fold into fresh minimal medium (YNB-D) containing 10 μg/ml ethidium bromide. The culture was aerated by bubbling at 28°C for 24 h and then diluted with 0.67 M potassium phosphate buffer (pH 7.0) and plated on YEPD agar plates. S288C EB1 was a clonal isolate from one such plate and was shown to be respiration deficient by the tetrazolium overlay technique of Ogur et al. (1957).

Table 1. Description and Source of Strains of S. cerevisiae Used.

Strain Designation	Genotype[a]	Reference	D_{10}[b] (J m^{-2})
S288C	α RAD		80
S277-11d	α rad1-2 (uvr-9)	Snow, 1967	5
S223-6a	α rad4-2 (uvr-5)	Snow, 1967	
S960-1a	α rad4-3 (uvr-12)	Snow, personal communication	9
	α rad2-5 ($uvs_{2,1}$) ade2-1	Cox and Parry, 1968	6
	α rad3-2 ($uvs_{13,1}$) ade2-1	Cox and Parry, 1968	6
S288C EB1	α RAD		80

[a]Designation in parentheses are the original designations assigned prior to standardization of nomenclature for radiation-sensitive strains of yeast (Game and Cox, 1971).

[b]Fluences of UV radiation required to reduce the colony forming ability on YEPD agar plates to 10% of that found in the absence of UV irradiation as determined for this study.

Figure 1. UV survival curves for the colony forming ability of the wild-type strain S288C and for the radiation-sensitive strain rad4-3.

Log-phase cells were diluted in potassium phosphate buffer to a density which would allow the survival of approximately 100 colonies per plate. After spreading duplicate samples on YEPD agar plates the plates were irradiated with UV radiation, emitted from two germicidal lamps, at 254 nm and with an average incident fluence rate of 1.08 J m^{-2} sec^{-1}. Colonies were counted after five days of incubation at 30°C in the dark. Error bars for S288C represent standard deviations for three independent experiments.

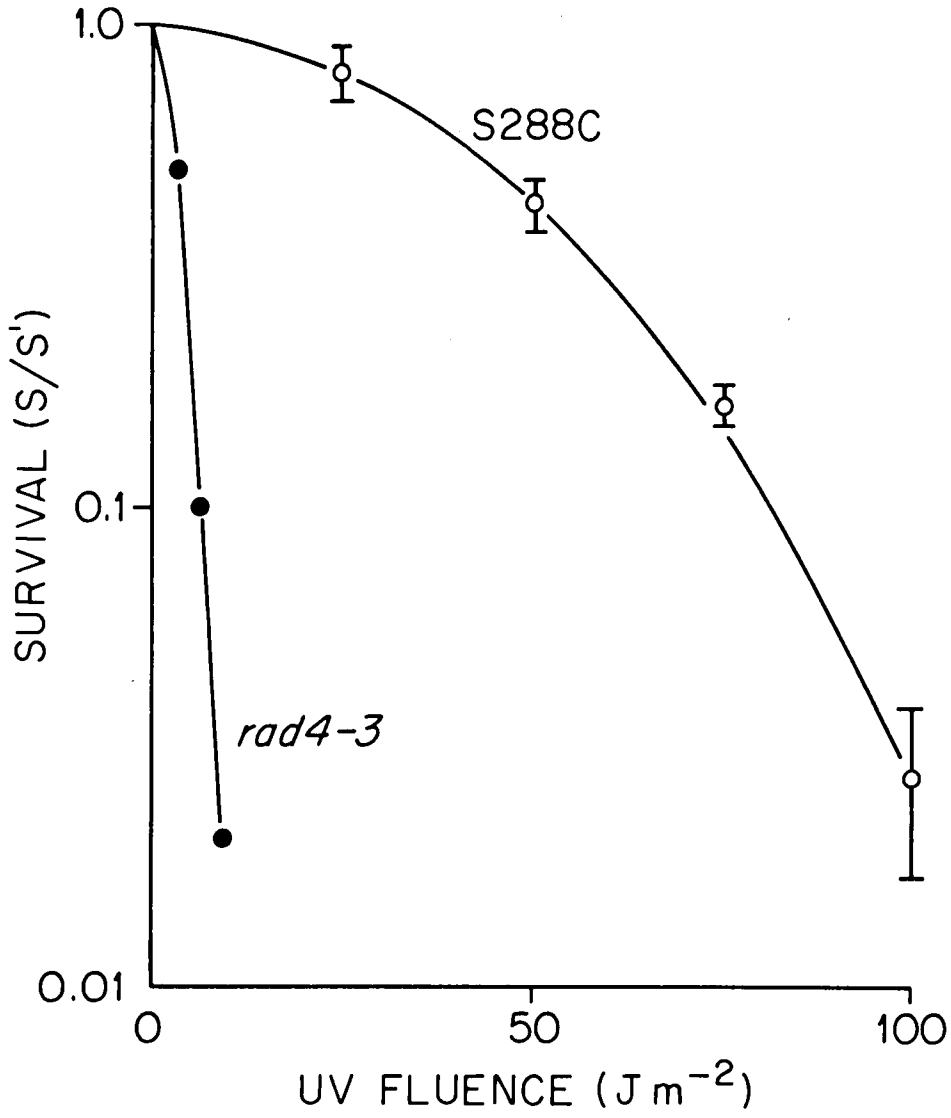


Figure 1

Media and Buffers

The following growth media were used:

1. YNB-D (a minimal synthetic medium used for liquid cultures) contained 0.67% Bacto Yeast Nitrogen Base without amino acids (Difco Laboratories, Detroit, Mich.), and 2% dextrose. This medium was supplemented with 20 µg/ml adenine for the growth of adenine auxotrophs.

2. YEPD Agar (a solid medium used for stock plates and viability determinations) contained 1% Bacto Yeast Extract, 2% Bacto-Peptone, 2% dextrose, and 1.5% Bacto Agar.

The following buffers were commonly used:

1. Potassium phosphate buffer contained 0.067 M potassium phosphate adjusted to pH 7.0.

2. UV endonuclease buffer (a buffer used in the UV endonuclease reaction mixture) contained 0.04 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), and 0.01 M tris (hydroxymethyl) aminomethane (Tris) chloride (pH 8.0).

Growth and Labeling Conditions

Cultures, inoculated from YEPD agar plates, were grown in YNB-D at 28°C, and were aerated by vigorous bubbling. To label cellular DNA log-phase cultures were diluted into fresh YNB-D containing either 5-8 µCi/ml [6-^{3}H] uridine with a specific activity of 19 or 23 Ci/mmole (Schwarz-Mann, Orangeburg, N.Y.), or containing 2 µCi/ml [2-^{14}C] uridine with a specific activity of 60.5 mCi/mmole (Amersham-Searle, Arlington Heights, Ill.). Cells were labeled for approximately 5-7 generations to a final

titer of 2-5 x 10^7 cells/ml. Cultures at this density were found to be in late log phase, the limiting stationary-phase titer being approximately 10^8 cells/ml in this medium. Labeled cells were held on ice and were used as soon as possible.

UV Irradiation Procedures

Labeled cells were harvested by centrifugation (400 x g, 4°C) and resuspended in 0.067 M potassium phosphate buffer (pH 7.0) at 6-8 x 10^6 cells/ml. The transmittancy of cell suspensions at 254 nm was determined with a Beckman DU spectrophotometer, model 2400, and absorption corrections were made according to Morowitz (1950). Cell suspensions during irradiation were normally less than 0.5 cm thick, and corrected fluence rates were normally greater than 80% of the uncorrected incident rate at the suspension surface. Cell viabilities after irradiation on plates or in suspension were comparable when corrections were made in this way. It is therefore unlikely that incident fluences were overcorrected due to light scattering during the measurement of transmittancy (Jagger et al., 1975).

UV radiation was emitted by two 15 watt, low-pressure mercury, germicidal lamps (General Electric, G15T8), housed in a desk lamp holder (Dazor Mfg. Corp., St. Louis, Mo.). Incident fluence rates were determined with a calibrated "Jagger Meter" (Jagger, 1961), and were either 1.08 or 1.35 J $m^{-2}sec^{-1}$. Cell suspensions were kept on ice and stirred with a magnetic stirring bar during irradiation. Irradiation and all subsequent operations with irradiated cells were performed under General Electric "gold" fluorescent lamps to prevent photoreactivation.

Postirradiation Treatments

For liquid-holding treatment, cells in 0.067 M potassium phosphate buffer (pH 7.0) were transferred to 17 x 150 mm test tubes and aerated by vigorous bubbling at 28°C for the desired incubation period. After the liquid-holding treatment, individual samples were kept on ice until cells were harvested for conversion to spheroplasts.

Photoreactivation by illumination with near UV radiation (see below) was done by one of the two following procedures:

1. Cell suspensions in potassium phosphate buffer were placed in 49 mm diameter, covered Pyrex petri plates. The samples were given photoreactivation treatment in an incubator (28°C) and were mixed periodically to reduce settling and self-shielding.

2. Cell suspensions in potassium phosphate buffer were given photoreactivation treatment in 17 x 150 mm test tubes. Aeration and mixing were provided by continuous bubbling, and culture temperatures remained at 24°C.

In both procedures photoreactivating light was provided by two General Electric, 15 watt, F15T8 BLB fluorescent lamps. These lamps emit 97% of their radiation at wavelengths between 300 and 400 nm (Jagger, 1967). Wavelengths below 300 nm were eliminated by the use of a 9.5 mm thick filter of window glass. Incident energy fluence rates, measured with a "Jagger Meter" and converted as suggested by Jagger (1961), were 2.3 J $m^{-2}sec^{-1}$.

Formation of Spheroplasts

Cells were converted to spheroplasts by a procedure modified from that of Duell et al. (1964) as suggested by M. A. Resnick (personal communication). Cells were pelleted by centrifugation in an International refrigerated centrifuge, model PR-6 (International Equipment Co., Needham Heights, Mass.) with a 253 rotor at 1500 rpm and 0°C. (centrifugal force approximately 400 x g). The pelleted cells were resuspended in 0.1 M Tris-SO_4 (pH 9.3) at a concentration of 1-4 x 10^7 cells/ml. After the addition of 40 µl of 14.3 M 2-mercaptoethanol per ml of resuspended cells, the suspension was incubated at 26°C for 10 min. The cells were then centrifuged as above and resuspended at the same concentration in a solution containing 20% (w/v) D-sorbitol, and 0.01 M EDTA (pH 7.0). The suspension was made 2% (v/v) with glusulase (Endo Laboratories, Inc., Garden City, N.Y.), and incubated 20 min at 26°C. Spheroplasts resulting from this procedure were pelleted by centrifugation as described for cells above and the pelleted spheroplasts were held on ice until resuspended for sedimentation on alkaline sucrose gradients or for lysis and DNA purification prior to the determination of UV endonuclease-sensitive sites.

Alkaline Sucrose Gradient Sedimentation and Molecular Weight Calculations for the Determination of Endogenous SS-Breaks

For the determination of endogenous ss-breaks, spheroplasts (0.2-1 x 10^7 spheroplasts) were resuspended in 100 ul of a solution containing 0.1 M NaCl and 0.02 M EDTA (pH 7.0). The resuspended spheroplasts were layered onto a 150 ul lysis layer (0.5 N NaOH, 0.02 M EDTA, and 3%

sarkosyl) on top of a 3.7 ml 5-20% alkaline sucrose gradient (2.0 M NaCl, 0.01 M EDTA, and 0.33 N NaOH). At the bottom of each gradient was a 0.1 ml cushion (60% sucrose and 0.33 N NaOH). Sedimentation was done in a Beckman model L 3-50 ultracentrifuge (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) at 20°C with a SW-56 rotor at 30,000 rpm for 150 min unless otherwise noted.

After sedimentation gradients were pumped from the bottom of each centrifuge tube with a proportioning pump (Technicon Corp., Tarrytown, N.Y.) and onto 1 x 57 cm Whatman 17 paper strips marked into fractions every 2 cm (Carrier and Setlow, 1971). The pumping rate was assumed to be constant with time and fractions were collected for fixed periods such that 24-25 fractions per gradient were obtained. After gradient fractionation the paper strips were washed twice in 5% trichloroacetic acid (TCA), and twice in 95% ethanol after the method of Bollum (1966). Radioactive uridine incorporated into cellular RNA was allowed to diffuse out of the paper strips during the first TCA wash which had a duration of 8-12 h. The strips were dried, cut into sections corresponding to individual fractions, and put into shell vials (Kimble Products, Toledo, Ohio) with approximately 4 ml of a counting solution containing 12 g of Permablend I (2,5-diphenyl-oxazole, 91%; 1,4-bis-2-(4-methyl-5-phenyoxazolyl)-benzene, 9%) per liter of toluene. The samples were counted with a Packard scintillation counter equiped with a Teletype paper-tape punch (Packard Instrument Co., Inc., Downers Grove, Ill.).

Data were analyzed with a computer program, written by J. E. Donnellan, Jr., and modified by R. J. Brake, which calculates weight

average and number average molecular weights (M_w and M_n, respectively) from the distribution of radioactivity through the gradient. Molecular weight calibrations for "high salt" alkaline sucrose gradients were made with T2, T7, lambda, and nicked PM2 bacteriophage DNA's (R. B. Setlow, personal communication).

Calculation of Endogenous SS-Breaks

Endogenous ss-breaks in the DNA of unirradiated or irradiated cells during liquid-holding treatment were calculated by the following formula:

Induced endogenous ss-breaks per 10^8 daltons =

$$\left[\left[\frac{2}{M_w}\right]_{\substack{\text{treated}\\\text{cells}}} - \left[\frac{2}{M_w}\right]_{\substack{\text{untreated}\\\text{cells}}}\right] 10^8$$

The number-average molecular weight was considered to be half the weight-average molecular weight. This relation holds for DNA that has been broken randomly, and the calculation of M_n from M_w reduces the sensitivity of molecular weight calculations to small changes at the top of gradients. Results computed in the above manner were not found to differ appreciably from results determined by the direct calculation of M_n from gradient profiles.

Alkaline CsCl Buoyant Density Gradients

Alkaline CsCl gradients (Vinograd et al., 1963) were used to identify putative mt-DNA from alkaline sucrose gradients. Alkaline sucrose gradients were prepared as described under "Alkaline Sucrose Gradient

Sedimentation and Molecular Weight Calculations for the Determination of Endogenous SS-Breaks," except gradients were centrifuged at 30,000 rpm for 330 min. Fractions were collected in Limbro plates and 10 µl samples from each fraction were assayed for radioactivity to determine the distribution of DNA through the gradients.

Alkaline CsCl step gradients were prepared as two 1.5 ml layers of different densities (Brunk and Leick, 1969). The solution for the bottom layer was brought to a density of 1.8900 g/cm^3 by the addition of solid CsCl (optical grade, The Harshaw Chemical Co., Solon, Ohio) to 0.04 M sodium phosphate (pH 12.3). The solution for the top layer contained (^{3}H) DNA from the alkaline sucrose gradient fractions of interest, and (^{14}C) DNA from whole cell lysates in 0.04 M sodium phosphate (pH 12.3). The solution for the top layer was brought to a final density of 1.5863 g/cm^3 by the addition of solid CsCl. The total average density for the 3 ml gradient was 1.7382 g/cm^3. The step gradients in 7/16 x 2-3/8 inch polyallomer tubes (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) were centrifuged in a Beckman model L 3-50 ultracentrifuge with an SW-56 rotor for 38 h at 30,000 rpm and 24°C. Gradients were fractionated by a procedure similar to that described for alkaline sucrose gradients except the alkaline CsCl gradients were pumped from the top with a Buchler Densi Flow apparatus (Buchler Instruments Division, Nuclear Chicago, Fort Lee, N.J.) and six drop fractions were collected on paper strips. The procedures for washing the paper strips and counting samples were as described for alkaline sucrose gradients.

DNA Purification for UV Endonuclease Treatment

Pelleted spheroplasts (0.2-1 x 10^7) were resuspended in 150 µl of a solution containing 0.02 M Tris-Cl (pH 8.0), 0.08 M NaCl and 0.002 M EDTA at 0°C and were quickly lysed by the addition of 150 µl of a solution containing 10% sarkosyl NL-97 (Sarkosyl was kindly provided by CIBA-Geigy Corp., Ardsley, N.Y.). Degradation of DNA by endonucleases was a problem during prolonged holding of the resuspended spheroplasts prior to, but not after, lysis by sarkosyl. To the lysates was added approximately 0.5 ml of phenol that had been equilibrated with UV endo buffer. After extraction with the phenol for 2 h at 24°C, the aqueous phase was recovered and extracted twice for 20 min each with two volumes of diethyl ether which had also been equilibrated with UV endo buffer. After the removal of most of the ether following the second ether extraction, residual ether was eliminated by evaporation at 35°C. The ether-extracted samples of 200-250 µl each were transferred to nitrocellulose bags and dialyzed for 30 min at 4°C against 4 ml of UV endo buffer. The dialysis procedure was repeated three more times against fresh UV endo buffer. Nitrocellulose bags were made by dipping a conical glass centrifuge tube into a solution of 7% Parlodion (Mallinckrodt Chemical Works, St. Louis, Mo.) in equal volumes of anhydrous diethyl ether and anhydrous ethanol. The resulting film was air dried and after being loosened by dipping successively into water and 95% ethanol was removed from the tube. It is known that single-strand DNA adsorbs to nitrocellulose (Billen and Hewitt, 1966) and that high UV fluences cause denaturation. (For a recent discussion see Rahn,

1973.) In this study label loss was noted during dialysis of samples irradiated with more than 50 J m^{-2}, but control experiments in which the dialysis step was omitted indicated the losses were not selective since irradiated, enzyme-treated samples had the same number of UV endonuclease-sensitive sites per unit length with and without dialysis.

Preparation of UV Endonuclease

The UV-specific endonuclease used in this study was partially purified by a procedure modified from that of Setlow and Carrier (1970). One gram of spray-dried Micrococcus luteus (Miles Chemical Co., Elkhart, Ind.) was suspended in 50 ml of a solution containing 0.2 M sucrose, and 0.01 M Tris-Cl (pH 8.0). The addition of 9 mg of lysozyme (General Biochemicals, Chagrin Falls, Ohio) to the cell suspension was followed by a 35 min incubation at 30°C. The suspension was then cooled to 0°C, and the cells were lysed by the addition of 50 ml of cold, distilled water. To precipitate nucleic acids, 8 ml of 10% streptomycin sulfate (Nutrtional Biochemical Corp., Cleveland, Ohio) were added slowly to the stirred lysate over a 30 min period. The precipitate was pelleted by centrifugation at 10,000 x g for 60 min. Ammonium sulfate, special enzyme grade (Mann Research Labs., Orangeburg, N.Y.) was added to the supernatant to give a 65% solution, and after stirring for 1 h at 4°C the resulting precipitate was pelleted by centrifugation at 10,000 x g for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in 20 ml of a solution containing 0.001 M 2-mercaptoethanol, 10% (v/v) ethylene glycol, and 0.01 M Tris-Cl (pH 7.6). This preparation was divided into smaller samples and frozen at -20°C.

