

PULSED FIELD GEL ELECTROPHORETIC STUDY OF THE REPAIR OF
X-RAY-INDUCED DNA DOUBLE-STRAND BREAKS IN YEAST

BY

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A thesis submitted to the Postgraduate School, Ahmadu
Bello University, Zaria, in partial fulfillment of the
requirements for the award of the degree of Master of
Science in Radiation Biophysics.

November, 1997

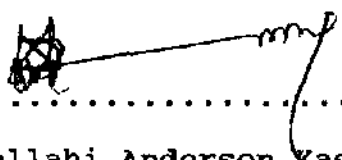
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I hereby declare that:

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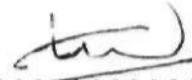
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CERTIFICATION

This thesis entitled "PULSED FIELD GEL ELECTROPHORETIC STUDY OF X-RAY-INDUCED DNA DOUBLE-STRAND BREAKS IN YEAST" by Abdullahi Anderson Kassimu meets the regulations governing the award of a degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literal presentation.


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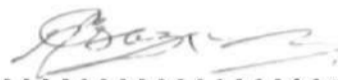
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DEDICATION

For dad, Mallam Kassimu Umar Akpele; mum, Hajia Rabietu
Kassimu; my junior brothers and sisters; my wife, Nafisatu
Abdullahi; and to the glory of ALLAH (S.W.T.).

ACKNOWLEDGEMENT

In the name of Allah, the compassionate, the merciful. Praise be to Allah (S.W.T.)! May the peace and blessings of Allah (S.W.T.) be on our noble prophet Muhammad (S.A.W.), his family, companions and *Ummah* up to the last day (Amen).

I am very grateful to the Nigerian Air Force authorities for granting me a study leave and to Prof. Dr. Jürgen Kiefer of *Institut für Biophysik, Strahlencentrum der Justus-Liebig Universität, Gießen, Germany* for arranging for me to carry out my research work in his laboratory through a fellowship from the German Academic Exchange services (DAAD).

My profound gratitude go to the following: my supervisors, Drs T.C. Akpa and B.W. Jimba for their guidance, kindness and encouragement; my head of Department Prof. I.B. Osazuwa, his predecessors Profs. S.B. Ojo and Jacob Adetunji, the Dean Post-Graduate (PG) School, Prof. J.Y. Olayemi, the Dean Faculty of Science, Prof. C.O. Ajayi, the PG programme coordinator Dr. M.N. Umego, Prof. Dalhatu Mohammed, Sheikh Muyyideen, Chief (Alh) S.A. Afegbua, Mrs F.A. Palu-Mustapha, Mrs I.G.E Ibeanu, Drs. Abdullahi, Yunusa, Umar, L.A Dim, S.P Mallam, T. Ige, R. Kangkolo, Mr. Abdulkadir Ajuji, Mallam Rabi, Mr. Owoyele, Mrs. Nwosu, and other lecturers, Research fellows and non-teaching staff of the Department of Physics, Faculty of Science, PG School and Centre for Energy Research and Training (CERT) A.B.U. Zaria, for their support and encouragement.

During my course work and stay in Alexander Hall, A.B.U., the following friends cooperated a great deal: Garba

S. Uthman, Abdul-lateef Oyelade, Abdul-Matin Kassim, Abubakar Umar, Sameer C. Yusuf, Abubakar Bichi, Bala Rano, Adamu Imam, Ibrahim Potiskum, Umar Jalingo, Ahmad Tijani, Tukur Tahir, Aminu Abubakar and other brothers.

The following colleagues, staff, well-wishers or friends in Germany in or outside the *Strahlzentrum, Gießen* assisted me a lot: Dr. Siegfried Rase, Dr. Eckart Schneider, Helga Schneider, Beate Barth, Petra Bepler-Kranert, Nicole Scheerer, Barbara Beier, Ulrike Weidmann, Hans-Dieter Port, Frank Gutermuth, Koekoeh Santoso and other members of group "B3"; Frau Dr. Marlis Frankenberg-Schwagger of *Universität Göttingen* who provided some materials on Rad 54-3 yeast, Frau Kiefer, Dr. Ingmar Kiefer, *Priv. Doz. Dr. Reiner Johannisson* of *Medizinisches Universität, Lübeck*; Dr. Med. Michael Weber of *Universität Heidelberg*; Herr Jürgen Johannisson and family in *Rheine-Munster*; Opa Johannes Johannisson and wife Ilse Woynack in *Lübeck*; Herrn Josef Dietel and family of *Studentenwohnheim, Eichendorffring, Gießen*; Herrn Heinrich Wagner and family in *Buseck*; Mr. Abul Quadri and family from *Bangladesh*; Professor Dr. Chakraborty and family from *Sri Lanka* and fellow-students like Al-hajj Lonnol Brahmantio (back to *Indonesia*), Rashid Diia from *Egypt*, Putu Kutzer-Yusuf from *Bali, Indonesia*, Khalid Kanjoh from *Syria*, Abdallah Jaber from *Palestine*, Rainer George from *Vogelsberg*, Windsor Tanner from *USA*, Beate Gunther, Timo Traurig, Thekla and others too numerous to mention.

While away in Germany, the following persons assisted my family immensely: Mallams Muhammad Tahir and Sakarada

Musa; Alhaji Tajudeen Rabiou Ademoye, Capt Alban Obi and WO Mohammed Yusuf. May Allah reward them abundantly (Amen).

On return, I was provided accommodation at Basawa barracks, Zaria, courtesy of the commandant NASMP Col J T Ogundele. I am grateful to him, the CO 11 FER, Lt Col EO Ezeodum and their officers and men. I also thank the following for their moral support: Gp Capt AAA Omanaiye, the Commander 1 AMC, NAF Makurdi, Wg Cdr EM Eno and his officers & men, Wg cdr RS Gwani, Lts Col Jibrin Hassan, L Odunwa, Iko Ayuba and NO Hambolu (rtd) & families; Sqn Ldr Manu Ibrahim, Majs AJ Bello and AM Dikko & families; Capts AA Salako, CE Nwanolue, Y Musa, AS Ishaq, S Tsukwam, O Arigu and PC Ogbogbo & families; Flt Lts ADM Hamza, CA Ohwo, YA Isma'il, MA Yakubu, UM Sylvanus, JA Yusuf, CE Egwoba, A Iyamu, MO Idonibo, I Sinni and K Aliyu; Lts KC Okoro, AA Tobun, Bitrus, and Abdulkarim; Fg Offrs TO Yusuf, MA Suleiman, Idris Sani, Danzangi, Abdullahi and AbdulAkeem; Sub Lts MK Bello and K Tenimu; Cpl Abdullahi, wife and Yunus; Sapper Mustapha & family; Hassan Sharafadeen, O.S.A Apampa, Lawal Dalhatu Mohammed, Sani and Saminu Ningi, Gambo, Umar Adamu Basawa, Aliyu, Salisu Abdullahi, Ibrahim Bala, Abdulhamid Jibir, Aliyu Kawu, Nike and Kemi Ajayi and other friends in Nigeria and abroad who helped in one way or the other.

Special thanks are due to my wife, parents, brothers, sisters, relations, and In-laws for their love, patience, support, encouragement and understanding.

Finally, thanks be to ALLAH (S.W.T.) for seeing me through the entire programme and for guiding me so far in the pursuit of knowledge for the benefit of mankind.

ABSTRACT

Diploid yeast cells Rad 54-3 of strain *Saccharomyces Cerevisiae* were prepared for survival assay, double-strand break (DSB), repair and budding delay analysis after irradiation with various doses of X rays.

The survival results provide indirect evidence for the repair of DNA DSB at the permissive temperature 23°C and inability to do so at the restrictive temperature 36°C.

Using the Clamped Homogeneous Electric Field (CHEF) electrophoresis method, the DNA DSB induction frequency was found to be $(7.43 \pm 0.34) \times 10^{-12} (\text{g/mol})^{-1} \text{Gy}^{-1}$. Repair investigation using same method appear to show the occurrence of secondary DSBs and also provided direct evidence for the repair of DSB at 23°C post-irradiation incubation in growth medium and inability to repair the lesions at 36 °C. The secondary DSBs could be due to enzymatic incisions at the damage sites enhanced by incubation in growth medium while the monophasic repair, found to lack the fast repair component, could be due to the relatively low dose used (maximum of 82 Gy).

A parallel experiment to the repair investigation - budding delay analysis - showed that budding delay is dependent on irradiation dose and incubation temperature.

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ABBREVIATIONS

CCD	Charge-Coupled Device
CHEF	Clamped Homogeneous Electric Field
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Breaks
EB	Ethidium Bromide
EDTA	Ethylenediamine tetra-acetic acid
FIGE	Field Inversion Gel Electrophoresis
L.E.T	Linear Energy Transfer
mbp	Megabase pairs
NDS	Sodium-Detergent-Sarcosyl buffer
OFAGE	Orthogonal Field Alteration Gel Electrophoresis
RNA	Ribonucleic Acid
ROFE	Rotating Field Electrophoresis
rpm	Revolutions per minute
TAFE	Transverse Alternating Field Electrophoresis
TBE	Tris-HCl, Boric Acid and EDTA buffer
UV	Ultra-Violet light

1.1 DNA Damage and Survival

It is generally accepted that Deoxyribonucleic acid (DNA) is a primary cellular target for lethal damage by X- and other ionizing radiations (Kiefer, 1990). A variety of lesions induced by ionizing radiation in biological systems exposed to it include DNA strand breakage, base damages and DNA-protein crosslinks. Of these, DNA double-strand breaks (DSBs) are considered to be the main lesions with regard to cell killing (Bedford *et al.*, 1978 and Blöcher and Pohlit, 1982) and a number of other biological endpoints (Bender *et al.*, 1974; Bryant, 1984 and Frankenberg-Schwager *et al.*, 1988). The repair of these lesions is an important factor that determines cell survival (Budd *et al.*, 1982).

1.2 Background To Previous Works

DNA DSB induction by X-rays and α -particles in yeast cells was introduced by Frankenberg *et al.* (1981) while that by different ions of various Linear Energy Transfer (LET) was done by Akpa *et al.* (1991). The mutant Rad 54-3 of yeast strain *Saccharomyces cerevisiae* was found to be temperature-conditional in DSB rejoining. It is able to rejoin X-ray induced DSB at 23°C but unable to do so at 36°C (Budd and Mortimer, 1982).

Determination of the induced DSB by different types of ionising radiation has been done using sucrose gradient sedimentation (Budd *et al.*, 1982 and Blöcher, 1982) and elution technique (Bradley and Kohn, 1979). These standard methods have been shown to be either fairly insensitive (Lehmann and Stevens, 1977), very laborious (Blöcher, 1982) or ambiguous (Okayasu *et al.*, 1988). Moreover, they usually require radioactive labelling of the DNA (Blöcher *et al.*, 1989).

