

UNIVERSITY OF LEICESTER

**DNA STRAND BREAKS INDUCED  
BY GAMMA-RAY IRRADIATION**

A THESIS SUBMITTED FOR THE DEGREE  
OF DOCTOR OF PHILOSOPHY

BY

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DEPARTMENT OF CHEMISTRY  
LEICESTER, ENGLAND, 1992

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## DNA Strand Breaks Induced By Gamma-Ray Irradiation

Saijun Fan

### ABSTRACT

#### Part I: Plasmid DNA System

The effects of a range of buffers and additives on the radiation damage in frozen aqueous plasmid DNA have been studied. In studies of various buffers, the results show that phosphate buffer system sensitise radiation DNA damage, EDTA and Tris present protections against DNA damage, in comparison with pure water system. In studies of other additives, radioprotection by NaI and LiCl increase with increasing concentrations, whilst radiosensitivity of DNA with Na<sub>2</sub>SO<sub>4</sub> and NaClO<sub>4</sub> increase with increasing their concentrations. DMSO shows a radioprotection.

A range concentrations of spermidine and spermine are used to probe the radioprotection of DNA by polyamines. The results suggest that the protection efficiencies of polyamines increase with increasing their concentrations, moreover, spermine has a greater effect than spermidine.

#### Part II: Cell system

10 mM concentration of spermine shows a radioprotection against DNA DSB and cell death. Metronidazole acts as a sensitiser in the induction of DSB and cell killing. However, spermine-linked metronidazole (AM1229) acts as radioprotectors against DSB under the condition of free-oxygen, and as sensitiser in induction of cell killing under the condition of atmospheric oxygen.

The yields of DSBs are compared between cells irradiated at 77K and 0°C. The results show that there is a reduction of DSB in cells exposed at 77K, approximately 35% less than that in cells exposed at 0°C. It may suggest that ca. 65% DNA DSBs formed from direct effect, 35% from indirect effects.

There is a difference of DSB yield in cells exposed to gamma-rays in the presence of hypotonic (0.05M) and hypertonic (1.5M) NaCl solutions. The results show that there is 20% increase in hypotonic solution, 8% reduction in hypertonic solution. However, these influences disappear when the cells are irradiated at 77K. The results suggest that the water concentration within cells has an effect on the radiation damage to DNA.

There is no evidence to show that an adaptive response of DNA DSB is induced in cell pre-exposed to low doses and subsequently to high doses. The results might suggest that there is no a simple link between repair of DNA DSB and the induction of adaptive response which is found in chromosomal aberration.



#### STATEMENT

The experimental work described in this thesis has been carried out by the author in the Department of Chemistry of the University of Leicester between November 1989 and October 1993. This work has not been submitted, and is not currently being submitted, for any other degree at this or any other University.

Signed: *Jan JS* ..... Date: *Feb. 8th 1993* .....

*To my lovely Son, Mengbo*

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## PREFACE

When tissues and cells are subjected to ionizing radiation, many physical and chemical reactions are induced to the effect that a large number of ions, free radicals, and excited molecules are created. These unstable species react chemically with one another and further with components of cells, producing biological molecular lesions that can express themselves in a variety of biologically significant changes, such as transformation, mutation, chromosome aberration and cell death. It is now widely accepted that the molecular basis for these biological consequences involves radiation damage to the nuclear genome or more specifically the deoxyribonucleic acid (DNA) which is regarded as a critical target. There have been strong experimental data for supporting this consideration.

It is therefore of great importance to analyze DNA damage and repair in providing a molecular base and background explanation for biological effects of ionizing radiation and the clinical application of radiation. Overall, the current studies on radiation-induced DNA damage and repair have been mainly carried out at the following levels: (1) measuring different DNA damage and repair kinetics, *in vivo* and *in vitro*, using different assays under the exposure of different radiation quality; (2) investigating effects of various additives and other factors on DNA damage and repair, such as radioprotectors, radiosensitizers and cell growth factors; (3) establishing the possible links between DNA lesions/repair and radiobiological effects.

This thesis summarises the findings obtained by the author on DNA damage and repair induced by gamma-ray in the system of plasmid DNA and cultured Chinese hamster V-79 cells in order to get more informations concerning radiation damage on DNA. Chapter 1 provides the literature on

the nature and structure of DNA, ionizing radiation, and its interaction with the DNA molecule, and the DNA damage/repair mechanism. In Chapter 2, the detailed experimental techniques and protocols employed are presented for detection of radiation damage in plasmid DNA and animal cellular DNA using electrophoresis and assay of neutral filter elution. Chapter 3 presents the experimental results and discussions of (1) plasmid DNA damage in different buffers and the effects of additives, (2) radioprotective effects of polyamines, (3) cellular DNA double strand break at low temperature, (4) cellular DNA damage in the presence of hypotonic and hypertonic salt solution, and (5) the effect of low dose radiation on rejoining kinetics of double strand break.

## ACKNOWLEDGEMENT

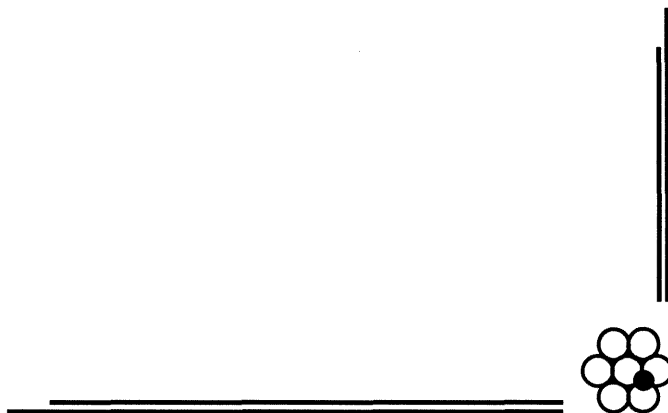
I would like to express my sincere thanks to my supervisor, Professor Martyn C. R. Symons FRS for his excellent guidance and advice throughout this project and Professor Paul M. Cullis for his valuable suggestions and kind help. Many thanks to the Cancer Research Campaign for financial support and the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom for an ORS Award that made it possible for me to do this work at the Department of Chemistry, Leicester University. I also acknowledge Professor D. DeBono in the Glenfield Community Hospital for allowing me to use the liquid scintillation analyzer at his laboratory. Most of the plasmid DNA extraction and purification work were done at the Department of Genetics, I am also grateful to the Director of the Genetics Department and many workers at Laboratory 145 for their help. Finally, I would like to thank my wife for her patience and encouragement.

Saijun Fan  
Leicester, October 1992



# Chapter 1

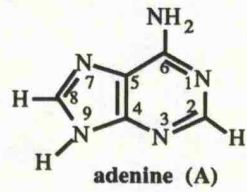
## *INTRODUCTION*



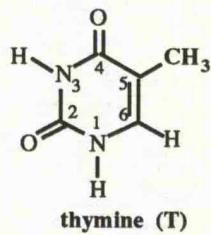
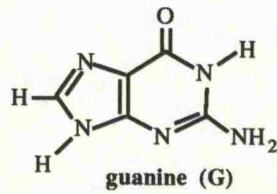
**1.1****Nature and Structure  
of DNA**

It has been well understood that DNA is a genetic material found in mammalian cells. The primary structure of the DNA molecule was defined by Watson and Crick in 1953 when they published their double-helix model based on X-ray diffraction analysis of DNA crystal patterns. It is a polymer which is highly ordered and twisted double stranded helical macromolecule, if the double helix is unwound, it would resemble a stepladder. This polymer is composed of three components; a 'back-bone' of alternating sugar and phosphate units rejoined by ester bonds, and a nitrogenous base attached to each sugar group. This structure is called **nucleotide (Figure 1.1.1)**.

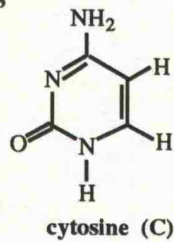
Each sugar unit has a ring structure with five carbon atoms. The third carbon atom ( $C_3$ ) in the sugar unit is joined to phosphate group which is further joined to the fifth carbon atom ( $C_5$ ) of the following sugar unit, ( $\cdots P-5'-\text{Sugar}-3'-P \cdots$ ), and so on, so that each strand carries the sugar units polarized in one direction and strand itself is polarized, starting at  $C_3$  and ending at  $C_5$ . The two strands are wound around each other in opposite polarity, from one end of the double stranded molecule one strand runs from  $C_3$  to  $C_5$  ( $3' \rightarrow 5'$ ), as the other strand runs from  $C_5$  to  $C_3$  ( $5' \rightarrow 3'$ ). Thus in a linear double strand there is one 3'-OH and one 5'-P terminus at each end of the helix. The first carbon atom ( $C_1$ ) of each sugar unit is joined to a complex



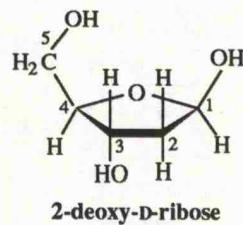
PURINES



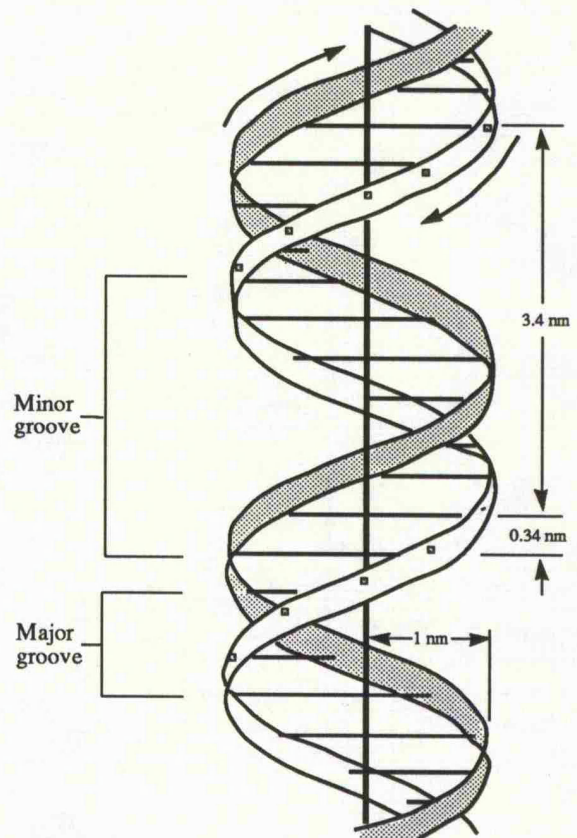
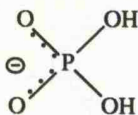
PYRIMIDINES



SUGAR



PHOSPHATE



(1 nm =  $10^{-9}$  meter)

**FIGURE 1.1.1**

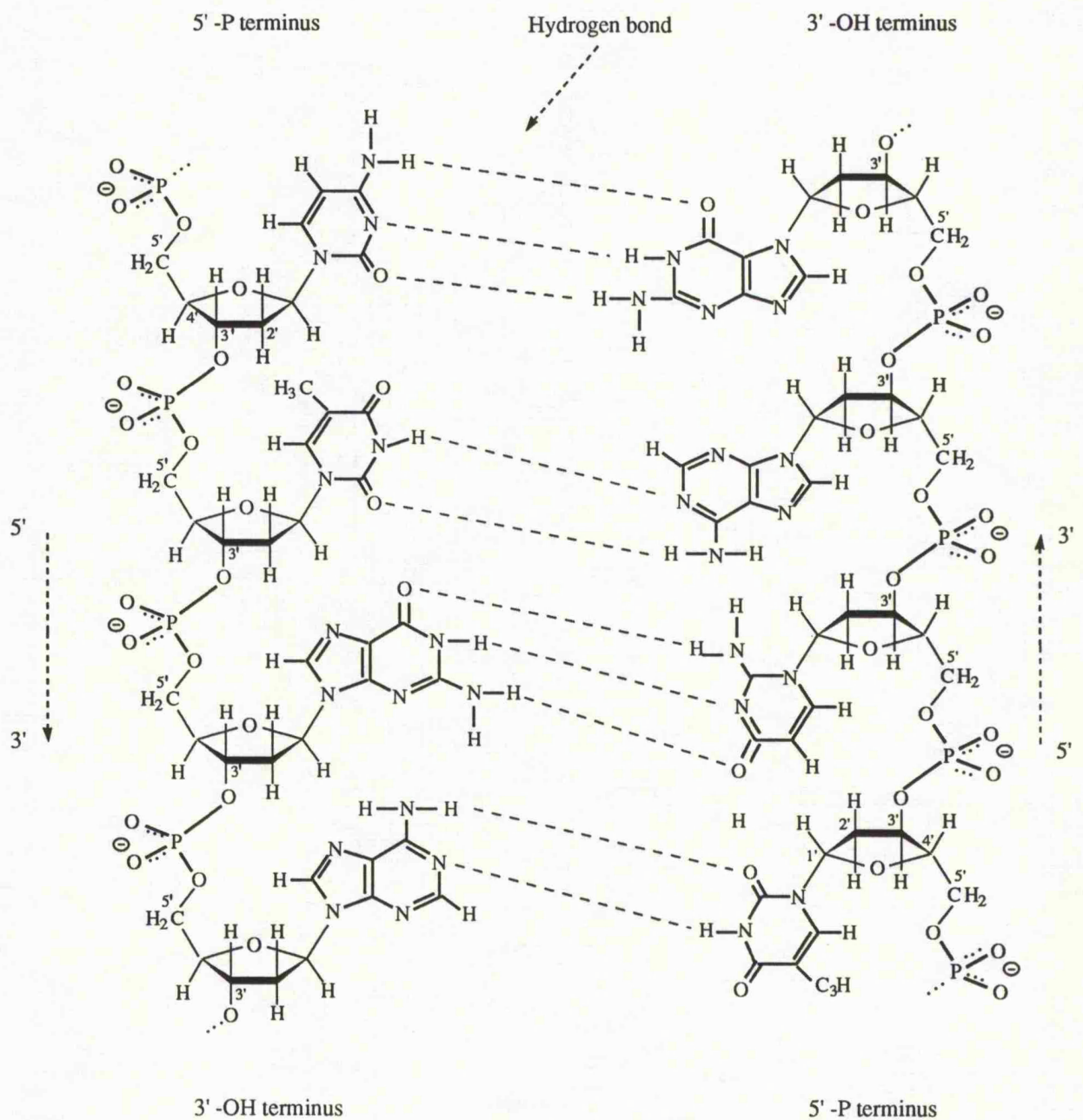
The basic components and a simplified model showing the helical structure of DNA (Adapted from Chadwick and Leenhouts 1981 and Coggle 1983).

organic base, either two purines, adenine (A) and guanine (G); or two pyrimidines, cytosine (C) and thymine (T). The sugar-phosphate chain is on the outside and the bases on the inside in the DNA double stranded helix (Figure 1.1.2).

Each unit of base is attached to the C<sub>3</sub> of the sugar, and with phosphate attached to the C<sub>5</sub> of the sugar forms a nucleotide. The single strand DNA is known as a polynucleotide chain. Nucleotides are identical except that each contains a different nitrogen base. In the absence of the phosphate unit, the base and the deoxyribose sugar form a nucleoside (Figure 1.1.3).

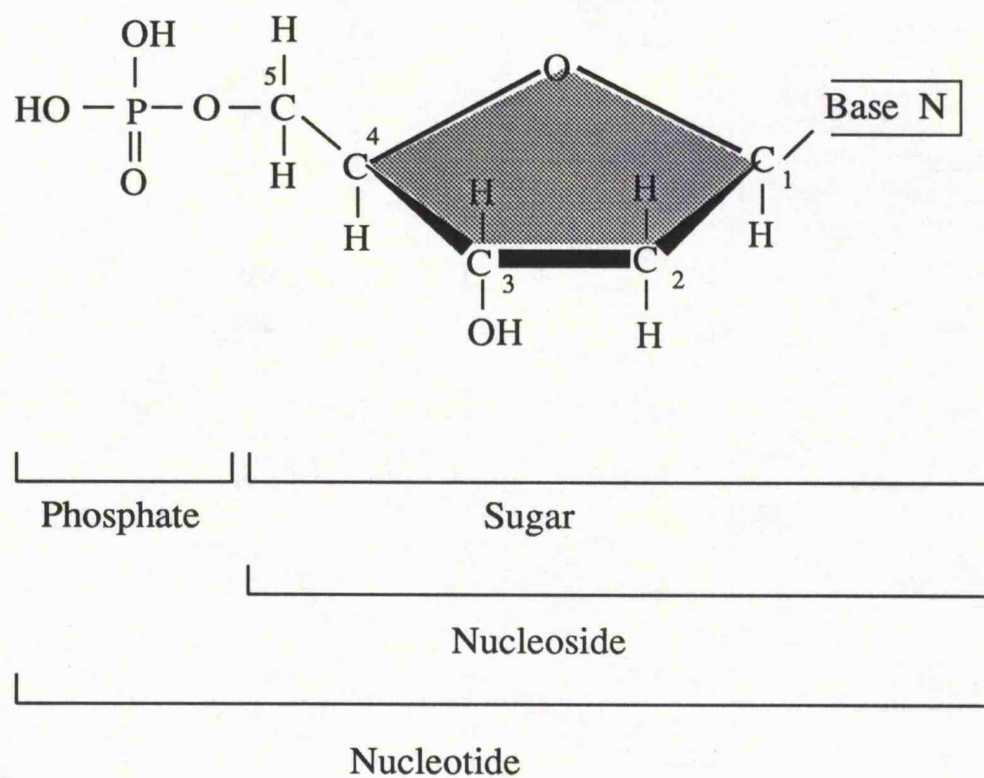
The two DNA strands are held together by hydrogen bonds between bases following a complementary pairing rule which was first defined by Watson and Crick (1953), i.e., A in one strand always pairs with T in the other by two hydrogen bonds, and C pairs with G by three hydrogen bonds, so that these four bases must always be present in equivalent amounts in double stranded DNA. Thus both purine and pyrimidine pairs fit into the double stranded helix without giving any geometrical distortion (see Figure 1.1.2). The structure repeats after 10 residues (base pairs), that is at interval of 3.4 nm, called 1 helical turn. The diameter of the DNA double helix is 2 nm. In addition the DNA helix has two external helical grooves, a deep wide one (the major groove) and a shallow narrow one (the minor groove). The major groove is the site of binding of specific proteins, the histones.

There are four division phases in a cell cycle (up to 24 h), pre-synthesis (G<sub>1</sub>, 8-12 h), DNA synthesis (S, 9-12 h), post-synthesis (G<sub>2</sub>, 4-6 h) and mitosis (M) phase. The DNA replication only occurs at a S division phase and is semi-conservative. During S phase the DNA replication initiates from a particular point and proceeds in the opposite direction. The two helically wound strands of DNA need to be separated and each serves as a model for the synthesis of a new complementary strand in the 5' to 3' direction, this process is mediated by DNA polymerase enzymes (mainly polymerase  $\alpha$ ,  $\beta$ ,  $\gamma$  in mammalian cells). There are also other DNA polymerases required for replication, such as



**FIGURE 1.1.2**

A stylized drawing of a segment of a DNA duplex showing the antiparallel orientation of the complementary chains. The arrows indicate the 5' → 3' direction of each strand (Adapted from Chadwick and Leenhouts, 1981).



**FIGURE 1.1.3**

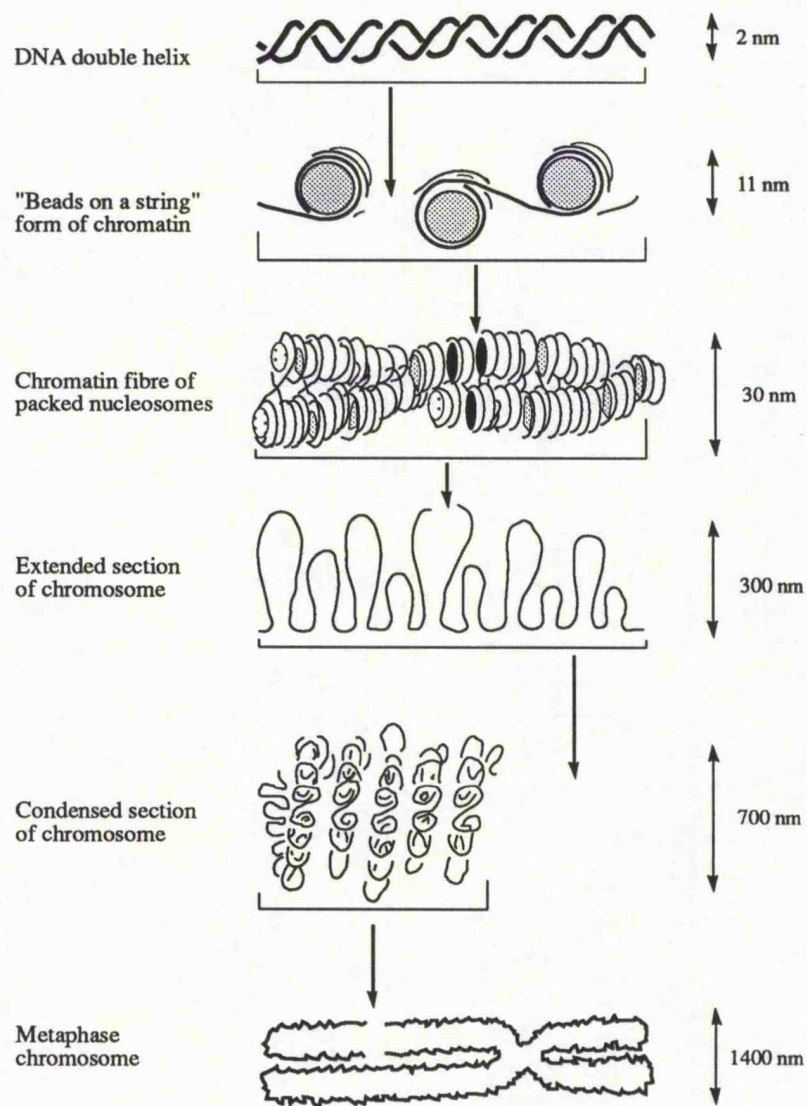
A typical nucleotide showing the three major components and the distinction between a nucleoside and a nucleotide.

helicases (effecting unwinding at the replication fork), topoisomerases (helix-destabilization, rewinding) and ligases (joining fragment on the lagging strand). In  $G_2$  phase the DNA undergoes the complex coiling that leads to the formation of chromosome. Figure 1.1.4 shows a simple model in which the DNA double helix is organized into a metaphase chromosome. The DNA is duplicated in its entirety such that at cell division each daughter cell will receive a copy of the full genomic content of the parent cell.

Unless replication is taking place, the fundamental unit of DNA from eukaryotes is known to be packaged with histones, forming repeating subunits called **nucleosomes** which have the dimensions of  $11 \times 11 \times 5.5$  nm, and compacted into chromatin within the cell nucleus. Chromatin isolated from the cell is defined as a complex which contains 1 unit of histone, 1 unit of non-histone chromosomal protein and 1 unit of DNA (Baserga and Nicolini 1976). Accordingly, the primary structure of chromatin is the repeating nucleosomes consisting of about 200 base pairs of DNA associated with a globular complex of histone made of two molecules each of H2a, H2b, H3 and H4. Approximately 140 base pairs of DNA are tightly associated with these histones and are known as the nucleosome core particles, whereas the remaining 60 base pairs are loosely associated or unassociated with these histones and serve as "linker" between adjacent core particles, as shown in Figure 1.1.5 (Kornberg 1980). Furthermore, it has been shown that isolated chromatin can be separated into "condensed" and "dispersed" fractions and their distribution in the inter-phase nucleus is non-uniform (Evans 1984). A schematic diagram of the chromatin distribution within the cell nucleus is shown in Figure 1.1.6, it is clear that a major part of the DNA is located near the nuclear membrane and some of it is associated with the nucleolus, and a small fraction (around 0.1%) of the total DNA is tightly bound to nuclear membrane components (Blackburn *et al.* 1978).

DNA is the same in almost all cells in the body of individuals (Wolpert 1988). A diploid mammalian cell contains ca. 10 pg of DNA or the equivalent

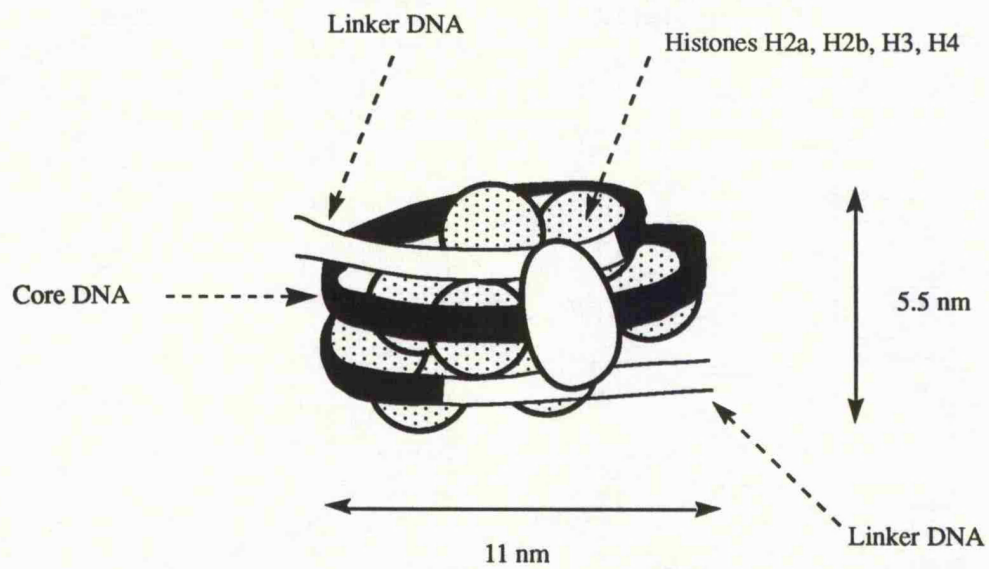




**FIGURE 1.1.4**

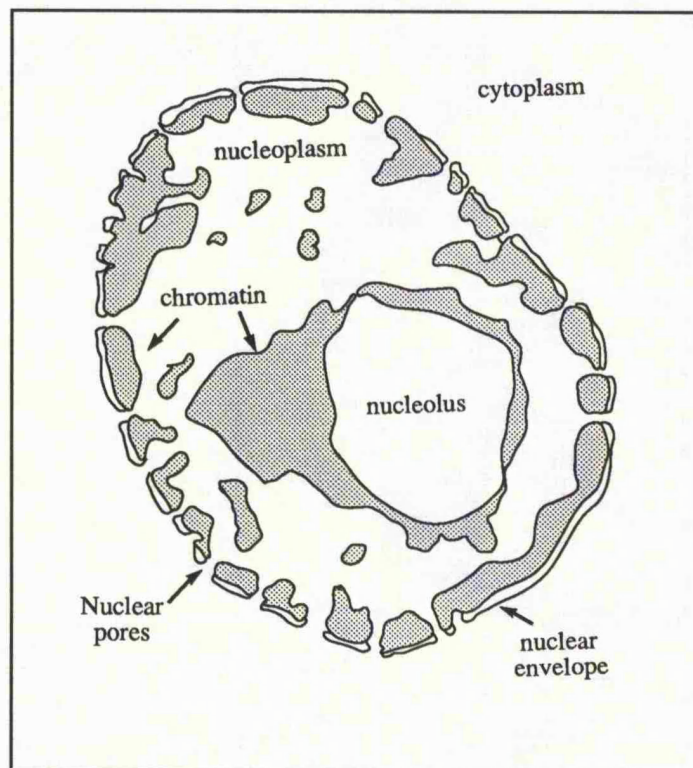
A schematic representation of how a very long DNA double helix molecule is organized via many different orders of chromatin packing to give rise to the highly condensed metaphase chromosome (Taken from Alberts *et al.* 1977).





**FIGURE 1.1.5**

Schematic diagram of a region of chromatin containing a nucleosome (Kornberg 1980).



**FIGURE 1.1.6**

Schematic representation of the distribution of chromatin within the nucleus (Taken from von Sonntag 1987).

of roughly  $3.5 - 4.8 \times 10^{12}$  dalton of DNA (Ostashevsky 1989). The extended DNA in the nucleus amounts to a single duplex some 1.9 m long, which contains around  $3 \times 10^9$  base pairs, and is arranged in haploid complement of 23 chromosomes in human cells (Evans 1984). The DNA and binding protein contain hydration bound water (0.3 g/g), also 60% of the particle volume is water.

The central role which the DNA molecule plays in the cell is to form the base sequence genetic code which determines the function and phenotype of the cells and to organize via its own mechanical integrity the correct transmission of the sequence of genes through mitosis to the daughter cells. Therefore, the continuing integrity of the DNA molecule is a prerequisite for the normal functioning and heredity of the cell. The alterations in the DNA molecule may obviously lead to damages in the base sequence and give an altered phenotype, mutation of the cell, or even cell death.

## 1.2

# Ionizing Radiation

The term "radiation" is very broad, it includes visible, infra-red, ultraviolet light, and radio waves, however, it is also used to mean "ionizing" radiation. **Ionizing radiation** is the radiation which changes the physical state of atoms at which it strikes, and causes them to become electrically changed or "ionized". It is also any radiation consisting of directly or indirectly ionizing particles or of a mixture of both (IAEA 1979, 1989). **Directly ionizing particles** are charged particles having sufficient kinetic energy to produce ionization by collision. **Indirectly ionizing particles** are uncharged particles which can liberate directly ionizing particles or can initiate nuclear transformation. Therefore, the phenomenon of ionization forms the basis of the definition of ionizing radiation. Biologically, ionizing radiations are non-specific damaging agents and can disrupt, like many alkylating agents, mutagens and carcinogens, normal biological processes in mammalian cells. However they act indiscriminately on all molecules in a treated sample and produce clusters of adjacent damages over short distances of a few nanometers.

There are many sources of ionizing radiation, including naturally-occurring radionuclides contained in the earth, building materials, air food and water, cosmic rays as well as man-made radiation. There are also different types of ionizing radiation, which are mainly classified as sparsely ionizing

radiation, such as X and  $\gamma$ -ray, and densely ionizing radiation, such as neutrons and  $\alpha$ -particles. Some characteristics of commonly used ionizing radiations are given in Table 1.2.1. In general, most of the radiation-chemical and radiation-biological work were done using  $^{60}\text{Co}$  gamma-source, X-ray machines and accelerators.

When ionizing radiation passes through matter, the interaction between the ionizing radiation and matter (including living matter) is a purely physical transfer of radiation energy. The energy of ionizing radiation is transferred to electrons of matter and ionization occurs. The ejected electrons usually possess sufficient energy to further cause the ionization and excitation of nearby molecules producing secondary electrons. Most of the radiation energy transferred is carried away as kinetic energy by the secondary electrons. The resulting molecular species possessing unpaired electrons are known as free radicals and are usually highly reactive. They tend to react further until chemically stable products are obtained, by a process of radical recombination. Therefore, the energy lost by these secondary electrons are the processes by which the energy of radiation is transferred to the matter, and this follows a sequence of events. Ionizing radiation interacts with matter through highly localized interactions leading to small but discrete volumes of excitation and ionization, the nature and extent of which depends on the quality of radiation, the absorbed dose and the dose rate as well as the chemical and physico-chemical composition of the irradiated materials (ICRU 1983).

Radiation damage to an intracellular molecule can be traced through several temporally distinct stages, which can be roughly divided into four parts (Dertinger and Jung 1970, Boag 1975):

(1) The physical stage ( $10^{-18}$  to  $10^{-12}$  seconds). The passage of an ionizing particle through an atom, in which energy deposition takes place via ionization and excitation processes;

(2) The physico-chemical stage ( $10^{-12}$  to  $10^{-9}$  seconds). The excited or ionized atoms rapidly bring about excited and ionized molecules which are

very unstable and reactive. Energy transfer, dissociation, relaxation and molecular rearrangement lead to the establishment of longer-lived diffusible radical species;

(3) The chemical stage ( $10^{-9}$  to  $10^0$  seconds). The radical species can diffuse to interact with radical scavengers or critical bio-molecules to give, eventually, stabilized molecular damage;

4) The biological stage ( $10^0$  to  $10^9$  seconds). The stabilized molecular damage, which is not repaired by the metabolic functions of the cell, can reveal itself in biological effect.

The separation of these time stages is somewhat arbitrary, but biological effects of ionizing radiation obviously result from a complex sequence of physical, chemical and cellular processes occurring over a protracted time scale.

Biologically, ionizing radiation is usually characterized by two factors: the linear energy transfer (LET), i.e., describing the energy deposition by different types of ionizing radiation. LET is defined as the average energy locally imparted to a medium by a charged particle of specified energy along a suitably small element of its path. High LET, such as  $\alpha$ -particles and fission neutrons, produces a high density of ionization along its path through the matter, whereas low LET, such as  $\gamma$ -ray and X-ray, produces relatively few ionizations (Adams 1987). And the relative biological effectiveness (RBE), i.e., describing the effectiveness of different types of ionizing radiation for inducing a particular biological end-point. RBE is defined as the ratio of the dose of the reference radiation (usually 250 KVp X-rays) to the dose of the particular radiation being studied that produces the same biological effect. The value of RBE depends not only on the types of radiation but also on the particular biological effects. For many biological effects, the RBE varies with the LET so that a hump-shaped response curve is obtained (Figure 1.2.1). The amount of radiation energy absorbed in any sort of matter is expressed in

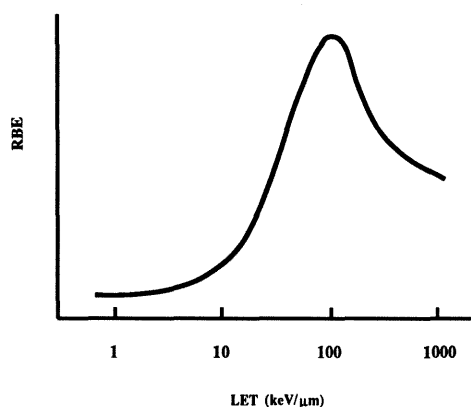
Table 1.2.1 Characteristics of some commonly used ionizing radiation

Symbol	Radiation	Source	Wavelength(mm)	Energy(MeV)	Charge	Mass <sup>a</sup>	Material
$\gamma$	gamma	radioactive nuclei	0.0005-0.1	10 - 100	0	0	photon
X	cathode	disintegrating atoms	0.01-10	0.01 - 10	0	0	photon
$e$	electron	accelerators & disturbed atoms	-	0.1 - >100	-1	1/1843	electron
$\beta$	beta	radionuclides	-	0.2 - 8	-1	1/1843	electron
$\alpha$	alpha	radionuclides	-	2 - 10	+2	4	Helium nucleus
n	neutron	accelerators & nuclear reactors	-	- <sup>b</sup>	0	1.01	photon + electron

<sup>a</sup> Mass relative to hydrogen;

<sup>b</sup> Thermal neutrons have < 0.04 KeV  
Intermediate neutrons have 1 - 500 KeV  
Fast neutrons have > 1 MeV.  
Adapted from Lucky, 1980.

