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RADIATION EFFECTS ON MOUSE LYMPHOMA

CELLS IN CULTURE

Thesis submitted
by
PAULINE J. WOOD, B.Pharm., M.P.S
for the degree
of
Doctor of Philosophy

This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of S. H. Moss, M.Sc. Ph.D. M.P.S. and D. J. G. Davies, M.Sc. Ph.D. F.P.S.

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SUMMARY

The thesis opens with a review of the current knowledge of the mechanisms for the repair of radiation induced damage in bacteria and mammalian cells.

The experimental work is presented in Chapters 2 to 6, each chapter beginning with an introduction and concluding with a discussion pertinent to the work described.

Chapter 2 is concerned with the development of the culture of the mouse lymphoma cell lines L5178Y, JL, L5178YS and AIV, together with a soft agar suspension procedure for assessment of viability.

The inactivation of the four cell lines, in two stages of growth, to gamma radiation and to 254 nm, 313 nm and 365 nm ultraviolet radiation has been compared in Chapter 3. The enhanced sensitivity of L5178YS cells to gamma radiation over that of the parent, L5178Y line is shown to UV radiation of all the wavelengths, as is the increased resistance exhibited by the AIV cell line.

Chapter 4 describes a preliminary investigation into the mutagenic response of L5178Y cells to 254 nm and 313 nm radiation in two mutation assay systems, ouabain resistance and 6-thioguanine resistance.

The sensitisation of 313 nm radiation inactivation and mutation of L5178Y cells by the sunscreen agent, para-amino benzoic acid PABA was investigated and the results are presented in Chapter 5. The sensitisation of 313 nm radiation inactivation was found to be dependent upon PABA concentration, and 0.2% PABA was found to enhance 313 nm radiation induced resistance to both ouabain and 6-thioguanine.

A method of producing mutants of L5178Y cells which are sensitive to 254 nm radiation is described in Chapter 6. The isolates were characterised in terms of their sensitivity to 254 nm radiation, with respect to the time in culture after isolation.
General conclusions concerning the use of L5178Y cell line for the elucidation of mammalian cell repair mechanisms are discussed in Chapter 7.
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CHAPTER ONE

INTRODUCTION
This thesis is concerned with the effects of radiation upon mammalian cells in culture. The radiation types used include gamma (γ) radiation and ultraviolet (UV) radiation. Both of these radiations form part of the electromagnetic spectrum, illustrated in figure 1.1. The boundaries of each region are not clear cut, and are dependent upon the system being defined. For biological purposes, the ultraviolet radiation region begins at 100 nm and ends at 380 nm. The vacuum UV radiation region is from 100 nm to 190 nm, so-called because these wavelengths are absorbed by water and air. The wavelengths from 190 nm to 290 nm are termed the far UV radiation region, and 290 nm to 380 nm, the near UV radiation region. The boundary between far and near UV radiation regions has arisen from the cut-off point of solar UV radiation absorption by ozone in the upper atmosphere. The shortest wavelength recorded at the earth's surface is 286 nm, and the relative intensity of UV radiation above this wavelength is dependent upon the thickness of the ozone layer (1). Recently, the term near UV radiation has been used for wavelengths of 320 - 380 nm, and the 290 - 320 nm region has been called the mid UV radiation region. An alternative definition of the regions stems from the reactions produced in human skin by UV radiation. The wavelengths from 340 nm to 380 nm induce pigment production without causing erythema and this region is often referred to as UVA. The wavelength component of sunlight from 290 - 340 nm, capable of producing erythema is referred to as UVB. Finally, the region from 190 to 290 nm corresponding to the far UV radiation region, which will produce erythema, but is not a component of sunlight at the earth's surface, is referred to as UVC.

The effects brought about by the various types of radiation giving rise to the above classifications of the electromagnetic spectrum are dependent upon the energies associated with the radiation.

The Quantum Theory

According to the Quantum theory, radiant energy is transmitted in discrete units called quanta or photons. The energy of a photon is related to the wavelength of light by the Planck Equation.

\[ E = h \nu = \frac{hc}{\lambda} \] (1.1)
**Figure 1.1 The Electromagnetic Spectrum.**

Energy (eV)

Wavelength (nm)

Long waves

Medium waves

Short waves

Microwaves

Infrared

Visible

Ultraviolet

Soft X-rays

Hard X-rays

Gamma rays

Cosmic rays

10^{-5}

10^{-4}

10^{-3}

10^{-2}

10^{-1}

10^0

10^1

10^2

10^3

10^4

10^5

10^6

10^7

10^8

10^9

10^{10}

10^{11}

10^{12}

Wave

length (cm)
where: $E$ is the energy of a single photon in joules
$c$ is the velocity of light, $3 \times 10^{10}$ cm per second
$\nu$ is the frequency of radiation per second
$\lambda$ is the wavelength of radiation in cm
$h$ is Planck's constant $6.62 \times 10^{-34}$ joules per second.

The energy of a photon is small, so often the Einstein is used, defined as the energy of a mole of photons.

$$E = N\nu$$  \hspace{1cm} (1.2)

where $N$ is Avogadro's number ($6.022 \times 10^{23}$)

Another way of expressing the energy of a photon is as the electron volt (eV), defined as the kinetic energy acquired by an electron when passing through a potential difference of one volt.

$$1 \text{ eV} = 1.602 \times 10^{-19} \text{ joules.}$$

Thus each wavelength has a specific energy and will cause a specific effect when absorbed by a molecule or atom. Wavelengths in the infrared region have photon energies of $0.01 - 0.1$ eV and when absorbed, cause molecular vibrations or rotations, and thus do not cause any chemical changes, although reactions may proceed at faster rates due to heat associated with these wavelengths. At the other end of the scale, wavelengths in the X-ray region and below have photon energies of $10^3$ to $10^8$ eV which are capable of removing or adding electrons to the outer orbitals of atoms or molecules, causing them to ionise, and these radiations are therefore termed ionising radiations. The upper boundary of the ionising capability is at about 100 nm and so wavelengths above this in the ultraviolet region are termed non-ionising radiations. The energies associated with non-ionising UV radiation photons are of the order of $10^1 - 10^3$ eV and are sufficient to excite the valency electrons to higher energy levels, thus increasing the probability of chemical reaction.
The first law of photochemistry states that for a photochemical reaction to occur, light must be absorbed by a molecule. If no light is absorbed, no photochemical effect takes place. The second law of photochemistry, the Stark-Einstein Law, states that if light energy is absorbed it need not have an effect, but if it does only one photon is required for each molecule affected. This is valid due to the short lifetime of the excited molecule and the low concentrations of photons that occur in most systems. Recently however, biphasic excitation has been observed, and the case of triplet-triplet energy absorption, an example of such a reaction will be discussed in Chapter 5.

The knowledge that a chemical change will not be induced by every quantum of energy absorbed by a molecule leads to the concept of the quantum yield, the efficiency of producing a chemical reaction. Thus:

\[
\phi = \frac{\text{Number of molecules reacting chemically}}{\text{Number of photons absorbed}} \quad (1.3)
\]

\[
= \frac{\text{Number of moles reacting chemically}}{\text{Number of Einsteins absorbed}} \quad (1.4)
\]

The Effects of Radiations on Molecules

Ionising radiation is non-selective in its effect and may ionise any molecule in its path. The effect may be direct, by ionisation of relevant molecules themselves, or indirect, by ionising other molecules in the system which then react with the relevant molecule(s). Water is the predominant constituent of cellular systems and so the ionised species of water are important. As the number of electrons in the ionised water molecule is uneven, the ions formed would be free radicals and as such are highly reactive:

eg. \[ \text{H}_2\text{O} + \text{hv} = \text{H}_2\text{O}^+ + \text{e}^- \quad (1.5) \]

\[ \text{e}^- + \text{H}_2\text{O} = \text{H}_2\text{O}^- \quad (1.6) \]
and \[ H_2O^+ = H^+ + OH^- \] (1.7)
\[ H_2O^- = OH^- + H^+ \] (1.8)

Free \( H^- \) can react with oxygen in the living cell to produce \( H_2O_2 \) and \( HO_2^- \). These, and other oxidising agents will interfere with balanced chemical activities. Organic peroxides may also be formed in biological systems and hydrogen bonds, double bonds and \(-SH\) groups may split to form free radicals.

In contrast to ionising radiation, UV radiation effects are brought about by excitation of valency electrons from low energy ground states to higher energy singlet and triplet excited states. These two states differ in that electrons in the singlet state have paired or anti-parallel spin directions and thus return easily to the ground state with re-emission of the photon. These states have lifetimes of \( 10^{-8} \) seconds. The triplet state electrons have parallel spin directions, due to crossing-over from the singlet state, and thus transitions to the ground state are less likely to occur, giving these electrons a lifetime of the order of \( 10^{-4} \) seconds. This increases their probability of undergoing a chemical reaction. Since each molecule has a characteristic set of energy levels it follows that photon absorption in the UV radiation region will cause only specific molecules to be excited.

The Biological Effects of Radiation

Radiation will cause a variety of effects to biological systems but it is generally accepted that the main target is cellular DNA. The effect most often studied is cell killing but mutagenesis and carcinogenesis are also extremely important. Other effects include cell division delay, interference with RNA, protein synthesis and changes in cell permeability and motility (2).

At the molecular level, radiation damage to DNA may be divided into two categories. 1. Breakage of the DNA backbone ie. single and double strand breaks and 2. Damage involving bases. The latter is most easily subdivided into lesions which cause varying amounts of
distortion to the DNA helix structure. Those lesions causing little
distortion include monofunctional ring saturation products such as
the addition of OH', oxygen or nitrogen to the 5, 6, double bond of
pyrimidines upon exposure to ionising radiation (3) or the addition
of a water molecule to the 5, 6, double bond upon irradiation, known
as pyrimidine photohydration (4). Products of the 5, 6, dihydroxy,
dihydrothymine type are a major lesion produced by γ radiation in
living cells (5) and removal of this lesion by a specific excision
process in bacteria and mammalian cells will be discussed later. The
elimination of the base to leave an apurinic or apyrimidinic site (AP
site) has been reported after γ irradiation (6).

The lesions causing major distortion of the DNA helix may be further
divided into monofunctional and bifunctional lesions. Monofunctional
lesions usually involve large molecule substitution and thus are not
usually induced by radiation. Bifunctional lesions include intrastrand
cross-links, caused by UV radiation (4, 7) which require a change in
conformation of the molecule, the lesion resulting in local helix
distortion. The most commonly occurring intrastrand cross-link is
the cyclobutane pyrimidine dimer, mainly between adjacent thymine
molecules, although thymine-cytosine and cytosine-cytosine dimers are
formed to a lesser extent by UV radiation, both in vitro (8) and
in vivo (9). Interstrand cross-linking of bases can be produced by
psoralen plus UV radiation (10, 11) and by ionising radiation (12).

Finally, cross-links between DNA and protein can be induced both by
UV radiation and ionising radiation in vivo and in vitro, but little
is known of the biological consequences (13).

As the above summary suggests, UV and ionising radiations may produce
the same types of lesions, but the difference between the two
categories of radiation lies with the quantities of the lesions
produced. The predominant lesion produced by ionising radiation is
the strand break (14) either single or double strand breaks, with
different repair susceptibilities (15). The major lesion induced by
UV radiation as has already been stated is the pyrimidine dimer.
However, it must be emphasised that the biological significance of each lesion is dependent upon the susceptibility of the lesion to the repair mechanisms of the cell.

The Biological Effects of Near UV Radiation

The development of phototherapy techniques involving near UV radiation (290 - 380 nm) has necessitated a detailed knowledge of the effects of these wavelengths at the cellular level. This has been reviewed extensively (16, 17). Most of the damage induced by near UV radiation appears to be in the DNA, but damage to other sites also occurs which has some significance. Near UV radiation induced growth delay appears to be caused by damage to 4-thiouridine residues in transfer RNA (18, 19) and near UV radiation also inhibits oxidative phosphorylation and macromolecular synthesis (20). Recently, membranes and membrane-binding proteins and transport systems have been implicated as sites of near UV radiation damage (17). It is not clear which chromophores are the targets for near UV radiation damage and they must include direct UV radiation absorption. However, Tyrrell suggests that sensitised reactions involving endogenous molecules, such as furcocumarins and porphyrins, may be the major mechanisms for near UV radiation damage (21).

The damage to DNA by near UV radiation is similar to that by far UV radiation but differs quantitatively. Irradiation at 365 nm produces approximately $10^6$ less pyrimidine dimers per unit dose, than at 254 nm (22). At 365 nm, the number of single strand breaks produced is just above half that of dimers (23). Ley et al (24) have shown that over 80% of these are true breaks and not alkali-labile lesions. In addition, Tyrrell (25) has suggested that near UV radiation may cause damage to the repair systems themselves, which enhances the lethal effect of the near UV radiation. However, this would not totally account for the strong oxygen dependence of near UV radiation induced lethality in all but the repair deficient mutants of _E. coli_ (26). Thus Tyrrell proposed a type of near UV radiation induced lesion which becomes fixed as a lethal event due to damage to the repair systems (25).
The Repair of DNA Damage

The first hint that radiation damage was repairable was by Hollaender in 1935 (27) seven years after Gates had correlated the nucleic acid absorption spectrum with the action spectrum for killing bacteria (28). The majority of the work on elucidation of repair systems has been carried out with isolated DNA or bacterial cells, in particular Escherichia coli. The development of biochemical techniques has led to an understanding of the functions of repair systems at the molecular level, while the production of specific E. coli mutants, deficient in certain steps of the repair pathways have given insight into the effects of repair systems upon the sensitivity of cells to radiation treatments. It is accepted that the basic concepts of repair of DNA damage are the same in both prokaryotes and eukaryotes, but that the detailed mechanisms of the repair pathways vary considerably. As would be expected, the eukaryotic cell presents a much more complex picture than that found in the bacterial cell.

Thus the evidence presented in the following section is concerned with the knowledge to-date of repair mechanisms in bacterial systems and in isolated DNA. This is followed by a review of the limited knowledge gained into the repair mechanisms in mammalian cells.

The repair mechanisms recognised to-date may be categorised under the following general headings:

1. Excision repair
2. Post-replication repair
3. S.O.S. repair
4. Photoreactivation

1. **Excision Repair**

A scheme for excision repair was first suggested by Setlow and Carrier in 1964 (29) and consisted of four main steps.

1. Recognition of damage and insertion of a nick in the phosphodiester backbone by an endonuclease.
2. Removal of the damaged section plus a number of bases by an exonuclease.

3. Resynthesis of the missing nucleotides by a polymerase using the intact DNA strand as template.

4. Joining the new nucleotides to the pre-existing DNA by a DNA ligase.

This basic scheme has proved to be valid but the detailed mechanisms and enzymology are more complex both in prokaryotes and eukaryotes, with considerable species variation.

Perhaps the most significant discovery was that for some types of damage the first incision step was not in the phosphodiester backbone, but in the glycosyl bond between the damaged base and the sugar, by a specific glycosylase. This glycosylase released the base, leaving an apurinic or apyrimidinic (AP) site, which was subsequently attacked by an AP endonuclease, thus severing the DNA backbone and allowing resynthesis to continue in the usual manner. The incision step appears to be rate limiting, and bacterial cells have a number of endonucleases, some of which have lesion specificity.

