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THE GENETICS OF X-RAY-INDUCED DOUBLE-STRAND-BREAK
REPAIR IN SACCHAROMYCES CEREVISIAE

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Ph.D. Thesis

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The Genetics of X-ray Induced Double Strand Break Repair in Saccharomyces cerevisiae

Martin Ellsworth Budd

This thesis examined the possible fates of x-ray induced double strand breaks in *Saccharomyces cerevisiae*. One possible pathway which breaks can follow is the repair pathway and this pathway was studied by assaying strains with mutations in the RAD51, RAD54, and RAD57 loci for double strand break repair using neutral sucrose sedimentation. rad54-3 strains were sensitive to x-rays at 36° and resistant at 23°, while rad57-1 strains are sensitive to radiation at 23° and resistant at 36°. In order of increasing radiation sensitivity one finds: rad57-1(23°) > rad51-1(30°) > rad54-3(36°). At the restrictive temperature 36°, rad54-3 cells are unable to repair double strand breaks, while at the permissive temperature, 23°, these strains are able to repair double strand breaks. On the other hand, strains with the rad57-1 mutation appear to be able to rejoin broken chromosomes at both the permissive and restrictive temperature. However, the low survival at the restrictive temperature shows that the sedimentation assay is not distinguishing large DNA fragments which allow cell survival from large DNA fragments which cause cell death. A rad51-1 strain also appeared able to rejoin broken

chromosomes, and is thus capable of incomplete repair. The data can be explained with the hypothesis that rad54-3 cells are blocked in an early step of repair, while rad51-1 and rad57-1 strains are blocked in a later step of repair.

The fate of double strand breaks when they are left unrepaired was also investigated with the temperature conditional rad54-3 mutation. If breaks are prevented from entering the RAD54 repair pathway they are modified and become uncommitted lesions. The rate these uncommitted lesions are repaired is slower than the rate the original breaks are repaired. One possible fate for an uncommitted lesion is conversion into a fixed lesion, which is likely to be an unreparable or misrepaired double strand break.

The presence of protein synthesis after irradiation increases the probability that a break will enter the repair pathway rather than the fixation pathway. Evidence is shown that the increased probability of repair probably results from an enhanced synthesis of repair proteins shortly after radiation.

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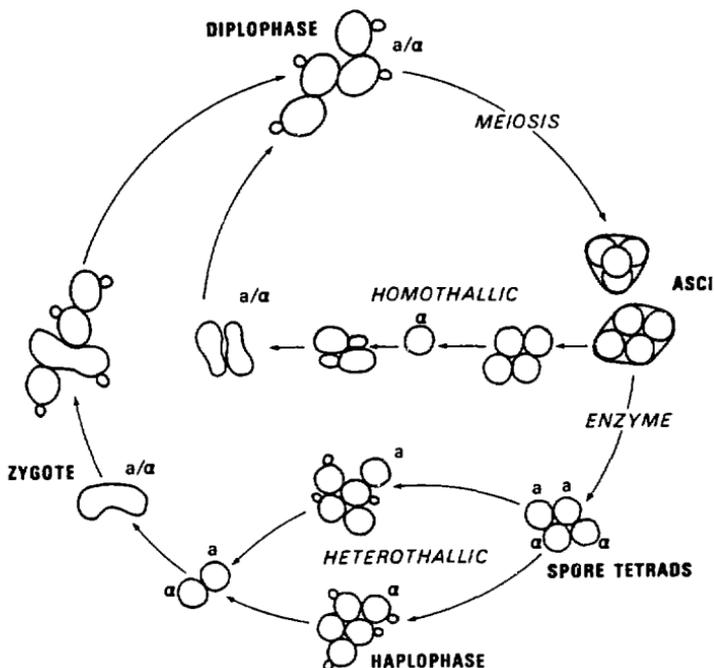
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INTRODUCTION

The yeast Saccharomyces cerevisiae has been extensively used in radiobiological studies. Figure 1 is taken from Mortimer and Hawthorne(1969) and illustrates the life cycle of Saccharomyces cerevisiae. Most yeast cultures from nature exist in the diploid phase. Under nitrogen starvation conditions diploid cells go through meiosis and yield 4 spored asci. The haploid asci can possess either one of two distinct mating types, MATa or MAT α . Haploid MATa and MAT α strains can mate to form a/ α diploid zygotes. The a/ α diploid zygote does not mate with either a MATa or MAT α strain and is able to undergo meiosis. Heterothallic strains of yeast can exist stably in the haploid state. On the other hand, homothallic strains of yeast can switch mating type from MATa to MAT α or MAT α to MATa as often as every generation. Thus, a single cell from a strain which is a homothallic haploid MATa or MAT α can give rise to a colony of a/ α diploid cells. Diploid cells can also arise from rare matings of haploid cells of the same mating type or endomitotic division giving rise to MATaa or MAT $\alpha\alpha$ diploids. Diploids which are aa or $\alpha\alpha$ at the MAT locus instead of a/ α are unable to undergo meiosis but are able to mate. Yeast cells possess 17 known chromosomes and genetics of this organism can easily be done using micromanipulation.

X-rays are one agent which causes both single and double strand breaks in the DNA (Friefelder, 1965). The idea



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FIGURE 1. Life cycle of *Saccharomyces cerevisiae*. The diagram illustrates the alternation of haploid and diploid phases, and the distinctions between heterothallic and homothallic strains. Taken from Mortimer and Hawthorne (1969).

of this thesis is to examine the possible fate of double strand breaks after irradiation in yeast. The first possible fate is repair of the break restoring the DNA to its original form resulting in a viable cell. The second conceivable fate could be interaction of the break with another broken or possibly unbroken chromosome resulting in a dicentric. The third possible fate would be the break remains unrepaired and does not interact with another chromosome. The likely phenotypic effect of the second and third possible fates would be cell death.

The final fate of double strand breaks in mammalian cells is inferred from the aberrations seen during the first mitotic division after irradiation. These are of two types (Lea, 1955; Okada, 1969; Wolff, 1972). One type is believed to result from 1 ionizing event, and are called one hit aberrations. These are the type of aberrations one would expect if the break has remained unrepaired and did not interact with another chromosome. The second type of aberrations are believed to result from 2 ionizing events and these are called 2 hit or exchange type aberrations. Examples of these are dicentrics, translocations, rings, and inversions.

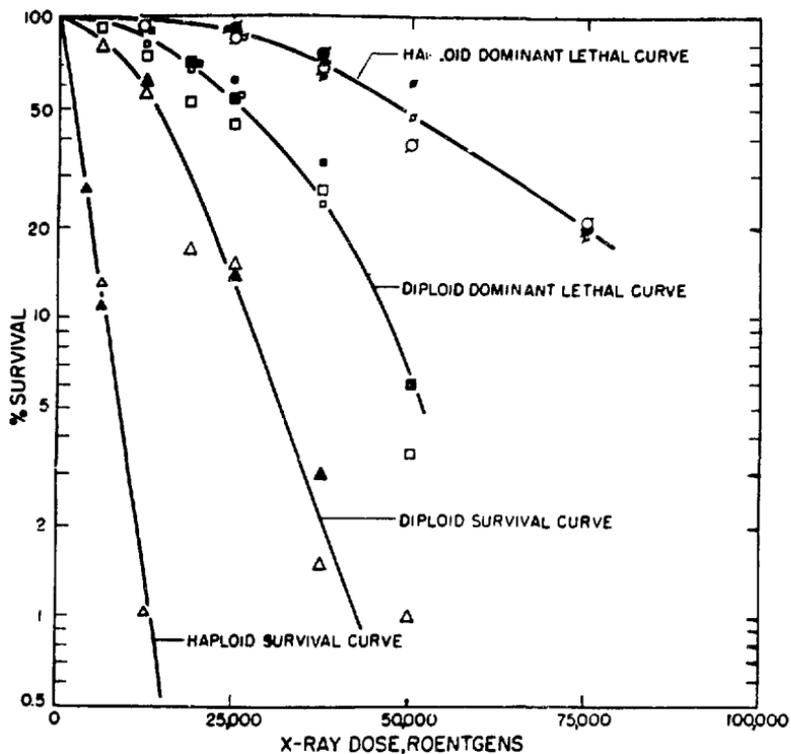
Latarjet and Ephrussi (1949) observed that haploid yeast cells exhibit an exponential x-ray survival curve, while diploid cells exhibit a more resistant survival curve characterized by a shoulder. Later it was shown that the

haploid survival curves are biphasic, and possess a radioresistant tail which represents the resistance of budding cells to x-rays (Beam et al., 1954). Furthermore the resistance of budding cells to x-rays correlates with cells at the DNA synthesis stage rather than with the appearance of the bud (Bird and Manney, 1974).

Since dominant lethality is an important concept in understanding the fate of induced breaks, a detailed introduction to previous experiments will follow. Dominant lethal damage can be studied by irradiating a haploid cell, mating it to a unirradiated cell, and then examining the zygote for viability (Mortimer, 1955). If recessive lethal damage is the predominant mode of cell death in diploids, then irradiation of one of the haploid parents should not affect zygote viability. A recessive lethal is one which kills the cell if present in a homozygous condition, either in a haploid or diploid state, but if present in a heterozygous state in a diploid allows cell survival. If the diploid zygote dies then the irradiated haploid parent has exerted a dominant lethal effect on the viability of the zygote. The amount of dominant lethal damage of a haploid is studied by irradiating a MAT^α haploid, mating it to a MAT^a cell, then assaying the zygote for viability. The amount of dominant lethal damage of a diploid is studied by irradiating MAT^{αα} diploids, mating to MAT^a or MAT^{aa} cells, then assaying the triploid or tetraploid zygotes for viability. Figure 2 which is taken from Owen and Mortimer (1956)

illustrates a haploid survival curve, a diploid survival curve of a strain homozygous at the mating type locus, a haploid dominant lethal curve, and a diploid dominant lethal curve. The data shows that MAT_{aa} or MAT_{QQ} diploid cells are more resistant to radiation than haploid cells. The haploid dominant lethal curve shows that recessive lethals are not the only form of lethal damage in yeast. The curves also show that diploids are twice as sensitive to dominant lethal damage as haploids. An interpretation of the result is that a diploid genome has twice as many sites for dominant lethal damage as a haploid genome. The sensitivity of the resulting zygote depends only on the ploidy of the irradiated parent, not on the ploidy of the unirradiated parent. The survival curve of the a/Q diploid strains is similar to the dominant lethal survival curve of a diploid.

Since the dominant lethal damage increases as the ploidy changes from haploid to diploid, then one might expect an increase in radiation sensitivity as the ploidy increases beyond diploid. This was observed by Mortimer (1958) when he showed that diploid strains are the most resistant to radiation, triploid strains are somewhat more sensitive to radiation than diploid strains. Tetraploid strains are about twice as sensitive to radiation as diploid strains, and hexaploid strains are significantly more sensitive to radiation than tetraploid strains. In order of increasing radioresistance one observes haploid < hexaploid < tetraploid < triploid < diploid. Mortimer found that for



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FIGURE 2. Haploid dominant lethal curve: ϕ , \circ , ϕ , \circ , ϕ
 Diploid dominant lethal curve: \square , \circ , \square , \circ , \square , \circ
 Diploid survival curve: \bullet , \circ as diploid \triangle , \circ as diploid
 Haploid survival curve: \blacktriangle , \bullet as haploid \triangle , \bullet as haploid

Taken from Mortimer and Owen (1950)

diploid, tetraploid, and hexaploid cultures, if LD_{10} is the dose required to give 10% survival, then $[LD_{10}]^x$ is a constant. This relationship can be explained if one assumes that dominant lethal damage plays the major role in cell death in cultures with a ploidy of diploid or greater.

It has been observed that heterozygosity at the mating type locus also plays a role in radiation resistance (Mortimer, 1958). a/a or aa diploid strains are about twice as sensitive to radiation as a/a diploid strains.

One of the key mutations in understanding the fate of double strand breaks and their relation to dominant lethality is the rad52-1 mutation. Cells with the rad52-1 mutation exhibit a severe radiation-sensitive phenotype (Game and Mortimer, 1974). The survival curve of haploids with a mutant rad52 allele lack the radioresistant tail, and diploids homozygous for rad52 exhibit an exponential x-ray survival curve, in contrast to the sigmoidal survival curve of wild-type diploids.

The role of the RAD52 gene product in the repair of lesions which could result in dominant lethal damage has been examined by Ho and Mortimer (1973). They examined rad52 dominant lethal survival curves by x-raying a rad52 haploid, mating with an unirradiated rad52 cell, and assaying the zygote for viability. The curve generated when both the irradiated and unirradiated haploid parents have a rad52 genotype is much steeper than when either of the parents is

RAD+. Irradiated haploids are much more likely to exert a dominant lethal effect when the resulting zygote is rad52/rad52 than when the zygote is RAD+/rad52. Ho and Mortimer (1973) proposed that the RAD52 gene product may be involved in the repair of lesions which may become expressed as dominant lethals. Thus, dominant lethals refer to non-repairable end products of radiation and they are more likely to occur if a rad52 mutation is present.

Early studies of dominant lethal damage were done using Drosophila. In Drosophila, dominant lethal damage was studied by irradiating males, mating to unirradiated females, and observing the portion of fertilized eggs which do not give rise to flies (Lea 1955). The resulting curve generated was sigmoidal and had a D_{37} of about 4 krads. In this experiment the corresponding number of females hatched was less than the number of males. Hence, the XX zygote appeared to be more sensitive to dominant lethals than the XY zygote. Since the Y chromosome is smaller than the X chromosome, less dominant lethal damage can be introduced. Alternatively interaction between a damaged X and its undamaged homologue may be required to cause lethality.

The structural form of dominant lethal damage was inferred from observations of the chromosomal structures which appeared in the salivary glands of the descendents of irradiated flies. Only small deletions less than 50 bands were observed in the heterozygous condition and were

inferred to behave as recessive lethals. Other aberrations, such as deletions larger than 50 bands, lost chromosomes, acentric fragments, and dicentrics were not observed and were inferred to be causally related to dominant lethals. The other possibility is that these aberrations did not occur in the resulting zygote. Recessive lethals were studied in the X chromosome. They increased in proportion to dose, and their yield was independent of the dose rate of radiation (Lea, 1955).

Further evidence of the nature of dominant lethal damage is provided by the work of Dewey et al (1971) and Carrano (1971). Dewey observed that at the D_{37} dose of Chinese Hamster Ovary cells there is an average of 1 aberration per cell. These aberrations included both the type one would expect if the broken chromosome was not repaired and did not interact with another chromosome, and the type where the break interacts with another chromosome to produce a dicentric. In mammalian cells chromosomal aberrations such as dicentrics and acentric fragments appear to behave in a dominant lethal fashion. The presence of a chromosomal bridge or dicentric prevented the cell from completing the first mitotic division (Carrano, 1971). Carrano also observed that the presence of acentric fragments correlated with cell death since cells with these aberrations completed the first mitotic division, had a high probability of being unable to complete succeeding cell divisions. Unfortunately, cytological observations of dominant lethal damage in yeast are

precluded since the chromosomes are not resolvable using light microscopy. However, the initial lesion leading to chromosomal aberrations may be a double strand break, and such initial lesions can be studied in yeast (Resnick and Martin, 1976; Ho, 1975).

The development of an assay for the repair of double strand breaks provided one of the key tools to understand the fate of these lesions, and the relationship of the repair of these lesions with survival. The assay used to study induction and repair of double strand breaks is neutral sucrose sedimentation as developed for yeast by Petes and Fangman (1972). The protocol involves lysing glucosylase treated radioactively labeled yeast cells on the top of linear 15-30% sucrose gradients, centrifuging at 10,000 rpm for 26 hrs, collecting fractions, and assaying for radioactivity. T4 phage is used as a molecular weight marker. Calculations from sucrose sedimentation demonstrated that chromosomal DNA from yeast has a number average of molecular weight of about 6×10^8 daltons. Measurements of chromosomal DNA determined by electron microscopy yielded an average length of 165 microns, corresponding to a molecular weight average 3.2×10^8 daltons. Furthermore Petes and Fangman (1973) extracted DNA from various fractions of a neutral sucrose gradient and established that the contour length of DNA increases with increasing distance down the gradient. These measurements suggest that the sucrose sedimentation technique, when applied to yeast, measures the size of

unbroken linear chromosomes.

The next step in understanding the fate of double strand breaks was provided by Ho (1975) and Resnick and Martin (1976). Ho (1975) using haploid strains, and Resnick and Martin (1976) using a diploid strain, used neutral sucrose sedimentation to assay for the repair of double strand breaks. Both groups showed that in RAD+ cells harvested from log phase, one possible fate of double strand breaks is their repair, and a functional RAD52 gene product is required for this repair. Ho (1975) observed extensive degradation of the DNA in log phase haploid rad52 cells incubated 3.5 hours after irradiation, thus illustrating that one possible fate of unrepaired breaks is degradation of the broken chromosome. Resnick and Martin (1976) observed that the profile of DNA from rad52 diploid cells irradiated and incubated in YEPD was at the same position as DNA extracted from irradiated cells with no incubation. By comparing the D_{37} value of diploid rad52 rad18 strains with the dose required to induce one double strand break per cell, Resnick and Martin established a 1:1 correlation of unrepaired double strand breaks and cell lethality. The repair of double strand breaks and the repair of lesions causally related to dominant lethal damage both require a functional RAD52 gene product. This correlation is consistent with the hypothesis that the initial lesion causally related to dominant lethal damage is the double strand break. Thus, it is not unreasonable to think that double

strand breaks which have been left unrepaired by a RAD52 gene product can lead to dominant lethal damage, but in yeast these experiments cannot be done.

The repair of x-ray induced double strand breaks in yeast and a corresponding relation to higher survival has also been observed by other investigators. Frankenberg-Schwager et al (1980) have shown that extensive repair of double strand breaks occurs in diploid yeast cells held in G-1 phase by liquid holding. The double strand break repair observed upon liquid holding of G-1 cells correlates with an increase in survival also observed upon liquid holding.

By using a diploid strain which can be easily synchronized, Brunborg et al(1980) examined the extent of cell survival and the amount of double strand break repair in various stages of the cell cycle. The strain NCYC-239 is most sensitive to x-rays in G-1 with resistance rising through S-phase, and reaching a peak in G-2 phase (Brunborg and Williamson,1978). This cell cycle pattern of radioresistance to x-rays correlates with the ability of the strain to repair double strand breaks. Cells irradiated in the G-2 stage are capable of complete repair of double strand breaks at doses of 50 krads while cells irradiated in the G-1 stage exhibited no repair. Brunborg and Williamson(1978) examined the G-2 block induced by x-rays in the strain NCYC-239. They observed that the strain exhibited a G-2 block of around 3-7 hours, which gives extensive time for the repair

of damaged DNA.

Besides x-rays, another DNA damaging agent which causes single and double strand breaks is methyl methane sulfonate, or mms. Chlebowicz and Jackmyczyk(1979) compared the survival after mms treatment of log phase MATa cells with those cells which have been synchronized in G-1 phase by α factor. The authors found that log phase cells were significantly more resistant to mms than cells synchronized in G-1 phase. The efficiencies of single strand break repair and double strand break repair were compared with G-1 and log phase cells. An analysis of single strand break repair using alkaline sucrose sedimentation shows that both log phase and G-1 phase cells are capable of repairing single strand breaks. However, neutral sucrose sedimentation shows that although log phase cells are capable of extensive but not complete repair in 8 hours, G-1 cells are severely defective in repair. The differential survival of G-1 and log phase haploid cells to mms correlates with the inability of G-1 cells to repair double strand breaks and the ability of log phase cells to repair double strand breaks. The results also provide evidence for the presence of two homologous DNA duplexes as a necessary condition for double strand break repair.

In summary, the previous investigators have shown that in RAD+ cells breaks have a high probability of being repaired, thus allowing the cells to survive. In strains

which are radiosensitive, such as rad52 strains, or strains which are in a radiation sensitive stage of the cell cycle, these breaks remain unrepaired. The fate of cells with unrepaired double strand breaks is cell death.

The neutral sucrose sedimentation technique measures the conversion of small DNA fragments to large DNA fragments. Thus, the assay demonstrates whether strains are able to rejoin broken chromosomes. However, neutral sucrose sedimentation does not distinguish between complete and incomplete repair, or correct and incorrect repair. Presently, the experiments discussed are consistent with a hypothesis which states that the conversion of small DNA fragments to large DNA fragments as measured by sucrose sedimentation, constitutes complete repair of the breaks. This hypothesis needs further testing and one way of testing the hypothesis is assaying other rad mutants in the RAD50-57 pathway for double strand break repair using neutral sucrose sedimentation. In particular, temperature conditional mutants provide a system to further examine the relationship between survival after irradiation, and the ability of a cell to rejoin broken chromosomes.

A model for the repair of double strand breaks was first proposed by Resnick(1976) and the main features of this model are a requirement for a homologous or sister chromosome which can pair with the broken chromosome, and the ability of the cell to undergo recombination. The model

is illustrated in figure 3A. The model proposes that 5' → 3' nuclease digestion of single stranded DNA from the broken chromosome initiates repair. Next, a single strand endonuclease nicks the DNA strand on the unbroken sister chromosome near the position of the break. The next step in repair is the transfer of single stranded DNA from the unbroken to the broken chromosome to form a heteroduplex. This is followed by strand displacement, DNA synthesis, endonuclease cleavage of the Holliday structure which has been formed, and ligation which completes repair. One possible outcome of repair of a double strand break is the unbroken crossing over between the broken chromosome and the chromosome involved in repairing the break. The model explains the observation that double strand break repair does not occur in G-1 haploids because there is a lack of a sister chromosome, and that no repair occurs in rad52 strains because such strains are unable to undergo recombination.

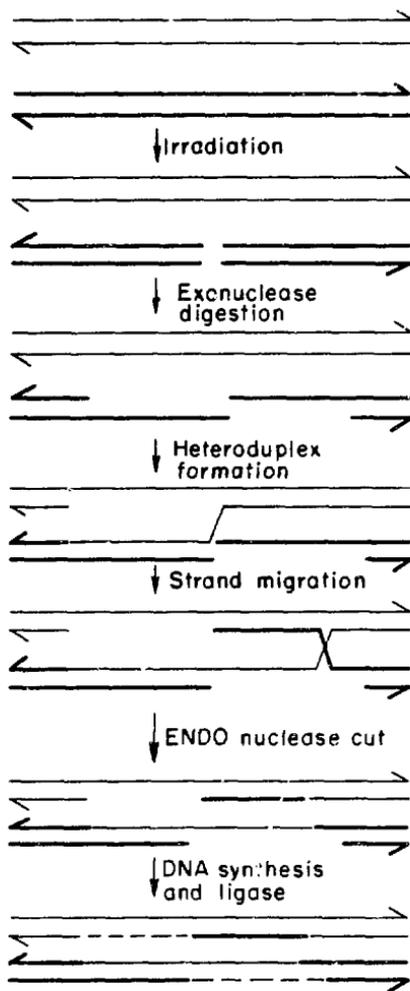
Another model of double strand break repair is the Szostak model (Orr-Weaver et al, 1981). The model is illustrated in figure 3B. The model differs from the Resnick model in the heteroduplex formation step. Thus, instead of strand invasion from the unbroken to the broken strand, there is strand invasion of the single stranded tail of the broken strand into the unbroken strand thus forming a heteroduplex. The single stranded end has a 3' primer and the next step is strand displacement by a pol I type

polymerase. The strand which is displaced then pairs with the other single stranded tail of the broken end. Repair is completed by polymerase and ligase. This model provides a mechanism whereby deletions can be repaired, which will be important in understanding the possible fate of breaks when they remain unrepaired for several hours after irradiation.