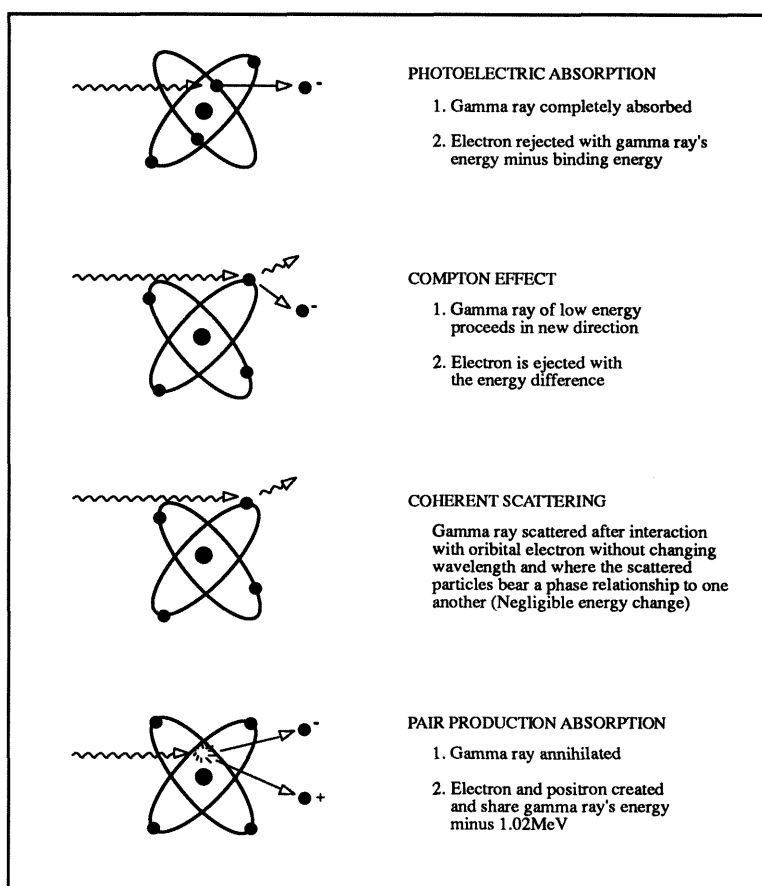
absorbed doses in the term of a International System Unit called the **Gray** (Gy, 1 Gy = 100 Rad).



**FIGURE 1.2.1** Generalized relationship between RBE and LET (Taken from IAEA 1986)

For all experiments presented in this thesis, a  $^{60}\text{Co}$  gamma-ray source was employed. Gamma-rays are low LET, sparsely ionizing radiations, and possess no mass, nor charge and penetrate tissues readily. The ionization at any particular dose is randomly distributed between molecules, particularly since there are a very large number of tracks. They typically travel long distances (many centimetres) from the emitting radionuclide before they interact with the tissue and cells to eject low LET electrons with ranges of micrometers to millimetres. Four types of attenuating events are considered (**Figure 1.2.2**) in the gamma-ray interaction with matter, photoelectric absorption, Compton effect, coherent scattering, and pair production absorption (IAEA 1979). It has been measured that on average each track of gamma-rays produces about 70 ionizations, 1 DNA single strand break, 0.04 double strand breaks, 0.01 initial chromatin breaks, about  $10^{-8}$  mutagenic

events and less than  $10^{-3}$  lethal events in a single gene of the cell, when cell nuclei are considered as spheres of  $8\ \mu\text{m}$  diameter (Goodhead 1991).



**FIGURE 1.2.2**

Gamma-ray interactions (Taken from IAEA 1979).



## 1.3

# Interaction of Ionizing Radiation with DNA Molecule

The interaction mechanism of ionizing radiation with DNA is very complicated and may involve many chromophores. The chemical and physical modifications in DNA may play a primary role (Ward 1981, Adams 1987, von Sonntag 1987). At present, it is generally considered that there are two action mechanisms for primary radiation damage to DNA (Ward 1985, Schulte-Frohlinde and von Sonntag 1985, von Sonntag 1987), the **direct effect** and the **indirect effect**.

There have been many attempts to produce definitions of these phenomena, none of which is entirely satisfactory. It becomes very philosophical at times, with ideas that are almost impossible to check. It is known that there are two major classes of damage to DNA molecules. One is observed in 'dry' DNA and in frozen aqueous DNA, where the significant damage is formed in the DNA itself in the form of electron-loss and electron-gain centres. These are certainly base centres, loss being from the purine bases and gain by the pyrimidine bases. These centres become 'fixed' in the DNA strands by proton transfer (Cullis *et al.* 1992). We think of such damage as being primarily "direct", we think of indirect damage in term of electron ejection from water molecules followed by proton loss to give 'OH radicals, and diffusion to the DNA to give base and sugar damage. Evidence suggests

that this is the most important mode of damage by the "indirect" process, and it is noteworthy the  $e^-$  and  $H^\bullet$  atoms do not seem to attack DNA so readily.

Returning to the "direct" mechanism, some confusion centres around the ability of  $H_2O^{+\bullet}$  radical cations to undergo electron-transfer prior to proton loss. It seems that for water molecules an average of about four electron transfer steps may occur in fluid water before the proton is lost. In that case, the 'hole' may move from  $H_2O^+$  into the DNA, and thus be indistinguishable from holes formed directly. Since no one as yet can detect these  $H_2O^{+\bullet}$  precursors, we include the route with the "direct" mechanism, since it is a charge-transfer process. We stress that, even without water damage, many "local" processes of this type must occur prior to the formation of the detected radical ions. Thus  $e^-$ -loss from phosphate should give  $(RO)_2PO_2^\bullet$  centres, which has been well characterised by electron spin resonance (ESR) spectroscopy (Sevilla *et al.* 1991, Hüttermann *et al.* 1992), but are never detected in DNA. Similarly the sugar unit must also lose electrons, but, like the phosphate centres, these are passed on to the base before any relaxation and trapping can occur. The same applies to the electrons.

These can reach DNA from  $H_2O^{+\bullet}$  ejection prior to solvation and give rise to pyrimidine radical-anions. We count such events as "direct". Again, such electrons could react with phosphate and sugar groups, but only the base-anions are detected by ESR spectroscopy.

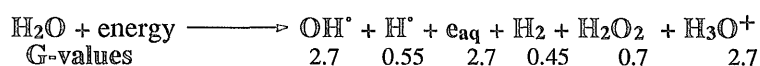
This is our "working" definition based on the results of ESR. We could produce new words, such as charge-transfer damage and radical damage etc, but should also briefly cover other concepts concerning "direct" and "indirect effects".

Baverstock and Cundall (1988a) proposed that "indirect effect" involves the interaction of genomic DNA with free radicals generated from the deposition of ionizing radiation in molecular components of the cell other than DNA, and "direct effect" involves the deposition of ionizing radiation energy

into the DNA molecule and the resulting damage without the mediation of an extrinsic radical or other clearly mobile species. Moreover, the damage due to indirect effect is supposed to be caused by  $\cdot\text{OH}$  radicals produced in the water sheath around the DNA molecule containing bound water (Michalik 1992).

The models of direct DNA damage were studied in different experimental conditions and assays (Baverstock 1985, Christensen *et al.* 1972, Roots and Okada 1975, Schulte-Frohlinde 1985, Ward 1985)

The data concerning indirect effects come mainly from studies of DNA irradiated in dilute aqueous solutions (Schulte-Frohlinde 1979, von Sonntag *et al.* 1981). In this case, the effects of irradiation are caused by the free radicals formed by the action of ionizing radiation on solvent water, which consists of the following three steps: (1) Direct action of radiation on water molecules which results, effectively, in ionization of water molecules to develop a serial 'primary radiolytic products of water', mainly  $\text{H}_2\text{O}^{+\cdot}$  and  $\text{e}^-$ . These primary processes do not occur separately in space, but as 'clusters' of small volume, called 'spurs' probably about 1 to 2 nm in diameter, containing 2 to 3 ion pairs (Mozumder and Magee 1966). The formation and the reactions of highly reactive water radiolytic products has been extensively studied (Draganic and Draganic 1971, von Sonntag 1987). In general, these molecules and radicals are summarised as:



The yields of these radicals are expressed as G-values, the G-value is defined as the number of product molecules formed per 100 eV of absorbed energy in the deoxygenated sample; (2) Reactions of reactive primary radiolytic products with DNA molecule; (3) Reactions of resulting radicals to form final stable products.

It has been concluded that the OH radical is the major effective one of these products in dilute aqueous solution of DNA (Block and Loman 1973,

Hutchinson 1985, Block and Loman 1986) and in exposure of cells (Roots and Okada 1972, Greenstock 1981, Ward 1981) to ionizing radiation, partly due to its high yield so that it attacks the sugar and base units frequently (von Sonntag 1987). In aqueous solution of DNA approximately 80% of the OH radicals attacking the DNA are expected to react with the bases and 20% with the sugar moieties (Scholes *et al.* 1960, Scholes 1983, Chatterjee and Magee 1985). The OH radicals may be responsible for 70% of the single-strand breaks produced in mammalian cells (Roots and Okada 1972). The solvated electron ( $e_{aq}$ ) and/or hydrogen atoms ( $H^{\cdot}$ ) can also inactivate biologically active DNA, but only by a relatively small amount (Feldbergard and Carew 1981).

Another definition is that, for practical purposes, the indirect effect mechanism can be considered to be that which, can be modified by radical scavengers, and the direct mechanism is that which cannot be modified (Baverstock and Will 1989).

In fact, the contribution of direct and indirect action to radiation damage are complementary, both are no doubt important. Thus the ultimate effect of ionizing radiation on DNA molecules must be the sum of direct action and of indirect action. The reactions and products in direct and indirect effects of radiation on DNA and their relationship are schematically shown in Figure 1.3.1. The main damage mechanism of radiation on the DNA molecule is different in the solid state, dilute aqueous solution, and cells or nuclei. In other words, the relative contribution of these two effects varies depending on the environment surrounding the DNA molecules being studied.

It is a long-standing question in radiobiology as to whether radiation effects on DNA in the cell nucleus occur mainly as the result of the attack by free radicals generated from water radiolysis or whether it is the result of direct ionization or excitation of DNA. Radiation damage to cellular DNA is closely related spatially to the distribution of energy deposit sites, due to its high concentration in the cell nucleus. In the nucleus of a cell, the concentration of DNA is considerably higher than that in model systems



because other surrounding components, such as protein and RNA which are tightly packed and highly organised together with DNA, result in a structure of semi-solid state solution (Révész 1985, Biaglow *et al.* 1983). Moreover, DNA-bound proteins are thought to protect the DNA inside chromatin against the damaging effects of radiation by scavenging hydroxyl radicals and by participating in chemical repair of DNA radical intermediates (Johansen and Howard-Flanders 1965, Roti Roti *et al.* 1974, Wartens and Childers 1982, Pritz 1988, Lett 1990, Ljungman 1991). Hydroxyl radicals are extremely reactive and will generally react with any organic material on contact. Thus, only diffusible water radicals close to the DNA molecules have a chance of reaching the target molecule to contribute to DNA damage. These facts may greatly limit the effective aqueous target volume and reduce the probability of indirect effects on the DNA.

Free DNA in a non-protective aqueous solution is far more susceptible to radiation damage than DNA within isolated chromatin and mammalian cells (Roti Roti *et al.* 1974, Swinehart *et al.* 1974, Mee *et al.* 1978, Hagen 1986, Ljungman 1991). A frequency of strand break per unit dose similar to that observed with DNA molecules in cellular environment was obtained when radiation-induced DNA strand breaks were measured in experimental conditions where there was no free water so that the 'direct' mechanism is maximized (Baverstock and Will 1987). These results indicate that the direct effect mechanism may be of considerable importance for DNA damage in cells, at least for the formation of strand breaks. It has been shown that a substantial fraction (30 to 70%) of DNA damage *in vivo* is due to the direct effect of radiation (Roots and Okada 1975, Ward 1981, 1985, Goodhead 1987, Lett *et al.* 1987).

However, in ESR studies of frozen aqueous studies of DNA, it was originally thought that the primary damage centres were confined to  $G^{\cdot+}$  and  $T^{\cdot-}$  units (Gräslund *et al.* 1971, Gegoli *et al.* 1982, Hüttermann *et al.* 1984, Herak *et al.* 1985, Cullis and Symons 1986). But recent work has established

that C is also a major product and A may also be of some importance (Barnes *et al.* 1991, Sevilla *et al.* 1991, Cullis *et al.* 1992, Hüttermann *et al.* 1992). Thus, it seems that there is no specificity which accords with end-group and sequence specificity results under these conditions (Cullis *et al.* 1992)

Finally, it should be stressed that although the primary lethal target for ionizing radiation within mammalian cells is always thought mainly to be nuclear DNA, the importance of other damage to cellular components, particularly as potential radiosensitisers or radioprotectors of DNA, cannot be ignored.

**1.4**

## **DNA Damage and Repair**

DNA is not a stable polymer and is subject to constant degradation damage as a result of spontaneous processes, largely resulting from the physiological conditions. The principal types of spontaneous damage are depurination, alkali-labile lesions and SSB which occur in total at a rate of about 100/min in the mammalian diploid genome (Vilenchik 1971, Lindahl and Nyberg 1972). 300 to 700 DNA strand breaks per unirradiated normal cell were reported for Hela cells (Rydberg 1980) and Chinese hamster ovary (Dikomey 1982). The important consequence of this chemical instability is the presence of continually operating repair systems capable of restoring the DNA to its undamaged state with a high degree of fidelity.

The general outline of the effects of ionizing radiation on DNA is based largely on identification of products. A spectrum of radiation-induced DNA structural modifications has been understood (Hüttermann *et al.* 1978, Lett 1990, Wallance and Painter 1990). Depending on the damage component and nature, it is usually accepted that the principal types for structure alteration of DNA molecule include: (1) chemical alteration in the four heterocyclic bases or sugar moieties without disruption of the backbone (Mattern *et al.* 1975, Hagen 1986); (2) DNA crosslinks between DNA-DNA, DNA-protein and protein-protein (Mee and Adelstein 1979, 1981); (3) DNA strand breaks, including physical disruption of



phosphodiester backbone of one chain of the DNA duplex, i.e., single strand break (SSB) and disruption of both strands of helix at the same or neighbouring sites, i.e., double strand break (DSB) (Humphrey *et al.* 1968, Lett and Sun 1970, Dugle *et al.* 1976, von Sonntag *et al.* 1981, Cadet and Berger 1985). Figure 1.4.1 illustrates the types of DNA damage induced by ionizing radiation, and the yields of some damages in relation to radiation dose in mammalian cells are summarized in Table 1.4.1. The ratio between the various lesions probably depends on the condition of irradiation quality, the state of DNA irradiated and the presence or absence of various radiomodifiers. Furthermore, these damages may also differ with respect to the repair pathways involved in their repair and to their biological significance.

Table 1.4.1 Measured numbers of damaged sites per cell per Gy

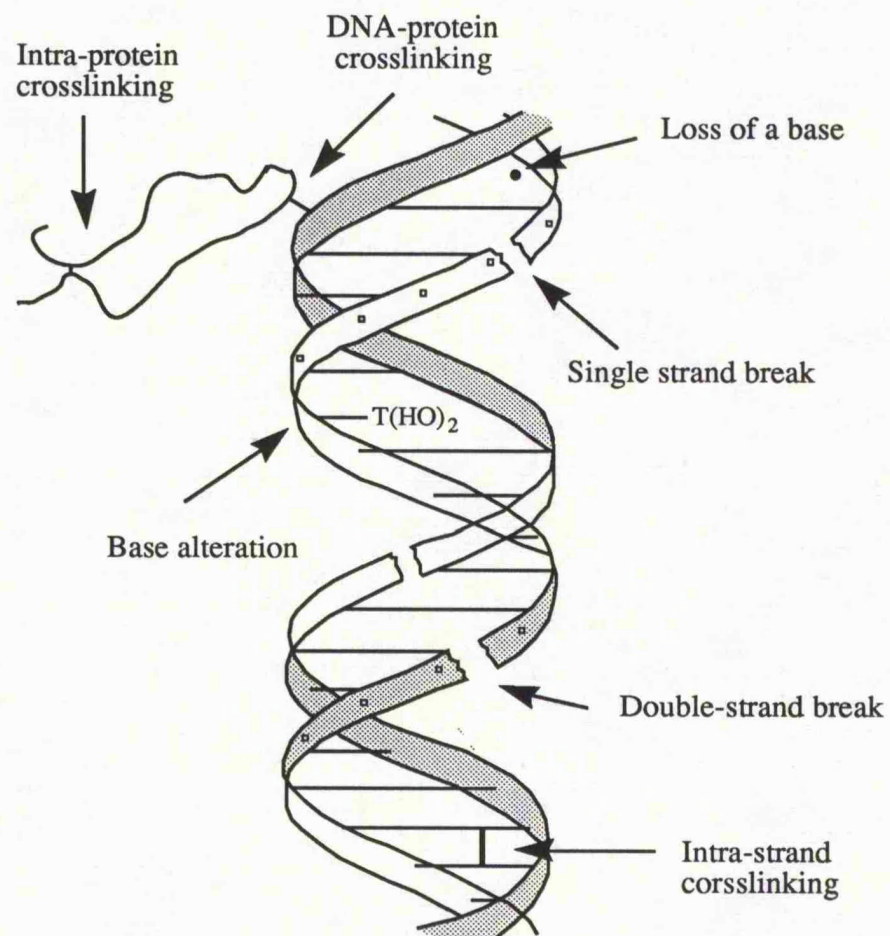
Base damage	1000
SSB	1000
DSB	40
DNA-protein crosslink	150

Data derived from Ward 1988.

SSB and some base lesions are regarded as 'irrelevant' DNA damages which are repairable with high fidelity and non-contributory to biological effects, but DSB and DNA-protein crosslinks are 'relevant' to DNA damages, which possibly contribute to biological effects (Baverstock 1991).

#### Base damage

After irradiation there is a great variety of altered DNA bases to be observed (Téoule 1987, Fuciarelli *et al.* 1990), and some of these base modifications can result in the disruption of the sugar-phosphate backbone



**FIGURE 1.4.1**

Predominant primary lesions identified in DNA molecule following exposure of ionizing radiation.

that the efficiency of this process was estimated to be about 10% (Schulte-Frohlinde 1990). Radiation-induced base damage can be rapidly and completely repaired via excision repair pathway in normal mammalian cells (Remsen and Cerutti 1977, van Loon *et al.* 1991).

The main difficulties encountered in the determination of radiation-induced base damages lie mostly with their low quantity and the wide range of compounds, together with the instability of some of the modified bases (Téoule 1987, Teebor *et al.* 1988). Much effort has been made to study chemical characterization and quantification of DNA base modifications, mainly the yield of radiation-induced purine and pyrimidine products, in different environments using chromatographic assay (Téoule *et al.* 1977, Breimer and Lindahl 1985), immunochemical assay (West *et al.* 1982a, 1982b), biochemical enzymatic analysis (Hariharan 1980) and gas chromatography-mass spectrometry (GC/MS) with selected ion monitoring (Fuciarelli *et al.* 1989, Dizdaroglu 1992). However, all of these techniques are capable of assaying only a very limited number of modified DNA products and no insight is given for the amount of total base damage. The oxidation products of thymine, thymineglycol (HO-T-OH), thyminehydroperoxy and 5-hydroxy-6-hydro-thymine (H-T-OH) are the best known radiation products of DNA bases (Dizdaroglu 1992).

### DNA crosslinking

In general, little is known about radiation-induced DNA crosslinks. It was found that DNA-protein crosslinks are formed spontaneously and produce a relatively high background of DNA-protein in non-irradiated chromatin (Mee and Adelstein 1981, Chiu *et al.* 1986, Teebor *et al.* 1988). Radiation increases the amount of DNA-protein crosslinking, the formation of which is thought to involve the initial interaction of hydroxyl radicals either with chromatin proteins or with constituents of DNA (Ramakrishnan *et al.* 1987). Some of such damages may be repaired at a slow rate (Chiu *et*

*al.* 1990). However, The chemical nature of these crosslinks and the mechanism of their repair are still not understood.

### DNA strand breaks

In all radiation-induced DNA damages, strand breaks predominate both in frequency and importance, since the covalently linked backbone of the DNA can be directly broken at the time of radiation exposure (called frank breaks) and a wide variety of lesions in the structures of base or sugar moieties can also lead to strand breaks via the intermediary of certain enzymes during repair of such lesions. DNA strand break is not site-specific but could be formed at any base (Henner *et al.* 1982). About 25% of DNA radical ions are converted into strand break (Symons 1987). DNA strand breaks may be classified according to three ways, that is, (a) the DNA strand damage formats which may usually distinguish SSB from DSB, this is the most common classification; (b) their end-groups by biochemical assays, i.e., DNA strand breaks (i) with phosphate groups at both the 3' and 5' terminals with sugar and the base missing and (ii) with an altered sugar on 3' terminal and 5' phosphate end group (von Sonntag *et al.* 1981); and (c) their repair profiles, fast, slow and non-repairable strand breaks are mainly considered by simulating DNA repair kinetics by various models (Nelson 1982). The chemical nature of these strand breaks in DNA has been reviewed by Schulte-Frohlinde and von Sonntag (1985). The RBE for DSB induction is close to unity, whereas that for SSB is less than unity (Maki *et al.* 1986, Grdina 1989, Peak *et al.* 1991).

### *Single and double strand breaks*

Single strand break (SSB) is the most abundant radiation lesion in DNA, and defined as the break in just one of the phosphodiester strands. Directly induced SSB can occur at the level of the phosphate diester bond, between the phosphate and the deoxyribose, or more frequently at the level

of the bond between the base and deoxyribose. The mechanism for the production of SSB in oxygen is well established, it is initiated by the production of a radical on the deoxyribose by loss of a hydrogen atom (either by direct ionization and proton loss, or abstracted by a  $\cdot\text{OH}$  radicals), this radical reacts with oxygen forming a peroxy radical and subsequently a SSB (von Sonntag 1987).

Despite the indicated technical differences, the formation and repair of SSB have been studied with success, the frequencies of SSB in the DNA of irradiated whole cells are nearly 100-fold less than that in DNA aqueous solution irradiated, and 10 fold less than that in isolated chromatin (Lett *et al.* 1967, Sawada and Okada 1970, Ormerod and Stevens 1971, Elkind and Chang-Liu 1972, Heussen *et al.* 1987, Feingold *et al.* 1988). It has been shown that the number of SSB is linearly related to the dose of radiation over a very wide dose range, from 0.2 Gy to 60,000 Gy, both in DNA aqueous solution (Bopp and Hagen 1970, Ward and Kuo 1978) and in cells (Dugle and Gillespie 1975, Ono and Okada 1974, Loggle 1983). The rate of 0.1 - 0.2 breaks/10Gy/10<sup>8</sup>dalton was reported widely in the literatures (Lehmann and Ormerod 1970a, 1970b, Garel and Axel 1976, Chiu *et al.* 1982, Chiu and Oleinick 1982, Graubmann and Dikomey 1983, van der Schans *et al.* 1983, Oleinick *et al.* 1984, Charlton *et al.* 1989).

About 0.07 to 0.1 SSBs per cell per Gy are produced in mammalian cells exposed to X-rays (Lett and Sun 1970, Roots and Okada 1972), and the same number of SSB in all phases of the cell cycle (Humphrey *et al.* 1968). The number of SSB in well oxygenated mammalian cells is 3 to 4 times that found in cells irradiated under hypoxic conditions (Heussen *et al.* 1987). As a rule, the yield of SSB per unit dose has been found to decrease as the LET of radiation increases (Ahnström and Edvardsson 1974, Ritter *et al.* 1977, McWilliams *et al.* 1983, van der Schans *et al.* 1983, Mirzayans *et al.* 1988).

With the increasing knowledge of radiation-induced DNA damage, DSB has been considered to be the most important and critical damage

resulting in some biological genetics effects, unrepaired or unreparable DSB has obvious deleterious effects on the ability of dividing cells to successfully pass genetic information from one generation to the next.

Double strand break (DSB) is defined as the break at the same or neighbouring place in each of two phosphodiester strands so that the DNA duplex molecule is cut into two pieces. At least two mechanisms concerning the DNA DSB formation have been proposed. If the pattern of radiation energy deposition by a single track is such that it forms DSB during its passage through the DNA as a result of a single hit process, which is formed linearly with dose (van der Schans *et al.* 1973, van Touw *et al.* 1985, Chatterjee and Magee 1985), the number of DSB will be very small compared to the number of SSB. On the other hand, DSB can result from two separate SSB either at directly opposite sites or at close proximity on opposite strands. This increases with the square of dose (van der Schans *et al.* 1973, Siddiqi and Bothe 1987).

The separation of two SSBs forming a DSB in opposite strands depends on the stability of hydrogen bonds between the DNA two strands (von Sonntag *et al.* 1981, Michalik, 1992). It is assumed that the maximum distance is 30 base pairs in irradiated supercoiled DNA in dilute aqueous solution at normal temperature (van der Schans 1978, van Touw *et al.* 1985) or 60 base pairs (Hempel and Mildemberger 1987) by using neutral gel electrophoresis. In the presence of high and low ionic strength, it is 2.64 and 15.8 base pairs, respectively (Freifelder and Trumbo 1969). But, using sucrose gradient centrifugation van der Schans (1978) reported only 16 base pairs. In addition, less than 10 base pairs was also reported by others (Chatterjee and Magee 1985, Charlton *et al.* 1989). The lack of close agreement probably stems from the fact that this number increases substantially with temperature during irradiation (Lindenau *et al.* 1976), post-irradiation heat treatment (van der Schans 1978, van Touw *et al.* 1985) and salt concentration (Freifelder and Trumbo 1969, van der Schans 1978).

Moreover, DSB may also be formed by enzymatic conversion of other types of damage (Bender *et al.* 1974, Craig 1980, Schulte-Frohlinde 1987). Ward (1985) and Téoule (1987) invoked a concept of multiple radical attack to explain the possible formation of DSB, i.e., locally multiply damaged sites (LMDS) which occur when clusters of ionization overlap the DNA. This indicates that the precursors of these lesions consist of pairs of independent radical sites formed one on each strand. It was indicated that high LET radiation is more likely to generate DSB by local massive destruction (Tobias *et al.* 1980, Coquerelle *et al.* 1987).

The dose relationship for the induction of DSB in dilute aqueous solution was in early experiments reported to be quadratic (Cox *et al.* 1958, Peacocke and Preston 1960), but later more careful analysis yielded a mix linear-quadratic dependence,  $aD + bD^2$ , (Freifelder and Trumbo 1969, Block and Loman 1973, van der Schans *et al.* 1973, Lindenau *et al.* 1976). However, DSB in dry DNA irradiated *in vitro* exhibits a linear dose relationship (Neary *et al.* 1970).

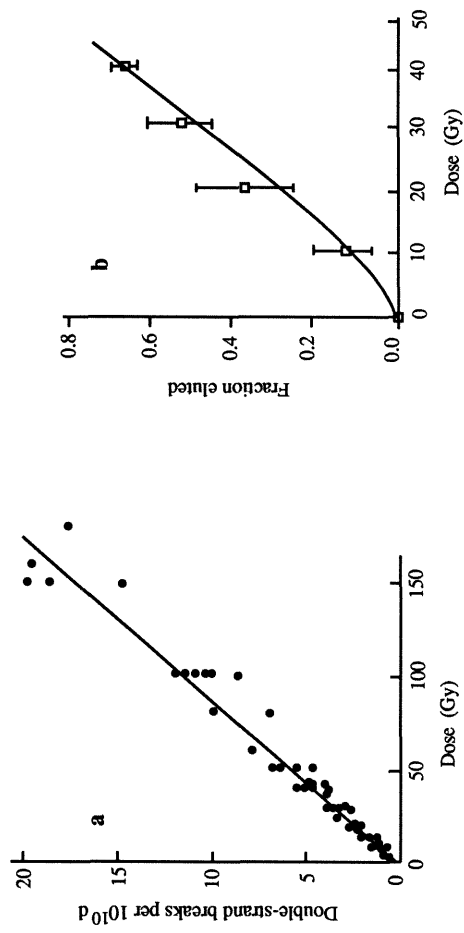
The relationship between dose and the number of DSB induced remains in doubt in mammalian cells, especially at low doses. Different methods gave different results which have led to a controversy concerning the shape of the dose-response curve and the importance of initial and resident DNA damage. In some works, the yields of DSB were found to be proportional to dose for DNA from eukaryotic (Bonura and Smith 1976, Sakai and Okada 1984, Roots *et al.* 1985) and prokaryotic (Bohne *et al.* 1970, van Touw *et al.* 1985, Roots *et al.* 1985) sources over a broad range of radiation doses. A linear dose-response was observed at 5 Gy to 250 Gy of radiation doses, with a frequency of about 0.01 to 0.02 breaks/ $10^9$ daltons/Gy in mammalian cells using neutral sucrose gradient sedimentation, PFGE and DNA precipitation under oxygen conditions (Corry and Cole 1968, Lehmann and Ormerod 1970a, Lett 1970, Corry and Cole 1973, Lehmann and Stevens 1977, Frankenberg-Schwager *et al.*

1979, Blöcher 1982, Olive 1988, Blöcher *et al.* 1989). This linear relationship indicates that DSB may arise as a results of single ionizing events or clusters (Siddiqi and Bothe 1987).

However, it has been found that there was a shoulder on the dose-response curve for induction of DSB when using neutral filter elution. Only the initial part of the curve at the lower dose is linear and at higher dose the number of DSB increases with some power of the dose, i.e., linear-quadratic response curve (Radford 1985, Prise *et al.* 1987, Peak *et al.* 1988a, Eguchi-Kasi *et al.* 1991). This shows that DSB may be produced by the passage of one ionizing event or as a result of two independent SSBs. Along with the suggestions of the non-linear yield-dose response, such a threshold would be expected for induction of DSB (Blazek *et al.* 1989). This threshold in DNA elutability might be attributable to variations in the structure of DNA or chromatin with individual cells, in various phases of the cell cycle, or to a saturation of 'chemical' repair process (Radford 1985, 1987, Wlodek and Hittelman 1987, Okayasu *et al.* 1988, Sweigert *et al.* 1988, Radford 1990). However, there were also experiments to obtain a linear dose effect curve through the modification of condition and composition of lysis and eluting solution (Ross and Bradley 1981, van der Schans *et al.* 1982, Prise *et al.* 1987, Okayasu *et al.* 1988, Flick *et al.* 1989, Okayasu and Iliakis 1989, Warters and Lyons 1990). Tilby *et al.* (1984) have reviewed a number of reports of qualitative and quantitative difference between DSB results obtained by neutral velocity sedimentation and neutral filter elution. Figure 1.4.2 shows the typical curves obtained using these two popular methods.

The ratio of the yield of SSB to DSB varies with the type of ionizing radiation used (Siddiqi and Bothe 1987) and is influenced by the measuring techniques and the state of the DNA during the irradiation. It appears that the ratio of SSB to DSB is in the order of 10 to 1 for low LET radiations, whereas with high LET radiation DSB are almost as frequent as





**FIGURE 1.4.2**

Kinetics of induction of DSB (represented in b by fraction of DNA eluted from filter) in X-irradiated Ehrlich ascites tumour cells by: a) the neutral velocity sedimentation technique (adapted from Blocher 1982), and b) the neutral filter elution technique (data from Costa and Bryant 1990a).

SSB *in vitro* (Alexander and Lett 1967, van der Schans 1981, Roots *et al.* 1985). Therefore, high LET radiation has a higher ratio of DSB to SSB than low LET radiation. For example, after X-irradiation DSB are 10 to 20 times less frequent than SSB in mammalian cells (Coquerelle *et al.* 1973, Cole *et al.* 1975, Matsudaira *et al.* 1977, Bryant and Blöcher 1980, van der Schans *et al.* 1983), 10 to 100 in the case of exposure to gamma-rays (van der Schans *et al.* 1982) and 10 to 30 *in vivo* (Bohne *et al.* 1970, van der Schans and Block 1970, Setlow and Setlow 1972).

### Repair of single and double strand breaks

A lethal radiation dose causes thousands of DNA strand breaks and other damages, but the cell has very competent repair systems to excise the damaged section and to survive. A normal capacity of the cell to repair DNA damage is critical to survival (Kemp *et al.* 1984). Damage repair may be carried out by one or more repair processes (chemical or/and enzyme repair). Before final damage products in DNA are formed, DNA radicals produced by direct and indirect effects of ionizing radiation may be reversed to normal DNA for some kinds of damage (Figure 1.4.10), which may be accomplished by various H atom and electron donors such as ascorbate and antioxidants and transfer of alkyl group to a recipient protein (Olesson and Lindahl 1980, Mehta *et al.* 1981). This kind of repair is called **chemical repair** (Nygaard and Simic 1983, Simic 1986). Chemical repair may lead to complete restitution of the original material by either endogenous or exogenous repair agents (radioprotectors) and can also lead to the formation of a variety of different products (DNA-P', as shown in Figure 1.4.10).