One of the most well-known endonucleases is the endonuclease V from T4 phage in infected E. coli cells. This is a low molecular weight protein, shown by many workers (30 - 33) to have two activities, both an N-glycosylase and an AP endonuclease, the latter cleaving the phosphodiester backbone 3' to the AP site. This activity has also been shown by an enzyme from Micrococcus luteus and both enzymes are specific for pyrimidine dimers (33). Other enzymes which appear to have lesion specificity include the E. coli endonuclease III which is specific for 5, 6, hydrated thymine in heavily irradiated DNA (34, 35). Evidence suggests that endonuclease III also has separate glycosylase and AP endonuclease activity (35), and enzymes specific for the removal of altered bases such as uracil and hypoxanthine, arising from spontaneous hydrolysis of cytosine or adenine (36) all incorporate the two-step incision mechanism (33).
In contrast, non-infected *E. coli* contains a high molecular weight protein with endonuclease activity which is dependent upon the uvr ABC gene products (37). Seeberg (38) has used an *in vivo* complementation assay for partially purified uvr ABC gene products and shown that neither products uvr A nor uvr B/C, the latter co-comatographing, show endonucleolytic activity alone, but together show an ATP dependent endonuclease activity specific for irradiated DNA (39). None have glycosylase activity (40). The role of ATP is not known but is suggested (38) to aid complexing of the uvr ABC gene products, thus suggesting that one of the uvr* +* gene products is an ATPase, as such enzymes have been isolated which use ATP as an energy source to unwind the DNA duplex (41). The uvr ABC enzyme is not specific for pyrimidine dimers as are the phage T4 endonuclease V or *M. Luteus* enzyme, and can recognise other forms of damage. Thus it is effective towards a number of chemical mutagens such as 4 nitroquinoline-1-oxide (4NQO) which produces biadducts in DNA (42). This includes interstrand crosslinks which the uvr ABC enzyme can unhook by nicking both 5' and 3' to the lesion on one strand of the DNA (43).

The uvr ABC enzyme complex is thought to act by the traditional pathway of direct incision into the DNA backbone, 5' to the lesion (33). Thus there now appears to be two distinct pathways for the incision step in the excision of DNA lesions.

1. A lesion-specific, two step incision involving an N-glycosylase, followed by AP endonuclease, characterised by T4 endonuclease V, *M. Luteus* enzyme and *E. coli* endonuclease III.

2. A non lesion-specific system, involving classical single-step incision in the phosphodiester backbone of DNA, characterised by the uvr ABC gene product of uninfected *E. coli*.

The two pathways are represented diagrammatically in figure 1.2.

Once the initial incision step has been made, there are a number of pathways in bacteria capable of repair resynthesis. However, the removal
Figure 1.2a  Schematic Diagram for Base Excision Repair.
Figure 1.2b  Schematic Diagram for Nucleotide Excision Repair.
of AP sites after glycosylase activity may be brought about by one of two mechanisms.

1. The AP endonucleases which may remove the sites produced by spontaneous hydrolysis or by glycosylase activity on altered bases, may cleave the DNA backbone on the 5' side of the AP site and such enzymes include *E. coli* endonuclease IV (44) and exonuclease III (45). Thus the subsequent steps of repair resynthesis can continue.

2. The UV specific endonucleases i.e. T4 endonuclease V and *E. coli* endonuclease III cleave the DNA backbone on the 3' side of the AP site, leaving a 3' deoxyribose terminus. This is not a primer for DNA polymerase and so the deoxyribose may be cleaved by endonuclease IV or to a lesser extent endonuclease VI (46) or a 3' - 5' exonuclease (33), leaving the 3' - OH primer for DNA polymerase. A diagrammatic scheme, taken from Warner (46) for the incision pathways is given in figure 1.3.

Livneh et al (47) have shown an endonucleolytic activity in *M. luteus* extracts which lacks dimer excising activity. This activity was a single incision and the substrate was possibly a substituted purine induced by ionising radiation or UV radiation. Further, Livneh et al (48) have postulated a pathway for removal of apurinic sites by direct insertion of the correct base by an insertase requiring Mg\(^{++}\) ions and duplex DNA structure.

The three enzymes involved in the excision of incised UV - irradiated DNA *in vitro* are the 5' exonuclease activity of DNA polymerase I (49) polymerase III (50) and the single strand specific exonuclease VII (51). Dimer excision is more efficient when simultaneous polymerisation occurs (52). But the pol A\(_1\) mutant of *E. coli*, deficient in polymerisation activity is less proficient at dimer excision than the pol A\(_{ex}\) mutant, which is deficient in 5' exonuclease activity (53) suggesting that polymerisation can act with other unassociated exonucleases. Each of the three polymerases in *E. coli* are known to perform resynthesis. Polymerases I and III have associated exonuclease
Figure 1.3 A Model for the Integrated Pathways of Excision Repair in Bacteria (46)
activity. Polymerase I is unique however, in that it can bind *in vitro* to nicks generated by dimer specific endonucleases unlike polymerases II and III.

Cooper and Hanawalt (54) have shown that resynthesis of the DNA after excision leads to 'patches' of variable size. Most lesions *in vivo* result in short patches of approximately 30 nucleotides with values from 13 to 30 (55 - 57) but some long patches do occur, and the two groups of patch size appear to result from two separate repair pathways. This suggestion arose from the observation that pol A strains of *E. coli* carry out more repair synthesis than pol^+^ strains and as the number of sites for repair is the same in both cases, the extra synthesis may be attributed to the formation of long patches. It is therefore suggested that DNA polymerase I is involved in short patch repair and polymerases II and III in long patch repair (42). An alternative classification of the two pathways is by growth medium dependence or independence (58) which suggests that the growth medium dependent pathway requires protein synthesis, since measurements of incision-break rejoining show the same results for cells in growth medium containing chloramphenicol as for cells in buffer. Repair in the absence of protein synthesis was dependent upon polymerase I and thus related to short patch repair, whereas growth medium dependent repair required the rec A rec B and lex A gene products. The rec A gene has been implicated in long-patch repair (59) and as chloramphenicol reduces long patch repair (53) this suggests that growth medium dependent and long patch repair may be related. Further, the requirement for the rec A gene by both pathways suggests that both may be part of the S.O.S. repair system (53). At the least, it suggests that long-patch repair is dependent upon a protein induced in response to UV radiation damage.

A diagram of the growth medium dependent and independent pathways for excision resynthesis is given in figure 1.4.

Finally, excision repair is completed by joining the repair patches to the pre-existing DNA by the polynucleotide ligase (60).
Figure 1.4 The Genetic and Physiological Control of the Excision Repair Pathways in Bacteria
Adapted from Smith (2)
Post-Replication Repair

Since for the most part, replicating DNA does not have a duplex structure, there is no template on which excision repair can take place. Thus unrepaired lesions in the parental strand of DNA at replication will have an effect upon DNA synthesis.

In bacteria, unexcised pyrimidine dimers will halt the replicating fork progression and give rise to gaps in daughter-strand DNA (61). The gaps are closed by a process of recombination and strand isomerisation (62, 63) to finally give intact daughter strands. The pathway is complex but has an absolute requirement for the recA gene product. In general terms postreplication repair is a means by which a lesion in DNA is by-passed, rather than removed. It was first described in all excision-deficient K12 strains of E. coli (61, 64) using the following protocol. Tritiated thymidine was incorporated into the cells after UV irradiation, over a given growth period and the DNA from the cells assayed. Irradiated cells gave a higher sedimentation rate than unirradiated controls, which gradually decreased with increased growth time after irradiation, until a co-sedimentation stage was reached. This experimental procedure has formed the basis for most post-replication repair studies with bacteria and mammalian cells.

The basic mechanism for post-replication repair uses the gap left opposite a lesion after replication, sister strand exchanges fill the gap with undamaged DNA, thus giving an intact template for repair resynthesis. The repair synthesis occurring here is distinguishable from excision repair in that it occurs in excision deficient (uvrA or B) mutants of E. coli thus permitting cells to tolerate the primary lesions by repairing the secondary lesions produced by the replication of damaged templates. Excision deficient E. coli cells after UV irradiation produced daughter strands of DNA of lengths approximating the distance between two pyrimidine dimers (60, 65). Sister strand exchanges were shown to accompany daughter strand gap repair. However, such repair will not lead to a dimer-free copy directly and it has been shown that dimers become equally distributed
between the parental and daughter-strand DNA (63, 66) and thus in the absence of excision repair, several replications are required to produce dimer free DNA (66).

The gene products in bacteria, required for daughter-strand gap repair include rec A (67) lex A (60, 66, 68) rec B, rec C, rec F, uvr D (60). pol A or pol C mutants repair gaps, but the double mutant does not, which suggests the requirement for either polymerase I or III. So far, the measurements of daughter-strand gap repair have involved the final ligation step, so nothing can be deduced regarding the intermediate reactions. However, recent work with more sophisticated chromatography techniques (69) measuring gap filling without ligation has suggested that lex A and rec A mutants block at a stage when the gap is partially filled (70).

As daughter-strand gap repair requires the rec A gene function, and in bacteria involves DNA exchange, it is often called recombinational repair: but it does require functions other than recombination since rec F and lex A mutants inhibit daughter-strand gap repair (66, 71, 72) but not genetic recombination (73, 74).

S.O.S. Repair

The term S.O.S. repair has been used to describe the responses in E. coli induced by agents such as radiation and chemicals. These responses include inhibition of cell division, of post-irradiation DNA degradation, induced bacterial mutagenesis, Weigle reactivation and Weigle mutagenesis (42). These responses all require the rec A and lex A gene products. The rec A protein, formerly known as protein X (75 - 77), synthesis is controlled by the lex A gene, the latter acting upon an operator to suppress the rec A gene (78), and by the rec A protein itself. Unirradiated cells contain a low level of rec A protein, which upon irradiation is thought to derepress the repressors by a protease activity (79) derepressing itself plus a number of other proteins. It has been suggested that degradation products of DNA produced at the replication fork, or incision sites may be the inducing signal for rec A protein synthesis (80, 81), and
oligonucleotides, by whatever mechanism may be responsible (42, 82).

Weigle reactivation has been thought to occur due to the modification of the normal DNA polymerases by an inducible protein which allows synthesis to continue past the lesion in an indiscriminate way, and thus increasing the possibility of error (83). This has been backed up by a number of lines of experimental research (84, 85). The ability of polymerase III to perform the error-prone transdimer synthesis (86) may be due to some unknown inducible factor, and Hanawalt (42) asks the question as to whether the altered polymerase would function only in repair, or take part in normal replication; that is, would the mutator effect be only at damaged sites or would it be random?

The inhibition of the 3'→5' exonuclease activity of polymerase III may be partly responsible for the cessation of "idling" at dimers by polymerase III, thus allowing synthesis to proceed. Alternatively Schroeder (87) has suggested that rec A protein induction may permit the reverse action of the 3'→5' exonuclease of polymerase III, allowing random insertion of nucleotides. Both suggestions are hypothetical, at this time.

Photoreactivation

The mechanism of photoreactivation involves the direct monomerisation of pyrimidine dimers in DNA, is specific for the dimer and requires the phr gene product. The process occurs in two stages, the first being complexation of the photoreactivating enzyme with the dimer substrate, which takes place in the dark. The second stage is the monomerisation of the dimer, using light of 310 - 480 nm wavelength, with release of the enzyme. (88) Sutherland (89) has shown by spectroscopic methods that the enzyme-dimer complex is the chromophore. A recent review of photoreactivation is given by Sutherland (90).
The complex nature of higher cells, in particular mammalian cells, and the lack of knowledge of the microstructure of the nucleus and thus the mechanisms of replication, have limited the progress in research in the field of mammalian cell repair. However, the discovery that the cells cultured from patients with certain heritable diseases show increased sensitivity to radiation and chemical treatment, and that this sensitivity is attributable to deficiencies in repair systems, has helped to understand the mechanisms of mammalian cell repair.

Excision Repair

It is generally accepted that the basic mechanism for excision repair in mammalian cells is the same as that for bacteria, but that the detailed processes show considerable variation. The differences in recognition of damage in the DNA of mammalian cells may lie in the structural differences of the DNA. Bacterial DNA consists of a single loop of duplex DNA, whereas the genetic material of the eukaryote is tightly packed within a discrete nucleus. The DNA itself forms a compact structure, associated with specific nuclear proteins. A detailed description of the structure of mammalian cell DNA thus far is given by Felsenfield (91). Briefly, the DNA is divided into specific areas, called nucleosomes of approximately 200 base pairs length. The nucleosome core is an invariant 140 base pairs long with as associated histone octamer, and between cores are linker or spacer regions which vary in length according to species. Thus variation in the repeating unit of the nucleosome is due to the spacer region. The DNA of the nucleosome can become coiled around the core histones. The nucleosomes in turn are involved in the highly ordered structure of chromatin found in the nucleus. Chromatin may take one of two structural forms, thin filaments or thicker fibres, due to a linear arrangement or coiling of the nucleosomes respectively. The presence of an H1 histone associated with the spacer region has a direct effect upon chromatin structure. Whatever the structure, the spacer regions are always found on the exterior. Little information is available regarding the relationship of chromatin structure to biological activity, although it is thought that during replication the
histones do not dissociate and thus the nucleosome structure need not be broken down. The recognition of damage to the DNA via a change in structural orientation has already been discussed in prokaryotes. There is now evidence that enzymes exist which aid in unwinding the tightly knit chromatin structure in eukaryotic cells, allowing access to damaged DNA regions.

Recent reports have shown that pyrimidine dimers are produced evenly between core and spacer regions, whereas psoralen adducts occur more often in spacer regions (92). It has also been shown that patches of repaired DNA are more sensitive to digestion by a staphylococcal nuclease. As this enzyme preferentially digests spacer regions, this infers that either repaired regions occur in the spacer regions, or that the repaired DNA patches are more susceptible to nuclease digestion (42). The latter hypothesis is supported by demonstrating that the nuclease sensitivity of repaired DNA after UV irradiation decreases on subsequent incubation (93). The repaired DNA is also more sensitive to DNase I (94, 95). Lehmann and Karran (92) suggest that the DNA region being repaired has a more open structure at the time of repair, and is thus more susceptible to nuclease activity. After repair the region is incorporated into the nucleosome once more and acquires the nuclease activity of the bulk DNA. Recent work by Collins and Johnson (96) has shown that novobiocin, a specific inhibitor of DNA gyrase in bacteria (97) inhibits the incision of UV-irradiated DNA in HeLa cells, and thus may suggest that the complex chromatin structure may be involved in the incision step. There has also been great interest recently in the enzyme poly (ADP ribose) polymerase which polymerises ADP ribose of NAD and eliminates nicotinamide (98, 99). Poly (ADP ribose) is attached to chromosomal proteins and the polymerase is dependent upon DNA and histones for its activity. As far back as the 1960's it was known that treatment of animals or cells with DNA damaging agents led to decreased levels of intracellular NAD. This and other indirect evidence suggests that DNA damage gives rise to an increase in poly (ADP ribose) polymerase activity, but no indication as to the role of the enzyme. Recently, Durkacz et al (100) have shown with L1210 cells that treatment with
dimethyl sulfoxide (DMS) plus 3-amino benzamide (a specific inhibitor of poly(ADP ribose) polymerase) prevented the rejoining of DMS induced single strand breaks. Thus poly(ADP ribose) polymerase has a function in the recovery of DNA damage, although the mechanism is still open for debate.

The first inference of the existence of excision repair in mammalian cells was by Rasmussen and Painter in 1964 (101) who found low levels of DNA synthesis after UV irradiation in non-S-phase cells, a phenomenon they called unscheduled DNA synthesis (UDS), which was thought to be due to filling of gaps after removal of damaged DNA, independent of the cell cycle. Duncan et al (102) have demonstrated that extracts of human cells can excise thymine dimers from DNA, and Waldstein et al (103) isolated an enzyme extract from calf thymus which has dimer specific UV endonuclease activity. The incision step is not well understood, but appears to be the rate-limiting step. The subsequent steps of excision repair occur very rapidly, demonstrated by the sensitive techniques required to detect the resulting single strand breaks (104).

