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REPAIR OF DNA DAMAGE  
IN HUMAN LYMPHOCYTES

by

Ron S. McWilliams

A Thesis submitted to the School of Graduate Studies  
of the University of Ottawa, as partial  
fulfilment of the requirements for the  
M.Sc. degree in Biology

Ottawa, Ontario, 1981

Ron S. McWilliams, 1981

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## ABSTRACT

Human peripheral blood lymphocytes (HPBL) are highly radiosensitive. A possible reason for this sensitivity could be a deficiency in the ability of HPBL to repair damage to genetic material. The induction and rejoining of DNA strand breaks after  $^{60}\text{Co}$  gamma- or 14.6 MeV neutron irradiation at low doses (1-2 Gy) was followed by use of a new sensitive assay for the detection of DNA strand breaks (Fluorescence Analysis of DNA Unwinding) in order to test this hypothesis. Rejoining after  $^{60}\text{Co}$  gamma-irradiation was rapid and biphasic with 70% of the breaks being rejoined with a  $t_{1/2}$  of 2.8 minutes, the remaining fraction (30%) being rejoined with a  $t_{1/2}$  of 35 minutes. The ability of HPBL to rejoin DNA strand breaks does not change appreciably over 72 hours of culture. 14.6 MeV neutrons are a high LET radiation with a greater biological effectiveness than  $^{60}\text{Co}$  gamma-rays. It was thought that the yield of strand breaks and their rejoining after 14.6 MeV neutron irradiation might be different than those after  $^{60}\text{Co}$  gamma-irradiation. The apparent yield of DNA strand breaks induced by 14.6 MeV neutrons is 0.34 that induced by  $^{60}\text{Co}$  gamma-rays, in contrast to the high biological effectiveness of 14.6 MeV neutrons. Experiments

were done to test for protein-DNA crosslinks which could be responsible for the low apparent yield of DNA strand breaks. No positive evidence for the existence of extensive cross-linking was found. A model involving the clustering of DNA strand breaks within chromatin structure is presented as a possible mechanism for the low yield. Rejoining of DNA strand breaks after 14.6 MeV neutron irradiation is rapid and biphasic, the kinetics differing from those after  $^{60}\text{Co}$  gamma-irradiation only in that there are fewer of the rapidly rejoined DNA strand breaks.

The extreme radiosensitivity of HPBL is probably not due to a deficiency in their ability to rejoin DNA strand breaks. There is no obvious relation between the induction and rejoining of DNA strand breaks after 14.6 MeV neutrons and the biological effects seen at these doses.

## RESUME

Les lymphocytes périphériques humains sont très sensibles aux radiations. Une des raisons pour une telle sensibilité pourrait être une déficience de l'abilité des HPBL de réparer les dommages du matériel génétique. L'induction et le raccommodage des scissions des chaînes d'ADN après irradiation à faibles doses de  $^{60}\text{Co}$  - gamma ou 14.6 MeV neutrons ont été suivis en utilisant une nouvelle méthode sensible pour détecter les scissions des chaînes d'ADN (Fluorescence Analysis of DNA Unwinding) afin de tester cette hypothèse. Le raccommodage après irradiation avec  $^{60}\text{Co}$  - gamma était rapide et biphasique avec 70% des scissions raccommodées à une demi-vie de 2.8 min., la fraction restante (30%) étant raccommodée à une demi-vie de 35 min. L'abilité des HPBL à raccommoder les scissions de l'ADN ne varie pas significativement en 72 heures de culture. 14.6 MeV neutrons sont des radiations à hautes LET avec une plus grande efficacité biologique que les rayons gamma  $^{60}\text{Co}$ . On a pensé que le taux de scissions et leur raccommodage après 14.6 MeV irradiation aux neutrons pourrait être différents de ceux après irradiation aux rayons gamma -  $^{60}\text{Co}$ . Le taux apparent des scissions induites par 14.6 MeV neutrons est 0.34 fois plus bas que celui causé par les rayons gamma -  $^{60}\text{Co}$  à l'inverse de la grande efficacité biologique de 14.6 MeV neutrons. Des expériences ont été entreprises dans le but de détecter la

présence de liens croisés entre les protéines et l'ADN, qui pourraient être responsable du taux apparent, peu élevé des scissions d'ADN. On n'a pas trouvé d'évidence pour l'existence d'un grand nombre de liens croisés. Un modèle incluant l'em-  
paquetage des scissions d'ADN à l'intérieur de la structure chromatique est présente comme mécanisme possible pour expliquer les taux peu élevés. Le raccommodage des scissions après irradiation avec 14.6 MeV neutrons est rapide et biphasique. Les cinétiques n'étant différentes de celles observées après irradiation avec gamma -  $^{60}\text{Co}$  que par le nombre moins important des scissions raccommodées rapidement. L'extrême sensibilité des HPBL aux radiation n'est probablement pas due à une déficience de leur capacité à raccommoder les scissions des chaînes d'ADN. Il n'y a pas de relation évidente entre l'induction et raccommodage des scissions après 14.6 MeV neutrons et les effets biologiques observés à ces doses.

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### ABBREVIATIONS

B cell	bone-marrow derived lymphocyte
ConA	concanavalin A
DNA	deoxyribonucleic acid
FADU	Fluorescence Analysis of DNA Unwinding
FBS	fetal bovine serum
HN2	nitrogen mustard (2,2'-dichloro-N-methyldiethylamine)
<sup>3</sup> H-TdR	tritiated thymidine, (methyl- <sup>3</sup> H)-thymidine
LET	linear energy transfer
AAAF	N-acetoxy-2-acetylaminofluorene
4NQO	4-nitroquinoline-1-oxide
PHA	phytohemagglutinin
RBC	red blood cell(s)
RBE	relative biological effectiveness
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
T cell	thymus derived lymphocyte
UDS	unscheduled DNA synthesis
WBC	white blood cell(s)

## DEFINITION OF TERMS

The relative biological effectiveness (RBE) is the ratio of the dose of one form of radiation needed to cause a given effect to the dose of a second form of radiation used as a standard (usually X-rays) to achieve the same effect. The RBE need not be the same for any given radiation form at different doses and is also dependent on the cell type and endpoint measured.

Linear energy transfer is the amount of energy deposited by a particle, in its passage through a given material, per unit distance (e.g. KeV/ $\mu$ m).

## CHAPTER I

### Introduction

#### 1.1 Statement of Thesis

DNA strand breaks induced by ionizing radiation are reported to be rejoined efficiently by cultured mammalian cells. The ability of lymphocytes to carry out this form of repair is less well defined. It is my intention to study the rejoining of DNA strand breaks in resting human peripheral blood lymphocytes after irradiation with either  $^{60}\text{Co}$  gamma-rays or 14.6 MeV neutrons. The purpose of this study will be twofold: first, to determine whether the cause of the extreme radiosensitivity of lymphocytes results from a deficiency in their ability to rejoin DNA strand breaks; second, to determine whether the high biological effectiveness of 14.6 MeV neutrons is related to the number of strand breaks induced in DNA or to the rate of rejoining of strand breaks, as compared to  $^{60}\text{Co}$  gamma-rays, using the lymphocyte as a model system.

In order to accomplish this I have isolated human peripheral blood lymphocytes by standard methods and irradiated them in vitro with low doses ( $\leq 4$  Gy) of  $^{60}\text{Co}$  gamma-rays or 14.6 MeV neutrons. I have determined the relative yields of DNA strand breaks after irradiation of

the cells with each radiation type by the use of an assay for DNA strand breaks in non-dividing cells recently developed in Chalk River by Dr. H.C. Birnboim and J.J. Jevcak. I have tested for the rejoining of strand breaks in resting human peripheral blood lymphocytes with time at 37°C after irradiation with either  $^{60}\text{Co}$  gamma-rays or 14.6 MeV neutrons.

### 1.2 The Lymphocyte

The lymphocyte is an integral part of the immune system, which is the body's major defence against infection. The human peripheral blood lymphocyte (HPBL) represents about one quarter of the leukocytes in blood, the remainder being neutrophils, monocytes, macrophages, and minor granulocytes. HPBL are generally divided into two classes, T cells and B cells. The T cell or thymus derived lymphocyte is responsible for cell mediated immunity (cell cytotoxicity, allograft rejection, etc.), and the B cell or bone marrow derived lymphocyte is responsible for humoral immunity (soluble antibody production) (Marchalonis, 1977). In the blood the HPBL is an inactive cell, carrying out little or no DNA synthesis, transcription or translation (Cline, 1975; Mitchell and Schäfer, 1979). In the presence of antigen, individual cells will respond by differentiating

and dividing once or twice before expressing their immune function (e.g. antibody production). It is possible to treat these cells with a polyclonal mitogen which will stimulate the majority of HPBL to undergo metabolic activation and perform one or two mitoses (Hume and Weidemann, 1980). During this time the lymphoblast, as it is called, presents a striking contrast to the resting lymphocyte. The cell increases over three times in diameter and the mitochondria, ribosomes, and most other organelles increase in number. While the nucleus of the resting HPBL is almost completely heterochromatin, that of the lymphoblast is dispersed. This is evidence of the marked increase in transcription which precedes DNA synthesis. The activities of several DNA-related enzymes increases markedly from barely detectable levels; these include DNA polymerases (Loeb et al. 1968), DNA ligase and single strand endonuclease (Pedrini et al., 1972). Some of these enzyme activities could be used in repair of damage to the DNA of cells as well as participating in the replication of the cell. As resting HPBL have very low levels of these enzymes, it is possible that they may be deficient in their ability to repair damage to the genetic material.

### 1.3 Description of Radiation Types Used

This project will deal with the effects of two

different forms of radiation as DNA damaging agents. These are described as ionizing radiations as they are capable of producing energetic charged particles in matter. The first type is gamma-rays from  $^{60}\text{Co}$ , a radioisotope with a half-life of 5.24 years. The principal products of each radioactive disintegration are two photons having different kinetic energies, 1.17 and 1.33 MeV. Gamma-rays (photons) lose energy primarily by interaction with orbital electrons of atoms in target matter. A photon may scatter inelastically to give rise to Compton electrons which have a maximum energy of 1.117 MeV; but the majority will have an energy of less than 200 keV. Compton electrons are responsible for over 90% of the total energy deposition from  $^{60}\text{Co}$  gamma-rays. Compton electrons have enough energy to cause a large number of further ionizations by collision with other orbital electrons (Dertinger and Jung, 1970).

The major source of these ionizations in biological tissues is the water molecule. An energetic electron can excite a water molecule which, as a result, may cleave to form a hydroxyl radical and a hydrogen atom. The electron can also add on to a water molecule, which will dissociate to form a hydrogen atom and a hydroxyl ion. It can also combine with a proton to produce a hydrogen atom. If it does not react before losing most of its kinetic

energy, it will remain in solution as an aqueous electron ( $e_{aq}^-$ ). The charged water molecule left behind after the ejection of an electron will dissociate to produce a proton and a hydroxyl radical.

The other form of radiation which will be used in this study is the fast neutron. To generate a beam of neutrons, either the fission reaction or a particle accelerator is commonly used. Fission neutrons have a broad spectrum of kinetic energies, usually centering around 2 MeV. They are produced in nuclear reactors or by the bombardment of fissionable materials by neutrons. In a particle accelerator a beam of particles of low mass such as protons or deuterons is directed onto a target of some element of low atomic number (e.g.  $^2\text{H}$  or D,  $^3\text{H}$  or T, or Be). The reaction of these particles with the target atom is accompanied by the emission of a high energy neutron, often leaving a stable by-product (Arena, 1971). Fast neutrons produced by any one reaction are usually monoenergetic and can have kinetic energies ranging from 150 keV to over 20 MeV. In this study 14.6 MeV neutrons will be used. Energy from this source is deposited in target material by several mechanisms. Over 80% of the energy deposited in biological tissue is due to elastic collision with a proton ("proton recoil"). Kinetic energy is

imparted to the proton which is capable of causing large numbers of ionizations by knocking out orbital electrons in a similar fashion to Compton electrons of gamma-irradiation before coming to rest. A lesser amount of the energy deposited is due to carbon, oxygen and nitrogen recoils; less commonly a non-elastic interaction with an atomic nucleus will cause the formation of alpha particles.

The average amount of energy deposited per unit length of particle track is defined as the linear energy transfer (LET). LET has become an important concept in radiation biology because many biological effects have different dose relationships depending on the LET of the radiation used. The general rule is that higher LET radiations have a greater effect at similar doses.  $^{60}\text{Co}$  gamma-rays are a sparsely ionizing radiation with a LET of 300 eV/ $\mu\text{m}$ . X-rays used in biological experimentation are generally considered to be similar to gamma-rays and have a LET usually below 3 keV/ $\mu\text{m}$ . The LET of neutrons is not usually discussed as the neutron does not give up its energy as evenly spread ionizations along its path but rather as discrete clusters of ionizations. These clusters are due chiefly to proton recoil, which gives neutrons their high LET characteristics. It is appropriate to consider the distribution function of

protons of different LET and the contribution of other particle types. For 14.6 MeV neutrons the LET derived from proton recoil is centered around 10 keV/ $\mu\text{m}$  with a maximum energy of about 100 keV/ $\mu\text{m}$ . There is also a higher LET component derived from interactions of neutrons with heavier atoms producing a LET of up to about 1000 keV/ $\mu\text{m}$  (Edwards, 1975).

#### 1.4 Radiolysis Products of Water in Damage of Genetic Material

The primary interaction of ionizing radiation with living organisms is the radiolysis of water, as water is the major constituent of cells. The significant products of the radiolysis of water are generally considered to be three highly reactive radicals, the hydrogen atom, the hydroxyl radical and the aqueous electron, the formation of which has been discussed. The hydroxyl radical is implicated as the most biologically damaging of the three. This radical will react almost as soon as it encounters an organic molecule in solution. The most important molecule in the cell and the one considered to be the most sensitive to attack by radicals is the genetic material, DNA. There are several possible sites for radical attack in the DNA leading to a large number of different products. Many of these have been identified by use of physical and chemical techniques

on model compounds related to native DNA. These include free bases, nucleosides and nucleotides. Only products of their reaction with free radicals in the presence of oxygen which are important to our discussion will be listed here. Radicals preferentially attack bases (80%) over the deoxyribose moiety of the nucleotide, pyrimidines being more sensitive than purines (Hems, 1960). Pyrimidines, their deoxynucleosides and deoxynucleotides are attacked at the 5,6 double bond, forming various degradation products. The cleavage of the glycosidic linkage of nucleoside or nucleotide is a common reaction. In a polynucleotide this reaction could produce an alkali-labile bond in the phosphate deoxyribose backbone. Purine derivatives are not as well studied but the major reactions of deoxynucleoside are release of free base or partial degradation of base by oxidation of the imidazol ring. Abasic sites are known to be unstable, particularly in alkaline media; the sugar phosphate bond may cleave forming a strand break (Shapiro, 1967). Another way a strand break may form is by the attack of deoxyribose directly by hydroxyl radical. The radical can abstract a hydrogen atom from the furanosyl ring leaving a radical on the sugar which can lead to the breakage of both the 3' and 5' phosphate linkages releasing inorganic phosphate and

and unaltered base. A less common event is the rupture of a carbon-carbon bond within the deoxyribose, also causing a strand break (Téoule and Cadet, 1978).

Aqueous solutions of DNA have been irradiated in air and the relative yields of various products which could lead to strand breaks as assayed for under alkaline conditions have been tabulated (Ullrich and Hagen, 1971). Seventy percent of the breaks are induced in neutral irradiation medium, the remainder requiring alkali for expression. A little less than half of the breaks induced in neutral solution are accompanied by the release of intact or altered nucleoside. The remainder arise from attack on the 3' carbon of the deoxyribose leaving a 3' hydroxyl group, or less often from the hydrolysis of the 5' phosphate ester linkage. Approximately one quarter of the bases which are chemically altered are released directly from the DNA molecule leaving an abasic site. Of the strand breaks which require alkali for expression, 60% arise from the loss of sugar by  $\beta$ -elimination at abasic sites: the rest result from alkali-labile alteration of the sugar.