Once final damage products are formed within the DNA, they are amenable to enzymatic repair (Friedberg 1985). Experiments with metabolic inhibitors have shown that the enzymes responsible are present in the cell prior to irradiation. DNA strand breaks present immediately after

irradiation are repaired quite rapidly (Ahnström and Bryant 1982). The repair of the different types of damage may involve different repair pathways, such as, excision repair (Henawalt *et al.* 1979), post-replication repair (Rupp *et al.* 1971), photoreactivation (Sutherland 1974) and recombination (Coggle 1983).

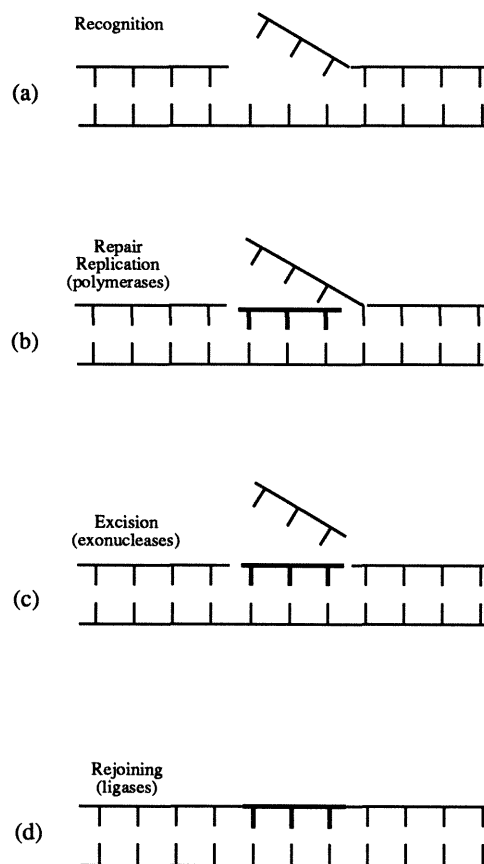
The models of damage repair in DNA can be classified in the following simplified way: 'error-free' repair, mainly excision repair, which leads to normal mitosis and causes no lethality and no mutation; 'error prone' repair which leads to chromosome and chromatid aberrations and may produce non-lethal or lethal mutations. The damage is not itself immediately repaired but is by-passed during DNA replication leaving gaps in the daughter strands. There is also 'incomplete' or lack of repair which leads to deletions and, inevitably, to loss of a part of genetic material in the first or subsequent mitosis where the continuity of the DNA strands is not re-established. In any case, a complex family of repair enzymes are needed in order to remove complex lesions from damaged DNA. The cell repair efficiency may decrease with increasing dose due to partial saturation of repair system (Goodhead 1985). Therefore, the repair of DNA damage may be an even more important factor than the lesion yield in determining the overall effect of radiation on cells and tissues.

Since the first report by Lett *et al.* (1967), the repair of SSB has been extensively studied by the use of different methods under alkaline condition. SSB in DNA produced by ionizing radiation could be rapidly and efficiently repaired *in vivo* or *in vitro* by repair experiments through cellular repair capability (Fox and Fox 1973, Ormerod 1976, Loggle 1983), although the enzymatic mechanism has not been completely worked out.

The repair of SSB is usually explained by the mechanism of 'excision repair', involving the removal of damage regions or inappropriate bases and re-synthesis of DNA using the undamaged complementary strand as a

template. The sequential steps briefly include: precision recognition of damage; incision of the damaged DNA strand at or near the lesion cleaving the phosphodiester bond; excision of the lesion and some of the surrounding nucleotides; repair replication to replace the excised region with a corresponding stretch of normal nucleotides ligation linking the newly synthesized repair patch to the parental DNA strand (Leadon 1990), as indicated in Figure 1.4.3. This repair cannot occur at 0°C since the repair process is enzymatically controlled and temperature dependent.

The kinetic analysis has been shown that the processes of SSB rejoining include at least two components, a fast component (half-time,  $t_{1/2}$ , of 2 - 10 minutes) and a slower component ( $t_{1/2}$  of 20 to 300 minutes) using different radiation doses in various mammalian cell lines (Ahnström and Edardsson 1974, Ben-Hur and Elkind 1974, Dugle and Gillespie 1975, Koch and Painter 1975, Roots and Smith 1975, Furumo *et al.* 1979, Fornace *et al.* 1980, Blöcher and Pohlit 1982, Dikomey 1982, van der Schans *et al.* 1983, Wheeler and Nelson 1987, 1991). The fast repair part of damage was estimated to comprise more than 75% of the observed SSB in cells immediately after irradiation (Ormendo 1976) and approximately the same amount of the breaks was rejoined by the fast repair process (Koch 1975, Yoshida *et al.* 1985). This biphasic nature of repair implied that at least two different molecular mechanisms may be involved in the repair of the DNA SSB. The fast and slow phase represent the repair of SSB and DSB, respectively (Bryant and Blöcher 1980, Blöcher 1983, Evans *et al.* 1986, van Ankeren and Meyn 1987). Alternative interpretation is that different repair rates may also represent the repair of lesions in very accessible and less accessible regions of the chromosomal DNA (Chiu and Oleinick 1982, Wheeler *et al.* 1988) or the involvement of different enzymes (such as polymerase  $\alpha$  or  $\beta$ ) as suggested by Miller and Chinault (1982) and Wheeler *et al.* (1992).



**FIGURE 1.4.3**

Diagram of the excision repair mechanism for the removal of such radiation induced lesions as DNA base damage and single-strand break (Adopted from Coggle 1983).

In addition, a few indications suggested that there is an intermediate repair component with half-time of 10 to 30 minutes present between the fast and slow repair of SSB, this part of repair was also considered to represent other classes of SSB (Korner *et al.* 1978, Bryant and Blöcher 1980, McWilliam *et al.* 1983, Bryant *et al.* 1984, Dikomey and Franzke 1986). Therefore, up to now, the repair kinetics of radiation-induced SSB in DNA of mammalian cells is generally considered to be best described by a sum of three exponential components (Figure 1.4.4). For instance, for X-ray irradiation in CHO cells, the half-times of the three components are about 2, 17 and 200 minutes and initial fraction, 0.7, 0.25 and 0.05, respectively, and these values are independent of radiation doses in the range from 1 to 100 Gy (Dikomey and Franzke 1986).

As for the repair of DSB, it is now believed that DSB can be repaired, at least in L1210 cell (Bradley and Kohn, 1979), CHO cells and primary human fibroblasts (Lehmann and Stevens 1977, van der Schans *et al.* 1982), V79 cells (Weibezahn and Coquerelle 1980), and Ehrlich ascites cells (Bryant 1980, Bryant and Blöcher 1980, Blöcher 1982, Ahnström and Bryant 1982). It was shown that mammalian cells are able to repair radiation-induced DSB at rates similar to those for SSB without an induction period (Corry and Cole 1973, Lehmann and Stevens 1977, Blöcher and Pohlit 1982, Blöcher *et al.* 1983) and are very efficient in repair of DSB in all phases of the cell cycle (Blöcher 1982). DSB is repaired significantly faster in quiescent cells than in proliferating cells for CHO cells (Dikomey 1990), for V79 cells (Weibezahn and Coquerelle 1981) and for EAT cells (Bryant and Blöcher 1980). There is a marked difference in DSB repair between repair-proficient cells and repair-defective cells (Ikialis *et al.* 1992). The amount of DSB rejoining also declines with increasing radiation dose, like SSB repair (van der Schans *et al.* 1982, Koval and Kazmar 1988, Ikialis *et al.* 1991b, 1992).



Repair kinetics of X-ray induced DNA strand breaks. data was taken from 1: Ahnstrom *et al.* (1974), V79, 9Gy; 2: Bryant and Blocher (1980), Ehrlich ascites, 100Gy; 3: Bowden and Kasunic (1981), U210, 3Gy; 4: Jorristma and Konings (1983), Ehrlich ascites, 6Gy; 5: Korner *et al.* (1978), V79, 100Gy; 6: McGhie *et al.* (1983), NHIK, 18.5Gy; 7: McWilliams *et al.* (1983), Hum. lym., 1Gy; 8: Sakai and Okada (1981), V79, 45Gy; and 9: Dikomey and Franzke (1986), CHO, 9Gy. Data are fitted by a sum of free exponential components as indicated by the indices I, II and III.

The kinetics of DSB rejoining in mammalian cells is an unresolved puzzle, because the repair patterns are different by using different methods. The repair curve could be described by a single exponential component with a half-time of some 2 to 4 hours using the method of premature chromosome condensation, alkaline unwinding or neutral sedimentation technique (Bryant and Blöcher 1980, Blöcher and Pohlitz 1982, Johnson *et al.* 1982, Cornforth and Bedford 1983, Iliakis *et al.* 1988, Wlodek and Hittelman 1988a). With neutral filter elution the repair kinetics has been consistently reported with a sum of two exponential components, an initial steep repair component of 70 to 90% ( $t_{1/2}$ , of less than 15 minutes) at low dose range, followed by a shallow component with an initial fraction of 10 to 30% ( $t_{1/2}$ , of 0.5 - 2 hours) at high dose range (Bradley and Kohn 1979, Weibezahn and Coquerelle 1981, Woods 1981, Blöcher and Pohlitz 1982, McGhie *et al.* 1983, Radford 1983, van der Schans *et al.* 1983, Radford 1985, Prise *et al.* 1987, Okayasu *et al.* 1988, Okayasu and Iliakis 1989, Swiegert *et al.* 1989).

This biophasic repair kinetics of DSB obtained by neutral filter elution could possibly be interpreted to reflect two distinct repair components, i.e., an initial component reflecting fast repair followed by a slower repair component. But a hypothesis suggests that the fast phase represents repair in very accessible regions of the chromatin and the slow phase represents repair in less accessible regions of the chromatin (Wheeler and Wierowski 1983).

This strong dependence of the DSB rejoining results on the methods applied is a well-documented fact (Peak *et al.* 1988b, Hutchinson 1989), but the reason is not completely understood up to now. Possible explanations are that (1) different methods may monitor different types of DNA lesions (existence of more than one type of DSB) and different mammalian cells may repair DSB at different rates (Costa and Bryant 1990b); (2) neutral filter elution differentiates between DSB of different complexity, either in

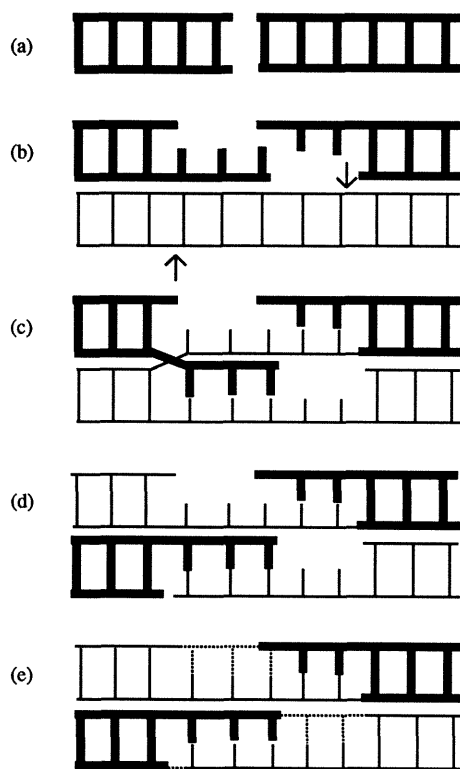


the repair enzymes required (Weibezahn and Coquerelle 1981) or in the location within the DNA (van der Schans *et al.* 1983); (3) the substrate used in different methods are different (Costa and Bryant 1990a). More recently, Iliakis *et al.* (1991b) reported that for all major techniques currently used, the rejoining of DNA DSB gave a similar initial rate under experiments of same initial cell population.

In contrast of the repair of SSB, the repair of DNA DSB does not have a template to copy and the biochemical mechanisms and processes involved in DSB repair are not yet known, although some possible repair models and mechanisms of DSB have been proposed (Lennartz *et al.* 1975a, Weibezahn and Coquerelle 1981, Kemp *et al.* 1984, Cox *et al.* 1986, Radford 1987). Figure 1.4.5 shows a theoretical model proposed by Resnick (1976) to explain how radiation-induced DNA DSB might be repaired, but there is as yet no experimental evidence to prove or disprove the recombinational process for the repair of DSB in DNA.

### Unrepaired DNA strand breaks

Although the important role of the unrepaired fraction of DNA breaks and their relation to some biological effects has been suggested (Painter *et al.* 1974, Painter and Young 1977, Sakai and Okada 1984, Koval and Kazmer 1988), quantitative study of these lesions in cultured cells has been very limited, partly because of the insufficient sensitivity for this damage detection and the small amount of unrepairable damage. Remaining SSBs after various periods of repair incubation were found to be linearly related to dose below a certain level, 50 Gy for neutrons and 10 Gy for gamma-rays, above these dose levels the curves bend upwards (Ahnström 1982), whereas unrepaired SSBs are only observed for X-ray doses exceeding 40 Gy and after very long repair intervals (Blöcher and Pohlitz 1980, Sakai and Okada 1981, 1984). These unrepaired SSBs can be considered to take part in the formation of DSB. It is generally assumed



**FIGURE 1.4.5**

A theoretical model for the repair of DNA double strand break. → = endonuclease incisions (Redrawn from Coggle 1983).

- (a) Double strand break;
- (b) Enzymatic excision of parts of a single strand for each of the broken ends, leading single-stranded pieces;
- (c) Terminal unjoined segments of each strand;
- (d) Reciprocal exchange between the double strand of DNA;
- (e) Final repair.

that SSB is unlikely to play an important role in the formation of subsequent biological effects, until they are inverted into DSB or unrepaired. For example, Ritter *et al.* (1977) reported that high LET radiation produced non-rejoined DNA SSB after 12 h exposure and was proportional to the enhanced cell killing produced by high LET radiation.

The curves of the frequency of unreparable DSB plotting against dose were shown to be concave (Sakai and Okada 1984, Eguchi-Kasai *et al.* 1991). High LET radiation produces high levels of non-rejoined DSB (Furuno *et al.* 1979, Blöcher 1988, Fox and McNally 1988), which may suggest that high LET radiation-induced DSB may be different in nature to those induced by low-LET radiation (Fox and McNally 1990).

### Determination techniques

At present, there is a wide range of quantitative assays available for the determination of radiation-induced DNA damage and the damage repair in cultured cells, based on widely different biophysical principles and relevant conditions that they are performed under (reviewed by von Sonntag 1987, Ahnström 1988, Radford 1988), such as light scattering method (Jones and O'Neil 1990), electrophoresis (Henner *et al.* 1982, Schwartz and Cantor 1984, Roots *et al.* 1985), velocity gradient sedimentation (McGrath and Williams 1966, Lehmann and Stevens 1977), DNA unwinding with hydroxylapatite chromatography (Ahnström and Erixon 1973, Ahnström and Edvardsson 1974), filter elution (Kohn and Grimek-Ewig 1973, Bradley and Kohn 1979), DNA precipitation assay (Olive 1988), pulsed-field-gel electrophoresis (Schwarz and Cantor 1984), flow cytometric assay (Item and Burkart 1987), enzymatic methods (Breimer and Lindahl 1985, Bryant 1985, Hagen 1986) and immunological methods (van der Schans *et al.* 1989). These biochemical methods are mainly utilised to measure DNA strand breaks, since, in addition to primary

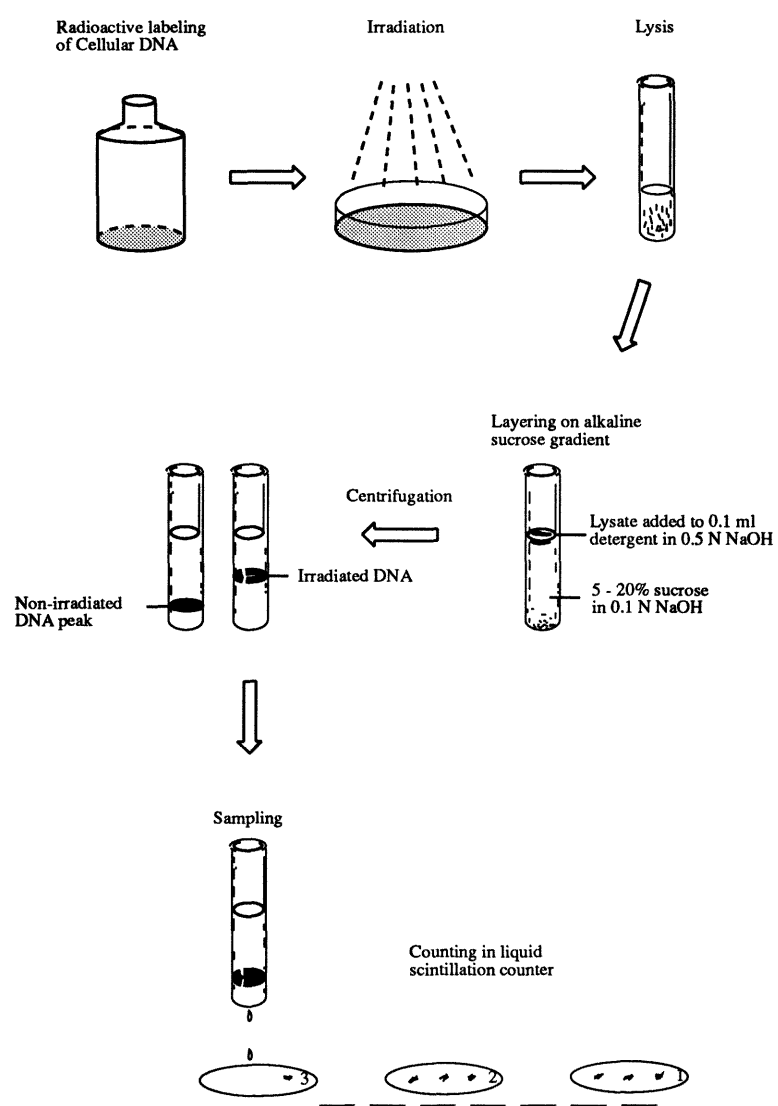
breaks, most other lesions in DNA can be transformed to strand breaks. They are also generally applicable to the study of other types of DNA lesions. Qualitative analysis of DNA DSB now is to use restriction enzymes to introduce a range of DSB in a selectably specific gene sequence so that the nature of the DNA repair event can be more closely characterised (Cox *et al.* 1984, Natarajan and Obe 1984, Bryant 1985, Cox *et al.* 1986).

In all available techniques, at present, the more used methods are sucrose gradient sedimentation, assay of DNA-unwinding with hydroxylapatite chromatography and filter elution. Herein a simple introduction concerning the three techniques is presented.

#### *Assay of sucrose gradient sedimentation*

Gradient sedimentation was originally introduced by McGrath and Williams (1966) to study SSB of bacteria under alkaline condition, and subsequently applied to test SSB induced by X-ray in mammalian cells (Lett *et al.* 1967). The measurement of the induction and repair of DSB in eukaryotic cells was also studied based on the sedimentation distance by the DNA in a neutral sucrose gradient under centrifugation to determine its relative molecular mass (Lehmann and Ormerod 1970, Corry and Cole 1973, Hartwig and Handschack 1975).

This technique is, at present, the only assay that enables direct measurement of the molecular weight of the DNA and thus of actual numbers of SSB and DSB present in the DNA. Figure 1.4.6 simply shows a flow procedure of the alkaline gradient sedimentation. However, this technique is only capable of distinguishing between relatively small pieces of DNA and so a relatively large radiation dose has to be given (50 to 100Gy) to chop the DNA into pieces small enough to be detectable. In addition, it is not only tedious but also lacks sensitivity and the result might be influenced by many parameters, such as speed effects, wall effects, gradient stability and capacity (Lett 1981, Ahnström 1988).



**FIGURE 1.4.6**

A typical procedure for alkaline sucrose gradient sedimentation analysis of DNA from cultured cells exposed to ionizing radiation to determine the number of breaks induced (Adapted from Kaplan 1972).

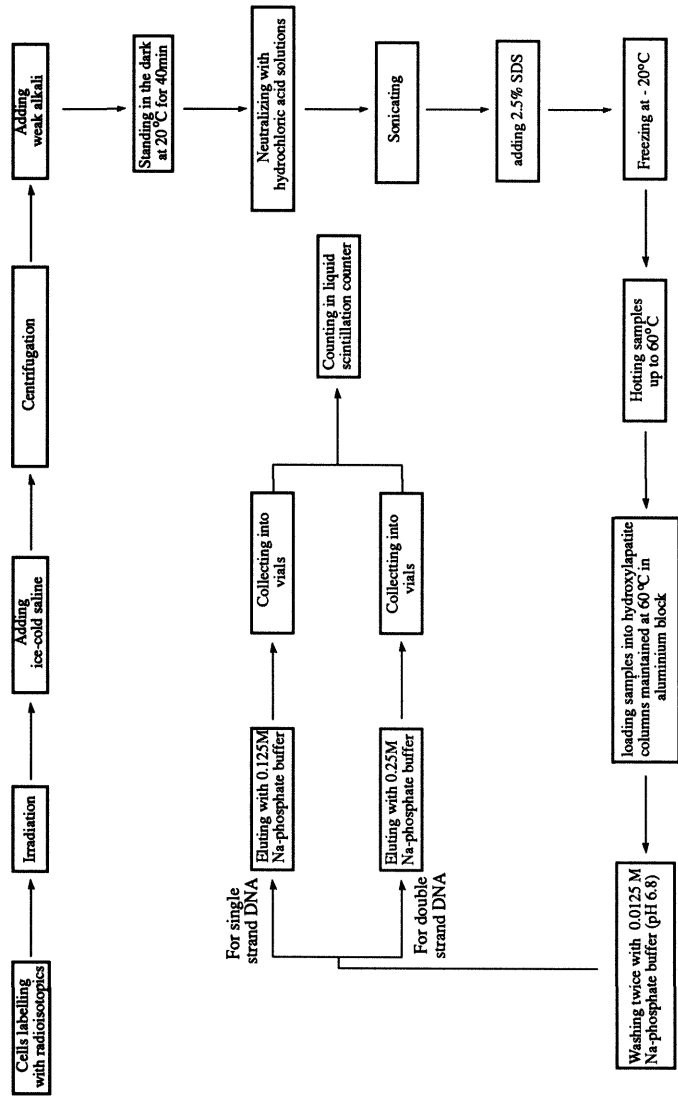
*DNA unwinding technique*

This method is based on the denaturation rate of DNA in alkaline solution which is enhanced when strand breaks are present, single and double strand DNA is separated by using different concentrations of phosphate buffers, neutralized and sonicated, the fraction of remaining DNA double-stranded after denaturation for a fixed time interval correlates with the number of strand breaks present per cell (Ahnström and Erixon 1973, 1981), this fraction can either be determined by hydroxylapatite chromatography (Ahnström and Erixon 1973) or by fluorometric assay (Birnborm and Jevcak 1981). Therefore, this method is to detect a total number of breaks (SSB plus DSB). Figure 1.4.7 presents a flow protocol of DNA unwinding assay with hydroxylapatite chromatography. The DNA molecule is usually determined by radioactive label. With hydroxylapatite chromatography the radiation effect on the DNA can be expressed in term of the relative mass fraction  $M_{ds}/M_{DNA}$  where  $M_{ds}$  represents the mass of DNA remaining in double stranded form and  $M_{DNA}$ , the total mass of DNA. This relative mass fraction can be expressed according to

$$F = d.p.m._{ds} / (d.p.m._{ds} + d.p.m._{ss})$$

where  $d.p.m._{ss}$  and  $d.p.m._{ds}$  are the disintegrations per minute measured in the samples containing single- and double-stranded DNA, respectively (Bryant and Blöcher 1980, Bryant 1990). This fraction depends on the alkaline solution used, the temperature during denaturation and the time of denaturation.

An improvement in sensitivity of the method by an order of magnitude can be obtained by introducing a double-labelling procedure (Rydberg 1980), which eliminates the variation between different columns in the separation procedure. Because the DNA unwinding technique is simple and sensitive, it has been considerably applied for the measurement of total strand breaks induced by ionizing radiation in the DNA of



**FIGURE 1.4.7** The protocol of DNA unwinding and hydroxylapatite methods of Ahnstrom and Erixon 1973 and Rydberg 1975.

mammalian cells (Ahnström and Edvardsson 1974, Roots *et al.* 1979, Bryant and Blöcher 1980, 1982, Iliakis *et al.* 1982, Dikomey and Franzke 1988). Furthermore, this method can be used to detect DNA strand breaks produced by doses of  $\gamma$ -radiation as low as 0.01 Gy (Rydberg 1980), these levels of irradiation would be expected to produce only 1 to 3 breaks per  $10^{12}$  Dalton (Kohn and Grimek-Ewig 1973).

It is known that irradiation of DNA produced not only frank strand breaks, but also alkali labile sites which convert to strand breaks at high pH, the frequency of SSB after alkaline treatment increased by a factor of about 1.5 (Bopp and Hagen 1970), therefore, the practical amount of DNA strand breaks should be accurately estimated with certain modification.

#### *Filter elution assay*

This assay utilizes filters to discriminate DNA sizes, but the filters do not absorb DNA and act mechanically to impede the passage of DNA 'fragments' under the condition employed. For constant flow rates with the same filter, the elution profile depends on both the molecular weight of DNA and the average dimension of the DNA colical (Nicolini *et al.* 1983, Balbi *et al.* 1986). SSB or DSB, which reduces molecular weight and increases the rate at which DNA passes through the filters at different pHs. The amount of DNA remaining on the filter is considered to represent the induction of SSB and DSB, at "neutral" conditions (pH 7.4 or 9.6) DNA remains in its native duplex, the relative number of DSB is measured, at "alkaline" conditions (pH 12.1) the DNA opens up to form single strands, therefore the relative number of SSB can be measured (Kohn and Grimek-Ewig 1973, Kohn *et al.* 1976, Bradley and Kohn 1979, Kohn *et al.* 1981).

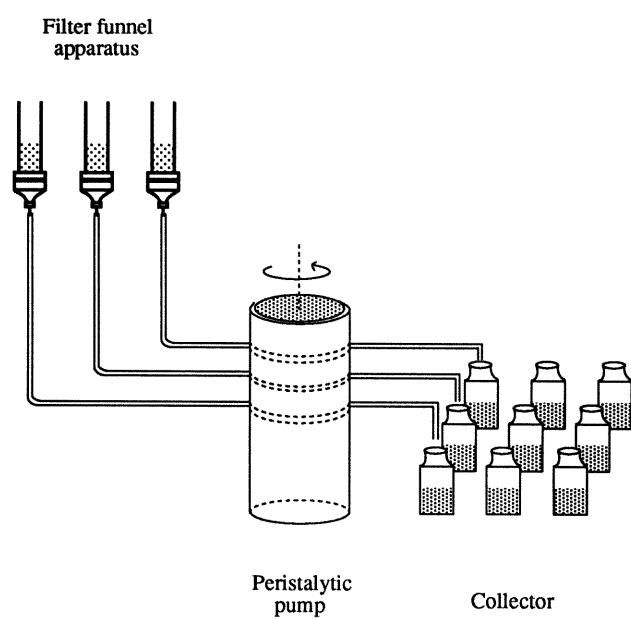
In the standard filter elution assay, labelled cells are collected on the filter, lysed at alkaline or neutral conditions and subjected to a steady and continuous flow of alkaline or neutral eluting solutions through the filter. Finally equal-volume fractions are collected over a period of up to 20



hours. DNA is eluted from the filter as a function of molecular weight, with low-molecular-weight DNA eluting rapidly and high-molecular-weight DNA eluting at later times. The elution rate of DNA from filters is normally expressed as the percent break relative to unirradiated controls. The layout of the apparatus used is illustrated in Figure 1.4.3. The detailed procedures of neutral filter elution is described in Chapter 2. Since double-strand DNA is not fully denatured below pH 11.6 (Vinograd *et al.* 1965), the 'neutral' filter elution (relative to 'alkaline' filter elution) is also called "non-denaturing" elution.

Since this technique was proposed (Kohn and Grimek-Ewing 1973, Bradley and Kohn 1979), owing to its high sensitivity and simple application, filter elution has now become one of common methods for assaying radiation-induced DNA damage, especially DSB, in mammalian cells. However, it was found that the modification of elution fraction is considerably influenced by the pH value, lysis conditions, the composition of elution buffer, amount of proteins associated with DNA and the presence of molecules which can influence chromatin swelling on the filter (Evans *et al.* 1987, Koval and Kazmar 1988, Peak *et al.* 1988b, Sweigert *et al.* 1988, Fox and McNally 1988, van Ankeren *et al.* 1988, Okayasu and Iliakis 1989, Radford 1990).

In neutral filter elution, the rate of elution of the DNA was more rapid when elution was performed at pH 9.6 rather than at pH 7.2 for the same dose (Koval and Kazmer 1987), this may be because more cellular materials that would interfere with the elution of DNA are removed at higher pH. But the rate of repair was the same at both pHs (Schwartz *et al.* 1987, Flick *et al.* 1989). In addition, it has been shown that the rate of DNA elution from irradiated or unirradiated cells was independent of the number of cells deposited on the filters in the range of  $0.1 - 2.0 \times 10^6$  cells (Kohn *et al.* 1976), however the induction of initial damage varies with cell types and the cell cycle phase of the population assayed distribution (Radford 1986b,



**FIGURE 1.4.8** Arrangement of apparatus during filter elution.

Okayasu *et al.* 1988, Radford and Broadhurst 1988, Sweigert *et al.* 1988, Wlodek and Hittelman 1988a). As described above, the results of DSB measured using neutral filter elution are different from those observed using other methods in DNA of mammalian cells, i.e., a non-linear relationship between fraction of eluted DNA and dose (an initial threshold) as well as biophasic repair kinetics. The accuracy of the elution assay can be increased by the use of internal standards, i.e., cell labelled with a different isotope from that of the control cells, irradiated with a fixed X-ray dose (usually using 45 to 50 Gy) and mixed with the control cells (Kohn *et al.* 1981, Radford 1985, Radford and Hodgson 1985, Evans *et al.* 1987, Prise *et al.* 1987, Wlodek and Hittelman 1987, Peak *et al.* 1988a, Prise *et al.* 1989).

Recently, there has been an increasing interest in the use of pulsed-field-gel electrophoresis (PFGE) at neutral pH as it is a sensitive and specific assay for measurement of DSB in high molecular weight DNA of irradiated eukaryotic cells (Contopoulou *et al.* 1987, Blöcher *et al.* 1989, Ager *et al.* 1990, Stamato and Deuko 1990, Ahn *et al.* 1991, Whitaker and McMillan 1992). Attempts have been made to measure yields of DSB at biologically significant dose of radiation, 1 - 10 Gy (Radford 1985, Prise *et al.* 1987, Peak *et al.* 1988a, Blöcher *et al.* 1989, Cederval and Erixon 1989). The dose response curve for radiation-induced DSB was also biphasic with an apparent reduction in rate of DSB induced with dose (Blöcher *et al.* 1989, Ahn *et al.* 1991, Whitaker and McMillan 1992). There are also reports to show linear induction of DSB employing this assay (Blöcher 1990a, Stamato and Denko 1990).

In summary, it is fairly obvious that the results of induction and repair of SSB and DSB depend largely on the assay that was employed. This leaves one to question the accuracy and/or validity of each assay and the possible interpretation of the results. An ideal technique for detection of radiation-induced DNA damages should have following essential features:

(1) that this method can measure DNA damage and repair, in particular DSB in a dose range corresponding to that used in studies of survival, chromosome aberration and mutation; (2) it should be easy to use and possibly measure large samples at the same time; and (3) it should be accurate and able to produce results quickly.

### Modification

The final probability of radiation-induced DNA damage and repair is a function of both the physical 'quantity and 'quality' of the radiation and the particular biological condition or environments in which DNA is present. Many endogenous and exogenous factors can modify the damage and repair of DNA, including: *physical*: radiation quality, dose and dose rate; *chemical*: the ambient and physiological environment, also including redox competition (sensitisers and protectors); *biochemical*: repair enzyme inhibitors and competing agents; *biological*: temperature, oxygen availability, cell cycle phase and proliferation.

It was shown that mammalian cells repair DNA breaks at a slower rate after high LET than after low LET radiation (Ahnström and Edvardsson 1978, Körner *et al.* 1978, Bryant and Blöcher 1980), maybe because high LET radiation produce dense clusters of damage involving both bases and sugars (Holley and Chatterjee 1990). A large number of residual breaks remain after a fixed time for irradiation with high LET than following low LET radiation, but there is no significant difference in DNA damage between the same LET radiation (Ahnström and Edvardsson 1974, Ritter *et al.* 1977, Roots *et al.* 1979, Bryant and Blöcher 1980, Coquerelle *et al.* 1987, Holley and Chatterjee 1990).

It is known that many chemical compounds can affect DNA damage or repair through two possible of interactions: the addition action, where the agents and radiation affect DNA molecule in an additive manner; and the synergic interaction, where the agents potentiate the effects of

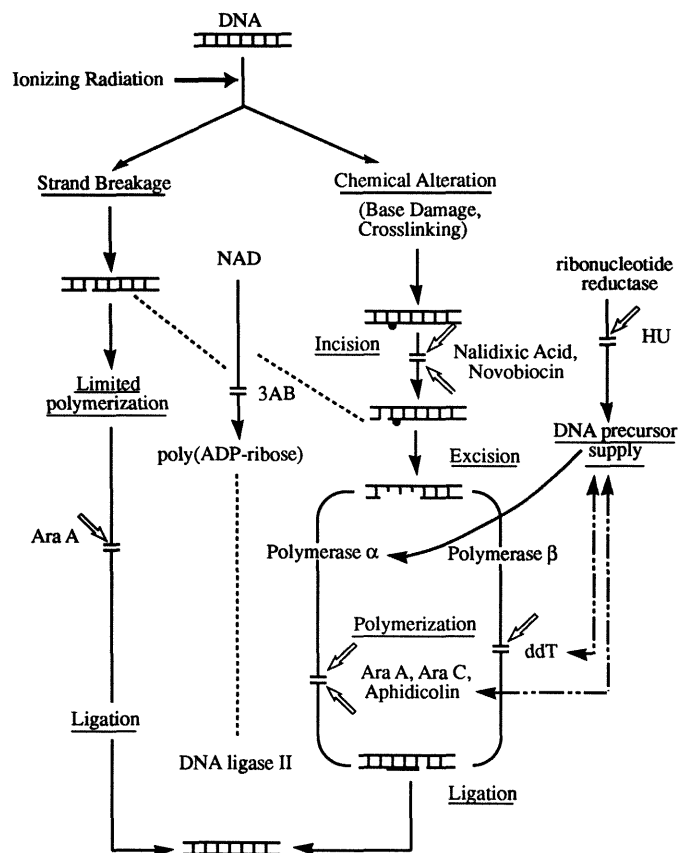
radiation. It is proposed that a compound can effectively protect DNA against the strand breaking actions of radiation by two mechanisms, scavenging of hydroxyl radicals and chemical repair of DNA radical in aqueous solution (Held *et al.* 1981), therefore, the vast majority of radioprotective compounds act on the indirect damage of DNA.