The mechanisms of excision repair in mammalian cells have been better understood by the use of cells from patients with the disease Xeroderma Pigmentosum (XP). Such patients show sun sensitivity and fibroblasts from these patients are defective in excision repair, with associated sensitivity to the toxic effects of UV radiation (105 – 107). Complementation has shown there to be at least seven distinct genetic types of XP (A to G), defective in excision repair, and the XP variants which appear normal in excision repair but with defects in daughter-strand gap repair. The introduction of phage T4 endonuclease V into permeabilised XP cells restores normal levels of UDS after UV irradiation in groups A to E (108) and this led to an increase in cell survival (109). Further studies have demonstrated that the addition of the T4 endonuclease V enzyme to XP cells restores repair replication to a near-normal level (110), and thus the ability of an endonuclease to allow cells from different complementation groups to perform excision resynthesis, suggests that the defect is in the incision step of the repair system. Each complementation group has its own set of repair characteristics, and these tend to be similar within the groups, with
the exception of group D, where UDS is higher than in the other groups up to 50% of normal, but survival after UV-irradiation is as low as group A, where UDS is only 10 - 25% of normal (111). This as yet has not been explained. In XP variants, no defect in excision repair has as yet been found with conventional methods (112) although some workers (113, 114) have detected a defect late in excision repair which is more marked if longer wavelength UV radiation is used (115). This has been suggested as the primary defect in XP variant cells, and the repair replication abnormalities may be consequences of the excision defect. Further complications involve the kinetics of complementation, groups A and E show rapid complementation without protein synthesis, whereas groups C and D require protein synthesis and complementation is slower (see 92).

Thus the incision step in mammalian cell excision repair is a complex one, and the study is not helped by the instability of the enzymes involved (116).

The excision of dimers and large adducts from mammalian cell DNA results in repair patches of 15 - 100 nucleotides in length, this being defective in XP cells (117). The enzymes involved are little known at present, some exonucleases having been isolated which can excise dimers from incised DNA, but none have been demonstrated in vivo.

The mammalian cell polymerases, α, β, γ or δ, do not have associated 5' - 3' exonuclease activity (118 - 120). However, evidence suggests that mammalian cell excision and resynthesis may be a coupled event, as they appear to be in E. coli, for example a 5' exonuclease activity from human lymphoblasts, purified with an AP endonuclease was stimulated by a polymerase (121) and the combined activity of the two enzymes resulted in repair resynthesis of depurinated DNA in vitro (122).

Other exonuclease activities have been described: hNase IV from rabbit tissue acts on double stranded DNA by 5' - 3' degradation of UV-irradiated DNA treated with UV-specific endonuclease from M. luteus (123). Cook and Friedberg (124, 125) have isolated three exonucleases from human KB cells using selective loss of dimers from DNA incised by T4
endonuclease V. A further exonuclease activity has been isolated from both placenta and tissue culture cells (126).

Of the four mammalian cell polymerases, γ polymerase is thought to be that for mitochondrial DNA (127), but it is not known whether it functions in the nucleus. δ polymerase has not been well characterised and may prove to be a form of α polymerase. Purified α polymerase is unable to initiate synthesis at a nick, but requires a gap of approximately 20 - 70 nucleotides (128), whereas the less purified enzyme, together with an exonuclease promotes insertion of a few nucleotides at a nick. The activities of both α and β polymerases have not yet been studied in vivo and chromatin structure may modify the enzyme activities considerably.

Two DNA ligases have been isolated from rat liver, DNA ligase I is found at higher levels in rapidly dividing cells, as is polymerase I, DNA ligase II levels remain relatively constant (129).

Some mammalian cells show capability to repair single strand and double strand breaks induced by ionising radiation (130) and up to 90% of these can be repaired in one hour, faster than dimer repair. Ritter et al (131) have suggested that single strand breaks may be the major lesion for ionising radiation induced lethality. Lett has shown that many tissues can repair single strand breaks, both in vivo and in vitro (132, 133) and Hattern et al (134) have shown human cells capable of repair of damage of the 5, 6, dihydroxy-dihydrothymine type.

Another genetic disorder Ataxia Telangiectasia (AT) described by Patterson and Smith (135) shows increased sensitivity to ionising radiation (136) although the UV radiation response is normal, and chemical response varies between strains (135). The biochemical defect in AT cells is not understood. AT cells in culture rejoin single and double strand breaks at normal rates (135), and repair 5, 6, dihydroxy-dihydrothymine normally, both in whole cells and cell free extracts (137, 138). AT cells also show normal levels of AP endonuclease, superoxide dismutase and catalase (139). Inoue et al have shown reduced levels of an activity in AT cells which renders γ-irradiated DNA a primer for exogenous DNA polymerase (140) but it is now thought that
AT sensitivity may be due to a small but significant portion of single strand breaks which are not repaired after ionising radiation (92).

Mammalian cells are capable of removing cross-links produced by, for example psoralen plus UV radiation. The disease Fanconi's Anaemia (FA) is characterised by cells having sensitivity to cross-linking agents and Sasaki (141) suggests that normal cells have an enzyme capable of recognising the cross-link, absent from FA cells.

The limited excision repair capabilities of rodent cell lines and adult animals may be due to loss of ability present in the embryos (142).

DNA-protein cross-links produced by UV and ionising radiations can be removed by normal human fibroblasts, but not by XP-A cells (143) which suggests these lesions are recognised in a similar manner to other large adducts.

**Replication Repair in Mammalian Cells**

A response in mammalian cells, similar to the daughter strand gap repair in bacterial cells, has been demonstrated using alkaline sucrose gradient techniques (144), the response being reduced in XP variant cells (112). Difficulties arise however, due to the nature of replication in mammalian cell DNA which takes place at a number of sites, called replicons, at the same time and replication can be both bidirectional or less frequently, monodirectional (145). Low molecular weight fragments called Okasaki fragments are joined within each replicon, then adjacent daughter strands are joined. This causes problems with sucrose gradient techniques, as if damage is present the distance between replicons may be exceeded by the resultant gaps produced by lesions. Also, the rate of joining of daughter strands may be similar to that for elimination of gaps (144). Discontinuities in daughter strands may arise from gaps left opposite lesions after replication, but may also be due to inhibition of initiation at the replicon, or of strand elongation. Strand elongation inhibition causes discontinuities between adjacent replicons, whereas initiation inhibition causes discontinuities between adjacent replicons, or between active replicons which are not adjacent.
and have one or more inactive replicons between them. The views held in 1978 regarding the mechanism for producing intact daughter strands were as follows:

1. Doniger (146) postulated that gaps were left in daughter-strand DNA opposite lesions, to be filled in by strand exchange or synthesis past lesions. Figure 1.5.

2. Edenberg (147) has suggested that replication forks are blocked for long periods of time at lesions, and do not reinitiate beyond them.

Lehmann (148) emphasised the controversy concerning this mechanism, and that one particular mechanism may not be generally applicable. However, a number of points have been elucidated since 1978.

The amount of sister strand exchange in mammalian cell is far less than for bacteria (144, 149) and experiments have shown gaps to be filled by de novo synthesis (144). As sister-strand exchange is infrequent, gap filling may occur by transdimer synthesis (150) or by using the complimentary daughter strand as template (151, 152) see figure 1.6.

Higher UV radiation fluences have been shown to slow the progression of the replicating fork, by delays at damaged sites (153), and to reduce the molecular weight of newly synthesised DNA (112). The latter may be due either to blockage at the replication fork by a lesion (147) or synthesis past a lesion, by the daughter strand DNA thus producing a gap (146). In both cases the newly synthesised DNA approximated the distance between lesions. Since the replicons which remained active after UV irradiation produced smaller strands than in unirradiated cells, the general opinion is that DNA synthesis continues after irradiation but leaves gaps in the daughter strands opposite lesions. In many cell types, newly synthesised DNA is the same length or only slightly smaller than that in undamaged cells. It has been postulated that forks cannot be blocked, and if gaps are
A. Dimers on both DNA strands.

B. Synthesis past dimers, leaving gaps in the daughter strand.

C. Synthesis of DNA to fill gaps.

Figure 1.5 A Model for Postreplication Repair: Gap Filling.
A. Dimers on both DNA strands.

B. Migration of the daughter strands.

C. Synthesis using complementary daughter strand as template.

D. Continuation of the replication process.

Figure 1.6 A Model for Postreplication Repair: Replicative Bypass.
formed in daughter strands, then they are sealed rapidly (112). Whatever the mechanism of replication repair, there is some effect on DNA synthesis which is eventually overcome, as normal sized DNA is synthesised after a time.

Lehmann et al (112) showed that XP-variant cells were deficient in post-replication repair, as the eventual synthesis of high molecular weight DNA took longer than in normal cells. It was first thought to be due to a reduced rate of gap filling, but Park and Cleaver (154) have shown that the difference is in the size of DNA synthesised immediately after irradiation. When UV radiation fluences were given, to produce the same size of nascent DNA in both XP variant and normal cells, the rate of increase in molecular weight was the same in both cases. Thus Cleaver and his colleagues have questioned the existence of post-replication repair. The answer given by Lehmann and Karran (92) briefly is to the effect that recovery to high molecular weight daughter DNA after UV irradiation means that post-replication repair does exist, and the presence of dimers in replicated DNA (155, 156) suggests that overcoming the effects on DNA synthesis cannot be due solely to excision of damage from parental DNA.

The reduced rate of DNA synthesis takes several hours to recover after UV irradiation. Little is known about this recovery mechanism but it is not seen in excision deficient XP cells nor in cells from patients with Cockayne's Syndrome, a neurological disorder with associated sun-sensitivity (157).

The size of newly synthesised DNA after irradiation increases more rapidly than the recovery of DNA synthesis, and this process does not require protein synthesis (158) a phenomenon as yet not understood.

Thus the events occurring during replication after treatment with UV or ionising radiation present a complex picture, that is at present far from being clearly understood.
Photoreactivation

The presence of photoreactivating enzyme in bacteria has been known for many years (159) with subsequent establishment of the mechanism of action. Although such enzymes have been found in metazoa and higher animals, including birds, fish and reptiles (160), they could not be found in mammalian cells for many years, nor could the expected biological effect be demonstrated, i.e. increase in cell survival (161). In 1974 however, Sutherland (162) reported the presence of photoreactivating enzyme in human leukocytes, which was specific for cyclobutyl pyrimidine dimers in isolated DNA, and attributed the lack of discovery of the enzyme to inadequate exposure to UV radiation, or to insensitive assay systems. It was also emphasised that the difficulty in demonstrating the photoreactivation in vivo, may be due to low enzyme levels or inaccessibility to DNA, the latter attributed to the results of Cook (163).

It appears that the mammalian cell photoreactivating enzyme may differ from bacterial or yeast enzyme, and there may be different levels of activity of the enzyme in mammalian cells dependent upon the cell type, or tissue type within a particular species (162, 164, 165).

Evidence for photoreactivating enzyme activity in mammalian cells has been contradictory, but one conclusion drawn has been the dependence on growth conditions of the photoreactivation effect (166 - 168). This phenomenon was finally confirmed by Mortelmans (169). Most evidence for activity has been from in vitro systems, such as photoreactivation of biological activity in UV irradiated transforming DNA, by mammalian cell extracts (170, 171). Wagner et al (172) used plaque forming ability of UV-irradiated virus particles adhered to unirradiated XP fibroblasts, and exposed to photoreactivating light, as a demonstration of photoreactivation activity. These methods show activity on heterogenous DNA, so it is necessary to demonstrate action upon the cell's own DNA. Sutherland and Oliver (166) demonstrated photoreactivation of the decrease in DNA synthesis in XP cells after low UV radiation exposures. Further, Harm has demonstrated the action of photoreactivating enzyme from marsupials, by host cell activation of
Herpes simplex virus (173), and in mammalian cells, including human cells, by competitive inhibition of in vitro photorepair of Haemophilus influenzae virus (174).

It is important to note that although many studies have demonstrated the presence of photoreactivating enzyme in placental mammals, and that these enzymes can monomerise dimers from DNA of the same species, as well as that of other systems, no demonstration has yet been made of a direct increase in survival, due to the enzyme activity. Cook (163) has suggested that excision proficient cells may mask the photoreactivation system, or that the DNA may be inaccessible to the enzyme. These and the low levels of photoreactivating enzyme in mammalian cells are all factors which need to be accounted for before the measurement of photoreactivation activity can be demonstrated in vivo.
The Relationship of DNA Damage and Repair to Mutagenesis and Carcinogenesis

It is now generally accepted that mutagenesis and carcinogenesis are associated with damage to DNA and its subsequent repair. There are several lines of evidence to support this. The studies of DNA repair mechanisms in bacteria have demonstrated that repair of lesions produced by UV radiation, by the excision repair pathway, is essentially error-free, whereas post-replication repair processes appear to be error-prone, leading to mutagenesis (175, 176).

Cells from XP variant patients, with a defect in post-replication repair, show a higher than normal mutagenic response to UV radiation. XP patients suffer a higher incidence of skin cancer than normal, as well as hypersensitivity to sunlight.

The link between mutagenesis and carcinogenesis has also been made by McCann and Ames (177) who have demonstrated that most carcinogens are mutagens. Finally, the carcinogenic nature of UV and ionising radiations in experimental systems, together with the involvement of sunlight in the induction of basal cell and squamous cell carcinoma in epidemiological studies, when considered with the previously stated evidence, suggests that the first step towards carcinogenesis may be through the error-prone repair of DNA damage.

It is worth considering the damage produced not only by exogenous agents, such as UV radiation and chemicals, but also damage arising spontaneously within the cell. Smith has suggested that the same repair genes act upon damage produced in either of the above ways (178). It follows that the implications of this system in spontaneous cancer production (179) may be explained by defects in metabolic functions within the cell as well as the genetic defects in repair systems, already mentioned.

Thus the importance of the role of repair of damage to DNA cannot be over-emphasised, in terms of the stability of the metabolic function of the organism, both in the face of attack from exogenous damaging agents, a situation which today is cause for much concern, and from metabolic alterations within the environment of the cell itself.
CHAPTER TWO

THE DEVELOPMENT OF MAMMALIAN CELL CULTURE TECHNIQUES
INTRODUCTION

The first successful explant of tissue into culture was in 1885, when William Roux (180) maintained the medullary plate of a chick embryo in warm saline for several days. In 1903, the first detailed observations of cell survival and division in vitro were carried out by Jolly (181), maintaining salamander leukocytes in hanging drops for up to one month, followed in 1907 by Harrison (182) who demonstrated beyond doubt the continuation of normal function in vitro using medullary tube explants from frogs grown in lymph clots. The use of the plasma clot to replace the lymph clot was introduced by Burrows, and thus the first tissue culture techniques were established (183).

The problem of bacterial contamination of tissue cultures was realised at an early stage, and one pioneer in the field, Alexis Carrel, applied stringent aseptic techniques familiar to him through his work as a surgeon, to his cultures, and was able to maintain a strain of cells in active multiplication for 34 years without the use of antibiotics (184). Such continuous cultivation of cells led to the realisation of large quantities of cells being grown over long periods of time, thus lending themselves to all kinds of experimental work. Lewis and Lewis (185) started to look for factors in medium necessary for growth, and attempts were made to analyse the media by Carrel and co-workers (186). Work carried on by Fischer, Waymouth, Eagle and many others (187, 188) has led to the development of growth media used today.

As early as 1913, it was shown that explanted tissue could be used for successful growth of virus (189), but the work of Enders (190) in 1948, which showed that poliomyelitis virus could be cultivated in the HeLa cell line, gave most impetus to work in this field.

In contrast to cell culture, Strangeways and Fell developed the technique known as organ culture (191), where tissue fragments were maintained in as close as possible state to that in vivo. This type of culture has proved invaluable in the studies of endocrinology.
Thus there are three main culture methods.

1. Tissue culture
2. Organ culture
3. Cell culture

The first two involve small fragments of tissue being put into growth medium; in tissue culture, the cells from the tissue are permitted to grow and divide in a disorganised manner, not so for the organ culture. In cell culture, the tissue is intentionally disorganised by disrupting the individual cells. Cultures produced in this way are called primary cultures.