These models are consistent with the data presented to date. In particular, the role of DNA synthesis in repair can be assayed using a system developed in this work. However, the result from this thesis does not adequately determine the role of DNA synthesis in repair. Also, if any other rad mutants are discovered to be defective in double strand break repair, then one might examine if they are defective in induced gene conversion.

Although only the RAD50-57 pathway is being discussed in this thesis, one should be aware that there are other pathways involved in repair. *Saccharomyces cerevisiae* possesses 2 other pathways of repair besides the rad52 pathway. The RAD1,2,3,4,7,14,and22 pathway has been shown to be involved in excision repair (Resnick and Setlow,1972). Strains with mutation in this pathway are UV sensitive but display normal x-ray resistance. The RAD6,9,18,REV1,2,3 pathway is involved in repair which is believed to occur during replication of the DNA (Lawrence et al,1974). Strains defective in steps in the RAD6,9,18, REV1,2,3 pathway are sensitive to UV and x-rays. The RAD6 gene product

RESNICK MODEL FOR DOUBLE STRAND BREAK REPAIR



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FIGURE 3A

SZOSTAK MODEL FOR REPAIR OF DOUBLE STRAND BREAKS

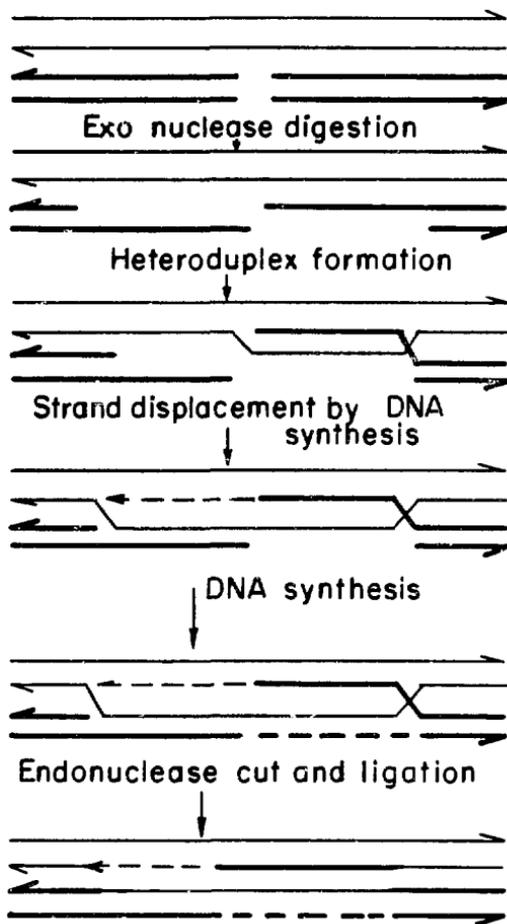


FIGURE 3B

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is required for wild type levels of mutagenesis. Strains with mutations in the RAD50-57 pathway are x-ray sensitive and exhibit normal sensitivity to uv. The phenotypic characteristics of one mutant from this group, rad52, include: an inability to undergo mitotic recombination (Prakash et al,1980), reduced sporulation (Game and Mortimer,1974), inviable spores, inability to undergo homothallic switching (Malone and Esposito,1980), and an elevated frequency of spontaneous and induced chromosome loss (Mortimer et al,1981).

One should be aware that some of the studies of the repair of double strand breaks have not involved DNA damaging agents. One such study is that of Weiffenbach and Haber (1981), who have also shown that breaks can be repaired in RAD+ cells or remain unrepaired in rad52-1 strains. Heterothallic yeast strains have stable mating types, MATa and MAT α , which switch from one to another at a frequency of 10^{-6} . Haploid homothallic strains which express the HO gene product are able to switch mating types at a frequency of almost once per cell generation. These mating type switches are believed to result on transpositions of silent copies of a or α mating type information from the right or left arm of chromosome III to the MAT mating type locus, which maps near the centromere of chromosome III. Thus, when MAT α HO strains switch to MATa, the α information at MAT is removed and replaced by bonafide MATa information which resides at the end of one of the arms of chromosome III. Weiffenbach

and Haber's evidence shows that the lethal event occurring in HQ rad52 cells is an unrepaired double strand break at the MAT locus on chromosome III. The double strand breaks, when not repaired, result in large deletions and chromosome loss. They have provided evidence that homothallic switching occurs through a double strand break, followed by rejoining of the chromosome in RAD+ cells.

Temperature sensitive mutants are a very useful tool in understanding metabolic processes. This type of mutant may provide a system to determine the fate of breaks induced by radiation. Studying repair in rad52-1 strains can only provide information about what occurs when repair is blocked indefinitely after irradiation. It does not provide information about what occurs when repair is turned off, turned on, then turned back off again. Several alleles of mutants in the RAD50-57 pathway have been shown to be temperature sensitive. One mutation, rad54-3 confers sensitivity to x-rays at 36°, and exhibits nearly normal resistance at 23°. Another mutation rad57-1, confers sensitivity at 23° and resistance at 36°. Survival curves of rad54-3 haploids, at the permissive temperature, 23°, retain the radioresistant tail associated with budding cells; however, at the restrictive temperature 36°, the survival curves are exponential (Ho and Mortimer, 1975).

Ho and Mortimer (1975) used the rad54-3 mutant to study the rate of repair of x-ray induced lesions and the time

rate fixation of the initial lesions into lethal lesions. Repair is studied by post-irradiation incubation of the cells at the permissive temperature, 23° , which allows repair, followed by shifting the cells to the restrictive temperature, 36° , which stops repair. Comparison of the survival of cells which have been incubated continuously at the restrictive temperature after irradiation gives the total amount of repair possible. Cell death or fixation is studied by incubating the cells at the restrictive temperature, 36° . After various times at 36° , the cells are shifted to the permissive temperature, 23° . Comparison of cell survival of cells shifted from the restrictive temperature to the permissive temperature with cells incubated continuously at the permissive temperature after irradiation shows the percentage of initial lesions which are fixed into lethal lesions. The experiments of Ho and Mortimer will be extended in the results section, since the rad54-3 mutation provides a very useful tool to understand the possible fates of double strand breaks after irradiation. This mutation will also be used as a tool to understand various factors which might change the probability whether or not a break will be repaired or left unrepaired. These factors include protein synthesis and DNA synthesis after irradiation.

The phenotypic expression of unrepaired double strand breaks is cell death and it usually occurs during the first or second mitosis after irradiation. Most frequently yeast

cells die as doublets that usually contain one nucleus (Mortimer, 1953). However, many cells which are damaged by radiation are capable of dividing once, twice, or even more times before death. In mammalian cells there is a sharp increase in eosin-stained cells (dead cells) following the first, and second mitosis after irradiation (Okada, 1970). The liquid holding experiments provide further evidence that attempt at nuclear division plays a major role in fixing initial lesions into lethal lesions. If yeast cells are irradiated, then held under non-growth conditions before plating, the survival is higher than if the cells are plated immediately after irradiation (Frankenberg-Schwager et al, 1980). The interpretation is that delayed plating after irradiation allows the cells a longer time to repair the damage before attempt an at nuclear division or another cell cycle event fixes the damage into lethal lesions. An attempt at nuclear division may cause potentially repairable broken ends to separate thus reducing the probability the DNA will be restored to its original form. Hence, the time between irradiation and attempted division may set a time frame during which repair can occur. This time may be the time remaining in the cell cycle from x-ray treatment to mitosis plus the time of the x-ray induced division delay. Burns (1956) observed with yeast that the mean time of the induced division delay increased with dose, and the variance of division delay also increased with dose. After 2.5 krads the mean value of the division delay in a diploid was 111

minutes with a variance of 55 min. After 10 krads the mean value of the division delay increased to 290 minutes and the variance increased to 113 min. Okada (1970) has located the point of the division delay in mammalian cells as being about 20 minutes past the end of the DNA synthesis phase in G-2, hence the name G-2 block. Brunborg and Williamson (1978) have hypothesized that the resistance of the diploid strain, NCYC-239, is due to repair occurring during the G-2 block. Thus, the G-2 block probably alters the fate of double strand breaks by allowing the cell time for their repair. However, there is extensive evidence that an attempt at nuclear division or some other cell cycle event is involved in a fixation event. In particular, a starting point for the analysis of cell death was to see if the fixation observed by Ho and Mortimer could be explained as a result of a cell cycle event, possibly DNA synthesis. DNA synthesis might be involved in fixation since Bender (1974) has proposed that some chromosomal aberrations are created through DNA synthesis.

There are also various modifying factors which can alter the possible fates of chromosomal breaks. One of these is protein synthesis as has been shown by Brunborg et al (1980). As previously discussed, the authors observed complete repair of double strand breaks 2 hours after irradiation in diploid cells synchronized in G-2 stage. Cycloheximide, at a concentration of 100 μ grams/ml, significantly inhibited repair, but did not completely block it.

Thus, in the presence of protein synthesis the breaks are more likely to be repaired than in the absence of protein synthesis.

Inducible repair was first observed by Weigle (1953) when he showed that UV irradiated phage λ showed an increased survival if plated on UV preirradiated hosts. This phenomenon is called Weigle reactivation and requires a wild type RECA and LEXA gene product (Defai et al, 1976). Using low speed neutral sucrose sedimentation Krasin and Hutchinson (1981) have shown that proteins required for the repair of double strand breaks in E. coli are synthesized in response to UV treatment.

Fabre and Roman (1977) have shown that cellular competence to undergo recombination is inducible by UV and x-rays. X or UV-irradiated MAT α ade6-21,45 haploids were mated with unirradiated MAT α ade6-21/ade6-45 heteroallelic diploids. The resulting zygote is triploid and has the genotype ade6-21/ade6-45/ade6-21,45*, where the * refers to the irradiated chromosome. One way of generating ADE+ prototrophs is a single event gene conversion between two unirradiated chromosomes; the ade6-21 and ade6-45 chromosome. Another way is through a two event process. The ade6-21,45* chromosome experiences two independent gene conversion events with both the ade6-21 and the ade6-45 chromosome. The probability of this two event process is very low. Thus, the most common way ADE+ prototrophs are generated is

through recombination between the two unirradiated chromosomes, the ade6-21 and ade6-45, of the triploid. They have further shown that the ADE+ prototrophs are generated by a one event process by using crosses which the double mutant ade chromosome is in a kar strain. The presence of the ade6-21,45* chromosome makes the cells competent to undergo recombination. Fabre and Roman found that x-ray doses of 5 krads induced competence to undergo recombination.

Because Brunborg et al (1980) have shown that double strand break repair has both a protein synthesis independent component, and a protein synthesis dependent component, further experiments were performed to investigate the nature of the protein synthesis dependent component of repair. The rad54-3 mutation was used as the tool in these investigation. The component of repair dependent on protein synthesis might be inducible repair. The hypothesis that repair involves recombination coupled with the observation of Fabre and Roman (1976) that recombination competence is inducible further suggests that repair might be inducible. The experiments developed in this thesis have examined the question whether the protein synthesis component of repair is inducible.

The central idea of this thesis is to understand the cellular pathway which double strand breaks follow after irradiation. To examine the repair part of the pathway the neutral sucrose sedimentation technique was used, and

rad54-3, rad51-1, and rad57-1 strains were assayed for the repair. However, the technique can only provide information about the repair pathway. The type of question one would like to answer is: what is the fate of double strand breaks in RAD+ cells? Because of the difficulty in answering this question, this thesis instead concentrated on studying the fate of breaks in rad54-3 cells. Hence, in order to find out what happens to breaks if they do not enter the repair pathway, post-irradiation temperature shifts of rad54-3 cells from the restrictive to the permissive temperature were used.

Questions concerning the fate of x-ray induced double strand breaks apply to both yeast and mammalian cells. However, these questions are much more difficult to tackle in mammalian cells. There is not a very good assay for double strand break repair in mammalian cells. Thus, the relationship between double strand break repair and survival in mammalian cells remains an unanswered question.

MATERIALS AND METHODSA) StrainsT-7 α tup7-1 adel-1 mal gal2-1X754-9D a rad54-3 tup7-1 adel-1 leu2-1X757-1D a rad57-1 tup7-1 tyr1-1 lys1-1X751-3A α rad51-1 adel-1 his7-1 lys1-1 ade4-499T77 a tup7-1 + his7-1 gal2-1
α tup7-1 adel-1 his7-1 gal2-1X754-6D3B a rad54-3 tup7-1 his1-1 trp2-1 + ade4-501 leu2-1 +
α rad54-3 tup7-1 his1-1 trp2-1 adel-1 ade4-501 + ura3-g713A a rad54-3 tup8-1 his1-1 + trp2-1 + + +
α rad54-3 tup8-1 his1-7 hom3-10 + ura3-1 ade2-1 ade8-18X1LC a rad57-1 leu2-112 his3-1 l + + +
α rad57-1 leu2-112 + his4-1 ura3-52 ura4-1XBC2 a rad51-1 lys1-1 lys2-1 trp5-1 his4-1 tyr1-1
α rad51-1 + + + + +

The symbols are described in Mortimer and Schild (1980).

The radiation sensitive strains were obtained from the Yeast Genetic Stock Center. The tup7-1 mutation was provided by Linda Bisson from Dr. Thorner's laboratory. The strain g713A was kindly provided by John Game. The tup8-1 mutation of g713A is an uncharacterized tup, and it may be allelic with any of the tup1 to tup7 mutations. Both ade4-499 and ade4-501 mutations are uncharacterized. The method used for genetic crosses is described in Mortimer and Hawthorne (1969).

B) Media

YEPD media is as follows: 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar. The minimal synthetic media is composed of trace elements, salts, a nitrogen source, biotin, and a carbon source. The synthetic complete and dropout media is minimal media plus appropriately added amino acids, purines, pyrimidines, and vitamins. The water agar plates are plastic dishes which contain a mixture of water and 2% agar. The plates used in the gene conversion experiment are deficient in histidine but contain the remaining essential amino acids.

C) X-ray source

The X-ray source was a Machlett OEG 60 tube with a beryllium window, operated at 50kVP and 20ma. Under these conditions, the dose rate was 190 rad/sec at shelf 10. A standard dose of 47.5 krads was used in all of the experiments involving sucrose gradients.

C) X-ray survival curves

Cells were grown up overnight in liquid synthetic complete media, and harvested in mid-log phase at about 10^7 cells/ml. Approximately 55-60% of the cells were budded. For the survival curves, cells were washed once with distilled water, diluted, and plated on YEPD media. Plates were exposed to radiation, incubated, and counted after a period of time

which allowed all possible colonies to grow up. For wild type cells this was 3 days and for rad54-3 cells at 36° this was 9-10 days. All the temperatures listed are in degrees Centigrade.

For the experiments which involved examination of inducible repair, rad54-3 cells were subcloned, grown up overnight at 23° collected onto 0.45 micron membrane filters, placed onto water agar petri dishes, and irradiated. Filters were then added to 50 ml flasks containing 10 ml of liquid YEPD media with or without cycloheximide [100 micrograms/ml]. During the split dose experiments cells were collected onto membrane filters, and again placed onto water agar dishes and re-irradiated. Total incubation time for all samples in liquid media was 5 hours at either 23° or 36°. After the incubation in liquid media, the plating at the restrictive temperature, 36°, always occurred on pre-warmed plates.

Cycloheximide is believed to inhibit the movement of the polysomes along the m-RNA (Cooper et al, 1967). At a concentration of 0.2 μ grams/ml there are about 1.4 molecules of the drug per ribosome, and at this concentration cycloheximide inhibits protein synthesis by 50%. At a concentration of 10 μ grams per ml cycloheximide inhibits protein synthesis by about 97%. Thus, the concentration of cycloheximide used in the experiments, 100 μ grams per ml, should block protein synthesis in both irradiated and

unirradiated cells. During the experiments which involved examination of the effects of hydroxyurea on repair and cell death, cells were incubated in liquid media with a hydroxyurea concentration of 0.075 M. At this concentration, hydroxyurea reversibly stops DNA synthesis in yeast, although protein synthesis remains at 90% the control level (Slater, 1973).

SEDIMENTATION EXPERIMENTS

The neutral sucrose gradient procedure used is similar to that of Petes and Fangman (1972), with minor modifications. The nuclear DNA was labeled for 3 or 4 generations with [³H]thymidine at 100 μ Ci/ml in liquid synthetic complete medium. The tup7-1 mutation incorporated in the strain permitted uptake of this labeled compound. Cells were harvested in mid-log phase. They were then treated in one of the following ways: (1) converted to spheroplasts, (2) placed on membrane filters, irradiated, and then converted to spheroplasts, or (3) placed on membrane filters, irradiated, and then incubated in YEPD media at the appropriate temperature for 6 hours by placing the membrane filter on top of a YEPD 1% agar dish followed by conversion to spheroplasts. The 0.45 μ pores on the filter allowed nutrients to reach the cells by diffusion. Spheroplasts were created by incubating the cells with 0.03 M mercaptoethanol, 0.2 M EDTA, 0.2 M Tris base (pH 9.5) for 15 min, then treating the cells with 10% glucylase in 0.9M sorbitol, 0.08 M sodium phosphate monobasic, and 0.016 M sodium citrate buffer for 30 min. 0.1 ml of a solution of spheroplasts were then layered on a pH 7, 15-30% sucrose gradient containing 0.01 M Tris, 0.015 M Na₂EDTA, 1 N NaCl and 1% SDS solution. The cells were lysed by passing them through a 0.1 ml 10% SDS layer. Gradients were then spun at 10,000 rpm for 26 hours, collected dropwise from the bottom of the tube onto numbered paper filters, washed twice with cold 5%

TCA, once with alcohol, dried, and then counted with a toluene-based scintillation fluid. The equivalent of about 200 counts/min of a marker T-4 C^{14} labeled phage was layered on the gradient. The number-average molecular weight was calculated using the relation

$$M_n = \frac{C_T}{\sum_{i=1}^n \frac{C_i}{M_i}}$$

where C_T = total activity, C_i = activity in the i^{th} fraction. M_i is computed using the relation (Freifelder, 1970)

$$\frac{M_i}{M_{T-4}} = \left(\frac{d_i}{d_{T-4}} \right)^{2.63}$$

where d_i = distance of the i^{th} fraction, and d_{T-4} = distance sedimented by T-4; $M_{T-4} = 1.2 \times 10^8$ daltons.

RESULTS

NEUTRAL SUCROSE GRADIENTS

As previously discussed, one of the key tools to understanding the fate of double strand breaks is the neutral sucrose sedimentation technique. This technique was used to further understand the steps involved in the repair of double strand breaks. The sedimentation technique was used to understand radiation sensitivities of rad54-3, rad51-1, and rad57-1 strains. The radiation sensitivity of rad54-3 strains can be explained by an inability to rejoin broken chromosomes. The radiation sensitivity of rad51-1 and rad57-1 strains do not appear to result from an inability to rejoin broken chromosomes.

Survival curves of haploid RAD+, rad54-3, rad57-1, and rad51-1 strains are presented in figure 4. The RAD+ haploid survival curve is biphasic, and this is a result of 2 subpopulations of differing radioresistance (Beam, et al 1954). The sensitive sub-population is composed mainly of G-1 cells, and the resistant sub-population is budding or S + G-2 phase cells which, in the survival curve in figure 4 represent about 60% of the population as obtained by extrapolation of the tail back to the vertical axis. The shapes of the rad54-3 haploid survival curve agree with those of Ho and Mortimer (1975). At the restrictive temperature, 36°, the survival curve is exponential and is missing the

radioresistant tail characteristic of haploid RAD+ strains. At 23° rad54-3 strains are more resistant to radiation than at 36°. The temperature sensitive rad mutants are more sensitive to radiation at both the permissive and restrictive temperature than the corresponding RAD+ strains. The survival curve of the rad51-1 haploid is a single hit exponential. The radiation sensitivity of the rad51-1 strain used for the gradient, J751-3A, is the same as that of g218/7A, the rad51-1 strain from the Yeast Genetics Stock Center. rad51-1 haploids are slightly more resistant to x-ray than rad54-3 haploids at 36°. The rad57-1 mutation is cold sensitive; 23° is the restrictive temperature, and 36° is the permissive temperature. At the restrictive temperature, the survival curve of rad57-1 strains is also a single hit exponential. rad57-1 strains at 23° are more resistant to radiation than either rad54-3 strains at 36° or rad51-1 strains. The survival curve of rad57-1 haploids at the permissive temperature has the same biphasic shape as a RAD+ haploid strain. The relationship which expresses the radioresistance of various rad mutants at their restrictive temperatures, where > means more resistant to x-rays, is rad57-1>rad51-1>rad54-3.

Diploid survival curves of RAD+, rad54-3, rad51-1, and rad57-1 strains are presented in figure 5. Diploid RAD+ cultures in log phase are a population with more uniform radioresistance than log phase haploid cultures and the survival curves of the RAD+ diploid exhibit a characteristic

shoulder. The survival curve of the rad54-3/rad54-3 strain at 36° is exponential with a D_{37} of 2.5 krad. However, at 23° the rad54-3/rad54-3 strain exhibits a shoulder on the survival curve and the D_{37} increases to 15 krad. The rad51-1/rad51-1 diploid exhibits a small shoulder on its survival curve. The D_{37} of the rad51-1 diploid the survival curve is about 5.5 krad which is about twice the D_{37} of the rad54-3/rad54-3 diploid at 36° . At the restrictive temperature there is a small shoulder on the rad57-1/rad57-1 diploid survival curve, and the D_{37} is about 6 krad. The relative radiosensitivities of rad54-3/rad54-3, rad51-1/rad51-1, and rad57-1/rad57-1 strains are the same in diploids as in haploids.

The results of neutral sucrose sedimentation experiments of a RAD+/RAD+ diploid, a RAD+ haploid, a rad54-3/rad54-3 diploid, a rad51-1 haploid, and a rad57-1 haploid are presented in Figures 5 to 9. Calculations from the data were performed for comparison with previously published data, to quantitate the extent of repair, and to arrive at a value for the efficiency of DNA strand breakage by radiation. Computation from 12 gradients gave an average M_n (control) of about 4.3×10^8 daltons. The value of M_n varied from gradient to gradient. The variation in M_n results from the fact that this value is determined not only by the position of the peak but also the distribution of DNA about the peak. The value of 4.3×10^8 compares with the value of 6.2×10^8 obtained by Petes and Fangman (1972), and the value

of 3×10^8 obtained by Resnick and Martin (1976). Petes et al (1973) extracted the DNA from the sucrose gradient and obtained values of DNA nuclear contour lengths varying from $50 \mu\text{m}$ (1.2×10^8 daltons) to $305 \mu\text{m}$ (7.2×10^8 daltons), with an average value of $165 \mu\text{m}$ (3.2×10^8 daltons). A calculation of the data from this thesis shows that 47.5 krad produces an average of 1 break/chromosome. A calculation of break production yields a value of 4.9×10^{-11} breaks/krad x dalton. This value compares with the value of 5.8×10^{-11} obtained by Resnick and Martin. The data of Resnick and Martin (1976) and Frankenberg-Schwager et al (1980) shows a linear increase of double strand breaks with dose. This fact suggests that double strand breaks are produced by a single event associated with the absorption of one photon.

If one assumes that yeast possess 17 chromosomes (Mortimer and Schild, 1980), then the dose of 47.5 krad yields an average of about 17 breaks in a haploid cell and 34 breaks in a diploid cell. The dose required to induce one double strand break in a haploid cell is about 2.8 krad and in a diploid cell is about 1.4 krad. Resnick and Martin (1976) calculate from their data that a dose of 1.1 krad will produce an average of one double strand break per diploid genome.

Thus, the sucrose sedimentation technique provides information about the efficiency of the initial breakage event. Until the technique was developed, the efficiency of

the initial breakage event was purely speculative. In order to understand the fate of breaks, and the relationship between unrepaired breaks and survival, an understanding of the initial event is necessary.