A number of endonucleolytic activities are known to be present in crude extracts of M. luteus prepared as described above. These include an activity for γ-irradiated DNA (Paterson and Setlow, 1972), one for uracil-containing DNA (Carrier and Setlow, 1974), and two activities for UV-irradiated DNA (Carrier, personal communication). Similar activities have been found in M. luteus extracts by other laboratories. (For a recent review see Grossman et al., 1975.) Despite the variety of enzymatic activities having damaged DNA as a substrate, the preparation used in this study was found to be completely inactive against native, undamaged DNA. This point will be discussed further in the Results chapter. Since in this study the crudely purified preparation is used only with unirradiated or with UV-irradiated DNA, the preparation will be referred to as the UV endonuclease, and sites cleaved by treatment with this preparation will be referred to as UV endonuclease-sensitive sites.

UV Endonuclease Treatment of DNA

Dialyzed samples of DNA were divided into two 100 µl samples. To the first no additions were made. To the second 5 µl of UV endonuclease were added. Both samples were incubated for 15 min at 35°C. The reaction was terminated by the addition of 10 µl of a solution containing 1.0 N NaOH and 0.02 M EDTA. Samples were held on ice until the determination of their size distributions on alkaline sucrose gradients.

Alkaline Sucrose Gradient Sedimentation and Molecular Weight Calculations for UV Endonuclease-Sensitive Sites

Size estimations of single-strand DNA were made by sedimentation through "low salt" alkaline sucrose gradients. The procedures used were similar to those used for the determination of endogenous ss-breaks, but differed with respect to the composition of the various solutions, the sedimentation conditions, and the gradient calibrations. One hundred µl samples of DNA incubated with or without UV endonuclease were layered onto 100 µl of a top layer (1 N NaOH, and 0.02 M EDTA) on top of a 5-2% alkaline sucrose gradient (0.5 M NaCl, 0.01 M EDTA, and 0.2 N NaOH) in a 4 ml polyallomer tube. Each gradient had a cushion on the bottom (60% sucrose and 0.33 N NaOH). Sedimentation was done in a Beckman model L 3-50 ultracentrifuge with an SW-56 rotor at 40,000 or 45,000 rpm for 80-300 min at 20°C. Gradients were fractionated and fractions were treated as described above. Gradients had previously been calibrated with T4, lambda and PH1X174 bacteriophage DNA's as standards (Friefelder, 1970; Sinsheimer, 1959).

Calculation of UV Endonuclease-Sensitive Sites

The following formula was used for the calculation of the number of UV endonuclease-sensitive sites:

$$\text{Number of sites per } 10^6 \text{ daltons} = \left[\left(\frac{2}{\bar{M}_w}\right)_{+enz} - \left(\frac{2}{\bar{M}_w}\right)_{-enz}\right] 10^6$$

As for endogenous ss-breaks, the number-average molecular weight was considered to be equal to half the weight-average molecular weight. In

addition to reducing the sensitivity of the calculations to small changes at the tops of gradients, the use of M_w reduced the contribution of mt-DNA to molecular weight calculations, and therefore the UV endonuclease-sensitive sites computed reflect sites in nuclear DNA.

CHAPTER III

RESULTS

Sedimentation of Yeast DNA on Alkaline Sucrose Gradients

General considerations. The presence of a thick cell wall complicates the gentle lysis of yeast cells for the purpose of obtaining large molecular weight DNA. The enzymatic digestion of the cell wall with glusulase produces spheroplasts amenable to gentle lysis and sedimentation procedures similar to those developed for the measurement of large molecular weight DNA from E. coli by McGrath and Williams (1966). In the procedure for yeast, cells were labeled with [6-^{3}H] uridine, treated with glusulase and lysed on top of alkaline sucrose gradients. Cells treated in this manner were found to release relatively large molecular weight DNA as shown by sedimentation through alkaline sucrose gradients (Fig. 2). For reference, bacteriophage T4 DNA labeled with [2-^{14}C] thymidine was sedimented on the same gradient. T4 DNA has a known single-strand molecular weight of 55 x 10^{6} (Freifelder, 1970).

In Fig. 2 $M_w/2$ for the main distribution of DNA is equal to 70 x 10^{6}. This value was one of the lowest values for DNA from unirradiated yeast to be determined during the course of this study. Normally $M_w/2$ was found to be between 80 and 90 x 10^{6} for DNA released from late log phase cells. Sedimentation of large molecular weight DNA from yeast through alkaline sucrose gradients has also been reported by Cryer et al. (1973).

Figure 2. Alkaline sucrose gradient sedimentation profiles of DNA released from S288C and bacteriophage T4.

Spheroplasts formed from log phase cells of S288C labeled with [6-^{3}H] uridine (●) were mixed with bacteriophage T4 DNA labeled with [2-^{14}C] thymidine (□) and were lysed on top of a 5-20% alkaline sucrose gradient. The gradient was centrifuged in a Beckman model L 3-50 ultracentrifuge with an SW-56 rotor at 20°C and 30,000 rpm for 130 min and was collected by being pumped from the bottom onto a strip of Whatman 17 paper which had been marked into fractions. The strips were washed overnight in 5% TCA and then for 30 min each in fresh 5% TCA and in two 95% ethanol washes. The strips were dried and the amount of radioactivity in each fraction was determined with a Packard scintillation counter and a toluene based counting solution. The percent of the total radioactivity recovered was determined and plotted as a function of the distance sedimented with the aid of a computer program. Arrows denote the positions of specific molecular weights in the gradient as determined from independent calibrations.

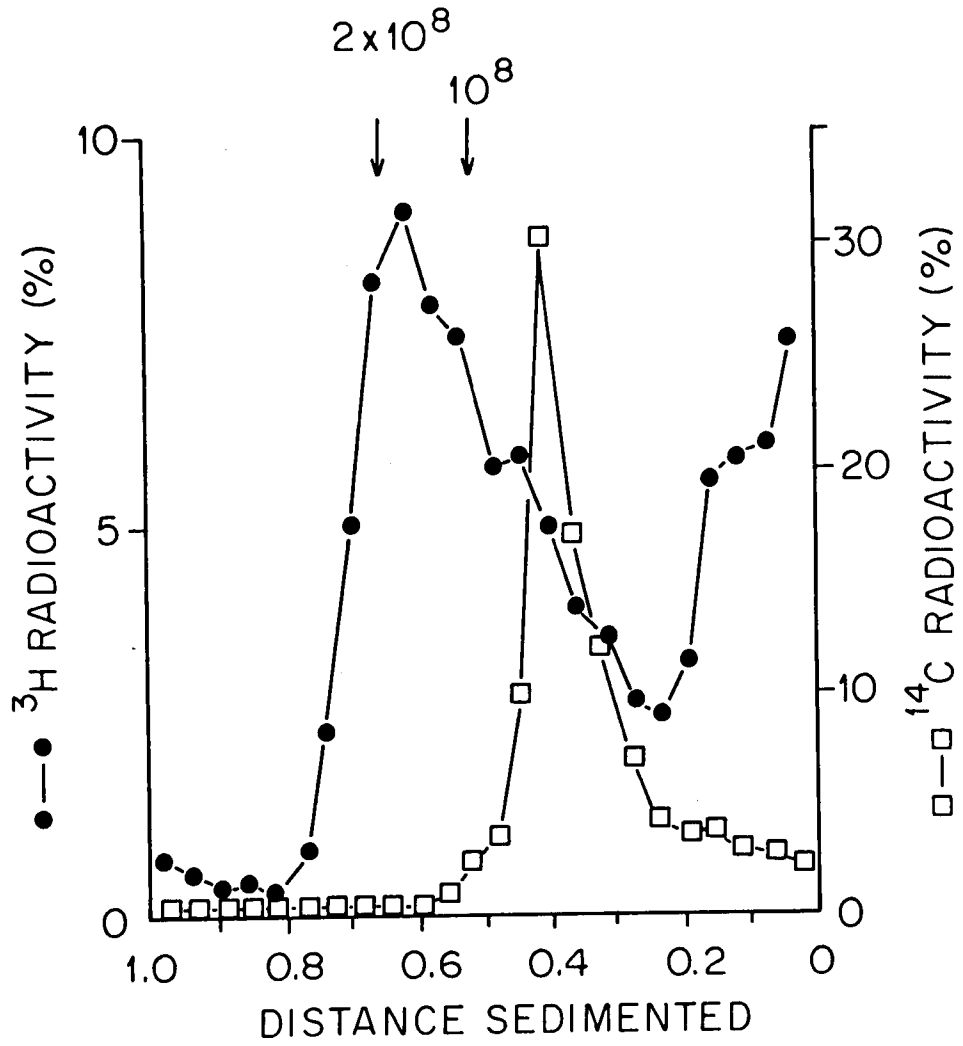

2x10^8
10^8
10
5
0
30
20
10
0
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED
●—● ³H RADIOACTIVITY (%)
□—□ ¹⁴C RADIOACTIVITY (%)

Figure 2

Currently available data suggest that yeast chromosomes contain a single, linear duplex molecule of DNA (Petes and Fangman, 1972; Petes et al., 1973; and Blamire et al., 1973). If the total haploid DNA content of approximately 10^{10} daltons (Hartwell, 1970) is divided among the nearly 20 chromosomes in yeast (Mortimer and Hawthorne, 1973), an average molecular weight of 500-600 x 10^6 can be expected for intact, nuclear DNA. Double-strand DNA of approximately this size has been detected following the gentle lysis of yeast spheroplasts on neutral sucrose gradients (Blamire et al., 1972; and Petes and Fangman, 1972). Assuming the absence of single-strand breaks and alkaline labile bonds, one could expect to find single-strand DNA with a molecular weight of about 250-300 x 10^6. If this value is assumed to be correct, the single-strand DNA in Fig. 2 has the equivalent of two breaks per single strand or four single-strand breaks per chromosome duplex.

Dependence of sedimentation rate on rotor speed. Petes and Fangman (1972) have observed anomalously low sedimentation rates when high rotor speeds were used to sediment large molecular weight DNA from S. cerevisiae through neutral sucrose gradients. However, smaller molecular weight DNA from bacteriophage T4 was unaffected in the range of rotor speeds examined. Similar results have been obtained with mammalian DNA sedimented through alkaline sucrose gradients (McBurney et al., 1971; and Ormerod and Lehmann, 1971). In each of the above cases, sedimentation independent of rotor speed, was found for a range of lower rotor speeds. Independence of rotor speed is a necessary condition for the determination of molecular weights. However, excessively slow rotor speeds

unnecessarily occupy ultracentrifuge time and may subject gradients to sedimentation artifacts arising from diffusion, convection and alkaline hydrolysis of DNA.

To determine if the sedimentation of large molecular weight, yeast DNA on alkaline sucrose gradients was affected by rotor speed under the standard sedimentation conditions expected to be used in this study, one radioactively labeled culture of S288C was split and sedimented through three gradients, one at 30,000 rpm and two at 15,000 rpm. Sedimentation times were adjusted to yield the same product of $(rpm)^2$ x time. All other conditions were identical. Table 2 shows that the weight-average molecular weight determined at 30,000 rpm is clearly in the range expected from the two samples sedimented at 15,000 rpm. It was therefore concluded that the sedimentation rate of denatured yeast DNA with M_w approximately equal to 160×10^6 was unaffected by rotor speed up to at least 30,000 rpm with the SW-56 rotor.

Dependence of sedimentation rate on DNA concentration. Under normal conditions, approximately 10^7 spheroplasts were layered on each alkaline sucrose gradient. Since a normal haploid cell contains 2.1×10^{-8} µg of DNA (Hartwell, 1970), approximately 0.2 µg of DNA was normally used for sedimentation analysis. To determine if DNA was sedimenting in a manner independent of concentration, different numbers of yeast spheroplasts (between 9×10^5 and 2.8×10^7, corresponding to between 0.02 and 0.6 µg of DNA) were sedimented under standard conditions (Fig. 3). Some gradient-to-gradient fluctuations were found during this

Table 2. Dependence of the Calculated Weight-Average Molecular Weight on Rotor Speed During Sedimentation of DNA in an SW-56 Rotor at 20°C through Alkaline Sucrose Gradients.

rpm	Time (min)	$(rpm)^2 t$	$M_w \times 10^{-6} \pm$S.E.
15,000	480	108×10^9	164±6.0
30,000	120	108×10^9	163

Figure 3. Dependence of the calculated weight-average molecular weight on the number of spheroplasts per gradient.

Log phase cells of strain S288C labeled with [6-^{3}H] uridine were spheroplasted and divided into portions containing from 9 x 10^{5} to 2.8 x 10^{7} spheroplasts and were layered onto individual alkaline sucrose gradients in equal volumes of a solution containing 0.1 M NaCl and 0.02 M EDTA (pH 7.0). Gradients were centrifuged as in Fig. 2, page 24, except the duration of the centrifugation was 150 min. All subsequent procedures were as in Fig. 2 and weight-average molecular weights were calculated with a computer program for the main distribution of DNA which corresponds to n-DNA.

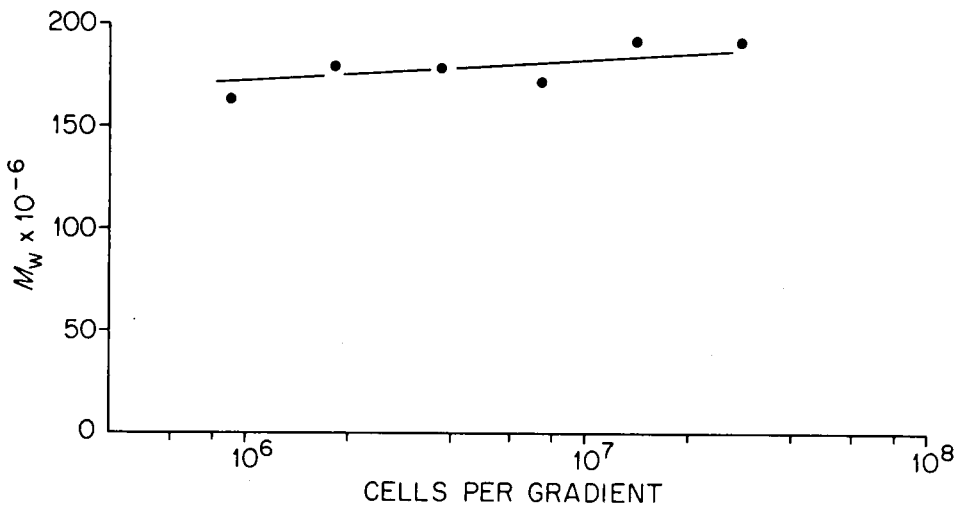


Figure 3

study even with cells from the same culture. Beyond this normal variation, no large dependence of sedimentation rate on DNA concentration could be discerned although the data are consistent with an approximate increase in apparent M_w of 5% over the range of concentrations studied.

Identification of mitochondrial DNA. As in other eukaryotes, the mitochondria of wild type yeast contain DNA. (For a recent review see Borst, 1972.) In S. cerevisiae mitochondrial DNA (mt-DNA) comprises approximately 10% of the total cellular DNA in log phase cells (Williamson, 1970). Mt-DNA is therefore present in sufficient quantity to be a potential problem during the determination of molecular weights for nuclear DNA (n-DNA).

Cryer et al. (1973) found mt-DNA to sediment slowly on alkaline sucrose gradients relative to n-DNA. To confirm this finding, sedimentation profiles obtained with the normal respiratory competent, wild-type strain S288C and a closely related respiratory deficient strain S288C EB1 were compared. S288C EB1 was isolated from a culture of S288C which had been grown overnight in YNB-D supplemented with 10 μg/ml ethidium bromide. Growth in ethidium bromide at this concentration has been shown to eliminate selectively and completely mt-DNA (Goldring et al., 1970). Both S288C and S288C EB1 were labeled with [6-^{3}H] uridine and spheroplasts were formed according to the usual procedure. Labeled spheroplasts from the two cultures were lysed on separate alkaline sucrose gradients and sedimented at 30,000 rpm for 130 min. The resulting sedimentation profiles are plotted together to facilitate comparison (Fig. 4).

Figure 4. Alkaline sucrose gradient profile of DNA released from spheroplasts of strain S288C EB1.

DNA from log phase cells of S288C EB1 labeled with [6-^{3}H] uridine (○) was sedimented on an alkaline sucrose gradient and the sedimentaion profile was determined as in Fig. 2, page 24. The sedimentation profile obtained with S288C (Fig. 2) (●) has been replotted to facilitate comparison with that of S288C EB1 DNA. The total acid precipitable counts recovered was 2844 cpm for S288C and 1015 cpm for S288C EB1.

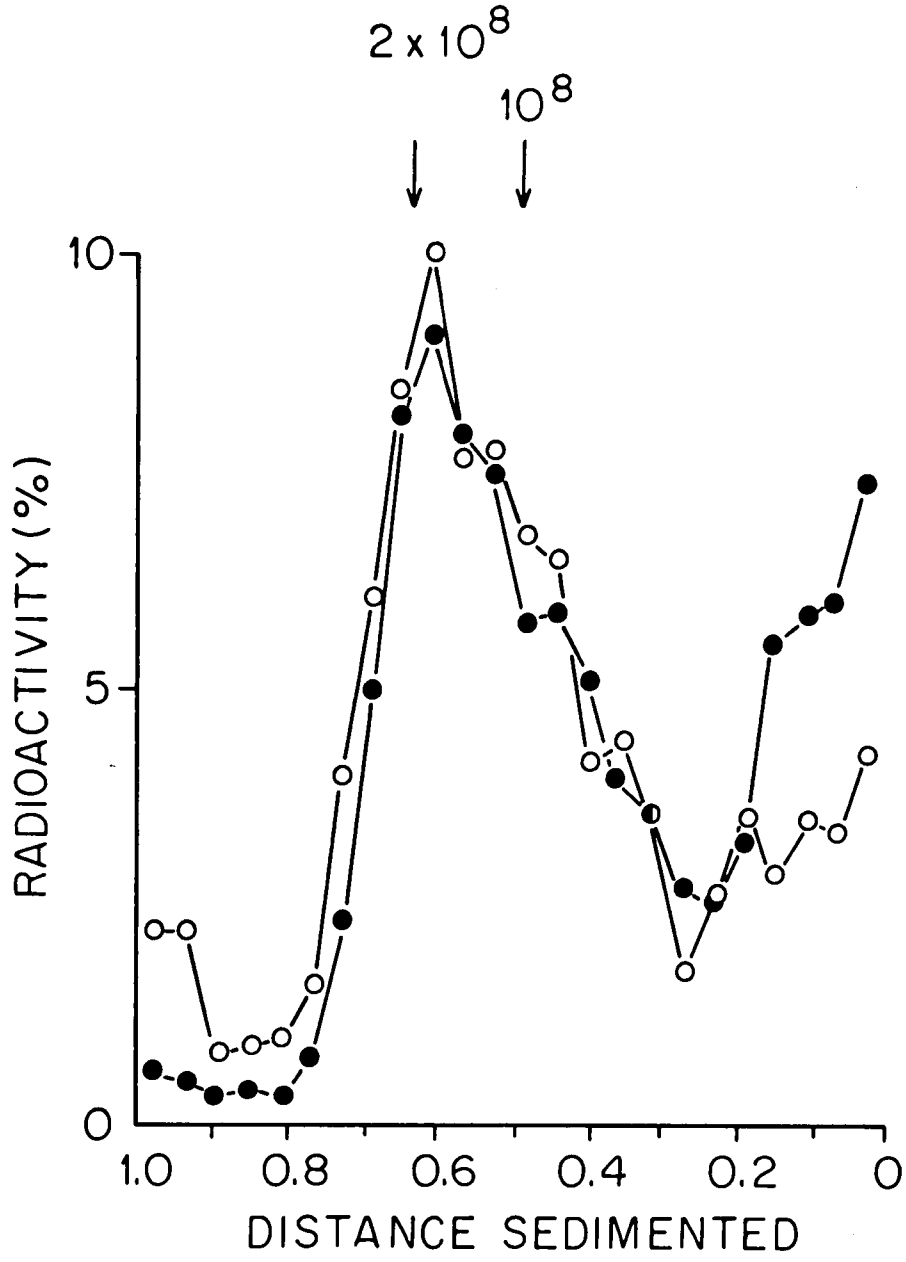

2 x 10^8
10^8
10
5
0
RADIOACTIVITY (%)
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED

Figure 4

The only reproducible difference found between alkaline sucrose gradient sedimentation profiles for S288C and S288C EB1 is in the top few fractions where S288C appears to have more DNA. To separate this material from nonsedimentable counts near the top of the gradients, samples from the same cultures of S288C and S288C EB1 as those used for the gradients in Fig. 4 were sedimented at 30,000 rpm for 330 min. With this longer sedimentation period the bulk of the DNA is found near the bottom in both gradients (Fig. 5), but DNA from S288C has an additional minor distribution sedimenting with $M_w/2$ equal to 1.5×10^6. This minor species of DNA is completely absent from strain S288C EB1. Because of the reported selective removal of mt-DNA by ethidium bromide (Slonimski et al., 1968; Goldring et al., 1970; and Clark-Walker, 1972), the slowly sedimenting DNA in S288C was tentatively assumed to be mt-DNA.