Any piece of tissue, however small, contains a number of different types of cell. For the first few days of tissue culture all of these cells survive, whereas after a time certain cells begin to migrate from the tissue. The first cells to migrate are usually white blood cells and macrophages, then fibroblasts. These are followed by epithelial cells. Many of the specialised cells, for example nerve cells, never move from the tissue. Even if cells migrate, how they survive in culture is variable. Blood cells disappear from the culture within a few days, while others such as muscle cells remain for months without dividing, then eventually die. Some cells divide rapidly for a time, then die after weeks or months. Cells which multiply repeatedly for a long time can often be passaged, that is put into suspension (eg. by treatment with trypsin) and inoculated into fresh medium, where they continue to grow. These cells then become a primary cell line. The primary cell line usually has two fates, it will either continue to multiply in culture for many months then die out, or will show the potential for indefinite growth in vitro, in which case it becomes an established cell line. Generally, a line is not established unless it has been cultured at least 70 times, with 3-day intervals between subculturing (184). The change from primary to established cell line may be gradual or may be dramatic. The term used to describe a more dramatic change is "transformation", which must not be confused with the application of the term in bacteriology. Transformation occurs usually in slow growing cultures, where rapidly growing colonies of
altered cells suddenly appear and outgrow the original culture. These established cells, unlike the primary cell lines, often no longer retain the characteristics of the cells from which they are derived. All primary cell lines have the normal number of chromosomes, whereas established cell lines often show tetraploidy in the early stages, becoming aneuploid eventually. These cells usually have short doubling times of 12 to 20 hours, and have similar nutritional requirements, whatever their origin. They will grow to higher cell densities than the primary cell lines, do not show specialised function, will grow from low inocula, and can be maintained in suspension, losing the requirement for surface contact exhibited by the primary cell line.

Primary cell lines can also be established from tumours as well as normal cells, in which case the cells act as if they have been transformed from the beginning. One paradox however, is that tumour cell lines often retain the specialised functions of the normal tissue.

The cells derived from a living cell in vivo or in vitro, will have one of two functions, it will "grow" by cell division, or it will begin to function in a manner peculiar to that cell or organ, ie. it will differentiate and thus cease to grow.

The recurring statement in tissue culture literature is that cells in culture dedifferentiate, ie. lose their specialised functions. This is well proven in many cases (192, 193). However, the opposite has been found (194, 195), with some cells exhibiting long term maintenance of a specialised function, but this is the exception rather than the rule. In particular, fibroblasts retain their ability to secrete collagen (196, 197). It is not easy to give a general rule and it may be that the retention of differentiating potential is not observed because of inadequate nutrients in the growth medium, supported by Doljansky, for melanin synthesis in retinal cells (198).
The Cell Cycle

For most eukaryotic cells there are four phases to the cell cycle. For mammalian cells, mitosis (M) takes up 30 to 60 minutes. This is followed by the first gap (G₁) with variable duration. DNA synthesis occurs at the end of G₁, giving rise to the synthetic (S) phase which lasts for 4 to 6 hours, followed by the second gap (G₂) of about 4 hours duration. G₁ may be extremely short or almost absent, giving a cell cycle of 8 to 10 hours, or may be several days in length with a correspondingly long cell cycle.

Growth Pattern of Established Cell Lines

Animal cells in culture show a growth pattern similar to that for bacteria. Cells from a stationary phase culture put into fresh medium will show an initial lag phase of hours or days, followed by the logarithmic phase until the population maximum is reached and cells enter stationary phase. This type of growth pattern is seen particularly in suspension cultures and at low inocula: other conditions may affect this pattern, such as slowing down of cell growth as a limiting density is reached which may result from an exhaustion of nutrients. Environmental effects upon growth of cells in culture is important, including pH, temperature and osmotic pressure. Small changes in these factors, outside the optimal range for the cells, can have a dramatic effect on cell growth. The usual temperature for mammalian tissue and cell culture is 37.5°C. The pH range is usually from 6.8 to 7.6, and the use of buffering systems to maintain pH will be discussed in the materials and methods.
MATERIALS AND METHODS

The Cell Lines

The cell lines used for the work presented in this thesis are as follows:

1. L5178Y: The mouse lymphoma cell line, established in culture by G. A. Fischer (199) from an ascitic tumour in the DBA/2 mouse. This line was kindly supplied by Dr. C. F. Arlett, M.R.C. Cell Mutation Unit, Falmer, Sussex.

2. L5178Y, JL: This cell line has the same origins as that in (1), but with a different history and cultured in a different growth medium. The cell line was kindly supplied by V. D. Courtenay, Sutton, Surrey, from John Lett, hence the referral throughout the thesis to this cell line as JL.

3. L5178YS: This cell line was obtained from a single clone of cells after exposure of L5178Y cells to X-irradiation, and cultured for six months (200). The cell line is described as radiosensitive with respect to the parent line. The cell line was kindly supplied by Dr. M. Fox, Holt Radium Institute, Manchester.

4. AIV: This cell line was obtained from a culture of L5178Y cells exposed to low levels of ionising radiation, by growing the cells in medium containing tritiated water. Over a period of many months, a culture of slow growing cells was obtained, which is described as radioresistant with respect to the parent line (201). The cell line was kindly supplied by V. D. Courtenay, Sutton, Surrey.

Culture Media

The culture media were prepared from two basic defined media. These were:

1. Fischers medium for leukemic cells of mice.
2. RPMI 1640 medium.
To the defined media were added a number of ingredients, and the formulae for the complete growth media are given in table 2.1, together with the source, form and storage requirements for each ingredient.

As all ingredients were prepared as sterile liquids or solutions, culture media were prepared aseptically with no final sterilisation, and stored at 4°C for up to one month.

The basic media are chemically defined and necessary for the culture of the cells concerned. The additions to the media are for specific reasons, and will be discussed individually.

**Serum**

Although many attempts have been made to culture mammalian cells in totally defined medium, serum is still considered essential for continuous cell culture, and as yet, the ingredients in serum which make it essential have not been elucidated. Thus it is still accepted practice to add a totally undefined substance to the defined medium. The type of serum suitable for cell culture varies with the mammalian cell type. Thus for mouse lymphoma cells, horse serum is employed. Further difficulties arise in that the ability to support cell growth varies between suppliers and also between batches from the same supplier. In order to maintain reproducible experimental data, a batch reservation system operates. Samples of serum are tested against a reliable batch in use, for the ability to support the growth of cells in maintenance culture, and of colonies formed in soft agar. An accepted batch, which will support adequate growth in maintenance culture and gives a plating efficiency of over 80% may be reserved for a six month period and supplied at regular intervals.

It is interesting to note that although the four cell lines cultured are related, their serum requirements are different. The L5178YS cells would not grow continuously in medium with the serum used for the other lines, and another suitable batch of serum was required for this line. The serum suitable for the growth of L5178YS cells also supported the growth of the other cell lines.
<table>
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<th>INGREDIENT</th>
<th>FORM AND VOLUME SUPPLIED</th>
<th>SUPPLIER</th>
<th>STORAGE TEMPERATURE</th>
<th>VOLUME IN MEDIUM (ml)</th>
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</tr>
<tr>
<td>Penicillin Streptomycin</td>
<td>100,000i.u./ml 100ml</td>
<td>Flow</td>
<td>-20°C</td>
<td>2.00</td>
<td>10.00</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>200mM Solution 100ml</td>
<td>Flow</td>
<td>-20°C</td>
<td>1.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>Autoclaved 500ml</td>
<td>-</td>
<td>R.T.</td>
<td>to 500</td>
<td>to 500</td>
</tr>
</tbody>
</table>

Table 2.1  Culture Media Formulae.
Sodium Bicarbonate and Buffering of Medium

Sodium bicarbonate is used as a buffer in the growth medium, designed to equilibrate with the atmosphere above the medium. Sodium bicarbonate breaks down in aqueous solution to form carbon dioxide and hydroxyl ions, the latter thus increasing the pH of the medium. In order to maintain the pH of the medium, the atmosphere above the medium is gassed with carbon dioxide to a level of 5% v/v, determined as that necessary to optimise culture conditions. This is achieved in one of two ways and will be discussed in the appropriate section.

Phenol red indicator is present in the medium and shows yellow at low pH, pink at high pH, orange at the optimal physiological pH range of 6.8 - 7.6. Thus the media are gassed with carbon dioxide to give an orange colouration, representative of the physiological pH.

The final concentration of sodium bicarbonate is 1 G per litre, in FM10 and FM20, and in RPMI-10 and RPMI-20 is 2 G per litre. No sodium bicarbonate is required to be added to RPMI 1640 basic medium because it is already included in the formulation.

Sodium Pyruvate

Sodium pyruvate is considered essential for the growth of cells from low inocula, even from a single cell, at a concentration of 200 µg per ml (202). The reasons for this requirement are not established.

Antibiotics

It is accepted that good aseptic technique is essential in the culture of mammalian cells. However, the problems of airborne bacterial contaminants are guarded against by the addition of antibiotics to the culture media. The combined use of penicillin and streptomycin provides cover against a broad spectrum of bacterial contaminants.

No cover can be provided against fungal contamination, and infected cultures are to be discarded, with new cultures started from stock.
Water

During the development of culture methods it became apparent that the quality of water used in the culture media was extremely important. Initially, double distilled water from a Fi-Stream 4 (Fisons Limited) was freshly collected into 500 ml Pyrex bottles (Schott), capped with plastic caps and autoclaved at 121°C for 15 minutes. The water produced in this manner had a pH of 4.5.

Due to equipment failure, it was necessary to find an alternative source of water. The single, glass-distilled water from a properly maintained still, collected and sterilised as described above, was found to be adequate. This water had a pH of 4.2, and was used for all subsequent work.

GENERAL MATERIALS

Dimethyl Sulphoxide

Dimethyl Sulphoxide, DMSO (Sigma Grade, Sigma Chemical Co) was sterilised by filtration through an 0.2 μm pore size, 29 mm diameter Millex FG hydrophobic disposable filter unit (Millipore) in 5 ml volumes into sterile bijou bottles with metal caps. The DMSO was stored at room temperature, warmed in the 37°C water bath when the laboratory temperature fell below 16°C, the melting point of DMSO.

Agar

It has been established that certain impurities in bacteriological grade agars notably sulphated polysaccharides can dramatically affect the growth of mammalian cell colonies. Thus a specially purified agar, Noble Agar (Difco) has been used throughout for the cloning procedures.

Five G. of Noble Agar was made up to 200 ml with glass distilled water in a 200 ml Pyrex glass bottle (Schott) and autoclaved at 121°C for 15 minutes. The agar was allowed to cool to approximately 70°C then remixed by gentle rotation and 20 ml volumes were distributed into...
25 ml sterile glass bottles with plastic caps. The agar was allowed to set at room temperature then stored at 4°C for up to one month. The final agar concentration was 2.5% w/v.

**Phosphate Buffered Saline**

Phosphate buffered saline (PBS) consists of the following two solutions (203)

**Dulbecco Solution 'A'**

<table>
<thead>
<tr>
<th></th>
<th>G. per Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>8.00</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>0.20</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>1.15</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Dulbecco solution A was supplied as solution tablets (Oxoid)

**Dulbecco solution 'B' (Mineral Salts Solution)**

<table>
<thead>
<tr>
<th></th>
<th>G. per Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Dulbecco solution B was supplied as a sterile solution in 5 ml ampoules (Oxoid).

Ten tablets of Dulbecco solution A were dissolved in glass distilled water and made up to 1 litre, clarified by filtration through an 0.45 µm pore size, 25 mm diameter Sartorius membrane filter using negative pressure and autoclaved in 100 ml volumes in metal capped glass bottles at 121°C for 15 minutes. The solutions were allowed to cool to room temperature, and 0.5 ml volumes of Dulbecco solution B were aseptically added to each 100 ml of solution A using a sterile syringe and needle.

The final solution, PBS has a pH of approximately 7.4.
Carbon Dioxide
Carbon dioxide gas, was used in two forms for equilibration with culture media.

1. Carbon Dioxide 5% v/v in Air (Air Products) was used to gas the atmosphere above the medium for cells in suspension culture. The system contained an in-line 0.2 μm pore size Swinnex filter unit. The gas was introduced into the tissue culture flask via a pre-flamed sterile, plugged pasteur pipette, for approximately ten seconds.

2. Carbon Dioxide (Distillers Co) was used to gas the boxes containing the plates for survival determination by soft agar suspension. The gas was delivered into the box with the lid ajar sufficiently to allow the delivery, by means of a pasteur pipette, for 30 seconds at a flow rate of 0.6 litres per minute. This has been previously determined to give sufficient carbon dioxide to maintain the medium pH at optimal for cell growth.

Tissue Culture Plastics
Twenty five cm² tissue culture flasks and 10 cm x 16 mm screw cap tissue culture tubes were supplied by Sterilin. Eighty cm² tissue culture flasks were supplied by Nunclon (Gibco). Petri dishes, 5 cm and 9 cm diameter, were single vented, bacteriological grade, supplied by Sterilin. Tissue culture grade Petri dishes were not necessary, as the cells did not come into contact with the plastic.

All plasticware was disposable, and passed through the decontamination autoclave after use.

Glassware and Re-usable Plastics
Glassware consisted of normal laboratory glassware (Fisons), plus bottles originally containing tissue culture media (Flow, Gibco).
All glassware, plastic and metal caps and plastic replicating pipette tips were subjected to a thorough washing procedure prior to use, and between each use. This procedure is described under general methods.

GENERAL METHODS

Washing Procedure

1. Glassware and re-usable plastics were rinsed with tap water after use.

2. All glassware except pipettes were soaked in a 2% v/v solution of RBS-25 in tap water for 30 minutes, ensuring complete immersion.

3. Surfaces were cleaned thoroughly with a brush, whilst rinsing under the tap.

4. Glassware was subjected to three tap water rinses, followed by three distilled water rinses.

5. A further soak in distilled water for 30 minutes.

6. A final rinse in freshly distilled water.

Pipettes were boiled in distilled water prior to a 30 minute wash in the pipette washer, then subjected to procedures 4 to 6.

Metal and plastic caps, and pipette tips were boiled three times in distilled water, then given three distilled water rinses before a final rinse in freshly distilled water.

All equipment was dried in the warm air cabinet prior to sterilisation.

Sterilisation Procedures

1. Glassware was sterilised by dry heat at 170°C for a minimum of one hour.
2. Plastic and metal caps, and pipette tips were sealed into D.H.S.S. specification sterilisation bags (D.R.G.) and autoclaved at 141°C for 5 minutes (Drayton Castle high vacuum autoclave).

3. Sterilisation of distilled water and Swinnex filter units (sealed into autoclave bags) was at 121°C for 15 minutes.

4. Sterilisation of solutions by filtration was with an 0.2 μm pore size 25 mm diameter Sartorius membrane filter in a Swinnex filter unit (Millipore). The unit was previously sterilised as described in 3.

All aseptic manipulations were carried out under laminar flow, using a Fell horizontal flow, laminar flow cabinet. The cabinet was allowed to run for 30 minutes prior to commencing work, and was swabbed liberally with 70% v/v alcohol before and after use, on all surfaces.

CELL CULTURE METHODS

Storage of Cultures

Stock cultures were prepared by centrifuging cell suspensions in logarithmic growth at 1500 rpm in the MSE minor centrifuge, for five minutes, decanting the supernatant and resuspending the cells at 10^6 per ml in FM10 or RPMI-10 with 5% v/v DMSO. One ml volumes were pipetted into 2 ml plastic sterile ampoules (Sterilin) and frozen in the Union carbide biological freezer unit (BF-6) set at ring 'E', fitted to the LR-33-10 refrigerator (Union Carbide). The ring 'E' setting allows a freezing rate of 1.2 to 1.4°C per minute, and the freezing time was a minimum of 1 hour 45 minutes. Ampoules were then transferred to the Union Carbide LR-40 liquid nitrogen refrigerator and stored in the vapour phase at below -130°C.