The neutral sucrose sedimentation data presented in Figure 6 illustrates that in diploid cells with a RAD+ genotype induced double strand breaks have a high probability of being repaired. A dose of 47.5 krads reduced the M_n (control) from 2.8×10^8 daltons to 1.7×10^8 daltons. After a 6 hour incubation in YEPD media the M_n (x-ray) increased from 1.7×10^8 daltons to 2.6×10^8 daltons. This increase represents apparently complete repair of double strand breaks. The survival of the strain at 47.5 krads is about 43%. This result agrees with that of Resnick and Martin (1976) and Brunborg et al (1980) who have already shown that in cells with a RAD+ genotype, breaks have a high probability of being repaired.

The results presented in figure 7 show that haploid cells with the RAD+ genotype are able to repair double strand breaks when the cells are harvested from log phase. The M_n calculated from the DNA profile of control cells is 3.4×10^8 daltons. After an exposure of 47.5 krads, the M_n is lowered to 1.3×10^8 daltons. Following a 6 hour incubation in YEPD media, the DNA sedimented at essentially the position of unirradiated DNA since the M_n is 3.4×10^8 daltons. The interpretation of the experiment is that apparently

complete repair of double strand breaks is also observed in haploid cells with a RAD+ genotype. The survival of the strain after 47.5 krads is high, 23%. This result agrees with that of Ho (1974) who has shown that in haploid cells with a RAD+ genotype breaks have a high probability of being repaired when the cells are harvested from log phase.

The possible fate of breaks induced in a rad54-3 diploid depends upon the temperature after irradiation. At 23° these breaks have a high probability of being repaired but at 36°, these breaks remain unrepaired as is illustrated in figure 8. Unirradiated DNA sedimented with an M_n of 4.9×10^8 daltons. A dose of 47.5 krads reduced the M_n to 2.9×10^8 daltons. A 6 hour post-irradiation incubation of rad54-3 cells at 23° resulted in a shift of the chromosomal DNA to a higher molecular weight, corresponding to an M_n of 3.9×10^8 daltons. Under these conditions the survival is 2.4%. At 23° the rad54-3 strain is capable of repairing double strand breaks. A post-irradiation incubation at 36° for 6 hours resulted in a DNA profile unchanged from the DNA profile seen immediately after the x-ray exposure. The survival of the strain at 36° after a dose of 47.5 krads is $10^{-6}\%$, as estimated by extrapolation. The lack of post-irradiation molecular weight changes in the DNA at 36° is analogous to similar molecular weight changes seen by Resnick and Martin (1976) in rad52-1/rad52-1 diploid strains. At 36° rad54-3 strains are unable to repair double strand breaks, however, at 23° such strains are able to

repair double strand breaks. The ability of rad54-3 cells to repair double strand breaks at 23° correlates with high survival and the inability of the cells to repair double strand breaks at 36° correlates with low survival.

It is to be noted that the number of breaks introduced in the DNA during the experiment of figure 8 is fewer than the number introduced in the DNA during the experiments of figures 6,7,9,10. Possible explanations are the dose might not have been 47.5 krads that day, or the strain might have a type of radiation protection effect from the initial lesion.

An example of a radiation sensitive strain which is able to convert low molecular weight DNA fragments into high molecular weight DNA fragments is a haploid rad51-1 strain. The sedimentation data of the rad51-1 strain is illustrated in figure 9. The control peak had a M_n of 4.9×10^8 daltons, and a 47.5 krad dose reduced the peak to one corresponding to an M_n of 1.9×10^8 daltons. However, a 6 hour incubation in YEPD raised the M_n to 2.7×10^8 daltons. Although a 47.5 krad exposure resulted in about 1.5 breaks/chromosome, a 6 hour post-irradiation incubation in YEPD media reduced the number of breaks per chromosome to 0.8. Thus, the assay shows that the rad51-1 strain is able to convert low molecular weight DNA fragments into high molecular weight DNA fragments. The interpretation of the experiment is the rad51-1 strain is able to rejoin broken chromosomes.

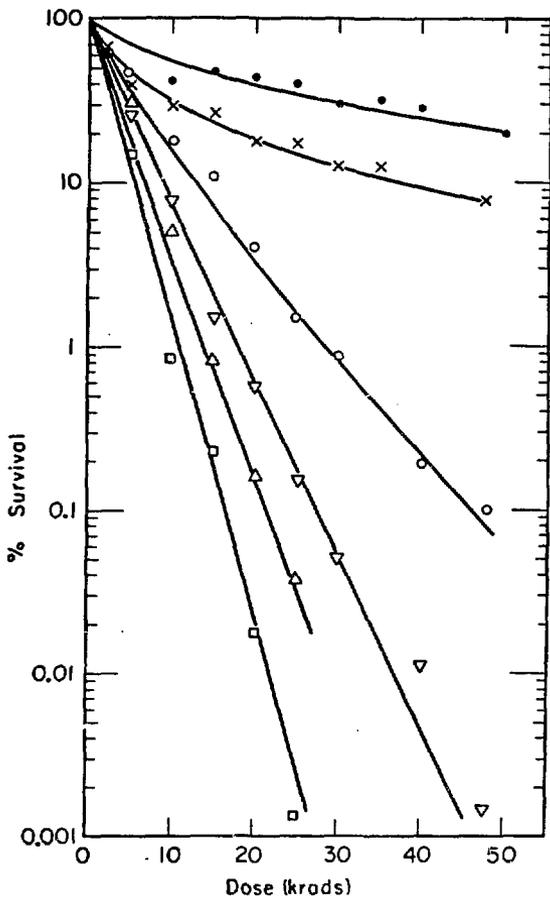
However, after 47.5 krads, the survival of the strain is very low, 2×10^{-5} as estimated by extrapolation. Thus, the sedimentation assay is probably measuring incomplete repair, but it may also be measuring inaccurate repair. Incomplete repair could result in intermediates in recombination, and unless these intermediates are resolved, cell death would eventually occur. Possible examples of incomplete repair are unresolved Holliday structures. A result of inaccurate repair might be dicentrics and translocations. Mowat and Hastings (1979) found no rejoining of broken chromosomes during a 3.5 hour post-irradiation incubation of rad51-1 strains. The difference between the result presented here and the result of Mowat and Hastings can be explained if one assumes that partial rejoining may have occurred between the 3.5 and 6 hour post-irradiation time period.

Another example of a strain which is able to rejoin small DNA fragments into large DNA fragments but exhibits low survival after irradiation is a rad57-1 haploid. The data for rad57-1 strains are shown in figure 10. Unirradiated DNA sedimented at a position corresponding to an M_n of 4.6×10^8 daltons. A 47.5 krad dose reduced the molecular weight of the DNA profile to 1.9×10^8 daltons. After a 6 hour incubation at the permissive temperature, 36° , the M_n increased to 3.0×10^8 daltons. This increase in the DNA molecular weight represents extensive repair of double strand breaks. At 36° , survival after 47.5 krads is 6%. At the restrictive temperature, 23° , the molecular weight of the

DNA also increased during a post-irradiation incubation. The M_n of the DNA profile of cells incubated at 23° after x-ray treatment is 2.9×10^8 daltons, and the survival is low, 0.002%. The extent of apparent double strand break repair which occurred at the restrictive temperature is similar to the amount occurring at the permissive temperature. The cold sensitive phenotype of rad57-1 strains is not a result of an inability to convert small DNA fragments into large DNA fragments. Again the sedimentation assay is not distinguishing large DNA fragments which allow cell survival from large DNA fragments which cause cell death. The large DNA fragments which cause cell lethality in rad57-1 cells at 23° might be intermediates in repair, such as unresolved Holliday structures. Alternatively, the large DNA fragments from rad57-1 strains incubated at 23° might be some end product of misrepair, such as a dicentric chromosome.

The strains which are most sensitive to radiation, i.e., rad52 and rad54 strains, are unable to convert small DNA fragments into large DNA fragments, and thus these strains are blocked in an early step in repair. rad57-1 and rad51-1 strains which are not as sensitive to radiation, appear at least partially able to rejoin small DNA fragments into large DNA fragments. Thus, these strains are blocked in a later step in repair. The survival of strains which are blocked in an early step of repair (rad54-3 strains at 36°) is less than those blocked in a later step (rad51-1 and rad57-1 strains at 23°). The results presented in this

section show that the neutral sucrose technique can detect whether or not cells are blocked in an early step in repair but cannot detect whether or not cells are blocked in a later step in repair.



XBL 8110-4263

FIGURE 4. Haploid survival curves
 • rad+ (30°); × rad57-1 (36°); ○ rad54-2 (23°);
 ▼ rad57-1 (23°); ▲ rad51-1 (30°); □ rad54-3 (35°)

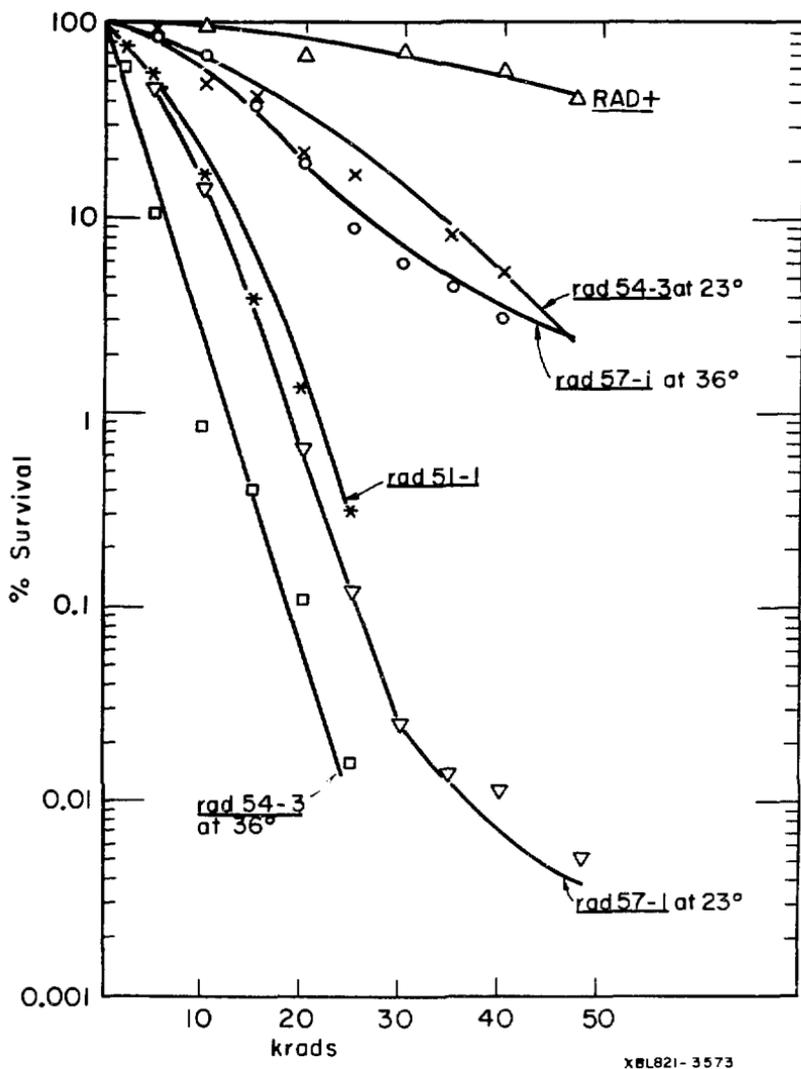


FIGURE 5. Diploid survival curve
 △ RAD+ (30°); × rad54-3 (23°); ○ rad57-1 (36°); * rad51-1 (30°);
 ▽ rad57-1 (23°); □ rad54-3 (36°);

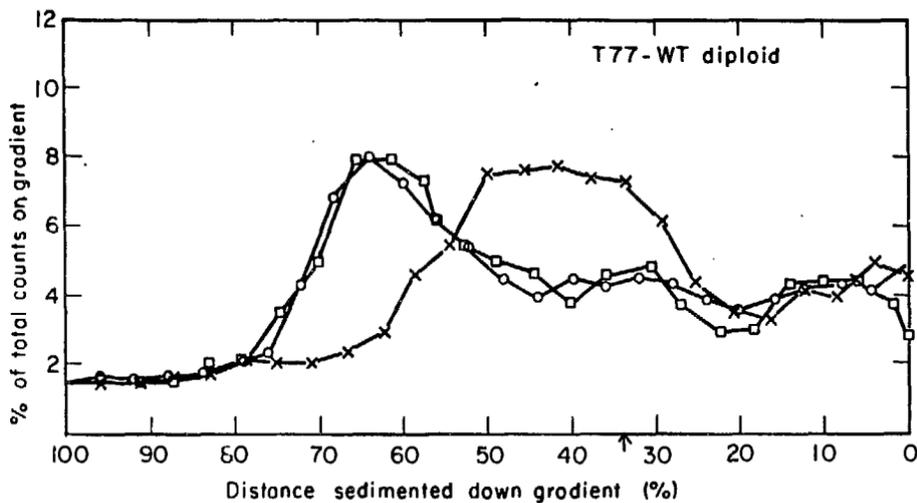


FIGURE 6. Neutral sucrose gradient of a diploid strain with a RAD+ genotype illustrating complete repair of double strand breaks

- , control
- ×, cells irradiated with 47.5 krad
- , cells irradiated with 47.5 krad and incubated for 6 hours in YEFD medium

Arrow points to the position of the T4 marker

XBL8110-4282

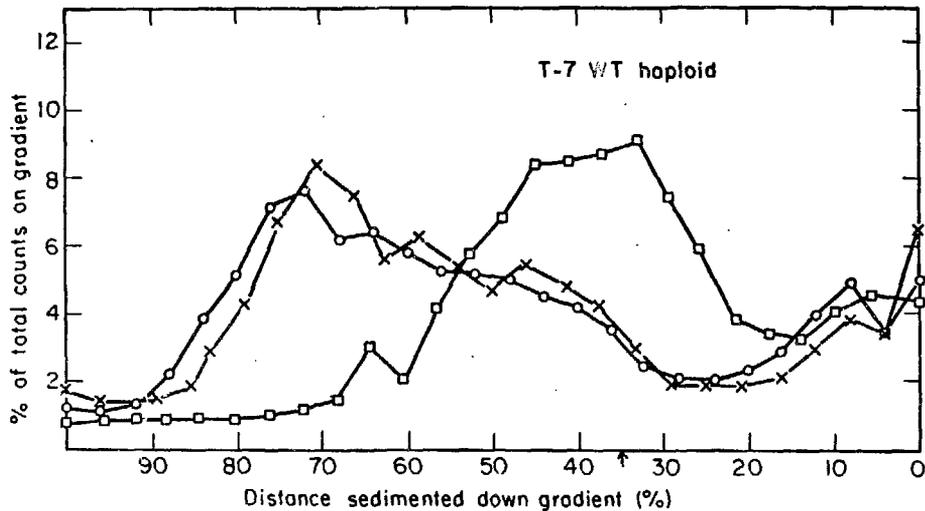


FIGURE 7. Neutral sucrose gradient of a haploid strain with a RAD+ genotype illustrating complete repair of double strand breaks

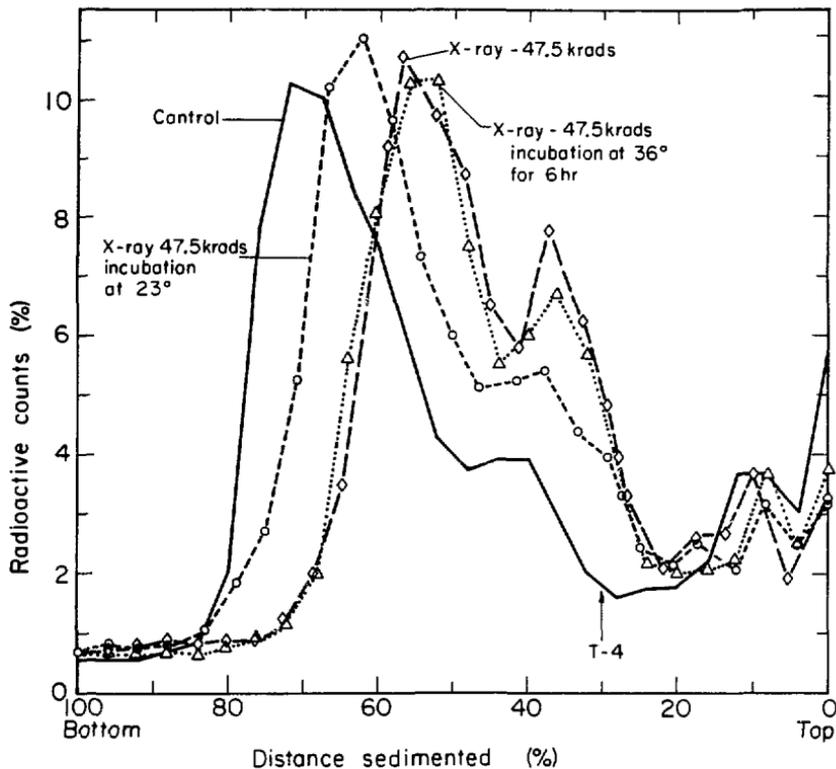
NBL 8110-4279

○, control

□, cells irradiated with 47.5 krad

×, cells irradiated with 47.5 krad and incubated for 6 hours in YEPD medium

Arrow points to position of T4 marker



XBL 812-3670

FIGURE 8. Neutral sucrose gradient of a diploid *rad54-3/rad54-3* strain illustrating temperature conditional repair of double strand breaks.

- , control
- ◊ , cells irradiated with 47.5 krad
- , cells irradiated with 47.5 krad and incubated in YEPD medium for 6 hours at 23° before assay.
- ▲ , cells irradiated with 47.5 krad and incubated in YEPD medium for 6 hours at 36° before assay.

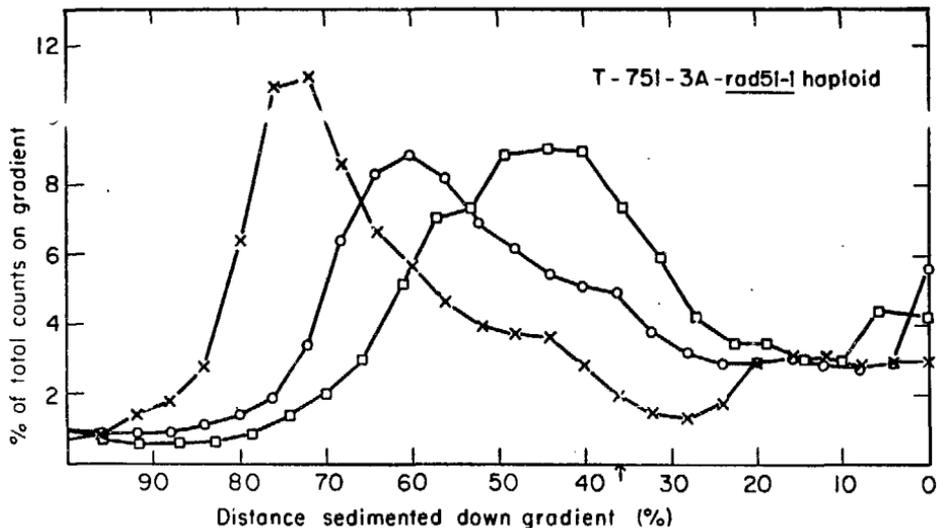


FIGURE 9. Neutral sucrose gradient of rad51-1 haploid strain illustrating incomplete repair of double strand breaks

- x, control
- , cells irradiated with 47.5 krad
- , cells irradiated with 47.5 krad and incubated for 5 hours in YEPD medium

Arrow point to position of T4 marker

XBL 8110-4281

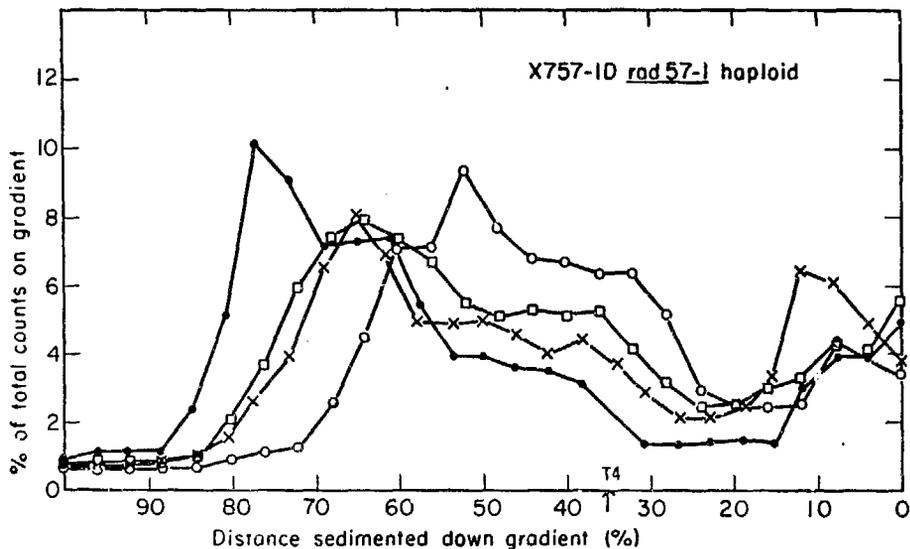


FIGURE 10. Neutral sucrose gradient of a cold sensitive haploid rad57-1 strain illustrating temperature independent rejoining of broken chromosomes. XBL 8110-4280

- , control
- , cells irradiated with 47.5 krad
- , cells irradiated with 47.5 krad and incubated in YEPD medium for 6 hours at 35° before assay.
- x , cells irradiated with 47.5 krad and incubated in YEPD medium for 6 hours at 23° before assay.

GENE CONVERSION

Since the results of the last section showed that the rad54-3 gene product acts at an early step in repair of breaks, this section investigated the possibility that this early step may be correlated with recombination. An experiment was done examining mitotic gene conversion at 23° and 36° in an a/q rad54-3/rad54-3 his1-1/his1-7 strain, g713A. The assay for recombination is reversion at the his1 locus. The cells were subcloned at 30°, grown up in liquid media at 30°, harvested on membrane filters while in log phase, irradiated while on the filter, resuspended in water, then plated onto histidine deficient medium. The dose used to assay for induced conversion is 5 krads. After higher dose of 10 krads, data on gene conversion at 36° were not obtainable as a result of a combination of low survival (0.15%) and low conversion frequency. The table below lists the data

<u>Dose</u>	<u>temp (°C)</u>	<u>% survival</u>	<u>conversion freq</u>
0 krads	23	100	2.7×10^{-4}
0 krads	36	64	8×10^{-6}
5 krads	23	89	3.2×10^{-3}
5 krads	36	2	5×10^{-6}

The conversion frequency is listed as revertants per survivor. There is about a 30 fold difference in the spontaneous gene conversion frequency when the cells are plated at 23° compared to plating at 36°. The difference in the

conversion frequency must have originated from events which occurred after plating the cells on selective medium because all the samples came from the same population. The possibility that the 30 fold difference in spontaneous conversion frequency results from a lethal effect of plating the cells at 36° is ruled out because the cells at 36° plated with an efficiency of 64% compared with those at 23° . Usually, the plating efficiency of rad54-3 cells is the same at 23° and 36° . Another possibility is that upon plating the cells at 23° , there might be a transient induction of meiotic enzymes due to histidine starvation, that might mimic nitrogen starvation used to induce sporulation. Upon plating the cells on histidine deficient plates at 36° , there still might be a transient induction of recombination enzymes, but recombination would be blocked due to the presence of a non-functional rad54-3 gene product. At 23° , the conversion frequency increased by about a factor of 10 after 5 krad. At 36° , there was no detectable increase in the conversion frequency after a dose of 5 krad. At the temperature which rad54-3 strains are unable to repair double strand breaks, 36° , there is no induced gene conversion. At the temperature the rad54-3 strains are able to repair double strand breaks, 23° , there is induced gene conversion. The result is consistent with a hypothesis that the early steps in the repair of breaks involve recombination.