### 1.5 Effects of Ionizing Radiation on Living Organisms

What is often studied, and is of practical importance in making decisions about radiation quality,

especially in medicine, is the effect of radiation on the living organism. There are many endpoints which are measured in vivo, such as survival, weight loss of specific organs (e.g. Di Paola et al., 1980a), lens opacification (e.g. Di Paola et al., 1980b), changes in tissue morphology and pathology (e.g. Zook et al., 1980), depression of DNA content in tissue (e.g. Geraci et al., 1975), and tumour induction (e.g. Jacrot et al., 1979). There is a body of information available on the effect of radiation on human beings. Exposure can be occupational, accidental, through radiotherapy or diagnostic X-rays. There are some data from survivors of the atomic bomb explosions. A large acute dose can have an almost immediate effect, while the effect of low doses may not be seen at all or have a latency of many years for expression. Exposure to doses as low as 10 mGy is estimated to increase the risk of genetic disease in offspring by 0.05% and also to increase the risk of fatal cancer by 0.1% (UNSCEAR, 1977). This dose is only 10 times the average natural radiation background dose received in one year (1.0 mGy). An acute dose of as low as 100 mGy can be detected by cytogenetic means, that is the analysis of chromosome aberration induction in lymphocytes. Several hundred mitoses are scored and generally the number of dicentric and ring

chromosomes are counted to come to an estimate of the dose received (Lloyd and Purrot, 1981). Doses above 1 Gy can cause a clinical disease known as Radiation Syndrome. The dose at which half of those individuals irradiated will survive for at least 60 days ( $^{60}\text{LD}_{50}$ ) is low, about 3.0 Sv. After a dose of as low as 2.0 Gy of X-irradiation the number of lymphatic cells is reduced substantially and the immune response will remain suppressed for months. The rapid drop in white blood cell (WBC) number is a reflection of the extreme radiosensitivity of the lymphocyte, while the long recovery time shows the extreme radiosensitivity of the rapidly dividing precursor cell in the bone marrow. Almost as sensitive is the gastrointestinal (GI) system. GI syndrome is caused by 5.0 Gy or more of irradiation. It is characterized by the depletion of the epithelial lining of the intestine. These epithelial cells and bone marrow cells follow the empirical rule that rapidly dividing cells are highly radiosensitive. In contrast, much higher doses (>50 Gy) are needed to induce deleterious effects on the central nervous system which is composed mainly of non-dividing cells (Prasad, 1974). The extreme sensitivity of the resting human peripheral blood lymphocyte is paradoxical if it is compared to the resistance of almost all other

non-dividing cells.

Damage to genetic material is generally considered to be the major cause of cell death at moderate doses of ionizing radiation. The ability of cells in culture to survive after irradiation is used as an indirect measure of damage to the DNA molecule and its repair. Several interesting conclusions have been drawn from the study of survival curves, some of which are relevant to our discussion here. The survival curve for cultured mammalian cells irradiated with low LET radiation is of increasing slope with higher dose, producing what is known as a shoulder. The low slope at lower doses is taken to mean that repair of some amount of sub-lethal damage can occur. At higher doses the multiplicity of the damage to the cell becomes too great for the capacity of the repair system of the cell and the curve enters an exponential phase. Survival curves generated by the irradiation of cells with high LET radiation have a very small shoulder, indicating that even at low doses there is no repair of sub-lethal damage, suggesting that if one radiation track interacts with a cell it will always put in enough damage to cause a lethal event (e.g. Railton et al., 1974; Broerse et al., 1968). When lymphocytes are irradiated with low LET radiation such as X-rays, the survival curve goes almost immediately into an exponential phase with a very small

shoulder, suggesting that there is no repair of what would be sub-lethal damage in a cultured mammalian cell (Stewart and Perez, 1976). In other words, the damage put in could be repaired by a cultured cell type at that low dosage. Twenty hours after 2.0 Gy of X-irradiation less than 50% of HPBL survive in culture. A further 30% die over the next two days (Hedges and Hornsey, 1978). A different study, using purified lymphocyte subpopulations, showed that T cells are moderately radioresistant, while B cell survival is affected by doses as low as 0.25 Gy of X-rays (Prosser, 1976). Both studies indicate that there is a small group of cells representing 20% of the total population which is quite radioresistant but that the majority, the remaining 80%, is extremely radiosensitive. Response of HPBL to mitogens such as PHA is somewhat reduced after moderate doses of X-irradiation (Serio et al., 1980; Ilbery et al., 1971), and the activation of this cell greatly enhances its survival after 5.0 Gy of X-rays (Sato, 1970). This again points out the paradox of the radiosensitivity of lymphocytes because soon after entering S-phase they start to degenerate and very few survive past 60 hours. This indicates that damage put in, though not immediately deleterious, is probably misrepaired and expresses itself as an inability to complete mitosis

or to survive thereafter.

Another indirect measure of DNA damage is the chromosome aberration. The analysis of chromosome aberrations in lymphocytes is routinely done in vivo for radiation accident dosimetry and in vitro as an assay for DNA damage. Chromosome aberrations have been detected in the HPBL of persons 30 years after they had received a dose of irradiation. Immediately after a radiation accident the aberration yield remains constant and high for weeks, suggesting that damage to chromatin is either not repaired at all or that it is misrepaired soon after the insult (Lloyd and Purrot, 1981). In split dose experiments, non-repair of damage leading to chromosome aberrations up to 12 hours after irradiation has been shown (Sasaki, 1978; Stefanescu et al., 1972). It would appear that lymphocytes repair damage leading to chromosome aberrations poorly.

The lymphocyte has been shown to be deficient in other forms of repair by the use of various chemical assays which may give a more direct indication of DNA repair activity in cells. An early study showed that HPBL perform low levels of repair synthesis after exposure to ultraviolet light, as compared to cells of chronic lymphocytic leukemia patients (Huang et al., 1972). As CLL cells and HPBL do not synthesize DNA semi-conservatively, density labeling of DNA

in order to separate strands in replicative synthesis from those in repair synthesis was not done. Lavin and Kidson (1977) carried out a study of the level of repair synthesis in resting and PHA stimulated HPBL. They used density labeling to differentiate between fragments of DNA which had incorporated label through semi-conservative replication or repair synthesis. They were unable to detect significant amounts of repair replication in the presence of 10 mM hydroxyurea in resting HPBL. The rate of repair replication in PHA stimulated HPBL follows that of replicative synthesis, the maximum rate occurring four days after addition of the mitogen. It would appear that HPBL are deficient in repair replication.

HPBL seem to be low in their ability to excise radiolabeled alkylating agents (N-acetoxy-2-acetylaminofluorene, AAAF) as compared to Con A stimulated HPBL or transformed lymphoblastoid cells (RAJI). Incorporation of radioactively labeled thymidine into material not retained on BND-cellulose was very low in resting HPBL treated with methyl methane sulphonate or AAAF. The highest rate of repair in stimulated HPBL was coincident with the peak of DNA replicative synthesis (Scudiero et al., 1976). Others have shown similar results (Lieberman et al., 1972).

Another extensively studied defect in the genetic

material of cells is DNA strand breaks induced by ionizing radiation. HPBL are reported to rejoin strand breaks introduced during the course of enzymatic repair processes.

In particular, they are capable of repairing DNA strand breaks introduced over the course of 12 hours during the repair of sub-lethal doses of ultraviolet light ( $0.5 \text{ J/m}^2$ ) (Yew and Johnson, 1979) as visualized by the nucleoid sedimentation technique of Cook and Brazell (1976).

Cultured human fibroblasts have been shown to rejoin strand breaks rapidly and efficiently by Hariharan et al. (1981) using the alkaline elution technique of Kohn (1979). There are a number of reports on the rejoining of strand breaks in HPBL after exposure to ionizing radiation. All have shown rejoining, but there is considerable variation in the findings. Hashimoto, Ono and Okada (1975) used alkaline sucrose gradient sedimentation to compare the rejoining rate of resting and PHA stimulated HPBL after a dose of 30 Gy of  $^{137}\text{Cs}$  gamma-rays. Stimulated HPBL rejoined strand breaks rapidly with a half-time of seven minutes, resting HPBL were less proficient, having a half-time for rejoining of 60 minutes. Repair kinetics for both cell treatments were reported to be monophasic. Cook et al. (1978) used a nucleoid sedimentation technique to find that resting HPBL rejoin strand breaks with a half-time of several hours

after a dose of 9.6 Gy of  $^{60}\text{Co}$  gamma-rays. Later, Fender and Malz (1980) used a similar technique to show rapid rejoining. They reported monophasic repair with a half-time of 10-15 minutes. These reports are conflicting as to the ability of HPBL to rejoin DNA strand breaks. It is possible that the radiosensitivity of lymphocytes is related to their capacity to rejoin DNA strand breaks.

#### 1.6 Biological Effects of Neutron Irradiation

Neutrons are a high LET radiation and will be used in this study. The RBE for neutrons is high (1.5-4) for in vivo effects (Field and Hornsey, 1971); Withers et al., 1970; Geraci et al., 1975; Barendsen, 1968). Neutrons of different energies have different efficiencies for cell killing in vitro. The RBE for 6.2 MeV neutrons in Chinese hamster cells is about 2.3 (Schneider and Whitmore, 1963; Tolkenndorf, 1978; Walika et al., 1979). The RBE for D-T (deuterium on tritium, average energy 14-15 MeV) neutrons, which are used also in my study, was 2.0-2.5 for Chinese hamster cells (Railton et al., 1974; Hall et al., 1975). For human cells the RBE was 1.6 to 2.3 at low survival levels (Broerse et al., 1968; Nias et al., 1967). The RBE rises sharply to 2.9-4.3 at higher survival rates. This is because neutrons kill in an exponential fashion even at low doses. D-T neutrons do not have an exceptional

effectiveness compared to neutrons of other energies. In the range of 0.2 to 1.0 MeV the RBE of neutrons is greater than four at low survival (Hall et al., 1975). The RBE for chromosome aberration inductions after D-T neutron irradiation is reported to be in the range of 1.1-1.45 (Bauchinger et al., 1975), 1.4-2.9 (Clare, 1978) and 2.7-13 (Lloyd et al., 1976). D-T neutrons have a moderately high RBE for all of the biological endpoints examined.

The biological effects of neutrons have been related to the differential ability of cells to repair damage in DNA induced by radiations of different qualities. Unfortunately, the repair of DNA strand breaks induced by fast neutrons and their yield has been examined only in cell types other than HPBL. Furuno et al. (1979) studied the rejoining of breaks in cultured mouse leukemic cells after a dose of 400 Gy of cyclotron generated neutrons (average energy, 13 MeV). They found an RBE for strand break yield of 0.6. The response was linear for strand break induction in this study as well as those which follow. The kinetics of rejoining was biphasic; the half-time of the first phase was 20 minutes, that of the second phase, about two hours. Ahnström and Edvardsson (1974) studied rejoining in Chinese hamster V79 cells after a dose of 40 Gy of fission neutrons (average energy, 2 MeV). They found a

half-time of rejoining of 10-15 minutes for a fast phase with the residual damage after 120 minutes of incubation representing less than a quarter of the total breaks. They also note an RBE for strand break induction of approximately 0.2. Kampf et al. (1977) note a low RBE, 0.7, for strand break induction after 6.2 MeV neutrons. Körner et al. (1978) using the same radiation facility found that 73% of the DNA strand breaks induced after 140 Gy of fast neutron irradiation were rejoined with a half-time of 2.9 minutes. The remainder were not rejoined after 80 minutes incubation. After irradiation with 100 Gy of 0.4 MeV X-rays 80% of the strand breaks were rejoined with a half-time of approximately 30 minutes. In contrast, Moss et al. (1976) found an RBE of 1.6 for the induction of strand breaks in mouse fibroblast cells by 10-600 Gy of fast neutrons. The rate of DNA strand break induction in cells after neutron irradiation appears to have little relation to the RBE of this high LET form of radiation, though there appears to be some difference in the pattern of rejoining of DNA strand breaks after fast neutron irradiation.

#### 1.7 Conclusion

The lymphocyte is of special interest as it is considered to be the key cell in the human immune system.

An understanding of the mechanism of the extreme radiosensitivity of this cell would be useful in medicine, immunology and fundamental research. The differences between the basic mechanisms of action of high and low LET radiations are also of interest. There are few studies on the capacity of resting human lymphocytes to rejoin DNA strand breaks as it has been difficult to detect strand breaks in cells which have not been radioactively labeled to show DNA. The assay developed by Dr. H.C. Birnboim and J.J. Jevcak (1981) is well suited for use with resting lymphocytes as the DNA is visualized by the use of a fluorescent dye. The technique follows DNA unwinding in alkali with time. The rate of unwinding is dependent in some complex fashion on the number of strand breaks in the DNA. The resting lymphocyte does not synthesize DNA or transcribe RNA. This ensures a minimum rate of unwinding in unirradiated cells, thereby maximizing the sensitivity of the assay, for there are few strand breaks in the native DNA. This assay will allow the investigation of the rejoining of DNA strand breaks in resting lymphocytes after very low doses of  $^{60}\text{Co}$  gamma-rays or 14.6 MeV neutrons.

This report will determine the capacity of resting human peripheral blood lymphocytes to rejoin DNA strand breaks, whether this capacity is dose dependent, and will

show the relationship of the kinetics of rejoining of DNA strand breaks for these two forms of radiation of different LET.

## CHAPTER II

### Materials and Methods

#### 2.1 Isolation of Total WBC

Blood from healthy adult volunteers was collected into 5 or 10 ml tubes (B-D Vacutainer) containing EDTA (3.6 mM) as an anticoagulant. Blood from 3-4 donors was pooled, chilled to 0°C, and then distributed into appropriate vessels for irradiation at 0°C. (See Irradiation sections.) After irradiation, WBC were isolated as follows. 9 ml of an ammonium chloride solution (0.87% NH<sub>4</sub>Cl, 10 mM Tris-HCl, pH7.2) was added to each 5 ml sample of blood and held at 0°C for 30 minutes to lyse the RBC (red blood cells).<sup>1</sup> WBC were pelleted in a centrifuge (0°C, 20 min., 400 g), and washed once by resuspension in the ammonium chloride solution and recentrifugation (0°C, 10 min., 400 g). The cells were then resuspended in solution B for analysis of strand breaks. (See details of FADU, described below.)

#### 2.2 Isolation and Culturing of HPBL

Blood from healthy adult volunteers was collected

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<sup>1</sup>For cells which were used in repair experiments the blood was diluted with an equal volume of RPMI 1640 at 0°C, then incubated at appropriate temperatures. The RBC lysis was done with three volumes of the ammonium chloride solution.

using an infusion set (Butterfly-19, Abbott, Montreal) and a 50 ml plastic syringe containing heparin (final concentration 60 IU/ml) as an anticoagulant. Lymphocytes were isolated after Bøyum (1976) as follows. Six volumes of a balanced salt solution (137 mM NaCl, 5.4 mM KCl, 5.6 mM  $\text{Na}_2\text{HPO}_4$ , 5.6 mM glucose, 0.14 mM  $\text{MgSO}_4$ ; pH 7.2-7.3) were mixed with four volumes of blood at 25°C; 25 ml of the diluted blood was carefully layered onto 15 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Dorval, Quebec) in a 50 ml polypropylene tube and centrifuged at 400g for 30 minutes at 18-20°C. RBC and granulocytes clump and sediment to the bottom of the tube. Mononuclear cells and some platelets band at the interface between the Ficoll-Paque and the diluted blood. This band of cells was drawn off with a pasteur pipette and washed twice by resuspension and centrifugation with balanced salt solution at 18-20°C. The washed cell pellet (containing >95% mononuclear cells) was resuspended in RPMI 1640 culture medium buffered with 25 mM HEPES (GIBCO, Grand Island, N.Y.) and supplemented with 10% fetal bovine serum (FBS) (Microbiological Associates, Bethesda, Md.) at  $2 \times 10^6$  cells per ml ( $1 \times 10^6$  per ml for  $^3\text{H}$ -thymidine uptake experiments and for radioautography); cells were incubated at 37°C in tightly stoppered 75 cm<sup>2</sup> plastic flasks for 18 hours in an air

incubator before repair experiments were carried out. Immediately prior to irradiation, non-adherent cells were transferred to plastic or siliconized glass vessels and chilled to 0°C. Cells were maintained at 0°C at all times, except during incubation, where indicated. After incubation samples were chilled rapidly to 0°C in an ice slurry. When the experimental treatments were completed the cells were centrifuged (10 min., 0°C, 400 g) and resuspended in a buffered solution (B, see FADU) for further analysis.