Mt-DNA in S. cerevisiae is known to have a lighter buoyant density in neutral CsCl gradients than n-DNA (Hartwell, 1970). Both denaturation (Berns and Thomas, 1961) and alkaline pH's (Vinograd et al., 1963) have been shown to increase the buoyant density of DNA. Therefore, to verify the identity of the slowly sedimenting DNA as mt-DNA, the density relation between the putative mt-DNA and the bulk of cellular DNA was investigated with alkaline CsCl gradients. A lysate of S288C labeled with [6-^{3}H] uridine was sedimented on an alkaline sucrose gradient as in Fig. 5. Gradient fractions were collected in the wells of a Limbro plate, and 10 µl samples were removed from each fraction and assayed for radioactivity to locate the slowly sedimenting DNA. Fractions containing the putative mt-DNA from the alkaline sucrose gradient were

Figure 5. Comparison of alkaline sucrose gradient profiles for S288C and S288C EB1 DNA's after prolonged sedimentation.

Cultures of S288C and S288C EB1 both labeled with [6-^{3}H] uridine were spheroplasted and treated as in Fig. 2, page 24, except individual gradients were centrifuged for 330 min. The total acid precipitable radioactivity recovered was 1573 cpm for S288C (●) and 697 cpm for S288C EB1 (○). As in Fig. 2 arrows denote the positions of particular molecular weights as determined from independent gradient calibrations.

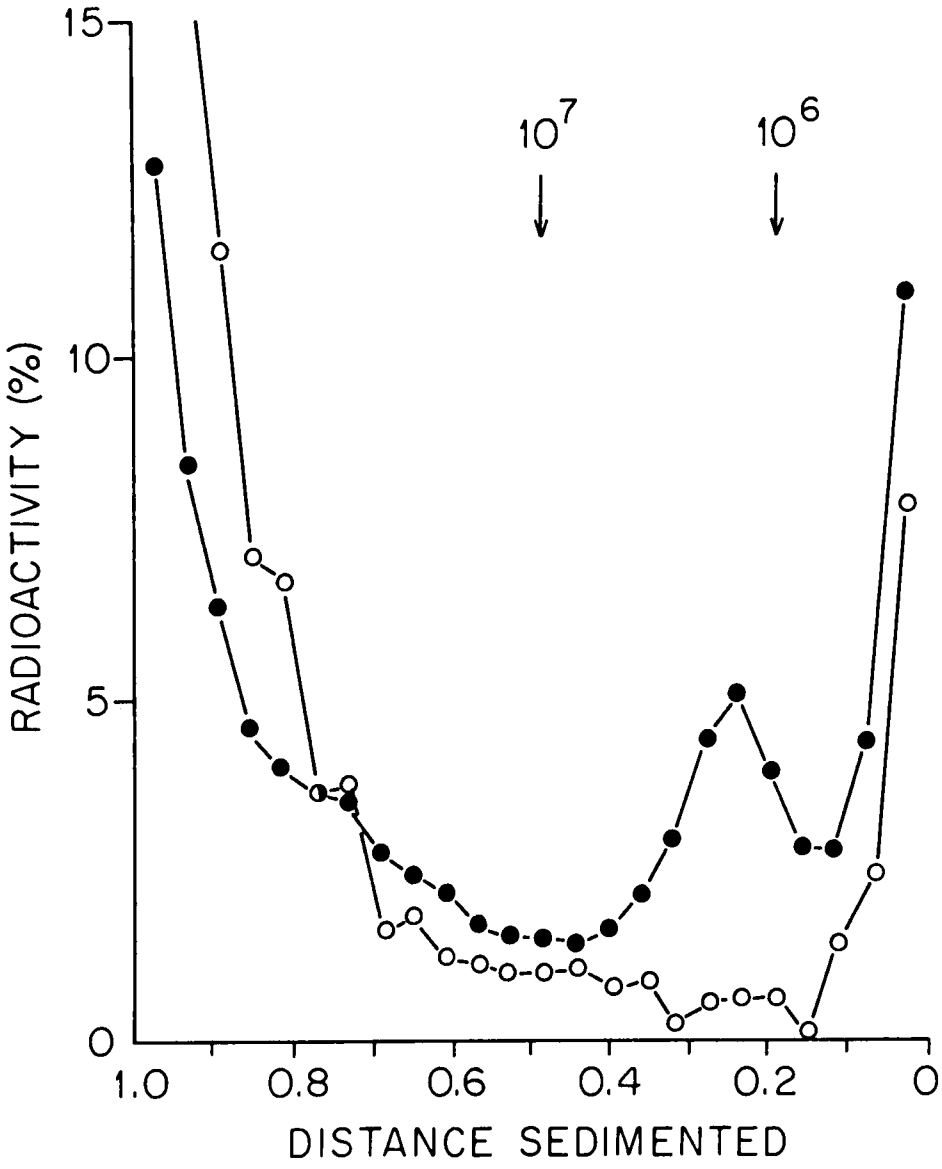

15
10
5
0
RADIOACTIVITY (%)
10^7
10^6
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED

Figure 5

pooled and banded on an alkaline CsCl buoyant density gradient together with a whole cell lysate from S288C labeled with [2-^{14}C] uridine (Fig. 6). The slowly sedimenting DNA is clearly of a lighter density than the bulk of the cellular DNA. This is the expected density relationship between mt-DNA and n-DNA (Hartwell, 1970) and confirms the identity of the slowly sedimenting DNA as mt-DNA.

Single-strand breaks in yeast nuclear DNA arising during incubation at alkaline pH. The sensitivity of the alkaline sucrose gradient method to small numbers of ss-breaks in large molecular weight DNA enabled Hill and Fangman (1973) to detect a slow accumulation of ss-breaks during the incubation of DNA in 0.3 N NaOH. The rate of accumulation was found to depend upon both temperature and pH. To investigate the sensitivity of yeast nuclear DNA to alkaline conditions, yeast spheroplasts were lysed on alkaline sucrose gradients with either the usual lysis solution containing 0.5 N NaOH or with a similar lysis solution at twice the usual NaOH concentration. Individual samples were layered at various times prior to sedimentation and spheroplast lysis was complete in 2-3 min. Samples were then incubated on top of the gradients until sedimentation, and molecular weights were determined as usual. With both 0.5 N and 1.0 N NaOH a slow, time-dependent reduction in molecular weight was found (Fig. 7). In terms of ss-breaks the reductions in molecular weight represent 0.20 ss-breaks/10^8 daltons per h with lysis layers containing 0.5 N NaOH and 0.36 ss-breaks/10^8 daltons per h with lysis layers containing 1.0 N NaOH. These rates

Figure 6. Alkaline CsCl buoyant density gradient profiles of DNA from a whole cell lysate of S288C and of mt-DNA rebanded from a high-salt alkaline sucrose gradient.

Slowly sedimenting DNA from S288C labeled with [6-^{3}H] uridine and recovered from an alkaline sucrose gradient, centrifuged as described in Fig. 5, page 35, was mixed with a whole cell lysate from S288C labeled with [2-^{14}C] uridine. The mixture was banded on an alkaline CsCl step gradient by centrifugation in an SW-56 rotor for 38.5 h at 30,000 and 24°C. Fractions were collected and radioactivity in each determined as described for alkaline sucrose gradients in Fig. 2, page 24, except the buoyant density gradient was pumped from the top with a Buchler Densi Flow apparatus. The total acid precipitable radioactivity recovered was 541 cpm for ^{3}H labeled, mt-DNA (O) and 1287 cpm for ^{14}C labeled, total cellular DNA (●).

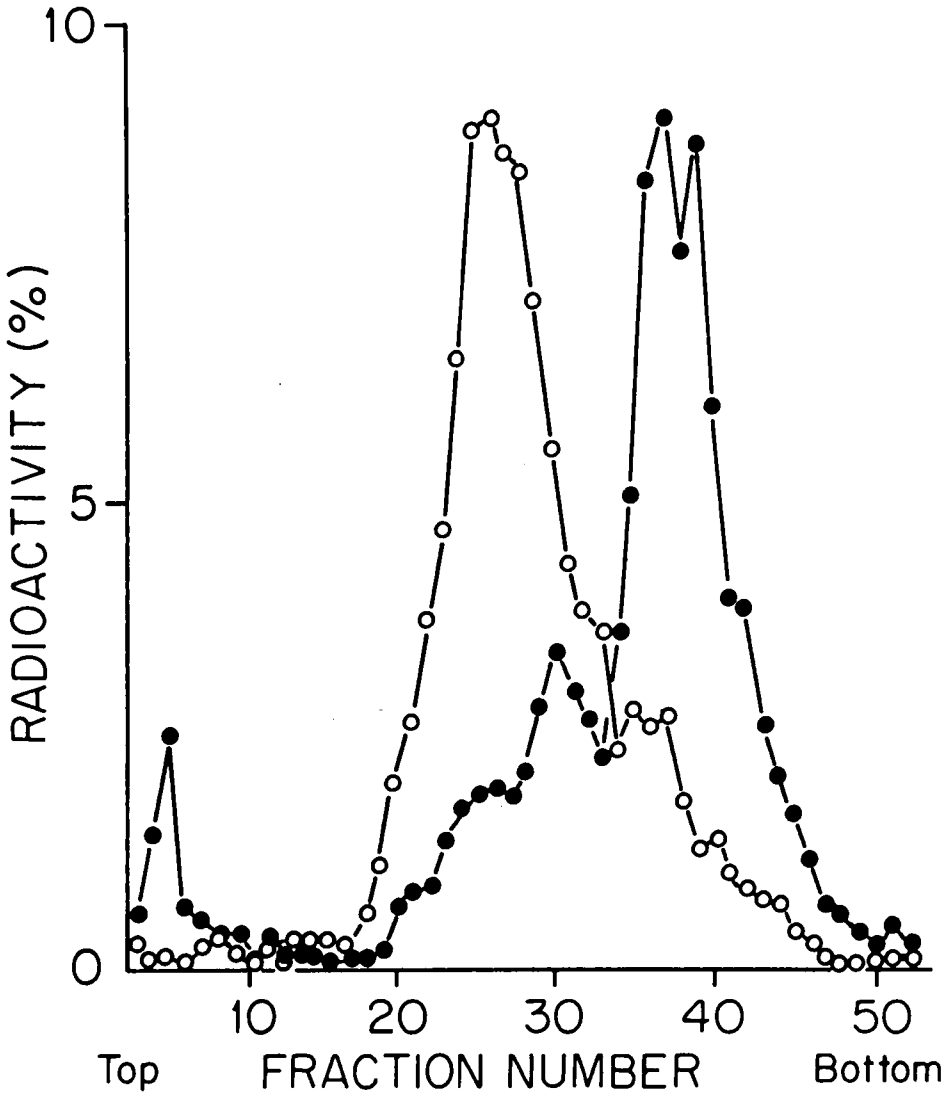

10
5
0
RADIOACTIVITY (%)
10
20
30
40
50
Top
FRACTION NUMBER
Bottom

Figure 6

Figure 7. Alkaline degradation of yeast n-DNA.

Yeast spheroplasts formed from log phase cells of S288C and labeled with [6-^{3}H] uridine were lysed on top of alkaline sucrose gradients in a lysis solution containing either 0.5 N (O) or 1.0 N (Δ) NaOH. Samples were incubated at 24°C for various periods of time prior to sedimentation as described in Fig. 3, page 29. Weight-average molecular weights were determined from sedimentation profiles with the aid of a computer program.

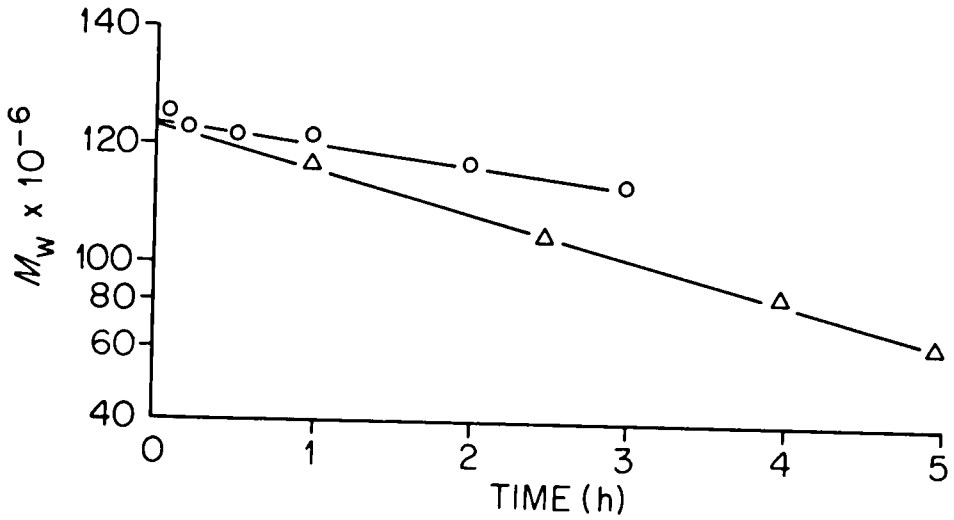


Figure 7

compare favorably with the 0.073 ss-breaks/10^8 daltons per h found by Hill and Fangman (1973) with E. coli DNA in 0.3 N NaOH.

Normally yeast spheroplasts were held for only 15-20 min in 0.5 N NaOH prior to sedimentation. Only 0.06 ss-breaks/10^8 daltons would be expected from this incubation prior to sedimentation. Sucrose solutions contained only 0.33 N NaOH and hydrolysis is probably retarded by the presence of sucrose (Cleaver, 1974a). Therefore fewer than 0.1 ss-breaks/10^8 daltons would be expected to result from alkaline degradation during lysis and sedimentation.

Endogenous Single-Strand Breaks in Nuclear DNA Following UV Irradiation of S. cerevisiae

Single-strand breaks in the wild-type haploid S288C after UV irradiation. Irradiation of strain S288C with 100 J m^{-2} followed by a 60 min incubation at 28°C in 0.067 M potassium phosphate buffer (pH 7.0) was found to result in a decrease in the sedimentation rate of DNA released from spheroplasts relative to an unirradiated control (Fig. 8). However, DNA released from spheroplasts after a 24 h postirradiation incubation of irradiated cells is nearly as large as that of the unirradiated control. This sequence of events is compatible with current models of excision repair which predict ss-breaks to arise from endonucleolytic attack near UV-induced photoproducts in the DNA, and to become closed after subsequent repair processes.

The relation between ss-breaks and postirradiation liquid holding was examined after several fluences (Fig. 9). Labeled S288C cells were irradiated and then aerated at 28°C in 0.067 M potassium phosphate

Figure 8. Alkaline sucrose gradient profiles of S288C DNA before and at various times after UV irradiation of cells with 100 J m^{-2}.

Cells from a log phase culture of S288C were converted to spheroplasts either without prior UV irradiation or after irradiation with 100 J m^{-2} followed by incubation in potassium phosphate buffer at 28°C for either 1 or 24 h. Alkaline sucrose gradient profiles were obtained as described in Fig. 2, page 24, except gradients were centrifuged for 150 min. The total acid precipitable radioactivity recovered was 7573 cpm for unirradiated sample (□), 7776 cpm for the sample irradiated with 100 J m^{-2} and liquid held for 1 h (○), and 6763 cpm for the sample irradiated and held for 24 h (●).

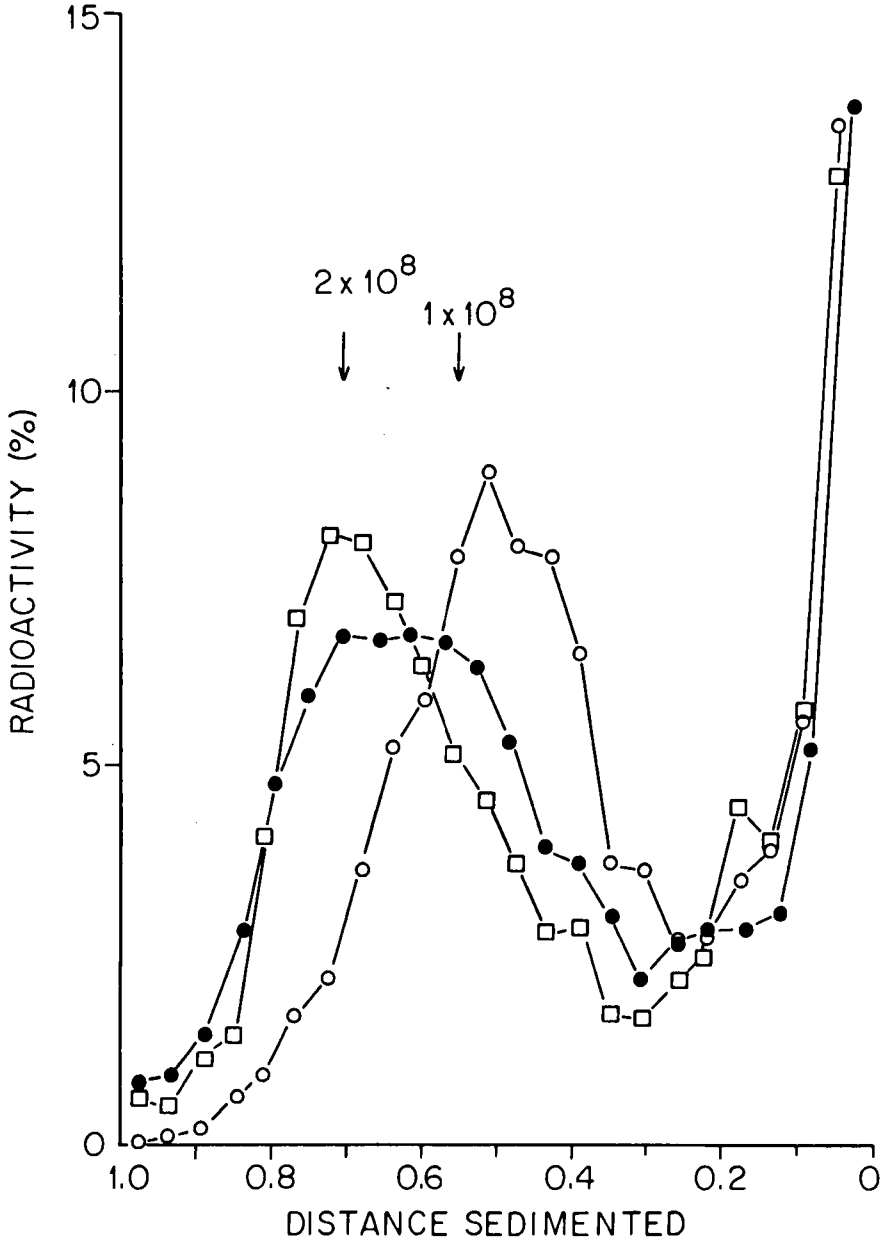

15
10
5
0
RADIOACTIVITY (%)
2 x 10^8
1 x 10^8
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED

Figure 8

Figure 9. Endogenous ss-breaks in S288C DNA during liquid holding in potassium phosphate buffer at 28°C after irradiation with 0, 50, 75, and 100 J m^{-2}.