THE EFFECTS OF CYCLOHEXIMIDE ON REPAIR

The presence of protein synthesis after irradiation increases the probability that a break will enter the repair pathway rather than remain unrepaired. Resnick and Martin (1976) and Brunborg et al (1980) have shown that cycloheximide (CYH) inhibited repair but did not completely block it. The results presented here will provide evidence that the effect of CYH on repair is indirect, it prevents the synthesis of repair enzymes induced by radiation. The results presented in the previous section showed that double strand break repair is a necessary condition for high survival. One can then ask whether the inhibiting effect of CYH on repair correlates with lower survival.

The rad54-3 strains provide a system with which one can use survival to analyze the effects of various drugs such as CYH on repair. Repair can be studied by irradiating the cells, incubating them at the permissive temperature, 23°, for a time, (t), which allows repair of the damage, then stopping repair by shifting the cells to the restrictive temperature, 36°. Comparison of survival with cells which have been continuously incubated at the restrictive temperature shows the amount of repair. Successively longer post-irradiation incubations at 23° before shifting to 36° results in successively greater survival. However, as shown by Ho and Mortimer (1975) the rise in survival maximizes after a 15 hour incubation at 23°, when the cells are

irradiated with 25 krads. It appears that all the lesions that can be repaired are repaired within 15 hours after irradiation.

Since extensive repair occurred during the first 5 hours at 23° after irradiation, a post-irradiation time of 5 hours at 23° was chosen to study the effects of CYH on repair. A dose of 25 krads was chosen because this dose produces more lesions than can be repaired in 5 hours at 23° . The evidence for this is that cells treated according to the protocol of irradiation with 25 krads, incubation at 23° for 5 hours, followed by plating at 36° , always had a survival much lower than cells treated according to the protocol of irradiating with 25 krads, incubation at 23° for 5 hours, followed by plating at 23° . In the experiment of figure 11 cells treated according to the first protocol (plating at 36°) had a survival of about 0.41%, while cells treated according to the second protocol (plating at 23°) had a survival of about 11%. Thus, although extensive recovery has occurred during a 5 hour incubation after 25 krads, complete recovery requires a longer time than 5 hours. This conclusion is also supported by the data in figure 20 in the section on fixation. When cells are treated according to the protocol of irradiation with 25 krads, incubation at 23° , followed by plating at 36° it takes about 12 hours before most of the breaks (90%) are repaired.

The logic behind the various protocols will now be presented. The survival of cells treated according to the protocol of x-ray treatment, incubation at 23° after radiation for 5 hours, followed by plating at 36°, presumably represents repair due to enzymes both present before irradiation and synthesized after irradiation. The survival of cells irradiated, then incubated with CYH at 23° for 5 hours, and plated at 36° in the absence of CYH represents repair due to enzymes present before irradiation but not synthesized after irradiation. The presence of CYH (100 micrograms per ml) after irradiation is expected to prevent the synthesis of new proteins after radiation, however this was not tested directly. Survival of cells irradiated, incubated for 5 hours in liquid medium at 36°, and plated at 36° represents no repair.

Figure 11 illustrates the effect of CYH on recovery in a rad54-3/rad54-3 diploid strain, X754-6D3B. Cells exposed to 25 krads, incubated at 23° for 5 hours, and plated at 36° had a survival 100 fold greater than cells exposed to 25 krads, then incubated continuously at 36° after irradiation. Cells exposed to 25 krads, then incubated at 23° for 5 hours with CYH and plated at 36°, exhibited a survival about 10 fold higher than cells incubated continuously at 36° after irradiation. These results are consistent with constitutive synthesis of the rad54-3 gene product. However, after the dose of 25 krads recovery occurring in the presence of CYH is significantly less than recovery occurring in the absence

of CYH. Thus, the inhibiting effect of CYH on repair observed by Resnick and Martin (1976) and Brunborg et al (1980) correlates with lower survival when the dose of 25 krads was used.

There are several possible explanations for the result that CYH depresses recovery. As figure 11 illustrates, a 5 hour incubation after 25 krads in the presence of CYH at the permissive temperature results in a 10 fold drop in survival compared with an incubation in the absence of CYH. If the effect of CYH on survival were exclusively a cytotoxic effect then unirradiated cells incubated with CYH for 5 hours should plate at 10% the value of the unirradiated cells incubated in the absence of CYH. However, unirradiated cells incubated with CYH for 5 hours have a survival value that is 75% (average of 20 experiment) that of controls. The standard deviation is 12%. The effect of CYH on repair involves effects other than cytotoxicity.

A steady state hypothesis is one possible explanation that CYH reduces recovery after irradiation. The steady state hypothesis states that the level of repair enzymes is the same before and after irradiation. There is a continuous degradation and replacement of repair enzymes which is not changed by a dose of radiation. When cells are incubated with CYH after irradiation, they are unable to replace the degraded repair enzymes. Since the cell contains fewer repair enzymes, fewer breaks should be repaired, and

survival should be lower.

Another possible explanation for the observation that post-irradiation incubation of rad54-3 cells with CYH lowers survival involves inducible repair. This hypothesis proposes that the level of repair enzymes increases after irradiation.

The following equations will be used to test the steady state model for its explanation of the effect of CYH on repair.

I) Assume 1 unrepaired break is a lethal event. If S = survival and B = average number of breaks/cell, then under the assumption that breaks are distributed in a Poisson fashion

$$S = S_0 e^{-B} \quad (1)$$

If S_1 and S_2 represent two different survivals and

$$\frac{S_1}{S_2} = e^{-(B_1 - B_2)} \quad (2)$$

then this equation can be re-written as

$$(B_1 - B_2) = -\ln\left(\frac{S_1}{S_2}\right) \quad (3)$$

II) Assume the number of breaks repaired per unit time is proportional to the level of repair enzymes, $[E]$, or the

cellular activity of the rad54-3 gene product:

$$-\frac{dB}{dt} = k[E] \quad (4)$$

At 36° , $\frac{dB}{dt} = 0$. Evidence for the relation $\frac{dB}{dt} = 0$ at 36° is illustrated in the sucrose gradient of figure 8.

III) Assume that in the absence of protein synthesis the level of repair enzymes per cell, $[E]$, decreases exponentially with time with a rate constant σ (Morowitz, 1978). Then,

$$[E] = [E_0]e^{-\sigma t} \quad (6)$$

$\frac{1}{\sigma}$ is the time it takes for the level of repair enzymes to be degraded to a value of $.37[E_0]$.

IV) Assume that CYH stops protein synthesis completely in irradiated cells.

V) Assume that radiation doses of about 25 krads do not cause any significant changes in total protein synthesis.

VI) Assume that the number of breaks repaired per cell during the 5 hour incubation of rad54-3 cells at 23° is

$$B_{\text{repair}} = \int_0^5 \frac{dB}{dt} dt = \int_0^5 k[E(t)] dt \quad (7)$$

This equation is believed valid because at the dose used, 25 krads, more breaks are induced than can be repaired in the 5 hour repair period.

The steady state hypothesis proposes that the level of repair enzymes remains unchanged by the dose of irradiation. Thus, $[E(t)]$ is constant during a 5 hour post-irradiation incubation at 23° in the absence of CYH. This hypothesis can be represented by the following equation:

$$[E(t)] = [E_0]$$

Figure 12 illustrates a geometrical model of the steady state hypothesis. The ordinate is labelled "Breaks repaired per unit time", and the abscissa is labelled "time". The area between each curve and the abscissa represents the number of breaks repaired per cell in 5 hours. The steady state hypothesis predicts that the number of breaks repaired when cells are treated according to the protocol of irradiation with 25 krad, incubation for 5 hours at 23° , followed by plating at 36° is given by the following relation

$$B = \int_0^5 k[E(t)]dt = \int_0^5 k[E_0]dt = k[E_0]5 \quad (8)$$

This relation is believed valid because the dose of 25 krad saturates the repair capacity of the cell. The number of breaks repaired when cells are treated with the protocol of irradiation with 25 krad, incubation with CYH for 5 hours at 23° followed by plating at 36° , can be predicted using the relation (4)

$$B(\text{CYH}) = \int_0^5 k[E(t)] dt = \int_0^5 k[E_0] e^{-\sigma t} dt = \frac{k[E_0]}{\sigma} (1 - e^{-5\sigma}) \quad (9)$$

This number is illustrated as the area under the solid lines of figure 12B. The value of σ can be calculated by experimentally comparing the number of breaks repaired in the presence of CYH with the number repaired in the absence of CYH.

Figure 13 represents the protocols of the next 4 experiments used to test the steady state model. All these experiments are based on the principle of allowing the cells a short time after irradiation to synthesize repair enzymes. Figure 13A illustrates the protocol in which cells are exposed to 25 krads, incubated at 36° for 5 hours, and plated at 36°. This protocol allows cells no time to repair breaks. The protocol of figure 13B represents cells exposed to 25 krads, incubated at 23° for 5 hours, and plated at 36°. The number of breaks repaired with cells treated according to this protocol is illustrated as the area under the solid lines of figure 12A. Figure 13C shows the protocol of cells irradiated with 25 krads, incubated at 23° for 5 hours with CYH, and plated at 36°. The number of breaks repaired with cells treated with this protocol is illustrated as the area under the solid lines of figure 12B. Figure 13D illustrates the protocol of cells irradiated with 25 krads, incubated at 23° for time (t) without CYH, then time (5-t) at 23° with CYH, followed by plating at 36°.

There is a short period of time after irradiation during which protein synthesis is allowed to occur. The time used are 7.5', 15', 20', 30', and 45'. Figure 13E shows the protocol of a split dose experiment. Cells are exposed to 10 krads, then incubated at 23° without CYH for a time (t) to allow protein synthesis to occur after irradiation. Then a dose of 15 krad is administered and CYH is added. The cells are incubated with CYH for (5-t) hours at 23°, and then plated at 36°. Figure 13F and 13G represent protocols of experiments where the doses were split into 2.5 + 22.5 krad and 1.5 + 23.5 krad fractions. These experiments were done to verify the conclusions of the previous experiments and to test whether repair is inducible at lower doses than 25 or 10 krads. The procedure of these split dose experiments is the same as the 10-15 krad split dose experiment. In all samples the total repair time in all samples is 5 hours. The total dose given to all the cells sums up to 25 krads.

The steady state hypothesis predicts the number of breaks repaired per cell when the cells are treated according to the protocol shown in figure 13D. The protocol is irradiating cells with 25 krads, incubating without CYH for time (t), incubating with CYH at 23° for time (5-t), and plating at 36°. For analysis the time of 30' will be chosen. After a 25 krad dose, [E] remains constant for 30', then CYH is added. Upon addition of CYH, [E] declines exponentially for the next 4.5 hours at 23°. The numerical

value of breaks repaired per cell, when cells are treated according to the protocol of figure 13D with $t=0.5$ hour, is predicted by steady state hypothesis to be

$$B = \int_0^{0.5} k[E_0]dt + \int_0^{4.5} k[E_0]e^{-\sigma t}dt = k[E_0] \left(.5 + \frac{1 - e^{-4.5\sigma}}{\sigma} \right) \quad (10)$$

This number is illustrated as the area under the solid lines of figure 12C. The value of $k[E_0]$ is computed using relation 8, and σ is computed by comparing the data which represents relation 8 with the data which represents relation 9. If cells are treated according to the protocol of figure 13D with $t=45$, then the number of breaks repaired per cell is given numerically by the relation

$$B = \int_0^{0.75} k[E_0]dt + \int_0^{4.25} k[E_0]e^{-\sigma t}dt = k[E_0] \left(.75 + \frac{1 - e^{-4.25\sigma}}{\sigma} \right) \quad (11)$$

This value is numerically illustrated as the area under the solid lines of figure 12D

The steady state hypothesis can also be used to predict the number of breaks repaired when the cells are treated according to the split dose protocols of figure 13E, 13F, and 13G. During the 10-15 krad split dose experiment the cells are allowed 5 hours to repair damage from the 10 krad dose, and $(5-t)$ hours to repair the damage from the 15 krad dose. Hence, if (t) is small compared to 5 hours, then the survival of cells treated according to the 10-15 krad split

dose protocol should be similar to cells exposed to a single dose of 25 krads. Thus, cells treated according to the protocol of figure 13E will be analyzed using relation 10. The predicted number of breaks repaired per cell is the area between the solid lines of figure 13C and the abscissa. The 2.5 + 22.5 krad and the 1.5 + 23.5 krad split dose experiments will be analyzed in a manner similar to the 10 + 15 krad split dose experiment.

Figure 14 represents data following the protocols of figure 13A, B, C, and D. The protocol is to irradiate the cells with 25 krads, incubate without CYH at 23° for times $t=7.5'$, 15', 20', 30', and 45', then incubate the cells at 23° with CYH at 23° for time (5-t), and plate at 36°. The data of figure 14 shows that adding CYH 7.5' to 15' after irradiation resulted in a significant increase in survival compared with adding CYH at the time of irradiation. The two points at 20' and 30' represent the values of survival determined with two different dilutions. Delaying the addition of CYH for 30' after irradiation resulted in a 4 fold increase in survival compared with the survival of cells in which CYH is added at the time of irradiation. When CYH is added 45' after irradiation survival is about the same as for cells irradiated, and incubated at 23° for 5 hours in the absence of CYH.

Table 1A shows an analysis of the data from this experiment. The first row states the actual value of survival,

the second row states the experimental number of breaks repaired per cell, the third row states the predicted number of breaks repaired per cell, and the last row states the predicted survival from the steady state model. Since the figures from the first 3 columns are used to compute the predictions of the steady state model, these predicted values of survival and breaks repaired apply only to the last column. The first column labels the protocol where cells were incubated for 5 hours at 36°, the second column 5 hours at 23°, the third column 5 hours with CYH at 23°, and the fourth column CYH is added 30' after irradiation. The columns 13A, B, C, D, E, and F label the value of survivals or breaks repaired when cells are treated according to the protocols of figure 13. The number of breaks repaired is computed using the relation (3),

$$B_1 - B_2 = -\ln\left(\frac{S_1}{S_2}\right) \quad (3)$$

The calculations for this and the other 2 experiments are performed using the relations 7, 8, 9, 10, and 11, and are shown in the appendix. When cells are treated according to the protocol of figure 13D, with $t=30'$, they repair about 2.1 breaks, and this value differs significantly from 1.0 breaks, which is the value predicted by the steady state model using relation 10. After treatment of cells according to figure 13D with $t=45'$, the number of breaks repaired is about 2.9 and this value differs significantly from the

TABLE 1

SUMMARY OF ANALYSIS OF EXPERIMENTS FROM FIGURE 15, and 16
13 A,B,C,D,E, and F refer to the protocols of Figure 13

SOURCE OF DATA	(5 hrs. at 36°)	(5 hrs. at 23°)	(5 hrs. at 23°)	(add CYH 30' after x-ray 13D)
<u>TABLE 1A</u> <u>FIGURE 14</u>	13A	13B	13C	
Survival %	.0152 ± .0011	.267 ± .044	.032 ± .002	.129 ± .012
Breaks Repaired per cell	0	2.9	.7	2.1
Predicted Survival %	-	-	-	.041
Predicted Nu. Breaks Repaired per cell	-	-	-	1.0
<u>TABLE 1B</u> <u>FIGURE 15</u>				13E
Survival %	.0042 ± .0009	.135 ± .02	.011 ± .002	.079 ± .006
Breaks Repaired per cell	0	3.3	1.0	2.9
Predicted Survival %	-	-	-	.015
Predicted Nu. Breaks Repaired per cell	-	-	-	1.3
<u>TABLE 1C</u> <u>FIGURE 16</u>				13F
Survival %	.0042 ± .0007	.151 ± .020	.026 ± .0044	.106 ± .010
Breaks Repaired per cell	0	3.6	1.7	3.2
Predicted Survival %	-	-	-	.034
Predicted Nu. Breaks Repaired per cell	-	-	-	2.1

predicted value of 1.2 breaks using relation 11. Thus, the results of this experiment are not compatible with the predictions of the steady state model.

Split dose experiments were done to further test the steady state hypothesis. Figure 15 shows the data of a 10 + 15 krad split dose experiment following the protocol of figure 13E. The cells were irradiated with 10 krads, incubated without CYH for time (t) at 23°, exposed to 15 krads, incubated with CYH for time (5-t) at 23°, and plated at 36°. Thus, protein synthesis was permitted to occur between the doses. The total time period of repair was 5 hours for all samples.

The data from figure 15 shows that by following the split dose protocol of 10-15 krads, and separating the doses by 15-20', a 6-7 fold increase in survival occurs compared with cells treated with 25 krads and incubated with CYH for 5 hours. Separating the 10 and 15 krad dose by 30' and allowing protein synthesis to occur between the doses resulted in a 7 fold increase in survival.

Table 1B shows an analysis of the data from this experiment using $t=30'$. The number of breaks repaired per cell in the experiment when cells are treated according to the protocol of figure 13E is 2.9, and this value differs significantly from 1.3, which is the value predicted by the steady state model using relation 10.

Other split dose protocols besides the 10 + 15 krad were attempted. These experiments were done to see if repair is inducible at lower doses than 10 krads. Figure 16 illustrates an experiment run in 2 separate parts, a 2.5 + 22.5 split dose experiment, and a 1.5 + 23.5 krad split dose experiment. The protocol of these experiments is shown in figures 13F and 13G. The experiment is the same as the 10 + 15 krad split dose experiment except the substitution of a lower dose for the 10 krad dose (2.5 or 1.5 krad dose) and a higher dose for the 15 krads (22.5 or 23.5 krads). Protein synthesis was allowed to occur for a time (t) between the doses, and was stopped by the addition of CYH at the time of the second dose.

The data of figure 16 shows that separation of the 2.5 and 22.5 doses by 7.5' resulted in over a 3 fold increase in survival and separation by 30' resulted in a four fold increase in survival. A similar result was obtained with the 1.5 + 23.5 krad split dose experiment. Table 1C shows an analysis of the data from this experiment. The experimental number of breaks repaired per cell when cells were treated according to protocol 13F, with $t=30'$, is 3.2 and this value differs significantly from 2.1 breaks, the value calculated using relation 10.

The data presented above when analysed using the assumptions 1-6 does not fit the hypothesis that the cellular activity of the rad54-3 gene product is constitutive and

is replenished but does not increase after irradiation. The inducible repair hypothesis proposes that the number of breaks repaired per unit time, abbreviated as $-(\frac{dB}{dt})$, increases after irradiation. This increase is diagrammatically illustrated in figure 17. 7.5' after irradiation, $-(\frac{dB}{dt})$ is increasing in the rad54-3 strain, and by 45' to 60' the increase has leveled off. The evidence for leveling off of $-(\frac{dB}{dt})$ 45'-60' after irradiation is shown in figure 14, where cells were treated with two protocols:

- 1) 25 krads, incubation at 23° for 5 hours, plate at 36°
- 2) 25 krads, incubation at 23° for 45' without CYH, incubate for 4 hours and 15' with CYH, plate at 36° for 4 hours and 15 minutes with CYH, plate at 36°,

Both protocols resulted in similar survivals, and presumably the same number of breaks repaired per cell. At least one of two events on the molecular level must occur in order to explain induced repair on the cellular level: 1. There is an increase in the number of rad54-3 enzyme molecules per cell after irradiation, 2. The cellular activity of the rad54-3 gene product increases after irradiation.

Ionizing radiation results in several possible signals which could lead to inducible repair: double strand breaks, single strand breaks, base damage, degraded DNA, single stranded DNA, and inhibited DNA synthesis. One of the possible signals, inhibition of DNA synthesis, can be investigated relatively easily. By using hydroxyurea it is possible to inhibit or stop DNA synthesis without affecting the

survival of cells. The role of inhibition of DNA synthesis in inducible repair can be examined by pre-treating the cells with hydroxyurea before x-ray treatment and the addition of CYH. Hydroxyurea (HU) at the concentration of 0.075M reversibly stops DNA synthesis. Upon removal of the drug, DNA synthesis resumes immediately. The diagram of figure 13H represents the protocol used to answer the question whether HU inhibition of DNA synthesis can be the signal for induced double strand break repair. It does not answer the question whether or not x-ray inhibited DNA synthesis induces repair. This protocol involves pretreating the cells with hydroxyurea 30' or 45' before irradiation, irradiating with 25 krads, incubating with CYH for 5 hours at 23°, and plating at 36°. The survival of cells treated according to this protocol is compared with the survival of cells treated with the protocol of figure 13C. To show that inducible repair was observed in the experiment, the protocol of figure 13D was repeated.

An experiment examining inhibition of DNA synthesis as a signal which leads to inducible repair is presented in figure 18. The figure presents two curves. One curve is a repeat of the protocol of figure 13D, and presents data similar to figure 14. When CYH is added 20' after irradiation, survival is 4 times greater than when CYH is added at the time of irradiation. The cells treated with the protocol of figure 13H had about the same survival as cells treated with the protocol of figure 13C. Thus, HU

inhibition of DNA synthesis does not result in enhanced cellular capacity for double strand break repair. Mimicking one of the effects of radiation, inhibition of DNA synthesis, does not lead to inducible repair.

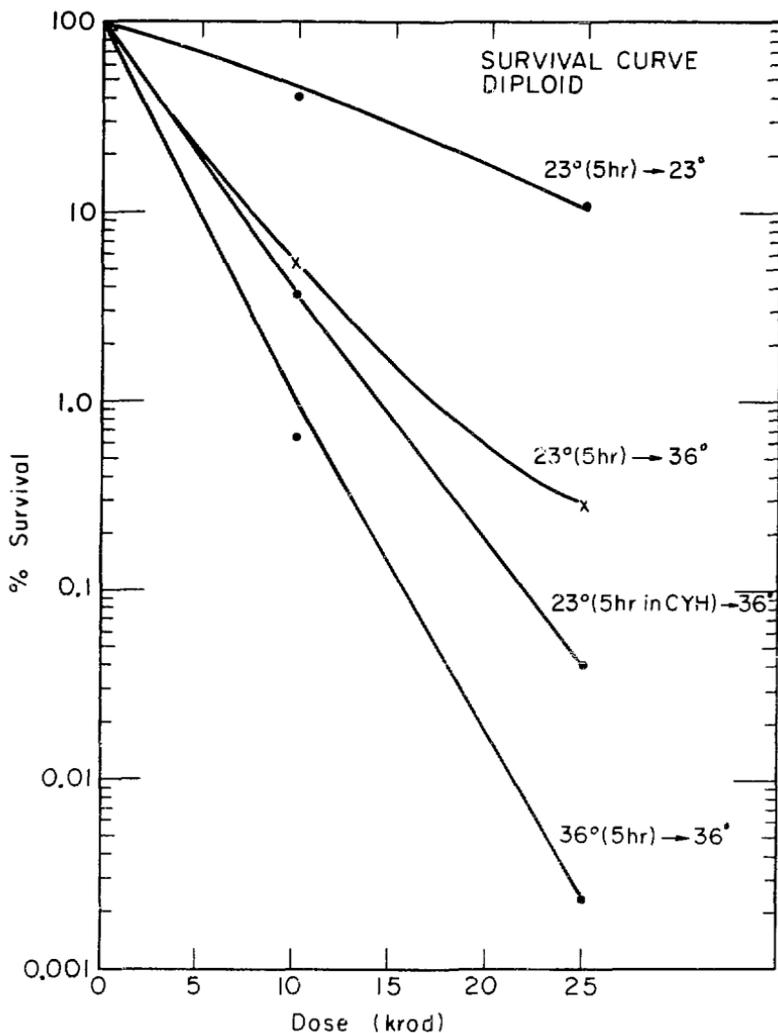
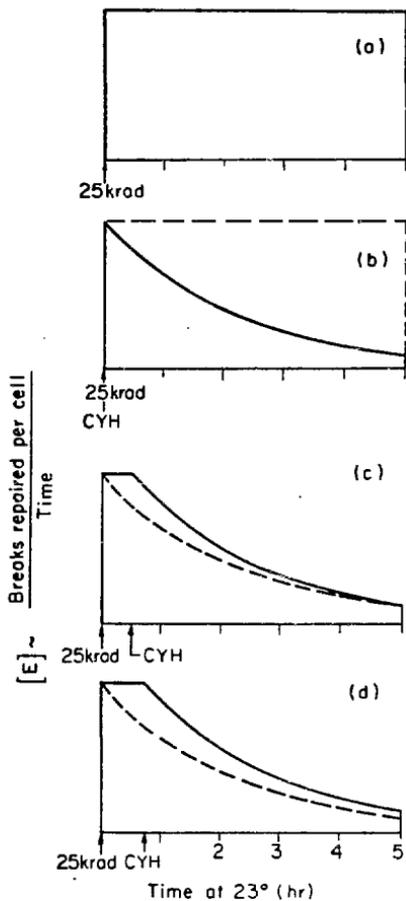


FIGURE 11. Data illustrating the effect of cycloheximide on repair as assayed with 23° (5 hr) to 36° post-irradiation temperature shifts of a rad54-3 diploid.