Hyperthermia can inhibit SSB repair (Ben-Hur and Elkind 1974, Clark *et al.* 1981, Dikomey 1982, Jorritsma and Konings 1983), base repair (Warters and Roti Roti, 1978), and DSB repair (Corry *et al.* 1977, Lett and Clark 1978, Radford 1983, Warters *et al.* 1987b, Dikomey and Franzke 1992). The inhibition extent depends upon the temperatures employed, especially at temperatures above or below 42.5°C (Warters *et al.* 1987, Warters and Axtell 1992). The yields of SSB, DSB and base damage induced by radiation were unaffected by prior heat treatments (Corry *et al.* 1977, Warters and Roti Roti 1978, Clark *et al.* 1981, Lunec *et al.* 1981, Dikomey 1982, Jorritsma and Konings 1983, Warters *et al.* 1987a). The basis for the incomplete repair of strand break with prior hyperthermic treatment appears to be an alteration in the binding of non-histone proteins to DNA (Clark and Lett 1978, Waters and Roti Roti 1979), the inhibition of repair enzyme access to, or function on, DNA lesions (Warters and Axtell 1992) or heat-induced change of chromatin structure (Jorritsma and Konings 1983).

Oxygen increases the initial yield of SSB induced by ionizing radiation (Dean *et al.* 1969, Dugle *et al.* 1972, Palcic and Skarsgard 1972, Koch and Painter 1975), but hypoxia decreases the yield of SSB and DSB induction (Lennartz *et al.* 1973, Radford 1985, 1986a). The oxygen enhancement ratio (OER) is 2 to 5 for SSB induction under hypoxia, 3 to 4 for DSB induction (Lennartz *et al.* 1973, Radford 1985, 1986b, Hentosh 1988, Whitake and McMillan 1992), but unchanged for base damages (Paterson *et al.* 1976, Skov *et al.* 1979).

In cell biological field, (1) there are more remaining DNA breaks in radio-sensitive cell lines, but in most cases resistant and sensitive cell lines cannot be distinguished on the basis of induction or repair of strand breaks (Fox and Fox 1973, Hesslewood 1978). It seems that sensitive and resistant mammalian cells have the same biochemical capacity to rejoin DNA breaks (Ahnström 1982). However, in tumour cells the radioresistant cells rejoin DNA DSB faster than radiosensitive cells and there are also comparable differences in induction of DNA DSB between different radiosensitive cell lines (Chen and Schwartz 1989, Schwartz and Vaughan 1989, Schwartz *et al.* 1990). These findings suggest that there might be an effect from the cells of different origin on DNA damage. (2) The number of DNA strand breaks is generally found to be independent of the state of growth (Wheeler and Wierowski 1983a, Iliakis and Seaner 1988, Wheeler *et al.* 1988, Dikomey 1990), and of the cell cycle position (Humphrey *et al.* 1968, Lett and Sun 1970, Sawada and Okada 1970, Blöcher 1982). (3) Repair of DNA strand break seems to occur in all phases of the cell cycle (Humphrey *et al.* 1968, Lohman 1968, Sawada and Okada 1970), and is fast in proliferating cells than in quiescent cells (Wheeler and Wierowski 1983a, Warters *et al.* 1985, Wheeler *et al.* 1988, Dikomey, 1990). (4) The alteration in chromatin structural organization (mainly chromatin proteins) could influence the yields of DNA lesion and repair (Mee *et al.* 1978, Chiu *et al.* 1982, Warters and Childers 1982). (5) There are a greater induction rate of DNA damage in cells irradiated in suspension than in cells irradiated as monolayer (Durad and Olive 1979, Hinz and Dertinger 1983, Hill and Hill 1991).

For the repair of radiation-induced DNA damage, the processes may be affected by serial inhibitors and enzyme-competing agents (Painter 1980). Figure 1.4.9 presents the pathway of some agents which affect the biochemical repair of radiation-induced DNA damage. The presence of these inhibitors will increase the yields of DNA damages induced by



**FIGURE 1.4.9**

Scheme to illustrate how various blocking agents can be used to interrupt the biochemical repair of radiation-induced lesions in DNA. → normal repair pathways; ..... activation; ——— competition; ⇨ blocking sites (Redrawn from Collins *et al.* 1983). 3AB, 3-aminobenzamide; Ara, arabinofuranosyl adenine, HU, Hydroxyurea; ddT, dideoxythymidine (Redrawn from Downes *et al.* 1983)

ionizing radiation (Bryant and Blöcher 1982, Iliakis *et al.* 1982, Bryant and Iliakis 1984).

### Relationship of DNA damage and repair to radiobiological effects

There have been a number of experiments and target models concentrating on DNA as the likely critical macromolecule in cell death and the cause-effect relationship between DNA strand breaks and some cytogenetic consequences of ionizing radiation, for example, chromosome aberrations, cell killing and mutation (Painter 1980, Iliakis 1988, Radford 1988, Ward 1988, Frankenberg-Schwager 1989, Breimer 1990, Pomplum 1991), but the exact biological significance of various kinds of radiation-induced DNA damage and repair is still controversial. Particular attention has been paid to DSB as the favoured critical lesion because many radiobiological effects are thought likely to be the result of either the failure of DNA repair systems to remove a small number of residual DSB in the DNA or misrepair of some DSBs.

### *DNA damage and chromosome aberration*

The close or linear correlation of DNA damage/repair and chromosome aberrations have been identified (Carrano 1973, Chadwick and Leenhouts 1981). It is believed that the major primary radiation damage responsible for aberration formation is the DNA DSB (Evans 1977, Wolff 1978, Natarajan *et al.* 1980, Obe *et al.* 1982).

The observation favouring the involvement of DSB as the primary lesion of chromosome aberration principally comes from that: (1) high LET radiation is more efficient in inducing chromosome aberrations than low LET radiation for a given dose, high LET radiation is also more efficient in inducing DSB than low LET radiation (Natarajan *et al.* 1986); (2) the data from split-dose and protracted exposure experiments showed



that the half-time for the interaction of lesions that result in chromosome aberrations is considerable longer than 10 minutes. The vast majority of SSB will be repaired within 10 minutes, and half-time for rejoining of DSB is longer than 1 hour (Preston 1983, Bedford and Cornforth 1987); (3) when X-irradiated cells are treated with neurospora endonuclease, which can convert SSB into DSB, there is an increase of the frequency of chromosome aberrations (Natarajan *et al.* 1980); (4) it was found that induction of DSB by restriction endonucleases, such as Alu I and Sau 3AI, efficiently induces chromosome aberrations in treated cells (Bryant 1984, Natarajan and Obe 1984); (5) inhibitors of DSB repair could enhance the yield of radiation-induced chromosome aberration (Iliakis *et al.* 1988, Mozdarani and Bryant 1989), moreover DSB repair deficient mutants had higher levels of chromosome aberration than the wild-type parent lines (Kemp and Ojaggo 1986, Darroudi and Natarajan 1987). However, due to the controversy surrounding induction and repair kinetics of DSB using different methods, it seems likely that there is more than one kinetics of DSB repair to be related to the induction of chromosome aberrations, the second slow component of DSB may underlay the observed joining of chromosome and chromatid breaks (Bryant 1990).

Chadwick and Leenhouts (1978) proposed that the yield (Y) of aberrations per cell might be related to the number of DNA DSB after a certain dose (D) irradiation by the equation

$$Y = k(\alpha D + \beta D^2)$$

where k depends on the types of aberration being scored and the scoring efficiency;  $\alpha$  represents the average number per unit dose of DSB induced in single radiation events and  $\beta$  represents the average number per unit dose squared of DSB that arise from the combination of two unrepaired SSBs.

### *DNA damage and cell killing*

Two processes of cell death have been well characterized following ionizing radiation, interphase death and reproductive death. There is evidence to suggest that DNA damage is responsible for reproductive death (Altoman *et al.* 1970, Hopwood and Tolmach 1979).

DSB, as unrepaired or as initial damage, is believed to be responsible for the lethal effect of ionizing radiation (reviewed by Painter 1980), and it was assumed that one unrejoined DSB is a lethal event (Blöcher and Pohlitz 1982), whilst the other major categories of radiation-induced DNA lesions (SSB, base damage and DNA-protein crosslinks) appeared to give little or no effect on the level of cell killing (Radford 1986a). This belief is supported by several facts that: (1) the correlation between cell survival and the rejoining of DSB was obtained using different cell lines, different phases of the cell cycle and different environmental conditions (Gillespie *et al.* 1976, Taylor 1978, Blöcher and Pohlitz 1982, Bryant and Blöcher 1982, Sakai and Okada 1984, Frankenberg-Schwager *et al.* 1985, Radford 1985, 1986b, Radford and Broadhurst 1986). The repair of DNA DSB markedly affects both the shoulder width and the slope of survival curves, increasing the shoulder width and decreasing the slope, (Dugle *et al.* 1976, Ritter *et al.* 1977, Resinick 1978, Painter 1980, Frankenberg *et al.* 1981, Bryant 1984, Resinick 1987, Wlodek and Hittelman 1987, Frankenberg-Schwager 1989). (2) Both the RBE of cell death and the induction of DSB increase with the LET of the radiation (Ritter *et al.* 1977, Cole *et al.* 1980). (3) The non-linear fraction of eluted DNA with doses obtained using neutral filter elution and biphasic dose-response curve by pulsed-field-gel electrophoresis, seemed a tenable explanation for shouldered survival curves (Radford and Hodgson 1985, Radford 1986b, Wlodek and Hittelman 1987, Koval and Kazmar 1988, Radford and Broadhurst 1988, Blazek *et al.* 1989, Costa and Bryant 1990b, Ahn *et al.* 1991, Whitaker and McMillan 1992). And (4) Chromosome aberration formed by the non-repair or

misrepair of DSB are speculated to be the mediating cause (Cole *et al.* 1980, Chadwick and Leenhouts 1981, Bryant 1985).

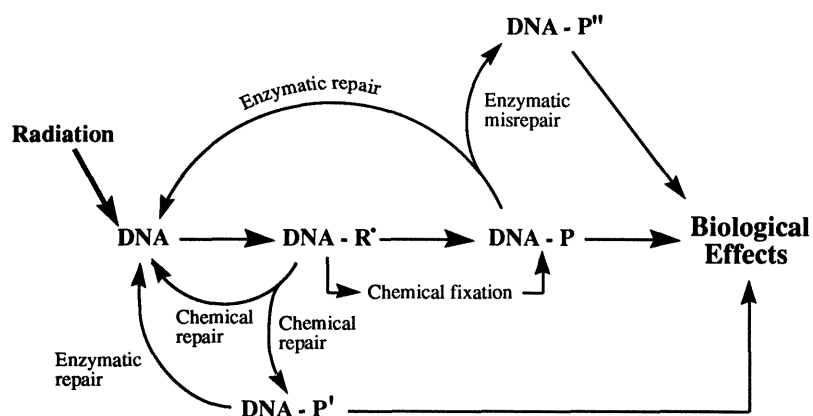
In addition, the importance of DSB in causing cell death, following exposure to ionizing radiation, is also emphasised by the following observations: (1) Radiosensitive mutant cell lines are found to be deficient in the repair of DNA DSB, with one exception of ataxia telangiectasia (Resnick and Martin 1976, Budd and Mortimer 1982, Jeggo and Kemp 1983, Kemp *et al.* 1984, Giaccia *et al.* 1985, Wlodek and Hittleman 1987, Jeggo 1990, Iliakis *et al.* 1992). (2) The production of DNA DSB by restriction endonucleases leads to the induction of chromosome aberrations which are known directly to correlate with cell death, especially unrepaired chromosome aberrations (Dewey *et al.* 1970, 1971, Grote and Revell 1972, Lloyd *et al.* 1975, Joshi *et al.* 1982, Bryant 1984, Natarajan and Obe 1984, Bryant 1985, Roberts and Holt 1985). However, Some authors also found no simple relationship between DNA strand breaks and cell death (Ono and Okada 1974, Lehmann and Stevens 1977, Hesslewood 1978, Kemp *et al.* 1984, Koval and Kazmar 1988, Sweigert *et al.* 1988).

There have been many theoretical and biophysical models to describe qualitative relationships between cellular DNA damage and lethal lesions (reviewed by Ostashevsky 1989, 1990), such as the 'molecular' theory of Chadwick and Leenhouts (1981), the 'saturable repair' model of Goodhead (1985), the 'repair-misrepair' model of Tobias (1985) and the 'lethal, potentially lethal' model of Curits (1986). In general, there are two modes by which DSB may confer cell death: firstly, an unrepaired DSB is lethal on its own; secondly, two DSBs interact to form a lethal lesion (binary misrepair) (Frankenberg-Schwager, 1990). The relationship between the number of DSB leading to cell survival (S) after a dose (D) of radiation and cell death was defined by the equation

$$S = \exp [-p(\alpha D + \beta D^2)]$$

where  $p$  is the probability that a DNA DSB leads to cell reproductive death;  $\alpha$  represents the average number of DSB per unit dose that induced in single radiation events and  $\beta$  represents the average number per unit dose squared of DSB that arise from the combination of two unrepaired SSBs (Chadwick and Leenhouts 1978). Dugle *et al.* (1976) reported that the value of  $p$  is approximately 0.7 in their experimental situation.

In conclusion, there are many types of DNA damages to be observed using a number of assays under exposure of different quality of ionizing radiation, DNA lesions of cell are induced linearly with dose of ionizing radiation, except that there is still a controversy over the linear or linear-quadratic induction of DSB. Repair is observed for all DNA lesions through various chemical and biochemical repair mechanisms, in general, base damage and SSB are repaired faster than DNA crosslinks and DSB. Induction and the repair rate of radiation-induced DNA damages may be affected by various factors. The misrepair and non-complete repair of DNA damages, especially DSB, may not yield a full recovery of all cell functions so that different radiobiological effects are produced (Figure 1.4.10). However, there remains considerable uncertainty and controversy as to the major aspect of critical mechanisms on DNA damages and their correlation to final radiobiological effects. Resolution of these may require further experimental and theoretical development.



**FIGURE 1.4.10**

The processes associated with the interaction of ionizing radiation with DNA in cells. Radiation-induced DNA radicals (DNA-R\*) can be repaired chemically, or enzymatically, or can be fixed into altered DNA products (DNA-P, DNA-P' and DNA-P'') by chemical fixation or chemical or enzymatic misrepair (Rojas and Denekamp 1989).

## Chapter 2

# ***MATERIALS AND METHODS***



## 2.1

### Extraction and Purification of Plasmid DNA

The basic requirement of plasmid DNA, which is suitable for analysis of strand break after exposure to ionizing radiation, is that plasmid DNA extracted should be as pure supercoiled as possible and as little nicked and as pure as possible. There is a wide range of methods available for isolation and purification of pure plasmid. In this thesis, the large-scale protocol of plasmid amplification in *Escherichia Coli* was used for preparation of pBR322 plasmid DNA. The lysis of cells and purification of plasmid DNA were carried out according to a modification of the alkaline lysis methods proposed by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

#### Amplification of plasmid in rich medium

- a. Maintained pBR322 plasmid in *Escherichia Coli* strain CSH26 by transformation, and then kept the strain as colonies in Luria-Broth agar plate at 4°C;

<b>Luria-Broth-Agar</b>	bactotryptone	10 g
	yeast extract	5.0 g
	NaCl	5.0 g
	agarose	16 g

Made up to 1.0 liter with distilled water and adjusted to pH to 7.0 with NaOH.

- b. Inoculated single bacterial colony carrying the pBR322 plasmid from an agar plate into a 30 ml Luria-broth medium containing with 100  $\mu\text{g/ml}$  ampicillin and 10  $\mu\text{g/ml}$  tetracycline, and grew with vigorous shaking to late log phase (an  $\text{OD}_{600}$  of 0.6) at 37°C;

Luria-Broth Medium	bactotryptone	10 g
	yeast extract	5.0 g
	NaCl	5.0 g
	D-glucose	1.0 g

Made up to 1.0 litre with distilled water and adjusted pH to 7.0 with NaOH. Because pBR322 plasmid contains the genes coding for resistance of ampicillin and tetracycline, the antibiotics was added into cultures to ensure pure pBR322 in amplifying. The mass of cultured cells was measured at  $A_{600}$  wavelength in Gilford 300 M micro-sample spectrophotometer.

- c. Transferred the culture into a 500 ml of Luria-Broth medium containing 100  $\mu\text{g/ml}$  ampicillin and 10  $\mu\text{g/ml}$  tetracycline pre-warmed at 37°C;
- d. Incubated the culture with vigorous shaking to an  $\text{OD}_{600}$  of about 0.4 at 37°C;
- e. Added 2.5 ml of a fresh solution of chloramphenicol (34 mg/ml in 100% ethanol) to the final concentration of 170  $\mu\text{g/ml}$ ;
- Chloramphenicol can complete inhibition of protein synthesis in order to achieve high yields of plasmid at the largest extent.
- f. Incubated the culture with vigorous shaking for a further 12 to 16 hours (overnight) at 37°C;

### Harvesting and lysis of bacteria

- a. Harvested the bacterial cells from the 500 ml culture by centrifugation in Sorvall GS3 centrifuge bottles for 20 minutes at 5000 rpm at 4°C;
- b. Discarded the supernatant quickly and inverted the open centrifuge bottle to drain away all of the supernatant (or under help of a vacuum pump);
- c. Resuspended the bacterial pellet in 100 ml of ice cold STE to wash;



STE        100 mM NaCl  
              10 mM Tris-Cl (pH8.0)  
              1.0 mM EDTA (pH8.0)

- d.    Recentrifuged and collected the bacterial cells for 20 minutes at 5000 rpm at 4°C;

- e.    Discarded the supernatant and resuspended the bacterial pellet in 10 ml of solution I for 5 minutes at room temperature;

Solution I (lysis buffer)        50 mM D-glucose  
    25 mM Tris-Cl (pH8.0)  
    10 mM EDTA (pH8.0)

- f.    Added 1 ml of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris-Cl, pH 8.0) and left to stand for 5 minutes at room temperature to weaken the cell wall;

- g.    Added 20 ml of freshly prepared Solution II, mixed the contents thoroughly and gently, and then left to stand for 10 minutes at room temperature to denature the DNA;

Solution II        0.2 N NaOH (freshly diluted from a 10 N stock)  
                          1% SDS (freshly diluted from a 20% SDS)

- h.    Added 15 ml of ice-cold Solution III, mixed the contents by shaking the bottle several times, and then left the bottle to stand on ice for 10 minutes to neutralize the DNA (a flocculent white precipitate was seen);

Solution III (pH 4.8)    5 M potassium acetate    60.0 ml  
                                  glacial acetic acid        11.5 ml  
                                  distilled water               28.5 ml

This step precipitated out chromosomal DNA, high molecular weight RNA and protein to form an insoluble clot, the plasmids remained in the solution. Solution III was stored at 4°C.

- i.    Centrifuged the bacterial lysate for 20 minutes at 5000 rpm at 4°C and allowed the rotor to stop without braking;

- j. Transferred the clear supernatant to a fresh centrifuge bottle (for Sovall SS34 rotor), added 0.6 volume of isopropanol (propanol-2-ol), mixed well and left to stand for 10 minutes at room temperature;  
This step precipitated out the plasmid.
- k. Centrifuged for 20 minutes at 13000 rpm at room temperature;
- l. Discarded all supernatant carefully and then washed the pellet and the wall of the bottle with ice-cold 70% ethanol at room temperature;
- m. Drained off the ethanol and removed any beads of liquid that adhered to the wall of the bottle using a mini pipette attached to a vacuum pump;
- n. Dried the pellet under a vacuum;
- o. Dissolved the pellet of nucleic acid in 4 ml distilled water;

#### **Purification of supercoiled DNA** (CsCl-ethidium bromide density gradient)

This method involves ultracentrifugation of cleared lysates in a mixing solution of cesium chloride (CsCl) with ethidium bromide (EtBr). Ethidium bromide binds by intercalating between the DNA base pairs and causes the DNA to unwind. A supercoiled plasmid DNA molecule has no free ends and can only unwind to a limited extent. It will not be bound by as much ethidium bromide as the open and linear form of DNA (also including fragmented chromosomal DNA), hence supercoiled plasmid DNA will have a higher density than the other types of DNA in the presence of saturated levels of CsCl. Because of the density differences, the supercoiled plasmid DNA can be separated from chromosome DNA, linear and open plasmid DNA.

- a. Added exactly 4.3 g CsCl into a 4 ml DNA solution;
- b. Transferred the 2 ml of mixture to a 2.1 ml of polycarbonate ultracentrifuge tube and added 50  $\mu$ l of a solution of ethidium bromide (10 mg/ml in water). Balanced exactly and sealed the tube carefully with aluminium screw cap;

- c. Centrifuged the density gradients at 100K rpm in a BECKAN TL-100 tabletop ultracentrifuge (Fixed Angle Rotor) overnight at 20°C;
- d. Cut the top of centrifuge tubes and collected the low band consisting supercoiled plasmid DNA into an Eppendorf tube using a 18-gauge hypodermic needle with a syringe under long wave ultraviolet light;
- e. Added an equal volume of iso-butanol saturated CsCl and mixed the two phases by vortexing;
- f. Centrifuged the mixture in a bench centrifuge for 3 minutes at 13000 rpm at room temperature;
- g. Removed the pink color supernatant containing ethidium bromide and repeated the extraction until the pink color was disappeared from both the aqueous phase and the organic phase;
- h. Measured the amount of remaining DNA solution, added 0.54 ml isopropanol and 0.5 ml distilled water per 0.4 ml DNA solution, and then left to stand for 5-10 minutes at room temperature;
- i. Spun the mixture for 10 minutes at 13000 rpm at room temperature;
- j. Drained away the supernatant carefully and washed the pellet with 1 ml 70% ethanol for 5 minutes at 4°C;
- k. Recentrifuged as above, removed ethanol, and finally dried the pellet in a vacuum;
- l. Dissolved the pellet of nuclei acid precipitated in appropriate amount of distilled water;
- m. Measured of the final solution of DNA at OD<sub>260</sub> in CE272 linear ultraviolet spectrophotometer, calculated the concentration of DNA as following the equation, and stored the DNA in aliquots at -20°C;

$$\text{DNA concentration (mg/ml)} = \frac{\text{OD}_{260}}{20} \times 500$$

20 OD<sub>260</sub> units are equivalent to the DNA at a concentration of 1 mg/ml. The ratio of the optical densities at different wave lengths (OD<sub>260</sub> and OD<sub>280</sub>) was used to indicate

the purification of the DNA sample. The rate of 1.8 indicates pure DNA, and more than 2.0, pure RNA or/and large amount of protein (Schleif and Wensik 1981).

- n. Ran gel using 10  $\mu$ l of the distilled solution of DNA as the method described in 2.3. section to check distribution of three forms of plasmid DNA obtained and possible amount of protein and RNA in the solution;

Above 90% supercoiled plasmid DNA (form I) without linear plasmid DNA (form III) was usually obtained following the protocol described as above, but there was a certain amount of RNA or/and protein appearing in the DNA solution extracted. Therefore, the methods described in the following sections were employed to remove the protein and RNA from the DNA solution.

#### Removal of RNA

- a. Added DNAase-free RNAase (10 mg/ml) to the DNA solution extracted to a final concentration of 10  $\mu$ g/ml, and then placed the tubes in water bath at 37°C for 1 hour;

10 mg/ml DNAase-free RNAase was prepared by dissolving 10 mg RNAase in 10 mM Tris-Cl and 15 mM NaCl, and then heated at 100°C in water bath for 15 minutes to inactivated DNAase in the solution. Stored for later use at -20°C.

- b. Removed the RNAase and protein from the DNA solution using phenol-chloroform-extraction in following section;

#### Removal of protein

(Phenol-chloroform-extraction)

- a. Added an equal volume of phenol to the DNA solution and mixed well with vortexing;

Phenol was redistilled and equilibrated with TE buffer and 8-hydroxyquinoline was added to a final concentration of 0.1% prior to storage at -20°C.

- b. Centrifuged in a bench microfuge for 10 to 15 seconds at 13000 rpm at room temperature;
- c. Transferred carefully upper aqueous layer containing plasmid DNA into a fresh Eppendorf tube;

If desired, re-extraction with phenol would be done.

- d. Added an equal volume of TE buffer to improve recovery of DNA;
- e. Added an equal volume of chloroform mixture;  
Chloroform mixture was prepared with mixing chloroform and isoamyl alcohol in the ratio of 24:1.
- f. Centrifuged in a bench microfuge for 10 to 15 seconds at 13000 rpm at room temperature;
- g. Removed upper aqueous phase containing DNA into a fresh Eppendorf tube;
- h. Added an equal volume of ether, mixed well with vortexing and standed for 1 to 2 minutes;
- i. Removed carefully the upper layer;
- j. Repeated step h. to i.;
- k. Precipitated the plasmid DNA in the solution by ethanol precipitation in the following section;

#### Ethanol precipitation

- a. Added 1/10 volume of 3 M sodium acetate (pH 5.5, adjusted with acetic acid) to the DNA solution;
- b. Added 2.5 volume of 100% ethanol and mixed well with vortexing;
- c. Cooled in dry ice-ethanol bath for 10 to 15 minutes;
- d. Centrifuged in a bench microfuge for 10 to 15 seconds at 13000 rpm at 4°C;
- e. Removed the supernatant very carefully under a vacuum pump;
- f. Washed the pellet and all walls of the tube with 70% ethanol;
- g. Centrifuged in a bench microfuge for 10 to 15 minutes at 13000 rpm at 4°C;
- h. Removed ethanol carefully and then dried the pellet in a vacuum;
- i. Dissolved the pellet of pure DNA in appropriate distilled water;
- j. Measured amount of DNA, ran gel and stored as m.-n. step described in the section of the purification of supercoiled DNA;

- k. Dissolved the DNA solution with distilled water to desired 100  $\mu\text{g/ml}$  concentration and stored at  $-20^{\circ}\text{C}$  for experiments;

In the procedures of plasmid DNA extraction and purification, all buffers and media were prepared with water that had been passed through a Fisons cartridge deioniser and followed by a millipore "Milli Q" cartridge system with a Millipore 0.2  $\mu\text{m}$  filter and autoclaved. RC-5-superspeed refrigerated centrifuge (BACKMAN) and bench microfuge 24 capable of generating 13000 rpm, and 1.5 ml sterile Eppendorf-type polypropylene tubes were used.

## 2.2

### **Preparation of Plasmid DNA Sample and Gamma-Ray Irradiation**

In most experiments of plasmid DNA presented in this thesis, a frozen aqueous solution system of plasmid DNA was employed unless otherwise stated. The sample size was 20  $\mu$ l, a final concentration of 50  $\mu$ g/ml plasmid DNA was used for all experiments.

#### **Preparation of plasmid DNA sample**

- a. Pipetted and mixed gently 10  $\mu$ l of 100  $\mu$ g/ml stock DNA solution and 10  $\mu$ l of 2 x concentration of chemical agent solutions or buffers being studied in a 1.5 ml sterile Eppendorf-type polypropylene tube;
- b. Pipetted the sample carefully onto an end of a 5 mm diameter, 3 mm bore, glass tubing and immediately submerged into liquid nitrogen until an ice sample pellet was formed;
- c. Pushed ice pellet of the sample out of the tubing onto liquid nitrogen and transferred it into a sterile little thin-wall glass screw topped bottle;
- d. Kept the bottle containing sample into liquid nitrogen for preparation of irradiation;

#### **Gamma-ray irradiation**

Irradiation was carried out using a 'Vikrad'  $^{60}\text{Co}$  source under ambient atmosphere unless otherwise stated. The dose rate of irradiation sources at any particular day was calculated and checked using the following equation:

$$D_t = D_0 e^{-\lambda t}$$

where:  $D_t$  is dose rate of irradiation at any particular day;  $D_0$ , dose rate where the source was calibrated;  $\lambda$ , decay constant;  $t$ , irradiation time in days after the source was calibrated. This  $^{60}\text{Co}$  source was calibrated on November 19, 1968, its dose rate was  $3.17 \times 10^4$  Gy/hour. The half life ( $t_{1/2}$ ) of  $^{60}\text{Co}$  is 1910.26 days. Thus the decay constant of the source is calculated,

$$t_{1/2} = \frac{0.693}{\lambda}$$

Therefore,  $\lambda = 3.298 \times 10^{-4}$  /day. Hence the dose rate of the  $^{60}\text{Co}$  was  $2.5 \times 10^3$  Gy/hour on January 1990.

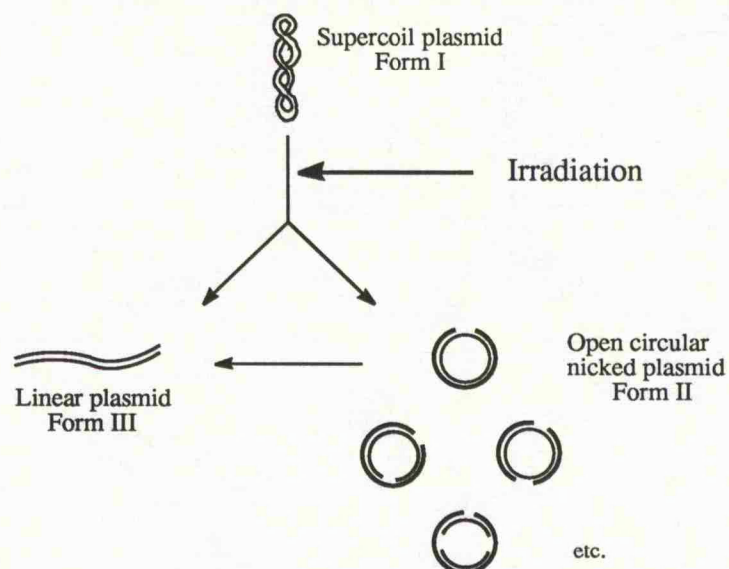
For the irradiation at 77K, the little thin-wall glass bottle containing plasmid DNA samples was placed in a Dewar vessel filled with liquid nitrogen in the presence of atmospheric oxygen. For the irradiation on ice or at room temperature, the same bottle and vessel were used without liquid nitrogen. In individual experiments different from the general methods described here, the details are provided under the Materials and Methods of the individual section in Chapter 3.



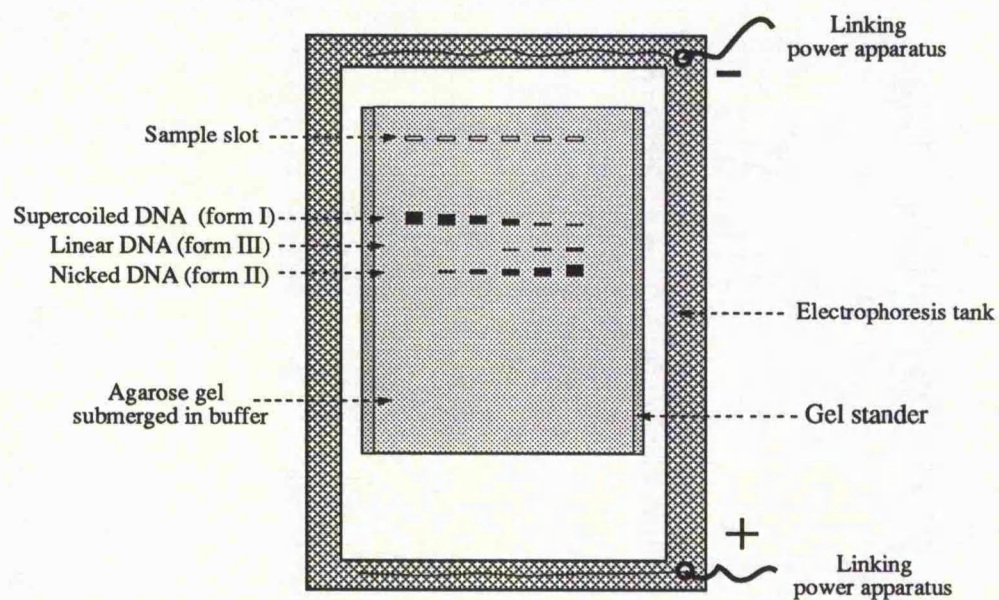
## 2.3

### Detection and Analysis of Strand Breaks in Plasmid DNA (Gel Electrophoresis with Scan)

It is known that pBR322 plasmid DNA molecules are normally small molecular and circular. This may be a covalently closed circle or twisted (called a **superhelix** or **supercoil**), which consists of two unbroken complementary single strands. Agarose gel electrophoresis was employed for analyzing gamma-ray-induced strand breaks using supercoiled plasmid DNA as the substrate. The assay is based on the conversion of the double stranded, supercoiled DNA (form I) into nicked circular (form II) and linear DNA (form III) under exposure to ionising radiation (as shown in **Figure 2.3.1**). The different form species of the plasmid DNA give rise to three separate bands in the gel after electrophoretic separation (**Figure 2.3.2**). The gel is stained with ethidium bromide, photographed under UV illumination, and finally densitometric tracings of the DNA bands on the negative are quantified using a computer program to obtain the quantity of DNA in each band. Under the experimental conditions used, other factors have no significant effect on the electrophoretic mobility of superhelical molecules since it depends solely on the size and superhelical nature of the DNA (Johnson and Grossman 1977). The density in each band is proportional to the amount of DNA present.