Cells from a log phase culture of S288C in potassium phosphate buffer were incubated at 28°C either without prior UV irradiation (▽) or after irradiation with 50 (□), 75 (△), or 100 (○) J m^{-2}. Samples taken at various times during the liquid holding treatment were held on ice until spheroplast formation. Spheroplasts were lysed on top of alkaline sucrose gradients and sedimented for 150 min and treated otherwise as described in Fig. 2, page 24. The number of ss-breaks relative to unirradiated, unincubated samples were calculated from sedimentation profiles as described in the Materials and Methods. Results after different fluences represent data from different experiments.

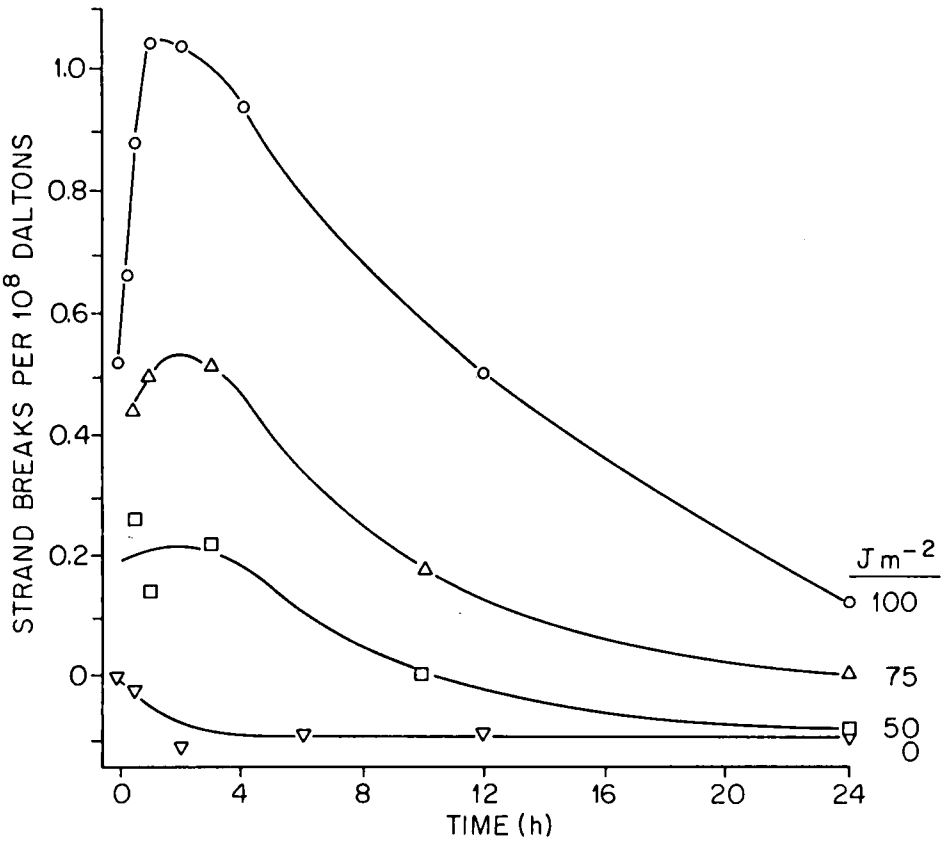

STRAND BREAKS PER 10^8 DALTONS
1.0
0.8
0.6
0.4
0.2
0
0
4
8
12
16
20
24
TIME (h)
$J\,m^{-2}$
100
75
50
0

Figure 9

buffer (pH 7.0). After 50, 75, and 100 J m^{-2} ss-breaks were found to increase rapidly in number to some maximum value, and then more slowly to decrease in number. The complete disappearance of ss-breaks relative to unirradiated controls requires longer postirradiation incubations after larger incident fluences. Thus a 10 h incubation after 50 J m^{-2} is sufficient to allow DNA from irradiated samples to return to a molecular weight approximately equal to that of DNA from unirradiated cells. But DNA from cells irradiated with 100 J m^{-2} and liquid held for 24 h is still smaller than that from unirradiated cells.

In Fig. 9 the maximum number of ss-breaks found during the liquid holding period appears to depend on the incident fluence. This was confirmed by irradiating individual samples from a single culture of [6-^{3}H] uridine labeled S288C with various fluences. Irradiated cells were then incubated at 28°C for periods of sufficient duration to allow the maximum numbers of ss-breaks to be present. The results of this experiment are presented in Fig. 10. The values obatined for the maximum number of ss-breaks after each fluence agree with values obtained in separate experiments in which more samples were examined after each fluence. The results in Fig. 10 show that the maximum number of ss-breaks is linearly related to the average incident fluence.

Early increases in the number of ss-breaks are more evident in Fig. 11 where measurements were made after shorter periods of liquid holding. From these data it is apparent that ss-breaks are present even in the absence of liquid holding at 28°C. There is also an additional

Figure 10. Endogenous ss-breaks in the DNA of S288C during potassium phosphate buffer liquid holding: the maximum number of ss-breaks observed after various fluences.

A single culture of S288C labeled with [6-^{3}H] uridine was divided into individual samples and irradiated at 254 nm with various fluences and then incubated at 28°C for periods of sufficient duration to allow the expression of the maximum number of breaks. Weight-average molecular weights were determined by sedimentation on alkaline sucrose gradients and the number of ss-breaks relative to an unirradiated sample were calculated as described in the Materials and Methods.

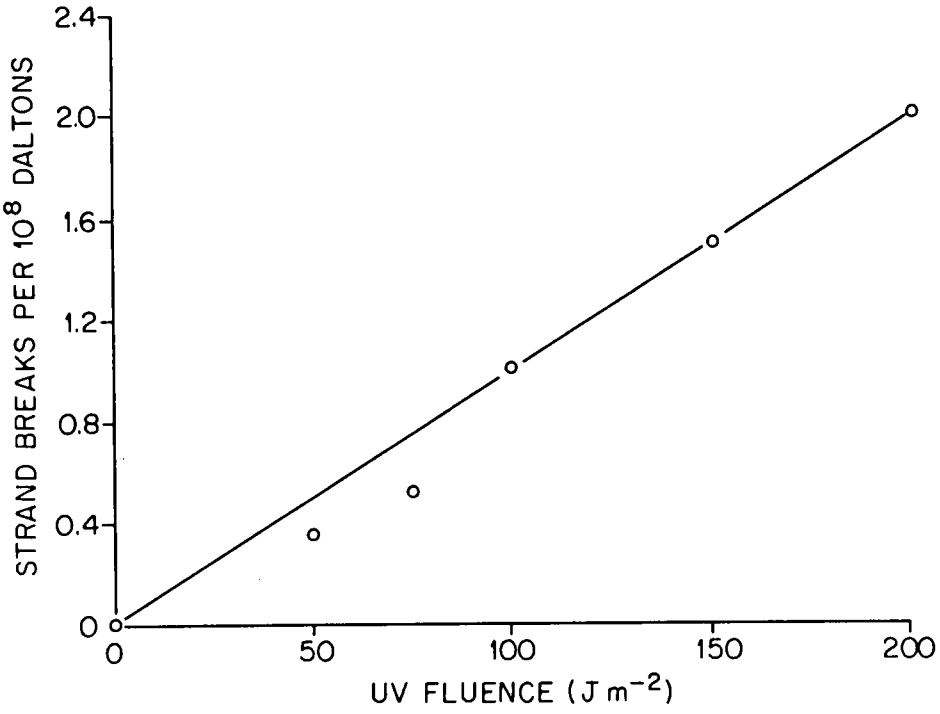

STRAND BREAKS PER 10^8 DALTONS
2.4
2.0
1.6
1.2
0.8
0.4
0
0
50
100
150
200
UV FLUENCE ($J\,m^{-2}$)

Figure 10

Figure 11. Endogenous ss-breaks in the DNA of S288C during liquid holding in potassium phosphate buffer at short times after the irradiation of cells with 50, 100, 150, and 200 J m^{-2}.

Endogenous ss-breaks were determined at various times after irradiation at 254 nm with 50 (■), 100 (●), 150 (▽), and 200 (▲) J m^{-2} as described in Fig. 9, page 45.

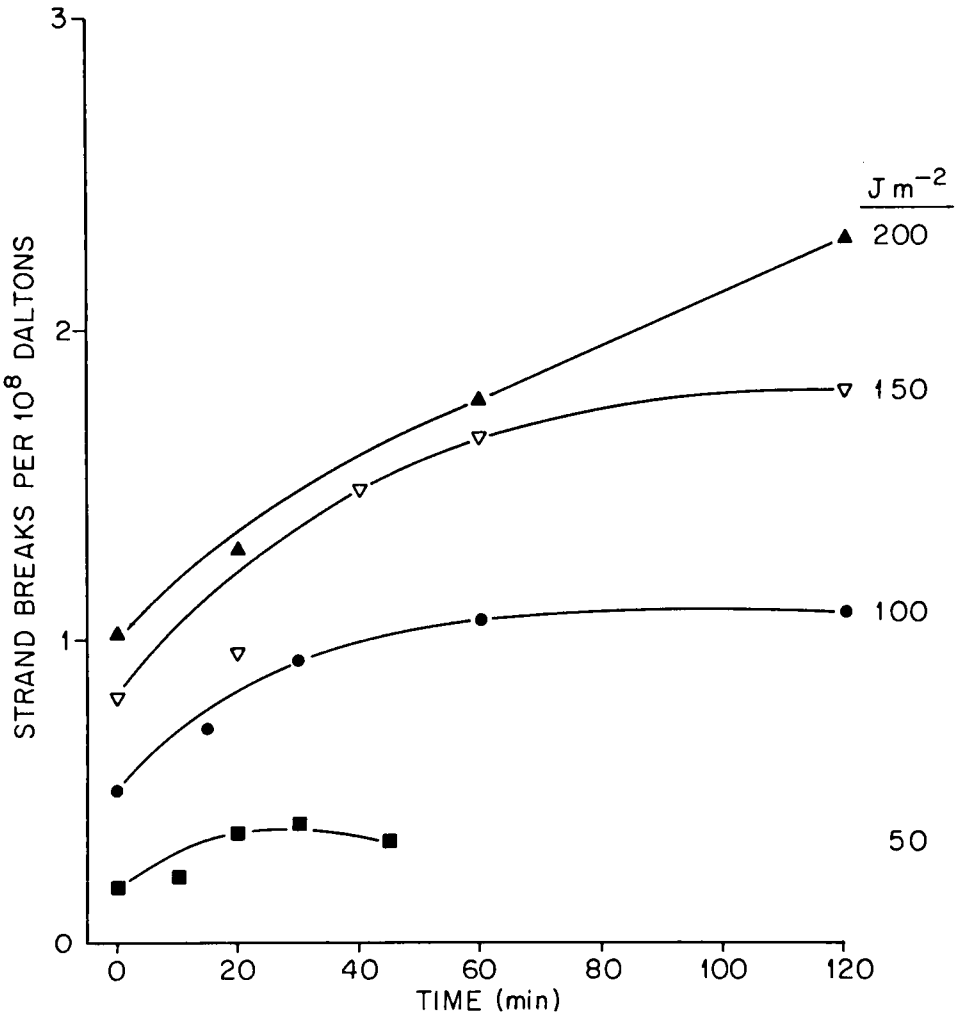

STRAND BREAKS PER 10^8 DALTONS
3
2
1
0
0
20
40
60
80
100
120
TIME (min)
J m^{-2}
200
150
100
50

Figure 11

increase in the number of ss-breaks which requires a postirradiation incubation at 28°C. Similar increases have been observed following UV-irradiation of E. coli (Setlow, 1967a, b), Lambda lysogens of E. coli (Shimada et al., 1968; Seeberg and Johansen, 1973), and HeLa cells (Ben-Hur and Ben-Ishai, 1971). But these findings are in contrast to results obtained with M. luteus (Okubo et al., 1971), bovine fibroblasts (Cleaver et al., 1972), and human fibroblasts (Cleaver, 1974b). In these latter studies ss-breaks were reported to be present immediately after irradiation and no further increase in number was observed.

Studies on the appearance of ss-breaks in the DNA of irraidated yeast cells are complicated by the absence of techniques for the immediate and gentle lysis of yeast cells. In addition to a period during which irradiated cells are normally held on ice prior to spheroplast formation, two 26°C incubations are necessary for the formation of spheroplasts susceptible to gentle lysis in the presence of ionic detergents.

To determine the effect of holding procedures other than the 28°C liquid holding period in potassium phosphate buffer (pH 7.0), the lengths of the various additional incubations were increased in duration to allow more time for changes in the number of ss-breaks to take place. Samples were irradiated on ice with 100 J m^{-2} and then held on ice for either 8 min or 24 h prior to spheroplast formation. Also during spheroplast formation, both the incubation period in 2-mercaptoethanol, Tris-Cl (pH 9.3) and in glusulase were doubled relative to their normal length. Table 3 shows that no significant

Table 3. Changes in the Number of Endogenous SS-Breaks During Ice Holding and During Spheroplast Formation.

UV (J/m^2)	Holding Conditions			$M_w \times 10^{-6}$
	Ice	Spheroplast Incubation 1 (min)	Spheroplast Incubation 2 (min)	
-	8 min	6	15	161
100	8 min	6	15	112
100	8 min	15	15	118
100	8 min	6	30	109
100	24 hr	6	15	110

differences could be detected in the M_w of the irradiated samples due to prolonged holding on ice or due to variations during the spheroplast procedure. These results suggest that the ss-breaks observed in the absence of liquid holding at 28°C are not the result of a slow enzymatic activity during holding periods on ice or during the preparation of spheroplasts, but do not rule out the possibility suggested by Cleaver et al. (1972) that a limited number of ss-breaks might be made by an activated enzyme complex.

Single-strand break data from Fig. 11, page 50, for irradiated samples not subsequently liquid held, has been replotted in Fig. 12 as a function of average incident fluence. A linear relationship clearly exists between the number of breaks measured in the absence of liquid holding and the average incident fluence. Wirths and Jung (1972) have detected ss-breaks in dehydrated ΦX174 irradiated at 254 nm. Setlow (1972) used the excision defective E. coli strain B_{s-1} to measure ss-breaks and alkaline labile bonds due to the direct photochemical action of UV radiation on DNA in vivo. He found 0.002 ss-breaks/10^8 daltons per J m^{-2} at 265 nm. In yeast approximately 0.005 ss-breaks/10^8 daltons per J m^{-2} at 254 nm are present in ice held cells. Because more breaks might be expected from equivalent fluences at shorter wavelengths (Wirths and Jung, 1972), at least a large proportion and perhaps all of the ss-strand breaks detected in DNA from UV-irradiated yeast, in the absence of postirradiation liquid holding, are probably due to the direct photochemical action of UV radiation.

Figure 12. Endogenous ss-breaks induced in the DNA of S288C by UV irradiation at 0°C.

Data for the number of ss-breaks in S288C n-DNA from cells irradiated with different fluences and kept at 0°C during and after irradiation until spheroplast formation was replotted from Fig. 11, page 50, as a function of fluence.

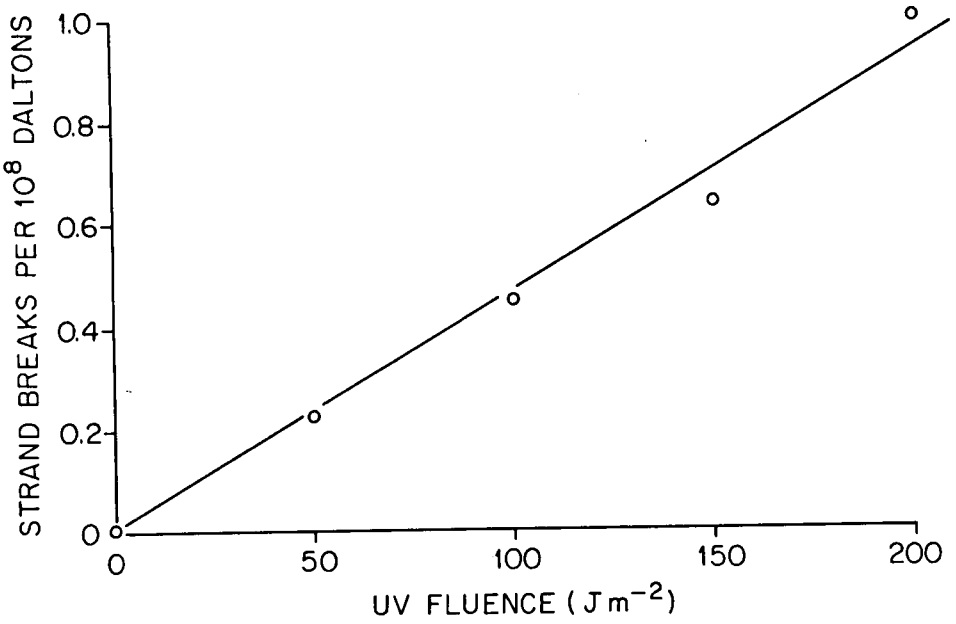

STRAND BREAKS PER 10^8 DALTONS
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
UV FLUENCE ($J\,m^{-2}$)

Figure 12

Photoreactivation of postirradiation ss-breaks. Although the postirradiation ss-breaks detected in yeast DNA are related to fluence, and are predicted by excision repair models, their relation to dimer excision is not readily established. The specificity of enzymatic photoreactivation for pyrimidine dimers (Setlow et al., 1965) was used to test for the dependence of the ss-breaks on dimers. For this purpose a culture of S288C was labeled with [6-^{3}H] uridine, UV-irradiated with 100 J m^{-2}, and then split into two parallel cultures. Subsequent incubations were identical for both cultures except one of the two was exposed to photoreactivating light by method 1 while the second was kept under identical conditions but in the dark. The first culture was photoreactivated in a 49 mm diameter, Pyrex petri plate for 1 h at 28°C. The total incident near UV fluence was 8300 J m^{-2}. For additional incubations both cultures were transferred to bubble tubes and vigorously aerated at 28°C in the absence of photoreactivating light. Viabilities following both treatments are shown in Fig. 13. Increases in viability due to photoreactivation are quite large when compared to increases due to liquid holding alone.