XBL 617-4056

FIGURE 12. A geometrical model of the steady state hypothesis

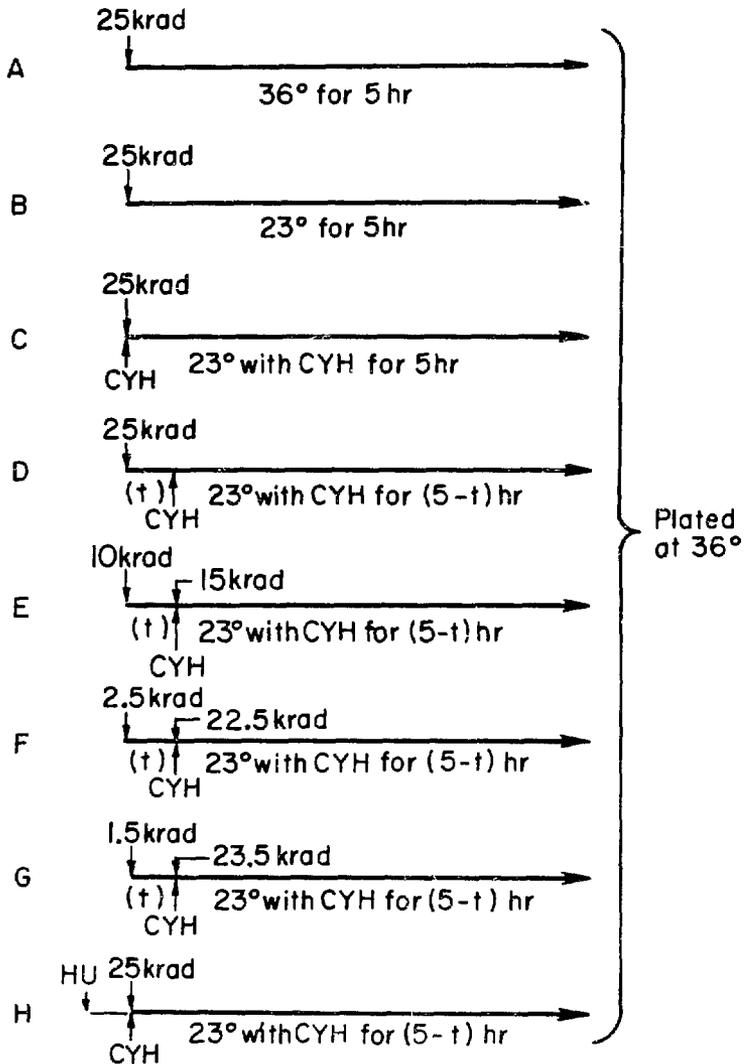
- A. The area under the solid lines is proportional to the number of breaks repaired when cells are exposed to 25 krads, incubated at 23° for 5 hours, and plated at 36°.
- B. The area under the solid lines represents the number of breaks repaired when cells are exposed to 25 krads, incubated at 23° with CYH for 5 hours, and plated at 36°.
- C. The area under the solid lines represents the number of breaks repaired when cells are exposed to 25 krads, incubated at 23° for 30 min without CYH, incubated at 23° with CYH for 4.5 hours, and plated at 36°.
- D. The area under the solid lines represents the number of breaks repaired when cells are exposed to 25 krads, incubated at 23° for 45 min without CYH, incubated at 23° with CYH for 4.25 hours, and plated at 36°.



XBL 822-3593

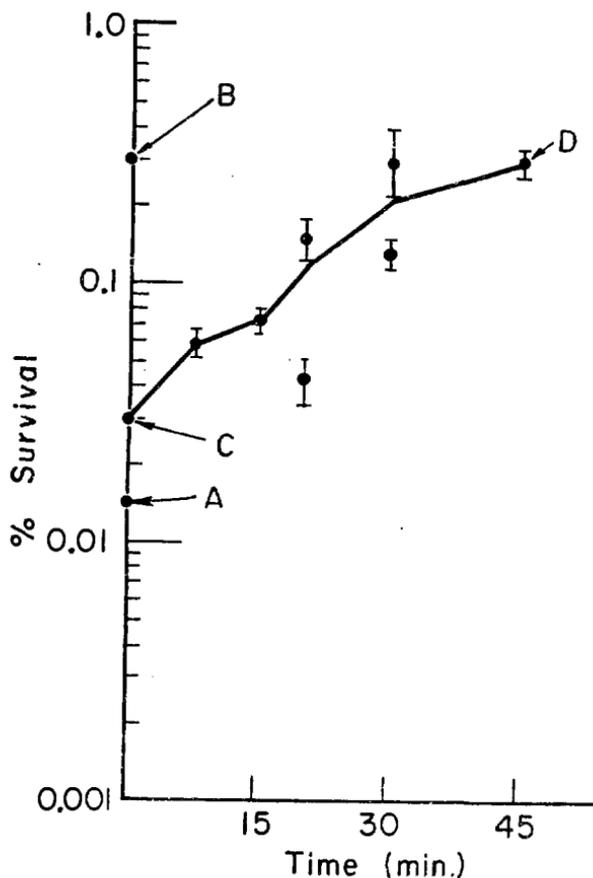
FIGURE 12

A geometrical model of the steady state hypothesis



XBL 822 - 3589

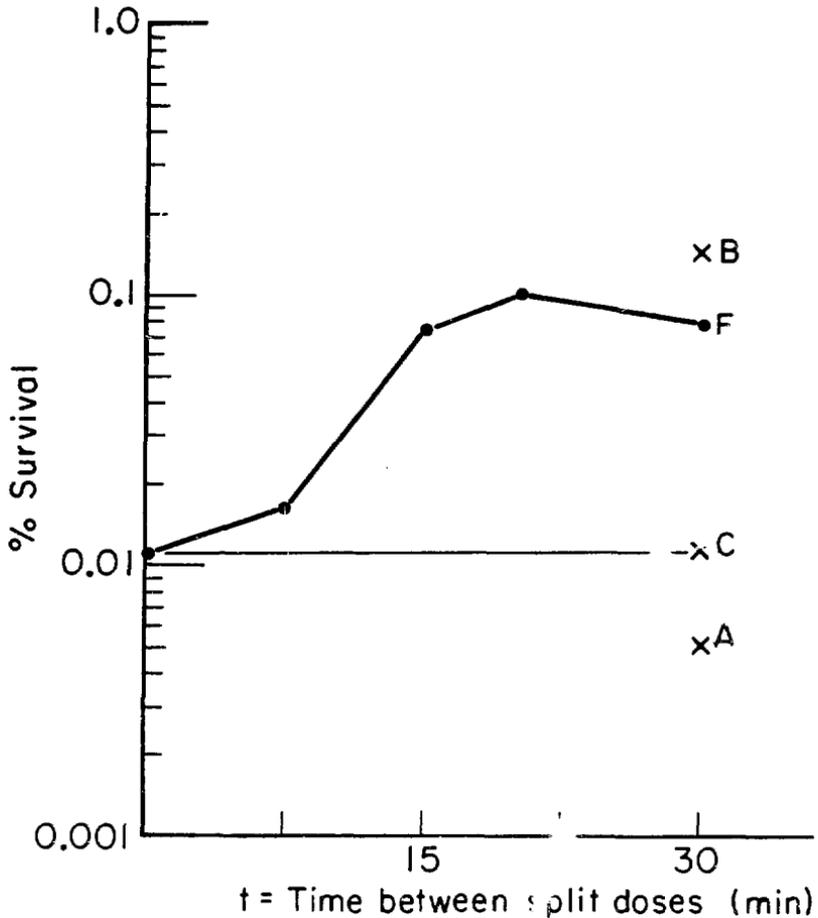
FIGURE 13 Protocol of experiments testing the steady state hypothesis, and the hypothesis that HU treatment will induce repair



XBL 822- 3586

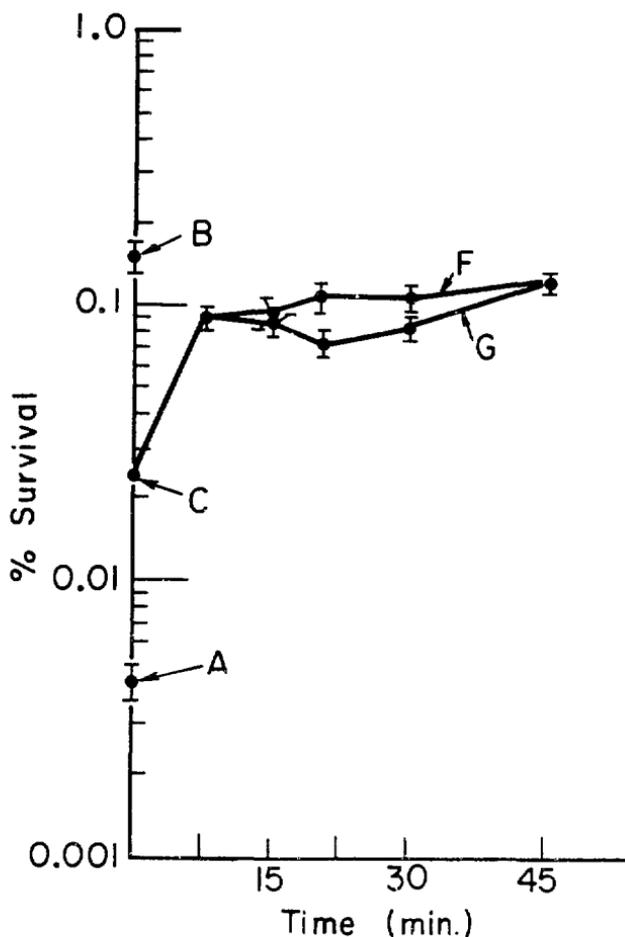
FIGURE 14. Data illustrating the effect of radiating rad54-3 diploids with 25 krads, delaying the addition of CYH for time (t) after radiation, incubating the cells at 23° for time (5-t) with CYH followed by plating at 36°. The letters A,B,C, D refer to the values of survival obtained by following the protocols of figure 13A,B,C,D.

The two points at 20' and 30' represent survivals determined with two different dilutions



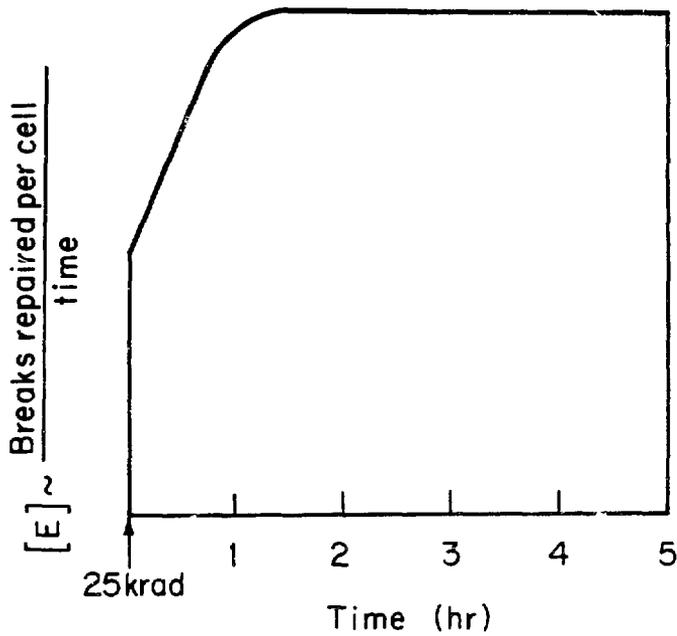
XBL 817-4054

FIGURE 15. Data illustrating the effect of splitting a 25 krad dose into a 10 + 15 dose fraction, separated by time (t), and delaying the addition of CYH until the second dose. The cells are incubated with CYH at 23° for (5-t) hours before plating at 35°. The letters A,B,C,E, refer to the values of survival obtained by following the protocols of figure 13/ 13, C, and E.



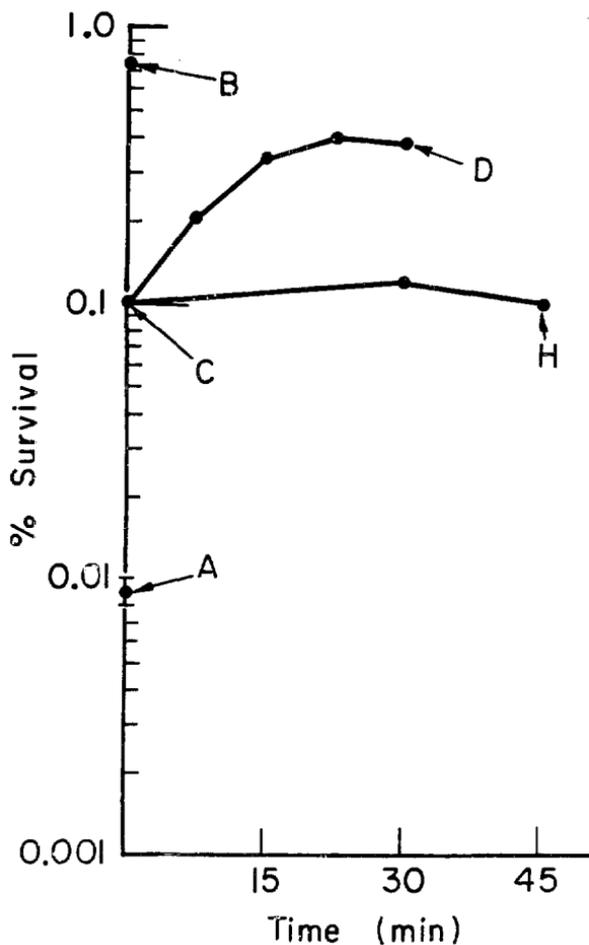
XBL822-3587

FIGURE 16. Data illustrating the effect of splitting a 25 krad dose into a 2.5 + 22.5 or 1.5 + 23.5 krad dose fraction, and delaying the addition of CYH until the second dose. Cells are incubated at 23° with CYH for (5-t) hours and then plated at 36°. The letters A,B,C,F, and G refer to the values of survival obtained by following the protocols of figure 13A,B,C,F,G.



XBL 822-3592

FIGURE 17. A model of the inducible repair hypothesis to explain data from figures 14, 15, and 16.



XBL 822 - 3588

FIGURE 18. Experiment examining HU inhibition of DNA synthesis as a signal for inducible repair. The letters A,B,C,D,H refer to the values of survival obtained by following the protocols of figure 13A,B,C,D, and H.

DNA SYNTHESIS AND REPAIR

Another question concerning the fate of induced double strand breaks is whether their repair is dependent on DNA synthesis. Resnick (1976) has proposed that DNA synthesis is involved in one of the steps in the repair of chromosomal breaks. The involvement of DNA synthesis in repair can be investigated with the same system used to examine the role of protein synthesis in repair. The protocol involves irradiating rad54-3 cells, incubating the irradiated cells at the permissive temperature, 23^o, in the presence of a drug that prevents DNA replication, removing the drug, and then stopping repair by plating the cells at 36^o. The drug used, hydroxyurea, selectively inhibits semi-conservative DNA synthesis by interfering with ribonucleotide reductase. The question of whether or not HU interferes with repair synthesis cannot be answered now since Resnick and Martin (1977) were unable to detect any repair synthesis in yeast after either UV or x-ray treatment. HU also has a small inhibiting effect on protein synthesis and mRNA synthesis. The concentration used in the experiment, 0.075 M, completely inhibits semi-conservative DNA replication in yeast (Slater, 1973).

Three experimental protocols which were used to analyze the role of DNA synthesis in repair are as follows:

1. x-ray with 25 krads, incubate the cells at 23^o for 5 hours without HU, plate at 36^o

2 x-ray with 25 krads, incubate the cells at 23° with HU for 5 hours, and plate at 36°.

3. x-ray with 25 krads, incubate the cells at 36° for 5 hours, plate at 36°

If DNA synthesis is involved in the RAD54 step in repair then the survival of cells treated according to protocol 1 should exhibit a survival significantly greater than cells treated according to protocol 2. The hydroxyurea treatment was performed in liquid YEPD medium and the cells were then plated onto YEPD medium. The number of plates counted was 8 at each point.

Four experiments were performed. Since the results of the experiments were not reproducible, all four experiments are listed in tables 2-5. The numbers in the first column indicate the temperature which the cells were incubated in liquid medium. The numbers in the second column indicate the temperature which the cells were plated. If the cells were plated immediately after radiation, then the treatment in the first column is labelled none. The figures in the third column indicate the cells per plate when unirradiated cells are incubated in the absence of HU. All the numbers are relative to unirradiated cells plated at 23° immediately after collecting. The figures in the fourth column indicate the number of cells per plate when unirradiated cells are incubated in the presence of HU. N.A. means non-applicable, and N.D. means not done. The figures in the fifth column indicate the % survival after irradiation in the absence of

HU. The % survival is relative to unirradiated cells plated at 23° immediately after harvesting. The figures in the sixth column indicate the % survival after irradiation and incubation in the presence of HU. The value of survival corresponding to protocol 1 is listed in row 4 column 5 and the value of survival corresponding to protocol 2 is listed in row 4 column 6. The t-test was performed to determine if these figures are statistically different. The values determined by the t-test are listed in the last column. The figures after \pm signs are the standard errors.

In two of the experiments, experiment 1 and 3, cells treated according to protocols 1 and 2, exhibited survival values which were statistically significant, $P < .01$. In the other two experiments, 2 and 4, the difference was not statistically significant, $P = .20$ and $P = .10$. Thus, the data are not reproducible and no real conclusions can be drawn from this experiment. Although HU at 0.075M blocks semiconservative replication, there still may remain small pools of DNA precursors which may be available for repair. HU also has a small inhibiting effect on mRNA and protein synthesis and as the previous section showed protein synthesis is required for maximal recovery. The small differences in survival may have resulted from different levels of protein synthesis. The experiment might have been redesigned to determine the role of HU in repair. Possible modifications are adding HU at a higher concentration, adding HU a short time after irradiation (to make sure that the HU effects seen are not a

result of inhibition of protein synthesis), and using a longer post-irradiation time at 23^o. Even with these modifications the experiment might lead to irreproducible results or results difficult to interpret. It appears that any *in vivo* technique to determine the role of DNA synthesis in repair must have a higher resolution than the experimental technique used here.

TABLE 2
EFFECT OF HYDROXYUREA ON PERCENT SURVIVAL - EXPERIMENT 1

5 hr	plating	Percent Survival				P(5%)
		0 krad		25 krad		
		-HU	+HU	-HU	+HU	
none	23°	100	NA	11.0 ±.6	NA	NA
none	36°	86	NA	.023 ±.002	NA	NA
23°	23°	200	130	10.7 ±.6	10.9 ±.3	NA
23°	36°	ND	ND	.361 ±.012	.172 ±.009	<.01
36°	36°	ND	ND	.011 ±.002	.020 ±.002	NA

TABLE 3
EFFECT OF HYDROXYUREA ON PERCENT SURVIVAL - EXPERIMENT 2

S hr	plating	Percent Survival				P(5:6)
		0 krad		25 krad		
		-HU	+HU	-HU	+HU	
none	23°	100	NA	8.4 ±.5	NA	NA
none	36°	87	NA	.0152 ±.001	NA	NA
23°	23°	170	140	7.1 ±.3	6.8 ±.3	NA
23°	36°	ND.	ND	.20 ±.02	.17 ±.02	.20
36°	36°	162	100	.007 ±.001	.010 ±.0005	NA

TABLE 4
EFFECT OF HYDROXYUREA ON PERCENT SURVIVAL - EXPERIMENT 3

5 hr	plating	Percent Survival				P(5:6)
		0 krad		25 krad		
		-HU	+HU	-HU	+HU	
none	23°	100	NA	7.1 ±.4	NA	NA
none	36°	101	NA	.0123 ±.001	NA	NA
23°	23°	179	132	7.1 ±.4	6.7 ±.6	NA
23°	36°	ND	ND	.31 ±.02	.17 ±.02	<.01
36°	36°	250	120	.018 ±.001	.023 ±.002	NA

TABLE 5
EFFECT OF HYDROXYUREA ON PERCENT SURVIVAL - EXPERIMENT 4

5 hr	plating	Percent Survival				P(5:6)
		0 krad		25 krad		
		-HU	+HU	-HU	+HU	
none	23°	100	NA	11.0 ±.6	NA	NA
none	36°	96	NA	.0037 ±.0004	NA	NA
23°	23°	156	118	8.9 ±.4	9.3 ±.6	NA
23°	36°	ND	ND	.57 ±.035	.50 ±.03	.10
36°	36°	ND	ND	.0022 ±.0004	.005 ±.0004	NA

RESULTS ON FIXATION

The temperature conditional rad54-3 mutation provides a tool for understanding the fate of double strand breaks when they remain unrepaired for various periods of time. These are the cell death experiments, and cell death is studied by irradiating the cells, incubating at 36^o, the restrictive temperature, for varying periods of time (t), then shifting the cells to the permissive temperature, 23^o. Comparison of survival with cells which have been incubated continuously at the permissive temperature after irradiation shows the percentage of initial lesions which have been fixed into lethal lesions.

This experiment has been done with rad54-3 haploid cells by Ho and Mortimer (1975). They observed that after a 3 to 5 hour post-irradiation incubation at the restrictive temperature, 36^o, about a 10 fold drop in survival occurred compared to cells incubated continuously at 23^o, the permissive temperature. After a further 5 hours at 36^o, another 10 fold drop in survival occurred. Finally, after about 15 hours at 36^o most of the initial lesions had been fixed into lethal lesions.

The experiment was repeated with the diploid strain, X754-6E3B, and the data is illustrated in figure 19. Incubation of irradiated cells at 36^o for 3 hours resulted in a 7 fold drop in survival compared with the cells incubated continuously at 23^o. After an additional 9 hours at 36^o, a

further 13 fold drop in survival occurred. The curve of figure 19 is biphasic, the sharpest drop occurs during the first 3 hours, and beyond 3 hours the cells die less rapidly. The shape of this curve is different from the haploid curve of Ho and Mortimer (1975) since they observed a biphasic component only after 12 hours.