The method of pulsed-field electrophoresis discovered by Schwartz and Cantor (1984) takes care of some of these shortcomings. The Clamped-Homogeneous Electric Field (CHEF) gel electrophoresis designed by Chu *et al.* (1986) which allows the separation of yeast chromosomes in straight lanes and distinct bands, has been used to determine DSB in mammalian cells (Blöcher *et al.*, 1986 and Löbrich *et al.*, 1994) and yeast cells (Löbrich *et al.*, 1993).

The induction and repair of DSB in Rad 54-3 has been investigated by sucrose gradient sedimentation (Budd and Mortimer, 1982). Electrophoretic techniques are yet to be employed in such studies.

1.3 The Present Work

The main objective of this work is to investigate the induction and repair of DSB in X-irradiated Rad 54-3 mutant of yeast strain *Saccharomyces cerevisiae* in growth medium at permissive (23°C) and restrictive (36°C) temperatures by

CHEF electrophoresis so as to compare the induction frequency and repair kinetics with earlier results obtained through other methods.

The work is designed to include survival and budding studies so as to establish a relationship between the percentage of budding cells in X-irradiated Rad 54-3 cells suspended in full medium (without Agar), incubation temperature and the radiation dose.

Chapter Two

INDUCTION, REPAIR AND MEASUREMENT OF DNA DSB

2.1 Induction of DSB

A DNA molecule is made up of double-stranded helix chain of deoxyribonucleic acid units joined by phosphodiester bridges formed between 5'-hydroxyl groups of one nucleotide and 3'-hydroxyl group of the next (see Figure 2.1).

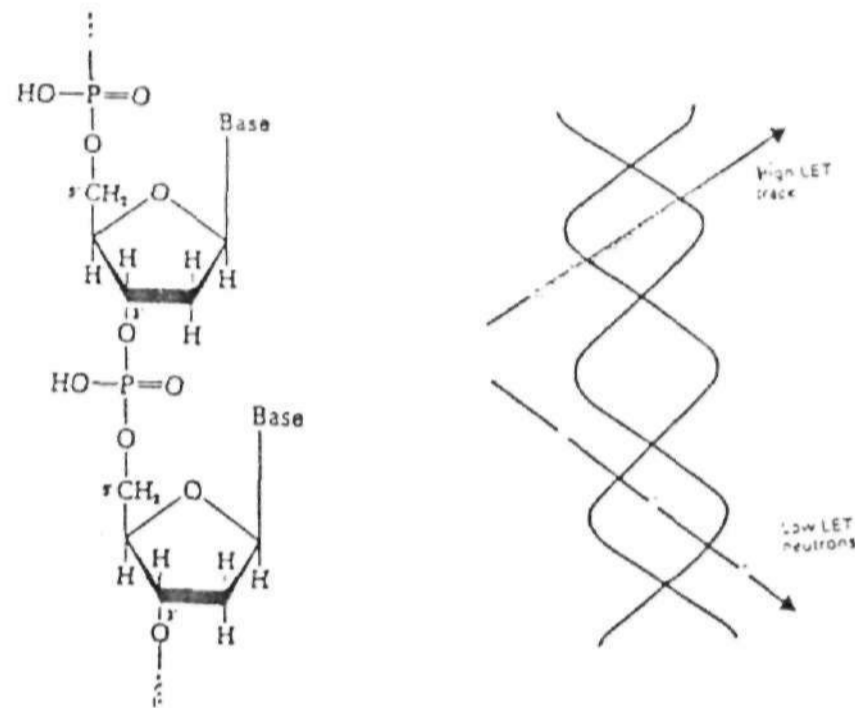


Figure 2.1 Schematic diagram of the DNA molecule and passage of radiation through it (after Akpa, 1992)

When the DNA is exposed to chemical or physical agents, energy is deposited at various points in the chain, culminating into some physical processes like radical formation and polymerization. Ionization, following energy deposition and chemical reactions occurring at and disrupting the backbone of DNA strand is called strand break. A single-strand break (SSB) occurs if only one of the two strands is broken. This may lead to internal deformation of the molecule but its size is maintained. However a double-strand break (DSB) occurs if two breaks in a molecule are within a distance of a few nucleotide pairs. The molecule becomes fragmented if originally linear or linearized if circular (Akpa, 1992).

2.2 Repair of DSB

DNA DSB is thought to be repaired by a recombinational process. One model, which still has to be proved experimentally, was suggested by Orr-Weaver and Szostak (see Kiefer, 1990).

As shown in Figure 2.2 one strand of DNA containing the DSB (heavy lines) invades the double strand of homologous DNA, displacing the other and creating a "D-loop". The gaps are enlarged and then filled by repair replication (broken lines). The newly replicated strands are ligated to the end of the DSB. Two cross points are thus formed which may be resolved by single strand breaks and ligation.

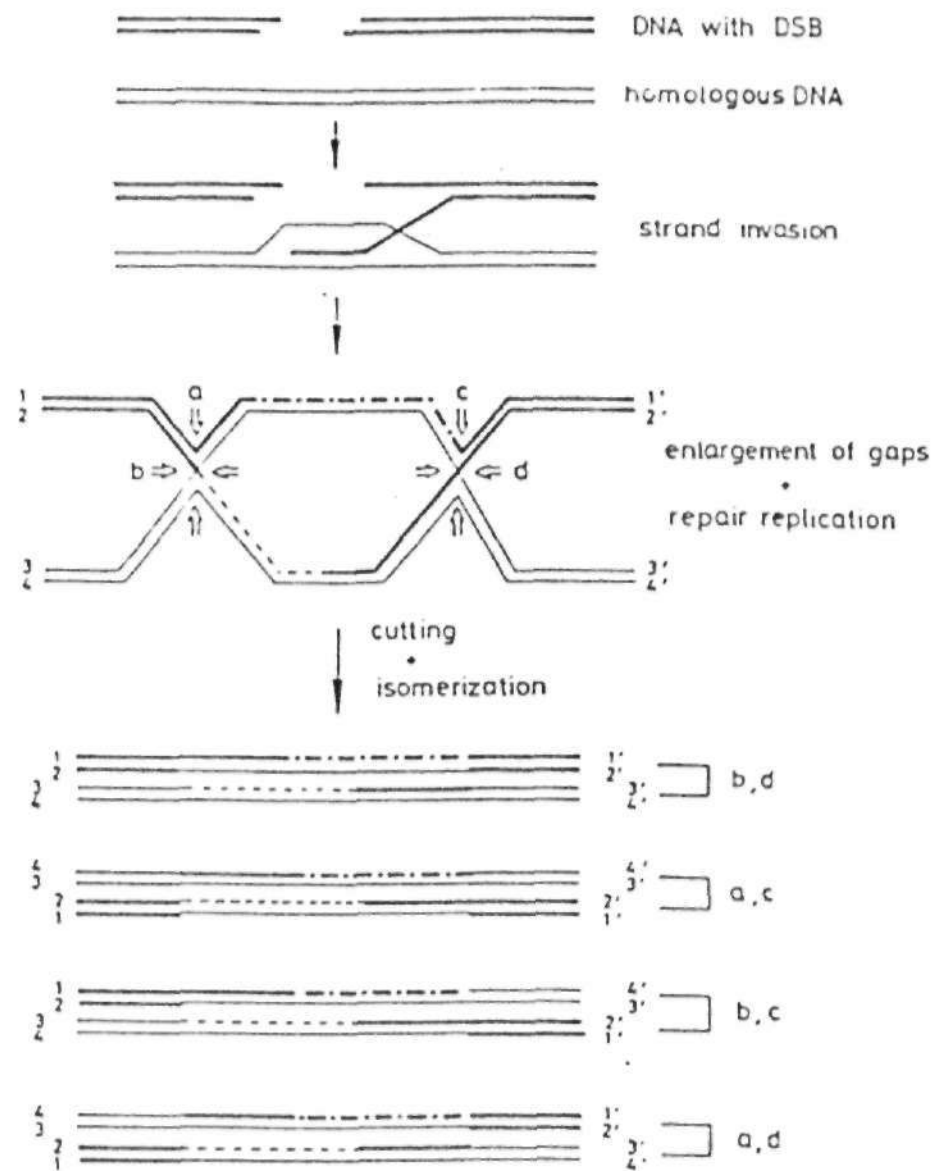


Figure 2.2 A proposed scheme for DSB repair by recombination. (after Kiefer, 1990)

The breaks may be in the inner or the outer strands. Isomerization may then lead to four different structures. If one inner and one outer strand is cut (b, c, and a, d), the larger part of the two DNA strands are exchanged (reciprocal recombination). Mutation results when DSBs are misrepaired while cell death occurs when DSBs are unrepaired (Akpa, 1992)

2.3 Measurement of DNA DSB

Methods of measuring DNA DSB include Sedimentation, Elution and Electrophoretic techniques.

2.3.1 Sedimentation Method

This method include velocity, boundary and nucleoid techniques.

i. Velocity sedimentation

In a sucrose gradient, the coefficient of sedimentation depends on the molecular weight. Anomaly occurs in the sedimentation pattern due to the distortion of the coiling chain by non-uniform frictional forces which depend on the square of the centrifuge speed and molecular weight of the particle. Such anomaly manifests in DNA of molecular weight of more than 10^8 , like yeast and mammalian

DNAs, for which centrifuging speeds of less than 10,000rpm are employed (Zimm and Schumacher, 1976).

ii. Boundary Sedimentation

DNA preparation with unbroken strands move in an ultra- centrifuge with a boundary detectable with UV-optical systems. Secondary boundaries occur as a result of impurities or presence of degraded molecules. The fraction of broken molecules in an initially homogeneous DNA sample is determined by quotient of the length of the slower or distorted boundary over the length of the sharp boundary in the optical trace. This represent the fraction of strand breaks (Davison and Freifelder, 1962).

The number of strand breaks determined with this method represents only minimal values, since breaks very close to the ends of the molecules can not be distinguished as separate.

iii Nucleoid Sedimentation

Nuclear RNA and DNA are released when cells are lysed in non-ionic detergents with high salt concentrations. The unbroken DNA is supercoiled and compact and sediment faster than broken DNA which are relaxed. Measurement of banding intensity can be made after staining with Ethidium Bromide. DSB frequency is estimated from the ratio of broken over unbroken DNA. Sedimentation behaviour is biphasic for

unirradiated DNA. The control must therefore be given a priming dose to remove the biphasic component (Cook and Brazell, 1976).