### 2.3 Irradiations

Gamma-irradiations were carried out using a Gamma-beam 150 (Atomic Energy of Canada Limited). This instrument consists of a  $^{60}\text{Co}$  source (150 TBq) housed in lead shielding. The dose to the sample is calculated by knowledge of the distance from the radiation source and the exposure time. The source had been calibrated in 1977 by L. Johnson of the Radiation Biology Branch using the ferrous sulphate method of Fricke (Bacq and Alexander, 1961). The dose was then calculated from tables which account for the decay of  $^{60}\text{Co}$  (half-life of 5.24 years). To deliver a dose of 1 Gy samples on ice were positioned 1 m from the source and exposed for approximately 6.5 minutes.

Fast neutrons were produced by the  ${}^3\text{H}({}^2\text{H},\text{n}){}^4\text{He}$  reaction by the bombardment of a tritium target with 0.15 MeV deuterons using the Health Physics Neutron Generator (Chalk River Nuclear Laboratories). The neutron generator was operated by A. Arneja of the Health Physics Branch. The neutrons produced had an average energy of 14.6 MeV and the dose rate was approximately 65 mGy/min. at a distance of 5 cm from the target. Care had been taken in design of the instrument to minimize contamination such that less than 2% of the total dose delivered to the samples was due to gamma-rays. Plastic vessels were used for the irradiations since the silicon in glass is activated by neutrons to produce  ${}^{28}\text{Al}$ , a beta-emitter. Glass was avoided because it posed a slight hazard, rather than because of the small contribution of  ${}^{28}\text{Al}$  to the final dose to the sample (less than 0.5%). Because of the low neutron fluences available, the cell sample to be irradiated had to be positioned close to the target and considerable care was necessary to ensure that the cells received a uniform dose which could be measured accurately. The irradiation vessel was a 25 cm<sup>2</sup> tissue culture flask supported upright inside a small polyethylene bucket containing a magnetic stir bar and an ice water slurry. The flask contained 30-35 ml of cells which were kept in suspension with a 13 mm magnetic

stir bar. The volume of the cell suspension was approximately  $5 \times 3 \times 2.2 \text{ cm}^3$ . The polyethylene bucket was placed upon an electric magnetic stirrer. Stirring was necessary to keep the cells in suspension, circulate cells within the non-homogeneous field, and maintain low temperature during the relatively long exposure time (20-40 minutes). The actual dose of neutrons was estimated by activation of several iron foils ( $5 \text{ } \mu\text{m} \times 1 \text{ cm} \times 1 \text{ cm}$ ) taped to the front and back surface of the flask; the  $^{56}\text{Fe}(n,p)^{56}\text{Mn}$  reaction was used and the  $^{56}\text{Mn}$  produced was detected by its gamma-ray emission using a calibrated sodium iodide spectrometer. The measurement of dose was done by A. Arneja under the supervision of Dr. W.G. Cross of CRNL. The cross-section for the reaction was taken to be 109 mb at this neutron energy. Fluences were converted to kerma values using the conversion factors of Caswell et al. (1980) by A. Arneja. The mean of the doses to the front and back foils was taken as the dose to the cells. This is not significantly different than the value which would be found by an integration over the distance between the foils, assuming a point source of neutrons.

#### 2.4 Tritiated Thymidine Uptake and Percent Cells in S Phase

One-half ml of HPBL were cultured in RPMI 1640 +

10% FBS ( $1 \times 10^6$  cells/ml) in tightly stoppered 12 x 75 mm culture tubes at 37°C in an air incubator. Con A was added to some tubes at a concentration of 20 µg/ml at time zero. At various times after initiation of the cultures, thymidine uptake was determined in triplicate by adding 1 µCi (37 kBq) (methyl-<sup>3</sup>H)-thymidine (New England Nuclear, 6.7 Ci/mM) and incubating for 60 minutes at 37°C, following which the samples were chilled and stored at -20°C for later analysis. For determination of acid-insoluble radioactivity, tubes were thawed and 0.5 ml of 1 mg/ml bovine serum albumen (BSA) in 0.2% sodium dodecyl sulphate (SDS) was added. One ml of 20% trichloroacetic acid (TCA) was added and the sample was held at 0°C for 30 minutes to allow precipitation of DNA. The precipitate was collected on a pre-wetted (10% TCA, 0°C) GF/C filter under low vacuum. The tube and filter were rinsed with 10 ml of 10% TCA at 0°C. The filter was again washed with 25 ml of 10% TCA at 0°C and then allowed to air dry under high vacuum. The filter was transferred to a glass scintillation vial and 0.2 ml of tissue solubilizer (NCS, Amersham, Searle, Arlington Hts., Illinois) was added to the filter and allowed to act at room temperature for thirty minutes. 5 ml of a toluene-based scintillator with acetic acid (1 l toluene, 4g Omnifluor (New England Nuclear), 1 ml glacial acetic

acid) was added and vials were counted in a scintillation counter for 10 minutes.

For determination of the percentage of cells in S phase, 0.5 ml cultures were used. The cells were pulsed with 2.5  $\mu$ Ci (92.5 kBq) of  $^3$ H-TdR (6.7 Ci/mM thymidine in RPMI 1640 and 10% FBS was added) and the cells were further incubated for 15 minutes.  $5-8 \times 10^4$  cells (100  $\mu$ l) of cells were sedimented onto a glass microscope slide with a cytocentrifuge (Cytospin, Southern Shandon, Runcorn, Cheshire) at 1500 rpm for 4 minutes. The slides were air dried and fixed in ethanol-acetic acid (3:1, vol/vol) for 2 minutes at room temperature. They were then washed twice in 95% ethanol for 5 minutes and air dried.

The slides were dipped in Kodak NTB-2 Nuclear Track Emulsion (diluted 1:1 with H<sub>2</sub>O) for two seconds at 45°C under a Wratten series 2 safelight. The slides were placed vertically to allow the emulsion to drain and dry following which they were placed into a light-proof box with silica gel dessicant for exposure at -20°C. After 2-5 days, slides were developed in Kodak D-19 developer made to half strength at 19°C for 2-5 minutes, stopped in distilled water for 10 seconds and fixed in Kodak fixer for 8 minutes. After washing in distilled water twice for 5 minutes, slides were stained immediately in 1% Giesma R66

at pH 6.8-6.9 for 10-12 minutes at 19°C. Destaining was carried out in dilute acetic acid solution (one drop of glacial acetic acid in 100 ml H<sub>2</sub>O) at 10°C for 1-2 seconds and then slides were washed twice with distilled water for 5 minutes. Finally, the slides were air dried and scored under a microscope. Low power (300X) was sufficient to visualize any silver grains which had developed in the emulsion. Any cell which had more than three grains directly over the nucleus was scored as being in S-phase.

#### 2.5 Assay for Proteinase K Activity

Proteinase K was used to digest any possible protein-DNA cross-links in an experiment more fully described in the Results section. It was necessary to show that the proteinase K was functional under the experimental conditions used. Cell lysate in solution B and C (see FADU) was incubated in the presence or absence of proteinase K (25-100 µg/ml) for various periods of time at 37°C and then chilled to 0°C and analyzed for acetone-soluble protein. 100 µl of the digestion mixture was added to 300 µl of acetone and held at 0°C for 30 minutes. The precipitate which formed was removed by centrifugation (2 minutes at 12,000 g) in an Eppendorf microfuge and 200 µl of water and 1.0 ml of borate buffer (0.3 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, pH 9.2, 0.1% Triton X-100) were added to each sample. For measurement

of total protein in the lysate, 10-100  $\mu$ l of lysate (containing 1-50  $\mu$ g of protein) and water (0.5 ml, final volume) was mixed with borate buffer. 0.5 ml of a fluorescamine solution (Fluoram, Roche, Nutley, NJ, 15 mg in 100 ml of acetone) was quickly added to the samples with vortexing. 0.5 ml of H<sub>2</sub>O was added and the fluorescence of the resulting solution read (excitation 390 nm, emission 475 nm). Bovine serum albumen, lysozyme and proteinase K were used to calibrate the assay. Without proteinase K, 20% of the cell lysate protein remained acetone-soluble during 90 minutes of incubation at 37°C; 100% of the cell lysate protein became acetone soluble within 10 minutes during incubation with 100  $\mu$ g/ml proteinase K.

## 2.6 Fluorescence Analysis of DNA Unwinding (FADU)

FADU was developed by H.C. Birnboim and J.J. Jevcak (1981) for the detection of strand breaks in total WBC. This method was found to be directly applicable to HPBL. The method is described here only briefly. The letter designations for the solutions are those used in the original paper. Cells ( $5-15 \times 10^6$ ) were suspended in 2.7 to 3.4 ml of a buffered isotonic solution (B) containing inositol as the osmotic stabilizer. This suspension was distributed by aliquots (0.2 ml) to 12

disposable glass tubes at 0°C which were labelled in groups of four as T (for total double stranded DNA), B (for blank, containing no double stranded DNA) and P (for partially unwound DNA). To each was added 0.2 ml of a lysing solution (C) containing urea and SDS. Cell lysis and chromatin disruption was allowed to proceed for 10 minutes at 0°C. The cell lysate at this point in solutions B + C was used to test for the effects of proteinase K as described elsewhere in this thesis. To the P and B tubes, two alkaline solutions were added (0.1 ml D + 0.1 ml E) to raise the pH sufficiently for DNA unwinding to begin. The samples were incubated at 0°C for 30 minutes to allow the alkali to diffuse into the viscous lysate. The contents of the B tubes were sonicated for 1-2 seconds to break DNA into short fragments. The P and B tubes were incubated at 15°C for 60 minutes (or other times as discussed in the Results) to allow partial (P) or complete (B) unwinding of DNA to occur. The tubes were then cooled to 0°C and 0.4 ml of solution (F) containing glucose was added to lower the pH sufficiently to stop DNA unwinding. The T tubes were treated differently. The buffering solution (F) was added before the alkaline solutions (D and E). Thus the pH was never high enough to allow any denaturation of the DNA. T, P and B tubes

were brought-up to room temperature after the addition of the buffering solution. The contents of each tube were briefly sonicated to render the samples homogeneous and 1.5 ml of a solution containing ethidium bromide (EtBr) (solution G) was added. The fluorescence of the tubes was read at room temperature in a Farrand Mark I spectrofluorometer (excitation 520 nm; emission 590 nm). The amount of double-stranded DNA,  $D$ , is given by  $(P-B) \div (T-B) \times 100$ . The quadruplicate values of P, T and B are averaged for each treatment to calculate a single D value. Unirradiated samples are designated  $D_c$ ; irradiated,  $D_r$ .

As will be discussed more fully in a later section, the data were converted to a parameter  $(-\log(D_r/D_c) \times 100)$  which appeared to be directly proportional to the dose. This parameter is used for discussion and can be considered to be the primary measurement of damage to DNA.



## CHAPTER III

### Results

#### 3.1 Pilot Experiments

##### 3.1.1 Purified HPBL Compared to Total WBC

At the beginning of this project FADU had been developed for use in detecting radiation damage to WBC in whole blood and had, at that time, been used with no other cell system. It was therefore necessary to carry out several pilot experiments before the major experiments could be carried out. First, it was necessary to determine the suitability of this assay for use with HPBL in culture medium.

Experiments were done to compare the response of WBC to 1.0 Gy of  $^{60}\text{Co}$  gamma-irradiation at  $0^{\circ}\text{C}$  in air either treated as whole blood or as WBC in culture medium (RPMI 1640 plus 10% FBS). Each treatment was done with  $15 \times 10^6$  cells suspended at  $5 \times 10^6$  cells/ml. The results shown in Table 1 indicated that DNA damage was the same when irradiation was carried out in either medium. Purified HPBL in culture medium were then assayed in a similar manner. These results, also shown in Table 1, indicated that HPBL have a similar response to 1.0 Gy of  $^{60}\text{Co}$  gamma-irradiation, but unirradiated HPBL seem to have more initial DNA damage (i.e. lower  $D_c$  value) than freshly isolated WBC.

TABLE 1

Response of White Blood Cells Irradiated

With 1.0 Gy of  $^{60}\text{Co}$   $\gamma$ -rays

As Blood or in Culture Medium

DNA Strand Breaks  
( $-\log(D_r/D_c) \times 100$ )

Unirradiated Control  
Values ( $D_c$ )

Medium  
of Irradiation

Cell Type

Experiment 1

WBC

Blood

85.6

14.6

WBC

RPMI 1640  
+10% FBS

83.1

15.2

Experiment 2

HPBL

RPMI 1640  
+10% FBS

68.4  $\pm$  2.4<sup>a</sup>

18.7  $\pm$  1.6<sup>a</sup>

<sup>a</sup> SEN (n=4)

### 3.1.2 Minimum Cell Number

As one aim of this research was to determine the repair capabilities of lymphocytes after low doses, it was apparent that, if  $15 \times 10^6$  cells were to be used for each data point, substantial numbers of cells would be needed. Since the volume of blood that can be taken from one donor is limited, I next determined if fewer cells could be used. HPBL were cultured 16 hours at  $2 \times 10^6$ /ml at  $37^\circ\text{C}$  in an air incubator, cooled to  $0^\circ\text{C}$  and irradiated with 1.0 Gy of  $^{60}\text{Co}$  gamma-rays. The cells were then distributed to tubes to give a final cell number of 15, 5 or  $3 \times 10^6$ . They were then centrifuged at  $0^\circ\text{C}$ , resuspended in 2.7 ml of solution B and FADU was carried out. The results (Table 2) indicated that the cell number has no discernible effect on the unwinding of DNA during incubation at  $15^\circ\text{C}$  in alkaline solution. In subsequent experiments between 5 and  $10 \times 10^6$  cells were used for each data point. It was found that higher cell numbers tended to give more stable fluorescence readings and somewhat less scatter in the replicates.

### 3.1.3 Maximum Holding Time for Irradiated Cells

At the outset of this project there was little information as to the ability of HPBL to repair radiation

TABLE 2

Effect of Cell Number on the Response of  
 HPBL to 1.0 Gy of  $^{60}\text{Co}$   $\gamma$ -rays

Number of Cells per Curve	Unirradiated Control Value ( $D_C$ )	DNA Strand Breaks ( $-\log(D_T/D_C) \times 100$ )
$15 \times 10^6$	74.8	17.96
$5 \times 10^6$	74.0	14.16
$3 \times 10^6$	76.8	15.22

damage and the effect of lengthy incubations on the ability to visualize repair was not known. An appropriate protocol for holding the cells after irradiation and incubation which would arrest any repair activity and also show a minimum of damage to the cells was needed. Two experiments were done to follow changes in DNA damage after 1.0 Gy of  $^{60}\text{Co}$  gamma-irradiation during incubation at different temperatures (Table 3). The first experiment using total WBC showed that after 20 minutes of incubation the amount of damage decreased markedly only above  $15^{\circ}\text{C}$ . If purified HPBL are held for 24 hours at these temperatures the estimate of damage also drops substantially; at  $15^{\circ}\text{C}$  and above no difference between irradiated and control cells was detectable. The  $D_c$  values fell after long incubation at the lower temperatures, indicating that low temperature may lead to some damage of cellular DNA. It appeared that holding irradiated cells below  $4^{\circ}\text{C}$  for short periods of time led to little or no rejoining of strand breaks and probably no change in the  $D_c$  values. On the basis of these results the following protocol for carrying out repair experiments was developed. Following irradiation at  $0^{\circ}\text{C}$ , cells were incubated for various times up to 80 minutes at  $37^{\circ}\text{C}$ . Unirradiated cells were

TABLE 3

## Effect of Temperature on Leukocytes in Culture Medium

After 1.0 Gy. of  $^{60}\text{Co}$   $\gamma$ -rays

Time of Incubation	Temperature of Incubation ( $^{\circ}\text{C}$ )	Unirradiated Control Value ( $D_C$ )	DNA Strand Breaks ( $-\log(D_T/D_C) \times 100$ )
Experiment One (WBC)			
20 minutes	0	81.2	12.9
20 minutes	4	80.8	11.7
20 minutes	15	79.8	10.9
20 minutes	37	79.3	3.84
Experiment Two (HPBL)			
0 hours	0	71.7 $\pm$ 3.8 <sup>a</sup>	18.7 $\pm$ 1.8 <sup>a</sup>
24 hours	0	43.0	12.2
24 hours	4	59.1 $\pm$ 14.1	10.5 $\pm$ 2.1
24 hours	15	56.2	-0.5
24 hours	37	61.1	2.9

<sup>a</sup>SEM, n=3

incubated in parallel. At each time point, cells were rapidly chilled to 0°C and held until the longest incubation was complete. Then all samples were centrifuged (10 min., 0°C, 400 g) and suspended in solution B for FADU. The  $D_r$  values for each time point were then compared to the average of all  $D_c$  values in that experiment.