Reconstitution of Frozen Cells

New cultures were started from stock every two months, with old cultures being discarded. Ampoules were removed from liquid nitrogen
storage and thawed in the 37°C water bath. The contents of the ampoule was mixed gently with a pasteur pipette and transferred to a 25 cm² tissue culture flask containing 9 ml of FM10 or RPMI-10 at 37°C. The atmosphere above the medium was gassed with 5% carbon dioxide in air, and the culture was incubated at 37°C.

Maintenance of Cultures

Cultures of L5178Y cells were maintained in FM10, cultures of L5178YS, JL and AIV were maintained in RPMI-10 at 37°C in 10 ml volumes contained in 25 cm² tissue culture flasks.

Cell counts were monitored by the use of the haemocytometer, and from these counts growth curves were constructed for each cell line. These are given in figures 2.1 (a) to (d). The cell doubling times were determined from the growth curves, during logarithmic growth, and are given in table 2.2. It was apparent that the cells in suspension gave a characteristic growth pattern, with definite growth phases. In order to maintain a healthy, logarithmically growing culture, cells were subcultured before the cell concentration exceeded 5 x 10⁵ per ml. Cells were subcultured into 25 cm² tissue culture flasks, by dilutions of 1 in 10 (1 ml cells + 9 ml medium) or 1 in 100 (0.1 ml cells + 9.9 ml medium).

EXPERIMENTAL

1. To determine the plating efficiencies of the cells in soft agar suspension

The mouse lymphoma cells characteristically grow in suspension. Therefore, in order to use the criterion of colony forming ability as a measure of survival, the cells are isolated by suspension in a semi-solid medium, after the technique devised by Puck and Marcus (204). This procedure has been adapted by workers to fit the requirements of each laboratory.
Figure 2.1a  Growth Curve for L5178Y cells grown in FM10 at 37°C.
Figure 2.1b  Growth Curve for JL cells grown in RPMI-10 at 37°C.
Figure 2.1c Growth Curve for L5178YS cells grown in RPMI-10 at 37°C.
Figure 2.1d Growth Curve for AIV cells grown in RPMI-10 at 37°C
Table 2.2  Cell Doubling Times for the Mouse Lymphoma Cell Lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Doubling Time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y</td>
<td>11.5</td>
</tr>
<tr>
<td>JL</td>
<td>10.5</td>
</tr>
<tr>
<td>L5178YS</td>
<td>12.0</td>
</tr>
<tr>
<td>AIV</td>
<td>15.0</td>
</tr>
</tbody>
</table>
Preparation of Agar Medium

The final formula for the soft agar suspension culture was as follows:

- FM20 or RPMI-20 (table 2.1) 22.0 ml
- Noble Agar 2.5% w/v 2.5 ml
- Cell suspension 0.5 ml

Thus from each cell sample, 4 x 5 ml plates were prepared, leaving a 5 ml overlap.

Twenty-two ml volumes of FM20 or RPMI-20 were pipetted into sterile 50 ml medical flat bottles with metal caps (Beatson Clark). The bottles were placed in the water bath to equilibrate to 37°C. The 25 ml bottles of 2.5% w/v Noble Agar were boiled in a beaker of distilled water. The molten agar was mixed gently and 2.5 ml volumes added to each bottle of medium using a sterile glass graduated pipette, while the agar temperature was above 70°C. The medium was mixed gently and the bottles returned to the 37°C water bath. The agar medium remains in a liquid state for at least three hours at 37°C.

Dilution of cell suspensions

Dilution of cells were carried out in PBS at room temperature. These were 1 in 10 (0.5 ml cells + 4.5 ml PBS) and 1 in 5 (0.5 ml cells + 2.0 ml PBS).

Plating the cells

An 0.5 ml volume of cell suspension from the final dilution in buffer was added to the 24.5 ml of agar medium, mixed thoroughly, and 5 ml volumes pipetted into four 5 cm diameter bacteriological grade Petri dishes. The plates were placed at -20°C for 15 minutes to rapidly set the agar thus preventing the cells from settling out of the medium. The plates were then left at room temperature for 5 minutes and placed in stacks of four plates, in clear polystyrene boxes (Griffin and George) up to a maximum of eight stacks per box, with a 50 ml beaker
of distilled water to humidify the atmosphere within the box. The box was gassed with carbon dioxide and the lid sealed in place with PVC British Standard gas-tight tape (Rotunda). The box was then incubated at 37°C.

Dilution Factors

A diagram of the dilution and plating scheme is given in figure 2.2.

It is apparent that the plating of 4 x 5 ml volumes from a cell suspension incorporating a 1 in 50 dilution (0.5 ml cells + 24.5 ml agar medium) will add a further factor of 10 to the total dilution factor.

Incubation Times

From the cell doubling times, see table 2.2 it was apparent that the AIV cell line would produce visible colonies less quickly than the other lines, and thus L5178Y, L5178YS and JL cells in soft agar suspension were incubated for 10 days, AIV cells for 14 days.

Colonies were scored by eye. Examination under low power magnification indicated no further colonies which were not detectable in this manner. A mean of four counts was taken for each sample.

Plating Efficiency

Preliminary experiments indicated that the concentration of agar used in the soft agar plating procedure was crucial in the plating efficiency obtained.

Initial experiments were carried out with L5178Y cells using 0.3% w/v Agar, and the plating efficiencies for three determinations, compared with three using 0.25% w/v Agar are given in table 2.3(a). It was therefore accepted that 0.25% w/v Noble Agar would be used in all subsequent soft agar suspension plating procedures.
Figure 2.2  Dilution and Plating Scheme for Soft Agar Suspension of Mouse Lymphoma Cells.
Plating efficiencies were determined for the four cell lines in two phases of growth:

1. Logarithmic, defined as cells at a concentration of \( 10^5 \) to \( 5 \times 10^5 \) per ml.

2. Stationary, defined as cells at a concentration of \( 10^6 \) to \( 2 \times 10^6 \) per ml.

Total counts were taken for each culture using the haemocytometer.

Viable counts were determined by diluting the cell suspension in PBS and plating in soft agar medium to give approximately 100 cells per 5 ml.

Colony counts per plate were determined and the mean taken. The plating efficiency was then calculated as follows:

\[
\text{Plating efficiency} = \frac{\text{Mean count} \times \text{dilution factor} \times 100\%}{\text{Total count}}
\]

The plating efficiencies obtained for the four cell lines, in two growth phases, are given in table 2.3(b).

A day-to-day variation in the plating efficiencies of the cell lines may occur, particularly dependent upon the treatment applied to the cells prior to plating. However, a plating efficiency of at least 80% would be expected for the parent line L5178Y, and it is apparent that this level is attainable by the sublines.

**Errors involved in Viability determination**

Errors involved in determining viability may be introduced in two ways.

1. The pipettes may not deliver precise or accurate volumes.

2. Insufficient mixing of cell suspensions during dilution.
### Table 2.3a Effect of Agar Concentration on Plating Efficiency.

<table>
<thead>
<tr>
<th>Agar Concentration (%)</th>
<th>Examples of Plating Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.</td>
</tr>
<tr>
<td>0.30</td>
<td>26.3</td>
</tr>
<tr>
<td>0.25</td>
<td>94.6</td>
</tr>
</tbody>
</table>

### Table 2.3b Plating Efficiencies of the Four Cell Lines in Two Phases of Growth.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Plating Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Logarithmic Phase</td>
</tr>
<tr>
<td>L5178Y</td>
<td>88.0</td>
</tr>
<tr>
<td>JL</td>
<td>81.8</td>
</tr>
<tr>
<td>L5178YS</td>
<td>69.5</td>
</tr>
<tr>
<td>AIV</td>
<td>63.8</td>
</tr>
</tbody>
</table>
1. Pipettes

Dilutions were performed using Gilson replicating pipettes (Anachem), fitted with plastic tips. These were used in three sizes:

- **P200**: Measures 0.01 - 0.20 ml
- **P1000**: Measures 0.10 - 1.00 ml
- **P5000**: Measures 0.50 - 5.00 ml

The volumes measured by the pipettes for viable count determination were:

- **P200**: 0.1 ml
- **P1000**: 0.5 ml
- **P5000**: 4.5 ml

The pipettes were set at the stated volume, a tip fitted and the volume of distilled water pipetted into a tared beaker. The weight of the sample of water was determined. In order to simulate experimental conditions, the pipette was reset, a new tip fitted and the procedure repeated. Ten sample weights were obtained for each pipette volume setting. The laboratory temperature was 20.4°C, and no further precautions were taken to assure a constant temperature.

The results for the determination are given in table 2.4.

The coefficient of variation in no case exceeds 1%, and the deviation from the theoretical value is a maximum of 0.802% for the 0.1 ml pipetted volume. This method of volume measurement was considered acceptable.

2. Dilution and Plating

In order to investigate the errors associated with the dilution and plating of cells, a series of separate dilutions of a sample cell suspension were carried out, giving a range of cell numbers per plate, according to the scheme given in figure 2.3.

The resulting data are given in table 2.5.
<table>
<thead>
<tr>
<th>Pipette Volume</th>
<th>Weight of Pipetted Volumes of Water at 20.4°C (g)</th>
<th>Mean Weight (g)</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation (%)</th>
<th>Theoretical Weight of Volume at 20.4°C (g)</th>
<th>Deviation from Theoretical Value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml. (P200)</td>
<td>0.0999 0.0984 0.0993 0.0984 0.0990 0.0986 0.0994 0.0987</td>
<td>0.0990</td>
<td>5.105 x 10^{-4}</td>
<td>0.516</td>
<td>0.0998</td>
<td>-0.802</td>
</tr>
<tr>
<td>0.5 ml. (P1000)</td>
<td>0.5000 0.4976 0.4984 0.4929 0.4990 0.4985 0.5008 0.4989 0.5005 0.4978</td>
<td>0.4984</td>
<td>2.230 x 10^{-3}</td>
<td>0.477</td>
<td>0.4990</td>
<td>-0.120</td>
</tr>
<tr>
<td>4.5 ml. (P5000)</td>
<td>4.4759 4.5137 4.5195 4.5462 4.4920 4.5073 4.5084 0.030 0.672 4.4910 4.4784 4.4890</td>
<td>4.5084</td>
<td>0.030</td>
<td>0.672</td>
<td>4.4910</td>
<td>+0.387</td>
</tr>
</tbody>
</table>

Table 2.4 Errors associated with Volumes pipetted from the Gilson Replicating Pipettes.
<table>
<thead>
<tr>
<th>SERIES</th>
<th>Dilutions in PBS</th>
<th>Dilution Factor</th>
<th>Cells per Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 in 10: 1 in 5</td>
<td>$5 \times 10^2$</td>
<td>200</td>
</tr>
<tr>
<td>B</td>
<td>1 in 10: 1 in 10</td>
<td>$10^3$</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>1 in 10: 1 in 5 : 1 in 5</td>
<td>$2.5 \times 10^3$</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>1 in 10: 1 in 5 : 1 in 10</td>
<td>$5 \times 10^3$</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td>1 in 10: 1 in 10: 1 in 5</td>
<td>$5 \times 10^3$</td>
<td>20</td>
</tr>
<tr>
<td>F</td>
<td>1 in 10: 1 in 10: 1 in 10</td>
<td>$10^4$</td>
<td>10</td>
</tr>
</tbody>
</table>

*Calculated from a starting cell concentration of $10^5$ per ml.

STARTING CELL CONCENTRATION
$10^5$ PER ML

1 in 10

1 in 5

A

1 in 5

C

1 in 10

D

1 in 10

B

1 in 5

E

1 in 10

F

Figure 2.3 Dilution Scheme for the Determination of Errors associated with Dilution and Plating of Mouse Lymphoma Cells.
<table>
<thead>
<tr>
<th>SERIES</th>
<th>DILUTION FACTOR</th>
<th>PLATE COUNTS</th>
<th>MEAN</th>
<th>STANDARD DEVIATION</th>
<th>COEFFICIENT OF VARIATION</th>
<th>CALCULATED CELL CONCENTRATION PER ML.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5 x 10^2</td>
<td>210 184 200 197</td>
<td>197.75</td>
<td>1.07 x 10^1</td>
<td>5.41%</td>
<td>9.89 x 10^4</td>
</tr>
<tr>
<td>B</td>
<td>10^3</td>
<td>92 95 107 93</td>
<td>96.75</td>
<td>6.95 x 10^0</td>
<td>7.18%</td>
<td>9.68 x 10^4</td>
</tr>
<tr>
<td>C</td>
<td>2.5 x 10^3</td>
<td>45 35 40 38</td>
<td>39.50</td>
<td>4.20 x 10^0</td>
<td>10.63%</td>
<td>9.88 x 10^4</td>
</tr>
<tr>
<td>D</td>
<td>5 x 10^3</td>
<td>24 16 19 17</td>
<td>19.00</td>
<td>3.56 x 10^0</td>
<td>18.74%</td>
<td>9.50 x 10^4</td>
</tr>
<tr>
<td>E</td>
<td>5 x 10^3</td>
<td>19 12 23 24</td>
<td>19.50</td>
<td>5.45 x 10^0</td>
<td>27.95%</td>
<td>9.75 x 10^4</td>
</tr>
<tr>
<td>F</td>
<td>10^4</td>
<td>9 6 13 12</td>
<td>10.00</td>
<td>3.16 x 10^0</td>
<td>31.60%</td>
<td>1.00 x 10^5</td>
</tr>
</tbody>
</table>

Table 2.5  Determination of Errors associated with Dilution and Plating of Mouse Lymphoma Cells in Soft Agar Suspension.
It is apparent that neither the order of dilution of the cell suspension, nor the number of cells plated significantly affect the calculated cell concentration per ml, and therefore the plating efficiency. However, the variation between counts is greater, when smaller numbers of cells are plated. Thus, in subsequent plating experiments, a plate count of 100 - 200 cells was aimed at.

DISCUSSION

The optimal conditions for the growth of mouse lymphoma cell lines have been determined.

The doubling times of the cell lines in suspension culture have been determined, and the differences in these times between cell lines have been taken into account in subsequent plating procedures.

The use of 0.25% w/v Noble Agar in the plating medium has been established as that which gives a satisfactory plating efficiency for the cells in soft agar suspension. The critical nature of the agar concentration may be attributed to the rigidity of the matrix produced by the agar, physically preventing the dividing cells from expanding as the colony forms, at higher agar concentrations.

In turn, the establishment of the optimal growth and plating conditions provides a means by which any factor altering the cell doubling time or plating efficiency can be rapidly determined, for example, the batch testing of serum.

The investigation of the accuracy of the dilution and plating protocols thus leads to the use of the soft agar plating technique for subsequent experimental work in this thesis.
CHAPTER THREE

RADIATION INACTIVATION OF MOUSE LYMPHOMA CELLS
INTRODUCTION

The effects of radiation on the cell which are the most easily detectable are:

1. Delay in cell division

2. Loss of proliferative ability

3. Induction of chromosome aberrations

The most widely studied effect of radiation is that on the proliferative ability of a cell. A cell is said to have recovered from the radiation effect if it reproduces to give a colony containing at least 50 cells, i.e. has undergone at least 6 cell divisions (205). The endpoint employed for the study in this chapter is cell survival, although the effect of radiation induced growth delay, together with post irradiation conditions and growth phase will also be taken into account. The inactivation of cells by radiation can be measured in one of two ways, both eventually giving rise to the survival-dose response curve.

1. Back Extrapolation of Growth Curves

This technique employs standard microscopic counting methods for cell populations particularly for cells which are cultured in suspension. Cells are exposed to graded radiation doses and put back into suspension culture after exposure. When exponential growth is attained, the growth curve is back extrapolated to give the cell count for survivors after irradiation, and the surviving fraction may be calculated with respect to the control count, after the counts have been corrected for the mitotic delay induced by the radiation. This correction has been demonstrated to be small (206).