2.3.2 Elution Method

There are two types - filter elution and unwinding technique.

i Filter Elution

This technique is based on the fact that free DNA in solution has been found to pass through a filter system at a rate which depends on its characteristic length. The method is applicable to large DNA (mammalian) in which sedimentation method has limitation (Balbi *et al.*, 1986).

ii Unwinding technique

During the unwinding process, the DNA rotates approximately like a rigid rod about a single axis. When broken, the molecules cease to rotate as a single rod, but different sections pursue independent rotation leading to unwinding. The fragments can be separated by eluting in hydroxylapatite column (hydroxylapatite chromatography) using alkaline solutions of different strengths (Walker and McLaren, 1965).

2.3.3 Pulsed Field Electrophoresis

Conventional electrophoresis with constant continuous electric field gives better results for small molecules than sedimentation technique but its application is limited to small molecules up to the size of hundred thousands of base pairs. Use of the technique for higher molecular weights require the application of priming doses to reduce the DNA lengths (Akpa, 1992). However, if at least one of the two directions has a pulsating electric field, larger molecular weights can be separated. All pulse-field electrophoresis techniques in use today are improvements on the system developed by Schwartz and Cantor (1984). The alterable parameters are the field angle, pulse time, applied voltage, gel concentration, run time and temperature.

This gives rise to various pulsed field orientation and systems like the Contour-clamped Homogeneous Electric Field (CHEF) electrophoresis, Orthogonal-field Alteration Gel Electrophoresis (OFAGE), Field inversion Gel Electrophoresis (FIGE), Transverse Alternating Field Electrophoresis (TAFE), and the Rotating Field gel Electrophoresis (ROFE)

i. CHEF Electrophoresis

This system, developed by Chu et al. (1986) with field angle alternating at 120° , can be used to separate entire yeast chromosomes and other molecules with lengths up to 10mbp in sharp bands.

ii Orthogonal Field Alteration Gel Electrophoresis (OFAGE)

This type of electrophoresis was first introduced by Carle and Olson (1984). In this system, the electric field alternates at 90°. It can be used to separate the entire chromosomes in yeast in bands within the gel slab. Differences in intensity of bands formed by the control and treated cells, suggested to be due to fragmentation, can be analysed for evaluating strand breaks (Contenpolou *et al.*, 1987).

iii. Field Inversion Gel Electrophoresis (FIGE)

The field inversion gel electrophoresis (FIGE) was found to be suitable for separating DNA by size. A system with electric field alternating at 180°, it was first described by Carle *et al.* (1986). A single modified form of FIGE, the asymmetric FIGE was claimed to be specific for DSB measurement (Stamato and Denko, 1990).

Chapter Three

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Yeast Strain

The diploid Rad 54-3 (X754-3860) mutant of yeast strain *Saccharomyces cerevisiae* which originated from Dr. Frankenberg-Schwager of GSF, Frankfurt, was used for all experiments and investigations carried out in this work. Its genotype is:

$$\frac{ahis1 \quad Leu2 \quad + \quad trp5 \quad + \quad tup7 \quad rad54-3}{ahis1 \quad + \quad ade \quad trp5 \quad Ura3 \quad tup7 \quad rad54-3}$$

At 36°C this mutant is unable to rejoin X-ray induced DSB, resulting in its maximum sensitivity, whereas at 23°C DSB rejoining occurs and cells are radioresistant (Budd and Mortimer, 1982).

3.1.2 Media

(i) Full, Complete or YPD Medium

Rad 54-3 diploid cells were grown to stationary phase on full medium in petri dishes at 30°C (3 to 5 days). Full medium consists of 1% Yeast extract, 2% Peptone, 2% Glucose

and 1.7% Agar dissolved in deionised water. For the repair experiments agar was excluded.

(ii) Succinate Medium

Incubation of yeast cells during survival experiment was done on Full and Succinate Media. The latter is obtained when the energy source in Full medium (Glucose) is replaced by 2.7% Sodium Succinate (Na-Succinate).

3.1.3 Stock Solutions for Electrophoresis Experiment

(i) Protoplasting Solution

This contains 2mg/ml Lyticase (SIGMA): activity 4000 U/ml and 0.01M Sodium Phosphate in 50% Glycerol (v/v).

(ii) LET-buffer (Prelysing solution)

This contains 0.5M EDTA (pH 8.0), 0.01M Tris (pH 7.5) and 7.5% 2-Mercaptoethanol.

(iii) NDS-buffer (Lysing solution)

This contains 0.5M EDTA (pH 8.0), 0.01M Tris (pH 7.5), 1% Sodium Laurysarcosine and 2mg/ml Proteinase K.

(iv) Gel Run Buffer (5xTBE)

This contains 0.45M Tris-base, 0.45M Boric Acid and 0.01M EDTA.

(v) Gel Stain Solution

This contains 0.5 μ g/ml Ethidium Bromide in 0.1M NaCl, 0.001M EDTA and 0.05M Tris-HCl (pH 7.5).

3.1.4 Irradiation Facilities**(i) X-ray Source**

The X-ray source used is a spectral analysis tube type FA 100/3 with tungsten anode and beryllium window (MÜLLER, HAMBURG, GERMANY). The power source is from an external high voltage generator type PW1140/00 (PHILLIPS, HAMBURG, GERMANY). The tube was operated at a constant condition of 80kV, 30mA with no filters. All irradiations were done under oxic conditions, in solution.

3.2 METHODS**3.2.1 Dosimetry**

Chemical (Fricke) dosimetry was used. The Fricke solution contains 0.4M concentrated H₂SO₄, 0.001M FeSO₄.7H₂O and 0.001M NaCl in double-distilled water. The NaCl desensitizes the system towards organic impurities, so that water of a lower quality may be satisfactorily used.

The high voltage power and X-ray sources were warmed up as specified. Four sets of 2.5ml Fricke solution in glass dishes were irradiated for 1min, 2min, 3min and 4min respectively. The extinctions of the control and irradiated

Fricke solution were measured with a PMQ3 Photometer (ZEISS, GERMANY) set to 304nm in 0.5 cm Quartz Special (QS) glass cuvettes.

The measured extinctions were plotted against the irradiation timings to give a straight-line graph as shown in Figure 3.1. The dose rate was calculated with the aid of the slope obtained.

Dose rate D (in Grays/min; Kiefer, personal communications) is given as:

$$D = \frac{E \times 1.207 \times 10^6}{\Delta\epsilon} \text{ Gy/min} \quad \dots\dots 3.1$$

where:

E = difference in optical density per sec between irradiated solution and control = 0.146/min (Figure 3.1).

$\Delta\epsilon$ = difference in molar extinction coefficient between Ferric and Ferrous ions at 304 nm.

At room temperature of 22°C $\Delta\epsilon = 2152$

Substituting,

Dose rate becomes 82 Gy/min

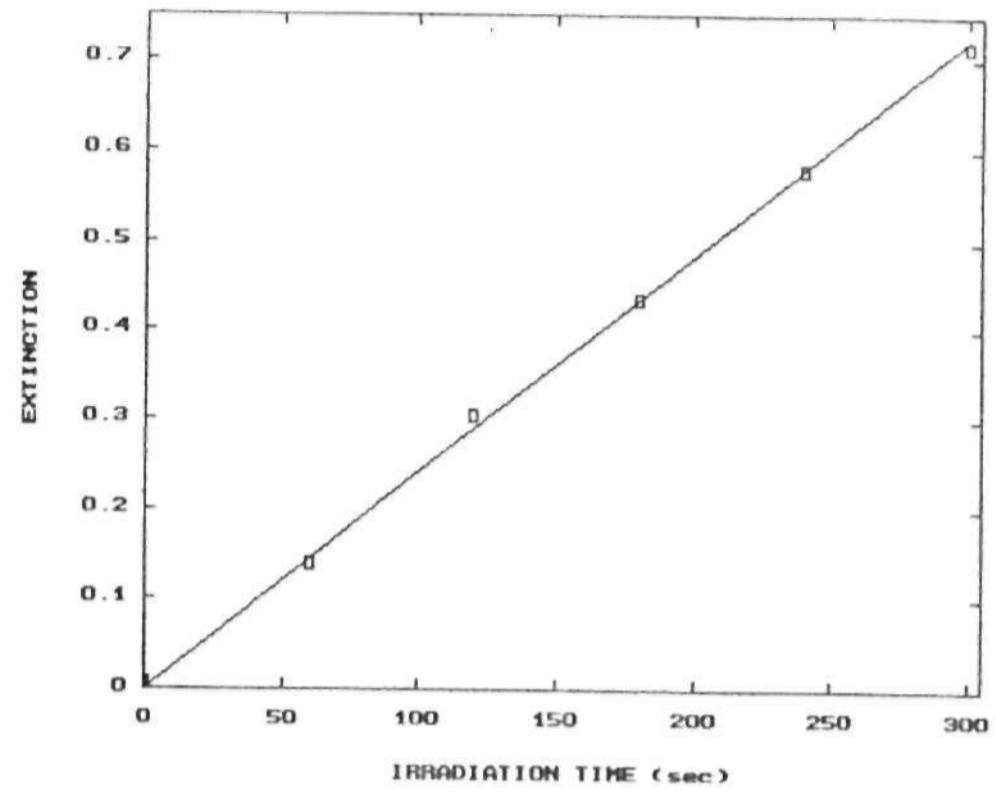


Figure 3.1 Fricke dosimetry curve: Extinction vs
Irradiation time

3.2.2 Cell Survival Investigation

Rad 54-3 cells were grown for between 3 to 5 days on agar plates containing complete medium. They were harvested, washed thrice with sterilized double-distilled water (bidest), counted and suspended in bidest on ice until ready for use.

2.5ml of cell suspension with concentration of 10^5 cells/ml was pipetted into each of 8 clean 2cm-diameter glass dishes placed on ice. The dishes were irradiated with X-rays at 80kv, 30mA up to 13.1, 39.3, 70.5, 117.8, 157, 235.6, 314, and 392.6 Gy at a dose rate of 70.5 Gy/min. After irradiation, the aliquots necessary to give spaced colonies were plated on lawns of labelled complete and succinate media. Unirradiated controls were also plated. For each dose, equal number of plated complete and succinate media dishes were incubated at 23°C and 36°C.

Macrocolonies of cells plated on full medium at 23°C and 36°C were counted after 4 and 8 days of incubation respectively, while those on Na-succinate medium at 23°C and 36°C were counted after 6 and 10 days of incubation respectively (Frankenberg-Schwager et al., 1987).

The surviving fraction for a given dose was estimated by dividing the number of counted macrocolonies at that dose by the number of counted macrocolonies for the unirradiated control. After normalizing the values, the surviving fraction is plotted logarithmically on the ordinate against the radiation dose on a linear abscissa

scale. The resultant curve, fitted to Equation 3.3 (Kiefer, 1990), is the survival curve.