The number of ss-breaks relative to unirradiated controls were determined in both cultures at various times after photoreactivation (Fig. 14). Relative to liquid holding alone both the maximum number of breaks during liquid holding and the length of holding time necessary for the disappearance of UV-induced ss-breaks are reduced by photoreactivation prior to liquid holding. Therefore, ss-breaks present in yeast DNA during postirradiation liquid holding are probably the result of the excision repair of pyrimidine dimers.

Figure 13. Colony forming ability of S288C at various times during liquid holding with and without photoreactivation treatment after irradiation at 254 nm with 100 J m^{-2}.

Log phase cells of S288C were irradiated in suspension with 100 J m^{-2} at an average incident fluence rate of 1.08 J m^{-2} sec^{-1}. Half of the irradiated cells were incubated at 28°C and half were photoreactivated for 1 h by method 1 as described in the Materials and Methods. The total incident fluence of photoreactivating light was 8300 J m^{-2}. Viabilities were determined by diluting cells in 0.067 M potassium phosphate buffer and by plating in duplicate on YEPD agar plates. Dilutions were made so as to allow approximately 100 colonies per plate. Plates were incubated in the dark at 30°C for five days and then counted.

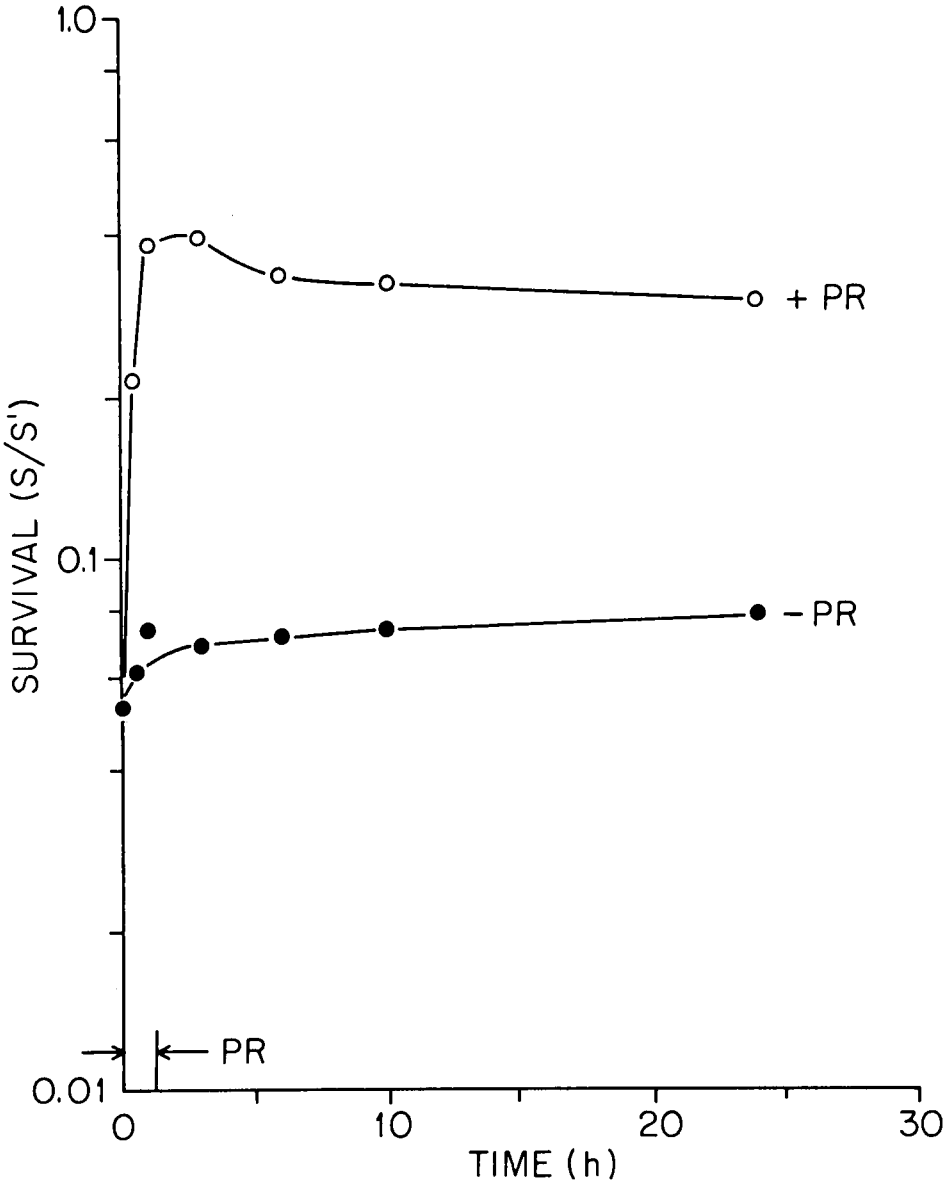


Figure 13

Figure 14. Photoreactivation of endogenous ss-breaks in the n-DNA of S288C.

Log phase cells from the same culture of S288C described in Fig. 13, page 58, were irradiated with 100 J m^{-2} and incubated with or without photoreactivation treatment as described in Fig. 13. Endogenous ss-breaks were determined from alkaline sucrose gradient sedimentation profiles as described in Fig. 9, page 45.

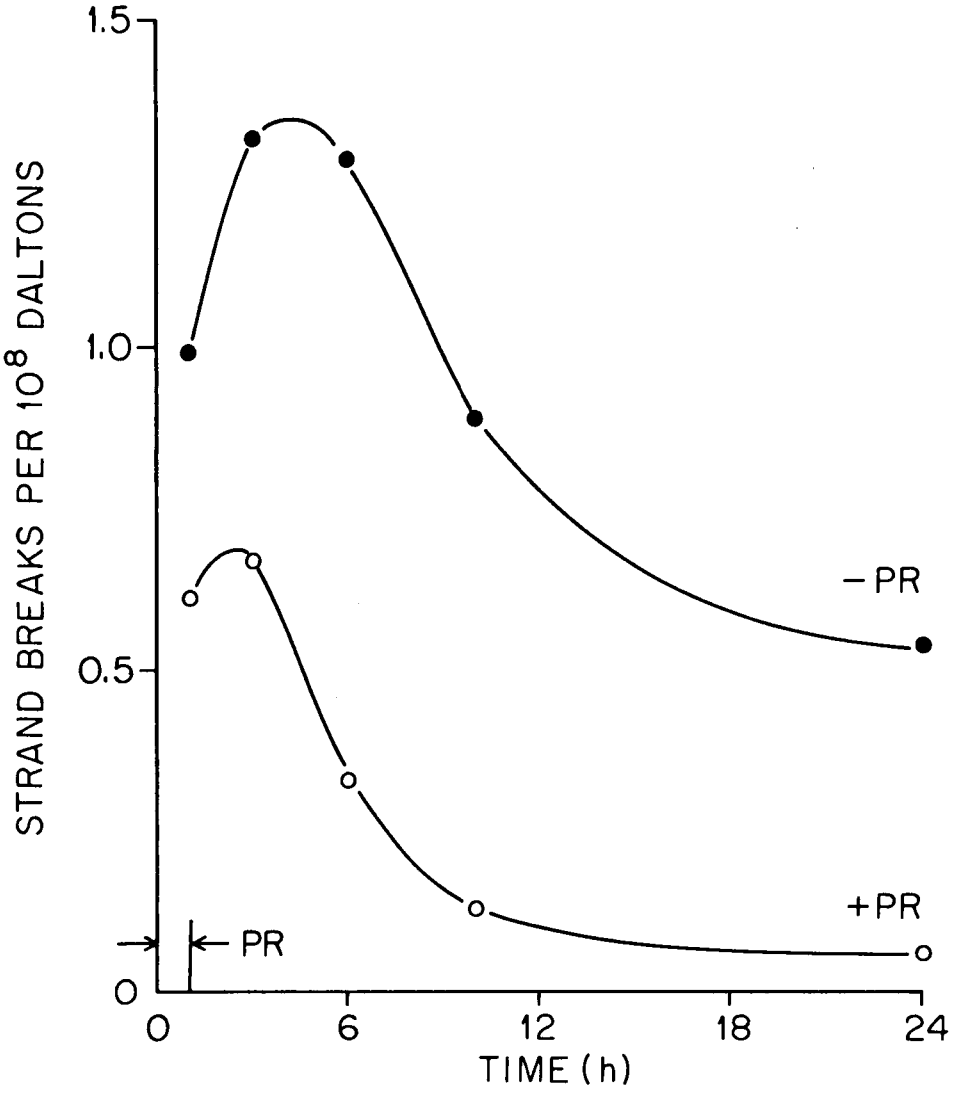

1.5
1.0
0.5
0
STRAND BREAKS PER 10^8 DALTONS
– PR
+PR
PR
0
6
12
18
24
TIME (h)

Figure 14

Postirradiation ss-breaks in UV-sensitive mutants of S. cerevisiae. Mutations at rad1, rad2, rad3, and rad4 are thought to confer UV-sensitivity through defects in excision repair (Cox and Game, 1974). In an attempt to understand better the roles of the various gene products, ss-breaks in DNA were examined in strains carrying mutations at each of these four loci. Strains most sensitive to UV were chosen because less sensitive alleles at each locus might be leaky. The results are presented in Fig. 15.

At early times during liquid holding each mutant has about the same number of ss-breaks as the wild-type. Prolonged liquid holding, however, did not allow the disappearance of ss-breaks that was observed with S288C. As in the wild-type strain S288C the number of endogenous ss-breaks observed was less than 1% of the number of dimers induced by the same fluence. Different patterns for the appearance of ss-breaks are found with the various mutants, but as shown for rad4, results with different alleles at the same locus may vary from each other also. From these data, all four loci appear to be proficient in the initial incision step but defective for some later step prior to ligation. It is unlikely that any of these mutants is defective in ligation since this would be expected to be a conditionally lethal mutation confering sensitivity to ionizing radiation as well as to UV radiation (Pauling and Hamm, 1968).

UV Endonuclease-Sensitive Sites in S. cerevisiae

Sedimentation of DNA extracted from unirradiated and irradiated cells in the absence of UV endonuclease treatment. Phenol extraction of

Figure 15. Endogenous ss-breaks in the DNA of radiation-sensitive strains of S. cerevisiae.

Log phase cells labeled with [6-^{3}H] uridine were irradiated incubated in potassium phosphate buffer at 28°C and the number of endogenous ss-breaks determined at various times by sedimentation on alkaline sucrose gradients as described in Fig. 9, page 45. Results with the wild-type strain S288C (○) irradiated with the same average fluence have been included with the results for the radiation sensitive strains (●) for comparison. Fluence A: 100 J m^{-2}, B, C and D: 75 J m^{-2}.

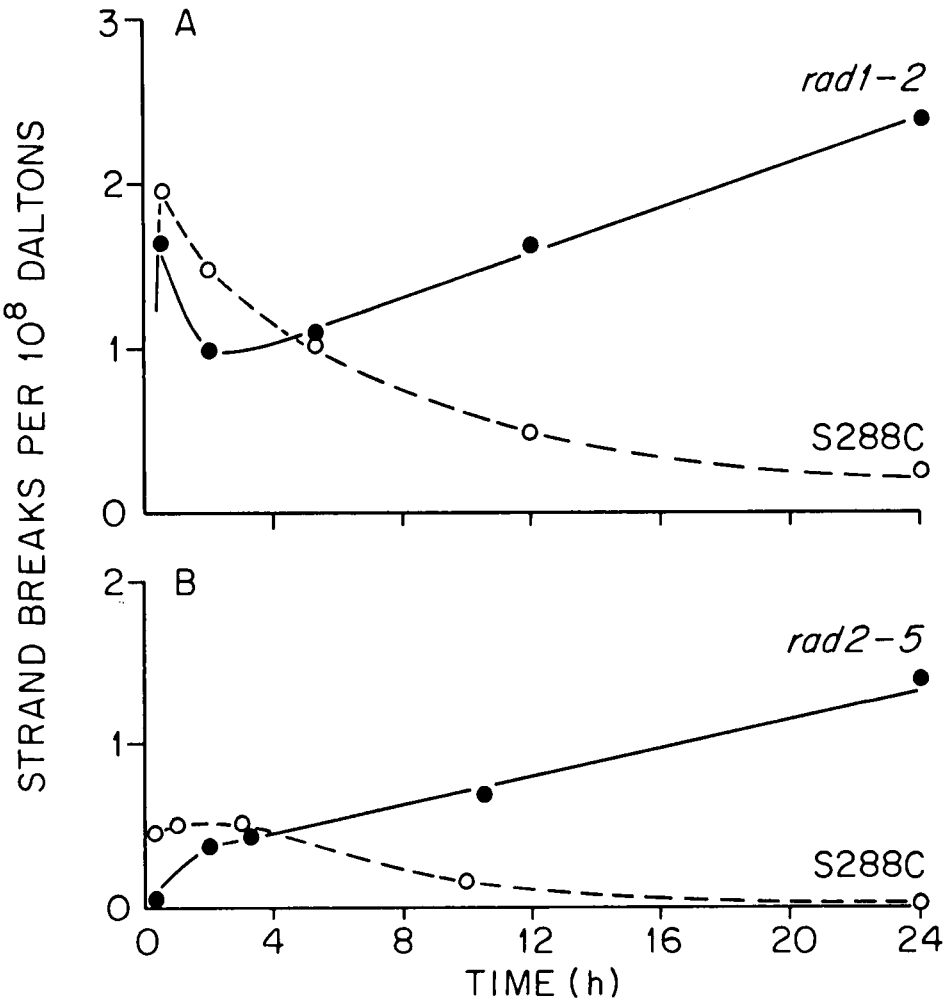

A
rad1-2
S288C
B
rad2-5
S288C
STRAND BREAKS PER 10^8 DALTONS
TIME (h)
0
1
2
3
4
8
12
16
20
24

Figure 15

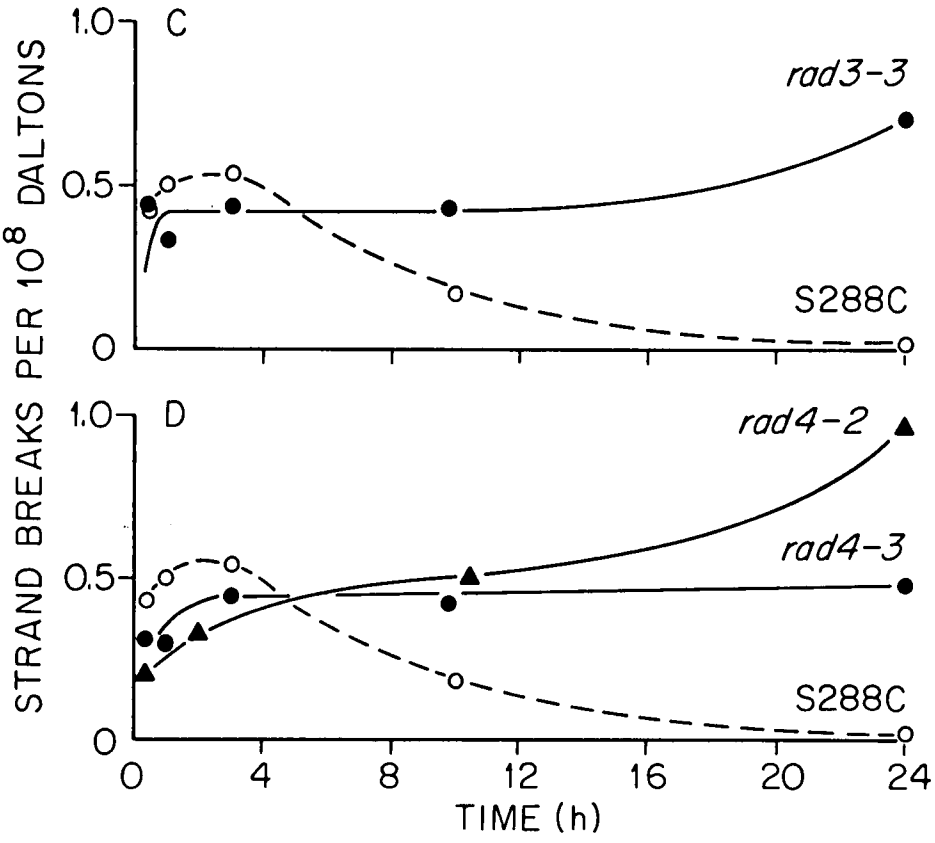


Figure 15 (continued)

yeast lysates was found to yield DNA with a single-strand weight-average molecular weight of 30-80 x 10^6. Subsequent ether extractions, dialysis, and incubation at 35°C did not significantly alter the size distribution. Some variation in size distribution was found between samples extracted separately, but beyond this background variability resulting from different amounts of shear, no further change in size could be attributed to prior UV irradiation in the absence of UV endonuclease treatment.

A small number of ss-breaks have been detected in DNA gently released from UV-irradiated cells. Such breaks may be either the direct result of photochemical reactions (Freifelder and Davis, 1963; Setlow, 1972) or the consequence of normal cellular DNA repair processes following UV irradiation (Setlow, 1967a, b; Cleaver, 1974a; see also "Single-Strand Breaks in the Wild-Type Haploid S288C after UV Irradiation" in the Results chapter). However, the number of endogenous ss-breaks due to both of these processes is small, 1% or less, when compared to the number of photoproducts produced by the same fluence. For the purpose of quantitating UV endonuclease-sensitive sites, each sample to be assayed for enzyme-sensitive sites was divided after dialysis but prior to UV endonuclease treatment. One half was then incubated with and the other half without the UV endonuclease. Half the weight-average molecular weight was determined for both halves from sedimentation profiles and the number of enzyme-sensitive sites calculated from the formula given in the Materials and Methods chapter. In this way all breaks, including those due to shearing of samples during extraction, produced prior to UV endonuclease treatment were subtracted from the

total determined with the enzyme treated sample during the calculation of the number of UV endonuclease-sensitive sites.

UV endonuclease activity against DNA from unirradiated and irradiated cells. Nonspecific endonucleolytic activity was also investigated. As discussed under the Materials and Methods chapter, the UV endonuclease preparation contains a wide variety of other nuclease activities. Therefore, it was important to determine the extent of activity against unirradiated DNA. Any such activity would increase background breaks, reducing the sensitivity of the assay, and complicating the interpretation of ss-breaks detected in irradiated samples. Unirradiated samples, divided after dialysis and incubated with or without UV endonuclease accompanied all experiments. Most unirradiated samples incubated with enzyme differed from their minus-enzyme control by less than 0.005 ss-breaks per 10^6 daltons (Fig. 16). There was no apparent correlation between the addition of enzyme to a sample and that sample's size distribution relative to the minus-enzyme control in the absence of prior UV-irradiation. These small deviations in molecular weight as determined from individual sucrose gradients are well within the expected gradient to gradient variability. It was concluded therefore that nonspecific activity was absent or at an undetectably low level under the assay conditions used.