2. Viable Counting

This method, whereby cells are separated from one another, and allowed to grow into discrete colonies, visible to the naked eye, as a measure of proliferative capacity was first used by Robert Koch to study
microorganisms. Puck and Marcus in 1955, carried out the first viable counts analogous to those in bacteria, using HeLa cells (207).

The colony counts obtained for the appropriately diluted samples after irradiation may be divided by that for the unirradiated control to give the surviving fraction, after correction of counts by the dilution factor. It follows that by this method, the effect of the plating efficiency of the cells is negated.

Survival Curves

The standard method of presenting survival data is to plot the surviving fraction on a logarithmic scale against the radiation dose on a linear scale. From this survival curve a number of parameters can be determined, which characterise the sensitivity of the cell population to the radiation treatment. The simplest survival curve is that which is a straight line, i.e. exponential inactivation of the cell population with increase in dose. This type of response was interpreted as implying a single target within the cell, requiring a single 'hit' to inactivate it. For even simple bacterial systems, the survival curve is rarely exponential over the entire dose range, and most survival curves exhibit an initial shoulder portion before an exponential response is reached. The presence of a shoulder to the survival curve was interpreted as the presence of a number of targets within the cell, required to be hit to inactivate the cell, and was described by the following equation

\[ \frac{N}{N_0} = n e^{-kD} \]

(3.1)

where

- \( D \) = radiation dose
- \( n \) = extrapolation number
- \( k \) = inactivation coefficient

\[ k = \frac{1}{D_0} \]

where \( D_0 \) is the dose for 1 mean hit per cell, giving rise to 37\% survival.

\( D_0 \) is also known as the \( D_{37} \) value or for UV radiation, the \( F_{37} \) value.
In recent years, with the increase in understanding of DNA repair mechanisms, the target concept has been largely replaced. However, the $n$ and $k$ parameters described above still describe in a non-interpretive manner, the response of a particular cell population to radiation.

**Survival curves for Mammalian cells**

The problems of attempting to fit survival data for mammalian cells to a formal equation may be due to one of two reasons.

1. A practical problem arising with most mammalian cell systems is the difficulty in attaining survival levels below $10^{-3}$ and so at the radiation doses required for this level of inactivation, an exponential response may not have been achieved. This has been demonstrated by Bryant (208) where the response for a green alga, at surviving fractions of $10^{-2}$ to $10^{-6}$ was exponential, whereas data points for up to $10^{-3}$ gave a better fit to a completely different equation, which would not approximate to exponential.

2. The complex structure of the mammalian cell, in particular its nuclear material, suggests the presence of many 'targets' and the damage of any one or combination of these is required for inactivation.

It is now generally accepted that the shouldered region of a survival curve for most mammalian cell systems and bacteria, is best explained by the action of repair mechanisms.

At low radiation doses, the repair mechanisms are able to function and so the inactivation rate is less than at high doses. As the dose increases, the inactivation rate increases, and may become exponential, or may not. The repair systems may become saturated at higher doses, gradually becoming less able to cope with accumulating lesions, or alternatively as implied with near UV radiation, the repair systems themselves become damaged by the radiation (21). Some mathematical formulae have been used to describe survival curves incorporating the
concept of repair, eg. Haynes (209) but due to the complexity of repair no acceptable general model is available. The survival curves obtained and presented in this thesis present a complex response, with few curves exhibiting exponential regions. With this in mind, the parameters used throughout are non-interpretive, and are either the $D_{10}$ values ($F_{10}$ for UV radiation) defined as the dose required to reduce the surviving fraction to $10^{-1}$, or the $D_{37}$ values ($F_{37}$ for UV radiation) defined as the dose required to reduce the surviving fraction to 0.37.

**Radiation Inactivation and DNA Repair**

It is now generally accepted that the response of an organism to radiation and chemical agents is dependent upon the ability of that organism to repair the damage caused by the agent.

The extensive work carried out to determine the structure of the *E. coli* genome, with mapping of the loci concerned with the enzymes involved in DNA repair pathways, has allowed the removal of these loci either singly or in multiples to produce mutant bacteria with specific repair deficiencies and consequently, characteristic responses to radiation or chemical agents.

The existence of a number of heritable genetic disorders such as xeroderma pigmentosum, with associated sunlight hypersensitivity and cellular deficiency in an incision step in the excision repair pathway, has helped considerably to elucidate the mechanisms of DNA repair in mammalian cells. However, a single defect as shown in the XP cells gives rise to a severe clinical disorder, and it follows that the number and variety of DNA repair deficiencies expressed among the human population will be limited.

The problem of relating altered sensitivity to a repair deficiency in mammalian cells may be approached in a number of ways.

1. The production of mutant cells with different sensitivities to radiation treatments. This procedure ideally would be analogous
to that for deficient mutants in bacteria. The procedure for deficient mutant selection in mammalian cells is still in its infancy. A method for isolating UV radiation sensitive mutants from a random population of chemically mutagenised cells will be presented in Chapter 6.

2. The study of the cross-sensitivity of different cell lines to a range of radiation treatments.

This chapter is concerned with the second approach, whereby four L5178Y cell lines, as described in Chapter 2, having varying responses to ionising radiation, were examined with respect to their sensitivity to 254 nm (far), 313 nm (mid) and 365 nm (near) UV radiation. The altered spectrum of lesions induced by these radiations, and by gamma radiation, together with the knowledge to-date of the repair capabilities of mouse cells in culture may help to further the understanding of the repair mechanisms and their relationship to the radiation sensitivities of the cell lines.

MATERIALS AND METHODS

Preparation of Cell Suspensions

The four cell lines L5178Y, JL, L5178YS and AIV were cultured as described in Chapter 2. For the experiments carried out and reported in this chapter, cells were taken in two phases of growth.

1. Logarithmic phase; where cell concentrations were between $10^5$ and $5 \times 10^5$ per ml.

2. Stationary phase; where cell concentrations were between $10^6$ and $2 \times 10^6$ per ml.

The concentration limits set for these two growth phases were determined from the growth curves, see figure 2.1 (a) to (d).
For both gamma and UV radiation exposure, cells were required to be in suspension in inorganic medium to prevent any effect from radiation induced toxic products of organic constituents.

A suspension of each cell line was centrifuged in a 10 ml screw-capped tissue culture tube at 1500 rpm for 5 minutes in an MSE minor centrifuge (15 cm diameter rotor). The supernatant was decanted and the cells resuspended in PBS. The suspension was recentrifuged, the supernatant decanted and the cells finally resuspended in PBS to give a concentration of $10^5$ cells per ml.

**Dilution and Plating**

The cell suspensions in PBS were subjected to the radiation treatments, after which they were appropriately diluted in PBS and plated in soft agar, with four replicates for each cell sample. L5178Y cells were plated in FM20 with 0.25% Noble Agar, JL, L5178YS and AIV cells were plated in RPMI-20 with 0.25% Noble Agar.

Plates were incubated at 37°C for 10 days in the case of L5178Y, JL and L5178YS cells, for 14 days in the case of AIV cells. Colonies were scored by eye, and the mean value of four counts determined for each sample. The surviving fraction was calculated by dividing that count by the control count.

A survival curve, as described in the introduction to this chapter was then constructed for each radiation experiment.

**Irradiation of Cell Suspensions**

Four radiation treatments were used:

1. Gamma radiation
2. 254 nm radiation
3. 313 nm radiation
4. 365 nm radiation
1. **Apparatus for Gamma Irradiation**

The apparatus for gamma irradiation is diagrammatically represented in figure 3.1. The 1\(\frac{1}{2}\) inch Gravatom source consisted of four six inch rods of Cobalt 60 in a lead container. A 1\(\frac{1}{2}\) inch steel tube sample cage could be lowered into or raised from the source using a pulley system. A metal jig which held five glass irradiation tubes fitted into the sample cage; when the cage was in position the tubes were exposed to the gamma rays from the Cobalt 60 source.

**The Radiation Tubes**

The tubes were prepared from four inch lengths of thick-walled Pyrex glass tubing, heat sealed at one end, the other end drawn out to give a narrow neck. The tubes were cleaned by the procedure for cleaning glassware given in Chapter 2 and dry tubes were plugged with cotton wool prior to dry heat sterilisation.

**Procedure for Gamma Irradiation**

The cell suspension at approximately \(10^5\) per ml in PBS was placed in the irradiation tubes in approximately 1 ml volumes using a sterile Pasteur pipette. The mouth of the tube was sealed using Nescofilm, until the tube was heat sealed at the narrow region. Six tubes were prepared in this way. One tube was kept as control and the other five were placed in the jig and exposed to graded doses of radiation at ambient temperature. One tube was removed after each dose. Subsequent manipulation of cell suspensions were carried out under red light. The tubes were opened, the cell suspensions remixed and 0.1 ml volumes removed using a Gilson P200 pipette with a sterile tip. The 0.1 ml volumes were appropriately diluted and plated in soft agar for survival determination.

**Dosimetry for Gamma Irradiation**

The method for determination of dose rate for the Cobalt 60 source is given in the Appendix.
1. Counter weight assembly.
2. Bottom shield assembly.
3. Cobalt 60.
4. Sample cage.
5. Central plug assembly.

7. Locking pin.
8. Shielding plus assembly.

Figure 3.1. Vertical Section of Gravatom Cobalt 60 Source.
Apparatus for 254 nm Irradiation

The apparatus for 254 nm irradiation is diagrammatically represented in figure 3.2. The UV radiation source was a 5 cm Penray lamp (UV products Inc. SC-1) which was fitted with a G275 filter, emitting approximately 95% at 254 nm wavelength. In order to maintain a steady fluence rate, a stream of air was blown over the filter surface to remove ozone, and the lamp was supplied through a voltage stabiliser. (Advance Electronics CV 100A). The lamp was positioned 15 cm above the irradiation vessel and exposure controlled by placing a 2 cm aperture shutter (GB Kershaw 630) between the lamp and the vessel. The shutter was controlled by hand and exposure timed using a stopwatch.

The irradiation vessel consisted of a straight sided glass bowl, with an internal diameter of 5 cm. In order to ensure continuous mixing of the cell suspension, a stream of humidified, filtered air was blown over the surface through a side jet. The irradiation vessel was designed to hold 10 ml of cell suspension, to give the distance between the source and the cell suspension of 15 cm.

Procedure for 254 nm Irradiation

The lamp was switched on for twenty minutes prior to irradiation to warm up. The fluence rate was measured before and after each experiment using the calibrated thermopile placed so that the window coincided with the level of the suspension surface, with the irradiation vessel removed. The irradiation vessel, cleaned and dried using 70% alcohol was put into position and sterilised by exposure to the Penray lamp for at least 15 minutes.

The cell suspension at approximately $10^5$ per ml in PBS was placed in the vessel, and an 0.1 ml volume removed. The cell suspension was then exposed to graded fluences of 254 nm radiation, with 0.1 ml volumes removed after each fluence. The 0.1 ml samples were appropriately diluted in PBS and plated in soft agar for survival determination.
Figure 3.2. Diagram of Apparatus for 254 nm Irradiation.

1. UV Radiation source.  4. Air inlet.
2. Shutter.  5. Position of thermopile.
3. Irradiation vessel.
Dosimetry for 254 nm Irradiation

The determination of fluence rate for the 254 nm source is given in the Appendix.

Apparatus for 313 nm and 365 nm Irradiation

The apparatus for 313 nm and 365 nm irradiation is diagrammatically represented in figure 3.3. The light source consisted of a 200 W super pressure mercury vapour lamp (Wotan) which had a fused silica envelope and an associated quartz condensing lens system. The lamp was fitted in a Bausch and Lomb housing and supplied through a voltage stabiliser. The lamp was allowed to burn for two hours prior to use, after which time the output was usually sufficiently stable. However the output of the lamp gradually decreased with use due to the darkening of the silica envelope. Lamps were therefore replaced after 100 hours of use or when the output fell below 600 µW at 313 nm. The high intensity Bausch and Lomb monochromator fitted directly to the housing for the light source. The monochromator operated by a 1350 groove per mm diffraction grating with a blaze wavelength of 300 nm and operating over the range 200 - 800 nm. Matched fixed slits were used, the entrance slit width being 2.68 mm, and the output slit width being 1.5 mm. Light entering the monochromator was focussed on the diffraction grating. Rotation of the grating allowed wavelength selection and the dispersed light was reflected onto the exit slit. The lamp and monochromator were enclosed in a wooden box to prevent stray light reaching the irradiation area. The exposure was controlled manually using an iris camera shutter (GB Kershaw 630) of 2 cm aperture. The light from the monochromator was focussed on the irradiation cuvette with a 40 mm diameter 55 mm focal length biconvex lens (Thermal Syndicate Ltd).

The irradiation cuvette is represented diagrammatically in figure 3.4, and consists of a Quartz 10 mm pathlength jacketed cuvette (Chandos Ltd) which, positioned at a distance of 65 cm from the monochromator, is fully illuminated by the beam of light when focussed. The cell suspensions were held at or below 0°C throughout irradiation to eliminate enzyme activity during this period. To achieve this, a
1. Mercury UV radiation source.  
2. Quartz collective lens.  
3. Monochromator.  
4. Shutter.  
5. Focussing Lens.  
6. Filter.  
7. Irradiation cuvette.  
8. Stirrer.

*Figure 3.3.* Diagram of Apparatus for 313 nm and 365 nm Irradiation.
1. Irradiation Cuvette.
   a. Entrance and exit ports for cooling solution.
   b. Position of suspension for irradiation.

2. Perspex Holder.

Figure 3.4. Diagram of Irradiation Cuvette for 313 nm and 365 nm Irradiation.
solution of antifreeze (Bluecol) of approximately 25% v/v was circulated through the jacket of the Quartz cuvette from an insulated metal beaker. The circulating antifreeze was maintained at -3.5°C by cooling with a U-Cool Bath Cooler (Neslab), and controlling the temperature with a heating unit (Grant Instruments). The antifreeze solution was circulated using a Multifix peristaltic pump. The conditions used gave a cuvette temperature of -0.3°C ± 0.2°C. The bath and cuvette temperature were checked periodically using a Comark temperature probe.

During irradiation, the cell suspension was stirred using a Quartz paddle rotating at 600 rpm.

Filters
The continuous nature of the light emitted from the mercury lamp is such that even after efficient dispersion, some stray light of shorter wavelengths may reach the exit slit of the monochromator due to scattering. The presence of the shorter wavelengths can significantly affect the response of cell systems, and so it is necessary to incorporate a filter into the optical arrangement, with a cut-off close to that of the selected wavelength. For 313 nm irradiation, a Mylar C 0.25 μm thickness filter was used. The absorption spectrum for this filter is given in figure 3.5 and was determined relative to air in a Pye Unicam SP1800 scanning spectrophotometer. It is apparent that the Mylar C effectively removes wavelengths below 300 nm. For 365 nm irradiation, a corning O-52 half-thickness filter (Precision Optical Instruments) was used. The absorption spectrum for this filter obtained in the same way as that for the Mylar filter is given in figure 3.6. It is apparent that the Corning O-52 filter effectively removes wavelengths below 350 nm.

Selection of Wavelengths
The mercury emission peaks at 313 nm and 365 nm were chosen for the irradiation wavelengths because of the relatively high energy output at these wavelengths.
Figure 3.6 Absorption Spectrum for Corning 0-52 Half Thickness Filter.
For irradiation experiments, with the filter positioned between the shutter and the irradiation cuvette, the peak emission was found by scanning the wavelengths on the monochromator, using the thermopile connected to the microvoltmeter to register the output. This was carried out prior to each irradiation experiment. The fluence rate was determined from the thermopile reading, prior to and at the end of each experiment.