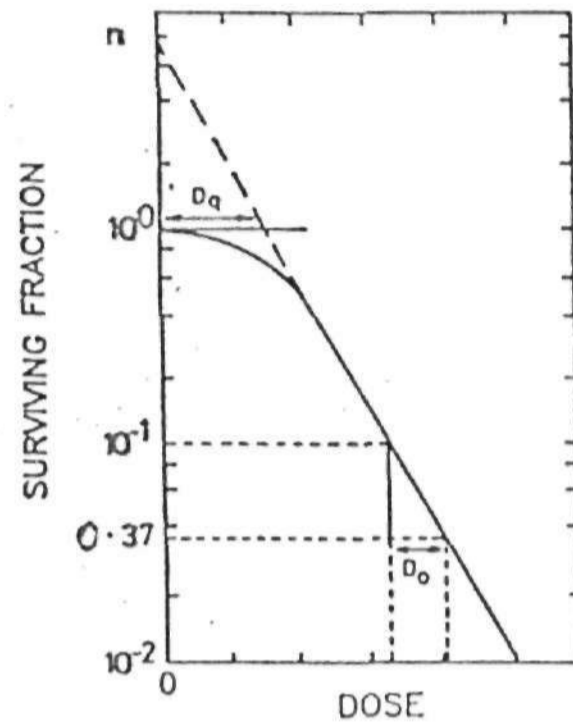


Figure 3.2 Characteristic parameters of shouldered survival curves (after Kiefer, 1990)

Homogeneous cell populations of Yeast display the survival curve shown in Figure 3.2. Due to the stochastic nature of the interaction processes, the radiation effects observed started from the small dose region, where a threshold-like behaviour is seen. As the dose increases, the plot approaches a straight line, indicating a simple exponential relationship (Kiefer, 1990). The curve may be characterised by the three parameters D_0 , D_q and n as shown.

- D_0 is obtained from the terminal part and is the dose necessary to reduce relative survival to e^{-1} or 37%. It is the reciprocal of the slope and characterises the sensitivity of a cellular system. Lower values indicate increasing sensitivity.

- n , the "extrapolation number", is the intercept with the ordinate of the backward extrapolation of the straight portion.

- D_q , the "quasi-threshold dose", is the dose where the backward extrapolation of the straight portion crosses the 100%-survival line.

The relationship between them is given by:

$$D_0 = \frac{D_q}{\ln n} \quad \dots 3.2$$

The survival equation for a homogeneous system is given by:

$$Y = ne^{-D/D_0} \quad \dots 3.3$$

where:

Y = Surviving fraction

n = Extrapolation number

D = Dose (Gy)

D_0 is a constant as shown above, and

$1/D_0$ = slope of exponential part of curve

3.2.3 DSB investigation

(i). Cell Preparation and Irradiation

Cells were harvested, washed, counted and suspended in sterilized double-distilled water on ice as in section 3.2.2 above. An aliquot of 2.5 ml of 1×10^8 cells/ml suspension was irradiated with 41 Gy X-rays. One millilitre of suspension was pipetted into a labelled eppendorf tube placed on ice to forestall repair. Five more dishes were irradiated with 82, 164, 246, 328 and 410 Gy of X-ray respectively and one millilitre of each pipetted into labelled eppendorf tubes on ice. The cells were pelleted by centrifuging with table centrifuge (HETTICH, TUTTLINGEN, GERMANY) for one minute. The supernatant was removed, the cells washed twice in 0.05M EDTA (pH 8) and then replaced on ice.

(ii) DNA Probe Manufacture for PFGE

About 10^8 cells were pelleted from each eppendorf tube after washing once more in 0.05m EDTA (pH 8). The supernatant was removed and traces of liquid carefully dried out with soft tissue paper. The tubes remained on ice.

Thirty microlitres of EDTA (pH 8) and $50\mu\text{l}$ lyticase (SIGMA), the protoplasting enzyme, were pipetted into the eppendorf tubes. After thorough mixing, the protoplast-enzyme mixture was incubated at 37°C for 20 min. Two hundred microlitres of 0.8% low melting agarose (BIORAD) was added to the mixture. The agarose was heated in a

microwave oven until it melted in 0.125M EDTA (pH 7.5). It was then kept in a water bath at 37°C. The gel-protoplast mixture was thoroughly mixed and moulded into PFGE sample blocks of dimension 20 x 9 x 1.2 mm (BIORAD MOULD). The moulds were immediately transferred horizontally into a refrigerator (4°C) for 20 min to enhance faster solidification and to forestall "cell sinking".

The probes were removed from the moulds and incubated in LOW-EDTA-Tris (LET) buffer containing 2-mercaptoethanol for 20 hrs at 37°C to allow the buffer to permeate the gels completely. The LET buffer was removed and replaced with NDS buffer containing the lysing detergent (Sodium Laurysarcosine) and protein digesting enzyme (Proteinase K) and incubated at 50°C for 22 hrs.

After removing the NDS solution, the probes were washed twice in 0.05M EDTA (pH 8), left for 30 min in 0.05M EDTA (pH 8) at room temperature, washed twice in same solution and then incubated overnight (20 hrs) at 37°C in EDTA.

The next day the probes were washed twice in EDTA, left for 30 min in EDTA at room temperature, washed twice again and left at room temperature for 24 hrs.

Finally, the probes were washed twice in EDTA, left for 30 min at room temperature, washed twice again and kept in 0.05M EDTA at 4°C for 4 days before electrophoresis. The above procedure was found to be optimal for electrophoretic investigation. The probes could remain intact for months under these conditions (Löbrich et al., 1993b).

(iii) Electrophoresis

Electrophoresis was done using CHEF-DR III BIORAD system. It consists of a power module, an electrophoresis chamber or cell with casting stand, 10-well comb, tygon tubing and levelling bubble, a variable speed pump, and a model 1000 mini-chiller.

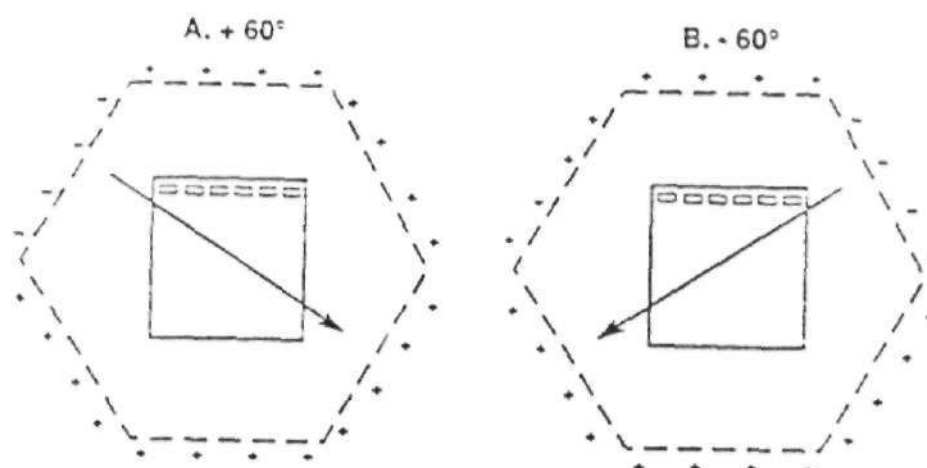


Figure 3.3 Voltage clamping by the CHEF-DR III system.

(A) Relative electrode potentials when the $+60^\circ$ field vector is activated. (B) Relative electrode potentials when the -60° field vector is activated.

(after BIORAD, 1992)

(a) Power Module

The power module contains all the electronics necessary to perform pulsed field electrophoresis. It contains a 350V power supply, the switching functions, and driver for the 24 electrodes. Clamped homogeneous electric field, which is maintained regardless of the field angle selected, is provided by the drivers. This dynamic regulation modulates the potentials so that the upper voltages are maintained regardless of gel size, fluctuation in buffer conductivity or temperature. The fused power supply operates with a maximum voltage gradient of 9V/cm or 300V and a lowest gradient of 0.6V/cm or 200V. Figure 3.3 shows how two pulses (+60° and -60° indicated by arrows) result in a 120° included field angle.

(b) Electrophoresis Cell

This is a 43 x 44 cm acrylic box in which 24 horizontal electrodes are arranged in a hexagon. A 14 x 13 cm gel cast on a platform in a separate casting stand is placed in the centre of the hexagon. The platform is held in place by a frame positioned on the chamber floor. Buffer enters the main chamber through six holes in the floor near the top and exits at the front through a T-fitting with one arm for draining and the other for circulation.

The lid contains an interlock for safety. Current flow is broken if lid is removed, and the high voltage to the chamber is disrupted. It also contains a mount at the upper

right for an external probe, connected to the mini-chiller which monitors buffer temperature in the chamber.

c) Pump and Accessories

The CHEF-DR III system includes a variable speed pump which provides a suitable flow rate of buffer through the chamber. Its power supply is electrically isolated within the power module for safety. It is connected by tygon or plastic tubing to the chamber and mini-chiller so that buffer flows from the chamber to the chiller through the pump and back to the chamber. Its dial is typically set at "70", for a flow rate of 11 per min.

d) Model 1000 Mini-chiller

This is a portable refrigerator specially designed for CHEF-DR III system. As the buffer is circulated through the unique heat exchanger, it is cooled rapidly and efficiently. This cooling is moderated by the external temperature probe, resulting in precise maintenance of set buffer temperature in the chamber. Figure 3.4 shows how the components of CHEF-DR III are connected.