#### 3.1.4 Stability of HPBL in Culture

The stability of HPBL in culture was also investigated as in other culture systems the behaviour of the cells can vary with culture time (Dougherty et al., 1980). A long-term culture of HPBL was done to determine the variation of the baseline of unirradiated HPBL ( $D_c$ ) with culture age. After dropping somewhat in the first 10 hours, the control values ( $D_c$ ) remained steady, rising slightly towards the end of the culture period (Table 4). It was decided that cells could be held 16 hours after isolation as is standard practice in other assay systems using HPBL, before experimentation.

#### 3.1.5 Effect of Anticoagulant or Culture Conditions

The initial  $D_c$  value presumably reflects the integrity of DNA within cells. Two factors which can easily be controlled are the anticoagulant used in the isolation of HPBL and the type and concentration of serum

TABLE 4

$D_c$  Versus Time in Culture Medium

<u>Time at 37°C (hours)</u>	<u>Unirradiated Control Value (<math>D_c</math>)<sup>a</sup></u>
0	68.02 <sup>b</sup>
9	66.0±0.6
22	63.9±1.1
35	64.7±0.4
52	67.9±3.1
58	69.1 <sup>b</sup>

<sup>a</sup> ± range, two determinations

<sup>b</sup> single determination

used in the culture medium. In one experiment (Table 5) the  $D_c$  values were the same at both 10 and 15% FBS in the culture medium. In a second experiment 10% autologous serum was compared to 10% FBS. The difference between the two seemed minimal. Use of EDTA (3.6 mM) or heparin (60 IU/ml) as anticoagulant also had no effect. It was decided that heparin be used as the anticoagulant and that the cells be cultured in 10% FBS. From this table it can be seen that there is little variation within an experiment among  $D_c$  values. However, among experiments a large variation in this baseline is seen. There has been no apparent correlation between  $D_c$  and the initial estimate of damage for 1.0 Gy of  $^{60}\text{Co}$  gamma-rays, nor on the rate of repair. These anomalies may be due to donor-to-donor variation or an as yet undiscovered inconsistency in the experimental protocol.

### 3.2 Experiments with $^{60}\text{Co}$ Gamma-rays

#### 3.2.1 Dose Response

From the preliminary experiments it was known that 1.0 Gy of  $^{60}\text{Co}$  gamma-irradiation induced a certain amount of damage which did diminish with time after incubation at 37°C. In order to study more closely the kinetics of repair it was necessary to have a measure of DNA strand breaks which was linear with dose. Other workers have shown,

TABLE 5

Effect of Culture Conditions on  $D_c$  of HPBL

<u>Culture Conditions<sup>a</sup></u>	<u>Unirradiated Control Value (<math>D_c</math>)</u>
Experiment One	
10% FBS <sup>b</sup>	68.3
15% FBS <sup>b</sup>	68.4
Experiment Two	
10% FBS <sup>b</sup>	81.1
10% FBS <sup>c</sup>	81.4
10% Autologous Serum <sup>b</sup>	78.6

<sup>a</sup>Cells cultured 16 hours at 37°C.

<sup>b</sup>Heparin (60IU/ml) used as anticoagulant.

<sup>c</sup>EDTA (3.6 mM) used as anticoagulant.

as discussed in the Introduction, that single strand breaks and alkali-labile lesions are put into DNA in a manner directly proportional to dose. As is shown in the Materials and Methods section, D values are calculated for unirradiated ( $D_c$ ) and irradiated ( $D_r$ ) samples. Initially, the method used to transform D values to a quantity which could be correlated to DNA damage was to take the arithmetic difference between  $D_c$  and  $D_r$ . Below 1.0 Gy the dose response is close to linear (Birnboim and Jevcak, 1981). In later experiments, when higher doses were used it became clear that the response was not linear. A dose response for  $^{60}\text{Co}$  gamma-irradiation of HPBL in air at  $0^\circ\text{C}$  was done in the range of 0.1 to 4.0 Gy. The line in Fig. 1 shows the results presented as  $D_c - D_r$  vs. dose. A definite curvature of the response line can be seen. When the data are transformed by the formula,  $-\log (D_r/D_c) \times 100$ , (described by Birnboim and Jevcak, 1981) the response becomes very close to linear ( $r = 0.9985$ ,  $p \leq 0.01$ ). This is shown in Fig. 2. It is clear that as little as 100 mGy can be detected. Other points about this line are taken from individual experiments and have been included to indicate the variation of the data obtained. Other data presented on this figure will be discussed later.

Figure 1 DNA strand breaks induced in HPBL by  $^{60}\text{Co}$  gamma-rays presented as the arithmetic difference between control ( $D_c$ ) and irradiated ( $D_r$ ) values.

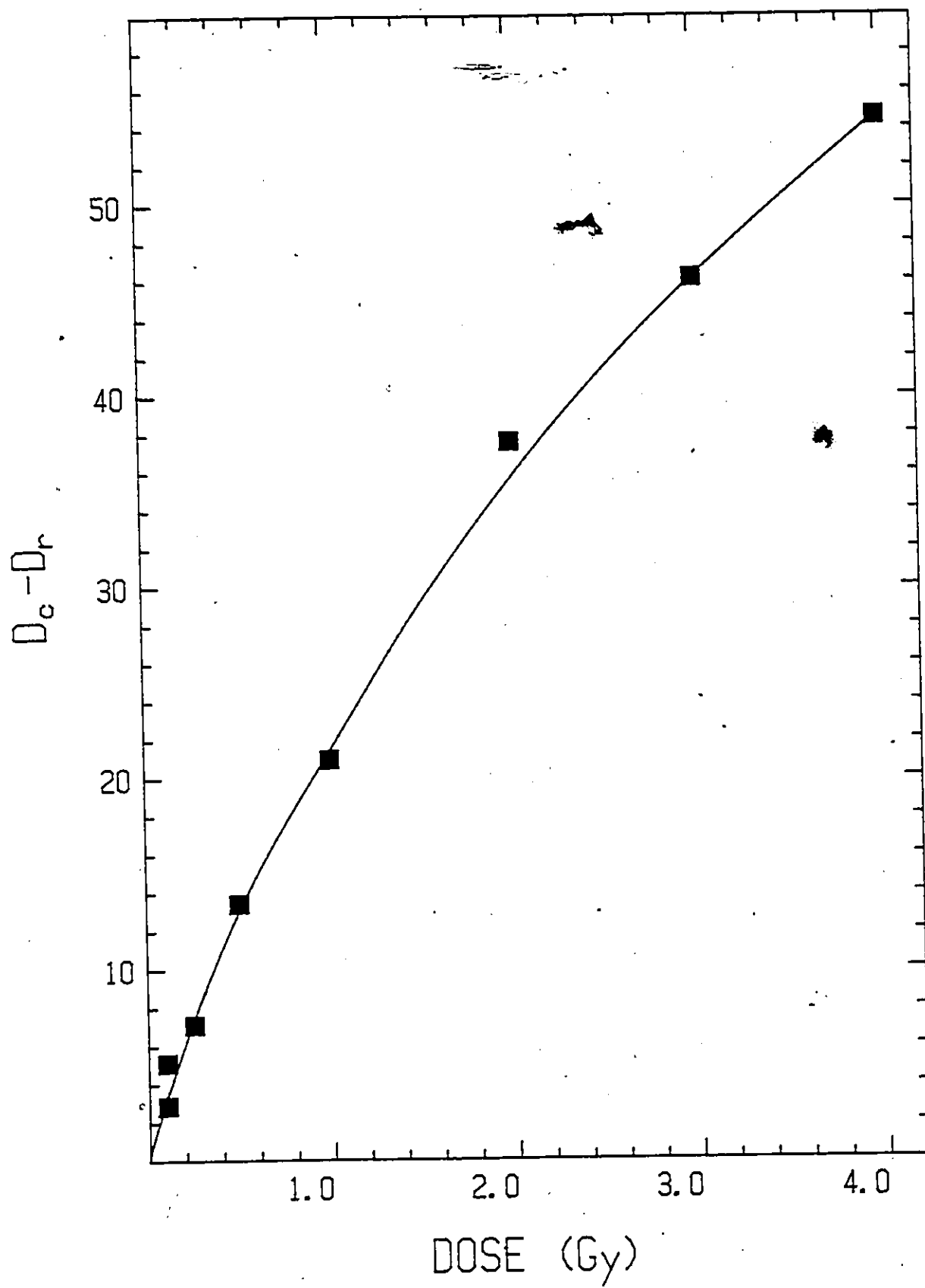
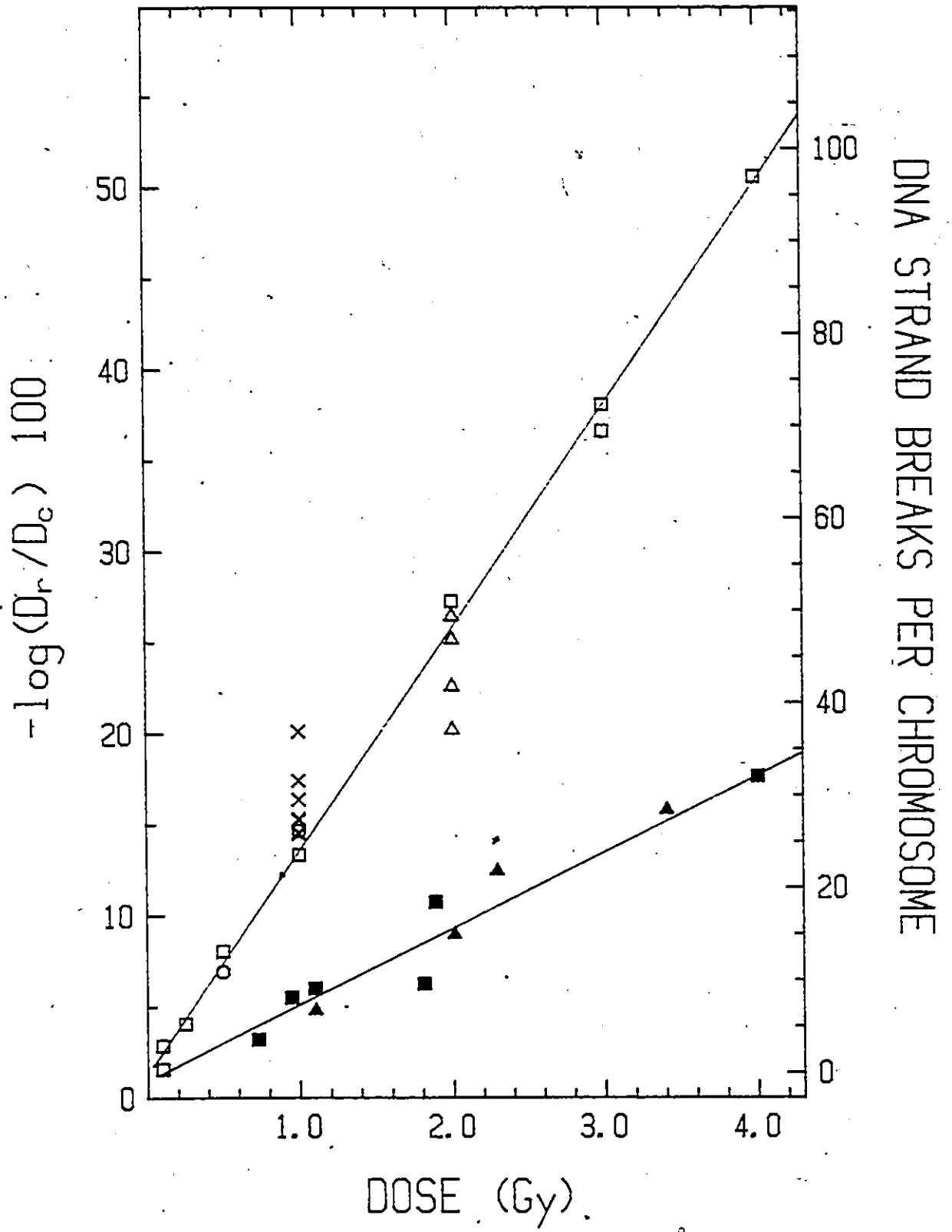


Figure 2 DNA strand breaks induced in HPBL by  $^{60}\text{Co}$  gamma-rays or 14.6 MeV neutrons at  $0^\circ\text{C}$ . The left ordinate shows the ratio of DNA unwinding rates for irradiated and unirradiated samples as measured by FADU. The right ordinate shows the average number of breaks per human chromosome this represents. Different symbols represent different groups of experiments carried out over a six-month period.  $^{60}\text{Co}$  gamma-rays (open symbols); 14.6 MeV neutron (closed symbols). Regression lines were drawn through data points from single experiments indicated by (  $\square$  ) and (  $\blacksquare$  ).



### 3.2.2 Conversion to DNA Strand Breaks per Chromosome

The efficiency of break induction in air for  $^{60}\text{Co}$  gamma-rays is usually reported to be about one break per human chromosome per 38 mGy (Ormerod, 1976). The slope of the dose response of Fig. 2 for the  $-\log (D_r/D_c) \times 100$  transformation is 12.27.<sup>2</sup> Therefore, 1.0 Gy will give  $-\log (D_r/D_c) \times 100 = 12.27$ , which can be converted directly to 26.3 strand breaks per chromosome.<sup>3</sup>

### 3.2.3 Rejoining of Strand Breaks After Gamma-Irradiation

Workers with other cell types, using cells labeled with radioactive markers and higher doses than those used in this report, have found rapid and nearly complete rejoining of strand breaks in  $^{60}\text{Co}$  gamma- or X-irradiated cells. Available reports on the repair capabilities of

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<sup>2</sup>These calculations assume that the non-zero intercept of this line is due to some variation in the  $D_r$  value, relative to the  $D_c$  values in this experiment. There is some indication that this may not be so and that the response is non-linear at low doses. If this is so, then the calculations used above may tend to overestimate the number of breaks per chromosome by one or two, which is near the limit of the assay system's sensitivity. (See Birnboim and Jevcak, 1981.)

<sup>3</sup>Early experiments were done with P values in triplicate taken at 60 and 75 min. of incubation for DNA unwinding at 15°C instead of in quadruplicate at only 60 min. This transformation is not equivalent for these experiments and those which are presented with damage as strand breaks have been scaled accordingly, using the same value of one DNA strand break per chromosome per 38 mGy of  $^{60}\text{Co}$  gamma-rays.