Although neither UV irradiation alone, nor UV endonuclease treatment of DNA from unirradiated cells was found to affect the size distribution of extracted DNA, the size of irradiated DNA was significantly reduced by UV endonuclease treatment after relatively small fluences. The

Figure 16. The effect of UV endonuclease treatment on the sedimentation rate of DNA extracted from S288C and sedimented on low salt alkaline sucrose gradients.

DNA labeled with [6-^{3}H] uridine and released from S288C spheroplasts was extracted once with phenol and twice with diethyl ether and then dialyzed against UV endo buffer. The dialyzed sample was divided and incubated with (□) or without (○) UV endonuclease from M. luteus for 15 min at 35°C. The samples were sedimented through low salt alkaline sucrose gradients for 155 min at 45,000 rpm in an SW-56 rotor at 20°C. Gradients were pumped from the bottom onto strips of Whatman 17 paper previously marked into fractions. After two washes in 5% TCA (the first for approximately 12 h) and two washes in 95% ethanol, the strips were dried and the amount of radioactivity in each fraction was determined with a Packard scintillation counter. Arrows indicate the positions of specific molecular weights as determined from independent gradient calibrations.

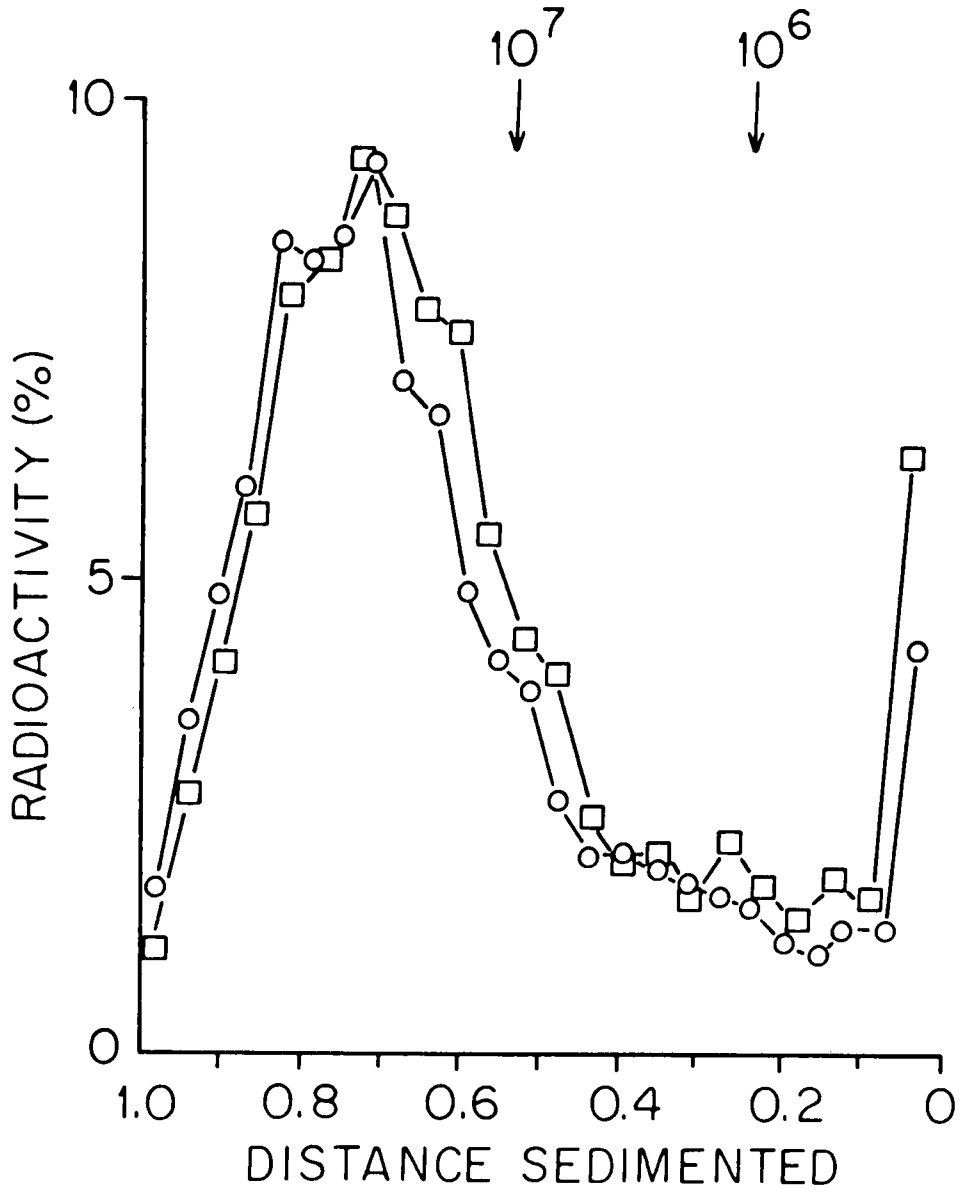

10^7
10^6
10
5
0
RADIOACTIVITY (%)
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED

Figure 16

sensitivity of the assay is evident from the reduction in sedimentation rate resulting from enzymatic treatment of DNA extracted from cells irradiated with only 5 J m^{-2} (Fig. 17). That this activity is specific and quantitative for cyclobutyl pyrimidine dimers will be discussed below.

Completion of UV endonuclease reaction. Reaction conditions must allow cleavage at all susceptible sites for the quantitation of enzyme-sensitive sites. To establish the reaction conditions necessary to allow the reaction to come to completion, 3 µl of UV endonuclease (approximately 21 µg of protein) were incubated with a 100 µl sample of DNA (approximately 0.1 µg of DNA) from cells previously irradiated with 80 J m^{-2}. The reaction was completed in less than 2.5 min at 35°C (Table 4). Neither a longer incubation nor a second 3 µl addition of enzyme following 20 min incubation at 35°C was found to increase the number of sites detected. The absence of any detectable nicking for 25 min in the unirradiated, enzyme-treated sample is in dramatic contrast to the complete nicking of DNA irradiated with 80 J m^{-2} in less than 2.5 min. For the standard assay conditions, the amount of UV endonuclease used was increased from 3 to 5 µl and the incubation period was 15 min at 35°C. The sample to be assayed always contained approximately 0.1 µg of DNA in 100 µl and was from cells irradiated with no more than 100 J M^{-2}. From the data in Table 4 it is evident that these reaction conditions are more than adequate to insure completion of the UV endonuclease reaction.

Figure 17. The effect of UV endonuclease treatment on the sedimentation rate of DNA extracted from S288C cells which had been previously irradiated at 254 nm with 5 J m^{-2}.

Procedures were the same as described in Fig. 15, page 63, except log phase cells of S288C were irradiated at 0°C with 5 J m^{-2} at 254 nm.

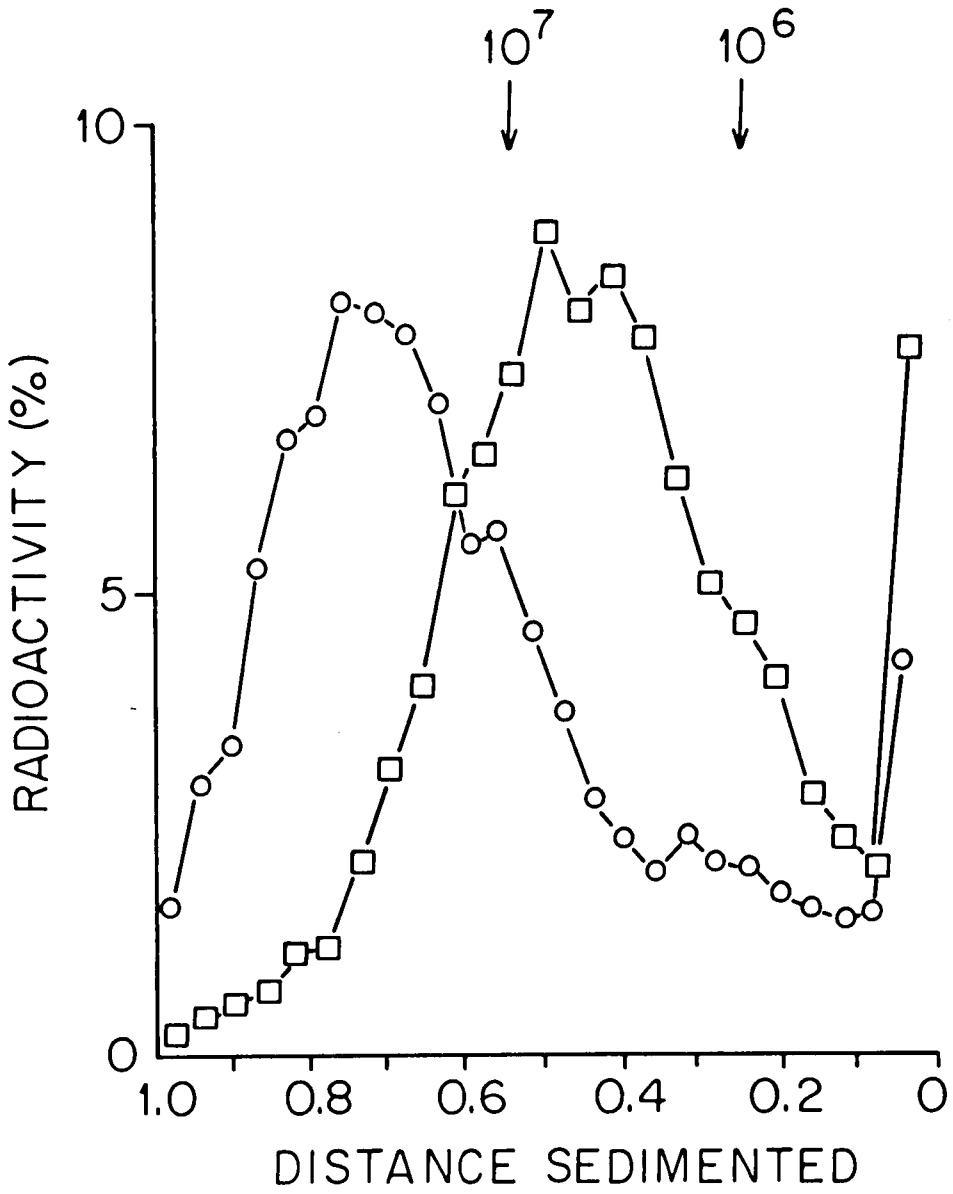

10^7
10^6
10
5
0
RADIOACTIVITY (%)
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED

Figure 17

Table 4. UV Endonuclease Kinetics (35°C).

UV (J/m^2)	UV Endonuclease (ul)	Incubation at 35°C (min)	(2/Mw) x 10^6 (sites/10^6 daltons)
-	-	0	.053
-	-	25	.051
-	3	0	.050
-	3	25	.059
80	-	0	.074
80	-	25	.081
80	3	2.5	2.59[b]
80	3	5	2.70[b]
80	3	10	2.30[b]
80	3	15	2.22[b]
80	3	20	2.45[b]
80[a]	3+3	20+10	2.36[b]

[a]Incubated 20 mins after the first enzyme addition followed by a second enzyme addition and another 10 mins incubation.

[b]Samples corrected for background breaks (0.078 breaks/10^6 daltons).

Fluence dependence of UV endonuclease-sensitive sites. At low fluences cyclobutyl primidine dimers are formed as a linear function of fluence (Setlow and Carrier, 1966; Unrau et al., 1973; Waters and Moustacchi, 1975). If UV endonuclease-sensitive sites are indicative of dimers, they too should be formed as a linear function of fluence. Furthermore, enzyme-sensitive sites should be equal in number to dimers after similar fluences. To demonstrate the relationship of UV endonuclease-sensitive sites to fluence, the following experiment was done. Individual cell samples in 0.067 M potassium phosphate buffer (pH 7.0) were irradiated with various fluences from 0 to 100 J m^{-2}. Cells were kept at 0°C during irradiation and until spheroplast formation. DNA was prepared from yeast spheroplasts and treated with UV endonuclease as described under Materials and Methods. Since the molecular weights for various samples were expected to range from 5×10^7 for the controls to less than 3×10^5 for irradiated, enzyme-treated samples, no one sedimentation condition could be used for the accurate determination of all molecular weights. Therefore sedimentation conditions were chosen to optimize molecular weight determinations by centering the various DNA distributions in their respective gradients.

In this experiment the total number of enzyme-sensitive sites induced by a given fluence is the desired quantity. Any loss of enzyme-sensitive sites after irradiation would yield some lower number and thus give an incorrect relation between the number of enzyme-sensitive sites and the number of dimers. Wild-type yeast have been shown to be capable of excising dimers from their n-DNA (Unrau et al., 1971; Resnick and

Setlow, 1972; Waters and Moustacchi, 1974a; Birnboim and Nasim, 1974). Therefore enzyme-sensitive sites should also be lost in wild-type yeast under conditions allowing the removal of dimers. That this is so will be shown below. To prevent loss of enzyme-sensitive sites, conditions of pH and temperature during and after UV irradiation were adjusted to those thought to prevent excision repair in yeast (Patrick and Haynes, 1964). As a check on this procedure and to insure that all enzyme-sensitive sites were retained, the radiation-sensitive strain S960-1a which is thought to be defective in excision repair (Game and Cox, 1972; Cox and Game, 1974) was also used in one experiment. S960-1a is a strain closely related to S288C and carries the super-suppressible allele rad4-3 (Snow, personal communication). As will be shown below this strain does not lose enzyme-sensitive sites for up to 6 h at 28°C.

In Fig. 18 the number of enzyme-sensitive sites detected is shown to be a linear function of fluence. The data presented is a composite of the results from three independent experiments, two using S288C and one using S960-1a. As can be seen, the results for the two strains were not significantly different. Approximately 0.026 sites per 10^6 daltons per J m^{-2} were found. This is in reasonable agreement with the 0.024 dimers per 10^6 daltons per J m^{-2} which may be calculated from the data of Unrau et al. (1973). Thus enzyme-sensitive sites are not only induced as a linear function of fluence, but also in numbers approximately equal to the numbers of dimers after equivalent fluences. Since pyrimidine dimers are the predominant photoproduct induced in UV-irradiated DNA (Setlow, 1972), the data strongly suggest that enzyme-sensitive sites are indicative of dimers.

Figure 18. Fluence response curve for UV endonuclease-sensitive sites in yeast DNA.

Two cultures of S288C (○,□) and one culture of rad4-3 (△), were irradiated at 0°C with various fluences at 254 nm. DNA was extracted from spheroplasts and treated with and without UV endonuclease from M. luteus. Molecular weight distributions were determined by sedimentation through calibrated low salt alkaline sucrose gradients and the number of enzyme-sensitive sites was calculated with the equation given in the Materials and Methods.

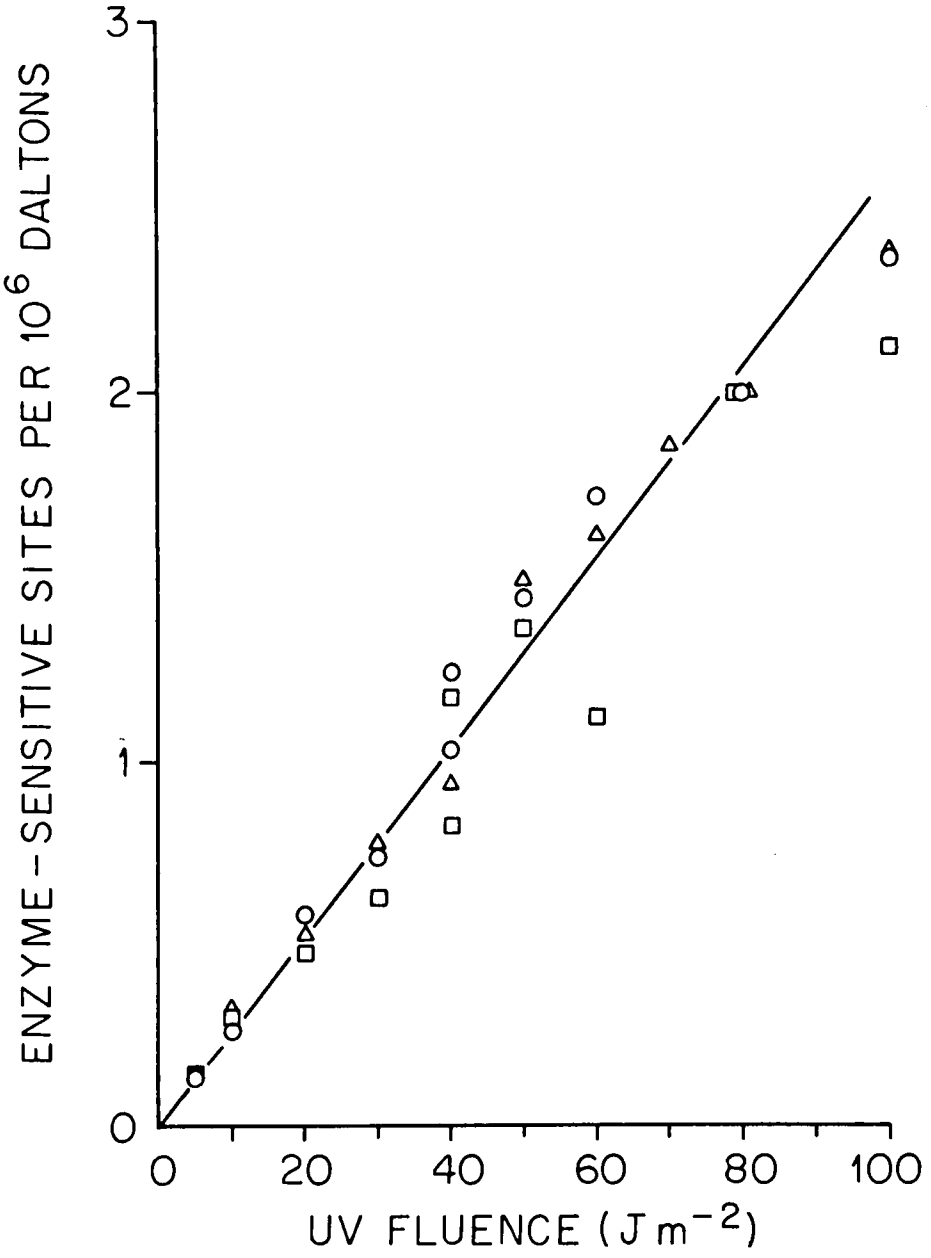

ENZYME-SENSITIVE SITES PER 10^6 DALTONS
3
2
1
0
0
20
40
60
80
100
UV FLUENCE ($J\,m^{-2}$)

Figure 18

Fate of UV endonuclease-sensitive sites during postirradiation holding treatment. The results of UV endonuclease treatment on DNA obtained from cells of the wild-type strain S288C and extracted in the absence of UV irradiation, after irradiation of cells with 5 J m^{-2}, or after irradiation of cells with 5 J m^{-2} followed by a short incubation at 28°C are presented in Fig. 19. When compared to an unirradiated control, the greatest susceptibility to UV endonuclease activity as shown by a reduction in the rate of sedimentation was found in the absence of any postirradiation liquid holding of cells. Susceptibility is rapidly lost if S288C is incubated at 28°C subsequent to irradiation, and after a 5 min postirradiation incubation at 28°C in potassium phosphate buffer (pH 7.0), essentially no difference between the irradiated and the unirradiated samples is detectable.