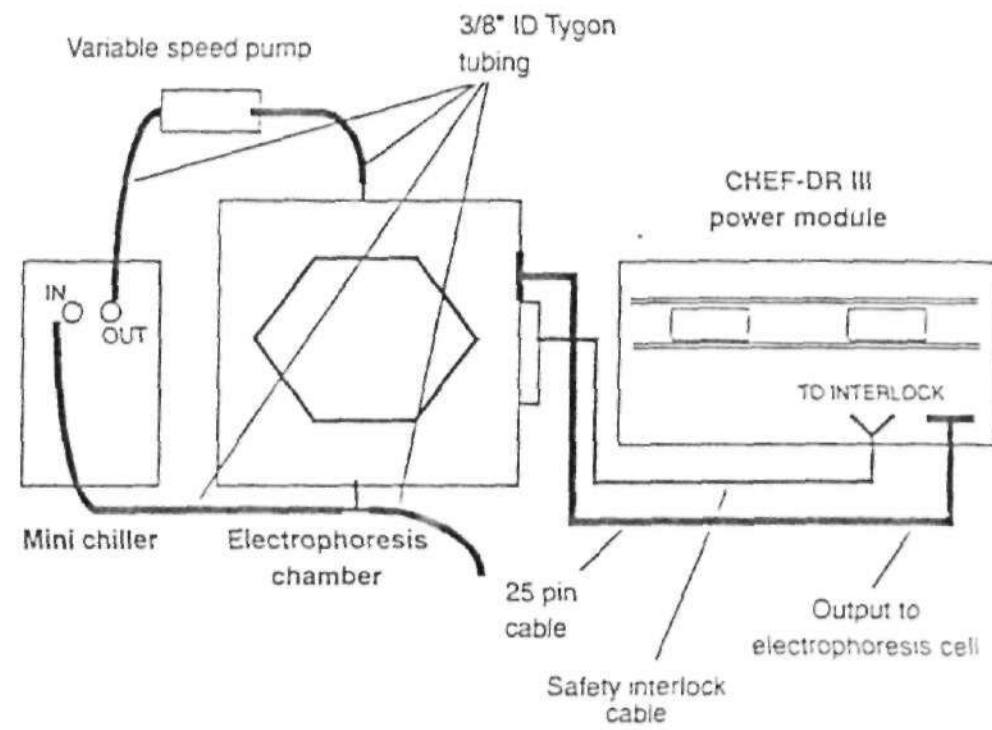


Figure 3.4 Interconnections between components of the CHEF-DR III system (after BIORAD, 1992)

iv. Running Gel manufacture and loading

The run gel was made with 1.0% pure grade agarose (BIORAD) in 80ml of 0.5 x TBE melted in a microwave oven and moulded in a 14 x 13 cm casting stand at a temperature of 50°C. Two 10-well combs were properly positioned in their comb holders and then inserted into the hot gel at

about one cm from both ends of the gels. The combs were properly aligned and the gel allowed to cool for 20 min at room temperature. Thereafter the gel was kept at 4°C for another 30 min to ensure good solidification. At this stage the combs were removed and a 5 mm thick gel with ten 2.5 mm- deep wells on both sides was formed on the platform. (Löbrich *et al.*, 1993b).

DNA probes for each irradiation point and unirradiated control were carefully cut into two small identical pieces (plugs) containing about 2×10^7 cells each (one was used in the run and the second placed, as a reference, in the agarose gel after electrophoresis). Two pieces of the unirradiated control were inserted into well numbers 2 and 9. Well numbers 3 to 8 were occupied by DNA plugs irradiated with 41, 82, 164, 246, 328, and 410 Gy respectively. Well numbers 1 and 10 were left empty as distortions usually occur on these lanes.

The plugs were covered with 1% low melt agarose in 0.05M EDTA (pH 8) and allowed to solidify for 10 min at 4°C.

v. Running Conditions

Two litres of running buffer (0.5 x TEB) was poured into the electrophoresis chamber and the power unit put on. The buffer was allowed to cool to 12°C (set on the mini-chiller) before the loaded gel on the cast platform was placed into the centre of the hexagon. The run parameters were then entered into the power module (Table 3.1)

Table 3.1 Electrophoretic run parameters

Parameter	Setting
BLOCK 1	
Initial switch time	60s
Final switch time	60s
Run time	15hr
Volts/cm	6V/cm
Included angle	120°
BLOCK 2	
Initial switch time	90s
Final switch time	90s
Run time	8hr
Volts/cm	6V/cm
Included angle	120°

vi. DNA Staining in Gel

After running the gel, it is stained so that the DNA fluorescence distribution under UV light is obtained. A fluorescent dye, Ethidium Bromide (EB), was used. Figure 3.3 shows the structure of EB molecule. EB binds to nucleic acids at two sites (Lepecq, 1971). The first is the electrostatic binding site which occurs as a result of electrostatic binding between positively charged EB and

negatively charged phosphate in single stranded polynucleotides.

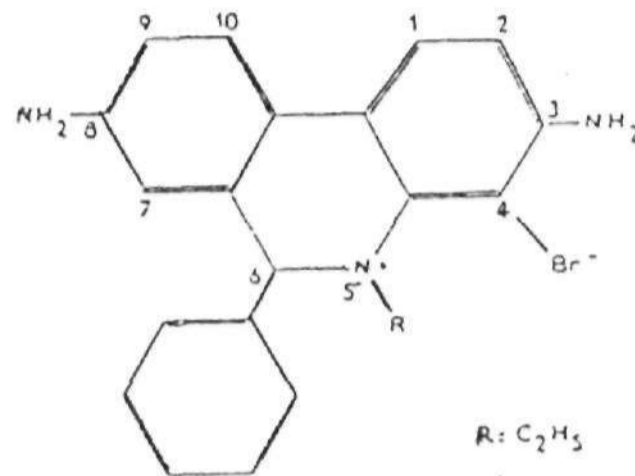


Figure.3.5 Structure of EB dye (Egenolf, 1994).

The EB fluorescence here decreases with ionic strength of the staining solution. The second is the intercalation binding site formed by noncovalent binding forces between the dye and double stranded molecules of the DNA. This leads to stacking of the planar groups of EB between DNA bases. Here, EB produces large and specific increase in fluorescence (21 fold for DNA and 25 fold for RNA with respect to free dye in solution). EB absorbs UV at between

300 to 360 nm and emits at 590 nm in the orange region of the visible spectrum. The UV absorbed by DNA at 264 nm is transmitted to the dye and also emitted at 590 nm (Lepecq and Padetti, 1967).

In order to achieve specificity of EB for double stranded structures of nucleic acids, a high ionic strength (0.1M NaCl) stain solution is used. The positive ions compete with the EB sites on the nucleic acid and reduces binding of EB to single stranded molecules.

In this work, the electrophoresed gel was immersed in staining solution containing 0.5 $\mu\text{g/ml}$ EB for 1 hr and destained overnight in 0.5 x TEB before photographing.

vii. Photographic technique

The stained agarose gel was put on a UV transilluminator with a wavelength of 302 nm. The fluorescence light (> 340 nm) is detected by a charge-coupled device (CCD) camera. The 8-bit camera (type CF 8 RCC, KAPPA, GLEICHEN) is liquid-cooled and delivers an evaluable picture of 512*512 pixel (chip size 739(H)*575(V)). The pixel-size is about $11\mu\text{m(H)}*11\mu\text{m(V)}$.

Due to the liquid-cooling system, the video-chip is cooled to about 50 K below room temperature. The electronic errors are reduced and so it is possible to increase the sensitivity from about 0.5 lux (live mode, 40 ms) up to some μlux (time integration up to 4 minutes). Since the integration does not behave linear under any condition, this effect is taken care of during the evaluation. The

camera picture is digitized with a frame grabber (LSF-AT, LEUTRON, GERNLINDEN), which is equipped with 768 kB in order to allow the storage of two pictures (262.144 byte) simultaneously. This option allows working with grids, target-crossed designations, storage of coordinates and most operations of image processing. The frame grabber is integrated in a PC-80286 with 2 MB on board. The camera-control program "VDIGC" written by Straaten, Weidmann and Pross, allows, in addition to the above mentioned options, the picture storage as "*.PIC" files for evaluation and "*.TIF" (Tagged Image Format) which are compatible to the MS-Windows software. The computer is linked with the DECNET network of the *Strahlzentrum* in order to transmit the pictures to the 80486-computers for evaluation and printing.

viii. Evaluation

To correct the fluorescence image data for background effects, a gel without DNA was recorded and subtracted from the sample image. The resultant image was stored on the computer for further analysis.

With the aid of a computer program "GELE", it was possible to calculate the fluorescent intensity of a whole lane, an electrophoresed well, its reference well and the first band. The problem of variations of DNA amount in the sample plugs was eliminated by the use of corresponding reference plugs.

The difference between the fluorescence of the reference well to the sample well was calculated for each

lane. The ratio of the fluorescence of the first band and the latter was calculated for the control lanes 2 and 9 and lanes 3 to 8 with irradiated samples. An average was obtained for the 2 unirradiated controls. This ratio represent the percentage of DNA in the first band of each lane with respect to the amount of DNA that migrated into the gel during electrophoresis. When normalised with the unirradiated control, this value for the irradiated samples in wells 3 to 8 becomes the relative fluorescence and serves as a basis for comparison. The results show an exponential decrease in the relative fluorescence intensity of the first band with various doses of 80 kV X-rays.

Based on the assumption that the dose which results in a decrease in fluorescence of the first band to 37% gives rise to one DSB per molecule (Löbrich et al., 1993b), the DSB induction frequency was calculated from the relation:

$$Y_{\text{DSB}} = \frac{F_r}{M_1} \quad \dots 3.4$$

where:

Y_{DSB} = Induced Double-strand break

F_r = Slope of relative fluorescence-dose curve

M_1 = Mass of chromosome in the first band

3.2.4 Repair Investigation

Rad 54-3 cells were prepared as in section 3.2.3 above. 10^8 unirradiated cells were pelleted, suspended on 0.05M EDTA and kept on ice.

Two hundred and fifty millilitres of 1x full medium (excluding Agar) in conical glass flasks were kept in shuttling 23°C and 36°C water baths respectively. 2.5×10^8 cells were X-irradiated in solution and some of these immediately introduced into both flasks such that their concentrations were about 5×10^6 cell/ml to ensure uninhibited cell growth. The remainder was kept on ice. 10^8 cells from this were pelleted into an eppendorf tube, suspended in 0.05M EDTA (pH 8) and kept on ice. After 1hr, 2hr, 4hr, 6hr, and 8hr incubation in full medium at 23°C and 36°C, 10^8 cells were pelleted from each flask into labelled eppendorf tubes suspended in 0.05M EDTA and kept on ice. DNA plugs were then manufactured from the probes. The procedure was repeated for 41 and 82 Gy X-irradiation. Pulsed field gel electrophoresis using CHEF-DR III system, was carried out as described in section 3.2.3.

3.3.5. Budding Delay Investigation

The percentage of budding cells of Rad 54-3 yeast strain was determined by counting the incubated sample with haematocytometer (Thoma chamber) at 0hr, 1hr, 2hr, 4hr,

6hr, and 8hr after incubation. The non-budding yeast cells were identified under microscope by their oval shapes while the budding ones had extra oval outgrowths.

3.2.6. Conduct Of Experiments

Stationary phase Rad 54-3 cells, grown for between 3 to 5 days at 30°C on agar plates containing complete medium, were harvested, washed several times, counted, suspended on sterilized double-distilled water and placed on ice until ready for use.

One hundred thousand cells were exposed to various doses of X-rays (0 to 400 Gy) and then plated on complete and succinate media to determine their survival.

To determine DSB induction, 2.5×10^8 cells were X-irradiated (0 to 400 Gy) out of which 10^8 were prepared for electrophoresis using CHEF-DR III system. The electrophoresed gels were stained in Ethidium Bromide which fluoresces under UV light. The gel pictures and fluorescence pattern were taken and stored for evaluation by a Camera-computer combination. Decrease of the fluorescence of the first band with increase in dose was used to calculate the DSB induction frequency.