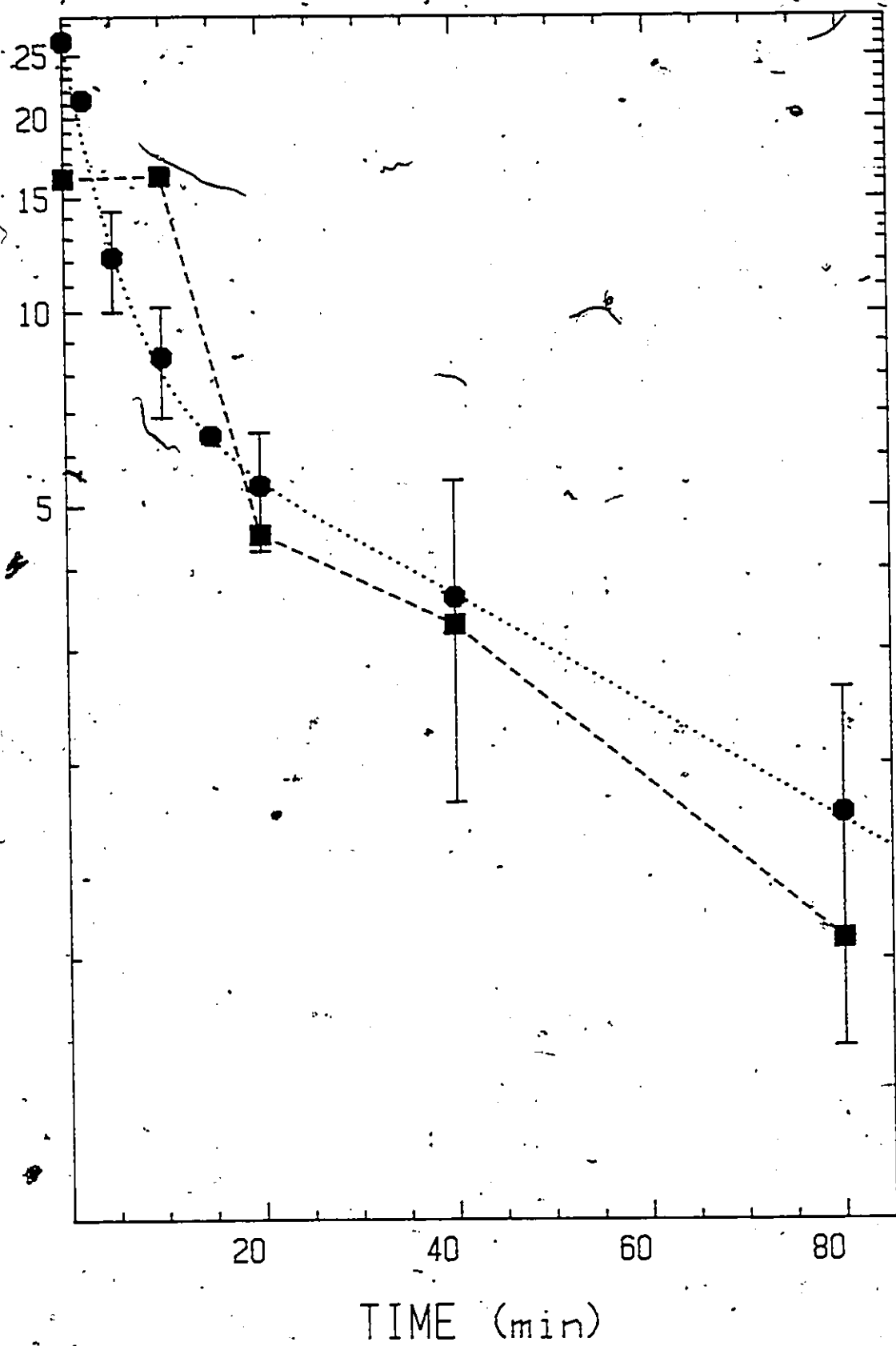
HPBL are generally inconclusive. It was considered of interest to determine the rejoining capabilities of HPBL after low doses (1.0-2.0 Gy) of  $^{60}\text{Co}$  gamma-irradiation in air. Several experiments were carried out 1.0 Gy to address this problem. Cell suspensions were irradiated in glass tubes at  $0^{\circ}\text{C}$  and those which were to undergo repair were warmed quickly to  $37^{\circ}\text{C}$  in a waterbath and incubated for various times, then chilled rapidly to  $0^{\circ}\text{C}$ . After the incubation period had been completed for all samples the cells were analyzed by FADU. As is evident from the results shown in Fig. 3, HPBL can rejoin strand breaks rapidly and efficiently. The kinetics of repair are biphasic; a rapid phase was complete after 20 minutes and by 80 minutes less than 10% of the initial damage remained. Biological and physical processes commonly follow exponential kinetics. Körner et al. (1978) had done a graphical analysis of data from a study of the rejoining of DNA strand breaks in Chinese hamster cells, assuming biphasic kinetics of repair. As the data were similar to those of Körner et al., it seemed appropriate to perform a non-linear regression analysis of the data using the formula:

$$Y = A \exp(-t \ln 2 / \lambda_1) + (100-A) \exp(-t \ln 2 / \lambda_2)$$
 where Y is the percent damage remaining after time, t; A is the

Figure 3 Rejoining of DNA strand breaks in HPBL at 37°C after 1.0 Gy of  $^{60}\text{Co}$  gamma-rays (●) or 1.1 Gy of 14.6 MeV neutrons (■). Error bars are SEM (n=4-6) for  $^{60}\text{Co}$  gamma-ray experiments.



DNA STRAND BREAKS PER CHROMOSOME



fraction of the total which is rapidly decaying, as a percent;  $(100-A)$  is the initial fraction of the slowly decaying parameter, as a percent;  $\lambda_1$  and  $\lambda_2$  are their respective half-lives, i.e. the half-time of rejoining of the two types of strand breaks. The half-time of the rapidly rejoined component, which represents 69.5% (60.1-78.9,  $p = 0.05$ ) of the total damage, is 2.8 min. (2.05-4.33,  $p = 0.05$ ). That of the slowly rejoined component (21.1-39.9%,  $p = 0.05$ ) is 35.0 min. (24.4-61.4,  $p = 0.05$ ).

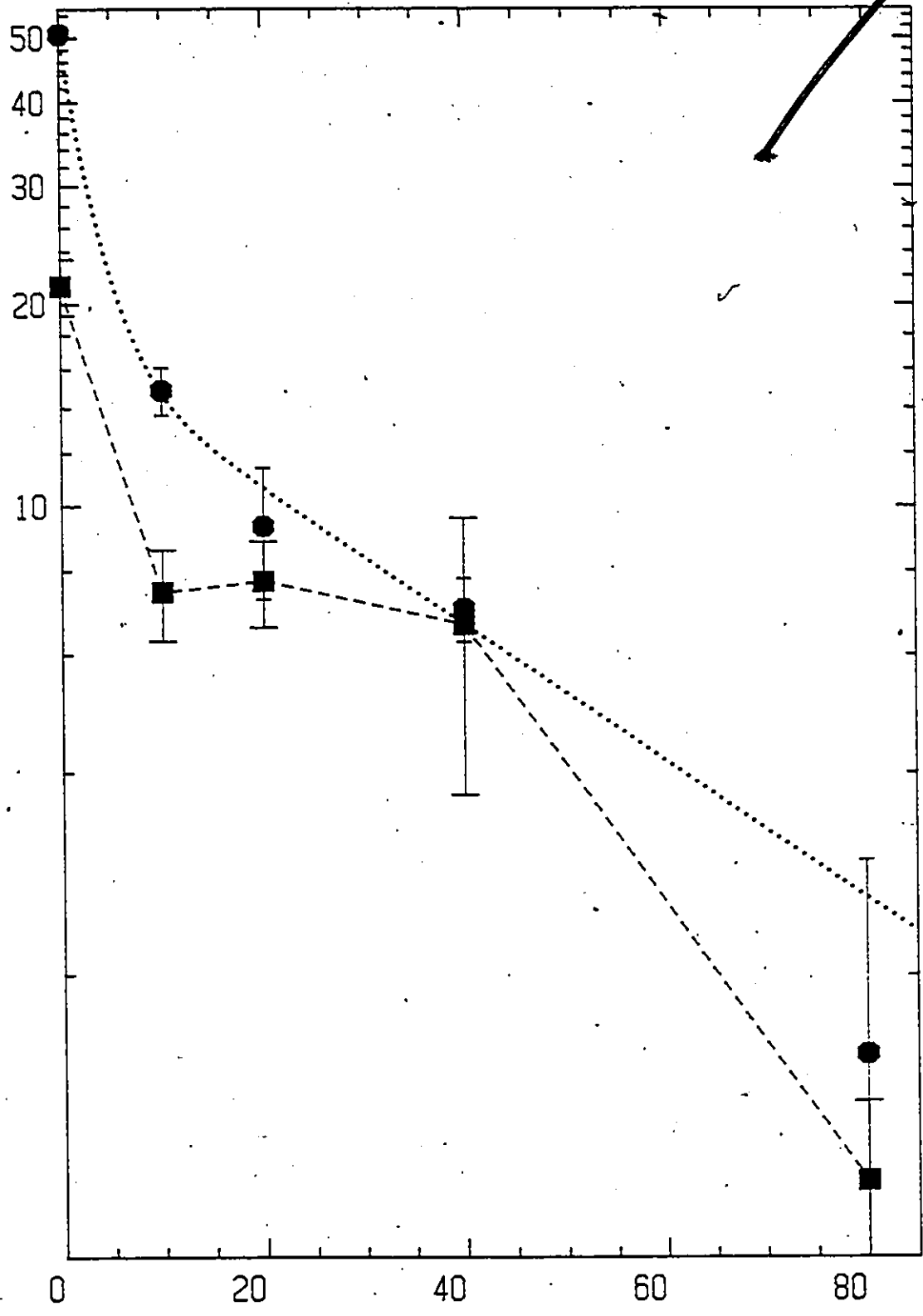
Four similar repair experiments were carried out on HPBL using a dose of 2.0 Gy of  $^{60}\text{Co}$  gamma-rays (Fig. 4). The results are similar to those for the 1.0 Gy experiment; the parameters differed only slightly ( $A = 66.6\%$ ,  $\lambda_1 = 2.2$  min.,  $\lambda_2 = 29.7$  min.). There were too few data points to give reasonable fiducial limits. The curves drawn through the experimental points for the two gamma-repair experiments were calculated from the parameters given above. Again other data presented in these figures will be discussed elsewhere.

#### 3.2.4 Rejoining of Strand Breaks: Effect of Culture Time

As there are reports of much longer half-times of repair than those given here, it was of interest to speculate

Figure 4 Rejoining of DNA strand breaks in HPBL at 37°C after 2.0 Gy of  $^{60}\text{Co}$  gamma-rays (●) or 2.0-2.3 Gy of 14.6 MeV neutrons (■). Error bars are SEM (n=4) for  $^{60}\text{Co}$  gamma-rays or the range for the 14.6 MeV neutrons (n=2). 2.3 Gy experimental data were normalized to 2.0 Gy.

DNA STRAND BREAKS PER CHROMOSOME



TIME (min)

on a possible cause for these differences. One could be that the time in culture of HPBL affects their competence to rejoin strand breaks. Some cell types markedly alter their ability to repair DNA damage with time in vitro (Dougherty et al., 1980). A pilot study was done to test for any change in the rejoining capability of HPBL after two different times in culture, 1 hour and 72 hours. After these times, HPBL were irradiated and placed into culture medium at 37°C for various periods, after which they were rapidly chilled to 0°C and analyzed by FADU. Fig. 5 shows that after 80 minutes the majority of the strand breaks are rejoined. The initial phase of repair is not as rapid as that for cells cultured for 16 hours, but this is more likely to be due to the slower warming of the flasks to 37°C in the air incubator. It is apparent, though, that there is no gross difference in the ability of HPBL cultured for 18 hours or one hour to rejoin strand breaks.

As HPBL are cultured for longer periods they may begin to deteriorate and die. Two experiments were done to assay strand rejoining in HPBL after 72 hours of culturing at 37°C in air. As is shown in Fig. 6 there is perhaps a greater variability in the values from time-point to time-point but these HPBL do rejoin half of the strand

Figure 5 Rejoining of DNA strand breaks in HPBL at 37°C after 1.0 Gy of  $^{60}\text{Co}$  gamma-rays after one hour in culture (■). Dotted line is curve for the same experiment with HPBL cultured for 16 hours (taken from Fig. 3).

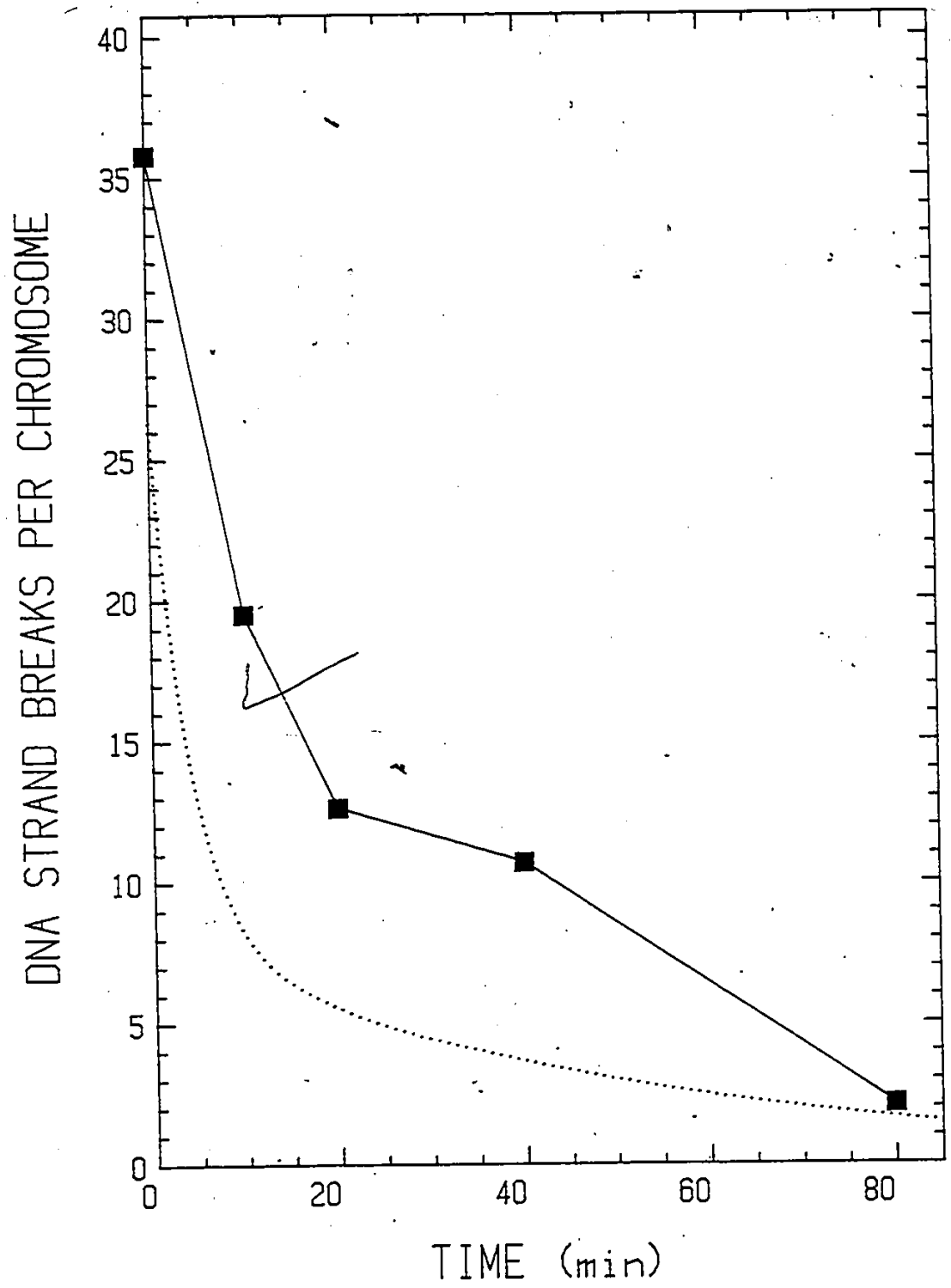
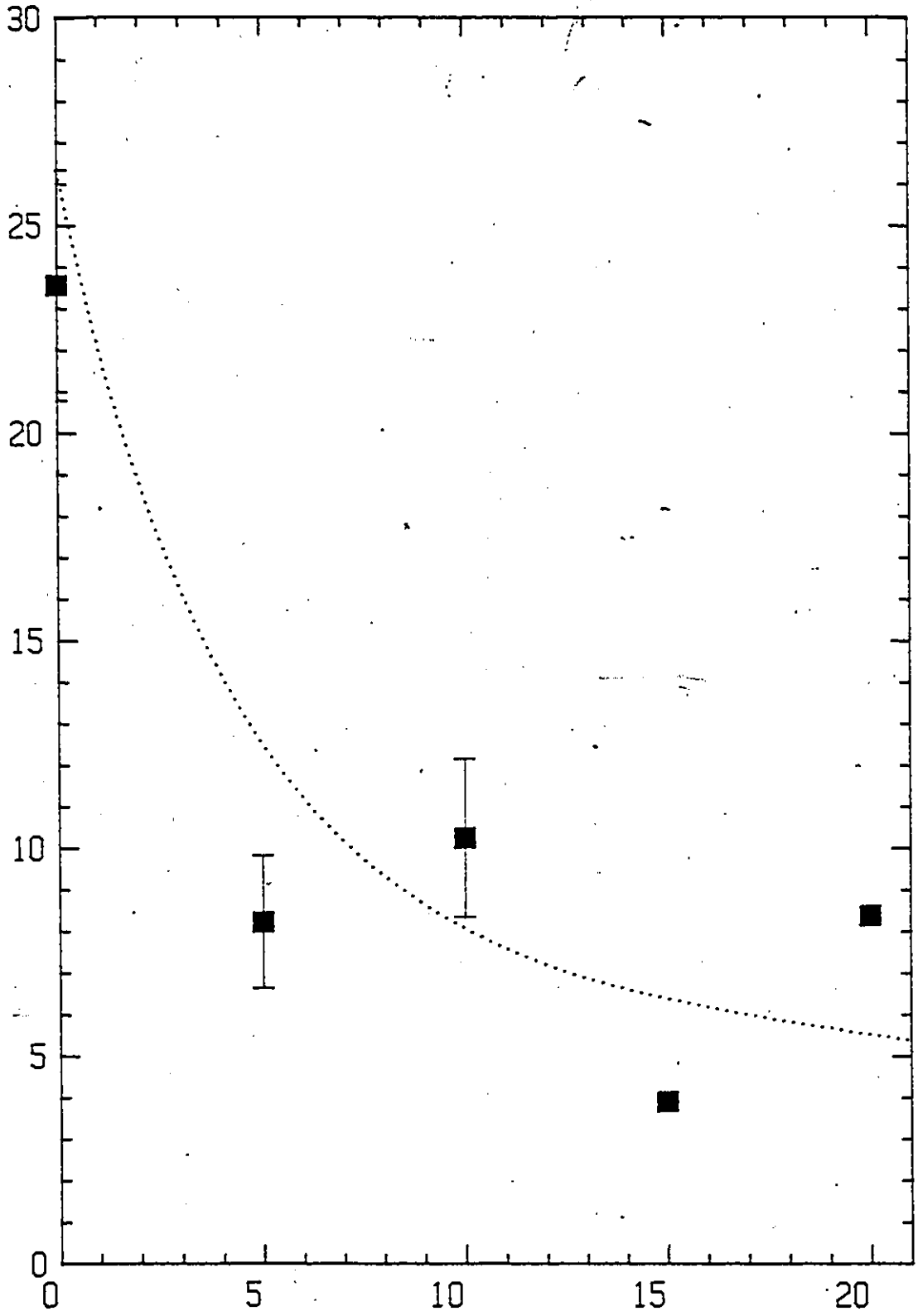