The numbers of UV endonuclease sensitive sites observed during incubation at 28°C at various times after irradiation with 5 J m^{-2} are presented in Table 5. The maximum number of sites was found after irradiation in the absence of any postirradiation incubation. No detectable sites remain in the DNA of cells held for longer than 5 min at 28°C after irradiation with 5 J m^{-2}. The elimination of UV endonuclease-sensitive sites is rapid and complete under these conditions in the wild-type strain S288C.

In Fig. 20 the response of S288C during holding in 0.067 M potassium phosphate buffer (pH 7.0) is depicted for several fluences from 5 to 100 J m^{-2}. After all fluences most of the sites are rendered insensitive to subsequent UV endonuclease treatment. However,

Figure 19. Sensitivity of DNA from S288C to UV endonuclease treatment after UV irradiation with 5 J m^{-2} and incubation at 28°C for various periods.

Cells of strains S288C in potassium phosphate buffer were either unirradiated or irradiated with 5 J m^{-2} and incubated at 28°C prior to spheroplast formation and DNA extraction. Each sample was divided and treated with or without UV endonuclease from M. luteus. Alkaline sucrose gradient profiles were obtained as described in Fig. 16, page 68. Profiles for the enzyme treated DNA after 0 J m^{-2} (●), 5 J m^{-2} (△), 5 J m^{-2} + 2 min at 28°C (▽), and 5 J m^{-2} + 5 min at 28°C (□) have M_w's of 73, 17, 27 and 67 x 10^6, respectively. Molecular weight distributions of DNA not treated with UV endonuclease were nearly the same and had M_w's of 73, 74, 75, and 66 x 10^6, respectively.

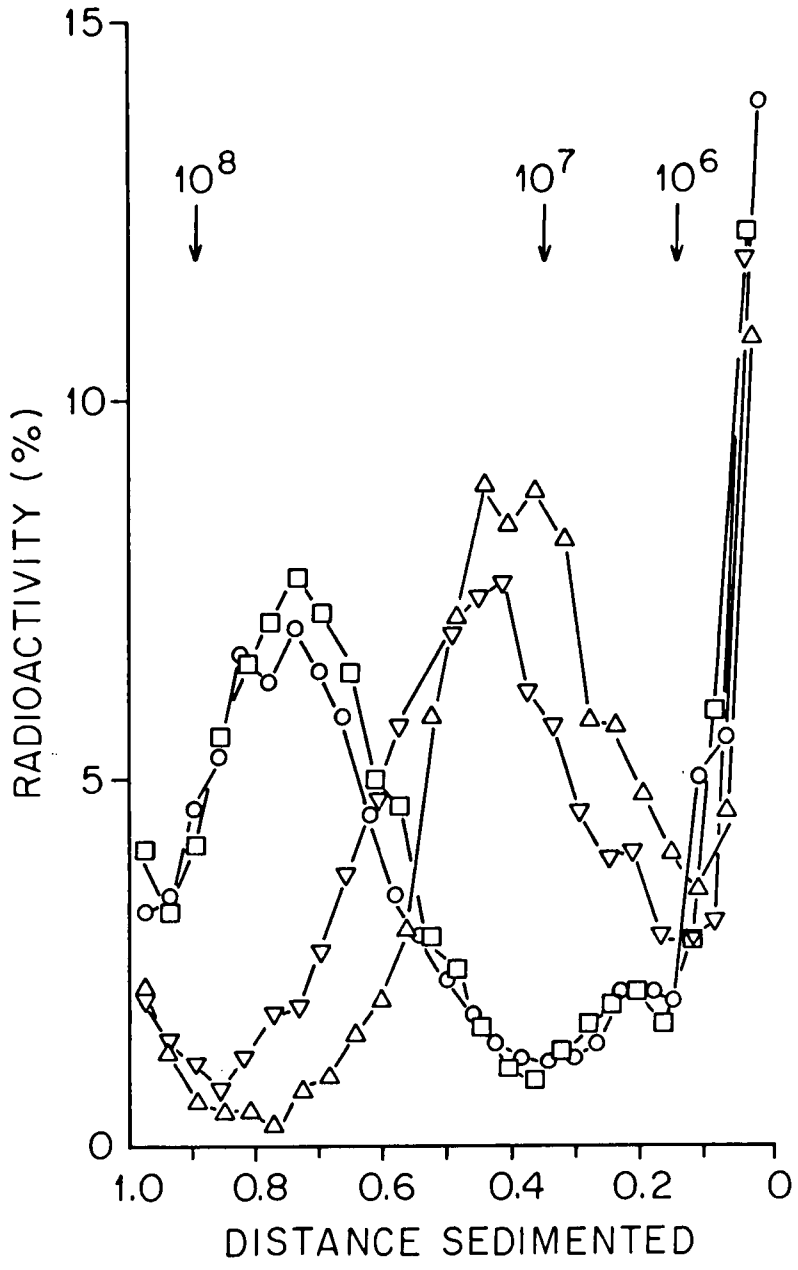

15
10
5
0
RADIOACTIVITY (%)
10^8
10^7
10^6
1.0
0.8
0.6
0.4
0.2
0
DISTANCE SEDIMENTED

Figure 19

Table 5. Removal of UV Endonuclease-sensitive Sites in S288C During Postirradiation Incubation at 28°C.

Fluence (J/m^2)	Postirradiation Incubation (min)	Sites/10^6 daltons	Total Sites (%)
0	-	0.000	-
5	-	0.103	100
5	2	0.056	54
5	5	-0.001	<1
5	10	-0.001	<1
5	20	0.002	2

Figure 20. Loss of UV endonuclease-sensitive sites from S288C DNA during liquid holding in potassium phosphate buffer at 28°C after UV irradiation with 5, 30, 50, and 100 J m^{-2}.

Cells from log phase cultures of S288C in potassium phosphate buffer were irradiated at 254 nm with 5 (O), 30 (□), 50 (Δ), or 100 (∇) J m^{-2} in independent experiments and then held for various periods of time at 28°C prior to DNA extraction. UV endonuclease treatment of the extracted DNA and sedimentation of the DNA through alkaline sucrose gradients were as described in Fig. 16, page 68. Enzyme-sensitive sites were computed from molecular weight distributions by the equation given in the Materials and Methods.

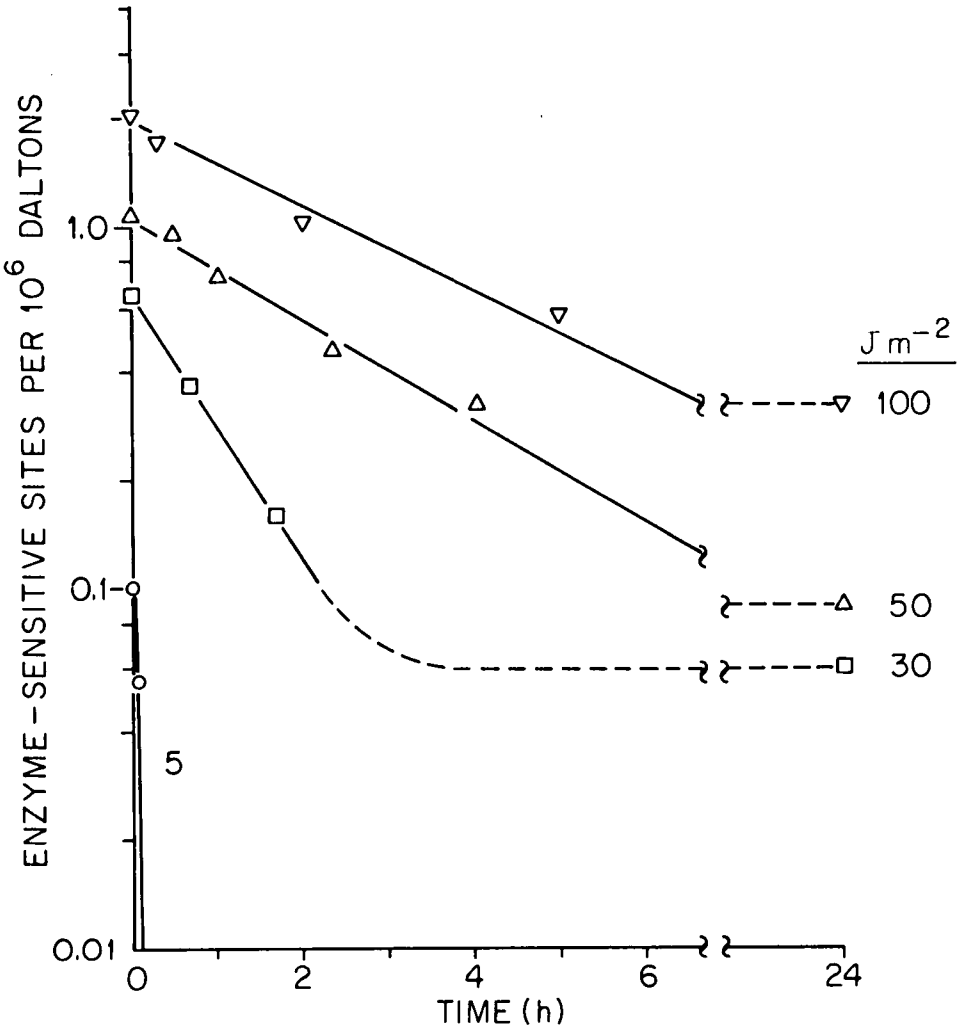

ENZYME-SENSITIVE SITES PER 10^6 DALTONS
1.0
0.1
0.01
0
2
4
6
24
TIME (h)
$J\,m^{-2}$
100
50
30
5

Figure 20

with increasing fluence an increasing number of sites seem to be resistant to cellular repair processes. Additional data after 50 J m^{-2} indicates that there is no further loss of enzyme sensitive sites after 12 h of incubation at 28°C. In this set of experiments 0.0, 9.1, 7.3, and 15.6% of the total number of sites induced by 5, 30, 50, and 100 J m^{-2}, respectively, remain after 24 h of incubation at 28°C. With the exception of the complete removal of enzyme-sensitive sites after 5 J m^{-2}, approximately 10% of the sites seem to be resistant to cellular repair processes under the conditions used.

The fate of UV endonuclease-sensitive sites in the radiation sensitive strain S960-1a (rad4-3) was found to be considerably different from that of sites in S288C. After 5 J m^{-2} (20% survival) the number of sites does not change significantly over a period of 6 h (Fig. 21). This is in contrast to the rapid removal of sites in S288C after the same fluence, and confirms the excision-repair deficient phenotype suggested by studies on the survival of multiple mutants (Game and Cox, 1972; Cox and Game, 1974).

Photoreactivability of UV endonuclease-sensitive sites. Enzymatic photoreactivation specifically monomerizes dimers in situ (Setlow et al., 1965). Since UV endonuclease-sensitive sites seem to be indicative of the presence of dimers, they too should be reduced in number after photoreactivation treatment. This reduction for the excision repair competent strain S288C is evident in Fig. 22. After 50 J m^{-2} of ultraviolet radiation at 254 nm, approximately 120 min are required for half the sites to be removed by excision alone, but with the addition of

Figure 21. Enzyme-sensitive sites in rad4-3 during liquid holding after irradiation with 5 J m^{-2}.

Log phase cells of rad4-3 labeled with [6-^{3}H] uridine and in potassium phosphate buffer were irradiated with 5 J m^{-2} (20% survival) and then held at 28°C for various periods. Enzyme-sensitive sites were determined as in Fig. 20, page 82, and results with S288C also irradiated with 5 J m^{-2} are plotted from Fig. 20 for comparison. The total number of sites after irradiation was 0.103 and 0.121 per 10^{6} daltons in S288C and rad4-3 respectively. No enzyme-sensitive sites were detectable in S288C after irradiation with 5 J m^{-2} when cells were incubated for 5 min or longer at 28°C.

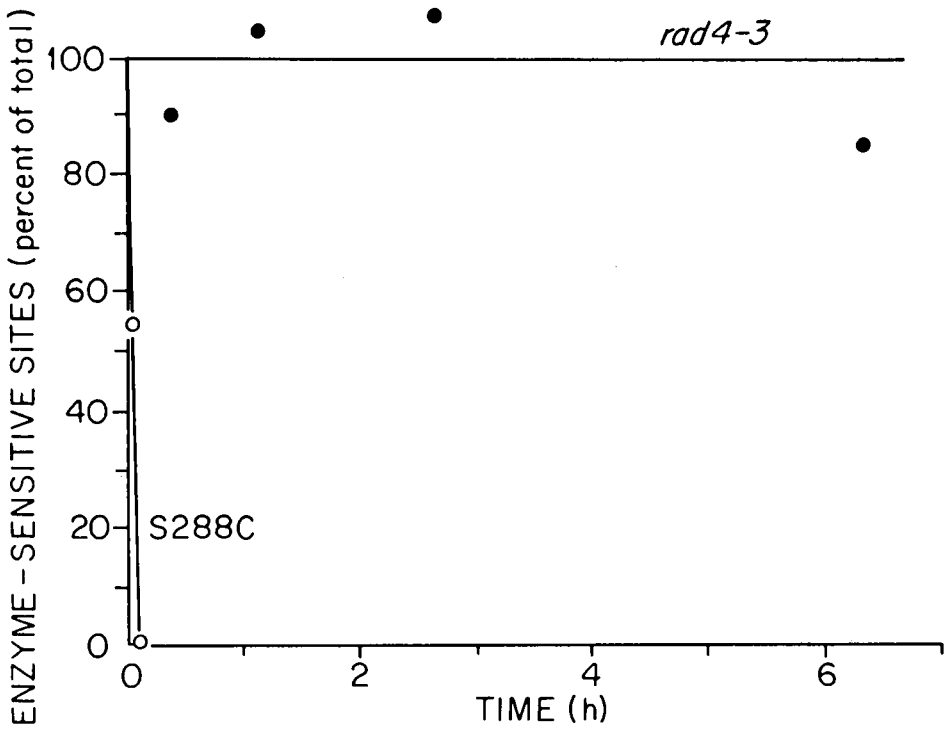

rad4-3
S288C
ENZYME-SENSITIVE SITES (percent of total)
100
80
60
40
20
0
0
2
4
6
TIME (h)

Figure 21

Figure 22. Photoreactivation of UV endonuclease-sensitive sites in S288C.

Log phase cells of S288C were irradiated with 50 J m^{-2} at 254 nm and then incubated in potassium phosphate buffer at 28°C with or without photoreactivation by method 1 as described in the Materials and Methods. The incident energy fluence rate for photoreactivating light was 2.3 J m^{-2} sec^{-1}. Photoreactivation was only for the first two hours after UV irradiation and more sites might have been removed by a longer treatment. Enzyme-sensitive sites were determined as described in Fig. 18, page 76.

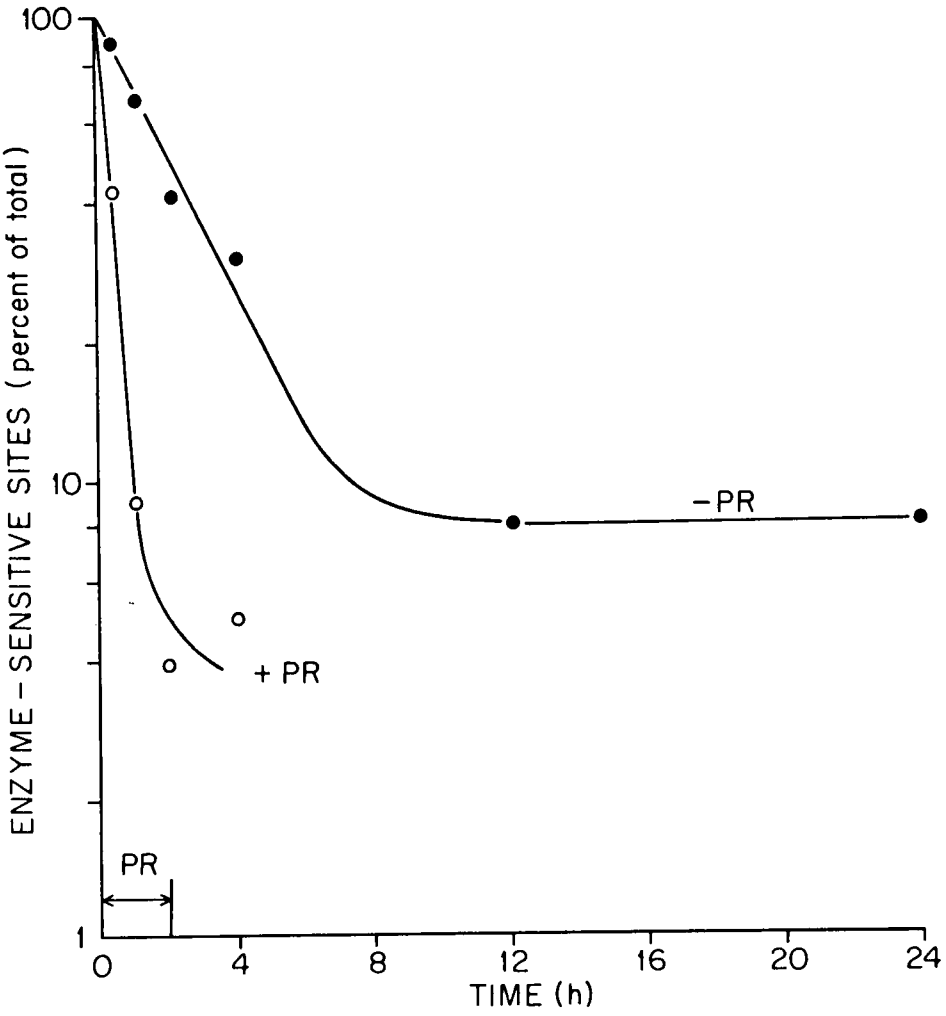

100
10
1
ENZYME-SENSITIVE SITES (percent of total)
-PR
+PR
PR
0
4
8
12
16
20
24
TIME (h)

Figure 22

photoreactivating light (Method 1 in the Materials and Methods chapter) half the sites are gone in 20 min.

The effectiveness of photoreactivating treatment is also evident in the excision-defective strain carrying the rad4-3 allele (Fig. 23). (Photoreactivation by method 2 was used in this experiment and so the results may not be strictly comparable to those presented for S288C in Fig. 22.) After 50 J m^{-2} at least two populations of photoreactivable enzyme-sensitive sites appear to be present in the DNA of the mutant rad4-3. The first population is readily photoreactivated with a half time for the loss of sites of about 25 min. The second population is photoreactivated at a slower rate with a half time for loss of sites of about 90 min. Samples were not photoreactivated for sufficiently long periods to determine if all sites were photoreactivable.