To investigate the repair Kinetics, control and irradiated samples of Rad 54-3 cells were incubated in suspension of sterilized 1 x complete medium without agar in water baths at 23°C and 36°C. After 0, 1, 2, 4, 6 and 8 hours of incubation, 10^8 cells were pelleted for DSB

analysis using CHEF electrophoresis. At the various timings, the percentage of budding cells were also determined.

RESULTS

4.1 Survival Analysis

Figure 4.1 shows the survival curves obtained when Rad 54-3 mutant of yeast *Saccharomyces cerevisiae* was plated on nutrient agar immediately after irradiation (immediate plating, IP) at the permissive (23°C) and restrictive temperatures (36°C).

At the restrictive temperature when DSB rejoining is disallowed, exponential survival curves were observed for both media. However, at 23°C when DSB rejoining is permitted, shoulder-type survival curves appeared, although the shoulder is more extended for cells plated on Na-succinate medium. On the complete medium, the "Quasi-threshold" dose, D_q , is 25 Gy while the dose necessary to reduce survival to 37%, D_0 , is 74Gy. The values for Na-succinate medium are D_q : 75Gy and D_0 : 128Gy. Thus, plating Rad 54-3 Yeast cells immediately after irradiation on succinate medium results in a D_q 3 times higher and a D_0 1.7 times higher than observed after immediate plating on full medium. This is an indication of a higher survival of the cells on succinate medium.

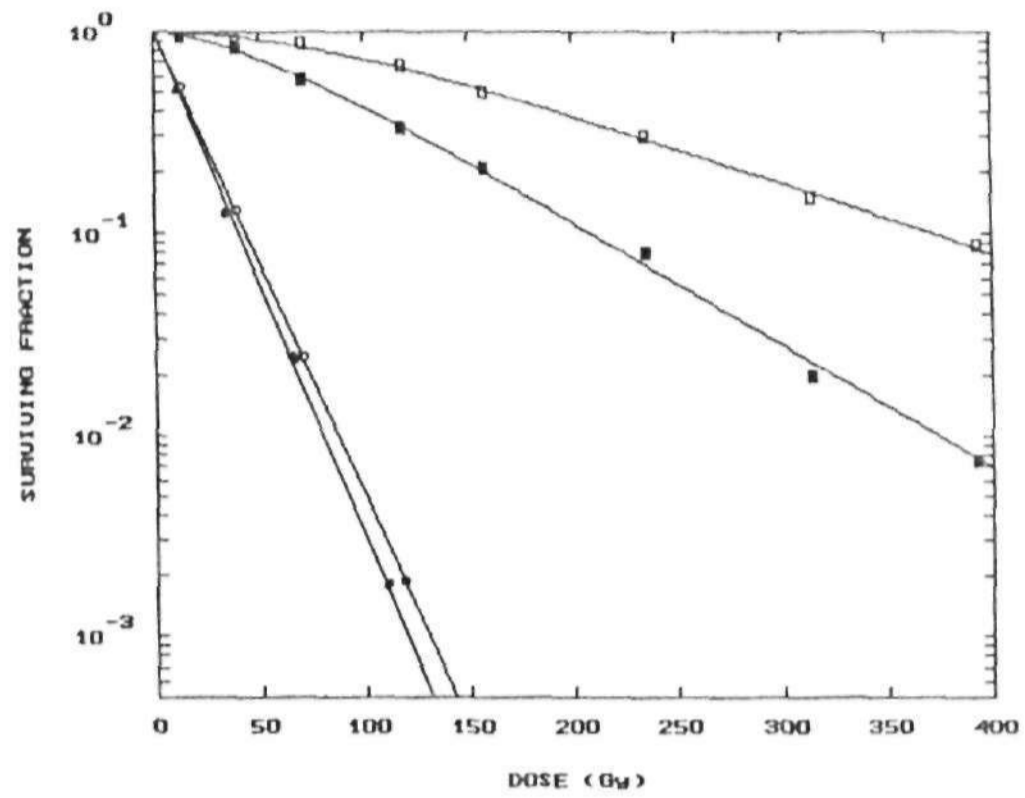


Figure 4.1 Survival curves of X-irradiated Rad 54-3 yeast cells plated on complete and succinate media at 23°C and 36°C (Legend: \square Succinate medium, 23°C; \blacksquare Complete medium, 23°C; \circ Complete medium, 36°C; \bullet Succinate medium, 36°C).

4.2 INDUCTION OF DSB

The relative fluorescence curve for 80 kV X-rays is shown in figure 4.2. The "relative fluorescence" is obtained by comparing the fluorescence of the first band of an irradiated sample with that of the unirradiated control after electrophoretically running the two in one gel. For various experiments, the relative fluorescence is plotted as a function of Dose and the slope. The slope was calculated from the equation:

$$\frac{Y}{A} = \text{Exp}(-D/B) \quad \dots 4.1$$

where:

$$\frac{Y}{A} = \text{Relative fluorescence}$$

$$D = \text{Dose (in Grays), and}$$

$$B^{-1} = \text{slope of graph}$$

$$\text{Since } B = (126.2 \pm 5.78) \text{ Gy}$$

$$\text{The slope} = \frac{1}{(126.2 \pm 5.78) \text{ Gy}}$$

$$= (7.924 \pm 0.363) \times 10^{-3} \text{ Gy}^{-1}$$

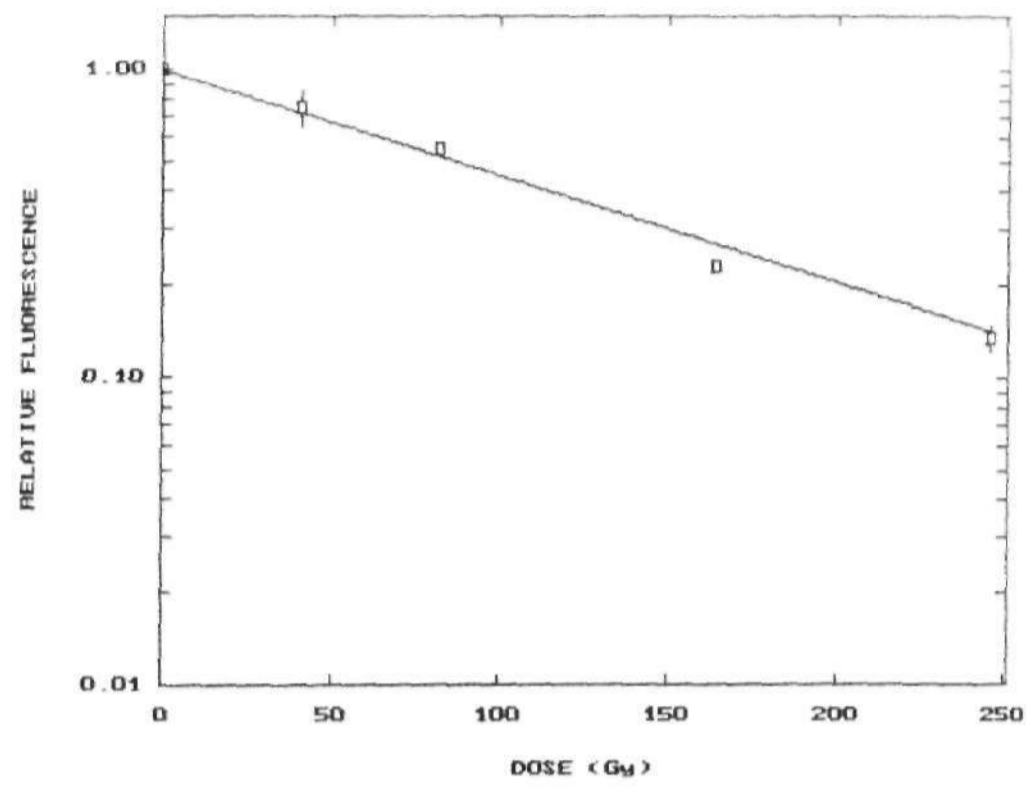


Figure 4.2 Relative fluorescence intensity of the first band (chromosome IV) after irradiation with various doses of 80 kV X-rays.

Based on the assumption made in equation 3.4 and the fact that it has been shown that with the electrophoretic condition used for the gels in this work, the first band actually corresponds to chromosome IV of length 1.6 mbp (molecular weight of $1.6 \times 10^6 \times 667$ g/mol) and not chromosome XII (Löbrich et al. 1993a),

$$\text{DSB} = \frac{(7.924 \pm 0.363) \times 10^{-3}}{1.6 \times 10^6 \times 667 \text{ g/mol}} \text{ Gy}^{-1}$$

$$\text{DSB} = (7.43 \pm 0.34) \times 10^{-12} (\text{g/mol})^{-1} \text{ Gy}^{-1}$$