**FIGURE 2.3.1** The principle of strand break analysis in plasmid DNA irradiated by gamma-ray.



**FIGURE 2.3.2** The equipment of electrophoresis in strand break analysis for plasmid DNA irradiated by gamma-ray.

The nicked form (II) reports numbers of SSB while the linear form (III) reports numbers of DSB (Bresler *et al.* 1979, Boon *et al.* 1984, 1985, Roots *et al.* 1985, Cullis *et al.* 1986, Hempel and Midenberger 1987, Manke *et al.* 1991, Miller *et al.* 1991), thus we can get quantitative assessment of three forms so that DNA damage can indirectly be measured. The assay of gel electrophoresis on plasmid DNA damage has several advantages: (a) it is conservative and easy to apply; (b) the amount of DNA for each experimental sample is small; (c) in this assay system the DNA is in a cell-free, protein-free solution, therefore any damage measured must be the result of radiochemical events (direct or indirect) and is not influenced by cellular responses to DNA damage.

#### Agarose gel preparation

- a. Dissolved 1.4 g agarose gels in 100 ml 1 x TBE buffer in a microwave oven;

Tris Borate EDTA Buffer (10 x)	0.89 M	Tris-Cl
	0.89 M	Boric acid
	25 mM	EDTA

Made up to 1.0 liter with distilled water and adjusted pH to 8.3 with glacial acetic acid.

- b. Added 10  $\mu$ l of 10 mg/ml ethidium bromide per 100 ml gel;
- c. Poured onto a perspex plate containing a moulding comb to set about 4 to 5 mm thick gel at room temperature;
- d. Put the plate with the cooled gel into an electrophoresis tank submerged in electrophoresis buffer (1 x TBE) in the horizontal position;

#### Electrophoresis run, staining and photography

- a. Added 4  $\mu$ l loading buffer (5 x) to 20  $\mu$ l of the sample;

Loading Buffer	0.25% bromophenol blue
	1.17 M sucrose

Loading buffer was prepared in TBE buffer.

- b. Loaded carefully 10  $\mu$ l of the DNA sample onto the slot of pre-prepared agarose gel soaking in the electrophoresis tank at room temperature;
- c. Applied constant 120 volts (50 mAmps) to electrophoresis tank with a LBK 2197 power apparatus for 1.5 to 2 hours;
- d. Photographed the gel using Kodak T Max professional film from illuminating of UV light at 300 nm using a transilluminator (UVP Inc.) with a red filter. Exposure time was 15 seconds at f4.5 under a Polaroid MP-4 land camera;
- e. Developed the negative film in developer for 3 minutes;
 

Developer	1.2 litre X-ray developer (Kodak)
	4.8 litre distilled water
- f. Stopped the development in stop solution for 30 seconds;
 

Stop Solution	140 ml glacial acetic acid
	14 litre distilled water
- g. Fixed the negative film in fixative for 2 minutes;
 

Fixative	1.2 litre FX 40 X-ray Fixer (Kodak)
	2.4 litre distilled water

Steps, d. to g., were conducted in completed darkness.
- h. Washed films with cold deionising water for 20 to 30 minutes to remove excess fixative and allowed to air-dry.

### Analysis and evaluation

The density of each band on the gel is proportional to amount of DNA presented in the plasmid samples, the density of the bands on the negative film can be analysed using a laser densitometer and the amount of three forms is calculated with the corresponding coefficient through the peak size fitted.

- a. Scanned the negative film using an LKB 2202 ultrosan laser densitometer (BROMMA) at 632.8 from a Helium-Neon laser linked to an Apple II microcomputer;

Absorption curve of the gel was stored on the disk using LKB 21900-001 gel scan interface and software package.

- b. Calculated peak size from the stored absorption curve in the data disks using gel scan program;
- c. Printed the fitted result of peak size in an EPSON FX-80+ printer;
- d. Calculated practical values of three plasmid DNA forms with relevant correct factors:

(1) The values for the supercoiled DNA (form I) should be multiplied by 1.2 to get actual amount, since the supercoiled plasmid DNA can unwind to only a very limited extent, it will not bind as much dye as will the linear and open circular forms, intercalating 20% less ethidium bromide per molecule in staining than other forms (determined by Hertzberg and Dervan 1982);

(2) The actual amount of nicked DNA (form II) was calculated according as the following equation. Because a background of certain level of nicked plasmid DNA is always present in the plasmid extracted, it is necessary to account for this background value in calculations of practical values of the nicked plasmid DNA;

$$N_p (\%) = \frac{N_t - N_b}{100 - N_b} \times 100$$

where:  $N_p$  represents practical percentage of nicked DNA formed by exposure;  $N_t$  represents total percentage of nicked DNA in the exposed plasmid samples;  $N_b$  represents percentage of nicked DNA in the non-exposed plasmid samples.

## 2.4

### Filter Elution Assay

For analysis of DNA DSB in cultured cells, the neutral filter elution developed by Bradley and Kohn (1979) was used with modifications in the type and concentration of the detergent. Three main procedures were involved: depositing cells on a membrane filter, lysing cell and digesting protein, and pumping DNA-denaturing solution through the filter.

#### Cell sample preparation and irradiation

- a. Cultured Chinese hamster lung fibroblasts, V79-379A in Eagle's Minimum Essential Medium supplemented with non-essential amino acids, 10%(v/v) fetal bovine serum and 2 mM glutamine at initial density of  $10^6$  cells in a 75 cm<sup>3</sup> flask and cultured in a 37°C incubator;

The cells stock culture were maintained routinely as monolayer in 75 cm<sup>3</sup>, by subculturing twice per week. The cell line and culture materials were purchased from Flow Laboratories.

- b. Labelled cells through adding 0.1 to 0.2  $\mu\text{Ci/ml}$  [<sup>3</sup>H]thymidine (Sigma, 56 mCi/mmol with 1mCi=37mBq) and an equimolar amount of unlabelled thymidine (1  $\mu\text{mol/l}$ ) for 20 to 24 hours the following day of culture;

Unlabelled thymidine was added in order to ensure uniform uptake and incorporation of labelled TdR into DNA over the time course of labelling. For DNA fluorometric assay, no-labelled cell was prepared.

- c. Pippetted the medium off the culture flask and washed with fresh warm medium twice;
- d. Trypsinized the cell layer by washing cells twice with 5 ml of cold trypsin/EDTA solution (Flow laboratories);
- e. Left the flask for some minutes at room temperature;
- f. Added 5 ml of fresh medium and pipetted cells gently to obtain a single cell suspension;
- g. Counted number of cells under a Hemocytometer;
- h. Added desired amount of fresh medium to obtain the final concentration of  $2 \times 10^6$  cells/ml;
- i. Pipetted the cells gently and transferred 1 ml into 1.5 plastic irradiation tubes;
- j. Placed the tubes on ice for 15 to 20 minutes;
- k. Irradiated the cells using gamma-ray source at dose rate described in section 2.2. under certain experimental conditions;

#### Neutral filter elution

- a. Placed polycarbonate filter (25 mm diameter, 2  $\mu$ m pore size, Nucleopore) pre-wetted in cold,  $Mg^{2+}$  and  $Ca^{2+}$ -free, PBS solution (Sigma) onto the cylinder/filter apparatus supported in a cylindrical plastic funnel;

The cylinder/filter apparatus consists of a 50 cm long, 45 mm diameter clear extruded acrylic cylinder mounted on a Millipore 'Swinnex' holder.

- b. Washed the cylinder/filter apparatus using 5 ml ice-cold PBS solution to dislodge any bubbles which might be present in holder and tubings;
- c. Diluted the cell samples in 5 ml ice-cold PBS;
- d. Deposited gently the cell suspension into the funnels;
- e. Dripped through the filter units under gravity so that the cells form a monolayer on the filter;
- f. Washed cells with a further 5 ml of ice-cold PBS;

- g. Pipetted 1 ml lysis solution (0.04 M Na<sub>4</sub>EDTA and 0.2% sarkosyl with fresh dissolved proteinase K at 0.5 mg/ml) into filter-holders just before the filter holders were empty, and left to stand for 60 minutes at room temperature in the dark;

Cells was lysed to remove non-DNA materials of the cells. pH value of lysis solution was adjusted to pH 9.6 with NaOH. lysis was carried out by wrapping the units with silver foil to avoid possible light damage (Kohn *et al.* 1976). Proteinase K was added to the lysis solution just prior to use to avoid loss of activity due to self-degradation. proteinase K was purchased from BDH Chemical Ltd. It was reported that proteinase K treatment can reduce the amount of protein on the filter from 4% of the total cellular protein to approximately 0.3% (Bowden *et al.* 1981).

- h. Pipetted gently 40 ml eluting solution onto the funnels;

Eluting Solution	EDTA(free acid)	0.02 M
	TPAH (20% in water)	ca. 0.06 M/litre

Adjusted pH to 9.6 using TPAH (abbreviation of tetrapropylammonium hydroxide, Fluk Chemie).

- i. Collected 10 fractions simultaneously and eluted separately into 4 ml plastic sample cups from cylinder/filter apparatus at the interval of 2 hours at 0.035 ml/min in the darkness;

### Analysis and evaluation

For the measurement of quantity of eluted DNA, two methods, radioactive DNA assay of [<sup>3</sup>H]dThd and fluorometric DNA assay of Hoechst 33258 were employed. It has been shown that the two assays gave comparable results for the quantitation of radiation-induced strand break in cellular DNA (Meyn and Jenkins 1983, Murray and Meyn 1987).

### *Radioactive DNA assay*

- a. Removed the filters from the apparatus into a scintillation vial and added 8 ml \*OptiPhase 'X' scintillation cocktail (LKB Scintillation Products);
- c. Transferred the collected fractions into scintillation vials, and added equivalent amounts of scintillation cocktail;
- d. Vortexed the mixture thoroughly for 10 seconds;

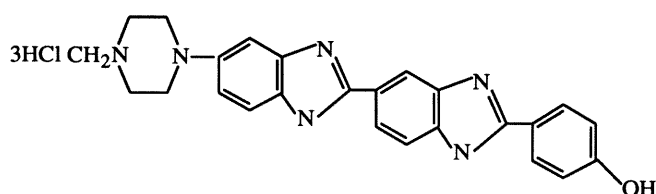


- e. Counted the radioactivity of DNA eluted in Packard model 1500 liquid scintillation analyzer (counts per minute, cpm);

The total radioactivity of each fraction was determined as the product of measured counts per minute (corrected for background) and the total volume of the fraction. The relative measure of DSB has been represented by a calculation of the increased elutability of DNA from irradiated cells relative to that from unirradiated cells. Assuming that the DNA takes up the  $^3\text{H}$ -TdR in a uniform manner, the fraction of radioactivity retained reflects the fraction of DNA retained. The relative fraction of DNA eluted is thus obtained using the expression:  $[1 - F_I] - [1 - F_C]$ , where,  $F_I$  represents the fraction of radioactivity retained on the filter after 12-hours of elution in the treated cell sample;  $F_C$  represents the fraction of radioactivity retained on the filter after 12-hours of elution in the untreated cell sample.

### ***DNA fluorometric assay***

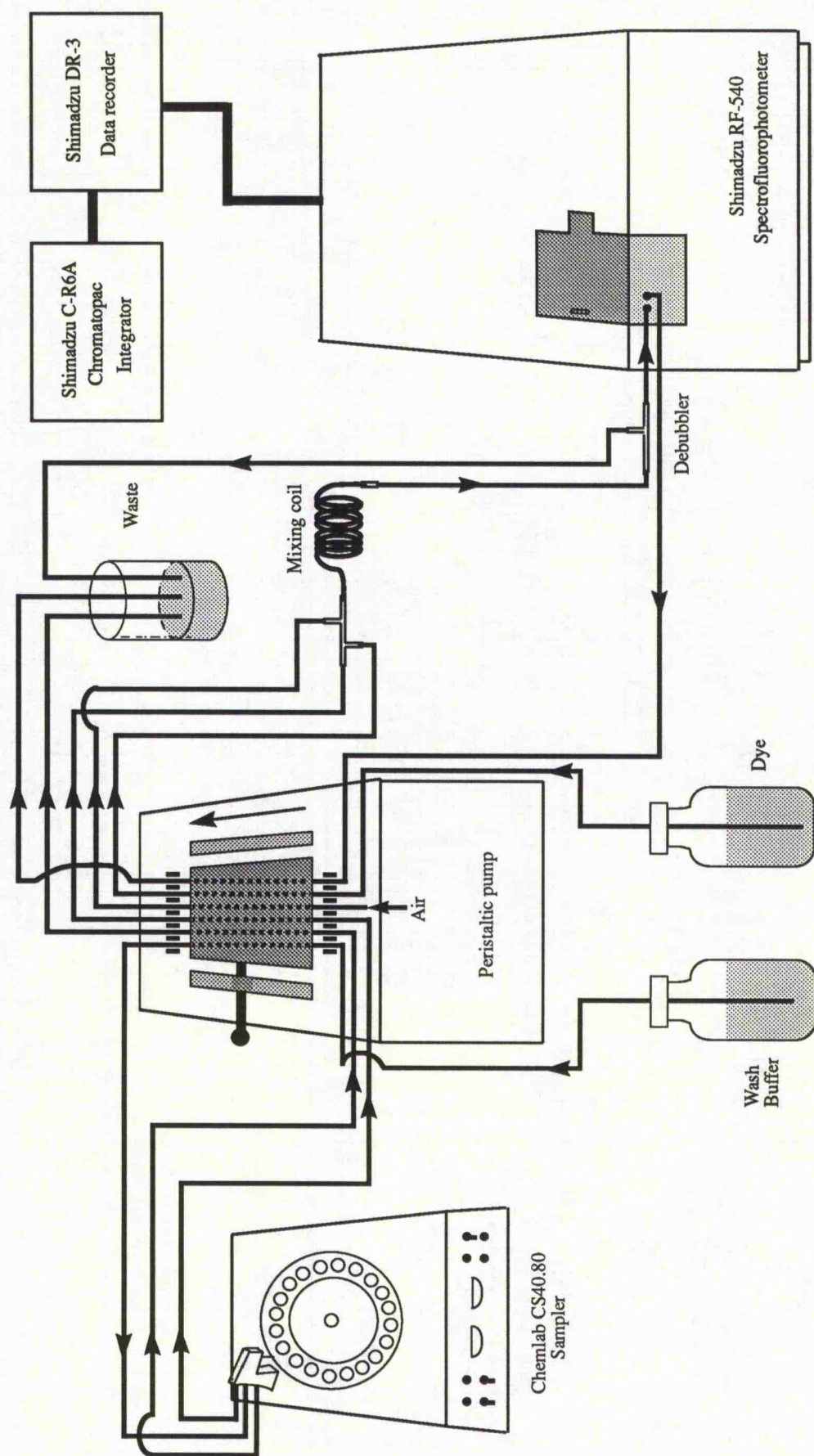
In 1979, Cesarone *et al.* presented the quantitative determination of DNA using Hoechst 33258 fluorochrome in neutral solution. This more rapid and reproducible technique of fluorometric assay following filter elution was used to test radiation-induced DNA damages (Stout and Beckert 1982, Sterzel *et al.* 1985, Murray and Meyn 1987). The relative amount of DNA in each fraction, including the filter, can be rapidly and simply determined spectrofluorometrically based on the Hoechst 33258 dye-DNA binding. Hoechst 33258 is a bibenzimidazole dye: 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazinyl)-benzimidazole trihydrochloride with the formula:



The stock solution of dye Hoechst 33258 was used as a  $2.33 \times 10^{-4}$  M in distilled water. The dye solution of the desired concentration was prepared by diluting the 200  $\mu$ l stock solution with 60 ml 0.2 M  $\text{KH}_2\text{PO}_4$  buffer and 20 ml 10 x concentration of standard saline citrate (1.54 M NaCl and 0.15 M sodium citrate) and making upto 300 ml with distilled water just before use.

The sample cups containing DNA eluting solution were transferred onto a Chemlab CS 40.80 autosampler which was set on 55 seconds for sample transferring and 65 seconds for washing with eluting solution. After washing the tubing, an aliquot of each sample was removed automatically by the sampler, passed through a chemlab CPP15 autoanalyser pump to a glass mixing coil where the sample was mixed with pre-buffered Hoechst 33258 so that a final dye concentration of  $5 \times 10^{-8}$  M was obtained. In the mixing coil, air was also added to prevent carry-over of sample or wash. The dye-DNA mixture was then flowed through a debubbler to remove any air bubbles, through a quartz flow cell in a spectrofluorophotometer-RF540 (Shimadzu corporation, Japan), and the fluorescence of DNA and dye mixture was measured at an emission wavelength of 475 nm with excitation wavelength at 360 nm. This was set up with the Shimadzu DR3 data recorder attached to the meter. The amount of fluorescence detected for each aliquot was recorded into the curve and calculated by a shimadzu C-R6A chromatopac integrator attached to the meter. Figure 2.4.1 presents a flow diagram of the layout of the equipment.

For the amount of DNA retained on the filter, the filter holder were removed from the syringe barrel and inverted over 25 ml universal bottle. The pump was reversed to order to push all of the possible DNA sticking to the tubing and filter holder out with 15 ml of the eluting solution. The filters were carefully removed from the filter holder into universal bottles which were heated to 120°C in a pressure cooker for 20 minutes. The bottle were vigorously shaken after cooling and the volume of eluting solution in



**FIGURE 2.4.1**

Flow diagram of the fluorescence autoanalyzer system for determination of DNA strand breaks

each bottles was noted. A 3 ml sample from each bottle was then assayed for DNA content using the procedures.

The sample containing calf thymus DNA (Sigma Company) at known concentration were used to produce a calibration curve of DNA concentration against fluorescence. Linear regression analysis was then carried out using the "minitab" facility (Minitab Inc.) on the University's DES Vax Mainframe computer.

By using the calibration curve it may possible to calculate the amount of DNA in each aliquot of filtrate, and the total amount of DNA on each filter prior to elution. The "total fluorescence" of each fraction was then calculated from the product of the corrected fluorescence intensity and the volume of fraction, and was proportional to the amount of DNA eluted in that fraction. Elution profiles relating the amount of DNA retained on the filter versus the volume eluted were then constructed. The relative fraction of DNA elution was calculated using the equation:

$$[1 - F_I] - [1 - F_C]$$

where,  $F_I$  represents the fraction of fluorescence retained on the filter after 12 h of elution in the treated cell sample;  $F_C$  represents the fraction of fluorescence retained on the filter after 12 h of elution in the untreated cell sample.

## 2.5

### Appendices

Plasmid is not replicated by itself, but it can co-exist in the host cell to replicate. In order to amplify plasmid in a few hours, it is necessary that the pieces of foreign plasmid are inserted into the host bacterial cells to give reproducible high yields. This process is called **transformation**. *Escherichia Coli* does not normally take up the foreign plasmid from its surrounding, but can be induced to do by prior treatment with  $\text{Ca}^{2+}$  ions under cold conditions, they are then known to be **competent**. The plasmid is added to the suspension of competent cells and taken up during a mild heat shock. Small, circular molecules are taken up most efficiently, whereas long linear molecules will not enter the bacteria. The method of Mandel and Higa (1970) was used to transform the *Escherichia Coli* strain CSH26 with 4362 base pair plasmid DNA derived from pBR 322.

#### **Preparation of competent *Escherichia coli***

- a. Streaked from frozen stock of bacterial cell strain, CSH26, onto a Luria-broth agar plate and grew at 37°C overnight;
- b. Picked a single fresh colony from the plate and inoculated in a 5 ml Luria-broth medium at 37°C to an  $\text{OD}_{550}$  of 0.3;

- c. Transferred the 5 ml culture into a 100 ml Luria-broth medium pre-warmed at 37°C at ratio of 1:20 volume and grew to optical OD<sub>550</sub> of 0.48;
- d. Took 30 ml aliquots and chilled on ice for 5 minutes into a polypropylene tube;
- e. Centrifuged the culture in a 'Chillspin' for 5 minutes at 6000 rpm at 4°C;
- f. Resuspended the cell pellet in 2/5 volume of Tfb1 and left the cells on ice for 5 minutes;
- g. Centrifuged the cells for 5 minutes at 6000 rpm at 4°C;
- h. Resuspended the cell pellets in 1/25 volume of Tfb2 and kept on ice for 15 minutes;
- i. Transferred competent cells as suitable volumes (50 to 200 µl) into cold microfuge tubes using a pre-chilled pipette;
- j. Froze cells in a dry ice-ethanol bath and stored at -70°C or in liquid nitrogen;

#### Transformation of *Escherichia coli* with plasmid

- a. Thawed the competent bacterial cells until just thawed at room temperature and immediately put them on ice for 10 minutes;
- b. Added plasmid up to 2/5 volume of the cell solution and left on ice for 40 minutes;  
Plasmid was not added more than 100 ng per 200 µl cell solution.
- c. Heated at 42°C for 90 minutes, and then returned to ice for 5 minutes;
- d. Added 2 - 4 volumes of Luria-broth culture (pre-warmed to 37°C) and incubated for 45 to 60 minutes at 37°C;
- e. Plated on Luria-broth agarose with 100 µg/ml ampicillin;
- f. Inoculated at 37°C overnight and finally stored for usage at 4°C;

## Chapter 3

# ***RESULTS AND DISCUSSION***



## 3.1

# Effects of Buffers and Additives on Radiation-Induced Plasmid DNA Damage

## Introduction

Pure DNA and its components have been the subject of numerous studies of genetic damage by ionizing radiation. In studies on DNA itself, two different approaches have been used. In one, damage is primarily indirect since dilute aqueous solutions are studied at ambient temperature. Under these conditions damage is largely confined to water molecules. OH radicals are believed to be the major water radical to attack DNA. In the other method, damage is almost entirely direct. The system used comprises either 'dry' DNA or frozen aqueous solution. In the latter, in contrast to the liquid, most of the water is present as ice crystals, damage to ice crystals is effectively confined, and can be ignored. Attention is therefore focused on direct damage to the saturated DNA phase (Gräslund *et al.* 1971, Schulte-Frohlinde 1979, von Sonntag 1981, Gregoli *et al.* 1982, Boon *et al.* 1984, 1985). Although the relative importance of indirect versus direct damage has been unclear, it is possible that direct ionization of DNA *in vivo* may constitute a major source of damage (Bohne *et al.* 1970, Boon *et al.* 1984, 1985, Cullis *et al.* 1985, Cullis *et al.* 1986).



$\gamma$ -irradiation of native DNA ultimately give rise to a number of well characterised lesions which include SSB and DSB, release of base from the intact chain and various base modifications.

Supercoiled plasmid DNA is particularly useful for this type of study due to the ease with which the yields of SSB and DSB can be quantitated by gel electrophoresis (Hempel and Mildenberger 1987). Electrophoresis of irradiated plasmid DNA under nondenaturing condition is one of the most sensitive and quantitative methods for measurement of strand breaks in studies of radiation damage on pure DNA. Plasmid DNA is small superhelical molecule (Form I) which may be converted to a linear molecule (Form III) and a nicked molecule (Form II). The formation of more than two SSBs will also lead to the linear form if closely spaced on opposite strands, the three forms without molecular weight change can readily separate in an electric field (Boon *et al.* 1984, 1985, Cullis *et al.* 1985, 1986, Blazek and Peak 1988, Miller *et al.* 1991).

It was shown that the most important results from plasmid DNA is that irradiation at liquid nitrogen temperature (77K, -196°C) under conditions that are close to those in the ESR spectroscopy studies does result, ultimately, in both SSB and DSB (Boon *et al.* 1984, 1985, Cullis *et al.* 1985, 1986). The goal of this research is to understand direct radiation damage on plasmid DNA in the presence of various buffers and additives at 77K.

### Methods and Materials

pBR 322 plasmid was isolated and purified from *Escherichia coli* by alkaline lysis methods which are described in Chapter 2 (section 2.1). The proportion of supercoiled DNA was greater than 90% in all samples. The stock solution of pure plasmid DNA was 0.1 mg/ml and stored at -20°C. The components of buffers and chemical additives studied include:

Buffers:

Phosphate buffer (13.06 mM  $\text{KH}_2\text{PO}_4$  + 56.6 mM  $\text{Na}_2\text{HPO}_4$ )

10 mM Tris buffer (tris(hydroxymethyl)methylamine)

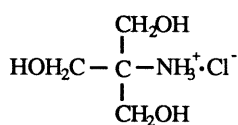
1.0 mM  $\text{Na}_4\text{EDTA}$  buffer (ethylenediaminetetraacetic acid)

Other additives:

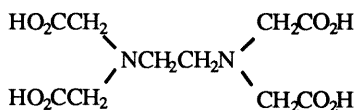
Sodium iodine (NaI) and Lithium chloride (LiCl)

Sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) and Sodium perchlorate ( $\text{Na}_2\text{ClO}_4$ )

Dimethyl sulfoxide (DMSO)



Tris·HCl



EDTA

All of these components of buffers and additives were purchased from Sigma Chemical Company. The buffer systems and DMSO solution were prepared by dissolving the materials in distilled water which was obtained by the method described in Chapter 2. NaI, LiCl,  $\text{Na}_2\text{SO}_4$  and  $\text{Na}_2\text{ClO}_4$  were dissolved in TE buffer (10 mM Tris and 1.0 EDTA). The pH value in all buffers was adjusted to ca. 7.4.

The plasmid DNA samples in different buffers and additive solutions for irradiation at liquid nitrogen temperature (77K) were prepared as described in Chapter 2 (section 2.2). Briefly, 10  $\mu\text{l}$  double concentrations of buffers and additive solutions were well mixed with 10  $\mu\text{l}$  stock solution of plasmid DNA, then these mixing solutions were frozen into small ice ball by directly submerging samples in liquid nitrogen and kept in irradiation bottles containing liquid nitrogen for irradiation.

Irradiation was carried out with a 'Vikrad'  $^{60}\text{Co}$  gamma source at a dose-rate of 40 Gy/min. During irradiation, samples were maintained in liquid nitrogen.

After irradiation, the ice samples were transferred into 1.5 ml sterile Eppendorf-type polypropylene tubes and maintained in an ice bath to allow melting. The samples were then added 4  $\mu\text{l}$  loding buffer and electrophoresed immediately they just thawed. The electrophoresis is described in Chapter 2 (section 2.3).

## Results and Discussion

### *Effects of buffers*

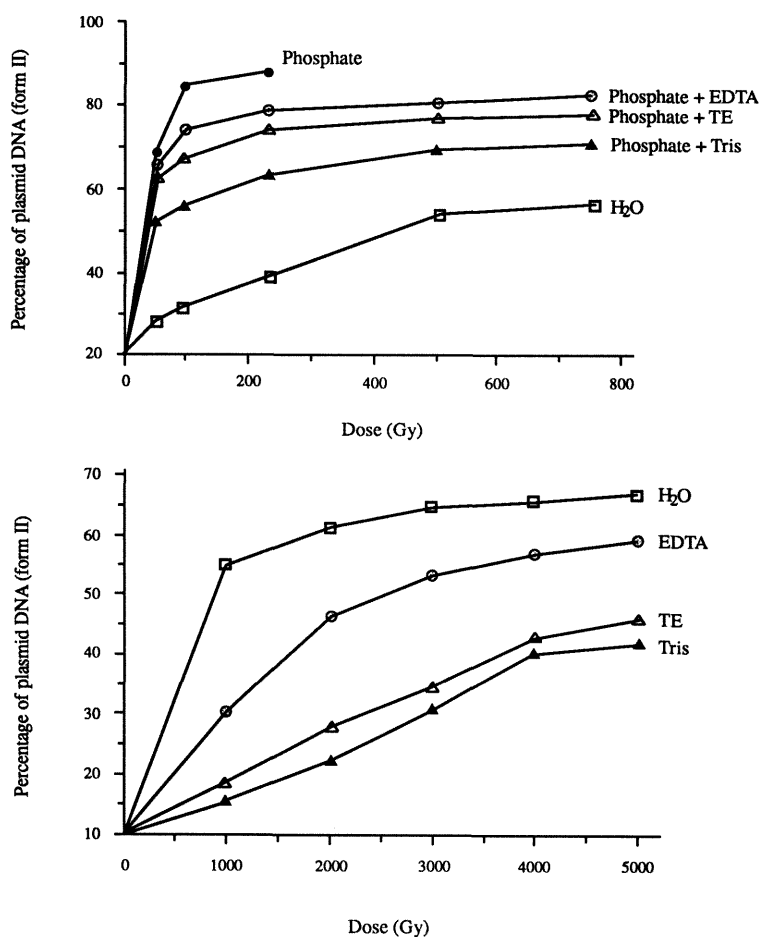
Table 3.1.1, Figure 3.1.1 and Figure 3.1.2 show the form II and form III of plasmid DNA induced by a wide dose range of  $\gamma$ -ray in the presence of different buffers. The percentages of each form are graphed against the radiation dose received. It was clearly shown that yields of damage by gamma-rays on frozen aqueous solutions of plasmid DNA depends on the presence of various buffer systems. As expected, when compared with the radiation damage of the DNA in the absence of added buffers (just in pure  $\text{H}_2\text{O}$ ), EDTA and Tris buffer systems acted as radioprotectors, whilst it is surprising to find that phosphate buffer system acted as a radiosensitiser. Moreover, the sensitising efficiency of phosphate buffer system was far greater than the protecting efficiencies of EDTA and Tris buffer systems. In comparison with the similar yield of DNA form II and form III (50%) induced by radiation, approximately 2500 Gy was needed in the presence of EDTA system, more than 5000 Gy, for Tris system. However, less than 50 Gy was needed to produce the same damage in the presence of phosphate system. The protection efficiency of Tris was around two-fold that of EDTA.

In addition, the sensitisation efficiency of phosphate buffer system was reduced by addition of EDTA and/or Tris in phosphate buffer system,

**Table 3.1.1** The palsmid DNA form II and form III and their ratio in different buffers after irradiation of gamma-ray.

Buffers & Doses	Percentage of form II		Percentage of form III		form II/form III
	Mean	S.D.	Mean	S.D.	
Phosphate					
50	68.9	0.80	3.1	4.2	22
100	85.8	0.72	7.2	3.7	11
220	88.1	0.76	12.3	3.9	7
Phosphate + EDTA					
50	66.8	1.7	2.5	2.5	26
100	73.9	2.5	4.1	2.6	18
220	79.7	2.3	7.6	1.1	10
500	81.3	1.9	11.9	3.2	6
750	85.1	2.2	12.5	1.7	6
Phosphate + TE					
50	62.6	3.5	2.0	3.2	31
100	68.7	3.7	2.8	4	24
220	73.4	4	5.9	5.0	12
500	78.3	3.6	7.2	4.7	10
750	79.1	3.7	9.6	5.2	8
Phosphate + Tris					
50	51.8	0.6	1.2	6.7	43
100	56.8	0.86	2.2	5.1	25
220	62.3	0.7	2.8	4.8	22
500	69.7	0.49	4.2	5.2	16
750	70.7	0.3	6.7	6.8	10
H <sub>2</sub> O					
50	28.6	1.6	0.5	7.6	57
100	32.2	0.8	0.8	8.2	40
220	39.1	0.6	1.7	5.6	23
500	52.7	2.8	2.4	2.1	21
750	54.2	2.1	3.3	3.7	16
1000	56.2	0.86	4.4	2.7	12
2000	62.2	2.7	8.2	4.2	7
3000	64.6	0.99	9.2	3.8	6
4000	66.4	0.6	11.7	1.9	5
5000	66.0	0.87	13.0	0.6	5
EDTA					
1000	30.1	2.5	1.6	3.5	19
2000	46.8	5.3	2.5	3.6	18
3000	63.6	2.1	3.4	2.6	18
4000	57.3	1.6	4.0	3.1	14
5000	58.8	2.1	4.3	3.7	13
TE					
1000	18.2	0.68	0		18
2000	27.4	0.7	1.1	6.2	25
3000	24.9	0.96	1.8	5.8	19
4000	44.5	1.5	2.2	4.8	20
5000	46.7	1.9	2.5	2.6	18
Tris					
1000	15.4	2.3	0		15
2000	23.4	2.7	0.9	6.8	26
3000	31.4	2.8	1.5	4.8	21
4000	40.7	5.2	1.7	3.9	24
5000	43.2	3.2	2.0	4.7	21

TE: 10 mM Tris and 1.0 mM EDTA.



**FIGURE 3.1.1**

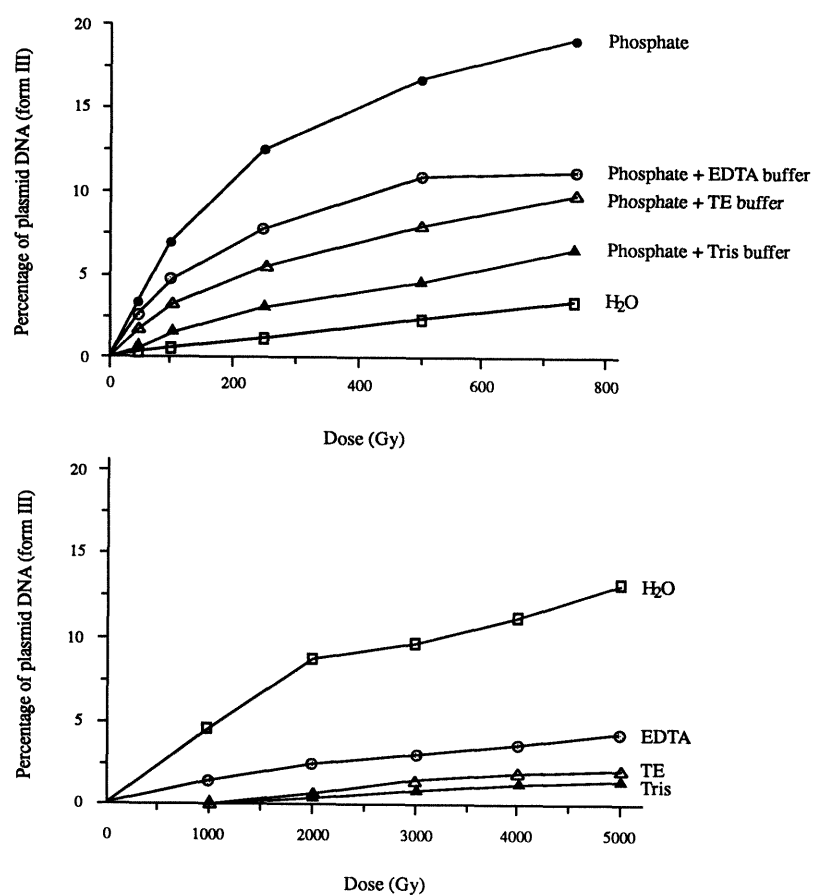
Dose-yield plot for the form II of plasmid DNA (pBR 322) by gamma-irradiation at 77K in different buffer systems.