Figure 6 Rejoining of DNA strand breaks in HPBL at 37°C after 1.0 Gy of <sup>60</sup>Co gamma-rays after 72 hours in culture (■). Error bars are range for 0, 5, 10 and 20-minute points (n=2). Dotted line is curve for the same experiment with HPBL cultured for 16 hours (taken from Fig. 3).

DNA STRAND BREAKS PER CHROMOSOME



TIME (min)

breaks introduced within five minutes at 37°C. There is no obvious difference in the ability of these cells to rejoin strand breaks from that of HPBL cultured for 16 hours.

### 3.3 Verification that HPBL are Normal Resting Cells

It is possible that the culture conditions used for these experiments somehow stimulated the cells to undergo some sort of activation, perhaps similar to that which HPBL undergo when mitogenically stimulated with a lectin such as PHA (phytohaemagglutinin) or Con A (Concanavalin A). This was tested using the percent of cells in S phase as a measure of cell activation. The results (Table 6) show that these cells were almost completely inactive, having a very low complement of S phase cells. The resting cells were of uniform, small size and densely staining in neutral Giemsa; there were no blasts except for the few cells that were in S phase. HPBL in this culture system underwent full mitogenic transformation with 25µg/ml of Con A (Table 6). The percentage of cells in S phase increased to over 30. <sup>3</sup>H-TdR uptake experiments were done, but no complete uptake curve was carried out. The stimulation index at 52 hours was approximately 35. This indicates that these cells were normal, resting but competent lymphocytes under these

TABLE 6

## Radioautography of Resting and Stimulated HPBL

Time in Culture (hours)	Resting HPBL		Percent in S-Phase <sup>c</sup>	Stimulated HPBL <sup>a</sup>	
	Number in S-Phase <sup>b</sup>	Number in Non-S-Phase		Number in S-Phase <sup>b</sup>	Number in Non-S-Phase
Experiment One					
22	1	1048	0.10±0.10	2	1140
46	7	1041	0.67±0.25	444	559
72	10	1014	0.98±0.31	398	714
Experiment Two					
72	8	1393	0.57±0.2	667	1528
					30.39±0.98

<sup>a</sup>25 µg/ml Con A<sup>b</sup>cell with 3 or more grains<sup>c</sup>±SE

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culture conditions, and that the rapid repair kinetics seen here is not a result of any activation of the cell or any other adverse effect.

#### 3.4 Preliminary Data: Con A Treated HPBL

Many studies have shown that the ability of resting HPBL to carry out several processes, including repair of DNA damage, is different from that of mitogenically stimulated HPBL. It was of interest to compare the rate of rejoining of DNA strand breaks after 1.0 Gy of  $^{60}\text{Co}$  gamma-radiation. Unfortunately, a number of experimental difficulties made any definite conclusions impossible.

The cell suspension could not be reproducibly dispersed due to the clumping of the cells after addition of the mitogen. This caused the baseline to vary as much as 20% or more. To circumvent this  $10 \times 10^6$  HPBL were cultured in  $25 \text{ cm}^2$  flasks for each data point. The reproducibility was only marginally improved. In addition, the variation of the pH of treated cells affected unirradiated control values and the estimate of initial damage. Attempts to eliminate changes in intercellular pH as an experimental variable were unsuccessful. However, the preliminary data (not shown) indicated that HPBL

cultured in the presence of Con A for 72 hours can rejoin DNA strand breaks, but much further experimentation would be needed to demonstrate any difference between resting and stimulated HPBL in their ability to rejoin DNA strand breaks.

### 3.5 Neutron Experiments

#### 3.5.1 Dose Response for 14.6 MeV Neutrons

As has been discussed in the Introduction, 14.6 MeV neutrons have a high RBE compared to  $^{60}\text{Co}$  gamma-rays for many biological effects. The induction or repair of strand breaks in lymphocytes after fast neutron irradiation may help explain this phenomenon. No previous study has been done of the ability of HPBL to rejoin strand breaks induced by 14.6 MeV neutrons. It was considered of interest to know the efficiency of strand break formation and repair following irradiation by 14.6 MeV neutrons as fast neutron facilities are in many medical centres and any information on this matter would be of use.

A dose response of HPBL for 0-4 Gy of 14.6 MeV neutrons is shown in Fig. 2. The response of  $-\log(D_T/D_C) \times 100$  was linear with dose, the regression line having a slope of 4.19. This is  $0.34 \pm 0.05$  of the slope of the  $^{60}\text{Co}$  gamma-ray dose response. It appears that fast neutrons give fewer breaks in spite of their being a high LET form

of radiation. There are several reasons that this may be so, and some will be presented in the discussion. One particular possibility was amenable to experimental testing and this was therefore explored further. Higher LET radiations may give a greater proportion of other types of DNA damage, one type in particular being cross-links. One can consider four classes of cross-links, any of which might decrease the rate of DNA unwinding in alkali:

(1) DNA-DNA, (2) DNA-protein-DNA, (3) protein-protein and (4) DNA-protein. Any of these cross-links could significantly impede the rate of unwinding of DNA and lower the estimate of damage. If these cross-links could be specifically broken, then unwinding should proceed more rapidly. Cross-links involving protein can be removed by digestion with a protease. Proteinase K has been used for this purpose in another assay system, the alkaline elution assay of Kohn (1979), where the proteins bound to the DNA are thought to inhibit the unwinding necessary to free the DNA from the filter or to be actually partly adsorbed to the filter itself and thus retard the elution of freed DNA molecules. The first experiment (Table 7) is a digestion of protein in the lysis solution (B + C, see Materials and Methods) with proteinase K before the unwinding step. The apparent number of breaks remained constant and low throughout the

TABLE 7

## Effect of Proteinase K on DNA Strand Break Yield

Dose (Gy)	Time <sup>a</sup> (min.)	Temperature (°C)	Proteinase kb	Unirradiated Control Value (D <sub>C</sub> )	Apparent DNA Strand Breaks (-log(D <sub>r</sub> /D <sub>C</sub> )x100)	Expected Value if Same Dose Given as <sup>60</sup> Co Gamma-rays <sup>c</sup>
Experiment One						
1.78	0	37	+	81.5	7.5	21.8
(Neutrons)	30	37	+	79.6	6.1	21.8
	60	37	+	80.2	7.3	21.8
	90	37	+	82.0	7.1	21.8
Experiment Two						
1.80	0	0	-	82.2	7.9	22.1
(Neutrons)	0	0	+	84.1	4.7	22.1
	90	0	-	84.3	7.0	22.1
	90	0	+	82.3	7.9	22.1
	90	37	-	80.7	5.6	22.1
	90	37	+	83.0	7.6	22.1

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<sup>a</sup>Incubation in B-C lysate (see Materials and Methods).<sup>b</sup>500 µg/ml in B-C lysate<sup>c</sup>from Fig. 1, 1 Gy 12.27

digestion period. If protein cross-links were responsible for the large difference between the response to fast neutrons and  $^{60}\text{Co}$  gamma-rays, then the value,  $-\log (D_r/D_c) \times 100$ , would have risen to 21.8. In a second experiment an incubation with or without proteinase K in the lysis solution was carried out over 90 minutes at  $37^\circ\text{C}$  or  $0^\circ\text{C}$ . There is some scatter of values, but it is clear that the values of the treated samples are not substantially higher than those for their untreated counterparts, nor are they near the value of 22.1 which would be found after a dose of 1.8 Gy of  $^{60}\text{Co}$  gamma-rays. Appropriate experiments were carried out to ensure that the proteinase K was capable of digesting the cell lysate proteins under the incubation conditions used, as described in Materials and Methods.

Cross-linking agents have been shown to decrease the apparent yield of gamma- or X-ray induced strand breaks in the alkaline elution method of Kohn (1979). If cells are treated with, for example, nitrogen mustard, and then irradiated with gamma or X-rays at  $0^\circ\text{C}$  the number of breaks seen is lower than what would be expected if the damage of the two agents acted independently. That is, the small number of breaks introduced by the bifunctional alkylating agent alone and the breaks introduced by radiation are not

additive if the two agents are administered together. Two experiments (Table 8) were done to determine if fast neutrons and  $^{60}\text{Co}$  gamma-rays were additive in their apparent strand break yield. In each experiment the data from samples which were irradiated with only one form of irradiation were used to predict the number of breaks which would ensue if the damages from the respective radiation types did not interact. The results show that there is no detectable difference between the predicted and actual values found for the sum of the two irradiations. Although cross-linking has not been ruled out as the mechanism for the lower than expected yield of strand breaks as assayed by FADU after neutron irradiation, neither experiment (proteinase K or neutron + gamma-ray) provided positive evidence implicating cross-links as the explanation for the observed results.

### 3.5.2 Rejoining of Strand Breaks after 14.6 MeV Neutron Irradiation

Three experiments were undertaken to determine the ability of HPBL to rejoin strand breaks after 14.6 MeV neutron irradiation. The results are shown in Fig. 3 and 4. In the first experiment, a dose of 1.1 Gy was used. HPBL did rejoin strand breaks induced by neutron irradiation (Fig. 3), but the number remaining at 80 minutes was too low

TABLE 8

Effect of Combined  $^{60}\text{Co}$  Gamma- and 14.6 MeV Neutron  
Irradiation on Strand Break Yield

Gamma Dose (Gy)	Neutron Dose (Gy)	Apparent DNA Strand Breaks $(-\log(D_r/D_c)) \times 100$	
		Observed	Projected (if additive) <sup>a</sup>
Experiment One			
1.0	-	11.8	-
-	1.23	5.0	-
1.0	0.92	14.0	15.8 (11.8+4.0)
1.0	0.38	13.2	14.0 (11.8+2.2)
Experiment Two			
0.5	-	4.6	
-	1.74	4.1	
0.5	2.17	10.9	9.7 (4.6+5.1)

<sup>a</sup>The sum of the contribution of the equivalent gamma-ray dose and the equivalent neutron dose as calculated from the control responses within the experiment.

to give an accurate indication of any residual damage. Two further experiments were done, using higher doses, 2.0 and 2.3 Gy. The doses were not exactly the same since the unfavorable geometry (i.e., a distance of only 5 cm from the target) makes prediction of exact dose difficult. The actual doses could accurately be determined after the irradiation from the activation of iron foils which had been positioned on the sample (see Materials and Methods). The results, Fig. 4, indicate that strand breaks induced by fast neutrons are rejoined rapidly. As in  $^{60}\text{Co}$  gamma-irradiation, kinetics of rejoining appear to be biphasic, the first phase being complete in less than 20 minutes. By 80 minutes the residual damage seen was so low that  $D_T$  was not significantly different from  $D_C$  of control cells and was not higher than that for  $^{60}\text{Co}$  gamma-irradiation at the same time. The disappearance of DNA damage after both types of irradiation follows very similar kinetics after 10-20 minutes of incubation. The principal difference in the curves is the lower amount of initial damage which is rapidly repaired after fast neutron irradiation.

CHAPTER IV

Discussion

4.1 Detection of DNA Strand Breaks in HPBL by FADU

In this project I have used a sensitive new assay, FADU, for the detection of DNA strand breaks in HPBL and have followed the rejoining of breaks after  $^{60}\text{Co}$  gamma-irradiation or 14.6 MeV neutron irradiation. All irradiations were carried out with air equilibrated samples. The yield of DNA strand breaks (Ormerod, 1976), cell inactivation (Tolkendorf, 1978; Broerse et al., 1968) or chromosome aberration induction (Watson and Gillies, 1970) is lower in the absence of oxygen than in its presence. The mechanisms of these oxygen-related effects are not well understood and this discussion will be confined to the effects of irradiation of cells in air. FADU uses an alkaline solution to cause DNA to unwind; the presence of discontinuities in the sugar-phosphate backbone allow unwinding to proceed more rapidly. These discontinuities (strand breaks) arise mainly from the indirect action of ionizing radiation through free radicals (Lohman et al., 1970; Roots and Okada, 1972). DNA strand breaks induced by gamma-irradiation, which are mainly single strand breaks (Ormerod, 1976), are easily detected by the use of FADU (Birnboim and Jevcak, 1981). Double

strand breaks would also be expected to be detected by FADU, since a double strand break is two coincident or nearly coincident single strand breaks on opposite strands of the DNA molecule (Hagen, 1967). The unwinding of DNA from strand breaks in alkali follows complex kinetics (Birnboim and Jevcak, 1981). Single and double strand breaks might be expected to unwind at different rates, but the relative rates of unwinding of the two break types is, at present, unknown. This means that both types of break will be detected in FADU as a drop in the relative fluorescence ( $D_T$ ), but the proportion of breaks constituting each type cannot be distinguished. Since alkali is used in FADU, some types of base or sugar damage and abasic sites may be converted to strand breaks and then detected.

4-Nitroquinoline-1-oxide (4NQO) induces almost exclusively alkali-labile lesions, possibly in the form of apurinic sites. (See Smith and Paterson, 1980.) McLean et al. (1981) have shown that breaks induced by 4NQO are detected by the use of FADU with great sensitivity. This provides evidence that FADU detects alkali-labile lesions. Gamma- or X-irradiation has been shown to induce single strand breaks, double strand breaks and alkali-labile lesions (Ormerod, 1976; Lennartz et al., 1976). FADU would be

expected to detect but not to discriminate among single strand breaks, double strand breaks and alkali-labile lesions.