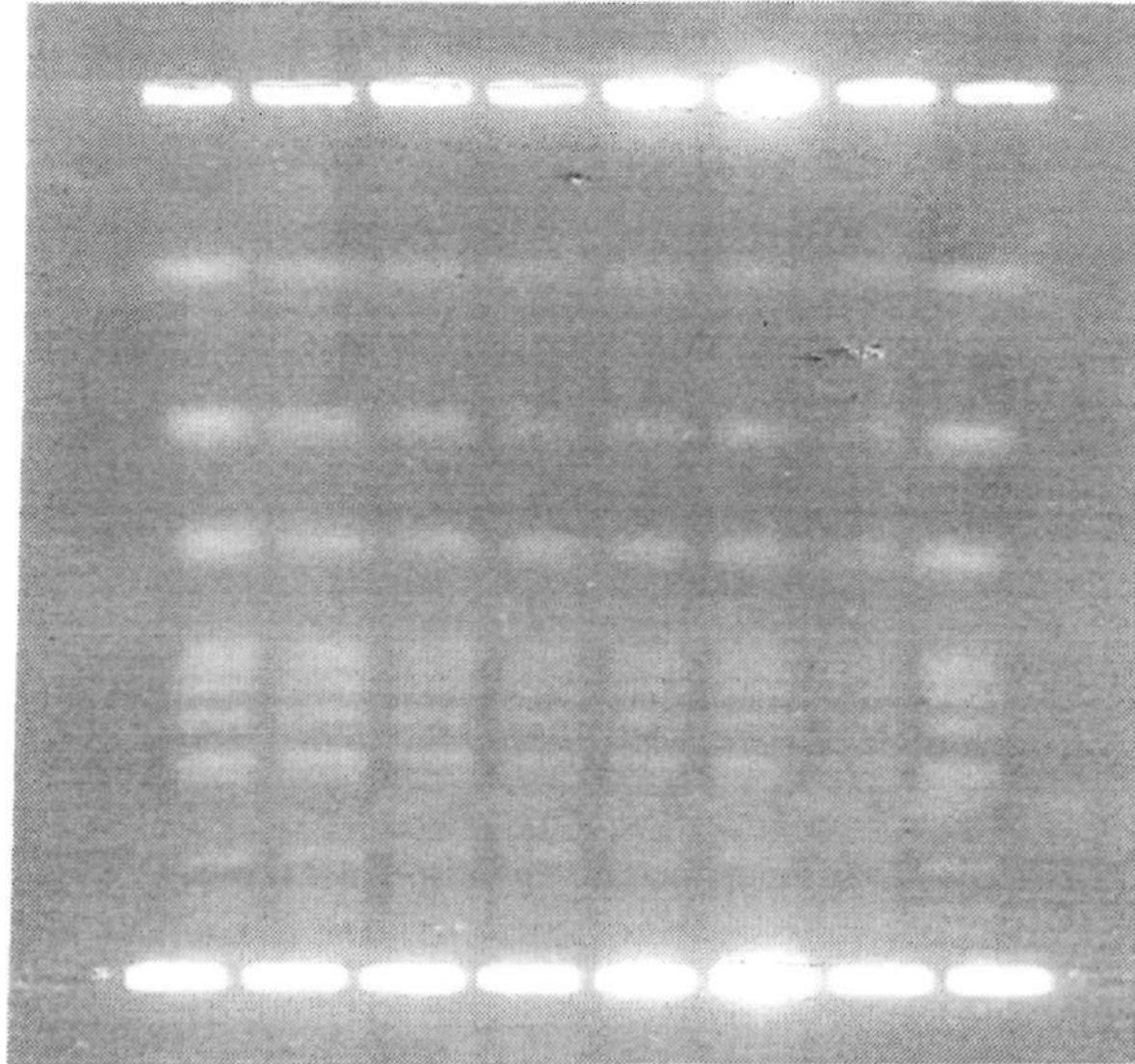


Plate 4.1 An uncorrected gel picture of unirradiated (lanes 1 and 9) and irradiated (lanes 3-8, with X-ray doses of 41, 82, 164, 246, 328 and 410 Gy, respectively) rad 54-3 mutant of yeast *S. cerevisiae* after electrophoresis.

4.3 REPAIR OF DSB

The dsb rejoining kinetics for Rad 54-3 strain of yeast cells exposed to X-rays under oxic conditions and incubated at the permissive (23°C) and restrictive (36°C) temperatures in growth medium are shown in Figure.4.3.

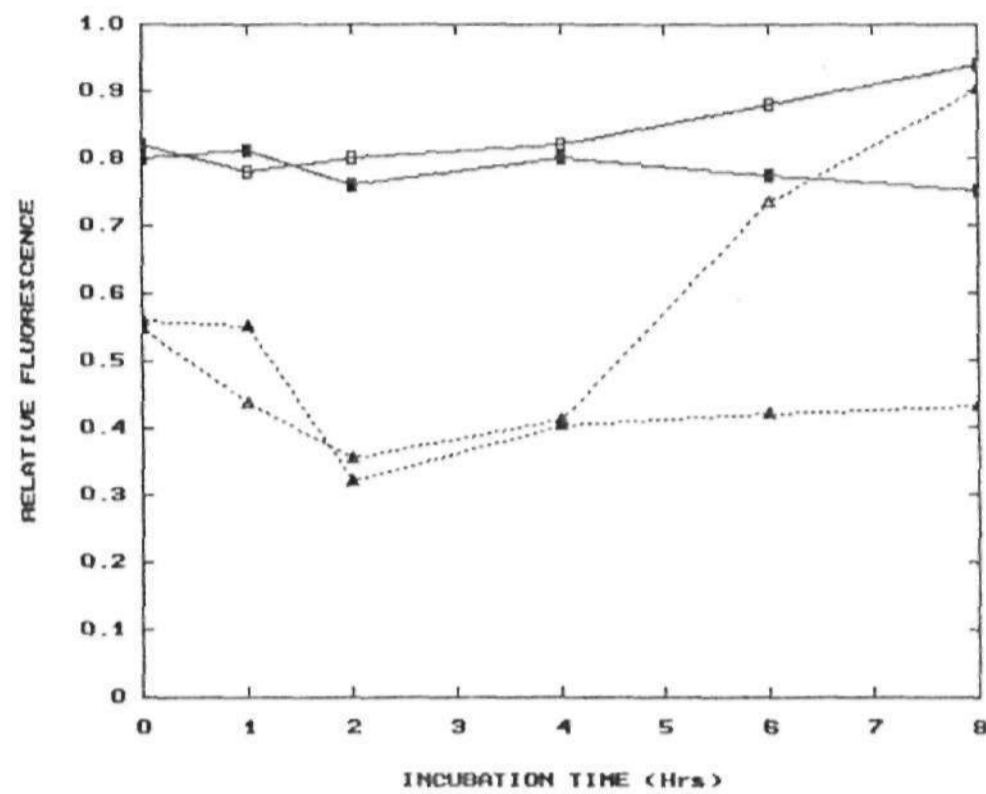


Figure.4.3 Kinetics of repair of X-ray induced dsb under growth conditions (Legend: □ 41Gy, 23°C incubation; ■ 41Gy, 36°C incubation; △ 82Gy, 23°C incubation; ▲ 82Gy, 36°C incubation).

Figure.4.3 shows the repair kinetics for DSB induced by 41 and 82 Gy X-rays incubated on nutrient agar at 23°C and 36°C. The 41 Gy repair curve shows that the relative fluorescence (percentage of DNA retained) of cells incubated at the permissive temperature reduced from 0.83 to 0.78 after the first hour but increased steadily to 0.94 after 8hr. This suggests that secondary DSBs were formed during the first hour of incubation, before DSB rejoining commenced, and then increased progressively until the percentage of intact DNA increased to 94%. However, for cells incubated at the restrictive temperature, there were small fluctuations about an almost constant value (0.8) of relative fluorescence throughout the 8-hr incubation period. This may be interpreted to mean that the 80% intact DNA left immediately after irradiation remained almost constant throughout the incubation period, implying that no repair took place.

The 82 Gy repair curve shows that cells incubated at the permissive temperature have a pronounced decrease in relative fluorescence (0.55 to 0.35) during the first 2 hours after which it increased up to 0.90 after 8hours. In other words, the percentage of intact DNA decreased from 55% to 35% after 2hr incubation due, maybe, to the formation of secondary DSBs. This eventually increased to 90% as the repair mechanism became activated. On the other hand, cells incubated at the restrictive temperature showed a constant relative fluorescence of 0.55 up to the 1hr incubation time. This dropped to 0.3 after 2hrs, increased to 0.4 after 4hrs and remained so for the remaining period.

A monophasic repair kinetics with repair half-life value (50% DSB repaired), $t_{1/2}$, of between 6 and 7hrs was observed.

4.4 BUDDING DELAY INVESTIGATION

Figures 4.5 and 4.6 show the budding curves when Rad 54-3 cells exposed to various doses of X-rays are incubated in full medium at 23°C and 36°C respectively for 8 hours. Per cent budding cells are plotted against incubation time.

After a lag phase in which only 9-16% of the cells incubated at 23°C formed buds, the index increased for several hours until it had reached a maximum value. The budding index appears to be more rapid in the control than in the irradiated cultures in which rate decreased with increasing X-ray dose. Generally, however, the budding curves of the irradiated cultures show similarity to that of the control after the various lag times. See figure 4.5.

Figure 4.6 shows that the budding curve for the control culture at 36°C incubation differs from that of 23°C incubation in two respects: one, the lag time is halved (1hr); and two, the budding index decreased at 8hrs incubation time to a value lower than those of the irradiated cultures. Even though the longest lag time of the irradiated samples at 36°C incubation appear to be half of that of the irradiated cultures at 23°C, their budding curves differ from that of the control culture thereafter.

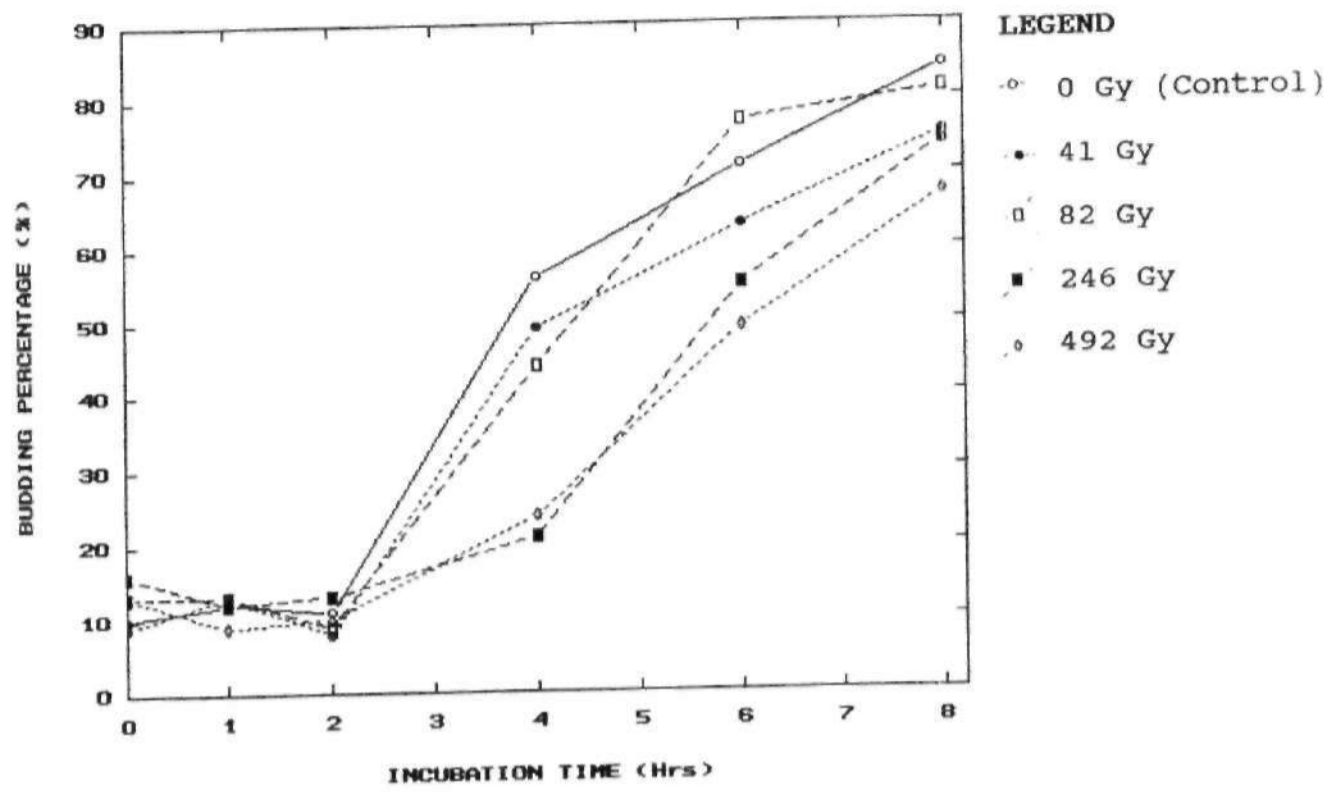


Figure 4.4 Budding curves for control and X-irradiated cultures of Rad 54-3 Yeast cells incubated in growth medium at 23°C.