Phosphate buffer: 13.06 mM NaH<sub>2</sub>PO<sub>4</sub>  
56.6 mM Na<sub>2</sub>HPO<sub>4</sub> ;

Tris buffer: 10 mM Tris-HCl;

EDTA buffer: 1.0 mM Na<sub>4</sub>EDTA;

TE buffer: 10 mM Tris + 1.0 mM Na<sub>4</sub>EDTA.

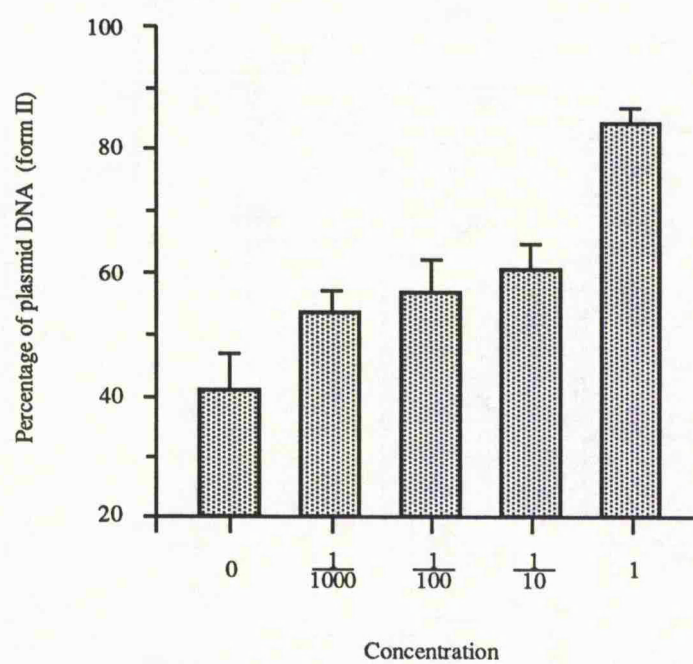


**FIGURE 3.1.2** Dose-yield plot for the form III from plasmid DNA (pBR 322) by gamma-irradiation at 77K in different buffer systems.

as indicated in the upper panel of Figure 3.1.1. And the effect of phosphate buffer system increased with decreasing dilution concentration (Figure 3.1.3). The results show a remarkable contrast between the effects of phosphate system in frozen aqueous solution of DNA irradiated at 77K and aqueous solution solution of DNA irradiated at room temperature where phosphate acted as a protector (Elsy 1991).

In an attempt to explain the difference of radiation-induced plasmid DNA damage in the presence of different buffer systems, a assumption of target-volume changes and attack on the DNA by radicals formed from the buffers is presented. Phosphates are excellent glass formers for water. This is because the extensive solvation of phosphate ions disrupts water structure which prevents many water molecules from entering the pure ice phase. As the glass phase, which presumably incorporates the DNA, increases, so the potential target volume increases. Thus, for example, electrons ejected at the periphery of the glass region may not react rapidly with phosphate ions or water, and hence may migrate to the DNA to give potential damage centres. This should give large increases in the centres relative to aqueous DNA, since most of the water is then present as ice crystals and the effective target volume is very small. The electron loss centres in the phosphate glass region are primarily  $\text{PO}_4^{2-\cdot}$  (or  $\text{HPO}_4^{\cdot-}$ ) radicals, as established by ESR spectroscopy (Langman 1992). These radicals ( $\text{PO}_4^{2-\cdot} + \text{HPO}_4^{\cdot-}$ ) are hydrogen atom abstractors and can attack DNA on annealing. Attack is most probably the peripheral sugar units giving hydrogen abstraction, and such centres frequently lead to SSB (Schulte-Frohlinde 1979).

These two factors, both of which stem from the large increases in target volume, may be responsible for the increase in damage that is observed. For tris buffer systems, the increase in the target volume is much less for equal concentrations. The tris,  $(\text{CH}_2\text{OH})_3\text{-C-NH}_3^{++}$  ions, are expected to react with ejected electrons,

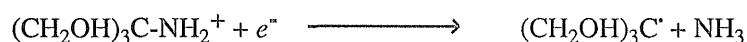


**FIGURE 3.1.3**

Induction of form II of plasmid DNA (pBR 322) by 90 Gy of gamma-rays at 77K in different concentrations of phosphate buffer system.

1 x concentration: 13.06 mM  $\text{NaH}_2\text{PO}_4$   
56.60 mM  $\text{Na}_2\text{HPO}_4$





preventing them from reaching the DNA. Also they will react with  $\cdot\text{OH}$  radicals, as well as undergoing direct electron loss. It seems that the resulting radicals, also detected by ESR spectroscopy (Langman 1992), do not attack the DNA with high efficiency on annealing, since the overall effect is slight protection.

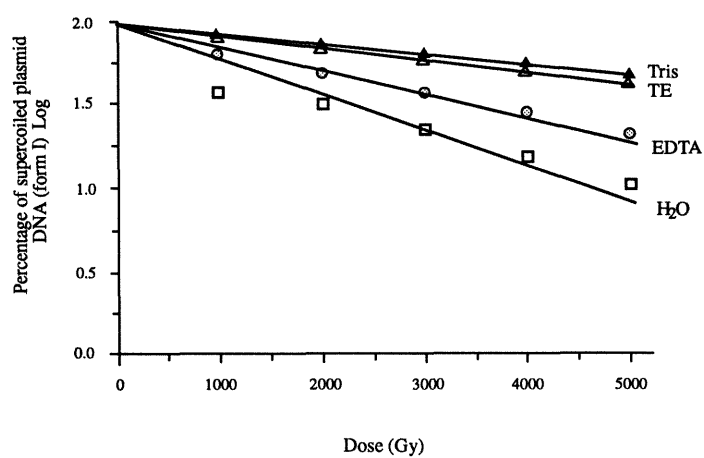
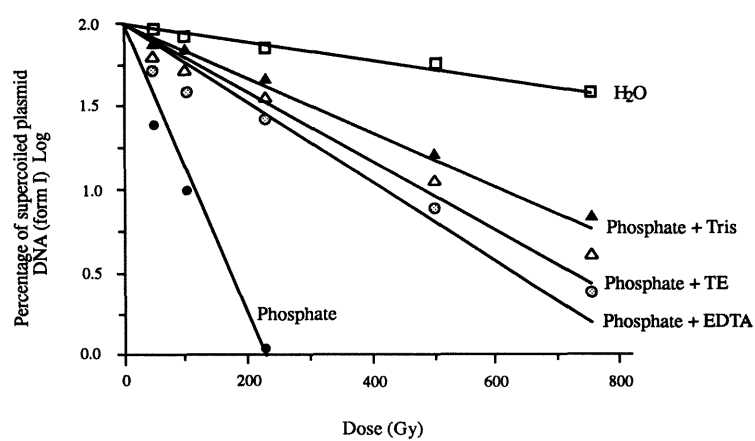
The ratio of plasmid DNA form II and form III induced by gamma-rays in different buffers is presented in **Table 3.1.1**. The ratio appears to decrease with increasing doses. This ratio changes in phosphate buffer system are greater than in other buffer system. **Figure 3.1.4** presents the proportion of supercoiled plasmid DNA (form I) remaining after exposure in different buffer systems and shows linear decrease in all buffers.

#### *Effects of other additives*

**Figure 3.1.5, 3.1.6 and 3.1.7** present the data concerning gamma-ray-induced plasmid DNA damage in the presence of NaI and LiCl, NaClO<sub>4</sub> and Na<sub>2</sub>SO<sub>4</sub>, and DMSO, respectively.

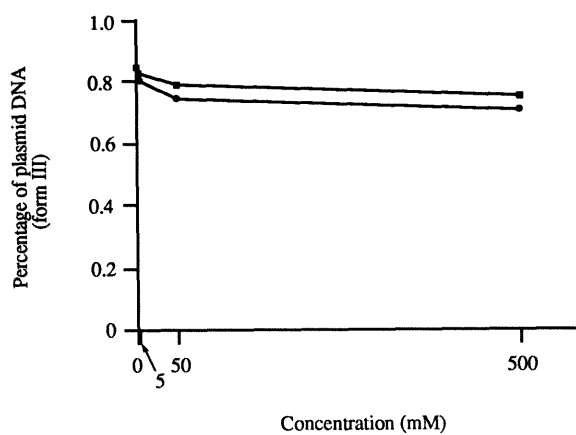
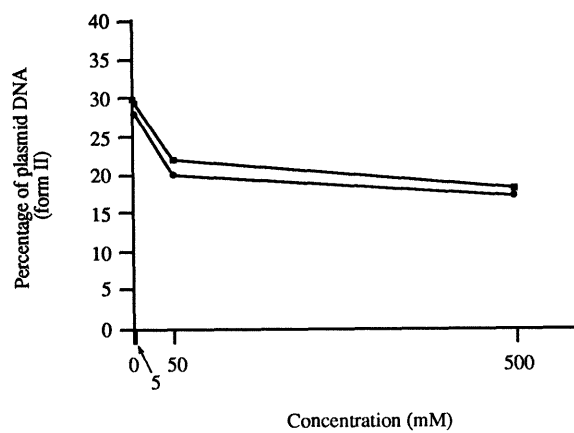
Since in frozen DNA solutions the DNA is fully solvated, it is easy to incorporate additives designed to modify the course of DNA damage. On freezing, pure ice crystal grow out until a glassy' DNA phase solidifies. Additives are totally rejected by the ice, and usually remain in the DNA phase and hence are close to the DNA.

It has been shown that increasing the concentration of salt can increase the radiation stability of the DNA due to the counterions shielding the negatively charged phosphate groups (Schildekraut and Lifson 1965), and



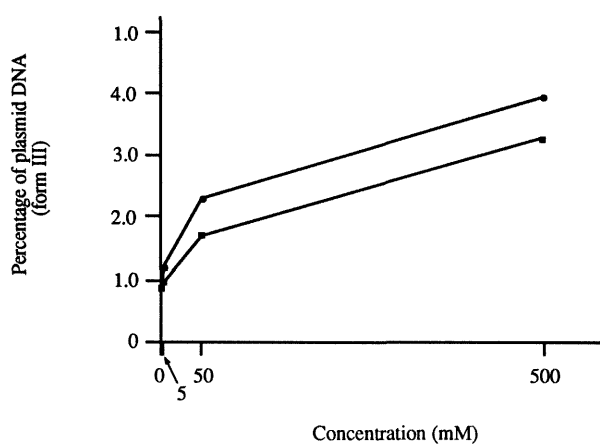
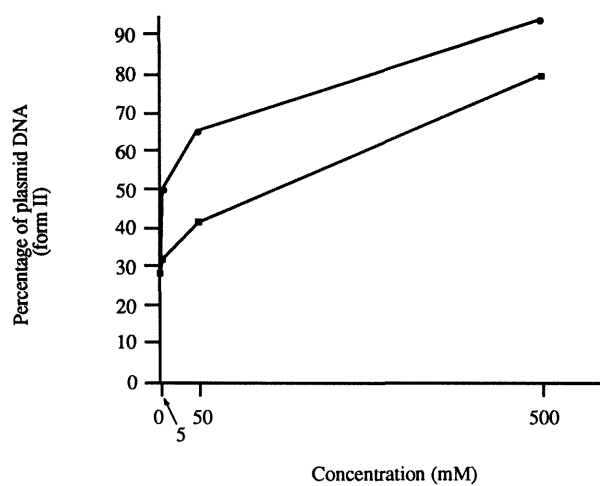
**FIGURE 3.1.4**

Semilogarithmic plot of the proportion of form I remaining after radiation at 77K in different buffer systems.



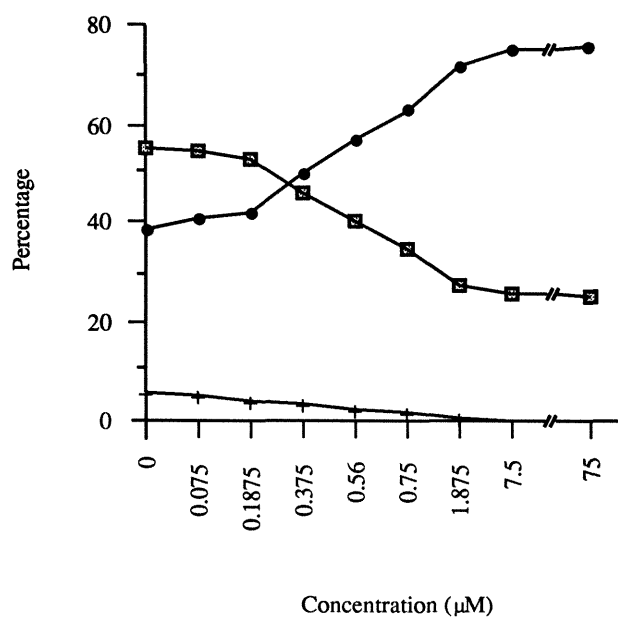
**FIGURE 3.1.5**

NaI ( ■ ) and LiCl ( ● ) effects on induction of plasmid DNA damages in TE buffer by gamma-rays at 2000Gy at 77K.



**FIGURE 3.1.6**

Na<sub>2</sub>SO<sub>4</sub> ( ● ) and NaClO<sub>4</sub> ( ■ ) effects on induction of plasmid DNA damages in TE buffer by gamma-rays at 250Gy at 77K.



**FIGURE 3.1.7**

Effects of DMSO with various concentrations on plasmid DNA damages induced by gamma-rays at 1000Gy at 77K.

- ( • ) Supercoiled DNA (form I);
- ( ■ ) Nicked plasmid DNA (form II);
- ( + ) Linear plasmid DNA (form III).

decrease the amount of supercoiling in PM2 and superhelical  $\lambda$  DNA (Wang 1969, Anderson and Bauer 1978).

In this research, both 50 mM of NaI and LiCl showed weak protection against radiation damage to the DNA, whilst 50 mM of NaClO<sub>4</sub> and Na<sub>2</sub>SO<sub>4</sub> had a greater sensitisation. Using frozen aqueous solutions, it has been established by ESR spectroscopy that the first detectable main radical centres are localised guanine and thymine ion radicals, G<sup>•+</sup>, T<sup>•-</sup> and C<sup>•-</sup>, therefore it implies that these radicals can be precursors of strand breaks (Barnes *et al.* 1991, Sevilla *et al.* 1991, Cullis *et al.* 1992). NaI and LiCl could reduce the total radical yields in the DNA which has been irradiated under direct damage condition at 77K (Bartlett 1985). Therefore, their protection can be explained by suggestion that they scavenge G<sup>•+</sup> to reduce DNA strand breaks.

As seen in Figure 3.1.5 and Figure 3.1.6, there are great differences between these salts after 50 mM concentration is used. NaI and LiCl show a radioprotective effect after 50 mM and this increase with increasing concentration, while NaClO<sub>4</sub> and Na<sub>2</sub>SO<sub>4</sub> still show radiosensitive effects after 50 mM and their efficiently increase with increasing concentration. Gregoli *et al.* (1982) found that the yield of DNA free radicals found with freeze-dried DNA irradiated at 77K was only half of that of frozen aqueous DNA irradiated at 77K. Therefore, this supports that the radiosensitisation increasing with higher concentration of NaClO<sub>4</sub> and Na<sub>2</sub>SO<sub>4</sub> is because contents of water in DNA double helical structures increases with increasing salt concentration, the target volume of DNA then increases.

Dimethyl sulphoxide (DMSO) is of great importance as an additive to cellular systems, since it acts as a cryoprotector. It is also found to be a radioprotector in that it suppresses radiation-induced cell transformation *in vitro*. DMSO is widely used as an OH free radical scavenger in biological

radiation studies (Chapman *et al.* 1979, Littlefield *et al.* 1988). The DMSO action on DNA has been examined by ESR (Cullis *et al.* 1990a)

In the present study, there is obviously reduction in the formation of DSB and SSB in the presence of DMSO. Estimating the the numbers of DSBs is somewhat inaccurate because of the relatively small amounts of the linear form of the plasmid generated under these conditions. However, there does appears to be a greater decrease in the numbers of form III as compared with form II. And the protective efficiency reached saturation in the range of more than 7.5  $\mu\text{M}$  (ca. 10:1 ratio of DMSO:DNA base pairs).

The mechanism of protection by DMSO is generally explained by the scavenging of OH radicals. But, in experimental condition used here, OH radical reactions are not an important source of DNA damage. At 77K only  $\text{G}^+$ ,  $\text{T}^-$  and  $\text{C}^-$  are initially detected by ESR, they are believed to be the major DNA damage centres (Barnes *et al.* 1991, Sevilla *et al.* 1991, Cullis *et al.* 1992). The presence of DMSO results in a clear loss of DNA radicals, a change of  $\text{G}^{+\bullet}$  and  $\text{T}^-/\text{C}^-$ , the rates of both  $\text{e}^-$  and hole capture are greater and can compete even with capture by DNA so that DMSO radicals dominate (Cullis *et al.* 1990).

## Summary

Exposure of frozen aqueous solutions of pBR322 plasmid DNA to  $^{60}\text{Co}$   $\gamma$ -rays at 77K gave different yields of form II and form III in different buffers and additives. In studies of buffer effects, it was found that in the presence of phosphate, the radiation sensitivity of plasmid DNA markedly increased, EDTA and Tris acted as protector against radiation damage. As for the effects of chemical additives used on radiation damage of DNA, it was found that  $\text{NaClO}_4$  and  $\text{Na}_2\text{SO}_4$  act as radiosensitisers and this efficiency increases with their concentration. NaI and LiCl act as a weak protecting agents. As expected, DMSO acts as a protecting agent. The

possible mechanisms related to the actions of these buffers and additives are discussed.



## 3.2

## Effects of Polyamines on DNA Damage Induced by Gamma-Rays

### Introduction

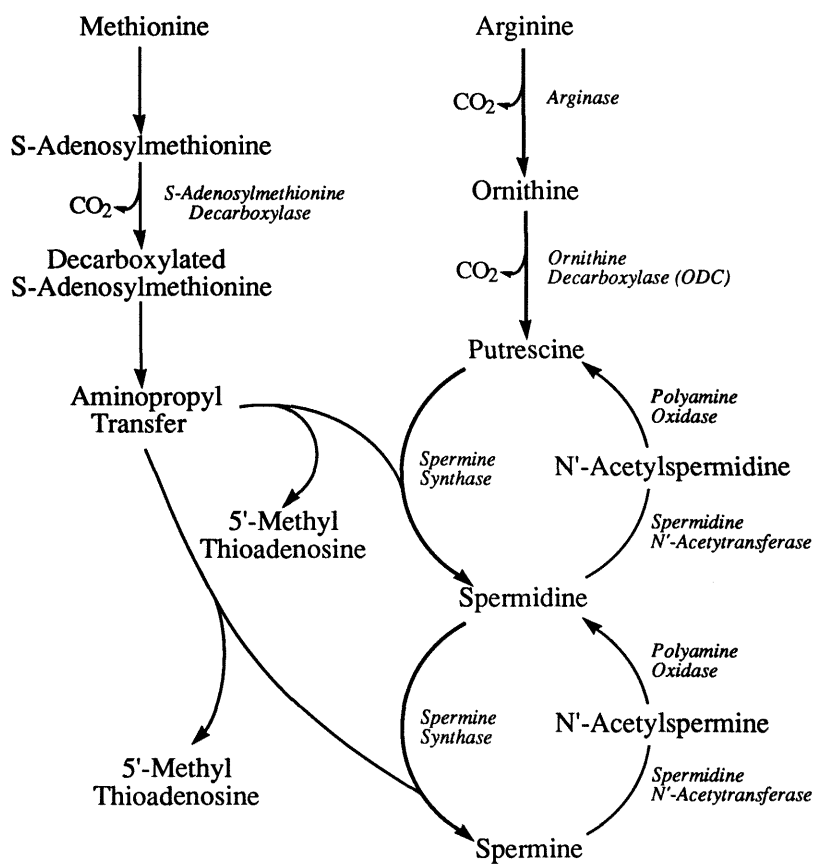
The polyamines, spermidine, spermine and their precursor, putrescine, are aliphatic polycationic compounds found in all cells.

putrescine	$\text{H}_2\text{N}-(\text{CH}_2)_4-\text{NH}_2$	$\log (K) = 5.2$
spermidine	$\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}(\text{CH}_2)_4-\text{NH}_2$	$\log (K) = 7.3$
spermine	$\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}(\text{CH}_2)_4-\text{NH}(\text{CH}_2)_3-\text{NH}_2$	$\log (K) = 9.2$

Where, K is the association constant for binding of the polyamine to DNA (Braulin *et al.* 1982). In fact, these polyamines are fully *N*-protonated into a group of small organic cations at physiological pH (ca. 7).

putrescine	$\text{H}_3\text{N}^+-(\text{CH}_2)_4-\text{NH}_3^+$
spermidine	$\text{H}_3\text{N}^+-(\text{CH}_2)_3\text{NH}_2^+-(\text{CH}_2)_4-\text{NH}_3^+$
spermine	$\text{H}_3\text{N}^+-(\text{CH}_2)_3-\text{NH}_2^+(\text{CH}_2)_4-\text{NH}_2^+(\text{CH}_2)_3-\text{NH}_3^+$

The biosynthetic pathways for putrescine, spermidine and spermine in mammalian cells are well established (Figure 3.2.1). Polyamine biosynthesis is highly regulated and intracellular levels of polyamines can change rapidly by orders of magnitude when cell growth is stimulated



**FIGURE 3.2.1**

General pathway for the biosynthesis of putrescine, spermidine and spermine in mammalian cells (Adopted from Pegg and McCann 1982). ODC is the rate-limiting enzyme.

(Tabor and Tabor 1984). Inhibition of polyamine synthesis has been used in the treatment of protozoal infection and is seen as a promising anti-tumor therapy due to its anti-proliferative effects (Walters 1987). The roles they play in biological systems have been dealt with extensively in the literature, mainly in the regulation of normal cell proliferation. Moreover, it has been proposed that polyamines have their biological consequences by interacting with the cellular nucleic acids (Bachrach 1973, Heby and Andersson 1984, Tabor and Tabor 1984). At low relative concentrations, cellular polyamines are present almost entirely on DNA.

The interaction of DNA and polyamines was first shown by the ability of spermidine and spermine to precipitate DNA and by the ability of these amines to protect DNA from denaturation by heat and alkaline denaturation (Abraham and Pihl 1981, Cavanaugh *et al.* 1984). As expected from polyelectrolyte theory (Oosawa 1971), this stabilizing effect was attributed to neutralization of the negative charges on the phosphate groups and the consequent increase of the effectiveness of various attractive forces (Tabor and Tabor 1984, Vertino *et al.* 1987), because polyamines are effectively fully protonated into polyammonium cations (PACs) in solutions buffered to a pH of ca. 7.

There can be no doubt that, in solution, PACs are associated with DNA, as reported in some experiments (Suwalsky *et al.* 1969, Richmond *et al.* 1984). Complexes formed between DNA and PACs appear to involve non-covalent linkages between the basic groups of the PACs and the acidic phosphate groups of the DNA (Tabor and Tabor 1964). The binding constant for DNA-polyamine complexes has been shown to decrease with increasing ionic strength ((Rubin 1977, Braulin *et al.* 1982), this indicates that polyamine binds to DNA in such a way that it can be displaced in competitive reactions.

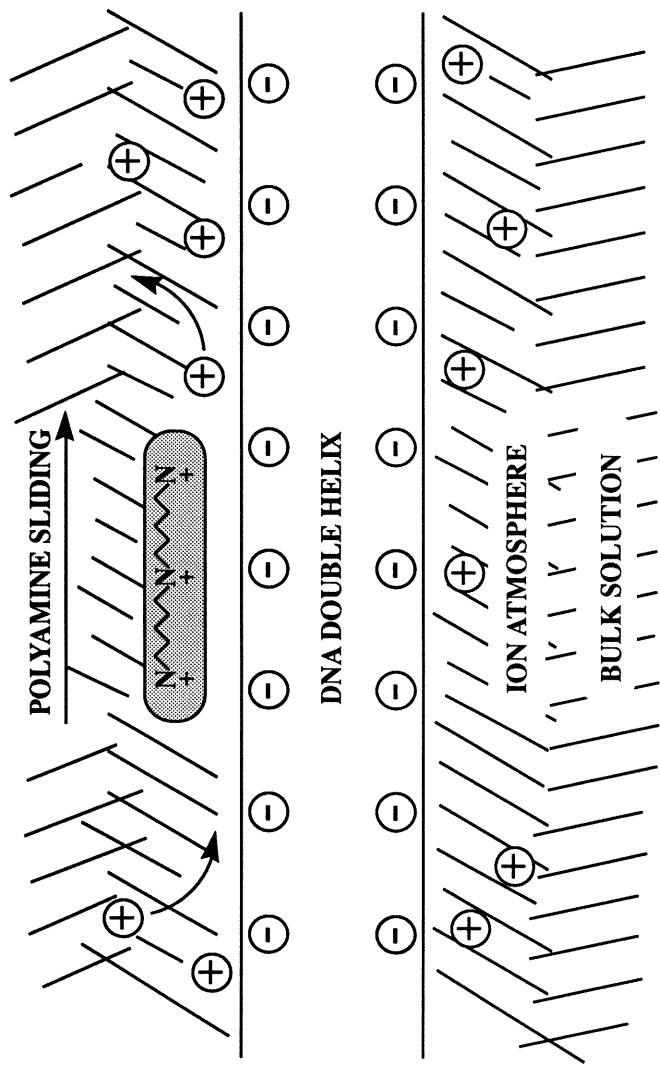
The NMR results probe the extent to which these PACs are strongly bonded to DNA (Manning 1978, Burton *et al.* 1981, Wemmer *et al.* 1985,

Besley *et al.* 1990, Besley 1991). Two possible binding modes can be envisaged for the DNA-PAC complex (Besley *et al.* 1990). The first is a tight binding mode in which the PAC is stationary at some site on the DNA during the period of interaction. The second is a loose binding mode in which PAC are constrained to be close to the DNA by simple electrostatic interactions yet they experience almost free diffusion along the DNA strands in such a way that they are close to the DNA for long periods.

When the sodium ions are close to the DNA the strong, asymmetric electric field (which has an effectively linear structure over small distances) causes a marked line broadening due to the alignment of the  $^{23}\text{Na}$  quadrupole with the electric field. Sodium ions close to the DNA feel this effect whilst sodium ions far from the DNA are unaffected, these two types of sodium ions are in rapid equilibrium and so the measured broadening is a time average. When PACs are added, they displace sodium ions from the vicinity of the DNA, thus causing a sharpening of the  $^{23}\text{Na}$  resonance. It was shown that the norspermidine cation, see Figure 3.2.2, is strongly bound to DNA (Besley *et al.* 1990, Besley 1991).

However, Liquori *et al.* (1967) and Suwalsky *et al.* (1969) have proposed a model for DNA-PAC binding which also has specific interactions, in which it was proposed that the tetracationic polyamines bridges the minor groove, with the two terminal ammonium groups being close to phosphate groups on either side of the groove. But this specific structure was not supported in some experiments (Quigley *et al.* 1978).

Despite the differences, all these studies can still be used to infer tight binding in DNA-PAC complexes. Therefore, radiobiologically, these polycations can be considered as potential protectors against radiation damage on DNA. When a drug is administered to cellular systems, there are a number of obstacles which have to be circumvented before the molecules reach their targets. If this target is nuclear DNA, the drug needs to penetrate the cellular and nuclear membranes and to reach the DNA before



**FIGURE 3.22**

A schematic representation of the model of the non-specific sliding interaction between a PAC and DNA phosphate groups (  $\ominus$  ) involving displacement of sodium ions (  $\oplus$  ).

being metabolised, and before it affects some other sensitive region of the cell. Thus it has been suggested that polyamines can also be used as a carrier to deliver drugs to DNA due to their high affinity for cellular DNA (Cullis *et al.* 1990b). This delivery minimises the time required for drug reaction and maximises the chance of reaching the require location on the DNA in order to avoid drug reaction with some other sensitive region of the cell.

In this work, the experiments are designed to examine the possible effects of polyamines as radioprotectors on radiation damage of plasmid DNA at liquid nitrogen temperature (77K), and effects of spermine and spermine-linked metronidazole on cellular DNA DSB and cell death induced by gamma-ray irradiation at 0°C.

## Materials and methods

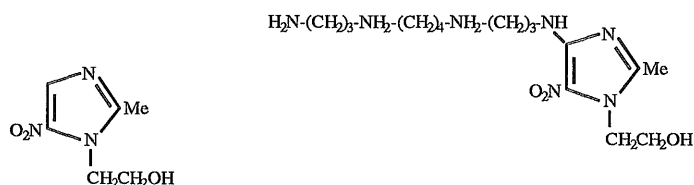
### *Analysis of plasmid DNA*

Spermidine and spermine were purchased from Sigma Chemical Company and used without further purification. The solutions of polyamines were freshly made up by dissolving the spermidine and spermine in phosphate buffer (13.06 mM  $\text{KH}_2\text{PO}_4$  and 56.6 mM  $\text{Na}_2\text{HPO}_4$ , pH = ca. 7) to a stock concentration of 20 mM just prior to use. pBR 322 plasmid DNA was used at a final concentration of 0.05 mg/ml, 0.075 mM with respect to base pairs, as described in Chapter 2. The appropriate amount of stock polyamine solution was diluted and mixed with the stock DNA solution (0.1 mg/ml) to obtain the desired ratio of polyamine/DNA base pairs. The volume of typical samples was 20  $\mu\text{l}$ . The polyamine-DNA complexes were incubated together for 20 min in a ice bath to allow time for the polyamines to intercalate into the DNA. Incubation time of 20 min did not produce any detectable difference in the results. After incubation, the polyamine-DNA complexes were frozen in liquid nitrogen and irradiated with gamma-rays at 77K in normal air, as described in Chapter 2 (section 2.2). The samples were placed in an in ice bath immediately the

irradiation was completed, and the resulting strand breaks were quantified using the electrophoresis techniques described in chapter 2 (section 2.3).

#### *Assay of cellular DNA DSB and cell survival*

Spermine and metronidazole (MET) were obtained from Sigma Chemical Company and used without further purification. Spermine-linked metronidazole (AM1229) was the kind gift of Professor P. Cullis, which was synthesized at the Chemistry Department, the University.



Metronidazole

Spermine-linked Metronidazole

Chinese hamster V79 cells were grown in culture flask and labeled with [<sup>3</sup>H]thymidine as described in section 2.4.

After trypsinization, the cells were washed with fresh medium and centrifuged. The cell pellets were resuspended in growth medium containing spermine, metronidazole, and AM1229, respectively. The spermine, metronidazole and AM1229 were dissolved in the growth medium at a concentration of 10 mM, and the pH was maintained at 7.2 to 7.4. The cells were grown for a period of 24 h at 37°C before irradiation. The medium was removed, the cell sheet was washed twice with fresh medium. The control cells were treated in the same way.

For the analysis of DSB, the cells were resuspended in desired amount of medium to obtain a final concentration of  $2 \times 10^6$  cell/ml. The cell suspensions were bubbled prior to irradiation with oxygen-free nitrogen for 30 min. After degassing, 1 ml portions of cell suspensions were gently dispensed into a 2 ml plastic irradiation tubes and kept on ice for

irradiation. The irradiation was carried out using 'Vikrad'  $^{60}\text{Co}$  source at a dose rate of approximately 35 Gy/min at 0°C. The DSB were measured by neutral filter elution as soon as the irradiation was finished, as described in chapter 2 (section 2.4).