The number of DNA strand breaks induced by gamma-irradiation has been experimentally established (Ormerod, 1976). The average value is calculated to be 26 total DNA strand breaks per Gy per human chromosome (Myers, 1977). The rate of DNA unwinding, which is measured as a change in fluorescence by FADU, has been correlated with the number of strand breaks induced by <sup>60</sup>Co gamma-rays (Birnboim and Jevcak, 1981). A dose which doubles the rate of DNA unwinding above the baseline rate of unirradiated cells is called the doubling dose. Ahnström and Edvardsson (1978) found a doubling dose of 1.5 Gy of X-ray with Chinese hamster cells using an unwinding technique which they had developed. 1.5 Gy is also the doubling dose found by the use of FADU with cultured HPBL after gamma-irradiation. This means that there are approximately 39 strand breaks per chromosome detected in the DNA of unirradiated HPBL by FADU. These breaks can arise spontaneously from alkali degradation of the DNA or from shearing of DNA during handling of samples. Unwinding presumably proceeds from the ends of linear DNA

molecules. It is possible that there are a certain number of naturally occurring breaks in the DNA, caused by normal enzyme activity or spontaneous degradation of the DNA in situ (Crine and Verly, 1976). Variation in the control values ( $D_c$ ) of unirradiated cells could be due to inconsistencies in handling, donor-to-donor variability, changes in culture conditions, or some, as yet undiscovered factor in the experimental protocol. These all play a part in determining the reproducibility of the assay, which is within 2-3 DNA strand breaks per chromosome. This has been dependent on the experience of the experimenter and developments introduced by H.C. Birnboim in the assay protocol over the duration of the thesis project. One can now reliably detect breaks caused by  $^{60}\text{Co}$  gamma-ray doses as low as 100 mGy in air.

#### 4.2 Rejoining of DNA Strand Breaks in HPBL After $^{60}\text{Co}$ Gamma-Irradiation

FADU can be used reproducibly to detect total DNA strand breaks in HPBL with great sensitivity. As discussed

in the Introduction, HPBL have been reported to be deficient in some forms of DNA repair. It was considered of great interest to determine the capacity of resting HPBL to rejoin DNA strand breaks induced by  $^{60}\text{Co}$  gamma-rays. Strand breaks in HPBL were found to be rejoined rapidly and effectively. The kinetics of rejoining is similar to that found after irradiation of cultured human fibroblasts (Hariharan et al., 1981) or Chinese hamster cells (Körner et al., 1978). Only a few studies on the ability of HPBL to repair DNA damage after gamma- or X-irradiation have been done previously. These studies differ widely in the reported kinetics of repair. I will speculate on some of the possible causes for these differences. Cook et al. (1978) found a half-time of about 4 hours for the restoration of the sedimentation velocity of a "nucleoid" structure. One factor in their protocol which could adversely affect the ability of HPBL to carry out repair is the pre-irradiation treatment of the cells. The cells were stored at  $4^{\circ}\text{C}$ . for several hours before experimentation.

There is indication from my work that keeping unirradiated cells at low temperature affects the integrity of the DNA (Table 3). Holding the cells at 4°C, as done by Cook et al. (1978) may have damaged the cells and rendered them unable to carry out repair as rapidly as has been seen by others. In addition, the high doses which were used could have induced in these cells an unspecified type of damage affecting the ability of HPBL to perform normal repair of DNA strand breaks, though repair in other cell types may not be appreciably inhibited at these doses. In the technique of Cook et al. (1978) changes in the sedimentation pattern of a "nucleoid" structure are followed at neutral pH. Alkali-labile lesions which are detected by FADU would probably not be detected by this assay. A second group of workers, Hashimoto, Ono and Okada (1975) observed a half-time of strand break rejoining of 60 minutes after irradiation of HPBL with a dose of (30 Gy) of <sup>137</sup>Cs gamma-rays. As suggested for the work of Cook et al. (1978) the high dose used may impair the ability of HPBL to rejoin strand breaks. Another explanation could be that the isolation technique they used to prepare cells for irradiation and subsequent incubation could cause a decrease in the repair capabilities of HPBL. In my preliminary experiments I have found that

DNA strand breaks are introduced in HPBL if the latter are concentrated to  $50 \times 10^6$ /ml at  $0^\circ\text{C}$  and then diluted to  $2 \times 10^6$ /ml and cultured at  $37^\circ\text{C}$  for 30 minutes (data not shown). Hashimoto et al. (1975) concentrated the cells, resuspended them in PBS for irradiation at  $0^\circ\text{C}$  and then centrifuged and resuspended the cells for incubation at  $37^\circ\text{C}$  for repair studies. This treatment may impair the ability of HPBL to carry out strand break rejoining. Lastly, Fender and Malz (1980) using an assay system similar to that of Cook et al. (1978), found rapid monophasic repair of DNA damage in HPBL with a half-time of less than 10 minutes. They used radiation doses and a cell handling protocol similar to those used in this study.

In my study, control experiments were carried out to show that HPBL used were not inadvertently stimulated after they were placed into culture. The cells showed neither increase in DNA synthesis nor morphological changes characteristic of mitogenically stimulated cells. Furthermore, I have presented data which indicate that the ability of HPBL to rejoin DNA strand breaks did not markedly change with time in culture. I suggest that the rapid kinetics of repair found by me and by Fender and Malz (1980) more nearly represents the ability of HPBL to rejoin DNA strand breaks in vivo.

4.3 The Nature of the Rapidly and Slowly Rejoined DNA Strand Breaks

4.3.1 Does the Slowly Repaired Component of DNA Strand Breaks Correspond to Alkali-Labile Lesions?

There have been a number of interpretations of the biphasic nature of the rejoining of DNA strand breaks after gamma- or X-irradiation observed by me and by others. The rapid phase has been equated with "clean" breaks which require little or no processing before they can be ligated (Hariharan et al., 1981). The slowly repaired damage is considered to be "dirty" breaks which are more difficult to rejoin. An alternative hypothesis put forward by van der Schans et al. (1981) is that the rapidly rejoined breaks are localized on the internucleosomal linker regions of the DNA in chromatin but there is little data to confirm this. Bryant and Blöcher (1980) have concluded that the slow phase of repair in Ehrlich ascites cells found by the use of their DNA unwinding assay for strand breaks represents the repair of double strand breaks. They found that the fraction of initial damage which is slowly repaired was about 10% of total strand breaks. This is near the generally reported frequency of 5% for double strand breaks (Lehmann, 1978). In Bryant and Blöcher's experiments the kinetics of the slow phase was closely related to.

the kinetics of rejoining of double strand breaks as determined by neutral sucrose sedimentation but only after a dose of 100 Gy of X-rays. This relation appears to be dependent on the dose used. This can be seen if one considers that the half-time of repair of the slowly rejoined breaks is only 40 minutes after the lower dose of 8 Gy of X-rays. It seems more likely that the slowly repaired component seen by Bryant and Blöcher is the same as that seen in my experiments and those of others (Körner et al., 1978; Ahnström and Edvardsson, 1974). In these cases the slowly rejoined breaks represent 30% or more of the total breaks. This is too high a fraction of total strand breaks to be double strand breaks.

It is my suggestion that these slowly repaired breaks represent predominantly alkali-labile lesions (which are detectable by FADU) and that the rapidly repaired damage is mainly due to frank strand breaks. The fraction (30%) of slowly repaired damage I have found is similar to the reported fraction of total breaks that is alkali-labile after X-irradiation (Ullrich and Hagen, 1971). This supports the possibility of a relationship between alkali-labile lesions and the slowly rejoined component of DNA strand breaks as measured by FADU. I further propose that the difference in the rates of rejoining of the two forms of

damage probably lies in the mechanism of their repair. Frank strand breaks would be repaired quickly because they can be either ligated directly or be rejoined through an excision repair process which requires no incision step since the strand breaks themselves would provide substrate for excision or polymerization. Alkali-labile lesions, which are not open strand breaks in the cell, would be repaired more slowly because they require recognition by a specific endonuclease to initiate excision repair by an incision step. Evidence for this will be found by examining the process of excision repair. Alkali-labile lesions consist of sites with damaged base or sugar, and abasic sites. They would be expected to be repaired by the same mechanism as alkali-stable lesions in the DNA which are similar in nature in situ. This process would include (i) incision at the damaged site; (ii) excision of the damaged nucleotide or residual sugar and possibly one or more nucleotides; (iii) incorporation of one or more nucleotides and (iv) ligation of the remaining single strand gap (Hanawalt et al., 1979; Günther et al., 1977). Since at least one nucleotide is added during the repair of each alkali-labile lesion, one would expect the uptake of labeled nucleotide to be rapid for the first hour after irradiation.

of cells with low LET radiation if this were similar to the repair of the slowly rejoined breaks. Spiegler and Norman (1969) found that the rate of  $^3\text{H}$ -TdR uptake in HPBL after irradiation with X-rays or 6 MeV electrons followed these kinetics, the uptake velocity falling to half the initial rate about 40 minutes after irradiation. This uptake of label would represent repair of both alkali-stable base damage and alkali-labile lesions. The observed kinetics of uptake of labeled DNA precursor into DNA of HPBL undergoing repair of gamma-ray induced damage shows a possible relation between alkali-labile lesions and the slowly rejoined breaks as seen by FADU, but cannot be used to prove identity of the two forms of damage.

There is other evidence that alkali-labile lesions and alkali-stable lesions are repaired with kinetics similar to that of the slowly rejoined component seen by FADU. Alkali-labile lesions induced by 4NQO are rejoined in human fibroblasts with a half-time of a little less than one hour (Smith and Paterson, 1980). This parallels the rate of rejoining of the slowly repaired damage seen in HPBL. In addition, one may also consider data gained by the use of gamma-endonuclease, isolated from Micrococcus luteus, which will cleave DNA at undefined sites of base damage in gamma-

irradiated DNA (Paterson and Setlow, 1972). After irradiation, gamma endonuclease-sensitive sites disappear in human fibroblasts with a half-time of approximately one hour (Paterson et al., 1976). This damage is probably alkali-stable but demonstrates that base damage, which is handled by excision repair requiring an incision step, has a half-life similar to that of the slowly rejoined strand breaks seen by FADU.

#### 4.3.2 Does the Rapidly Repaired Component of DNA Repair Correspond to Frank Strand Breaks?

I shall now consider the fast phase of repair as seen by FADU and its relation to frank strand breaks. Half of frank strand breaks involve the release of free nucleoside from DNA (Ullrich and Hagen, 1971) and are, therefore, not capable of being immediately ligated, but would require the addition of one or more nucleotides either through excision repair or directly by polymerization. These breaks should then also contribute to the kinetics of uptake of labeled nucleotide. An incision step would be unnecessary, for the strand break would provide substrate directly for exonuclease or polymerase activity. As there is no incision step required for the repair of frank breaks it is my suggestion that they should be repaired very quickly. The magnitude of uptake would be small as the number of frank strand breaks requiring excision repair represents less than one quarter of the

total damage requiring excision repair (Ullrich and Hagen, 1971). Spiegler and Norman (1969) found that the cumulative uptake of label had a positive intercept at time zero. This may represent the rapid initial uptake expected, but more extensive uptake experiments would have to be done to verify this observation.

Other experiments have been done which tend to support the argument that frank strand breaks are repaired rapidly. S1 endonuclease, isolated from Aspergillus oryzae, is capable of cleaving DNA opposite single strand nicks under certain conditions (Wiegand et al., 1975). When DNA from cells irradiated with moderately high doses of gamma rays is treated with S1 nuclease, DNA strand breaks detectable by alkaline sucrose gradient sedimentation are introduced. During repair the disappearance of S1 nuclease sites is rapid with over 90% of the breaks sites removed by 30 minutes (Paterson et al., 1978). It is possible that in this assay system frank strand breaks are made evident. If this is so, it would tend to support the proposition that frank strand breaks are rejoined quickly.

Excision gaps formed during the repair of alkali-stable base damage induced by ultraviolet light can be visualized by use of a DNA unwinding technique (Ahmström

and Edvardsson, 1978) or by "nucleoid" sedimentation (Yew and Johnson, 1979). It might be considered reasonable that the excision gaps formed during the repair of alkali-stable lesions which exist during the interval between incision and ligation could also be detected by the use of the unwinding method, FADU. It is my suggestion that this interval is short and that very few excision gaps are open at any one time during repair. Subsequently, they would not be detected by FADU. To demonstrate why this should be so, it is appropriate to consider excision repair in more depth. Excision repair of gamma-ray induced base damage requires the addition of 1-2 nucleotides per site as compared to 15 or more for that of damage induced by ultraviolet light (Günther et al., 1977). Excision gaps formed after ultraviolet light irradiation remain open for 3 minutes (Erixon and Ahnström, 1978). Those formed after gamma-irradiation would be expected to be much more short lived as there are fewer nucleotides added after the incision step (i.e., 1-2). The incision step is the slowest step in the repair of ultraviolet light induced DNA damage (Erixon and Ahnström, 1978). Even so, repair of damage caused by  $1.5 \text{ J/m}^2$  of ultraviolet light irradiation in human fibroblasts is completed with a half-time of

approximately 40 minutes (Fornace et al., 1976). It is my suggestion that only the disappearance of alkali-labile lesions is seen by FADU with a half-time of approximately 35-40 minutes, the excision gaps of other base damage being too few at any time to be detected.<sup>4, 5</sup>

My proposal that the slowly rejoined damage represents alkali-labile lesions is supported by the data of Fender and Malz (1980) who report rapid monophasic kinetics of repair in HPBL after X-irradiation. Solutions used in their assay system to visualize DNA damage are at or near neutral pH. The damage detected is probably only frank strand breaks and not alkali-labile lesions. They report only a fast phase of repair with a half-time of about 10 minutes. There is no obvious slowly repaired component which could represent either the excision of base damage, as seen by the use of this technique after ultraviolet irradiation (Yew and Johnson, 1979), or the

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<sup>4</sup> If the  $t_{1/2}$  of the repair of base damage were also 35-40 minutes and the amount of damage were three times that for alkali-labile lesions (Ullrich and Hagen, 1971) and the excision gap remained open for a full three minutes, I have calculated that the maximum proportion of strand breaks represented by these excision gaps would be 10% or less of the total breaks remaining at any one time. Since the estimates are all maxima for each parameter considered, it is more likely that the proportion is closer to 2% or less of the total breaks remaining.

<sup>5</sup> This would also reinforce the idea that frank strand breaks should be rapidly repaired, since the incision step is rate limiting in the repair of base damage (e.g. alkali-labile lesions).

slow repair of a possible subset of frank DNA strand breaks. This would suggest again that the slowly repaired component as detected by FADU represents alkali-labile lesions. The kinetics of nucleoid reconstitution after X-irradiation as seen by Fender and Malz (1980) appears to be slower than that for the rapid phase of strand break rejoining after gamma-irradiation as seen by the use of FADU. This could be due to small differences in the rate of temperature equilibration during post-irradiation incubation. Alternatively, this could indicate that there is a lag (after the rejoining of DNA strand breaks) in the reconstitution of the structure in chromatin which is detected by the use of this assay. At present, doses needed to allow alkali-labile lesions to be detected directly are above 10,000 Gy which is well above a dose at which normal repair kinetics could be observed (Lennartz et al., 1975). All of the experiments discussed here are consistent with the model that slowly repaired damage seen in this thesis by the use of FADU represents alkali-labile lesions and that the rapidly repaired damage represents frank DNA strand breaks.

#### 4.4 Relation of DNA Strand Breaks to Cell Inactivation

##### 4.4.1 Cultured Mammalian Cells.

Cell inactivation as a biological endpoint can result from damage to various sites and as such is very difficult to characterize biochemically. Different criteria of cell inactivation have been followed. One endpoint of cell inactivation is interphase death, characterized by a decline in metabolic function, degradation of the DNA, and eventual autolysis of the cell. Morphological changes, such as pyknosis or loss of membrane integrity, seen as the inability of the cell to exclude certain dyes. (e.g. trypan blue) are used as in vitro indicators of interphase death. Another endpoint is mitotic death, defined as the inability of cells to divide and form colonies on replating after treatment with radiation or other damaging agents. A dose given to induce this effect without interphase death occurring is low enough such that the initial insult is not so severe as to affect the cell morphology immediately; at a later stage the cell will be unable to complete mitosis or will fail to proliferate after one or a few further mitoses. This could be due to a failure of any number of systems in the cell. One possibility is that a rearrangement

of the DNA, seen as a chromosome aberration, will induce the loss of necessary genetic information or interfere with the redistribution of the chromosomes during mitosis. "Liquid holding" experiments have shown that damage which would otherwise lead to cell inactivation can be repaired if cells are held at sub-optimal growth conditions to inhibit replication synthesis of DNA (Belli and Shelton, 1969). Similar experiments have shown repair of damage leading to chromosome aberrations (Sasaki, 1978). These experiments and others have led some to suggest that damage to DNA and its repair are important events related to cell inactivation (Arena, 1971; Chadwick and Leenhouts, 1973; Williams, 1976). Because of interest in a possible role of DNA damage in cell inactivation several enzymatic pathways of repair of this damage induced by radiations or chemicals have been studied and elucidated (Hanawalt et al., 1979). It is of interest to know if DNA damage and its repair is directly related to cell inactivation.