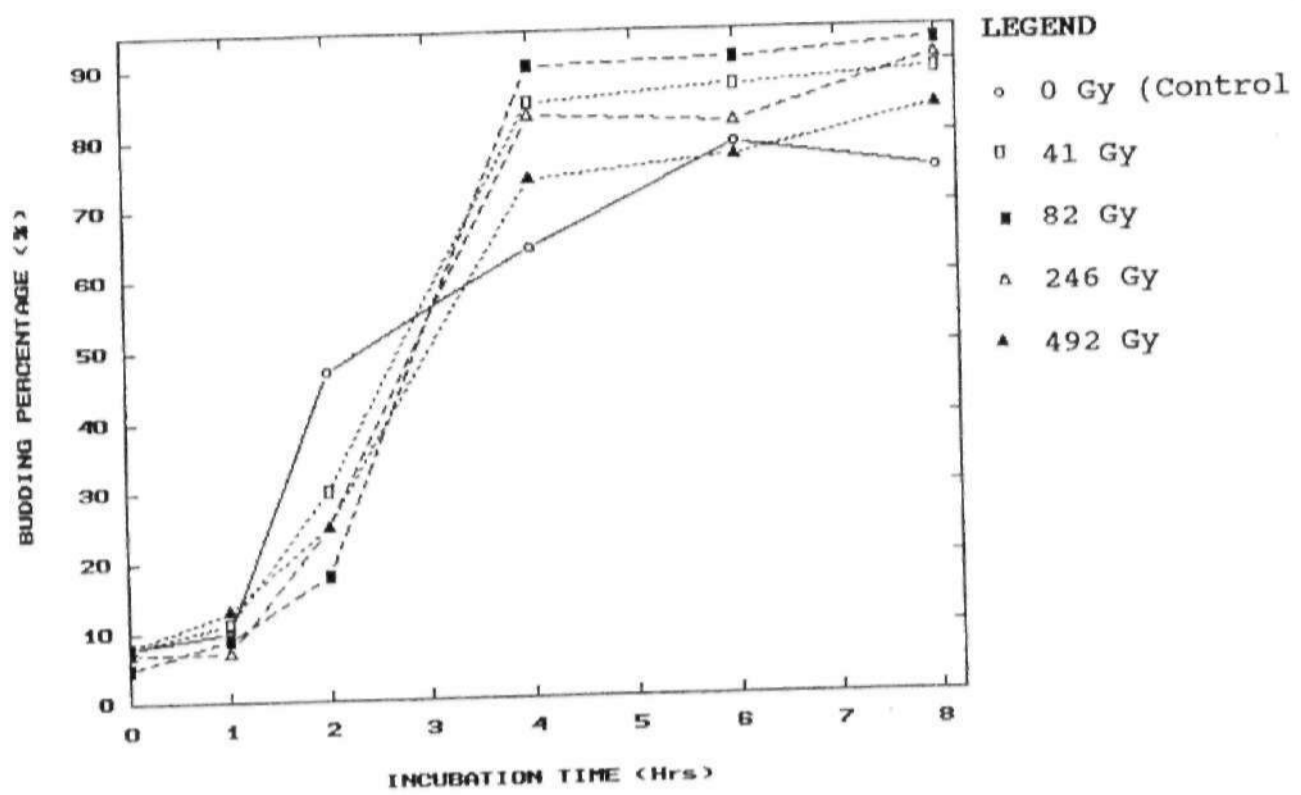


Figure 4.5 Budding curves for control and X-irradiated cultures of Rad 54-3 Yeast cells incubated in growth medium at 36°C.

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

The shouldered survival curves obtained at the permissive temperature (23°C) showed that with Rad 54-3 diploid mutant of yeast *S. cerevisiae*, X-ray-induced DSB is repaired only at that temperature but not at the restrictive temperature (36°C). This had earlier been observed by Budd and Mortimer (1982). Survival of cells plated immediately after irradiation depends on the energy source supplied in the nutrient agar (Frankenberg-Schwager, 1988). This explains the enhanced radioresistance of Rad 54-3 mutant at the temperature permissive for DSB repair when glucose in full medium is substituted with Na-succinate in succinate medium (Figure 4.1). It had been reported that yeast cells plated on nutrient agar containing Na-succinate exhibit an increased post-irradiation lag before entering S phase compared to cells plated on glucose containing nutrient agar. This increased lag before entry into the S phase provides more time for repair of potentially lethal lesions, thereby resulting in an enhanced survival of cells (Perper, 1975; Frankenberg and Frankenberg-Schwager, 1981). Earlier studies with Rad 54-3 mutant cells also suggest that it is DSBs that are repaired during this lag (Frankenberg-Schwager *et al.*, 1988).

A linear relation was found between dose and DSB induction pointing to the fact that DSB comes about as a result of energy deposition by a single track of radiation (Ward, 1990; Brenner and Ward, 1992; Löbrich et al., 1993). A DSB induction frequency of $(7.43 \pm 0.34) \times 10^{-12} \text{ (g/mol)}^{-1} \text{ Gy}^{-1}$ was found for Rad 54-3 mutant. This is close to the result obtained with the wild type yeast (WT 211*B) by Löbrich et al., (1993) using CHEF electrophoresis.

As shown in Figure 4.3, cells of Rad 54-3 irradiated with 41 and 82 Gy X-rays and incubated in complete medium at the permissive temperature (23°C) appear to show signs of secondary DSB production during the first 2 hours of incubation, to varying degrees (increased DSB induction is indicated by decreasing percentage DNA retained, the "relative fluorescence"). This is interesting but not a new phenomenon. Ahnström and Bryant (1982) found that DNA DSBs were generated by the repair of X-ray damage in Chinese hamster cells. While their result might explain the secondary DSB production at the permissive temperature when the repair mechanism is activated in Rad 54-3 cells, it certainly does not explain same observation at the restricted temperature when repair is deactivated.

Whitaker and McMillan (1992), suggested that the decrease in average fragment size with increasing repair time when DNA DSB repair was measured by PFGE could be due to DNA degradation within the gel. In order to eliminate the possibility from this work, time was given for the cells to repair before being encapsulated in agarose plugs. Moreover, the time-course of their appearance and the fact

that they finally disappear suggests that these DSBs presumably arise by enzymatic incisions at damaged DNA sites rather than by DNA degradation. This is in agreement with an earlier observation (Frankenberg-Schwager *et al.*, 1994).

Secondary DSB production has been found in anoxically irradiated yeast cells incubated in non-growth medium, but not in oxicly irradiated yeast incubated in same (Frankenberg-Schwager *et al.*, 1990, 1994a, and 1994b; Frankenberg-Schwager and Frankenberg, 1991). It therefore seems plausible to suspect that the growth medium enhanced the enzymatic incisions at damaged DNA sites during incubation at both permissive and restrictive temperatures. In addition, as the secondary DSBs disappear, the cells revert to their original characteristics of repair proficiency and deficiency at the permissive and restrictive temperatures respectively (see Figure 4.3).

The lack of fast repair component could be due to the relatively low dose used here - maximum of 82 Gy (Whitaker and McMillan, 1992; Frankenberg-Schwager *et al.* 1994).

Yeast cells usually require about 2 hours lag time before cell division commences in a favourable medium. The fact that it took about 4 hours for cell division to start when X-irradiated cells were incubated at 23°C in 1x complete medium (see Figure 4.5) may be taken to mean that energy that could have been used for growth was directed at the repair of DNA DSBs during that extra time.

The absence of maximum and minimum values on the budding curve of the control culture at 23°C incubation

(Figure 4.4) show that the cells are still in the first cell cycle after 8hrs. After the maximum prolongation of lag time for the irradiated cultures at same incubation temperature (2hrs) the irradiated cultures behaved like the control. From this, it may be concluded that the cells possess repair systems that allow them eliminate the radiation-induced damage in the DNA during the delay time of the first cell cycle. This is in line with the hypothesis of Phillips and Tolmach (1966) that progression delay is dependent upon the time necessary to repair the radiation-induced damage and Faber and Kiefer (1975).

The difference in behaviour of the budding curves of the control culture and the irradiated samples (after their lag times) during 36°C incubation as indicated in Figure 4.5 show that the irradiated cultures could not repair the radiation-induced damage in the budding delay time. Of course, repair is not expected at this incubation temperature (Budd and Mortimer, 1982; Frankenberg-Schwager *et al.*, 1987). This may simply suggest that the budding delay times in X-irradiated Rad 54-3 Yeast cells incubated at 36°C in growth medium are not governed by repair processes.

5.2 CONCLUSION

This work is made up of four parts. The first established a fundamental basis for the work in cell survival analysis. Results from survival analysis provided

indirect evidence for the repair of X-ray induced DSB in Rad 54-3 cells at 23°C and inability to repair the lesion at the restrictive temperature (36°C).

The second part involves the determination of DSB induction frequency using CHEF electrophoresis. This was found to be $(7.43 \pm 0.34) \times 10^{-12} \text{ (g/mol)}^{-1} \text{ Gy}^{-1}$ and in agreement with the result of Löbrich et al. (1993).

The third part of this work is the investigation of repair kinetics of X-irradiated Rad 54-3 diploid mutants of *S. Cerevisiae* in growth (1x complete) medium at the permissive and restrictive temperatures using the sensitive CHEF electrophoresis technique. The results of this investigation are important in two respects: (1) direct evidence for the repair of DSB in this mutant is provided; and (2) occurrence of secondary DSBs during the first two hours of incubation at both temperatures was observed. The latter has not been reported before. It is proposed that the secondary DSBs may be due to enzymatic incisions at damaged DNA sites enhanced by incubation in the growth medium.

The last part involves the investigation of the budding delay of cells of Rad 54-3 mutant of yeast *S. cerevisiae* incubated in growth medium at 23°C and 36°C. Dose- and incubation-temperature-dependent budding delays were observed.

5.3 RECOMMENDATIONS

It is recommended that more investigations should be done to find out the effects of higher concentrations of complete medium, and its substitution with Na-succinate and non-growth medium, on the repair kinetics obtained. It might also be interesting to experiment with UV rays and α -particles.

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