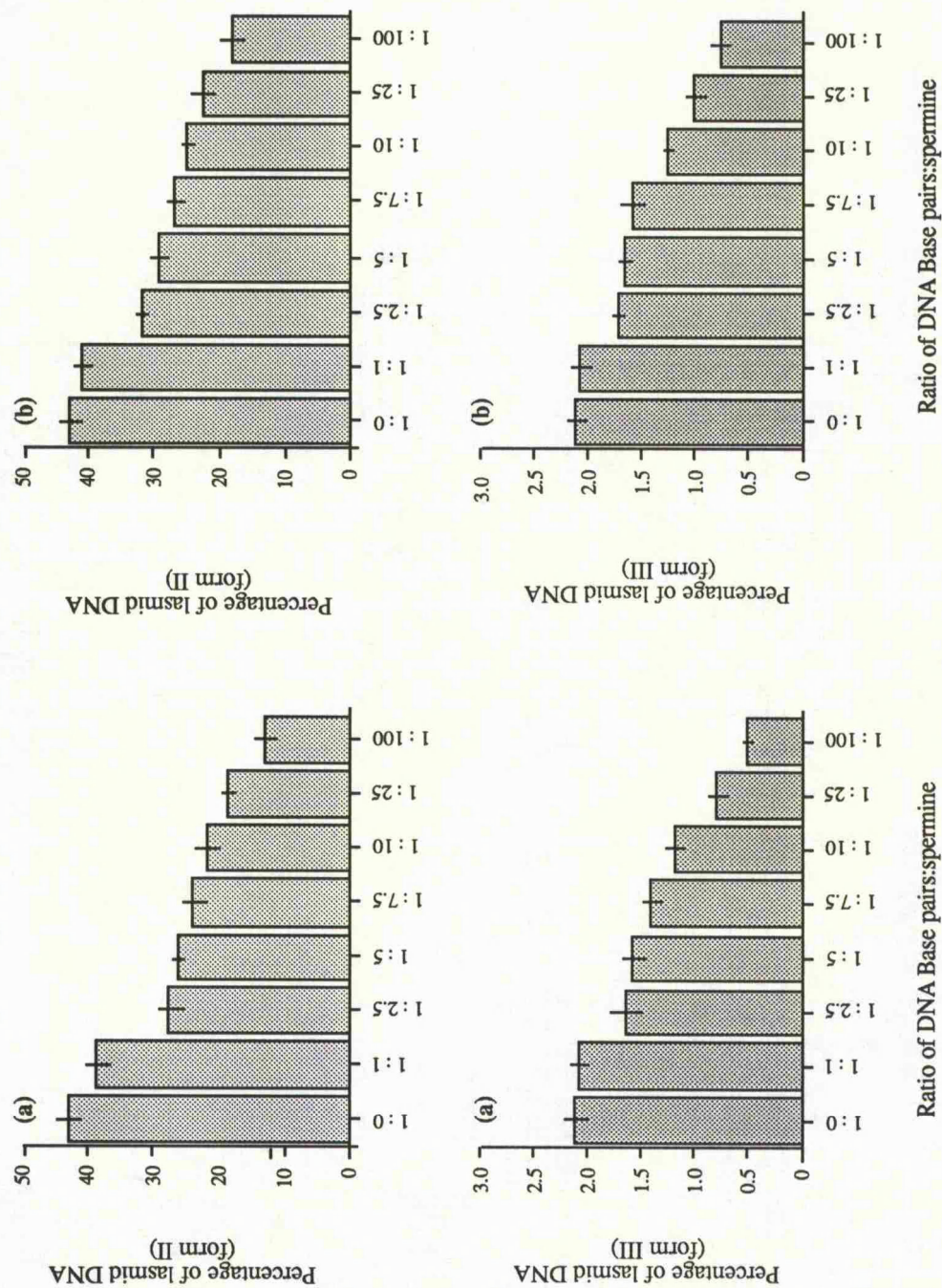
Compressed nitrogen gas from commercial sources usually contains trace amounts of oxygen. It was therefore necessary to remove the oxygen from any nitrogen that was used for pumping oxygen from samples. Nitrogen was passed through two flasks of chromous chloride to remove the oxygen, and a flask of dilute sodium hydroxide solution, followed by a flask of sterile water, to remove any acid that may escape from the first flask. Bubbling through this system also ensured that the nitrogen used was water saturated.

For the analysis of cell killing, clonogenic assays were used to measure cell survival. After cultured for 24 h in medium containing spermine, metronidazole and AM1229, the cells were resuspended in medium without drugs. The total number of test cells was determined and irradiated using gamma-rays at ice bath temperature. After irradiation the cell suspension was further diluted in warm fresh medium to give a concentration of 200 cells/ml. 1 ml volume of cell suspensions were transferred to tissue culture flasks containing 30 ml fresh medium, and these were incubated at 37°C for 7 days. The viable cells left formed colonies which were fixed and stained with 1.25% crystal violet in absolute ethanol. The colonies (50 cell or more) were then scored. The survival fraction was calculated as the ratio of colony forming efficiency (irradiated cells) to colony forming efficiency (unirradiated cells).

## Results and discussions

Figure 3.2.3 shows the effects of several concentrations (with respect to base pairs) of spermidine and spermine on the strand breaks of plasmid DNA induced by gamma-rays at liquid nitrogen temperature.





**FIGURE 3.2.3**

Protection effects of spermine (a) and spermidine (b) with various concentrations (as DNA base pairs:polyamines) in phosphate buffers on plasmid DNA damages induced by gamma-rays at 35 Gy at 77K.

Spermidine and spermine both reduce the yield of form II and form III. The magnitude of the effects is dependent on the polyamine concentrations, but only slightly increases with increasing concentrations. Moreover, there was a considerable increase of radioprotection efficiency after 2.5:1 concentration of polyamines:DNA base pairs is obtained.

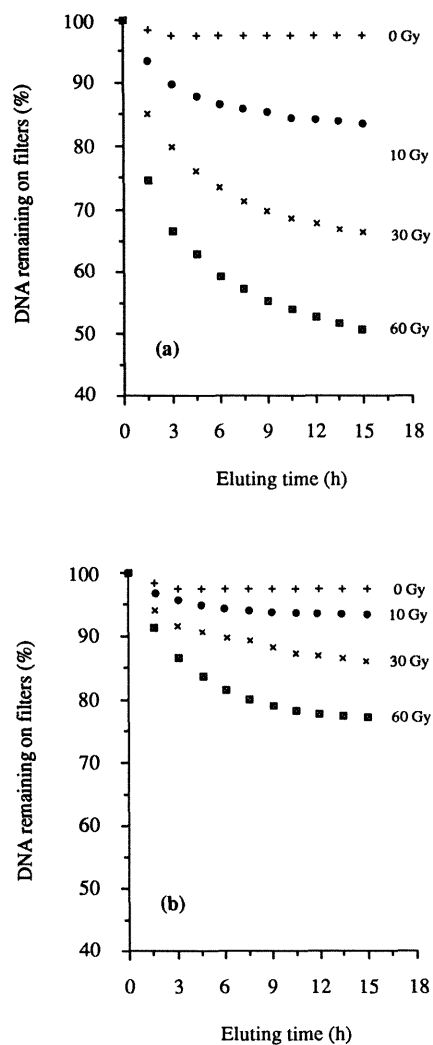
Spermine has a higher protection efficiency against both form II and form III than spermidine. It indicates that a polyamine carrying a higher number of positive charges is more efficient than the one carrying a lower number of charges, the protection increase with the association constant ( $k$ ) for binding of the polyamine to DNA, spermine<sup>4+</sup> > spermidine<sup>3+</sup>. This results is consistent with those reported (Rubin 1977, Braulin *et al.* 1982).

It is now believed that  $\gamma$ -irradiation of frozen aqueous solution of DNA at liquid nitrogen temperature (77K) give rise to radical cations and anions through direct damage (Cullis *et al.* 1992). The addition of spermidine and spermine in a wide range of concentration to frozen aqueous solutions of DNA may reduce yields of G<sup>•+</sup> and C<sup>•-</sup>/T<sup>•-</sup> following irradiation at 77K, because they are able to react with electrons and holes.

#### *Effect of polyamines with culture cells*

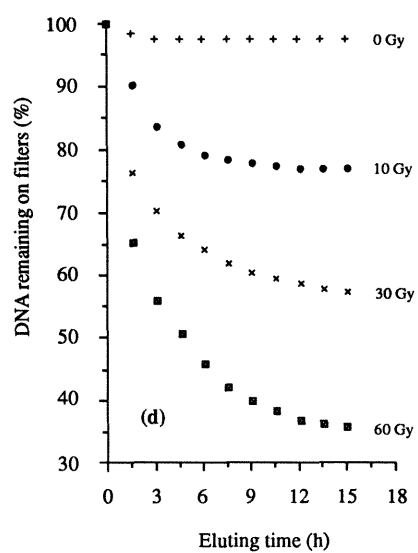
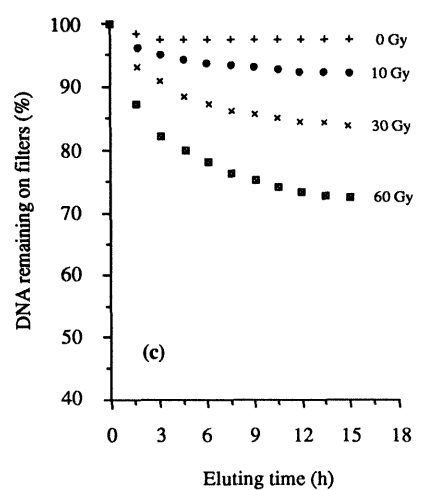
Figure 3.2.4, 3.2.5 and 3.2.6 show DNA DSB and cell death induced by gamma-rays in the presence of 10 mM spermine, metronidazole and spermine-linked metronidazole (AM1229). The following observations can be made regarding the data presented:

(1) For DSB induced by  $\gamma$ -rays under free-oxygen environment, metronidazole acts as radiosensitiser, the mean value of relative modifying factor (MF) is about 1.3. While spermine and AM1229 act as radioprotectors, the mean value of relative modifying factor (MF) is approximately 2.5 and 2.0, respectively. These relative efficiencies decrease with increasing radiation doses. There is a slight difference in protection efficiency between spermine and spermine-linked metronidazole;



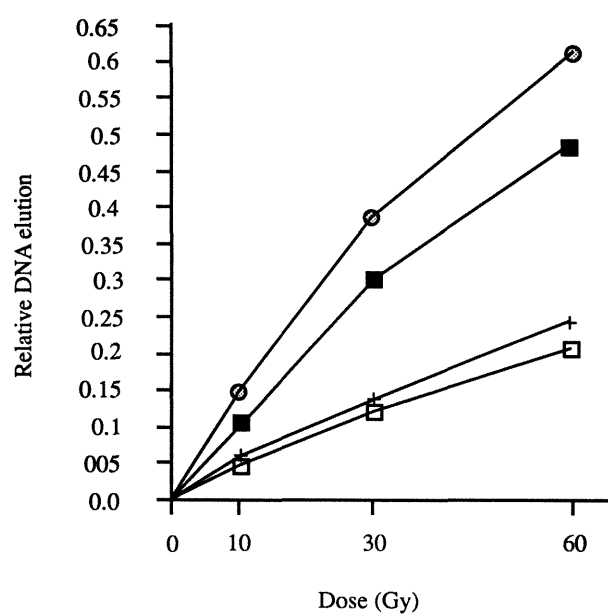
**FIGURE 3.2.4a**

Induction of DSB in DNA of V-79 cells by gamma-rays at ice bath temperature in medium without drugs (a) and medium containing 10 mM spermine (b). The percentage of DNA remaining on the filters is plotted as a function of the eluting fractions.



**FIGURE 3.2.4b**

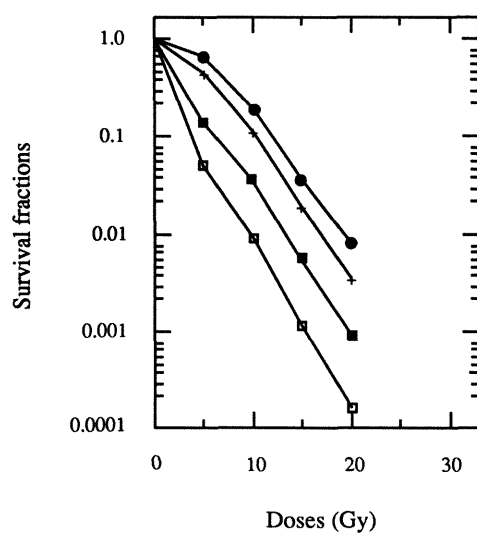
Induction of DSB in DNA of V-79 cells by gamma-rays at ice bath temperature in medium containing 10 mM AM1229 (c) and medium containing 10 mM metronidazole (d). The percentage of DNA remaining on the filters is plotted as a function of the eluting fractions.



**FIGURE 3.2.5**

Dose-response curves for gamma-irradiated cells under the condition of free-oxygen. Relative DNA elution is plotted as a function of radiation dose.

- ( ■ ) in medium without drugs;
- ( □ ) in medium containing 10 mM spermine;
- ( + ) in medium containing 10 mM AM1229;
- ( ⊗ ) in medium containing 10 mM metronidazole.



**FIGURE 3.2.6** Clonogenic survival for gamma-irradiated cells under the condition of atmospheric oxygen.  
 ( + ) in medium without drugs;  
 ( ● ) in medium containing 10 mM spermine;  
 ( ■ ) in medium containing 10 mM AM1229;  
 ( □ ) in medium containing 10 mM metronidazole.

(2) For cell death induced by  $\gamma$ -rays under atmospheric oxygen, metronidazole and spermine-linked metronidazole act as radiosensitisers, whilst spermine acts as a radioprotector.

As expected, it was demonstrated that spermine modified the cellular damages, both DSB and cell killing, as radioprotector, and metronidazole altered the cellular damages as a radiosensitiser. However, it is surprising to find that spermine-linked metronidazole (AM1229) protects the cells to the induction of DSB under free-oxygen, and sensitises the cells to cell killing under the condition of atmospheric oxygen.

Experiments on the direct effects of polyamines on DNA in cultured mammalian cells are particularly difficult to interpret because deprivation and increase of polyamines dose not occur rapidly; by the time a certain amount of polyamine has reached to nuclear DNA, many changes have been occurred. Moreover, little is known how polyamines enter the mammalian cells and reach the nuclear DNA. However, from the results presented here, it is clear that polyamines indeed have certain protection effect on cell damage induced by ionizing radiation, although the exact mechanism and pathway of this protection are unknown.

There were reports to explain the effects of polyamines on cell damage through a mechanism of anisotonic fixation effects, in which polyamines are considered as anisotonic agents which alter the water content inside cells (Rao and Johnson 1979, Raaphorst and Azzam 1981). If so, 10 mM concentration of spermine as a hypotonic solution decreases the intracellular water content so that indirect effects of radiation decreases. The mechanism concerning the effect of hypotonic solution is discussed in section 3.4.

When in contact with solvated DNA, metronidazole protects rather than sensitizes. Since metronidazole anions are relatively stable, they failed to induce DNA damage (Boon *et al.* 1985). However, in contrary with its effects on naked DNA, at cellular level metronidazole was shown to be an

efficient radiosensitiser (Foster and Willson 1973, Dische and Saunders 1978). The results obtained here are in agreement with these reports. The radiosensitising action appears to parallel the electron affinities of metronidazole as a hydroxyl-radical scavenger, the radical anions have been implicated as possibly significant intermediates (Adams *et al.* 1976). Furthermore, the yield of  $G^+$  centre, which is the precursor of strand breaks, is strongly enhanced by the presence of metronidazole with high electron affinity (Gräslund *et al.* 1977), the electrons are more deeply trapped. This may be of potential significance in terms of the radiosensitising action of metronidazole. All these results can be taken as supporting the most widely accepted theory for sensitising action (Adams and Cooke 1969).

As discussed in Chapter 1 (section 1.4), the cell death on exposure to ionizing radiation arises primarily as a result of DNA damage, especially DSB as initial damage. Therefore, here it is not hard to understand the respective effects of spermine and metronidazole on cell death in view of their actions in the formation of DSB induced by  $\gamma$ -rays.

The data illustrated in Figure 3.2.5 and Figure 3.2.6 show that there is a remarkable difference for the effects of spermine-linked metronidazole (AM1229) on cellular DNA DSB and cell killing induced by gamma-rays. It is unknown why the cell irradiated is protected from the formation of DSB in the presence of AM1229 which acts in terms of the characteristic of spermine, and sensitised for the cell killing in the presence of AM1229 which acts in terms of the characteristic of metronidazole. But the influencing efficiencies of AM1229 is lower than that of spermine or metronidazole alone. It is unclear whether this difference is from irradiation under different oxygen levels, because the cells were irradiated under the condition of free-oxygen for the analysis of DSB and under oxygenated condition for the analysis of cell survival.



### Summary

Major conclusions drawn from this study are (1) Polyamines, spermidine and spermine, are shown to protect plasmid DNA against radiation damage. Both the formation of SSB and DSB at liquid nitrogen temperature (77K) are reduced, and the protection efficiency increases with the concentration and with the association constant ( $k$ ) for binding of the polyamine to DNA; (2) Metronidazole (MET) increased the induction of cellular DSB and cell killing by gamma-rays at 0°C as a radiosensitiser, spermine reduced both as a radioprotector. (3) Spermine-linked metronidazole (AM1229) presents different influences, on the induction of DSB as radioprotector under the irradiation condition of free oxygen, and on the cell killing as radiosensitiser under the irradiation of atmospheric oxygen.

### 3.3

## DNA DSB of Cultured Cells induced by Gamma-rays at Low Temperatures

### Introduction

It is commonly supposed that damage to DNA is one of the most important modes of interaction between ionizing radiation and cellular systems. Furthermore, DSBs are often implicated as major lesions involved in some biological effects, such as chromosomal aberration, cell death and mutagenesis. In the conventional concept, the DNA radiation damage is due partly to a "direct effect" mechanism, leading initially to ionized centres in the DNA, and partly to an "indirect effect" in which water radiolysis radicals, especially hydroxyl radicals ( $\text{OH}^\bullet$ ), attack the DNA in various ways. This will depend on the various conditions in the studying system, which may favour one mode over the other. In pure dilute aqueous DNA, the indirect mechanism clearly dominates, whereas for frozen aqueous DNA, the direct mechanism is thought to dominate. This is strongly supported by ESR studies which establish that primary radical-cations and radical-anions are formed in the DNA bases, and that hydroxyl radicals are harmlessly trapped in the pure ice phase (Hüttermann 1982, Sévilla 1977, Symons 1987). Water molecules associated with DNA and alkali cations form  $\text{H}_2\text{O}^{+\bullet}$  initially, and these may undergo electron-transfer so rapidly

that this competes with proton loss to give hydroxyl radicals under these conditions (Boon *et al.* 1984, 1985, Cullis *et al.* 1986).

There is no clear consensus regarding the relative contribution of these modes to radiation-induced DNA damage in the cellular nucleus. A number of workers have drawn attention to the importance of the direct mechanism. The reason for this is that nuclear DNA is not only associated with histon proteins, but also packed in such a way that local water concentration is relatively low (Neary *et al.* 1972, Boon *et al.* 1984, Christensen *et al.* 1972, Baverstock 1985, Symons 1987, Baverstock and Will 1989). DNA is only 4% of the total mass of the cell nucleus, hydroxyl radicals formed from distant water molecules will largely be scavenged before they reach nuclear DNA, since hydroxyl radicals are extremely reactive and will generally react with any organic material on contact, only radicals generated in the primary acts close to the DNA have a chance of reaching the target molecule.

Our present knowledge of relative contributions of direct and indirect effect on components to cellular DNA lesion is rather limited by current assays available. Although studies concerning the above have been carried out in the presence of free radical scavengers (Roots and Okada 1972, Chapman *et al.* 1973, Baverstock, 1985, Cullis *et al.* 1985, 1986, Baverstock, 1989), only the results of the "indirect effect" have been obtained. Present experiments were designed to make it possible to directly compare these two situations for cellular DNA damage by irradiation at ice-bath temperature and at liquid nitrogen temperature (77K), following rapid freezing, in order to suppress the indirect damage mechanism.

### Materials and methods

The method of cultivation of Chinese hamster lung fibroblasts (V-79) and the composition of culture medium have been described already in detail in Chapter 2 (section 2.4). After trypsinizing and counting,

exponentially growing cells were resuspended in fresh medium to give a concentration of  $2 \times 10^6$  cells/ml for experiments.

#### *Sample preparation at low temperature and irradiation*

1 ml aliquots of cell suspension for each cell sample was directly and carefully dropped into liquid nitrogen using 1 ml sterile pipettes to produce around 30 small ice pellet balls, which were transferred into a sterile little thin-wall glass screw topped bottle. All bottles were kept in liquid nitrogen for irradiation. The irradiation was carried out using a 'Vikrad'  $^{60}\text{Co}$  source at a dose rate of approximately 34 Gy/min. After irradiation the cell samples were placed on ice to allow melting into aliquots or were thawed in a water bath at  $37^\circ\text{C}$ . When only a small ice fragment still remained in the thawing cell suspension, the samples were put into an ice-bath, this procedure took less than 1 min at  $37^\circ\text{C}$ . The two methods of sample thawing gave the same DSB results, the data comparing them are not shown here. Identical samples were irradiated at ice-bath temperature for the same doses.

#### *DNA DSB analysis*

The cell samples were diluted with 5 ml ice-chilled PBS and analysed for DNA DSB as rapidly as possible after irradiations was completed. The neutral DNA filter elution method with some modifications was used for measurement of DSB, while the relative amount of DNA was determined using fluorometric assays (Hoechst 33258), as described in Chapter 2 (section 2.4). The percentage of DNA remaining on the filter was plotted against eluting time. The relative number of DNA DSB inductions was calculated using relative DNA elution. Internal standards were not used.

Deoxygenated samples were prepared with oxygen-free nitrogen for more than 20 min prior to freezing. The oxygen-free nitrogen was prepared using the method described in section 3.2. Some samples were cooled slowly, with dimethyl sulfoxide (DMSO), which was achieved by

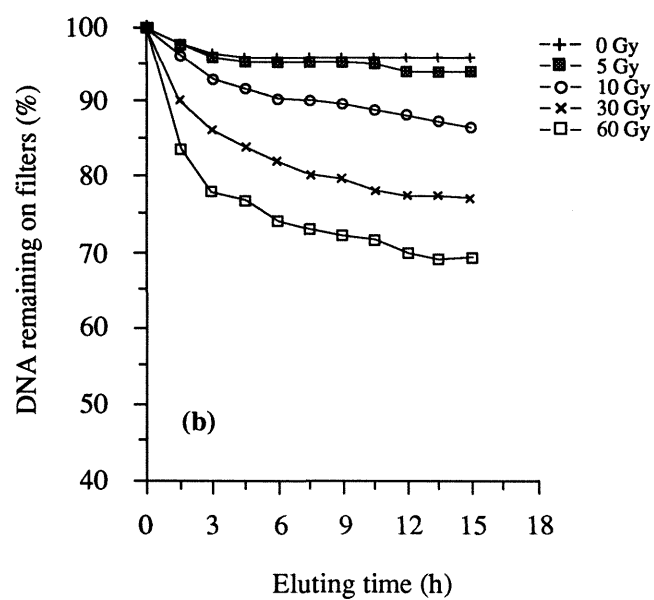
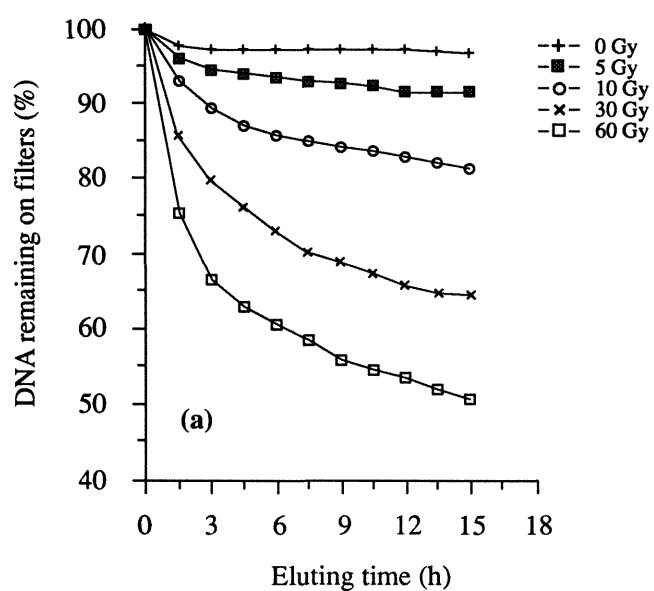
means of a normal cell storage method using a  $-20^{\circ}\text{C}$  thermostat for 2 h prior to cooling to liquid nitrogen temperature.

### Results and discussion

As in previous studies (Bradley and Kohn 1979), the dose-response curve for the reduction in DNA remaining on the filter is considered to represent induction of DSB. A series of elution profiles of DNA were obtained in Chinese human V-79 cells exposed to gamma-rays at  $0^{\circ}\text{C}$  and at liquid nitrogen temperature. These results are shown in Figure 3.3.1a and Figure 3.3.1b, respectively. The curves of relative DNA elution from neutral elution profiles versus doses are shown in Figure 3.3.2a.

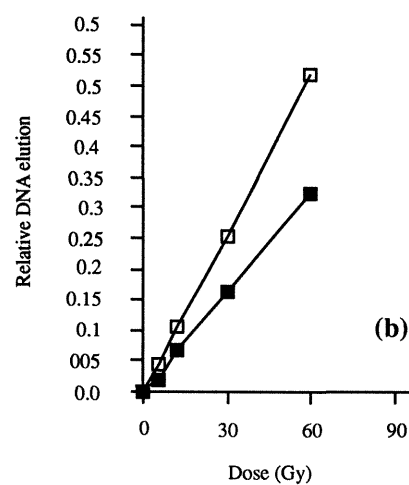
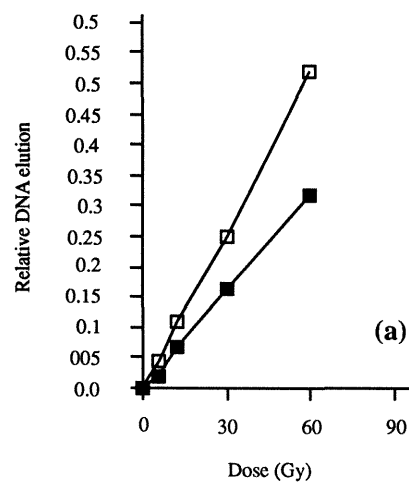
The freezing process itself did not result in observable cellular DNA damage. However, a difference of DSB induction was found between cells exposed at  $0^{\circ}\text{C}$  and cells exposed at liquid nitrogen temperature, representing around 35% less induction of DSB for frozen than for non-frozen cells. Moreover, for frozen cells exposed to the same dose of gamma-rays, an equivalent reduction of DNA DSB was found in normal air environment and in deoxygenated environment (Figure 3.3.3a).

The simplest explanation for the very small decrease in yields of DSB on going from fluid to frozen solution is that in both cases a major mechanism for DSBs is via 'direct' damage, that is via initial redox reactions rather than radical attack (Baverstock 1985, Cullis *et al.* 1986, Baverstock 1989). But, the results contrast remarkably with those obtained from freezing aqueous plasmid DNA in the absence or presence of hydroxyl radical scavengers, as discussed in section 3.1. In the case of pure DNA there was a very large reduction in the numbers of SSB and DSB on freezing. When the cells were frozen in liquid nitrogen and irradiated, the radiolysis products of water in the nucleus, which are normally responsible for the indirect effect of radiation, are largely trapped in the ice crystallite and cannot interact with DNA. Therefore, under this condition, The main



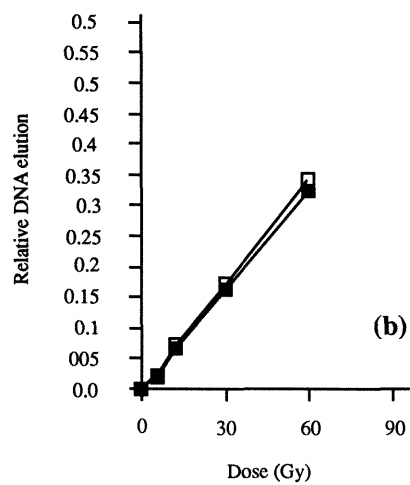
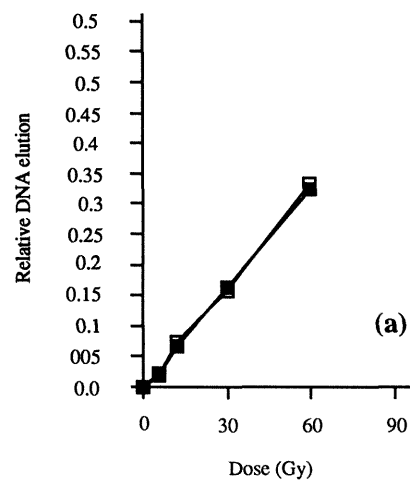
**FIGURE 3.3.1**

Induction of DSB in DNA of non-radiolabeled cells by gamma-rays at ice bath temperature (a) and at liquid nitrogen temperature (b). The percentage of DNA remaining on the filters is plotted as a function of the eluting fraction.



**FIGURE 3.3.2**

Dose-response curves for gamma-irradiated cells using assay of neutral filter elution.  
 (a) Freezing samples irradiated at 77K ( ■ ) and non-freezing samples irradiated at ice bath temperature ( □ );  
 (b) Non-melting freezing samples irradiated at 77K ( ■ ) and remelting freezing samples irradiated at ice bath temperature ( □ ).



**FIGURE 3.33**

Dose-response curves for gamma-irradiated cells using assay of neutral filter elution.

- (a) Samples irradiated at 77K in air ( □ ) and samples irradiated at 77K in oxygen-free environment ( ■ );
- (b) Freezing samples without DMSO ( □ ) and slow-freezing samples with DMSO ( ■ ).



DNA damage observed may only come from the effect of direct ionization. The indirect effect should be negligible, as the described by Mearyman (1966).

If considering the mechanism of both direct and indirect effect, the results suggest that the 35% unobtained DSB yields are likely to be compromised as a results of indirect effects in frozen cells with comparison of that in non-frozen cells, in other words, approximately 35% DSBs are formed from indirect effects, and 65% from direct effects. It is interesting to note that when the fast-freezing cell samples were melted into aliquots, and then irradiated at 0°C, the frequencies of DSBs were similar to those obtained from normal cell samples which were also irradiated at 0°C (Figure 3.3.2b). This can be interpreted as confirming the assumption that the decrease observed in fast-freezing cell system would be the result of indirect effects.

As shown in Figure 3.3.3b, The yield of DNA DSB were compared in fast-freezing cell samples without DMSO and slow-freezing cell samples in the presence of DMSO. The slow-freezing cell sample was obtained using routine cell store method;  $2 \times 10^6$ -cells/ml containing 100 $\mu$ l DMSO in 1 ml medium was placed in a -20°C freezer for 2 hours, and then immediately and rapidly transferred to liquid nitrogen. DMSO is a cryoprotective agent and radical scavenger. Again, there were no significant differences to be observed. This imply that the movement of free radicals was minimized in frozen cells in liquid nitrogen, therefore DMSO had no further influence on DNA damage produced by radiation in this case. Baverstock (1981) proposed that the dominant action of the scavenger molecules in the cell nucleus is to modify the response of the DNA to direct energy absorption, rather than modify the hydroxyl radical yield reaching the DNA, but no such suggestion could be made from our results. Burki *et al.* (1975) reported that there was also an efficiency of DNA SSB reduction by radiation with a dose-modifying factor of ca. 4 in cells which were slowly

frozen with DMSO than non-frozen cell at 37°C. By comparison with the results obtained here (a dose modifying factor of ca. 1.5), the reduction of SSB is more than that of DSB under similar experimental conditions. This might be because most of the SSB is as a result of the indirect action of the OH radical in DNA of cells irradiated (Roots and Okada 1972, Skov 1984). This comparison is further evidence that varying conditions alters the proportion of direct and indirect effect to the induction of cellular SSB and DSB.

The possible importance of direct damage has previously been stressed (Bavestock 1985, Cullis *et al.* 1986, Bavestock 1989). It was shown that one possible reason for the high yields of DSBs relative to SSBs under conditions of direct damage may be related to the mechanism for this type of damage. ESR results suggest that effective electron-transfer between bases is a dominating model of damage. At liquid nitrogen temperature, it was suggested that direct damage of ionizing radiation gives radical cations and anions in all bases, both electron-loss and electron-gain centres can give rise to strand breaks (Barnes *et al.* 1991, Cullis *et al.* 1992, Hüttermann *et al.* 1992). Given that the separation between these centres is often quite small (a few base-pairs only), the DSBs will result at relatively high frequencies. This phenomenon, if correct, may also contribute to the present results. In addition, Bavestock and Cundall (1988a, b) have recently established a mechanism for long-range energy transfer to explain direct deposition of radiation energy on DNA. This implies that DNA in which ionizing energy has been 'directly' deposited does not necessarily behave as a passive entity, but that it mediates between energy absorption by processes of long-range excitation transfer.

Finally, it is worth noting that the advantage of the cell system which is directly frozen in liquid nitrogen is that the direct damage may be effectively suppressed from the total radiation mechanism of DNA damage in cells, and the DNA strand breaks of cells will be not influenced by

enzyme repair systems of cells. Since there was low survival in frozen cells without DMSO, plating efficiency is only approximately 0.35% (the data are not shown here). In addition, we found that there was no difference in the observed results of DNA DSB from cell samples prepared by the protocols described in methods and materials or by directly submerging in irradiation bottles containing cell suspension in liquid nitrogen. We therefore propose that cell systems in which the cells are rapidly frozen will possibly provide a useful pathway for studies of kinetics of direct effects on radiation-induced DNA damage.

### Summary

It has been shown that there was a reduction of radiation-induced DSB in cells which were frozen at nitrogen temperature compared with that observed in cells irradiated at ice-bath temperature, and this change was not influenced by the presence of free radical scavengers (DMSO). These results suggested that 65% DSBs were produced due to the mechanism of radiation direct effect. This result is in marked contrast with the large decrease in yield of DSBs on dilute aqueous solution of DNA from room temperature to 77K which was discussed in section 3.1.

## 3.4

# Effects of Hypotonic and Hypertonic Buffers on DNA Double Strand breaks

## Introduction

The radiation-induced DNA lesions can be initiated three ways, (a) by direct hit only (radiation energy directly deposits in the DNA), (b) by indirect hits only (free radicals by water attack the DNA), and (c) by compound hits (von Sonntag 1987, Ward 1988) depending on the state of DNA being studied and irradiation environment. It is well known that direct effect of radiation is responsible for the DNA damage in solid state, and indirect effect for the DNA damage in dilute aqueous solutions (Hutchinson 1985, Cullis and Symons 1986, Ward 1988, Schulte-Frohlinde 1990). However, the relative contribution of both actions on cellular DNA damages is the subject of much debate, and is obviously different from that on pure DNA in solid state and in dilute aqueous solution because the spatial distribution of species produced within the spurs, blobs, and short tract must be considered in conjunction with the structure in which the DNA is packaged.

As described in Chapter 1, DNA is packaged within the cellular nucleus in a hierarchy of structures (nucleosomes), and surrounding components (such as protein and RNA) are tightly packed and highly organised with DNA. The DNA in chromatin can be considered to be a

condition more closely resembling the solid state. DNA bound protein is thought to protect the DNA acting as hydroxyl radical scavengers in order to diminish the yield of OH radical reaching DNA. Therefore, it is believed that the mechanism of radiation-induced DNA lesions in the cell and nucleus is dominated by direct effect of radiation.

However, it is suggested that in nucleosome crystals there is no protein extending round the outside of the DNA so that this region, at least, would be accessible to water and hence to OH radicals (Richmond *et al.* 1984), moreover, there were experiments to show a significant decrease of cellular DNA damage induced by radiation in the presence of free radical scavengers (Roots and Okada 1972, Chapman *et al.* 1973), this protecting effectiveness significantly depends on its concentration near DNA (Zheng *et al.* 1988).