Kilbey and Smith (1969) have shown that the photoreactivability of colony forming ability after UV irradiation is retained in rad1-1 during liquid holding. Under similar conditions photoreactivability was lost in a wild-type strain. In bacteria the retention of photoreactivability has been attributed to the absence of excision repair (Harm, 1968). Strains defective at rad1 are now known to be defective for excision repair (Unrau et al., 1971; Waters and Moustacchi, 1974a; Prakash, 1975) confirming the prediction of Kilbey and Smith (1969). Because photoreactivation acts through the monomerization of dimers, the photoreactivability of dimers would also be expected to be retained during liquid holding in an excision defective strain of yeast.

Figure 23. Photoreactivation of UV endonuclease-sensitive sites in rad4-3.

Log phase cells of strain rad4-3 were treated as described in Fig. 22, page 87, except photoreactivation was by method 2 as described in the Materials and Methods.

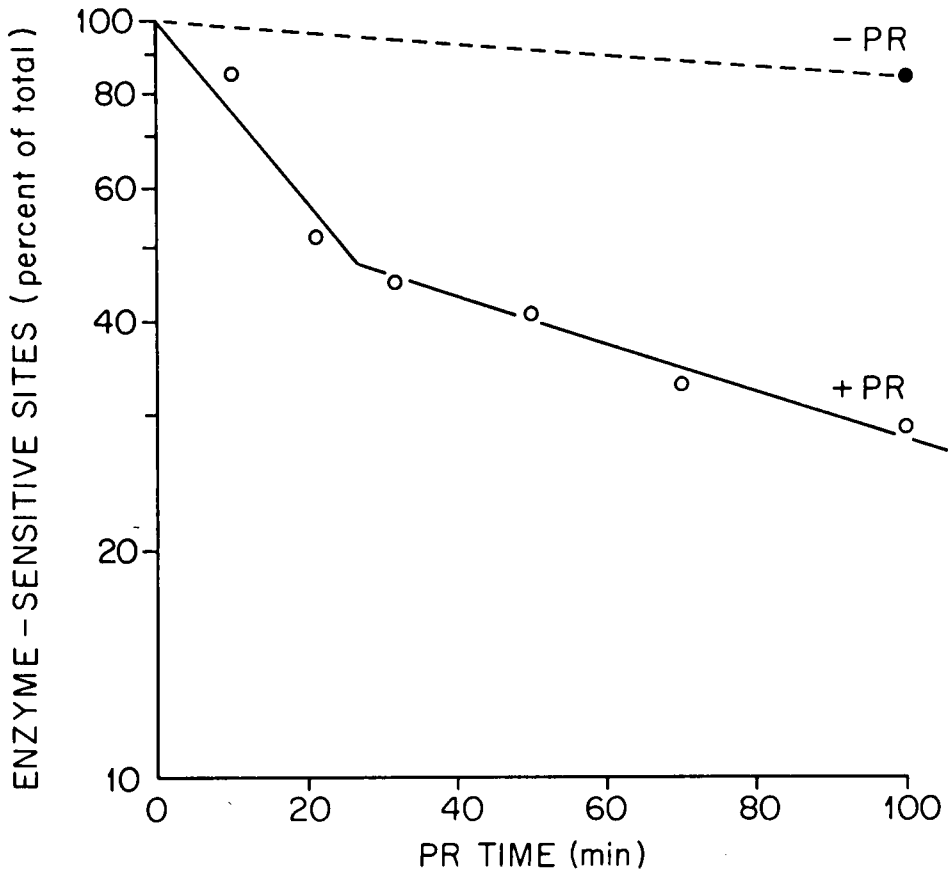

100
80
60
40
20
10
ENZYME-SENSITIVE SITES (percent of total)
-PR
+PR
0
20
40
60
80
100
PR TIME (min)

Figure 23

The effect of liquid holding in buffer on the photoreactivability of UV endonuclease-sensitivesites was investigated in the excision defective strain rad4-3 after irradiation with 5 J m^{-2} at 254 nm. In agreement with the excision defective phenotype of rad4-3 few if any sites were lost during liquid holding in the absence of photoreactivating light (Table 6). The colony forming ability was found to be maximally photoreactivated by 10 min of photoreactivating treatment, method 2, and no further increase was observed during longer exposures to photoreactivating light. It was found that liquid holding after UV irradiation had no effect on either the photoreactivability of colony forming ability or the photoreactivability of UV endonuclease-sensitive sites. Therefore UV endonuclease-sensitive sites remain unchanged not only in number but also in photoreactivability during holding in potassium phosphate buffer at 28°C after the irradiation of rad4-3.

Table 6. Effect of Buffer Holding on the Photoreactivability or UV Endonuclease-Sensitive Sites in rad4-3.

Conditions[a]	% Survival	Equivalent[b] Fluence (J/m^2)	DMF[c]	10^6 Sites daltons	Equivalent[b] Fluence (J/m^2)	DMF[c]
Control	100	0	-	.000	0	-
2.5h PR	86	1.0	-	.004	.2	-
UV	22	5.0	1.0	.124	5.0	1.0
UV+21 min PR	55	2.7	.54	.023	.9	.19
UV+120 min LH	22	5.0	1.0	.107	4.31	.86
UV+120 min LH +21 min PR	49	3.0	.60	.033	1.33	.27

[a]PR = photoreactivating treatment, UV = 5 J m^{-2}, LH = buffer holding.

[b]Equivalent fluence equals fluence which should give the same survival or number of sites as the original fluence (5 J/m^2) plus any additional treatments.

[c]DMF = Equivalent Fluence/original fluence.

CHAPTER IV

DISCUSSION

In this study single-strand, n-DNA released from yeast by the gentle lysis of spheroplasts on top of alkaline sucrose gradients was found to be slightly smaller than expected from previous results reported by Petes and Fangman (1972) and by Blamire et al. (1972). In these latter studies neutral sucrose gradients were used to determine the size of native n-DNA also released from yeast spheroplasts. In comparison to the expected single-strand, number-average molecular weight of 250-300 x 10^6, single-strand DNA released onto alkaline sucrose gradients had number-average molecular weights of approximately 80 x 10^6. Actual and apparent reductions in molecular weight may result from a variety of causes. Actual reductions in molecular weight have been attributed to alkaline degradation of DNA (Hill and Fangman, 1973) and apparent reductions in molecular weight have been found at high rotor speeds with yeast DNA on neutral sucrose gradients (Petes and Fangman, 1972). In my study insufficient ss-breaks were found to arise from alkaline degradation of DNA to explain the smaller than expected molecular weights and no rotor speed artifacts could be detected. Actual reductions in molecular weight could have also resulted from shear during spheroplast lysis or from the presence of alkaline-labile bonds. But at least part of the smaller-than-expected molecular weight was probably the result of label in partially replicated DNA molecules. Such molecules would be expected

in log-phase cells grown in radioactive medium and used without subsequent growth in nonradioactive medium. The presence of replicating molecules in neutral sucrose gradients would be expected to result in the detection of molecules larger than single chromosomes. But in alkaline sucrose gradients replicating molecules would be denatured and yield molecules of both chromosome and less than chromosome length. The presence of partially replicated molecules would therefore result in smaller average molecular weights.

The ability to sediment relatively large molecular weight DNA from yeast through alkaline sucrose gradients has allowed the detection of a small number of ss-breaks in the DNA of UV-irradiated cells that are not present in the DNA of unirradiated cells. Single-strand breaks have been found in the DNA of a wide variety of organisms from E. coli (Setlow, 1966) to human fibroblasts (Setlow et al., 1969; Cleaver, 1974b), and ss-breaks arising from incisions near pyrimidine dimers are predicted to be present in the DNA of UV-irradiated cells during excision repair. However, the relation between ss-breaks and excision repair is not always clear. In yeast I have observed that the number of ss-breaks present during liquid holding in potassium phosphate buffer is related to the fluence of previously delivered UV radiation. A dose reduction in the number of dimers may be accomplished through photoreactivation treatment. That the ss-breaks induced by UV irradiation are actually related to the presence of dimers and probably excision repair also is suggested by a similar reduction in the number of ss-breaks after photoreactivating treatment.

Since ss-breaks are expected to be the result of incisions made by UV endonucleases, they would not be expected to be present in strains deficient in UV endonuclease activity. This has been found to be true with lambda lysogens of E. coli (Shimada et al., 1968; Seeberg and Johansen, 1973). Mutations at uvrA and uvrB result in the loss of detectable UV endonuclease activity in extracts of E. coli (Braun and Grossman, 1974b). Single-strand breaks were found in UV-irradiated lambda DNA after the infection of wild-type E. coli but not after the infection of radiation-sensitive strains carrying mutations at uvrA and uvrB (Shimada et al., 1968; Seeberg and Johansen, 1973). In yeast radiation-sensitive strains carrying mutations at rad1, rad2, rad3, and rad4 are defective in excision repair. If one of these four genes coded for the UV endonuclease in yeast it might be expected that the defect would show up as an inability to make ss-breaks in DNA after UV irradiation. Representative strains carrying mutations at each of the four loci were examined for the ability to make ss-breaks after UV irradiation, but all strains examined were found to make ss-breaks after UV-irradiation.

In particular strains of yeast having mutations at rad3 have been thought to be likely candidates for defects at an early step in excision repair (Cox and Game, 1974). Findings in this study indicate rad3 mutants are able to make the initial incision. Cox and Game (1974) have also pointed out that their results are compatible with the involvement of the rad3 gene product in a later step in excision repair the substrate for which is converted from the original photoproduct

through reversible reactions. In E. coli and M. luteus the initial incision by UV endonucleases leaves a 3'-hydroxyl and 5'-phosphoryl termini (Grossman et al., 1975). This structure is a suitable substrate for polynucleotide ligase and unless altered in some additional way is therefore reversible through ligation, and it is quite possible that the first step of excision repair in yeast is also reversible. From the ability of rad3 mutants to make ss-breaks after UV-irradiation it would be predicted that the rad3 gene product is involved in some step after incision.

The detection of ss-breaks after UV-irradiation of radiation-sensitive strains of yeast does not necessarily rule out the presence of defects in the incision step of excision repair. The presence of more than one structural gene for UV endonuclease could mask the loss of one activity. But the presence of more than one UV endonuclease activity in yeast appears to be unlikely when the UV sensitivity of these strains is considered. Single-strand breaks might also arise in a nonspecific manner not permitting the selective excision of dimers. Suzuki et al. (1966) have reported extensive DNA degradation in E. coli strain B_{s-1}. This strain does not selectively excise dimers (Setlow and Carrier, 1964) because of a mutation at uvrB. Mutations at uvrB eliminate UV endonuclease activity (Braun and Grossman, 1974b). Therefore incisions leading to DNA degradation must arise by some other nonspecific mechanism. Similar phenomena could occur in radiation-sensitive strains of yeast. In this study apparently nonspecific reductions in molecular weight were frequently observed when radiation-sensitive strains of yeast were held

in potassium phosphate buffer for longer than 12 h after UV irradiation. If nonspecific ss-breaks were present, they could have prevented the detection of defective UV endonuclease activity.

In the second part of this study, extracts of M. luteus were found to introduce a limited number of ss-breaks into DNA extracted from UV-irradiated S. cerevisiae. Although a variety of photoproducts are produced in DNA by UV irradiation (Setlow and Setlow, 1972), the following evidence suggests that the enzymatic activity associated with M. luteus extracts and responsible for the ss-breaks in enzyme-treated, UV-irradiated DNA is specific for pyrimidine dimers:

1. Extracts are totally inactive against unirradiated DNA,
2. The predominant photoproducts at low fluences are pyrimidine dimers (Setlow, 1968),
3. Enzyme-sensitive sites are induced in equivalent numbers to dimers from 0 to 80 J m^{-2},
4. Sites are photoreactivable, a process known to be specific for dimers (Setlow et al., 1965).

Furthermore endonucleases specific for pyrimidine dimers have been isolated from M. luteus extracts (Grossman et al., 1975). Additional enzymatic attack at some lesion distinct from dimers, but present at much lower levels, cannot be excluded, but from the above considerations most, if anot all, enzyme-sensitive sites detected in UV-irradiated DNA treated with M. luteus extracts appear to be identical to pyrimidine dimers.

The specificity of enzymatic activity in M. luteus extracts has been used to develop a relatively simple and sensitive assay for pyrimidine dimers in S. cerevisiae. Assays based on the detection of UV endonuclease-sensitive sites have several advantages over chromatographic procedures currently used for the detection of dimers in yeast (Unrau et al., 1972; Birnboim and Nasim, 1974). First, the detection of enzyme-sensitive sites requires only a small number of cells, 10^7 or less. This reduces the number of cells that must be labeled. Detection of the small percentage of radioactivity in dimers by chromatographic techniques requires much larger sample sizes. Second, sedimentation on alkaline sucrose gradients during the measurement of enzyme-sensitive sites allows the use of general nucleic acid precursors for DNA labeling without also requiring extensive DNA purification to eliminate concomittantly labeled RNA. Such purifications are required prior to the chromatographic analysis of dimers when general nucleic acid precursors are used for labeling. Specific DNA labeling with thymine or thymidine is precluded in yeast by the absence of thymidylate kinase (Grivell and Jackson, 1968).

Recently strains capable of incorporating 5' TMP have been isolated (Jannsen et al., 1968; Jannsen et al., 1970; Brendel and Haynes, 1972), and such strains may potentially be used to eliminate complications arising from the simultaneous lableing of DNA and RNA with general nucleic acid precursors such as uracil or uridine. But although strains with improved incorporation have been isolated (Fäth and Brendel, 1974; Wickner, 1974) labeling with TMP is still relatively expensive and may

preferentially label mt-DNA thereby necessitating the separation of n-DNA and mt-DNA by banding in CsCl buoyant density gradients (Prakash, 1975).

Finally, the detection of enzyme-sensitive sites is considerably more sensitive to small numbers of dimers than currently used chromatographic techniques. The increased sensitivity results from the large changes in molecular weight caused by small numbers of ss-breaks in large molecular weight DNA together with the ability to gently isolate DNA for the assay of enzyme-sensitive sites. If care if taken to avoid excessive shear during DNA isolation, enzyme-sensitive sites may readily and reproducibly be detected after only 5 J m^{-2} and should be detectable after 1 J m^{-2} or less. This represents a 10-100 fold increase in sensitivity over most currently used procedures for the detection of pyrimidine dimers in yeast.

The presence of mt-DNA in yeast is a potential problem during the study of events in n-DNA. Postirradiation events in n- and mt-DNA appear to be considerably different (Waters and Moustacchi, 1974a; 1974b; Prakash, 1975). Excision repair is operative for n-DNA (Unrau et al., 1971) but not for mt-DNA (Waters and Moustacchi, 1974b). After large fluences of UV radiation mt-DNA may also be degraded (Waters and Moustacchi, 1974b). CsCl buoyant density gradients prior to the chromatographic analysis of dimers has been used to separate the two DNA species and to allow analysis of the results in n-DNA uncomplicated by different events in mt-DNA (Waters and Moustacchi, 1974a). The use of TMP and strains capable of incorporating it into their DNA has been

suggested to eliminate labeling of RNA, but this procedure may preferentially label mt-DNA. Any such increase in the production of label in mt-DNA relative to n-DNA would further complicate the analysis of events in n-DNA.

The separation of n- and mt-DNA was not found to be necessary in this study. When [6-^{3}H] uridine was used less than 10% of the label incorporated into cellular DNA was found in mt-DNA. Mt-DNA was found to be no larger than n-DNA under any conditions. During the detection of enzyme-sensitive sites mt-DNA in samples from unirradiated cells sediments more slowly than n-DNA even though the n-DNA is slightly sheared during purification. This is also true when DNA extracted from irradiated cells is not enzyme treated. UV irradiation introduces approximately the same proportion of dimers in both n- and mt-DNA (Waters and Moustacchi, 1974b). Since ss-breaks introduced by the M. luteus extract are equal to the number of dimers and additive to ss-breaks already present in the enzyme-treated DNA, mt-DNA in enzyme-treated samples from irradiated cells would sediment at a rate no faster than n-DNA. Loss of dimers due to excision repair occurs only in n-DNA and photoreactivation appears to occur to nearly the same extent in both n- and mt-DNA (Waters and Moustacchi, 1974b). Therefore under all conditions mt-DNA has a molecular weight as small as or smaller than n-DNA. The use of weight-average molecular weights in the calculation of enzyme-sensitive sites reduces and/or eliminates any contribution by the small percentage of radioactivity in mt-DNA. This enabled the study of events in n-DNA without separation of n- and mt-DNA's.

The detection of UV endonuclease-sensitive sites proved to be useful in following the fate of dimers during postirradiation liquid holding. This assay alone does not detect the actual removal of dimers from DNA since a site becomes no longer detectable after an incision is made near the dimer during normal excision repair. The reasons for this are as follows. It is unlikely that the UV endonuclease in M. luteus extracts recognizes dimers with closely associated ss-breaks. In any event an additional ss-break close to the original incision site could not be resolved by sedimentation on alkaline sucrose gradients. Thus the actual excision of dimers is not necessary to reduce the number of enzyme-sensitive sites. But the incomplete repair of damaged regions would leave ss-breaks and large accumulations of ss-breaks were not found during the investigation of endogenous ss-breaks. If the ss-breaks introduced during excision repair were simply resealed with the dimer still present, the dimer would again be expected to be a suitable substrate for the UV endonuclease in the M. luteus extract. From this it was concluded that the disappearance of enzyme-sensitive sites during postirradiation liquid holding was due to complete dimer excision soon after the initial incision event.

Through the detection of enzyme-sensitive sites log-phase, wild-type yeast were found to excise dimers rapidly and completely after 5 J m^{-2}. However, increasing numbers of dimers were found to be resistant to removal by excision repair in potassium phosphate buffer. At 30, 50, and 100 J m^{-2} approximately 10% of the sites initially produced by UV irradiation were found to be resistant to cellular repair

processes. Both Unrau et al. (1971) and Prakash (1975) have reported complete removal of dimers after 500 and 67 J m^{-2} respectively. In both cases irradiated cells were incubated in growth medium. In the first of these studies (Unrau et al., 1971) 10% of the original dimers could have been difficult to detect with the chromatographic procedure used. Prakash measured UV endonuclease-sensitive sites by a procedure similar to that described in this study. The increased sensitivity of this procedure enables the detection of small numbers of dimers. Since all enzyme-sensitive sites were found to be removed in wild-type yeast incubated in growth medium after irradiation, excision repair appears to be more nearly complete after low fluences when cells are incubated in growth medium than when cells are incubated in nongrowth conditions.

By contrast to results with the wild-type strain, mutation at rad4 was found to completely block excision repair in potassium phosphate buffer after only 5 J m^{-2} and with a surviving population of approximately 20%. The absence of excision repair in strains having mutations at rad4 was expected from the epistatic interaction of rad4 with rad1, rad2, and rad3 (Game and Cox, 1972). Despite the inability of strains carrying rad4 to excise dimers, this same strain was found to make ss-breaks at near the wild-type level after UV irradiation. Together the data suggest that mutations at rad4 block excision repair at some step after the initial incision.

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