Procedure for 313 nm and 365 nm Irradiation

The irradiation procedure was the same for both 313 nm and 365 nm exposure. The lamp was switched on and allowed to run for at least 30 minutes prior to exposure of the cells. The irradiation cuvette was aligned and the beam focussed. The cuvette was rinsed three times with PBS and sterilised by exposure to 254 nm radiation from a vertically mounted Penray lamp with a Quartz paddle in position.

The cell suspension at approximately $10^5$ cells per ml in PBS was prepared. A 3 ml volume was placed in the irradiation cuvette and the paddle and pump to circulate the antifreeze were switched on. At least 5 minutes were necessary to cool the stirred suspension to $0^\circ$C and so a standard preparation time of 10 minutes from the time of resuspension in PBS after centrifugation to the beginning of the exposure time, incorporating a minimum of 5 minutes to cool the suspension was employed for all experiments using the apparatus described.

Sampling of the suspension was with the Gilson P200 pipette with a fitted tip. The tips were sufficiently long and thin to permit a sample being removed from the cuvette without stopping and removing the paddle. This enabled the suspension to be maintained at a constant temperature throughout the experiment. A control sample, and samples after graded fluences, of 0.1 ml volumes were taken, diluted in PBS and plated in soft agar for survival determination.

Dosimetry for 313 nm and 365 nm Irradiation

The determination of fluence rate for 313 nm and 365 nm radiation is given in the Appendix.
EXPERIMENTAL

1. Survival of Cells in PBS

Before the responses of the cells to the radiation treatments could be determined it was necessary to assess the survival characteristics of the cells in PBS. This was carried out for the parent line L5178Y at 20°C (for gamma and 254 nm irradiation) and at 0°C (for 313 nm and 365 nm irradiation).

Suspensions of L5178Y cells in PBS were prepared as described for the irradiation procedures at approximately $10^5$ cells per ml and were held at 20°C or 0°C. Samples of 0.1 ml were removed at time zero as control and at intervals subsequently. Samples were diluted in PBS and plated in soft agar to determine survival. The surviving fractions obtained are given in table 3.1. It is apparent that the survival of the cells at 20°C is less than at 0°C, over the time interval examined. However, as total exposure times for gamma and 254 nm irradiation did not exceed 10 minutes, the PBS alone would not be expected to significantly affect the radiation survival results.

The holding of cells in PBS at 0°C appears to have a significant effect on survival only after about one hour. The total time that cells were held in PBS, including the 10 minutes preparation time as well as UV radiation exposure never exceeded 60 minutes, and so no correction was made to the survival levels. For this reason, because of the high fluences required to inactivate the cells at 365 nm wavelength, only a low level of kill could be achieved.

2. Effect of Cell Concentration on Sensitivity

When carrying out survival experiments it is often necessary to take into account the absorption of the incident radiation by the suspending medium, and its scattering and absorption by the irradiated cells themselves in order to obtain a true picture of the effect of the radiation on the cells. This may be carried out by taking absorbance measurements and calculating a correction factor to account for loss of incident radiation according to the equation devised by Morowitz (210).
Table 3.1 Survival of L5178Y cells after Holding in PBS.

<table>
<thead>
<tr>
<th>Holding Time (Minutes)</th>
<th>Surviving Fraction 20°C</th>
<th>Surviving Fraction 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>15</td>
<td>$9.69 \times 10^{-1}$</td>
<td>$9.84 \times 10^{-1}$</td>
</tr>
<tr>
<td>30</td>
<td>$9.00 \times 10^{-1}$</td>
<td>$9.12 \times 10^{-1}$</td>
</tr>
<tr>
<td>45</td>
<td>$8.19 \times 10^{-1}$</td>
<td>$9.19 \times 10^{-1}$</td>
</tr>
<tr>
<td>60</td>
<td>$7.36 \times 10^{-1}$</td>
<td>$8.47 \times 10^{-1}$</td>
</tr>
</tbody>
</table>
An alternative, simple approach is to study the effect of starting cell concentration on the survival curve obtained, for example after 254 nm irradiation. This wavelength is particularly suitable due to the strong absorption by the nuclear material of the cell.

Survival curves were constructed, after suspensions of L5178Y cells at $10^5$ and $5 \times 10^5$ ml in PBS were exposed to 254 nm radiation. The survival curves are given in figure 3.7.

It is apparent that the five-fold increase in cell concentration does not significantly affect the sensitivity of the L5178Y cells to 254 nm radiation.

Therefore, a standard cell concentration of $10^5$ per ml was used for all irradiation experiments, with no correction being made for absorption. The comparable survival curves at the two cell concentrations also allows for the variation in cell concentration when preparing the cell suspensions, where the cell concentration is usually between $10^5$ and $2 \times 10^5$ per ml. The absorption of 313 nm radiation by a cell suspension of $10^5$ per ml was shown to be negligible.
Figure 3.7 Effect of Starting Cell Concentration on Inactivation of L5178Y cells by 254 nm Radiation:
Starting Cell Concentration (●) $10^5$ per ml.
(▲) $5 \times 10^5$ per ml.
Gamma Radiation Sensitivity

The four cell lines, L5178Y, JL, L5178YS and AIV were exposed to gamma radiation as described in the materials and methods, at the two defined phases of growth.

The resulting survival curves are given in figure 3.8 (a) L5178Y, (b) JL, (c) L5178YS and (d) AIV.

The survival data are given in the Appendix.
Figure 3.8a  Inactivation of L5178Y cells by Gamma Radiation.

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.8b  Inactivation of JL cells by Gamma Radiation:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.8c  Inactivation of L5178YS cells by Gamma Radiation:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.8d Inactivation of AIV cells by Gamma Radiation:

(●) Logarithmic Phase.

(▲) Stationary Phase.
254 nm Radiation Sensitivity

The four cell lines L5178Y, JL, L5178YS and AIV were exposed to 254 nm radiation as described in the materials and methods, at the two defined phases of growth.

The resulting survival curves are given in figure 3.9 (a) L5178Y, (b) JL, (c) L5178YS, (d) AIV.

The survival data are given in the Appendix.
Figure 3.9a  Inactivation of L5178Y cells by 254 nm Radiation:

(○) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.9b  Inactivation of JL cells by 254 nm Radiation

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.9c Inactivation of L5178YS cells by 254 nm Radiation

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.9d Inactivation of AIV cells by 254 nm Radiation:

(○) Logarithmic Phase.

(▲) Stationary Phase.
313 nm Radiation Sensitivity

The four cell lines L5178Y, JL, L5178YS and AIV were exposed to 313 nm radiation as described in the materials and methods, at the two defined phases of growth.

The resulting survival curves are given in figure 3.10. (a) L5178Y, (b) JL, (c) L5178YS, (d) AIV.

The survival data are given in the Appendix.
Figure 3.10a  Inactivation of L5178Y cells by 313 nm Radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.10b  Inactivation of JL cells by 313 nm Radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.10c Inactivation of L5178YS cells by 313 nm Radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.10d  Inactivation of AIV cells by 313 nm Radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
365 nm Radiation Sensitivity

The four cell lines L5178Y, JL, L5178YS, and AIV were exposed to 365 nm radiation as described in the materials and methods at the two defined growth phases.

The resulting survival curves are given in figure 3.11 (a) L5178Y, (b) JL, (c) L5178YS, (d) AIV.

The survival data are given in the Appendix.
Figure 3.11a  Inactivation of L5178Y cells by 365 nm Radiation at 0°C:
(●) Logarithmic Phase.
(▲) Stationary Phase.
Figure 3.11b  Inactivation of JL cells by 365 nm Radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.11c  Inactivation of L5178YS cells by 365 nm radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
Figure 3.11d  Inactivation of AIV cells by 365 nm Radiation at 0°C:

(●) Logarithmic Phase.

(▲) Stationary Phase.
DISCUSSION

The survival curves obtained after 313 nm or 365 nm irradiation were such that in most cases, the surviving fraction did not reach $10^{-1}$, and so $D_{37}$ values were used as a measure of sensitivity. In order that a comparison could be made, the same parameter was used to describe the sensitivity of the cell lines to gamma and 254 nm radiation, although the latter survival curves covered at least two log cycles inactivation.

The relative sensitivities of the four cell lines, at the two growth phases, to the four radiation treatments will be discussed from two viewpoints:

1. Sensitivity with respect to growth phase.

2. Sensitivity to individual radiation treatments.

1. The Radiation Sensitivity with respect to Growth Phase

The cells irradiated in the stationary phase of growth showed increased sensitivity to all four radiation treatments over that for logarithmically growing cells, with the exception of AIV cells after 254 nm irradiation where there was no difference in response at the two phases and L5178YS cells after 254 nm irradiation where the stationary phase cells were slightly more resistant than the logarithmic phase cells. The data presented in the literature, with respect to the sensitivity of a cell population at different stages of the growth cycle, is contradictory, both for bacteria and mammalian cells with no evidence of particular trends in response with age of culture. It would appear that bacterial cells exhibit far less variation in response throughout stationary phase, than during exponential growth (211).

It is generally accepted for repair competent E. coli strains, that sensitivity to 254 nm UV radiation is greater during logarithmic phase than stationary phase (212) although there was little change in sensitivity throughout the entire growth cycle for the double repair deficient mutant AB2480. This increase in sensitivity has been associated with excision repair capability (213).
This sensitivity pattern appears to be similar for near UV radiation (365 nm) (214) for E. coli, but an increased sensitivity to broad spectrum UV radiation with increased age of culture has been reported for Neurospora crassa (215).

With respect to mammalian cells, sensitivity to X-irradiation has been demonstrated to be cell cycle dependent in HeLa cells (216) and rat tumour cells (217), and in both cases, the sensitivity to the radiation treatment was greater for cells in stationary phase than in exponential growth. In contrast, Berry et al (218) found little difference in sensitivity for the two growth phases, after X-irradiation of HeLa cells or Chinese hamster cells, although some small variations were attributed to growth media.

Chan and Little (219) have reported a greater sensitivity to 254 nm radiation for mouse embryo fibroblasts in exponential growth, than in stationary phase.

Recently, Griego et al (220) have demonstrated that mouse myeloma cells exhibit greater sensitivity to monochromatic 365 nm radiation in the stationary phase, than in exponential growth, in addition to which a wide range of sensitivities were shown throughout logarithmic growth phase. This sensitivity to 365 nm radiation, with respect to the age of culture is represented in figure 3.12.

The results presented in this chapter indicate a clear difference in sensitivity at the two stages of cell growth. The increased sensitivity in stationary phase may be attributed to growth medium. Cells enter stationary phase for one of two reasons, either, in the case of monolayer culture, confluency is reached, or certain nutrients in the growth medium become exhausted. Berry et al (218) noted the increased stationary phase cell sensitivity to ionising radiation only when cells were cultured in medium with low levels of folic acid and thymidine. Thus they suggested that stationary phase cells in this medium accumulated in G1 phase, since lack of nutrients such as folic acid and thymidine would prevent cells from producing DNA and entering S-phase. The enhanced sensitivity of the cells in stationary phase was considered
Figure 3.12  The Sensitivity of Mouse Myeloma Cells to 365 nm Radiation and the Relationship to Stage of the Growth Cycle (220)
to be due to a particular property of the cells in G1 phase of the cell cycle, as yet unknown.

Since the mouse lymphoma L5178Y cells grow in suspension culture, it is reasonable to suggest that cells would enter stationary phase due to exhaustion of certain nutrients, and so it may be possible that the above results would explain the increased sensitivity to all radiation treatments of L5178Y cells in stationary phase.

**The Response to Gamma Radiation**

The sensitivities of the four cell lines, in two phases of growth, to gamma radiation expressed as the D<sub>37</sub> values, together with values from the literature are summarised in table 3.2.

In both phases of growth, the AIV line showed increased resistance of 2.5 to 3 fold over that of the parent line. The L5178YS line showed approximately a two-fold increase in sensitivity over L5178Y. The JL line, showed sensitivity similar to that for L5178Y which would indicate that the plating medium did not affect the cell survival.

**The Response to 254 nm Radiation**

The F<sub>37</sub> values for the four cell lines, at both phases of growth, after 254 nm irradiation, are given in table 3.3.

At 254 nm wavelength, the responses of the cell lines were similar to those for gamma radiation, but the differences were less marked. The AIV line exhibited an increased resistance of about 1.5 over that of the parent line in both growth phases, and the L5178YS line showed little increase in sensitivity over L5178Y.

Again, the JL cell line showed similar responses to the radiation treatment, as the L5178Y, indicating that the difference in plating medium does not affect 254 nm radiation sensitivity, when comparisons within the same growth phase were made.
<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>D&lt;sub&gt;37&lt;/sub&gt; (Gy)</th>
<th>LITERATURE FOR LOGARITHMIC PHASE CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Logarithmic Phase</td>
<td>Stationary Phase</td>
</tr>
<tr>
<td>L5178Y</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JL</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>L5178YS</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIV</td>
<td>4.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Table 3.2** A Summary of D<sub>37</sub> Values for Inactivation by Ionising Radiation of Strains of Mouse Lymphoma L5178Y Cells.
Table 3.3  $F_{37}$ Values for the Four Cell Lines after 254 nm Irradiation.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$F_{37}$ (Jm$^{-2}$)</th>
<th>Logarithmic Phase</th>
<th>Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y</td>
<td>10.2</td>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td>JL</td>
<td>9.4</td>
<td></td>
<td>8.0</td>
</tr>
<tr>
<td>L5178YS</td>
<td>7.6</td>
<td></td>
<td>8.8</td>
</tr>
<tr>
<td>AIV</td>
<td>12.8</td>
<td></td>
<td>12.4</td>
</tr>
</tbody>
</table>
The analysis of the relative responses of the four cell lines at both 313 nm and 365 nm wavelengths was complicated by the change in response experienced during the experimental period. The L5178Y cell line was used originally, and the response of this line to 313 nm and 365 nm radiation was determined at an early stage. When the AIV and L5178YS lines were brought into the laboratory, and the series of experiments described in this chapter carried out, repeat survival experiments with the parent line indicated an increase in resistance to both 313 nm and 365 nm radiations.

A series of experiments were carried out to try and elucidate the reason for the sensitivity change.

1. The first variable considered was instability of the radiation source, or a fault in the fluence rate measurement. The fluence rate measurement with the thermopile was checked using potassium ferrioxalate actinometry (see the Appendix ), and showed no deviation from the determination made two years previously. The filters were also checked for malfunction and were found to be satisfactory.

2. Irradiation temperature was then considered. The increase in resistance to the wavelengths under discussion may have been due to inadequate temperature control. The apparatus was checked frequently, and no fault had been noted. But to check further, a 313 nm radiation survival experiment was carried out at 20°C. The resulting survival curve is given in figure 3.13. The two levels of survival of log phase L5178Y cells at 0°C are given in figure 3.14. It is apparent that the increase in sensitivity is not due to an increase in irradiation temperature.

3. The ingredients in the growth medium were then considered, and were probably the most likely cause of the problem. As a serum batch change had occurred at a time between the two sets of results, a comparison of survival of irradiated cells in plating
Figure 3.13  Inactivation of LS178Y cells by 313 nm Radiation at 20°C.
Figure 3.14 Inactivation of L5178Y cells by 313 nm Radiation at 0°C:

- (●) Early Response.
- (○) Later Response.
medium containing the two batches of serum was made. This was carried out for both 254 nm and 313 nm radiation. Each sample was diluted in PBS and plated from the same suspension into the two media. The results are given in table 3.4. It is clear that the serum was not responsible for the change in cell sensitivity to 313 nm or 365 nm radiation.

No other alterations in the ingredients for media, including the water, could be found that may account for the sensitivity change.