Since the diffusion distance of the OH radical in the mammalian cellular nucleus is several nm, the hydroxyl radicals which could attack the DNA molecule comes from a water layer around DNA, 2 - 3 nm in radius (Roots and Okada 1975, Hutchison 1985). Therefore, the damage due to the indirect effect is supposed to be caused by OH radicals produced in the water sheath around the DNA molecule containing bound water (Ward 1988, Michalik 1992). The quantity of water in the nucleus and its distribution must have a great influence on the amount of free radicals which may reach the DNA.

Variation of ionic strength and water content within the cell through the change of salt concentration in the cell culture medium resulted in hypotonic and hypertonic culture conditions (Rosenburg *et al.* 1972). These in turn should alter the amount or the structure of water associated with the coordinating shells of macromolecules (Raaphorst *et al.* 1975, Raaphorst and Kruuv 1977). It was found that hypotonic solutions of NaCl (less than 0.15 M) increased the cell radiosensitivity and hypertonic solutions of NaCl (0.5 to 1.5 M) decreased the cell radiosensitivity (Raaphorst and Kruuv

1976, 1977, Raaphorst and Dewey 1979). Focussing on DNA damage, cell irradiation in hypotonic solution led to a greater yield of DNA SSB than in normal solution and altered repair efficiency (Ward *et al.* 1983). These findings can be explained on the basis that the variation of ionic strength in the external medium has a significant effect in altering the total water structure and amount inside the cells. This structure change of water may further influence the number of radicals and the rate of free radicals accessible to the 'target site' and thus, the amount of damage (Raaphorst and Kruuv 1976, 1977, Ward *et al.* 1983). It has also been suggested that the quantity of cellular water and its distribution must be of great importance for indirect radiation effects, and this has been demonstrated experimentally using bacterial spores (Power and Tallentire 1968, Iwasaki *et al.* 1974).

In the present study, The yield of cellular DNA DSB induced by gamma irradiation at 0°C and at liquid nitrogen temperature (77K) was examined in the presence of hypotonic and hypertonic salt solutions. It was hoped to find out more about the contribution of direct effects and indirect effects from primary radicals of water radiolysis to radiation-induced cellular DNA damage by altering the quantity of intracellular water.

### Materials and methods

The method of cultivation of Chinese hamster V-79 cells, the composition of the culture medium and [<sup>3</sup>H]thymidine labeling have been described already in detail in Chapter 2 (section 2.4). After trypsinizing, the exponentially growing cells were resuspended in fresh medium.

### *Anisosotonic treatment and irradiation*

After centrifuging and removing the medium, the cells were washed once and resuspended in either 0.05 M hypotonic solution or 1.5 M hypertonic solution pre-warmed at 37°C, the cell number was adjusted to  $2 \times 10^6$  cell/ml. The cells in anisotonic solutions were incubated for 20 min in a 37°C water bath. The salt solutions were made by dissolving reagent

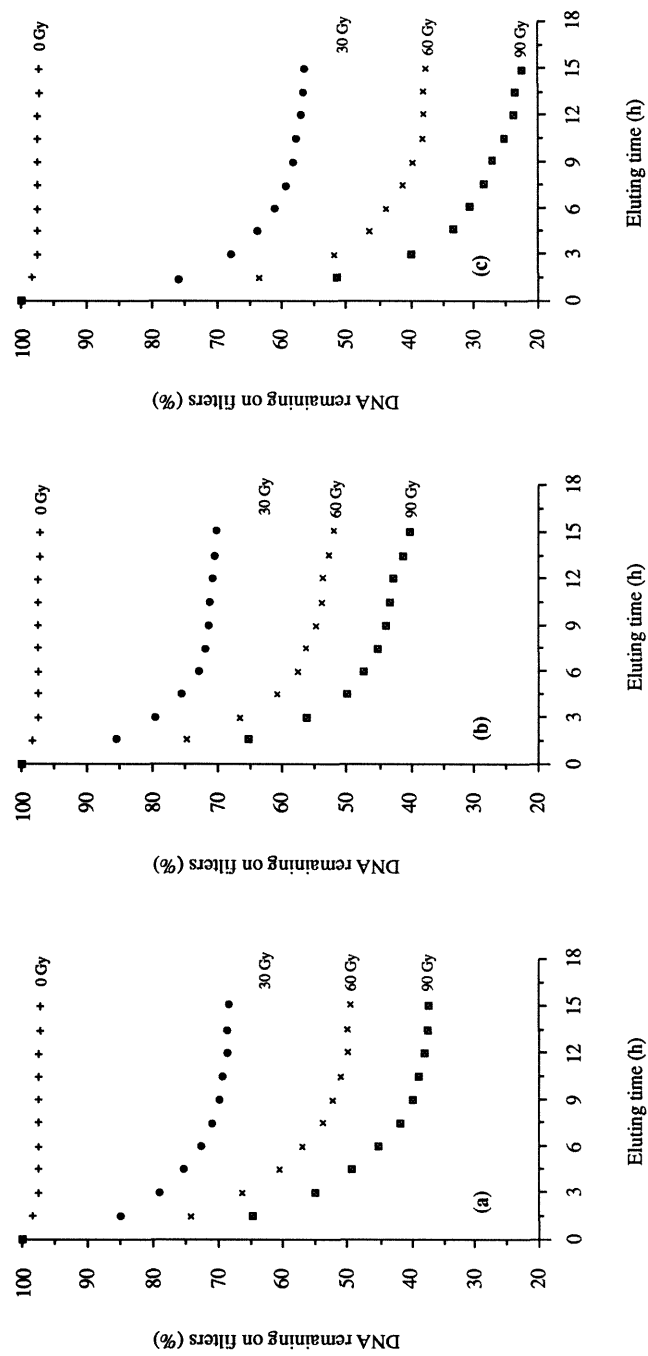
grade NaCl (Sigma) in water that had been passed through a Fisons cartridge deioniser followed by a millipore "Milli Q" cartridge system with a Millipore 0.2  $\mu\text{m}$  filter and autoclaved. The pH of NaCl solution was 5.5 independent of the concentration. After incubation, 1 ml portion of cell suspensions was gently dispensed into a 2 ml plastic irradiation tube and kept on ice for irradiation. Irradiations were carried out at ice bath temperature or at liquid nitrogen temperature with a 'Vickrad'  $^{60}\text{Co}$ -gamma source at a dose rate of 36 Gy/min in air. For samples irradiated at 77K, the tubes containing cells were directly submerged in liquid nitrogen to freeze the cells and were kept in liquid nitrogen for irradiation.

#### *DNA damage assays*

The cell samples irradiated were diluted with 5 ml ice-chilled PBS and analysed for DNA DSB immediately irradiation was completed. For the samples irradiated at liquid nitrogen temperature, the irradiation tubes were submerged into a 37°C water bath to thaw the samples and 5 ml ice-chilled PBS was added. DNA DSB was assayed by neutral filter elution as described by Bradley and Kohn (1979) with modifications in types and concentrations of the detergents used and radioactive DNA assay, which has been described in detail in Chapter 2 (section 2.4). It was found that there was no difference in eluting rate due to cells in hypotonic and hypertonic solution. The percentage of DNA remaining on the filter was plotted against elution volume. The control experiments without hypotonic and hypertonic salts were carried out in isotonic medium in the same way. Internal standards were not used.

#### **Results and discussion**

In our initial experiments the frequencies of cellular DNA DSB induced by gamma-rays at ice-bath temperature were examined in the presence of 0.05 and 1.5 M NaCl solution. Typical DNA elution curves are shown in Figure 3.4.1 and a relative DNA elution from neutral elution



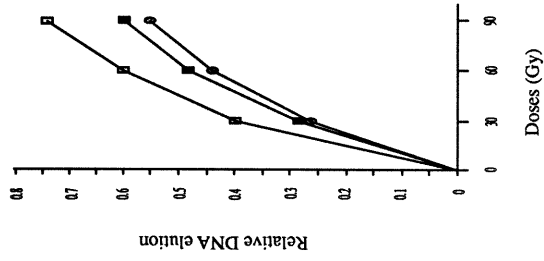
**FIGURE 3.4.1**

Induction of DSB in DNA of V-79 cells by gamma-rays at ice bath temperature in isotonic medium (a), in 1.5 M NaCl solution (b) and in 0.05 M NaCl solution (c). The percentage of DNA remaining on the filters is plotted as a function of the eluted fractions.

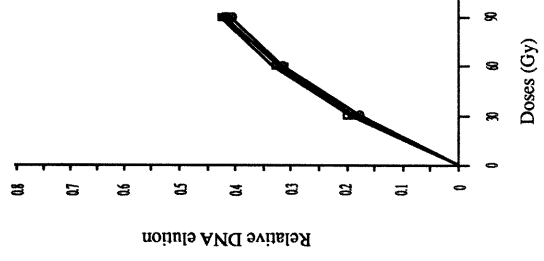


profiles versus dose is shown in Figure 3.4.2. As described previously (van Ankeren and Wheeler 1985), a 20 minute exposure to 0.05 or 1.5 M NaCl solution followed by incubation at 37°C has no lesion effect on the DNA of cells without irradiation (control samples). This is approximately equivalent to zero-dose elution base line. However, the frequency of DNA DSB induced by gamma-ray markedly increased in the presence of 0.05 M NaCl during irradiation, about 20% increase in DSB, whereas the frequency declined in the presence of 1.5 M NaCl, approximately 8% reduction in DSB when compared with the frequency of DSB induction by radiation in normal isotonic medium. There is a non-linear relationship between relative DNA elution and dose in the selected dose range, as shown in Figure 3.4.2, which is consistent with the results reported by other authors (Prise *et al.* 1987, Flick *et al.* 1989, Okayasu and Iliakis 1989, Warters and Lyons 1990). The results suggest that radiation damage by indirect processes are sensitive to the artificial change of cellular water concentration.

It have shown that the extent of damage and distribution of damaged sites were profoundly influenced by the constituents of chromatin in the nucleus which could modify DNA damage induced by ionizing radiation (Chiu *et al.* 1982, Warters and Childers 1982, Oleinick *et al.* 1984, Heussen *et al.* 1987, Warters *et al.* 1987, Ljungman 1991). In addition, it is also known that total cellular chromatin can divide operationally into actively transcribed regions (active chromatin) and non-transcribed regions (inactive chromatin), the former being more susceptible to certain damage (Patil *et al.* 1985). Therefore, as a possible reason, hypotonic salt treatment might cause dispersing of nuclear chromatin so that more DNA structure in chromatin, especially active chromatin, would be accessible to water and hence to OH radicals. In contrast, hypertonic salt treatment might cause condensing of chromatin to reduce the opportunity of free radical



**FIGURE 3.4.2** Dose-response curves for Gamma-irradiated cells in isotonic medium (■), in 1.5 M NaCl solution (●) and in 0.05 M NaCl solution (□) at ice bath temperature using assay of neutral filter elution.



**FIGURE 3.4.3** Dose-response curves for Gamma-irradiated cells in normal medium (■), in 1.5 M NaCl solution (●) and in 0.05 M NaCl solution (□) at liquid nitrogen temperature using assay of neutral filter elution.

interaction with DNA structure in chromatin (Robbins *et al.* 1970, Brasch *et al.* 1971, Detter *et al.* 1972);

When a DSB occur in naked aqueous DNA, the two SSBs may be formed either at directly opposite sites, or separated from each other or by no more than a certain number of nucleotide pairs in opposite strands. The critical distance, ' $\alpha$ ', beyond which two SSBs on opposite strands would no longer lead to a DSB, strongly depends on the ionic strength of the medium. At low ionic strength, the double-helical structure opens more readily than at high ionic strength. Freifelder and Trumbo (1969) and van der Schans (1978) reported in solutions containing 0.01 M NaCl, an ' $\alpha$ ' value of 34 which decreased to 16 in the presence of 0.01 M NaCl, and 3 in the presence of 1.0 M salt. The number of DSBs produced by a given dose was much lower at high ionic strength than at low ionic strength. Consequently, this would be a more satisfactory explanation of the present results if the situation still applies for nuclear DNA, hypotonic salt treatment increases the possibility of SSB formation, further the yield of DSB.

From the above considerations, it is evident that the mechanism involving the effectiveness of anisotonic salt solutions on radiation-induced cellular DNA damage is likely to be complex. In addition, it has been shown that the post-irradiation anisotonic treatment has no effect on the induction of DNA SSB and DSB, only delayed the onset of repair of DNA damage (van Ankeren and Wheeler 1985, Hinchliffe and McNally 1986, Kosaka *et al.* 1990, McNally *et al.* 1990).

A second experiment was carried out to compare the efficiency of DSB induction in the presence of hypotonic and hypertonic NaCl solution by applying the technique of cell irradiation at liquid nitrogen temperature (77K). It is believed that most migration of free radicals produced in both DNA-bound water molecules and free solvent water molecules surrounding DNA are restrained under this temperature (Boon *et al.* 1984, 1985). Therefore, the consequence of indirect effect of radiation on DNA damage

at 77K can be largely ignored, and the damage source will only be considered to be from direct effect of radiation. Under these conditions, it was of interest to find that not only the excess yield of DNA DSB due to the presence of hypotonic salt solution at 0°C was removed, but also the amount of radiation-induced DNA DSB in normal isotonic cell environment was reduced (Figure 3.4.3).

It is apparent that this decrease in temperature from 0°C to 77K brought about a reduction in the efficiency of DNA DSB produced by gamma-rays in treated cells with both hypotonic and hypertonic salts. If the mechanism of cellular DNA DSB induced by radiation are believed to be from the direct effect in the case of cells exposed to radiation at liquid nitrogen temperature, it can be surmised that the decreased yield of DSB at liquid nitrogen temperature arises from the indirect effect. According to the data presented here, it predicts that the indirect effect contributes approximately 35% of DSB induction, while from the direct effect contributes ca. 65%. This is consistent with those obtained using radiolabel-free cells (section 3.3) This result is also in approximate agreement with those reported by some other authors (Roots and Okada 1975, Michaelles and Hunt 1978, Baverstock and Will 1987). The explanation for DNA damage induced by gamma-rays at liquid nitrogen temperature is discussed in section 3.3.

### Summary

Induction of cellular DNA DSB induced by gamma-rays was examined at 0°C and at liquid nitrogen temperature (77K) in the presence of hypotonic (0.05M) and hypertonic (1.5M) NaCl salt solutions. At 0°C, the cells showed a marked increase (ca. 20%) of DSB induction by gamma-rays in the presence of 0.05 M NaCl, and a slight decrease (ca. 8%) of DSB frequency in the presence of 1.5 M NaCl. However, these influences on DNA damage yield, due to the presence of both hypotonic and hypertonic

solutions, disappeared when the cells were irradiated at liquid nitrogen temperature.

These results suggest on the one hand that the amount of water within the cell may modify the DNA damage by means of influencing yields of free water accessible to the DNA. On the other hand, both direct effects and indirect effects of ionizing radiation are of complementary importance in induction of DNA strand breaks when considering the overall cellular DNA damage, but have different contributions. Approximately 65% DNA damage in cells irradiated can be expected by direct electron hits from radiation energy deposition, therefore, the observation is consistent with the idea that the direct effect is mainly responsible for DNA damage induced by radiation, at least DSB induction. This also implies that the anisotonic treatment on radiation-induced cell killing, which was reported elsewhere, may not be due to changes in the amount of DNA damage.

## 3.5

### **Pretreatment effect of low doses on Repair Kinetics of DNA Double Strand Breaks**

#### **Introduction**

A phenomenon described as 'adaptive response' has been paid special attention in the radiation response of cultured mammalian cells. The procedure comprises the use of very low prior to exposure to high dose. This procedure was developed after Olivieri and co-workers (1984) first reported that the frequencies of chromosomal aberrations and cell killing induced by high doses (challenging dose) were less than the expected sum in mammalian cells pre-exposed to low doses (adapting dose) of ionizing radiation (Sanderson and Morley 1986, Shadley *et al.* 1986, Ikushima 1987, Bosi and Olivieri 1989, Wolff *et al.* 1988, Wolff *et al.* 1989, Fan *et al.* 1990, Wang *et al.* 1991, Wojcik *et al.* 1992). This response to ionizing radiation has also been demonstrated in laboratory animals (Liu *et al.* 1987, Cai and Liu 1990, Wojcik and Tuschl 1990, Liu *et al.* 1992)

Although the mechanism involved in this response is still not clear, it was suggested that the induction of repair capacity might be a good working hypothesis for the explanation of adaptive response. This is supported by inhibition experiments of 3-aminobenzamide (Olivieri *et al.* 1984, Wiencke *et al.* 1986, Shadley and Wolff 1987, Wolff *et al.* 1988). Also, the

observation of increased unscheduled DNA synthesis points in this direction (Wojcik and Tuschl 1990).

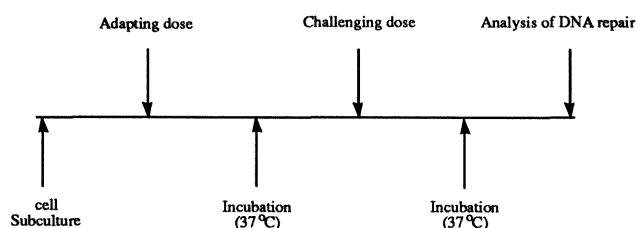
The close correlation of chromosomal aberrations and DNA damage/repair has been identified (reviewed by Chadwick and Leenhouts 1981), especially for DNA DSB (Natarajan and Obe 1978, Bryant 1984, Bryant *et al.* 1987). Wolff *et al.* (1988) found that the human lymphocytes exposed to low doses of ionizing radiation become refractory to chemical mutagens which induced DSB in DNA, but a challenge with an alkylating mutagen which produces SSB failed to show this effect. An elevated rate of DNA SSB repair in cells from different organs of mice adapted to high doses of  $\gamma$ -rays applied chronically at an extremely low dose-rate, has recently been reported by Gaziev *et al.* (1991). Consequently, the question arose as to whether the adaptive response to ionizing radiation might also hold true for certain responses in cellular DNA DSBs. The aim of present experiments was to determine whether or not Chinese hamster V-79 cells could be adapted by low dose pre-treatment for rejoining of DSB induced by subsequent high dose of gamma-rays.

### Materials and methods

It is considered that low concentrations of radioisotopes (such as [ $^3\text{H}$ ]thymidine), which are usually used in cell labeling, can induce such an adaptive response as adapting dose (Olivier *et al.* 1984, Sanderson and Morley 1986, Wolff *et al.* 1989). Thus, all experiments were carried out with exponentially growing Chinese hamster lung fibroblasts (V-79) without radiolabel. The method of cultivation of the cells and the composition of culture medium have been described in detail in Chapter 2 (section 2.4). Two days later after subculture, the cells were trypsinized, counted, and then resuspended in fresh medium to give a final concentration of  $2 \times 10^6$  cells/ml for experiments. The cell number was kept constant for all experiments. 1 ml aliquots of cell suspension was transferred into a 1.5

ml plastic irradiation tube and maintained in a ice bath for irradiation. The irradiation was carried out using a 'Vikrad'  $^{60}\text{Co}$  source at a dose rate of 35 Gy/min.

For experimental protocol of adaptive response, typically, after exposure to adaptive dose at ice-bath temperature, the tubes containing cell samples were immediately placed into a  $37^{\circ}\text{C}$  water bath to allow repair to occur (damage repair of adapting dose), then returned to an ice bath for irradiation of subsequent challenging doses. After a second dose of gamma-rays, the cells were again returned to  $37^{\circ}\text{C}$  for second repair (damage repair of challenging dose). Finally, the cells were maintained in the ice bath and 5 ml ice-chilled PBS was added to assay DNA repair (as shown in **Figure 3.5.1**).



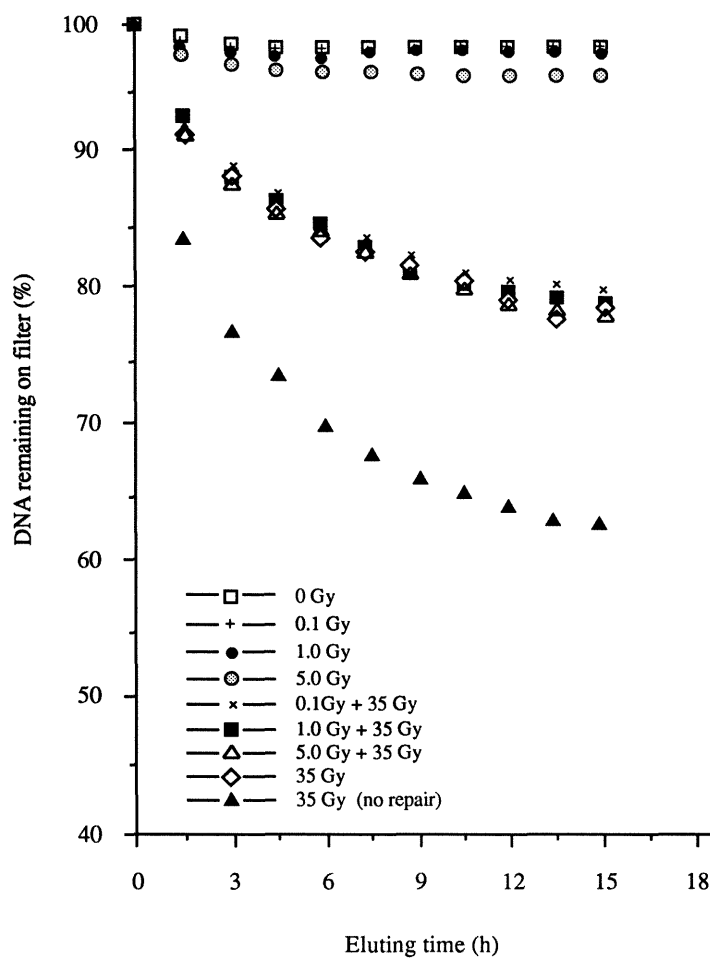
**Figure 3.5.1** Flow diagram of the procedure of adaptive response.

For measurement of DSB repair, the DNA neutral filter elution method and DNA fluorometric assays (Hoechst 33258) described in Chapter 2 (section 2.4).

## Results and discussion

The first set of experiments were performed to see if an adaptive response could be induced by pretreatment with an adapting doses of 0.1, 1.0 or 5.0 Gy, followed by a challenging dose of 35 Gy. The data for the repair kinetics of DSB with and without low dose pre-exposure are compared in **Figure 3.5.2**. To induce adaptive response, 10 min of repair





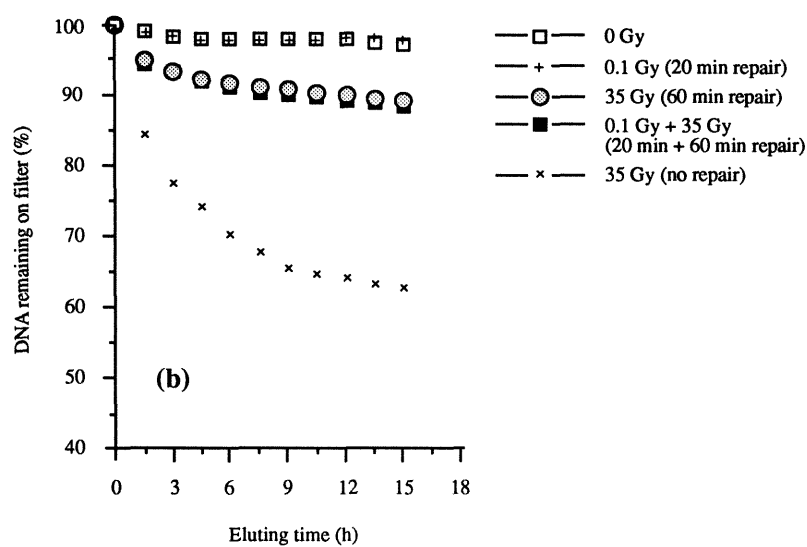
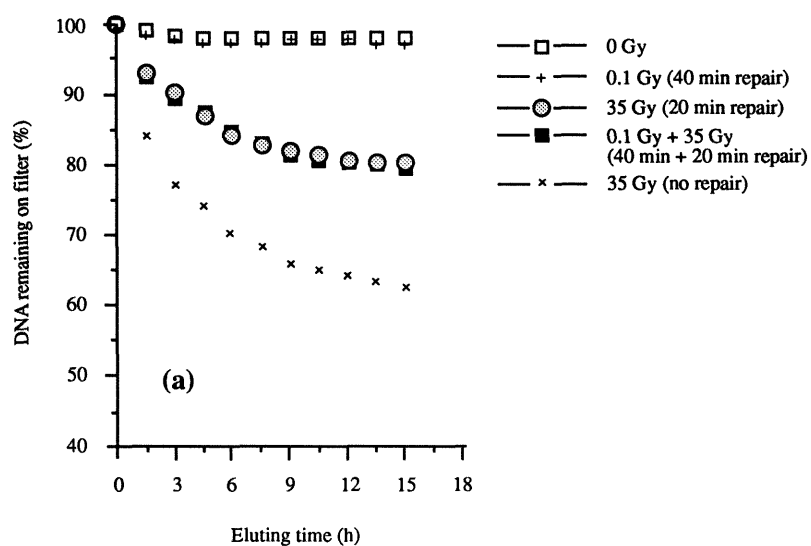
**FIGURE 3.5.2**

Neutral filter elution assay of DNA DSB rejoining after 35 Gy of gamma-rays in exponentially growing Chinese hamster V-79 cells pre-exposed by 0.1, 1.0 and 5.0 Gy. Typical procedure of adaptive response was that the cells were pre-exposed to low doses (adapting doses) and repaired in a 37°C water bath for 10 min, then were irradiated to a high dose (challenging dose) and had further incubation for 20 min. Cell repair was stopped by ice chilling.

time was employed after adapting doses, and 20 min, after challenging dose. These conditions were chosen as discussed in Chapter 1 (section 1.4). A rapid removal of 70 -90% of the DSB within the first 15 - 30 min. Plotted in the figure is DNA remaining on filters as a function of eluting time interval. As indicated in the figure, similar eluting profiles for the rejoining of DSB after the challenging dose, with and without adaptive dose, are obtained. Unlike the typical adaptive response in chromosomal aberration reported previously, pre-exposure to low adapting dose, which induces little or no DSB by itself, did not result in any further reduction in the frequencies of DNA DSB induced by subsequent high challenging dose, within different adapting doses covered here.

The second set of experiments were conducted to see whether this failure of induction of DSB to adaptive response in the first experiments were attributed to insufficient repair time. The effect of various repair periods of post-irradiation incubation at 37°C on the rejoining rate of DSB following adapting dose or challenging dose were studied. When the repair time following adaptive doses was extended to 20, 30 and 40 min or the repair time for challenging doses was extended to 30, 40 and 60 min, again, the cell did not show significant change in eluting profiles. For clearer results, the eluting curves are presented for only two sets of data, 40 min repair after adaptive doses and 20 min repair after challenging dose (Figure 3.5.3, the upper panel), 40 min repair after adaptive dose and 40 min repair after challenging dose (Figure 3.5.3, the low panel).

It has been shown that the time interval between adapting dose and challenging dose is an important factor in the induction of adaptive response and in intervals of less than 4 hours, no adaptation in chromosomal aberration to ionizing radiation was shown (Shadley *et al.* 1987, Ikushima 1989). Presumably, the repair enzymes necessary for the adaptation are expected to be synthesized within this time. Therefore, the next set of experiments were carried out using the 4 h interval between adapting dose



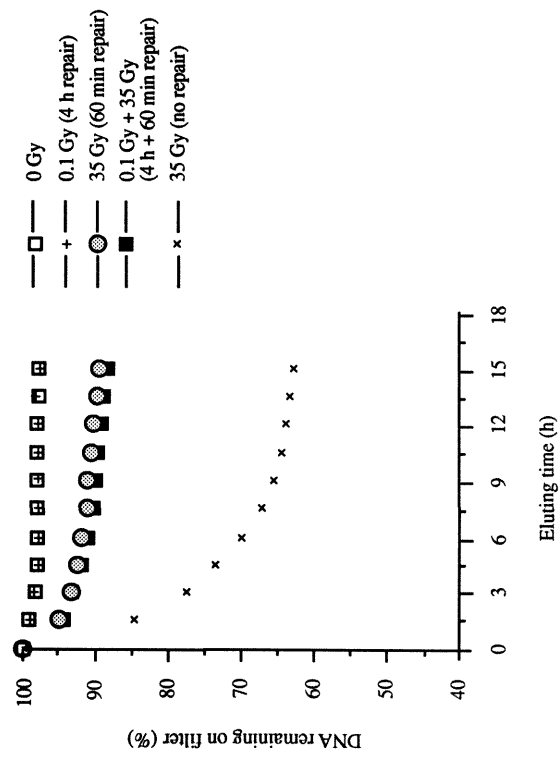
**FIGURE 3.5.3** Comparison of repair kinetics of DSB induced by 35 Gy challenging dose in cells pre-exposed to 0.1 Gy adapting dose. Cell repair was stopped by ice chilling.  
 (a) 40 min incubation at 37 °C after adapting dose, 20 min repair after challenging dose;  
 (b) 20 min incubation at 37 °C after adapting dose, 60 min repair after challenging dose.

and challenging dose (Figure 3.5.4). However, this also revealed no significant differences in rejoining rates of DSB.

Therefore, none of the conditions tested indicated the induction of adaptive response which has been reported for chromosomal aberration.

As yet, there are no agreed explanations for this apparent discrepancy in the induction of adaptive response between DSB and chromosomal aberration. Although it is believed that DSB is responsible for the formation of chromosomal aberrations (Bryant 1984, Natarajan and Obe 1986, Bryant *et al.* 1987), the results obtained here may imply that the mechanism of radiation-induced adaptive response in chromosomal aberration could be more complex and not simply linked to DSB. It is noteworthy that 3-aminobenzamide (3AB), an inhibitor of poly(ADP-ribose) polymerase, could negate the adaptive response (Wiencke *et al.* 1986, Ikushima 1987, Shadley and Wolff 1987), whereas the inhibition of poly (ADP-ribose)polymerase caused only a slight delay in the rate of rejoining the DNA strand break induced by radiation (Zwelling *et al.* 1982). So this inhibition of adaptive response due to the presence of 3AB illustrated that these enzymes might be needed in the development of adaptive response, and has made it difficult to associate DSB and repair as the direct molecular basis to the adaptive response of chromosomal aberration.

An adaptive response could occur in repair-proficient and repair-deficient strains of occytes (Fritz-Niggli and Schaeppi-Buechi 1991). Moreover, the adaptive response could be inhibited by the protein synthesis inhibitor which is present between adapting doses and challenging doses (Wolff *et al.* 1989, Youngblom *et al.* 1989). These suggest that the mechanism of adaptive response may involve protein synthesis and structure alteration of protein attached to nuclear chromatin, rather than direct induction of repair enzymes.



**FIGURE 3.5.4** Comparison of repair kinetics of DSB induced by 35 Gy of challenging dose (60 min repair at 37°C) in cells pre-exposed to 0.1 Gy adapting dose (4 h repair at 37°C). Cell repair was stopped by ice chilling.

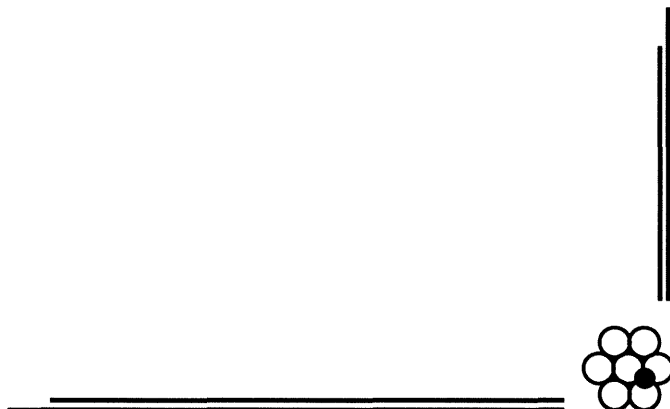
It is also worth noting that in the induction of adaptive response for chromosomal aberration, the challenging doses used were less than 3 Gy (all references related to adaptive response). However, only one high challenging dose of 35 Gy was used in the present study. The question arises whether such high challenging dose might inactivate the 'inducible' repair enzymes evoked by low dose so that the adaptive response could not be exhibited. Unfortunately, higher doses of radiation have to be applied to observe radiation-induced changes in cellular DNA using assay of filter elution. If so, it is difficult to induce adaptive response in DNA damage/repair using the same conditions for adaptive response of chromosomal aberration through available assays of DNA damage.

Many results concerning the adaptive response of chromosomal aberration remain controversial. Firstly, relatively stringent adapting conditions with regard to dose, dose-rate and irradiation time are necessary for the induction of adaptive response. (Shadley *et al.* 1987, Shadley and Wiencke 1989). In the present study only high dose-rate was used for both adapting dose and challenging dose due to reasons of availability. Secondly, the adaptive response to ionizing radiation was not observed using the same or similar experimental conditions to those at which the adaptive response has been determined by others (Bauchinger *et al.* 1989, Bosi and Olivieri 1989, Schmid *et al.* 1989, Sankaranarayanan *et al.* 1989, Greinert *et al.* 1991, Shadley 1991). These negative results implied that the precise condition necessary to induce an adaptive response to ionizing radiation might be expected to be different within various cultured cell types and individuals, physiological and/or genetic differences obviously have a considerable impact, but whether it is the case, at present, the answers to this question remain unclear. Therefore, The failure of induction of adaptive response in DNA DSB to ionizing radiation reported here must be treated with caution, and further work is required.

### Summary

Contrary to expectation, there was no evidence that an adaptive response of DNA DSB in Chinese hamster C-79 cells pre-treated with low doses and subsequently to a high doses compared with cells exposed to the high dose alone, using different low adapting doses (0.1, 1 and 5 Gy) and different repair times (up to 4 h). These results suggest that this is not a simple relationship between repair of DNA damage and the induction of adaptive response which is found in chromosomal aberration. The reasons for this is open to discussion, but may be due to the high challenge doses needed in this study.

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