There are two inherited disorders in man which are marked by an acute radiation sensitivity and have been linked to an inability to carry out repair of a specific type of lesion in DNA induced by radiation. Ataxia telangiectasia is an autosomal recessive disease

characterized by an extreme sensitivity to ionizing radiation and a high incidence of spontaneous cancers (Paterson and Smith, 1979). Each individual affected appears to have a defect in only one gene product, though there are different complementation groups which have the same clinical symptoms and an in vitro radiosensitivity. One strain of cells from an ataxia patient (AT3BT) is deficient in the ability to remove damage which is seen as gamma-endonuclease sensitive sites in DNA (Paterson et al., 1976). Xerodermosa pigmentosum, another genetic disease, is inherited as a single autosomal recessive gene and is associated with a sensitivity to ultraviolet light and a high incidence of spontaneous skin cancers. Cells from patients suffering from this disease are deficient in excision repair of DNA damage induced by ultraviolet light (Hanawalt et al., 1979). In cell lines from patients with either disease the radiosensitivity is postulated to be due to a defect in the repair of DNA damage.

It is of interest to consider whether cell inactivation or chromosome aberration induction is linked to the repair of DNA damage, in particular DNA strand breaks. It has been postulated that total DNA strand breaks which are left unrepaired are correlated with cell

death (Ritter et al., 1977). These authors used alkaline sucrose gradients to determine the number of DNA strand breaks remaining in Chinese hamster cells two hours after very high doses (30 Gy) of accelerated particles of various LET. Cell inactivation correlated well with the number of residual strand breaks after repair. However, Ahnström and Edvardsson (1978) using an alkaline unwinding technique with Chinese hamster cells have since shown that after moderate doses of X-rays the number of breaks drops to background levels after five hours of incubation. They further show that only after doses greater than 200 Gy of X-rays is rejoining incomplete. It is probable that the extensive non-rejoining of total DNA, which can occur at high doses, cannot be extrapolated to explain biological effects at lower doses. My results show low levels of DNA strand breaks remaining after 1-2 Gy of <sup>60</sup>Co gamma-irradiation and 80 minutes of repair. Because there are so few residual breaks it seems reasonable to conclude that the non-repair of strand breaks is a rare event which could be responsible for ~~only~~ a small minority of the effects seen at the cellular level. It has also been argued that residual double strand breaks are correlated with cell inactivation. The findings and conclusions by authors working in the field are similar to those for total strand

breaks. Dugle et al. (1976) found non-rejoining of double strand breaks in Chinese hamster cells after doses in excess of 100 Gy of X-rays. However, more recently, others have shown rejoining of double strand breaks even after very high doses. Handschack and Malz (1980) have shown that the number of double strand breaks goes to background levels within two hours after doses as high as 200 Gy of X-rays. More recently, ~~van~~ der Schans et al. (1981) have shown by use of the neutral elution technique of Bradley and Kohn (1979) that rejoining of double strand breaks is rapid and biphasic after 50 Gy of  $^{60}\text{Co}$  gamma-rays, with less than 10% of the initial breaks remaining after two hours. The residual damage seen after high doses could be interpreted as the result of some as yet undefined injury to the cell which renders repair enzymes ineffectual. At lower doses the last few breaks may in fact be repaired or they may be below the limit of detectability.

Further evidence has been generated by the study of chromosome aberration induction. Sasaki (1978) has studied the induction of chromosome aberrations in HPBL after irradiation with X-rays, gamma-rays or fast neutrons. He considers that the formation of an exchange aberration requires the interaction of two damaged sites ("events")

within a certain distance of each other. Knowing the dose, the LET of the radiation used and the aberration yield, he calculates that the mean energy loss per event which could lead to the formation of an aberration is 50 eV. This is approximately the energy required to form a single strand break by  $^{60}\text{Co}$  gamma-rays and would tend to rule out double strand breaks, since over 1 keV is required, on the average, per double strand break formed. The actual number of strand breaks induced per human genome after 1 Gy of  $^{60}\text{Co}$  gamma-rays is estimated to be 1250 (Myers, 1977). The yield of unstable chromosome aberrations after that dose is approximately 0.18 per cell (Lloyd and Purrot, 1981). The discrepancy between the actual number of DNA strand breaks and the detectable effect, be it cell inactivation or chromosome aberration induction, is not well understood. It could be that a small subset of the damage induced, which requires 50 eV to be formed, is responsible for the effects seen. It could also be suggested that the misrepair of any one strand break or other defect could lead, with equal probability, to cell inactivation or chromosome aberrations.

#### 4.4.2 Radiosensitivity of Lymphocytes

After 1.0-2.0 Gy of X-irradiation, the majority of HPBL in culture will undergo interphase death as measured by

pyknosis or trypan blue exclusion within a few days (Prosser, 1976; Hedges and Hornsey, 1978). This is in striking contrast to cultured mammalian cells when one considers the LD<sub>50</sub> for mitotic death in cultured human fibroblasts (Deschavanne et al., 1980) and in HeLa cells (Elkind and Whitmore, 1967) is also about this dose. This level of interphase death occurs only after doses of 10-50 Gy in HeLa cells (Elkind and Whitmore, 1967). It is probable that the radiation sensitivity of non-dividing HPBL is not the result of a deficiency in the ability to rejoin DNA strand breaks, as repair has been shown in this thesis to be as rapid as that in cultured cells. There is the possibility that some minor form of damage which is seen as strand breaks is poorly repaired in HPBL. There are several changes in the physiology of the cell after irradiation which indicate that the process of interphase death is very complex. DNA of HPBL will start to disintegrate within one-half hour of irradiation with higher doses (Timberlake et al., 1976). The major autolysis of HPBL will begin at 10 hours after 5 Gy of X-rays (Sato, 1970). The breakdown of the cell by autolysis is dependent on active metabolism in the cell (Scaife and Brohée, 1967). RNA content drops unless the cells are stimulated with a mitogen such as PHA (Cirkovic, 1969). Protection of HPBL from inter-

phase death can be achieved if mitogen is added as late as two hours after irradiation (Schreck and Stefani, 1964).

This indicates again that the effect seen is not the direct result of the damage induced which is detected, but rather, a specific metabolic event which can be triggered by damage to any number of sites. It has been proposed that some time after treatment with a DNA damaging agent or other toxic agent, cells undergo endonucleolytic death due, in some cases, to action initially affecting a non-DNA target (Williams et al., 1974). The low complement of organelles and other cell constituents in HPBL could render an unknown critical function hypersensitive which, if inactivated, is incapable of regenerating itself. It is unclear whether this target could be DNA.

It would be of interest to study further the rejoining of DNA strand breaks in HPBL after treatment with ConA to determine if there is a difference in rejoining rates of resting and stimulated cells. The repair would have to be substantially more rapid for a difference to be detected. The fate of the DNA during mitotic and interphase death could be followed in stimulated and resting HPBL over longer periods of culturing after irradiation in order to achieve a better understanding of these highly complex

processes. Another interesting possibility stems from the fact that different populations of HPBL could rejoin DNA strand breaks with different efficiencies. T and B lymphocytes have different radiosensitivities and could be relatively easily separated to test this possibility.

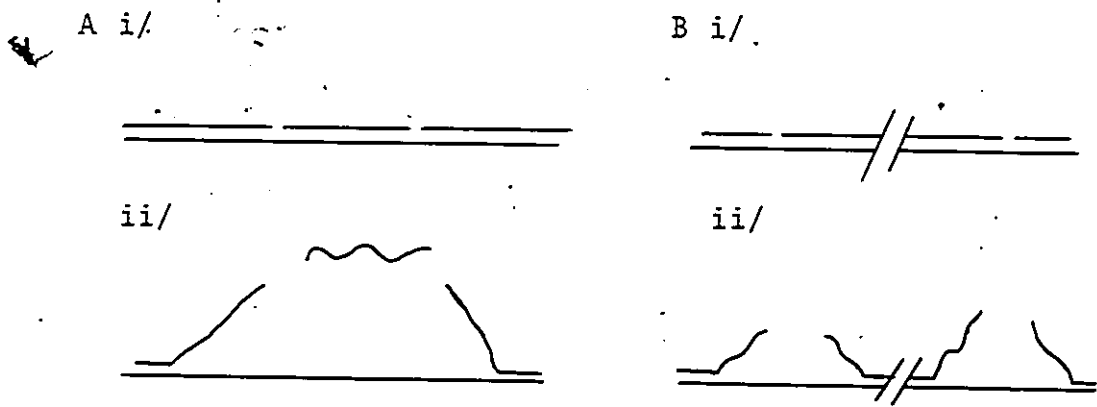
#### 4.5 Induction and Rejoining of DNA Strand Breaks After 14.6 MeV Neutron Irradiation

When HPBL are irradiated on ice with 14.6 MeV neutrons and analysed by FADU, the apparent yield of strand breaks is 0.38 that found after the same dose of  $^{60}\text{Co}$  gamma-rays. The dose is the amount of energy deposited in the target tissue per unit mass (e.g. 1 Gy = 1 J/kg). The observation that a neutron dose which deposits the same amount of energy as a similar dose of gamma-rays appears to induce fewer DNA strand breaks than gamma-rays is surprising, particularly when one considers that the RBE of neutrons for cell inactivation or chromosome aberration induction is greater than 1.5. In considering an explanation for this apparent difference, it is unlikely that there is some form of fast repair which allows some amount of undetected damage to disappear before the cells are assayed, as the cells were held at 0-2°C for a short time before analysis and cells irradiated with gamma-rays were treated in a similar fashion in parallel. An

alternative possibility which was considered is that neutrons may induce cross-links which could slow down the rate of DNA unwinding (in alkali, thereby lowering the apparent yield of strand breaks as detected by FADU. The bifunctional cross-linking agent, nitrogen mustard (bis(2-chloroethyl)-methylamine, HN2), will slow down the rate of DNA unwinding after gamma-irradiation as detected by FADU. The apparent number of strand breaks in WBC found after gamma-irradiation, which induces very few cross-links, is substantially lowered if cells are first treated with HN2 (H.C. Birnboim, pers. commun.). When neutrons were used in conjunction with gamma-rays to irradiate cells, the rate of unwinding was not significantly different from the sum of the rates expected if the damage from the two forms of radiation did not interact. Cross-links involving protein could still be present but those produced after neutron irradiation could be distributed in such a way that when the cells were irradiated in conjunction with gamma-rays the rate of unwinding would still be additive. Another method which can be used to detect cross-links has been developed for application in the alkaline elution assay of Kohn (1979). Cross-links involving protein induced by X-rays, HN2 or other cross-linking agents can be broken by treatment with proteinase K (Kohn, 1979). Consequently, the rate of elution will become more rapid

after treatment with this enzyme. Proteinase K has had no positive effect on the rate of DNA unwinding after neutron irradiation in my experiments. This indicates that it is unlikely that cross-links involving protein are responsible for the apparent low yield of strand breaks after neutron irradiation. It is possible that DNA-protein cross-links may not be detected by FADU.

A more likely explanation of the low apparent yield of strand breaks induced by fast neutrons stems from the hypothesis that these breaks are inhomogenously distributed in the DNA. The energy of fast neutrons is deposited in cells along densely ionizing tracks, large amounts of energy tending to be concentrated in a relatively small volume (Rossi, 1979). If two or more single strand breaks on the same strand are close enough together, they will be scored as one break by FADU. A mechanism by which two proximal breaks can be scored as one is illustrated below:



In A two breaks which are close together on the same strand are shown (i/). If this length of DNA is placed into alkali, the small intervening piece will completely unwind in a very short time and only the two remaining ends will determine the rate of unwinding and only one strand break will be detected (Aii/). If the two breaks are independent (i.e. very far apart, B) there will be four ends of the DNA molecule present throughout the assay period and the rate of unwinding will be twice that for the situation shown in A. If the frequency and number of breaks in close proximity were high, the apparent yield of strand breaks detected would be proportionally lower, as greater numbers of breaks would tend to mask each other.

The clustering of lesions may alter the severity of damage by affecting the subsequent repair. In this argument, damage induced by gamma-rays or neutrons is supposed to be due mainly to indirect effects through free radicals; thus, the types of damage should be similar for both radiation types. The distribution of the damage would be the only difference. KÖrner et al. (1978) have suggested that excision of damage due to "bad" breaks within clusters could induce a "catastrophe" leading to non-repair or the induction of a double strand break. To enlarge on this idea, there are a number of ways repair

could be affected: i/ the repair of one site may delay or preclude repair at another site, possibly by interfering with the binding of a repair complex to the second site by steric hindrance. During this interval the damage at the second site could be changed to an irreparable form of lesion. ii/ The action of a repair complex at one site could induce the unfolding of one or more nucleosomes. A double strand break may be stabilized within the nucleosome, such that it can be repaired relatively easily by ~~ligation~~ ligation. The disruption of the nucleosome structure could release the DNA double strand break allowing the two free ends to drift apart and thereby become irreparable. iii/ Exonuclease activity resulting from repair at one site may run over a strand break on the opposite strand inducing a double strand break which may not be reparable. Clustering has been implicated in the induction of chromosome aberrations. By the use of neutrons of different energies, Holmberg (1978) has shown that the chromosome aberration yield in HPBL is correlated with the amount of proton recoil, and is even more strongly correlated with oxygen recoil which would cause even more densely ionizing tracks and presumably denser clustering of strand breaks.

The kinetics of DNA strand break rejoining after

14.6 MeV neutron irradiation is different from that seen after  $^{60}\text{Co}$  gamma-irradiation. The rejoining is rapid and biphasic which is in agreement with that reported by Ahnström and Edvardsson (1974), Körner et al. (1978) and Hesslewood (1978). All previously published work indicates that the rate of repair of strand breaks induced by fast neutrons slows down to a negligible level after up to two hours of post-irradiation incubation. This is not seen after X- or gamma-irradiation. The dose range used in the previously published reports was 40-140 Gy. These high doses may affect the ability of the cells to repair part of the damage present.

If clustering of strand breaks were responsible for the low apparent yield of DNA strand breaks, the half-time of rejoining as assayed by FADU would be somewhat longer for each phase. This is because all of the breaks in a given cluster would have to be rejoined to be seen as the rejoining of what would be detected as one break by FADU. The relationship between the degree of clustering and rejoining kinetics would be very complex. It would be of interest to use neutron sources of different energies to explore further the relationship between repair kinetics and LET. Higher doses could be used, but care would have

to be taken to distinguish between radiation induced strand breaks and breaks induced by endonucleolytic degradation during autolysis.

#### 4.6 Conclusions

HPBL were irradiated with  $^{60}\text{Co}$  gamma-rays or 14.6 MeV neutrons. HPBL were found to rejoin DNA strand breaks rapidly with biphasic kinetics. The  $t_{1/2}$  for the fast phase was 2.8 minutes and that for the slow phase was 35 minutes. After  $^{60}\text{Co}$  gamma-irradiation the fast phase of repair comprised 70% of the initial breaks. After 14.6 MeV neutron irradiation the apparent yield of initial breaks was 0.38 that found after  $^{60}\text{Co}$  gamma-irradiation. The lower yield was due mainly to a reduction in the rapidly rejoined component. After 80 minutes the number of DNA strand breaks was not detectably different from background levels after either form of radiation. Cross-links involving protein were tentatively ruled out as an explanation for the low apparent yield of DNA strand breaks after neutron irradiation. The clustering of breaks in the DNA appears a more likely mechanism to explain this initially surprising finding. The major conclusions are i/ the extreme radio-

sensitivity of HPBL does not arise from a deficiency in their ability to rejoin DNA strand breaks and ii/ there is no obvious relation between the high RBE for 14.6 MeV neutrons and the apparent yield of DNA strand breaks or the ability of cells to rejoin them.

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