The data will be analyzed using the relation

$$S_1 - S_2 = e^{-(B_1 - B_2)} \quad (1)$$

where S = percent survival relative to unirradiated controls and B = average number of lesions per cell. The percentage of initial lesions which have been fixed into lethal lesions is computed using the relation

$$\% \text{Lesions Fixed} = 1 - \frac{\ln \frac{S_{36 \rightarrow 23}}{S_{36}}}{\ln \frac{S_{23}}{S_{36}}} \quad (2)$$

where S_{23} is the percent survival at 23° , S_{36} is the percent survival at 36° , and $S_{36 \rightarrow 23}$ is the percent survival when cells are shifted from 36° to 23° . The percentage of initial lesions which are fixed into lethal lesions is labeled on the right ordinate of figure 19. Thus, an analysis of the data shows that after a 3 hour post-irradiation incubation at 36° , about 22% of the initial lesions are fixed into lethal lesions, and after 12 hours at 36° about 50% of the

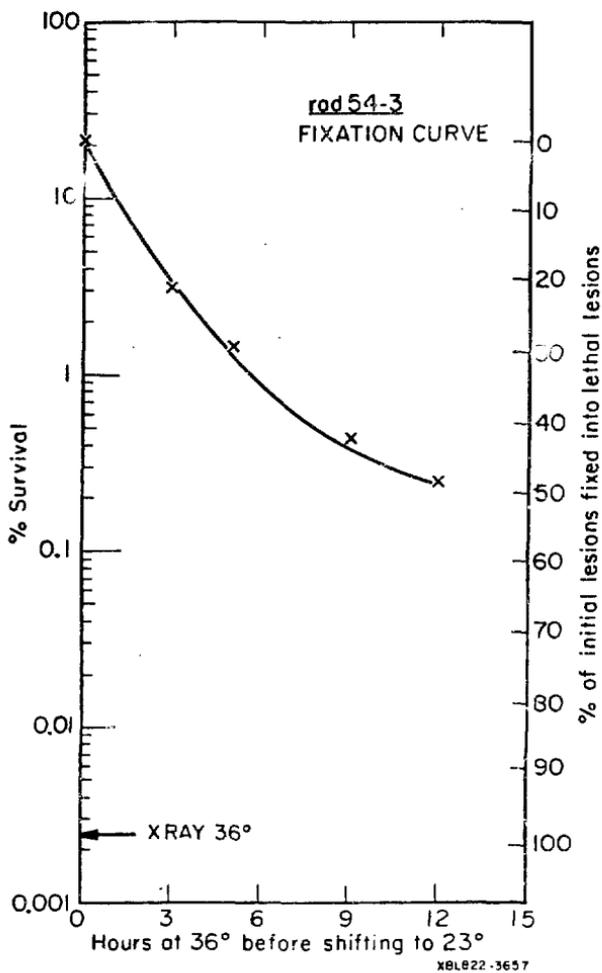


FIGURE 19. Experiment illustrating the effect of post-irradiation 36°→23° temperature shifts on survival. The strain is X754603B a rod 54-3 diploid.

initial lesions have been fixed into lethal lesions.

The initial lesions are thought to be base damage, single strand breaks, and double strand breaks. The lethal lesions are believed to be unrepaired or mis-repaired double strand breaks (Resnick and Martin, 1976; Dewey et al, 1971; Carrano, 1973). Fixation is defined as possible events which may convert an initial lesion into a lethal lesion as a result of 36° to 23° post-irradiation temperature shifts. Misrepair is defined as the creation of an unreparable state of the chromosomes other than what existed before radiation. Possible examples of misrepair are the rejoining of a broken end of one chromosome with the broken end of another chromosome resulting in a dicentric, homologous or non-homologous recombination of the broken chromosome with an unbroken chromosome, formation of telomere type structures at the ends of breaks, and the formation of unreparable deletions from breaks. Some of the examples of misrepair listed above, such as rejoining of broken ends of different chromosomes, are examples of misrejoining. An uncommitted lesion is an initial lesion which has been altered, but is potentially capable of being either repaired or being converted into a lethal lesion. Thus, an uncommitted lesion is an intermediate between an initial lesion and a repaired or fixed lesion. The idea of an uncommitted lesion is taken from the model of Tobias et al (1980).

At 36° rad54-3 cells are unable to repair double strand

breaks while at 23° these strains are able to repair such breaks. Thus, in the absence of double strand break repair initial lesions are fixed into lethal lesions.

Fixation can include a number of possible events: First, fixation may result from exonuclease digestion at the ends of a double strand break, thus converting an initial double strand break into an unreparable double strand break. Second, the formation of telomeres, or telomere like structures, at broken ends may be involved in fixation. Non-homologous recombination of the broken end of one chromosome with another chromosome may also be involved in fixation. An unanswered question is whether the frequency of non-homologous recombination in rad54-3 cells at 36° is high enough to convert initial lesions (breaks) into lethal lesions. Third, fixation may include an attempt at nuclear division since this cell cycle event would probably cause potentially repairable ends to separate, thus reducing the probability the DNA will be restored to its original form. Shortly before mitosis chromosome condensation occurs, and the process of condensation may alter breaks so that they become unreparable. Another role of attempted nuclear division in cell death would be to segregate previously fixed lesions such as dicentrics so that their lethal phenotype can be expressed. This role of nuclear division is not included among the examples of fixation. Fourth, if one proposes that single strand breaks and base damage are the initial lesions, then fixation may result from replication

of damaged DNA. It will be assumed that fixation does not include mis-repair via a RAD51 dependent rejoining mechanism, at least while the cells are being held at 36°. The first experiment tested directly whether DNA synthesis is involved in fixation.

The next experiment discussed was designed to answer the question: is the movement of the cell through the DNA synthesis stage of the cell cycle involved in fixation? DNA polymerases replicating on a DNA helix with a single strand break may be unable to replicate beyond the break in the damaged strand, leaving a gap. In the undamaged strand, DNA replication would not be hindered. One effect of a polymerase blocked on the damaged strand could be the creation of a double strand break. This mechanism for creation of double strand breaks from base damage and single strand breaks has been postulated by Bender et al (1974). Replication of damaged DNA could lead to an increased number of double stranded breaks per cell and possibly cell death.

The role of DNA synthesis in fixation was investigated by using three protocols. The drug used, hydroxyurea (HU), reversibly stops DNA synthesis in yeast when used at a concentration of 0.075M (Slater, 1973). The protocols are as follows:

1. x-ray with 25 krads, incubate the cells at the permissive temperature 23° for 5 hours, plate the cells at 23° ,
2. x-ray with 25 krads, incubate the cells at the

restrictive temperature 36° for 5 hours, plate at 23° ,
3. x-ray with 25 krads, incubate the cells at the restrictive temperature 36° with HU for 5 hours, plate at 23° .
Protocol 1 represents cells allowed to repair damage with a zero time period of fixation after irradiation. Protocol 2 represents cells given a 5 hour period of fixation before the cells are allowed to repair the damage. Protocol 3 represents cells given a 5 hour period of fixation while they are blocked in the S phase of the cell cycle. If the movement of cells through S phase is a significant component of fixation, then cells treated according to third protocol should exhibit a survival similar to those treated according to first protocol.

The data from this experiment is shown in table 6. The numbers in the first column indicate the temperature which the cells were incubated in liquid medium. The numbers in the second column indicate the temperature which the cells were plated. If the cells were plated immediately after irradiation, then the treatment in the first column is labelled none. The figures in the third column indicate the cells per plate when unirradiated cells are incubated in the absence of HU. All the numbers are relative to unirradiated cells plated at 23° immediately after collecting. The figures in the fourth column indicate the number of cells per plate when unirradiated cells are incubated in the presence of HU. N.A. means non-applicable, and N.D. means not done. The figures in the fifth column indicate the percent

survival after irradiation in the absence of HU. The percent survival is relative to unirradiated cells plated at 23° immediately after harvesting. The figures in parenthesis are the number of lesions per cell at the listed survival. The figures in the sixth column indicate the percent survival after irradiation in the presence of HU.

At 23° the cells harvested, incubated in liquid media for 5 hours at 23°, and then plated increased in number by a factor of about 1.7. The cells plated immediately at 23° after 25 krads, have essentially the same survival as cells incubated at 23° in liquid media after irradiation, then plated at 23°. This suggests that the survivors of the 25 krad dose are not dividing during the 5 hours at 23°. Table 6 shows that the presence of HU in unirradiated cultures inhibited multiplication, as would be expected of a drug which arrests the cells in S phase. Cells treated according to protocol 1 (x-ray with 25 krads, incubate at 23° for 5 hours, then plate at 23°) had a survival of 7.1%. Cells treated according to protocol 2 (x-ray with 25 krads, incubate at 36° for 5 hours, plate at 23°) exhibited a survival of 0.96%. Cells treated according to protocol 3 (x-ray with 25 krads, incubate at 36° with HU for 5 hours, plate at 23°) had a survival of 1.20%. Thus, when rad54-3/rad54-3 diploid cells are incubated under conditions where both repair of double strand breaks and DNA synthesis are blocked for 5 hours, the survival is significantly less than under conditions where repair of double strand breaks is permitted to

TABLE 6
THE EFFECT OF HYDROXYUREA ON PERCENT SURVIVAL

5 hr	plating	Percent Survival			
		No X Ray		X Ray (25 krad)	
		-HU	+HU	-HU	+HU
none	23 ⁰	100	NA	8.4 ±.5	NA
none	36 ⁰	88 ±5	NA	.016 ±.001	NA
23 ⁰	23 ⁰	170 ±7	107 ±7	7.1 ±.4 (2.65)	6.8 ±.4 (2.69)
36 ⁰	23 ⁰	180 ±7	121 ±7	.96 ±.12 (4.65)	1.20 ±.15 (4.42)
36 ⁰	36 ⁰	ND	ND	.0075 ±.001 (9.50)	.010 ±.001 (9.21)

occur, protocol 1. About 27% of the initial lesions are fixed into lethal lesions when the cells are blocked in S phase for 5 hours, and this compares with the value of 29% fixation when the cells are not blocked in S phase. The interpretation is that at 36° initial lesions are fixed into lethal lesions even when the cells are blocked in the S phase of the cell cycle for 5 hours. Therefore fixation does not depend on movement of cells through S phase.

The possible role of mitosis in fixation was inferred from this experiment. Incubation of cells with EU prevents S + G-1 cells, which are about 65% of the population, from entering mitosis. The percentage of cells dying as a result of a 5 hour post-irradiation incubation at 36° with HU is about 80%, and this value of cell death suggests that the subpopulation of G-1 + S cells which are unable to enter mitosis are also dying.

One action of irradiation is to cause cells to become temporarily arrested in the cell cycle, specifically in G-2 (Okada, 1970). Using a radiation resistant diploid with a generation time of about 90 min, the data of Burns (1956) fit the relation, where D equals dose in rads,

$$\text{Average Delay (min)} = 350(1 - e^{-0.0015D}) \quad (3)$$

If D=25 krads, then the Delay = 342 min. However, the relation may not apply to the strain X754-6D3B since the rad54-

3/rad54-3 diploid has a longer generation time than 90 min, probably 5-6 hours at 36^o, and it is a radiation sensitive strain. The evidence for a generation time of 5-6 hours is taken from the data of table 6, in which the cell culture multiplied by a factor of 1.8 during a 5 hour incubation at 36^o. The division delay of X754-6D3B was not tested. Brunborg and Williamson (1978) have proposed that much of the damage is repaired during the time period of the division delay.

One might still argue that mitosis plays a major role in fixing initial lesions into lethal lesions, even if the mitotic event is delayed for a time period of over 5 hours after irradiation. One needs to argue that the initial lesions are double strand breaks, the lethal lesions in rad54-3 cells are correlated with unrepaired double strand breaks, and the role of mitosis is to set a time limit, T hours, within which repair can occur. Arguing that mitosis is playing a major role in fixation is the same as arguing that the probability per unit time that a break is correctly repaired from the point of irradiation to the point of mitosis is relatively constant. If rad54-3/rad54-3 cells are irradiated with 25 krads, then incubated at 23^o, they will have a certain time T, hours to repair the breaks. After mitosis the remaining potentially repairable chromosomal ends would be separated, thus making the breaks unreparable. The data of Burns (1956) suggests that the division delay after 25 krads is perhaps at least 6 hours,

and may be longer. When irradiated cells are held at 36° for 5 hours before shifting to 23° , the repair time is presumably 5 hours shorter. A possible explanation for the lower survival of cells incubated at 36° for 5 hours post-irradiation vis a vis those incubated immediately at 23° is a shorter repair time of $T-5$ hours compared with T hours.

In order to test the validity of the above argument the following experiment was performed: Irradiate the cells with 25 krads, incubate the cells at 36° for time (t_{fix}), shift the cells to 23° for time (t_{repair}), then shift the cells to 36° indefinitely. In this experiment the cells were collected onto membrane filters, irradiated, serially diluted, plated, placed in the appropriate incubator, then shifted back and forth between the appropriate incubators.

The following represents the rationale for the above experiment. The first 5 hours after irradiation can be separated into a 3 hour time period at 36° and a 2 hour time period at 23° . If the time period between irradiation and mitosis is greater than 5 hours, and if the probability of a break being correctly repaired per unit time is constant during this 5 hour period, then the order of the 23° and 36° incubation should not affect cell survival. Hence, cells treated according to the following protocols should have similar survivals:

- 1) 25 krads, incubate 2 hours at 23° , incubate at 36°
- 2) 25 krads, incubate at 36° for 3 hours, incubate at 23°

for 2 hours, incubate at 36°

As illustrated in figure 20, when cells are given a 2 hour period of repair at 23° immediately after irradiation, protocol 1, the survival increases from 0.0022% (cells incubated continuously at 36° after irradiation) to 0.19%, thus resulting in 4.1 lesions repaired per cell. However, if cells are treated according to protocol 2, the survival is 0.016%, resulting in about 1.9 lesions repaired per cell. Thus, cells treated according to the first protocol exhibited a survival about 10 fold higher and repaired about twice as many lesions as cells treated according to the second protocol. The most likely interpretation is that the probability per unit time that a break is correctly repaired is not constant between irradiation and nuclear division.

Besides the data from protocols 1 and 2, a family of curves were generated by varying t_{fix} from 0 to 12 hours, and t_{repair} from 2 to 24 hours. The data is illustrated in figure 20. The label on the left margin of figure 20 is percent survival and the label on the right margin is number of breaks repaired. The curve (*) with $t_{\text{fix}}=0$ represents repair with 0 hours fixation. The data show that increasingly longer post-irradiation incubations at 23° results in increasingly greater survival. The resulting curve is similar to the curve of Ho and Mortimer (1975). Survival finally levels off after about 15 hours at 23° after irradiation. The curve (x) for 3 hours fixation was generated by varying t_{repair} from 2-24 hours. Increasingly longer post-

irradiation incubations at 23° result in increasingly greater survival. The repair curves for 5 (\square), 9 (Δ), and 12 (\times) hours fixation are generated in a similar manner. The data from figure 19 and figure 20 were taken from the same experiment. Thus, the data of figure 19 shows the survival when the cells are allowed about 9 days to repair the damage. The data from figure 19 will be used to compute the number of repairable lesions after various time periods of fixation.

Table 7 shows an analysis of the data from this experiment. The figures from the first column indicate the time period of fixation and are labelled (t_{fix}). The figures from the second column indicate the number of lesions repaired per cell and are labelled (B_{repair}). The figures of the third column indicate the time period of repair in hours and are labelled (t_{repair}). The earliest repair time was chosen since it is believed that calculations from this point will give the most reliable value for the initial rate of repair. The figures from the fourth column are the number of lesions repaired per cell (B_{repair}) divided by the time period of repair (t_{repair}). This figure represents an initial rate of repair of uncommitted lesions and is labelled $-\left(\frac{dU}{dt}\right)_0$. The figures from the fifth column are $\frac{-\left(\frac{dU}{dt}\right)_0}{10.6}$, where 10.6 is the total number of initially induced lesions. These figures represent an initial percentage rate of repair. The figures in the sixth column

represent the total number of repairable lesions after the various time periods of fixation. This number is computed using the relation

$$T = \ln \frac{S_{36 \rightarrow 23}}{S_{36}}$$

and the survival values are taken from figure 19. The figures in the seventh column are the initial rate of repair of uncommitted lesions divided by the total number of repairable lesions,

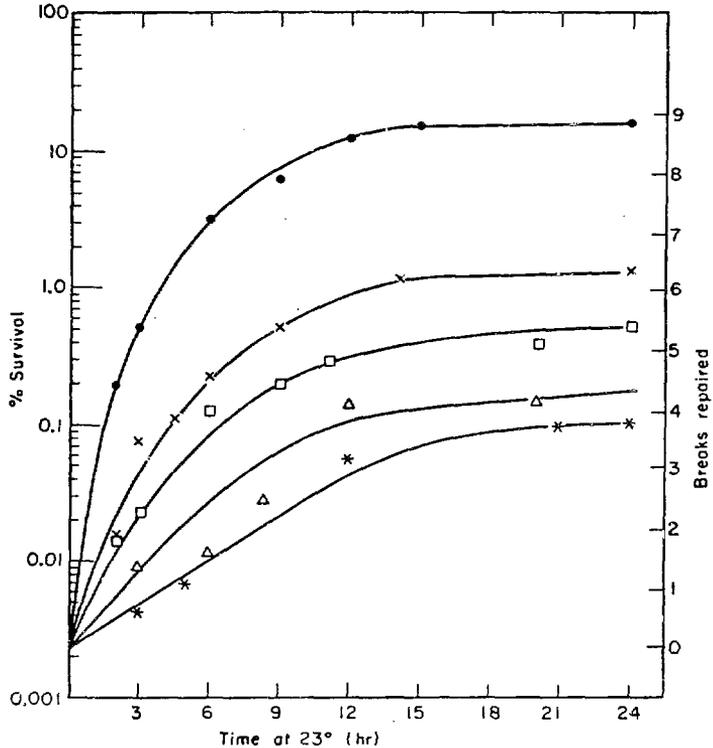
$$P_{\text{rate}} = \frac{-\left(\frac{dU}{dt}\right)_0}{T}$$

P_{rate} represents an initial proportional rate of repair of the remaining uncommitted lesions, and has units of $(\text{time})^{-1}$. The data shows that P_{rate} declines with increasing time periods of fixation, from 0.25 at 0 hours to 0.05 at 12 hours. This conclusion is believed valid even though P_{rate} has the same value at 3 and 5 hours fixation.

The initial recovery which occurs after the various time periods of fixation results from the repair of uncommitted lesions. As the fourth column of Table 7 shows this rate decreases with increasing time periods of fixation. The decrease in $-\left(\frac{dU}{dt}\right)_0$ can result from 2 possible factors. One is that there are fewer uncommitted lesions after the time period at 36⁰. This would occur if the initial lesions

have been either mis-repaired or are unreparable, and are thus no longer uncommitted lesions. Another possible explanation for fixation is that many of the lesions are still uncommitted but the rate of repair of the remaining uncommitted lesions is slower. If fixation can be completely explained with the proposal that after a post-irradiation shift of 36° there are fewer uncommitted lesions, then one would expect the ratio $\frac{-(\frac{dU}{dt})_0}{T} = P_{rate}$ to be constant for increasing time periods of fixation. However, the ratio declines with increasing post-irradiation times at 36° . For instance, when the cells are irradiated with 25 krads, then incubated at 23° , about 82% of the lesions are repairable and $-(\frac{dU}{dt})_0 = 2.2$. If the cells are irradiated with 25 krads and incubated at 36° for 3 hours, then 68% of the lesions are repairable but $-(\frac{dU}{dt})_0 = 0.95$. Thus, the reduction in $-(\frac{dU}{dt})_0$ from 2.2 to 0.95 after 3 hours at 36° cannot be completely explained as a result of a 2 fold decrease in the number of uncommitted lesions. Instead, the explanation is that there is a decline in the proportional rate the remaining uncommitted lesions are being repaired. Thus, a significant component of fixation is a process which converts initial lesions into uncommitted lesions, and the rate of repair of these uncommitted lesions becomes slower with longer post-irradiation times at 36° . One possible process which could alter breaks such that P_{rate} declines is slow exonuclease digestion at the ends of double strand breaks, creating a potentially repairable deletion. The resulting

lesion is hypothesized to be an uncommitted lesion, but the time the cell takes to repair this lesion would be longer than the time required to repair the original break. The data from the sucrose gradient of figure 8 shows that for at least 6 hours after irradiation extensive degradation is not occurring.



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FIGURE 20

- , cells exposed to 25 krad, incubated for time (t) at 23° followed by permanent incubation at 36°
- x, cells exposed to 25 krad, incubated for 3 hrs at 36°, incubated for time (t) at 23°, followed by incubation at 36°
- , cells exposed to 25 krad, incubated for 5 hours at 36°, incubated for time (t) at 23°, followed by incubation at 36°
- ▲, cells exposed to 25 krad, incubated for 9 hours at 36°, incubated for time (t) at 23°, followed by incubation at 36°
- *, cells exposed to 25 krad, incubated for 12 hours at 36°, incubated for time (t) at 23°, followed by incubation at 36°

TABLE 7

ANALYSIS OF REPAIR KINETICS EXPERIMENT

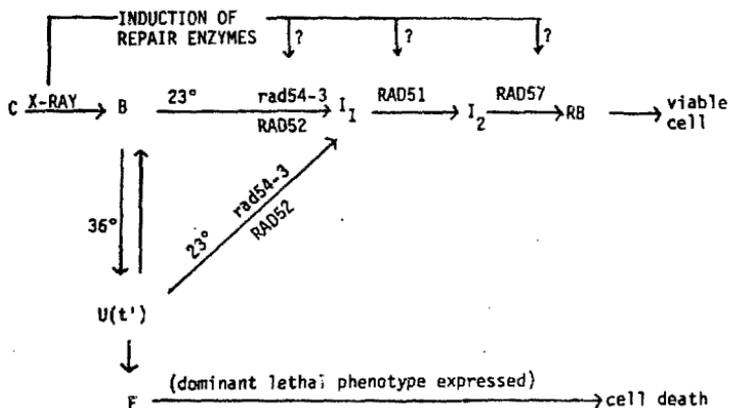
t_{fix}	B_{repair}	t_{repair}	$-\left(\frac{dU}{dt}\right)_0$	$-\left(\frac{dU}{dt}\right)_{\frac{10.6}{}}$	T	P_{rate}
0	4.4	2	2.2	.21	8.7	.25
3	1.9	2	.95	.09	7.2	.13
5	1.8	2	.9	.085	6.4	.14
9	1.4	3	.47	.044	5.2	.09
12	.64	3	.21	.02	4.6	.05

DISCUSSION

The next page presents a model consistent with the data of the results section, and illustrates the possible paths that double strand breaks can follow after irradiation. The break can be converted into a repaired break, $B \rightarrow RB$. This step is assayed with the neutral sucrose sedimentation technique. Since the technique only detects the conversion of small DNA fragments into large DNA fragments, it can only assay for an early step in this repair. rad54-3 strains are temperature conditional for repair of double strand breaks, since at 23° these strains are able to repair double strand breaks while at 36° these strains are unable to repair such breaks. rad51-1 and rad57-1 strains appear to be blocked in later steps of repair because the sedimentation assay shows that these strains are able to convert small DNA fragments into large DNA fragments. The intermediates of repair are labelled I_i . If breaks are prevented from entering the RAD54 pathway then the hypothesis is that they are modified and become uncommitted lesions. Since the data does not provide any evidence that the conversion of a break into an uncommitted lesion is irreversible, the process is drawn as reversible. There might be a dynamic equilibrium between B lesions and U lesions. If rad54-3 cells are shifted from 36° to 23° , the uncommitted lesions, U, are then able to enter the repair pathway. There may be 2 possible pathways for this reaction. In the first pathway, the U lesion is acted on by the rad54-3 gene

FIGURE 21

Model outlining the possible fates of double strand breaks after irradiation



C = Unbroken chromosome

B = Initial double strand break

RB = Repaired double strand break

U = Uncommitted lesion

F = Fixed lesion, Lethal lesion

I₁ = Intermediate of repair

t' = Time period of fixation

k = Rate constant for the reaction: B → RB

$$-\left(\frac{dB}{dt}\right) = kB$$

k(t') = Rate constant for the reaction: U → RB

$$-\left(\frac{dU}{dt}\right) = k(t')U$$

k(t') is equivalent to P_{rate} in the section on Fixation

As t' increases, k(t') decreases

23° and 36° represent the temperatures which the above reactions are favored in rad54-3 cells

product resulting in the formation of the I_1 intermediate, $U \rightarrow I_1 \rightarrow I_2 \rightarrow RB$. In the second pathway, the uncommitted lesion, U , is converted to the original break lesion B , and then the B lesion is acted on by the rad54-3 gene product resulting in the formation of the I_1 intermediate, $U \rightarrow B \rightarrow I_1 \rightarrow I_2 \rightarrow RB$. Both these pathways can be abbreviated, $U \rightarrow RB$, and this reaction is hypothesized to occur with a rate constant $k(t')$, where $k(t')$ fits the relation

$$-\frac{dU}{dt} = k(t')U$$

where t' is the time period of fixation, or the time period cells are held at 36° after radiation. In the section on fixation, $k(t') = \frac{-(\frac{dU}{dt})}{T(t')}$, where $T(t')$ is the total number of repairable lesions per cell after the various time periods of fixation. The interpretation of the decline of $k(t')$ with increasing t' is that the longer the lesions are prevented from entering the RAD54 pathway the slower is their proportional rate of repair once the block in the pathway is removed. Another fate of an uncommitted lesion is to become a fixed lesion. The fixed lesions are most likely unreparable or misrepaired double strand breaks. In some manner as yet unknown the fixed lesion behaves as a dominant lethal. Protein synthesis modifies the possible fate of breaks, since the data suggests that irradiation causes an induction of repair enzymes. The model on the last page illustrates the possible fates of breaks induced

by radiation. The first part of the discussion will discuss the fate of the breaks in yeast, and the model second part will discuss how the model relates to repair in other organisms.

Calculating the efficiency of the first step in which irradiation induces double strand breaks is necessary for understanding the fate of these lesions. In calculating the efficiency of the first step the numbers needed are the number average molecular weight of unirradiated DNA, the dose, and the number of breaks induced by the dose. The average M_n obtained from the 12 gradients was about 4.3×10^8 daltons. This value compares with the value of 6.0×10^8 daltons obtained by Petes and Fangman (1972), and 3.1×10^8 daltons obtained by Resnick and Martin (1976). The variation of the values of M_n are explainable by the fact that the calculation of M_n is determined not only by the position of the peak but also by the distribution of DNA about the peak. Calculations from this thesis showed that the dose of 47.5 krads produced an average of 1 break per chromosome. Thus, the efficiency of break production is 0.49×10^{-10} breaks/krad dalton. The efficiency of break production obtained by Resnick and Martin (1976) is 0.58×10^{-10} breaks/krad dalton. If 47.5 krads produces 1 break/ chromosome, then the dose which produces an average of 1 break per haploid genome is 2.8 krads under the assumption that yeast possess 17 different chromosomes (Mortimer and Schild, 1980). This calculation assumes that breaks are produced linearly with dose.

Resnick and Martin (1976) computed that a dose of 2.2 krads produced an average of 1 break per haploid genome. The D_{37} for a rad52 haploid is about 3 krads (Ho and Mortimer, 1973) which is consistent with an unrepaired double strand break being a lethal event in haploids.

Also, to understand the possible fate of induced double strand breaks it is necessary to know whether breaks are produced in proportion to the dose (D), or the square of the dose (D^2). One theory of cell survival postulates that double strand breaks are induced with significant D^2 kinetics (Chadwick and Leenhouts, 1973). The D^2 kinetics might result from 2 independently produced single strand breaks which are close enough together to produce a double strand break. The question of whether breaks are produced with D or D^2 kinetics cannot be answered with the data from this study since only one dose point was used. However, both Resnick and Martin (1976) and Frankenberg-Schwager et al (1980) observed that double strand breaks initially induced in yeast increase proportionally with dose, rather than dose squared. In E. coli, Krasin and Hutchinson (1977) also found a straight line relation between double strand breaks and dose. Freifelder (1965) using T-7 phage and Christensen et al (1972) using RF ϕ X-174 phage demonstrated that breaks increase linearly with dose when irradiated in broth. In these 3 systems, yeast, bacteria, and phage, neutral sucrose sedimentation measures the molecular weight of intact chromosomes. When Dugle et al (1976) applied neutral sucrose

sedimentation to assay for chromosome break repair in mammalian cells, they observed breaks being produced with D^2 kinetics. Interpretation of sucrose sedimentation experiments of mammalian cells is complicated by the inability of the technique to measure the molecular weight of intact chromosomes. The main body of evidence points to breaks being produced in a straight line relationship with dose, at least in viruses and lower eukaryotes.

The next part of the discussion analyzes the reaction whereby the break is repaired, $B \rightarrow RB$. The data from the results show that in cells with a RAD+ genotype, breaks produced by radiation have a high probability of being repaired after irradiation, and this high probability of repair correlates with high survival. When haploid and diploid RAD+ cells were analyzed for double strand break repair, the DNA profile of cells irradiated with 47.5 krads and incubated for 6 hours in YEPD medium was in the same position as DNA from cells not exposed to x-rays. Thus, apparently complete repair was observed in diploid log phase RAD+ cells. Using a diploid strain synchronized in G-2, Brunborg et al(1980) also observed apparently complete complete repair of double strand breaks during a 2 hour post-irradiation period. When the strain was synchronized in G-2, the survival was 80% at the dose used for the gradient. Rapid and apparently complete repair of double strand breaks in 2 hours correlated with high survival.

In cells with a rad54-3 mutation, the fate of initial double strand breaks depends upon the temperature after irradiation. At 36°, these breaks are not repaired while at 23°, they are repaired. The evidence for this is that the DNA profile of cells exposed to 47.5 krads, and incubated for 6 hours at 36°, remained unchanged from the profile of cells exposed to 47.5 krads with no incubation. The interpretation is that rad54-3 cells are incapable of repairing double strand breaks at 36°. However, upon a post-irradiation incubation at 23°, the DNA shifted to a higher molecular weight. Thus, at 23° these strains are capable of repairing double strand breaks. The RAD54 gene product is acting at an early step in repair. The survival of the rad54-3 strains is high at the temperature at which such strains are able to repair double strand breaks and low at the temperature at which they are unable to repair double strand breaks. However, the post-irradiation shift observed in the profile of DNA extracted from rad54-3 cells given a 6 hour incubation at 23° remained at a lower molecular weight than the control. One possible explanation is that the cells take longer than 6 hours to complete repair, probably 12 hours. The evidence for a 12 hour repair time is taken from figure 20. The rise in survival observed upon 23° to 36° post-irradiation temperature shifts of rad54-3 cells maximizes after about 12 hours at 23°. Also, rad54-3 strains at 23° are more sensitive to radiation than RAD+ strains, thus it is expected even at the permissive

temperature that such strains should exhibit defective repair in comparison with RAD+ strains.

Incubation of rad54-3 cells at 36° eliminated both the radioresistant tail on the haploid survival curves as well as the shoulder on the diploid survival curves. By shifting the cells to 23° , the temperature which they are able to repair double strand breaks, the shoulder on the diploid survival curve reappears. Thus, the x-ray resistant components of survival curves result from a cellular capacity to repair double strand breaks. The D_{37} of the rad54-3 haploid at 36° is about 2.5 krads, which is consistent with one unrepaired double strand break being a lethal lesion in a haploid. Thus, if left unrepaired the fate of one double strand break is to cause cell death in a haploid cell. The value for the efficiency of strand break production by x-rays was used to arrive at this conclusion, and now it is obvious why this number is so important. The D_{37} of the rad54-3 diploid 36° is about 2.5 krads, a dose which induces an average of 2 double strand breaks per diploid cell. Thus, there are about 2 double strand breaks per lethal event in a rad54-3 diploid at 36° . At 23° the D_{37} rises to about 15 krads, resulting in about 12 double strand breaks per lethal event.

The x-ray responses of rad52 and rad54 strains are very similar, and thus it is likely that they operate at the same early step in repair. The inability of rad54-3 strains to

repair double strand breaks at 36° is analogous to the same deficiency in rad52 strains. The x-ray survival curve of rad54-3 diploid strains at 36° and the rad52 diploid strains are very similar. The D_{37} of rad52 diploid strains is about 2.1 krads, a dose which induces an average of 2 breaks per diploid genome (Resnick and Martin, 1976). The diploid rad52 rad18 double mutant with a D_{37} of 1.5 krads is more sensitive to radiation than the diploid rad52 single mutant (Resnick and Martin, 1976). The D_{37} dose induces a little over 1 double strand break per cell. The interpretation of Resnick and Martin is that 1 unrepaired double strand break is a lethal lesion in a diploid cell. This calculation provides evidence that unrepaired double strand breaks behave as dominant lethals.

Although rad52 and rad54 strains are very similar in their x-ray response, they differ in one respect. rad52 diploid cells plate at low efficiency, less than 50%, and the control colonies are variable in size. Mortimer et al (1981) found a high rate of chromosome loss in rad52 diploids, about 1% per chromosome for control colonies. The frequency of chromosome loss increases upon irradiation of rad52 diploids. However, rad54-3 diploid cells plate with a high efficiency at both 36° and 23° . Also, except for a few petites seen, all the control colonies exhibit uniform colony size. Hence, the event which lowers the plating efficiency of rad52 cells, is either not occurring in rad54 cells or is occurring at an undetectably low level.

In this study a rad51-1 strain is an example of a strain exhibiting low survival and probably some incomplete repair of double strand breaks. The extent of apparent repair in 6 hours is similar to the rad54-3 strain at 23°. This result extends the result of Mowat and Hastings (1979) who in 3.5 hours found no repair of double strand breaks in a rad51-1 haploid. The structures observed in the gradients are probably intermediates in repair, maybe broken chromosomes stuck together by a Holliday structure. The diploid rad51-1 survival curve exhibits a small shoulder, unlike the rad54-3 strain at 36° for which no shoulder was seen. The D_{37} of the rad51-1 diploid is approximately 5.5 krads, about twice the D_{37} of the rad54 strain. At the D_{37} dose there are about 4 to 5 double strand breaks per cell. Since the rad51-1 haploid strain is able to convert small fragments into large large fragments, it presumably acts after the RAD52 RAD54 step in repair. This is shown in the model of figure 21.

If repair of double strand breaks is blocked at the RAD57 step, then the structures observed in the gradient are large DNA molecular weight intermediates which do not allow the cell to survive. This is because rad57-1 strains are presumably able to convert small DNA fragments into large DNA fragments with equal efficiency at both the permissive and restrictive temperatures. Thus, the cold sensitive phenotype of rad57-1 strains does not appear to be the result of an inability to perform the initial stages of

repair. rad57-1 strains at 23° are blocked in a later step of repair than rad54-3 or rad52-1 strains. Although rad57-1 strains incubated at 23° are very sensitive to radiation, they are not as sensitive to radiation as rad52 or rad54 strains. Thus, blocking the RAD57 step in repair results in higher survival after radiation than blocking the RAD52 or RAD54 step of repair. However, at neither the permissive nor restrictive temperature is the repair complete after the end of the 6 hour post-irradiation incubation time. It is likely that the rad57-1 strains are capable of further rejoining after 6 hours. The large molecular weight DNA structures observed in the gradient, of rad57-1 strains at 23° may be repair intermediates, possibly Holliday structures formed between broken and unbroken chromosomes. Alternatively, a defective RAD57 gene product may predispose a cell to misrepair. Mis-repair could be the rejoining of two broken ends of different chromosomes resulting in a dicentric or a translocation of an acentric fragment. In figure 21, the RAD57 step in repair is placed after the RAD51 step because the survival of rad57-1 haploid strains at the restrictive temperature is higher than rad51-1 haploid strains.

In summary, the following sequence represents the order of steps in repair of double strand breaks: RAD52 RAD54 (same step), RAD51, RAD57. The sucrose sedimentation technique can detect whether cells have completed an early step in repair, such as the RAD52 RAD54 step, but does not appear

to detect whether cells have completed a later step, such as the RAD57 step. Also, the survival of cells blocked at an early step (rad54-3 cells at 36°) is lower than cells blocked in a later step of repair (rad57-1 strains at 23°). The survival of the rad51-1 strain is intermediate between that of the rad54-3 strain and that of the rad57-1 strain.

The data of Saeki (1980) shows that rad52-1 and rad51-1 diploid strains did not exhibit x-ray induced gene conversion at the leu1 locus, however rad54-1 and rad57-1 strains did exhibit induced gene conversion. Since rad54-3 strains at 36° are more sensitive to radiation than rad54-1 strains, the rad54-1 allele may be leaky. In the rad54-3 diploid strain, g713A, x-ray induced gene conversion at the his1 locus did not occur at 36° , while at 23° , x-ray induced gene conversion did occur. It should be added that the combination of low survival after irradiation, and low conversion frequency in rad54-3 cells at 36° made the finding values of conversion frequency at this temperature difficult. Thus, it appears that cells which are blocked in the early steps of repair, such as the RAD52 RAD54 step, are unable to exhibit x-ray induced gene conversion. This correlation provides evidence the early steps in repair of double strand breaks involve recombination. rad57-1 strains presumably blocked in a late step of repair do exhibit some induced gene conversion. Thus, the later steps in repair might not involve recombination. This completes the discussion of the pathway of repair of induced double strand breaks.

One experiment studying the integration of broken plasmids into yeast will be helpful in understanding the fate of double strand breaks when they are not repaired soon after irradiation. Orr-Weaver et al (1981) have proposed a model of integration of linear plasmids into chromosomes which is equivalent to Resnick's model of double strand break repair. In yeast circular plasmids integrate at sites of homology with the yeast chromosome. If a circular plasmid carries the HIS3 gene then the plasmid will integrate at the HIS3 locus in the chromosome. They observed that when a circular plasmid carrying the HIS3 gene is cut in the middle of the HIS3 gene with an endonuclease, thus creating a double stranded break, the frequency of integration at the his3 locus in the chromosome increases 1000 fold. If the plasmid is cut at a site away from the HIS3 gene then the integration frequency approximates that of the uncut plasmid. Even when there is a deletion in the region of the HIS3 gene which is several thousand base pairs in length, the linear plasmid still integrates at a 1000 fold greater frequency than the circular plasmid. Also, the deletion is repaired when the linear plasmid with a deleted region of the HIS3 gene integrates into the chromosome. This result will be used to analyze the fate of induced breaks when not repaired soon after irradiation. Circular plasmids integrate at the same frequencies in RAD+ and rad52 strains. However, the integration of broken plasmids requires a functional RAD52 gene product. The authors have

concluded that the broken ends of plasmids are highly recombinogenic, and the recombination only occurs when a functional RAD52 gene product is present. The similarities between the integration of broken plasmids and the repair of chromosomal breaks has led to a model of plasmid intergration which is similar to Resnick's model for repair of double strand breaks. This model is illustrated in figure 3B.

The previous discussion has concentrated on the fate of double strand breaks when they are permitted to enter the repair pathway, $B \rightarrow RB$. The kinetics of repair is described by the following equation, where k is a rate constant of repair, and B is the average number of breaks per cell:

$$-\frac{dB}{dt} = kB$$

However, when the repair pathway is blocked, such as incubating rad54-3 cells at 36° , as figure 21 illustrates, the breaks become modified and are thus classified as uncommitted lesions. This step is independent of DNA synthesis and is abbreviated $B \rightarrow U$. Since there is no evidence that the step is irreversible, the reaction $U \rightarrow B$ is also hypothesized to occur. One can also conceive of breaks in RAD+ cells becoming uncommitted lesions if they do not enter the repair pathway shortly after irradiation. The model of figure 21 illustrates that uncommitted lesions have several possible fates. One fate is conversion into a lethal

lesion, $U \rightarrow F$. The fixed lesions are most likely unreparable or misrepaired double strand breaks. Another fate of uncommitted lesions in rad54-3 cells is possible if the cells are shifted to 23° after a period of time (t') at 36° . This fate is repair of the uncommitted lesions, $U \rightarrow RB$, and this repair is hypothesized to occur with a rate constant $k(t')$, where t' is the time period at 36° after irradiation before the cells are shifted to 23° . The kinetics of repair of uncommitted lesions occurs according to the following equation, where U is the average number of uncommitted lesions per cell:

$$-\frac{dU}{dt} = k(t')U$$

The data from the last column of table 7 show that with increasing time periods of fixation (t'), $k(t')$ progressively decreases. $k(t')$ represents a proportional rate of repair of the remaining uncommitted lesions and is equivalent to P_{rate} in the section of fixation. $k(t')$ has declined by about one half after 3 hours at 36° and after 12 hours at 36° it has declined about 5 fold.

Thus, fixation is a process which converts breaks into uncommitted lesions, and the longer lesions remain uncommitted, the longer it takes the cells to repair them. One possible process which could convert breaks into uncommitted lesions is exonuclease digestion. Single strand exonuclease digestion would result in a single strand terminus, and as

the terminus became longer it might take the cell a longer time to repair the lesion. Alternatively, a double strand exonuclease acting at the ends of the break may result in a deletion. As the deletion becomes increasingly longer, as it might with longer post-irradiation times of rad54-3 cells at 36°, then the time required to repair the deletion may be longer than the time required to repair the original break. The longer the deletion, the longer the time required to repair it. Orr-Weaver et al (1981) have provided evidence that these types of lesions are repairable.

No data which would directly support the above hypothesis was obtained. In particular, the sedimentation results of figure 8 show that measurable DNA degradation did not occur in the rad54-3 strain used in the experiments. Other experiments which might have provided evidence, such as checking the incubation medium for excreted [³H] were not performed. The hypothesized DNA degradation is postulated to be unmeasurable with the sucrose sedimentation technique.

Another possible explanation of fixation is the addition of short fragments onto the ends of the breaks, with the result that the cell would take a long time to repair these the resulting lesions.

Fixation does not appear to be explainable with the proposal that a type of misrepair is occurring with dicentric as a possible result. This is because misrepair would reduce the number of uncommitted lesions since it is likely

that a misrepaired lesion is a fixed lesion. As table 7 illustrates $-(\frac{dU}{dt})$ drops from 2.2 lesions repaired per cell/hour (0 hours fixation) to .21 lesions repaired per cell/hour after 12 hours fixation. At 0 hours fixation there are about 8.7 repairable lesions per cell and after 12 hours fixation there are about 4.6 repairable lesions per cell. The 10 fold drop in $-(\frac{dU}{dt})$ after 12 hours at 36° is not a result of a 10 fold drop in the number of repairable lesions per cell because there is only a 2 fold drop in the number of repairable lesions per cell. Thus, it is likely that the 10 fold drop in $-(\frac{dU}{dt})$ from 0 to 12 hours post-irradiation incubation at 36° is not a result of 90% of the initial lesions being misrepaired. Instead, the decline is explained by a progressive decrease in the rate the existing repairable lesions are being repaired. This completes the story about the direct fate of x-ray induced double strand breaks.

This thesis also investigated possible modifying factors which could alter the probability of whether a break enters the repair pathway, or whether it becomes a fixed lesion. The occurrence of protein synthesis shortly after irradiation increases the probability that the break enters the repair pathway rather than the fixation pathway. The interpretation of the data from the section of the effects of cycloheximide on repair there is an enhanced cellular capacity to repair double strand breaks. The enhanced cellular capacity to repair breaks is postulated to result from

an increase in the number of repair enzymes per cell. The repair proteins which are induced are most likely the ones whose availability is limiting the rate of repair. If the concentration of rad54-3 enzyme molecules in the cell is limiting the rate of repair, then it is likely that there is an increased synthesis of this gene product after irradiation. Thus, the rad54-3 gene product may be one of a number of repair enzymes synthesized after irradiation.

Ho (1975) has provided evidence that the availability of the RAD52 gene product limits the rate of repair in tetraploids. If < means more sensitive to radiation, and (+) is the wild type allele then the data is summarized as follows: rad52/rad52/rad52/rad52 < +/rad52/rad52/rad52 < +/+/rad52/rad52 < +/+/+/rad52 < +/+/+/+. Thus, with an increasing number of rad52 alleles per cell, tetraploid strains become more sensitive to radiation. The data can be interpreted with the hypothesis that repair is limited by the concentration of the RAD52 gene product.

Krasin and Hutchinson (1981) have shown that the induced synthesis of a required enzyme for repair can occur without induced repair occurring. The RECA gene product is required for repair of double strand breaks. However, some of the treatments which result in induced synthesis of RECA gene product such as treating cells with nalidixic acid do not result in enhanced repair.

Although the inducible repair hypothesis is the most likely explanation of the results, other explanations are possible. One possible explanation is that in the presence of protein synthesis after irradiation a protein may be synthesized that prevents the degradation of the RAD54 gene product, or another repair enzyme. Another possibility is that a protein may be synthesized after irradiation which alters the conformation of the DNA in such a manner so that the breaks are repaired more quickly. Also, after irradiation a protein may be synthesized that depresses fixation rather than enhances repair.

The inhibiting effect of CYH on repair of double strand breaks was first observed by Resnick and Martin (1976), but was more clear cut in the system of Brunborg et al(1980). They used a strain capable of apparently complete repair in G-2. In that system complete repair of double stranded breaks was observed during a 2 hour post-irradiation incubation period, and the presence of CYH caused a marked inhibition of repair. This provides evidence that survival is lower in the presence of CYH because fewer breaks are repaired.

The time appearance and the dose dependence of the enhanced survival was examined in the experiments. The data analyzed geometrically consisted of cells given post-irradiation treatments of 30' without CYH, and 4.5 hours with CYH. Post-irradiation incubation after irradiation

without CYH for time periods less than 30' also resulted in enhanced survival. Delaying CYH for 7.5' after irradiation resulted in significantly increased survival during the 1.5 + 23.5 and 2.5 + 22.5 krad split dose experiments. Thus, very shortly after irradiation a protein or proteins are synthesized which cause an increase in survival. The lowest inducing dose used, 1.5 krads, resulted in a little over 1 double strand break per cell and about 20 single strand breaks per cell. Hence, relatively small doses and short times are required to generate an enhanced survival response which results from altered protein synthesis after irradiation.

It is more likely that the inhibiting effect of CYH on repair results from preventing the synthesis of repair proteins after irradiation, rather than from some effect like CYH binding to DNA and preventing repair. During the 1.5 + 23.5 split dose experiments it was observed that delaying the addition of CYH 7.5' after irradiation results in a significantly higher survival than when CYH is added at the time of radiation. The cells treated with these two protocols had similar treatment times with CYH, 4 hours and 52' compared with 5 hours. Hence, if CYH prevented repair in some manner like binding to the DNA, then delaying the addition of CYH for 7.5' after irradiation should not result in a significant change in survival, than when CYH is added at the time of radiation.

The time periods which the repair enzymes appear, 7.5' to 45', are short compared to the time that double strand breaks are fixed into lethal lesions. Thus, during the immediate post-irradiation period during which there is an increased synthesis of repair enzymes, the lesions which the repair enzymes act on are not being transformed into lethal lesions.

Repair can be separated into a protein synthesis independent component (constitutive repair) and a protein synthesis dependent component (induced repair). After the dose of 25 krads, there is enough damage induced so that both components are required for repair. However, as shown in figure 11, after 10 krads the constitutive repair component is able to repair most of the damage. After 1.5 and 2.5 krads the constitutive repair component should be able to repair all the repairable damage without any assistance from the inducible repair component. Induced repair after 1.5 krads was found because the challenge dose used was high. Thus, the paradox is that at the doses of 1.5 and 2.5 krads there is an induced synthesis of repair enzymes, but at these doses the constitutive level of repair enzymes should be all that is needed to repair the damage. The statement that protein synthesis after irradiation increases the probability that breaks will enter the repair pathway is true at high doses, 25 krads, but is probably not true at lower doses of 1.5-5 krads, even though repair enzymes are inducible at these lower doses. The discussion of the data

supporting the model of figure 21 is finished.

In one interesting step whereby the fixed lesion most likely an unreparable or misrepaired double strand break, behaves as a dominant lethal no experiments were done. However, an analysis of the final fate of the lesion will be performed anyway. In these experiments a cell has a lethal lesion if it is unable to give rise to a colony visible to the naked eye. Probably about 1000 cells are required for a colony to be visible. Thus, cells which exhibit a delayed form of cell death, such as those which are able to divide only 5 times after irradiation are classified in the same category as those that die in the first mitotic division.

It is expected that cells with defective RAD genes would exhibit specific types of aberrations, because they are unable to rejoin broken chromosomal fragments. rad52 and rad54-3 cells at 36° would be expected to show aberrations such as fragments and deletions but not aberrations like dicentrics which would result from the joining of centric fragments of two broken chromosomes. Hence, it would appear that cell death in these mutants should result from the presence of chromosomal fragments and deletions. Comparison of break frequency with the D_{37} shows that only 2 double strand breaks are necessary to cause cell death in a rad54-3 diploid at 36°. If a diploid cell has 2 random breaks in its genome, then the breaks will most likely be induced in 2 non-homologous chromosomes. If the breaks are

rad52/rad52/rad52 genotype are more sensitive to radiation than cells with the qq rad52/rad52 genotype. In their experiments the D_{37} of the qq rad52/rad52 diploid is 2.5 krads, and this dose induces an average of 2 double strand breaks per 2n genome. The D_{37} of the qqq rad52/rad52/rad52 triploid is a dose, 1.7 krads, which induces about 2 double strand breaks per 3n genome. Although a qqq rad52/rad52/rad52 strain with 2 unrepaired breaks should have sufficient DNA for essential functions it is not viable. This observation shows that redundant genetic information does not protect a cell from the lethal effects of unrepaired breaks. In fact, redundant information sensitizes a cell to the lethal effect of breaks because there are more sites for breaks to occur.

The results of an experiment of Pontecorvo (1942) can be used to argue that the potentially lethal effect of deletions does not account for the increased sensitivity of qqq rad52/rad52/rad52 cells to radiation compared with qq rad52/rad52 cells. In *Drosophila*, deletions in the autosomes which are lethal in diploids, are not lethal in triploids. Hence, redundant genetic information should protect a cell from the lethal effects of deletions. Pontecorvo argues that dominant lethal damage at low doses results from union of sister chromatids.

Several possible speculations will be discussed to account for the observation that 1-2 unrepaired double

strand breaks are capable of causing lethality in a diploid. Two of the speculations propose how structures which are known to physically interfere with nuclear division (dicentric) are formed in the absence of RAD52 RAD54 gene products. However, the real explanation may include none of the below. Presumably the broken end is not going to rejoin with another broken end via the RAD52 RAD54 pathway thus resulting in a dicentric chromosome. One possible speculation involves the formation of hairpin loops at the ends of unrepaired double strand breaks. A mechanism for the formation of these structures is as follows: If 5' → 3' exonuclease digestion of the ends of the break occurs, the broken chromosome will have a single stranded tail with a 3' end. The single stranded tail then bends back and forms a hairpin loop. The hairpin loop could occur in the presence of a palindromic sequence, but may also occur in the absence of a palindrome. It is possible that hairpin loops could still form from 2 sequences which are inverted repeats of 4-5 base pairs in length, and separated by several hundred base pairs. A 3' → 5' exonuclease could then digest the remaining single stranded end up to the hydrogen bonded structure of the hairpin loop. The 3' end of the hairpin loop is now a substrate for a pol I type polymerase which might act to close a possible gap. The remaining single stranded break is then closed with a ligase. When this structure is replicated in a semi-conservative manner the result is a dicentric. The dicentric then causes cell death

by interfering with the completion of nuclear division. It was noted in the section on fixation that stopping DNA synthesis did not interfere with fixation. However, the steps talked about here are presumed past the stage where the initial break has been fixed into a lethal lesion. These steps concern the question of how the fixed lesion expresses its phenotype.

The above speculation proposes how 1 unrepaired double strand break can behave in a dominant lethal fashion. It is consistent with a linear relation between dose and lethality in rad52 or rad54-3 (36°) strains. If the above structures are formed with high frequency in rad52 and rad54 cell, then the above speculation would explain why qqq rad52/rad52/rad52 strains are more sensitive to radiation than qq rad52/rad52 strains.

Another possible speculation whereby unrepaired breaks could become transformed into lesions which interfere with cell division is suggested by McClintock's studies on spontaneous chromosomal aberrations in maize. In an early study McClintock (1941) constructed a dicentric chromosome from a cross in which one parent had a normal chromosome 9, and the other parent had a chromosome 9 with a duplication and inversion in the short arm. Recombination between the normal and abnormal chromosome generated the dicentric chromosome. McClintock observed that the fate of the resulting dicentric was breakage at anaphase, replication of the

chromosome, fusion of the broken ends of the sister chromatids thus recreating a dicentric, rebreakage of the dicentric during a subsequent anaphase, followed by refusion of the broken ends during prophase. The dicentric was constructed in such a manner that the cell did not die from the occurrence of deletions after the breakage-fusion-bridge cycle. The breakage-fusion-bridge cycle involving sister chromosomes can continue through several successive mitoses. The breakage of the dicentric chromosomes during anaphase is often at the point of fusion but also occurs elsewhere in the chromosome. Thus, duplications and deletions are created in chromosomes which go through the breakage-fusion-bridge cycle. However, if the broken chromosome enters the zygote where there is an unbroken chromosome, the break usually heals and the breakage-fusion-bridge cycle ceases.

Another observation of McClintock (1941) is that non-homologous pairing occurred between chromosomes under various conditions, such as the presence of a structural rearrangement occurring in a heterozygous condition, or the presence of an unbalanced chromosome complement. In particular a univalent chromosome can often pair with itself in monosomic or trisomic plants. The conditions under which non-homologous pairing occur are probably those in which homologous pairing are inhibited. Possible events which may arise from the non-homologous chromosomal pairing are inversions and translocations.

In a later publication McClintock (1951) observed that the breakage-fusion-bridge cycle occurred at Ds (for dissociation) sites, and were under control of the Ac (for activator) system. The genes where Ds elements were located were unstable in the presence of Ac, and variegated phenotypes were observed in the endosperm. However, in the absence of Ac, breaks did not occur at the Ds sites and the genes were stable. The instability of the genetic loci was related to the transposition of Ds elements from one chromosome site to another. The events associated with Ds transpositions resulted in the formation of translocations, deletions, inversions, ring-chromosomes, dicentrics, and acentric fragments. The Ds and Ac elements of maize are analogous to the recently observed transposable elements in yeast and bacteria.

In order to account for the lethal effect of 1-2 breaks in a rad52 or rad54 cell it will be proposed that there are 2 types of rejoining systems possible in yeast. The mechanism of type 1 involves homologous recombination and is dependent on functional RAD52 RAD54 gene products. The number of breaks per cell would decrease after irradiation if repair occurs via the RAD52 RAD54 pathway. In the absence of the RAD52 RAD54 pathway another pathway of rejoining could occur, possibly analogous to the Ds Ac system in maize. Possible Ds type elements in yeast are Tyl elements δ sequences and other transposable elements of

yeast (Cameron et al, 1979). Thus, if the RAD52 RAD54 pathway is blocked, this hypothesis proposes that there is an increase in the number of events occurring via a Ds Ac type mechanism. As discussed previously, McClintock (1941) observed non-homologous chromosomal pairing under conditions where homologous pairing was inhibited. Similarly in yeast if a rad52 mutation prevents homologous pairing in yeast, then one might observe an increase in the number of events associated with non-homologous pairing, such as Ds Ac type events. One such Ac Ds like event which could transform an unrepaired break into a dominant lethal would involve replication of two pieces of the broken chromosome followed by random fusion of the ends of the sister chromosome centromere fragments. The presence of a Ds like element near the end of one of the fragments might allow this end to fuse preferentially. The acentric fragments may also fuse, and might eventually become lost. However, the loss of acentric fragments is probably not the mechanism whereby an unrepaired break behaves as a dominant lethal. Also, it is possible that the presence of an Ac Ds type element might result in non-homologous recombination between a broken centromeric fragment and an unbroken chromosome with a dicentric as a possible result.

The above speculation also would explain the result of Ho and Mortimer (1973) that a rad52/rad52/rad52 strain is more sensitive to radiation than a rad52/rad52 strain. The basic question about the above hypothesis is whether or not

the frequency of Ac Ds type rejoining occurs at a high enough frequency in rad52 and rad54 cells to convert most of the unrepaired double strand breaks into lesions which interfere with nuclear division. It may also be possible that unrepaired breaks interfere with the cell cycle in yeast an as yet unknown mannner. Possibly, the presence of a chromosome with a centromere and only one telomere causes a cell cycle arrest. This completes an analysis of the possible fates of induced double strand breaks in yeast.

The next question concerns whether or not the model illustrated in figure 21 is applicable to other organisms. The step in which the break is repaired has also been demonstrated in other organisms, and may exist in all organisms. Double strand break repair was first demonstrated in the bacteria Micrococcus radiodurans. This bacterium requires high doses, on the order of 500 krads, to effect significant cell killing. Kitayama and Matsuyama observed complete repair of double strand breaks in 3 hours in a Micrococcus radiodurans strain after a dose of 220 krads, and repair did not occur in the presence of chloramphenicol.

E. coli has a D_{37} of 15 krads and is significantly more sensitive to x-rays than M. radiodurans. The D_{37} initially induces about 8 to 9 double strand breaks per cell. Using low speed centrifugation, Krasin and Hutchinson (1977) have shown repair of double strand breaks in E. coli. The repair of double stranded breaks did not occur in a reCA mutant

strain. The repair of double strand breaks was observed when the cells were grown in a rich medium containing glucose and amino acids, with the result that there are 4 to 5 genomes per cell. If the cells were grown up in medium which the carbon source was aspartate, then the D_{37} was 3 krads, the number of genomes per cell was 1.3, and double strand break repair was not observed. The conclusion of Krasin and Hutchinson was that repair of double strand breaks in E. coli requires the presence of duplicate genomes, and a functional RECA gene product. Krasin and Hutchinson (1981) have further shown that repair is inducible with UV pretreatment.

It is likely that double strand breaks induced in mammalian cells are also repairable. If the value for the efficiency of strand breakage, 0.58×10^{-10} breaks per krad dalton (Resnick and Martin, 1976), is multiplied by the mass of the human diploid genome, 4.2×10^{12} daltons (Kornberg, 1979), one finds that 4-5 rads will initially induce 1 break per diploid genome. Since the D_{37} of human cells averages about 150-200 rad (Okada, 1970), it is likely that most of the initial breaks are repaired. However, direct biochemical demonstration of this repair is complicated by the inability of sucrose gradient sedimentation technique to measure the molecular weight of intact DNA molecules with molecular weights larger than 2×10^9 daltons (Hutchinson and Krasin, 1977). In the sedimentation experiments involving mammalian cells it has been necessary to give the control

cells doses of 5 krads. The sedimentation of unirradiated DNA from mammalian cells produces results which are not reproducible (Corry and Cole, 1973). Experiments examining the repair of double strand breaks in mammalian systems have yielded conflicting results. Corry and Cole (1973) observed the repair of double strand breaks in Chinese Hamster ovary cells treated with doses less than 50 krads. However, Dugle et al (1976) and Lehman and Ormerod (1970) were unable to observe repair using doses from 20-50 krads. These doses used to study repair in mammalian cells are very high compared to a D_{37} of 150-200 rads. Thus, the negative results do not offer convincing evidence for the absence of double strand break repair in mammalian cells. The doses used to demonstrate repair in yeast are of the same order of magnitude as the D_{37} of RAD+ cells. The survival of the diploid RAD+ strain was 43% after 47.5 krads, which is the dose used to assay for repair.

The part of the model of figure 21 showing induced synthesis of repair enzymes is also applicable to other organisms. As previously stated the repair of double strand breaks in E.coli is inducible. It is likely but has not been shown that such enzymes that repair breaks are under LEXA control. Kenyon and Walker (1980) have evidence for the existence of a set of genes in E. coli, din A-F, which exhibit increased transcription upon treatment with DNA damaging agents. The role of protein synthesis in the repair of x-ray damage in mammalian cells is not known.

Probably the clearest demonstration in mammalian cells of an enhanced response to DNA damaging agents are the experiments of Samson and Schwartz (1980). They have shown an adaptive response to mutagens that produce alkylation damage in Chinese Hamster Ovary and human cells. The treatment of Chinese Hamster Ovary cells with low levels of 1-methyl-3-nitro-1-nitrosoguanine enhances their resistance to high levels of 1-methyl-3-nitro-1-nitrosoguanine.

As stated in the introduction the fate of induced double strand breaks can only be inferred from the chromosomal aberrations seen at the first mitotic division after irradiation. Thus, in mammalian cells the fixed lesions are directly observable. However, the fixed lesions observed in mammalian cells may not be the same as the fixed lesions in rad52 and rad54 yeast cells. Whether initially induced double strand breaks in mammalian cells enter an uncommitted state in a manner analogous to the model illustrated in figure 21 is not known.

Recently, Tobias et al (1980) have proposed a time dependent model to analyze x-ray survival curves. The model assumes that the rate lesions are repaired is proportional to the number of existing lesions, L , plus the square of the number of existing lesion, L^2 . Presented mathematically their formula is

$$-\frac{dL}{dt} = \lambda L + kL^2$$

L can be assumed to represent the number of double strand breaks per cell. The kL^2 term represents the repair of lesions that result from an interaction of independently produced breaks and cause cell death. The λL term represents repair which results in restoration of DNA to its original form. By representing L as double strand breaks per cell, the λ coefficient becomes proportional to $[E]$, where $[E]$ was represented in the results section as the cellular activity of the RAD54 gene product.

The experiments examining enhanced survival upon delaying the addition of CYH provide evidence in rad54-3 cells that λ is a function of time. 7.5' after irradiation λ begins increasing, and evidence has been presented in figure 14 that by 30'-45' the increase in λ has stopped. The 30'-45' time period is short compared to the time period which double strand breaks are fixed into lethal lesions.

The experiments of fixation using rad54-3 strains provide a further analysis of the dependence of λ on time. However, the process of fixation of lesions in cells incapable of repair (rad54-3 cells at 36°), may be different from the process of fixation of lesions which have not been repaired in a cell capable of repair (RAD+). If fixation in cells incapable of repair and cells capable of repair share common elements, then following an initial increase after irradiation, λ progressively declines.

In conclusion, the data from this thesis shows that x-ray induced double strand breaks have several possible fates. In RAD+ cells and rad54-3 cells at 23° a break is very likely to be repaired. However, at 36° in rad54-3 cells and possibly at a low frequency in RAD+ cells the break is not repaired and enters an uncommitted state. The longer the lesion is uncommitted, then the hypothesis is the slower is the proportional rate of repair of the lesion. If uncommitted lesions become fixed lesions, the result is cell death in some unknown manner. The presence of protein synthesis after radiation increases the probability the break will enter the repair pathway rather than the fixation pathway. This increased probability of repair is probably related to an inducible repair component.

APPENDIXCalculations from figure 14

The number of breaks repaired during the 5 hour post-irradiation treatment at 23° is computed using the relation

$$B_1 - B_2 = -\ln\left(\frac{S_1}{S_2}\right) \quad (3)$$

where S_1 and S_2 represent two different survivals, and B_1 and B_2 represent the corresponding number of breaks left unrepaired at survivals S_1 and S_2 . When cells are treated with the protocol of figure 13B, the number of breaks repaired is

$$B_{36} - S_{13B} = -\ln\left(\frac{S_{36}}{S_{13B}}\right) = \ln(.0152\%/.267\%) = 2.9 \quad (A.1)$$

S_{36} equals the survival at 36° and S_{13B} equals the survival of cells treated with the protocols of figure 13B. B_{36} is the number of breaks left unrepaired at 36°, and B_{13B} is the number of breaks left unrepaired when cells are treated according to the protocol of figure 13B. Hence, the number of breaks repaired when cells are treated according to the protocol of figure 13B is $B_{36} - B_{13B}$. When cells are treated with the protocol of figure 13C, a 5 hour incubation of the cells with CYH, the number of breaks repaired is equal to

$$B_{36} - B_{13C} = -\ln\left(\frac{S_{36}}{S_{13C}}\right) = -\ln(.0152\%/.032\%) = .7 \quad (\text{A.2})$$

When cells are treated with the protocol of figure 13D with $t=30'$, the number of breaks repaired is equal to

$$B_{36} - B_{13C} = -\ln(.0152\%/.129\%) = 2.1 \quad (\text{A.3})$$

The relation (8) from the text states

$$B_{36} - B_{13B} = k[E_0]5 = 2.9 \quad (8)$$

Dividing by 5 one obtains

$$k[E_0] = .58 \text{ breaks repaired/hour} \quad (\text{A.5})$$

By comparing the relations (A.1) and (A.2), one obtains the relation

$$B_{36} - B_{13C} = \frac{1}{4}(B_{36} - B_{13B}) \quad (\text{A.4})$$

By using the relations (8) and (9)

$$B_{36} - B_{13C} = k[E_0] \frac{(1 - e^{-5\sigma})}{\sigma} \quad (9)$$

of the text, and the relation (A.4), the value of $\frac{1}{\sigma}$ can be found.

$$k[E_0] \frac{5}{4} = k[E_0] \frac{(1-e^{-5\sigma})}{\sigma} \quad (\text{A.6})$$

$\frac{1}{\sigma} = 1.28$ hours is a solution. The predicted number of breaks repaired when cells are subjected to the protocol of figure 13D, with $t=30'$, is obtained using the relation (10) from the text and the solution of σ from equation (A.6).

$$B_{36} - B_{13D} = k[E_0] \left(.5 + \frac{1-e^{-4.5\sigma}}{\sigma} \right) = 1.0 \quad (\text{A.7})$$

The experimental value of breaks repaired is 2.1. To calculate the predicted value of survival if cells are treated according to the protocol of figure 13D, S_{13D} , one uses the relation

$$\frac{S_{13D}}{S_{36}} = e^{-(B_{13D} - B_{36})} \quad (\text{A.8})$$

or inserting the experimental value of S_{36} and the predicted value of breaks repaired one obtains

$$S_{13D} = (.0152) (e^{1.0}) = .041\% \quad (\text{A.9})$$

The actual value for survival is $.129\% \pm .012$.

If the cells are treated according to the protocol of figure 13D with $t=45'$, then the predicted number of breaks repaired is

$$B_{36} - B_{13D} = k[E_0] \left(.75 + \frac{1 - e^{-4.25\sigma}}{\sigma} \right) = (.58)(2) = 1.2 \text{ (A.10)}$$

The experimental value of breaks repaired with cells treated with the protocol of figure 13D, with $t=45'$ is

$$B_3 - B_{13D} = -\ln\left(\frac{.0152\%}{.289\%}\right) = 2.9 \quad \text{(A.11)}$$

The survival of cells treated according to the protocol of figure 13D with $t=5'$ is predicted to be

$$S_{1D} = (.0152)(e^{1.2}) = .050\% \quad \text{(A.12)}$$

The actual value of survival is $.289\% \pm .029$. The values of survival predicted from the steady state hypothesis differ significantly from the actual values.

The 10 + 15 krad split dose experimental calculations.

The number of breaks repaired when cells are treated with the protocol of figure 13B is

$$B_{36} - B_{13} = -\ln\left(\frac{S_{36}}{S_{13B}}\right) = -\ln\left(\frac{.0042}{.135}\right) = 3.3 \quad \text{(A.13)}$$

This result combined with the equation (8) from the text

$$B_{36} - B_{13D} = k[E_0]5 \quad (8)$$

gives the result

$$5[E_0]k = 3.3 \quad (\text{A.14})$$

dividing by 5 one obtains

$$k[E_0] = .65 \text{ breaks repaired/hour}$$

In the presence of CYH the number of breaks repaired is equal to

$$B_{36} - B_{13C} = -\ln\left(\frac{S_{36}}{S_{13C}}\right) = -\ln(.0042\%/.011\%) = 1.0 \quad (\text{A.15})$$

Hence in this experiment

$$B_{36} - B_{13C} = \frac{1}{3.3}(B_{36} - B_{13B}) \quad (\text{A.16})$$

This equation can be used to solve for $\frac{1}{\sigma}$ and the solution as shown previously is 1.6 hours. Thus the predicted number of breaks repaired when cells are subjected to the protocol of figure 13E, with $t=30'$ is equal to

$$B_{36} - B_{13E} = k[E_0] \left(1.5 + \frac{1 - e^{-4.5\sigma}}{\sigma}\right) = (.65)(2) = 1.3 \quad (\text{A.17})$$

The experimental value of breaks repaired when cells are treated according to the protocol of figure 13E is equal to

$$B_{36} - B_{13E} = -\ln\left(\frac{S_{36}}{S_{13E}}\right) = -\ln(.0042\%/ .079\%) = 2.9 \quad (\text{A.18})$$

The value of survival predicted from the steady state hypothesis can be obtained using the experimental value for survival at 36°, and the predicted value for the number of breaks repaired. The value of survival predicted from the steady state hypothesis can be obtained from the relation

$$S_{13E} = S_{36} e^{-(B_{13E} - B_{36})} = (.0042)(e^{1.3}) = .015\% \quad (\text{A.19})$$

The actual value of survival is .079% ± .006 which is significantly different from .015%. The predictions of the steady state hypothesis differ from the experimental data.

2.5 + 22.5 krad split dose experiment

In this experiment the number of breaks repaired when cells are treated with the protocol of figure 13B is equal to

$$B_{36} - B_{13B} = -\ln\left(\frac{S_{36}}{S_{13D}}\right) = -\ln\left(\frac{.0042\%}{.151\%}\right) = 3.6 \quad (\text{A.20})$$

Using this relation, and the relation (8) from the text

$$B_{36} - B_{13B} = k[E_0]^5 \quad (8)$$

one can solve for $k[E_0]$.

$$k[E_0] = \frac{3.6}{5} = .72 \quad (\text{A.22})$$

The number of breaks repaired when cells are treated according to the protocol of figure 13C is equal to

$$B_{36} - B_{13C} = -\ln\left(\frac{.0042\%}{.024\%}\right) = 1.7 \quad (\text{A.23})$$

By combining the equations (A.20) and (A.23) one obtains the relation

$$\frac{1}{2}(B_{36} - B_{13B}) = (B_{36} - B_{13C}) \quad (\text{A.24})$$

Using the relation (8) and (9) of the text one obtains the relation

$$k[E_0] \frac{5}{2} = k[E_0] \left(\frac{1 - e^{-5\sigma}}{\sigma} \right) \quad (\text{A.25})$$

Solving the equation yields a value of $\frac{1}{\sigma} = 3.1$ hours. The number of breaks repaired when the protocol of figure 13F is followed with $t = 0'$, is predicted to be less than or equal to

$$B_{36} - B_{13F} = k[E_0] \left(.5 + \frac{1 - e^{-4.5\sigma}}{\sigma} \right) = (.72)(2.9) = 2.1 \quad (\text{A.26})$$

The experimental value for breaks repaired when cells are subjected to the protocol of figure 13F is calculated to be

$$B_{36} - B_{13F} = -\ln\left(\frac{.0042\%}{.106\%}\right) = 3.2 \quad (\text{A.27})$$

This value is different from the predicted value of 2.1. The predicted value of survival if cells are treated according to the protocol of figure 13F is less than or equal to

$$S_{13F} = (S_{36}) (e^{2.1}) = .034\% \quad (\text{A.27})$$

This value differs significantly from the experimental value of $.106\% \pm .010$.

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