4. The L5178Y cell line, was also considered to be a possible source of the problem.

The sensitivity of the cells to 313 nm or 365 nm radiation was examined with respect to the concentration of the cells in the maintenance culture, and therefore with the stage of the cell growth cycle. Apart from the increase in sensitivity when cells entered stationary phase, no correlation could be made between the sensitivity and the age of culture. However, this assumes an alteration in sensitivity in proportion to the progress through the cell cycle of growth. If Griego et al (220) predict no such proportionate change in sensitivity, see figure 3.12, then variation in sensitivity could not be directly correlated with the age of culture. However, even if cells for logarithmic growth phase are taken between $10^5$ and $5 \times 10^5$ per ml, this range may still be wide enough to permit the variation in response seen with L5178Y cells.

It therefore follows that the responses to mid (313 nm) and near (365 nm) radiation may be strongly dependent upon the stage of growth of the culture. For this reason, the responses of the four lines presented here, may not be representative of the true response.

The alteration in response of L5178Y cells to 365 nm radiation is indicated in figure 3.15 for the log. phase cells.

Since a comparison is to be made between the cell lines, the later sensitivities in terms of $F_{37}$ values have been used. The $F_{37}$ values for the cell lines after 313 nm irradiation are given in table 3.5(a) and for 365 nm radiation, in table 3.5(b).
Table 3.4a Effect of Serum on Survival of L5178Y Cells after 254 nm Irradiation.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (Jm^-2)</th>
<th>Surviving Fraction.</th>
<th>Serum Batch</th>
<th>Serum Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.</td>
<td>2.</td>
</tr>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>4.50</td>
<td>7.22 x 10^{-1}</td>
<td>8.31 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td>9.00</td>
<td>4.31 x 10^{-1}</td>
<td>3.80 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td>18.00</td>
<td>2.90 x 10^{-2}</td>
<td>1.77 x 10^{-2}</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4b Effect of Serum on Survival of L5178Y Cells after 313 nm Irradiation.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJm^-2)</th>
<th>Surviving Fraction.</th>
<th>Serum Batch</th>
<th>Serum Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.</td>
<td>2.</td>
</tr>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>9.28 x 10^{-1}</td>
<td>8.45 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>6.31 x 10^{-1}</td>
<td>7.97 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td>3.89 x 10^{-1}</td>
<td>1.35 x 10^{-1}</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.15  Inactivation of L5178Y cells by 365 nm Radiation at 0°C:

(●) Early Response.

(O) Later Response.
### Table 3.5a  
**F<sub>37</sub>** Values for the Four Cell Lines after 313 nm Irradiation at 0°C.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Logarithmic Phase</th>
<th>Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y</td>
<td>10.5</td>
<td>7.7</td>
</tr>
<tr>
<td>JL</td>
<td>9.3</td>
<td>6.0</td>
</tr>
<tr>
<td>L5178YS</td>
<td>9.2</td>
<td>3.6</td>
</tr>
<tr>
<td>AIV</td>
<td>(12.3)</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Values in brackets indicate estimates by extrapolation of survival curves.

### Table 3.5b  
**F<sub>37</sub>** Values for the Four Cell Lines after 365 nm Irradiation at 0°C.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Logarithmic Phase</th>
<th>Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y</td>
<td>81</td>
<td>49</td>
</tr>
<tr>
<td>JL</td>
<td>(200)</td>
<td>77</td>
</tr>
<tr>
<td>L5178YS</td>
<td>68</td>
<td>51</td>
</tr>
<tr>
<td>AIV</td>
<td>(162)</td>
<td>57</td>
</tr>
</tbody>
</table>
The results indicate a similarity in response of the four cell lines at 313 nm wavelength, both for logarithmic phase and stationary phase cells, with perhaps the exception of AIV which shows a higher F37 value than the other lines, in both growth phases. At 365 nm wavelengths, again there are no distinct trends in sensitivity, except that the JL line shows a slightly higher resistance, which in this case may be attributed to a plating medium effect.

The survival curves produced at 313 nm and 365 nm have not covered more than one log. cycle kill, due to the requirement for high fluences at these wavelengths. Therefore, before any definite comparison may be made between the cell lines, for sensitivity to 313 nm or 365 nm radiation, many more survival experiments are required, with extension of the fluences to achieve a higher level of kill. This would require alterations in the optical system to obtain a higher fluence rate.

The growth conditions after irradiation must still be considered an important factor in variation of radiation response. Hahn et al (225) have demonstrated that the response of Chinese hamster cells to UV radiation is strongly dependent upon the nutritional content of the post irradiation growth medium. Work with human fibroblasts in this laboratory has supported this, with variation in response to mid and near UV radiation being attributed to serum and water.

Finally, the evidence supplied by the literature, together with the data presented, implicate the stage of cell growth, as well as post irradiation culture conditions as responsible for variation in response, and both are worthy of a more detailed investigation.
CHAPTER FOUR

ULTRAVIOLET RADIATION INDUCED MUTAGENESIS IN L5178Y CELLS
INTRODUCTION

Mutation is defined as a heritable alteration, either cytoplasmic, or within the nucleus of a cell (226). Such mutations may be spontaneous or induced. Most mutations are recognised through a change in phenotype, although some may be silent, that is, having no effect upon the phenotype, eg. the new nucleotide sequence may code for the same amino acid, or a different amino acid which does not alter protein function. Alternatively, the mutation may be suppressed.

Classification of Mutation

The primary concern with bacterial and mammalian cell systems is with alterations to nuclear material, and these may be classified into three general groups.

1. Changes in Chromosome Number

Changes in ploidy have been associated mainly with plant research, and have little consequence in animal cell culture, although attempts have been made to associate changes in ploidy with alterations in sensitivity to radiation (227).

2. Changes in Gene Number or Arrangement

Chromosome aberrations usually occur as a result of repair or exchange of damage to DNA, and may take a number of forms, such as duplication, deletion or inversion of genetic material.

3. Changes at the Molecular Level

Mutations of this type may be divided into two general groups.

(i) Point Mutations

(ii) Frameshift Mutations

(i) Point mutations may further be described as transitions or transversions.
Transitions involve the substitution of a purine for a pyrimidine or vice versa, in the DNA strand, and may be brought about by the incorporation of base analogues into the DNA molecule. For example, 5-bromouracil (5BU) which closely resembles thymine, may be incorporated into the DNA sequence instead of thymine. But 5BU can undergo tautomeration from the usual keto form, to a rare enol state, which will then pair with guanine (G) and not adenine (A) thus leading to a replication error, with mispairing of G for A, giving genetic instability and delayed mutation.

The tautomeration of 5BU may also lead to an incorporation error, where the 5BU is recognised as dCTP (deoxycytosine triphosphate) and subsequent copying of 5BU yields an immediate mutant. The dual action of 5BU can be used to recognise transitions, since 5BU causes the mutants to undergo reversion. Not all tautomeration leads to stable mutation since DNA polymerase can remove many mispairs.

Two-amino substituted purines can cause transitions as effectively as 5BU, as the tautomer can be read as adenine or guanine.

Transversions involve the substitution of a purine for a purine, or pyrimidine for a pyrimidine. They occur frequently as spontaneous mutations and are produced by only a few mutagens, eg. 4 Nitroquinoline-1-oxide (4 NQO). Some are attributed to the malfunction of DNA polymerase or to error-prone repair.

(ii) Frameshift mutations involve the insertion or deletion of usually one to four bases in the DNA molecule, thus causing a 'shift' in the reading frame or codon. This leads to misreading of the base sequence. Acridine dyes give rise to frameshift mutations by intercalating between the base pairs. This action requires single strand breaks, and therefore favours replicating DNA, and short runs of the same base pair. The acridine molecule is thought to act by stabilising the illegitimate pairing of bases of the free end and the uninterrupted DNA strand, until the gap is closed. The mechanism for this action is represented in figure 4.1. Deletions may be caused by interstrand crosslinking.
Figure 4.1 A Model for Frameshift by Acridine.
agents, where DNA around the crosslink is not replicated, while segments on either side replicate and join with each other.

Mutations caused by UV radiation are mostly base substitutions, both transitions or transversions, often affecting two consecutive bases, eg. opposite a dimer. UV light can also cause frameshifts and deletions. Such mutations, and those caused by ionising radiation, as previously described, result from imperfections of the inducible error-prone repair system.

Mammalian Cell Mutation Assay Systems

Although the use of mammalian cells in vitro for mutation assays had been considered for many years, the practical aspects were not investigated to any extent until Puck and co-workers (228, 229) established methods for the growth of colonies from single, isolated cells. This led initially to the determination of spontaneous mutation rates for a few established cell lines (230, 231). Early attempts to modify mutation rates by treating cells with known mutagens were not successful (232, 233). This was later considered to be due to the high background spontaneous rate (234). In 1968, however, two groups, Kao et al (235, 236) and Chu et al (237, 238) independently demonstrated that gene mutation could be induced in Chinese hamster cells by alkylating agents.

Work involving the use of cell lines drawn from human origin have been hindered by the nature of such cell cultures, presenting low plating efficiencies and cell senescence after 30 - 50 cell generations. The main work in this field has been developed using established rodent cell lines, Chinese hamster or mouse cells, and those from tumours, such as the HeLa cells line (239), although many other lines have been used.

Isolation of Mutants

The isolation of mutants from cell populations may be by either non-selective or selective means.
1. Non-selective isolation involves using large numbers of colonies which are tested individually or by a mechanical method such as replica plating (240).

2. Selective isolation may involve a number of techniques.

(i) A technique introduced by Kao et al (235, 236) involves the use of a large cell population, which is exposed to 5-bromodeoxyuridine (5 BudR) in a medium lacking certain nutrients. Those cells capable of growth in such medium would incorporate 5BudR, and would be killed on subsequent exposure to near-visible light. Those cells not capable of growth in the medium would not incorporate 5BudR, and would be unaffected by the light exposure. Subsequent transfer of the cells to whole medium would permit them to grow, and be assayed for growth requirements. In this way, mutants of Chinese hamster cells were isolated, requiring agents such as glycine, adenine and thymidine (241).

(ii) A technique modelled on the thymineless death method for bacteria, where DNA synthesis was blocked by a folic acid antagonist such as aminopterin, was used by a number of workers (239) to give auxotrophs for a number of amino acids.

From the latter technique developed the use of a selective agent in the growth medium for isolation of mutant colonies for both forward and reverse mutations. Table 4.1 illustrates a number of the genetic markers used in mammalian cells for mutant isolation by selection. The most commonly used genetic markers are those affecting molecules synthesised by the cell, for example:

1. Resistance to analogues of guanine and hypoxanthine is characterised by mutation at the hypoxanthine, guanine, phosphoribosyltransferase (HGPRT) locus.

2. Resistance to 6-diaminopurines is characterised by mutation at the adenine phosphoribosyltransferase (APRT) locus.
<table>
<thead>
<tr>
<th>Source</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Purine Analogue Resistance</td>
</tr>
<tr>
<td></td>
<td>Aminopterin Resistance</td>
</tr>
<tr>
<td></td>
<td>Virus Resistance</td>
</tr>
<tr>
<td></td>
<td>Glutamine Dependence</td>
</tr>
<tr>
<td>Monkey</td>
<td>Temperature Sensitivity</td>
</tr>
<tr>
<td>Mouse</td>
<td>Purine, Pyrimidine Analogue Resistance</td>
</tr>
<tr>
<td></td>
<td>Aminopterin Resistance</td>
</tr>
<tr>
<td></td>
<td>Radiation Resistance</td>
</tr>
<tr>
<td></td>
<td>Temperature Sensitivity</td>
</tr>
<tr>
<td></td>
<td>Asparagine Dependence</td>
</tr>
<tr>
<td>Rat</td>
<td>Asparagine Dependence</td>
</tr>
<tr>
<td>Chinese Hamster</td>
<td>Purine, Pyrimidine Analogue Resistance</td>
</tr>
<tr>
<td></td>
<td>Ara C Resistance</td>
</tr>
<tr>
<td></td>
<td>Temperature Sensitivity</td>
</tr>
<tr>
<td></td>
<td>Auxotrophy</td>
</tr>
</tbody>
</table>
Resistance to 5BudR is characterised by mutation at the thymidine kinase (TK) locus.

The genes coding for these three activities are considered to be recessive. The phenotypic expression of such recessive genes in mutant cells of diploid nature requires some explanation.

In the case of the HGPRT locus, this is known to be X-linked, and mutation at this locus gives rise to the condition of Lesch-Nyan syndrome in man (242). Thus the haploid nature of the X-chromosome in male animals permits expression of this gene.

In the case of autosomal recessive genes the expression of the phenotype may be due to a divergence of the chromosome complement from diploid, regularly seen in rodent cell lines in culture, which gives rise to deletions of regions of chromosomes or aneuploidy. Thus successful isolation of some recessive mutants may depend on specific monosomies. Alternatively, as in the case of the TK locus autosomal recessives may be detectable in the heterozygous state, in which case the double recessive is detected by resistance to an increased concentration of the selective agent, over that for the heterozygote. This applies for both the forward and reverse mutations at the TK locus (243).

The HGPRT Locus

As this locus has been used for studies in this thesis, the background theory will be discussed in more detail.

The phosphoribosyltransferase enzyme specified by the HGPRT gene converts guanine, hypoxanthine and analogues of these bases to nucleotides. Analogues such as 8-azaguanine (azg) or 6-mercaptopurine (6-mp) have no effect upon the cell until they are ribose-phosphorylated and then incorporation of the nucleotides into the DNA results in cell death. Forward mutation at the HGPRT locus gives rise to HGPRT deficient (HGPRT⁻) mutants, which cannot carry out the conversion and will therefore survive. The survival of the mutants is ascribed to the
production of nucleotides \textit{de novo}, which is the main pathway for
purine nucleotide synthesis. The HGPRT route is considered to be a
salvage pathway.

HGPRT\textsuperscript{−} mutants selected in this way can be checked for stability,
reversion to HGPRT\textsuperscript{+} by selection in THMG medium (thymidine, hypoxanthine,
methotrexate, glycine medium). Methotrexate, a folic acid inhibitor
blocks the \textit{de novo} synthesis of purines, and disrupts other one carbon
transfers, which lead to synthesis of thymidylate and some amino acids.
If cells are HGPRT\textsuperscript{−}, then THMG medium selects against them, while
HGPRT\textsuperscript{+} cells survive, the hypoxanthine in the medium supplying the basic
material for purine nucleotide synthesis. The reversion to HGPRT\textsuperscript{+} is
also dependent on an effective thymidine kinase (TK) since blocking
\textit{de novo} synthesis leaves this as the only means of synthesising
thymidylate, which is assured by the inclusion of thymidine in the
THMG selection medium. Thus, THMG medium may be used to select for
revertants of both TK and HGPRT loci.

Figure 4.2 illustrates the salvage pathways for bases and nucleosides,
involving the TK and HGPRT enzymes.

Figure 4.3 illustrates the \textit{de novo} synthesis of nucleotides.

Initial studies of mutation at the HGPRT locus used 8-azaguanine as
the selective agent (237, 245). However subsequent work by Clive \textit{et al} (234) has indicated a dependence of initial cell concentration
upon the spontaneous mutation rate, and so alternative selective
agents were sought. Subsequently, 6-mercaptopurine was found to be
unsuitable. Work by Cole and Arlett (246) established the suitability
of 6-thioguanine (6TG) as a selective agent, and this has been used in
the study of UV radiation induced mutagenesis in this thesis, at the
HGPRT locus.

When the mutagenic activity of an agent is to be determined in a
mammalian cell system, it is emphasised that more than one assay
should be used. Thus a number of assay systems have been developed,
including 1,8 arabinofuranosylcytosine (ara-c) resistance in L5178Y
Figure 4.2 Salvage Pathways for Bases and Nucleotides (244)
Figure 4.3  Folic Acid Involvement in de novo Synthesis of Nucleotides (244)
cells (247), and resistance to emetine, diphtheria toxin and ouabain in CHO cells (248). The resistance to ouabain has been studied by a number of other workers in mouse cells (246) and in human cell lines (249), and has also been employed as an assay in the present study.

**Mechanism of Ouabain Resistance**

Ouabain is a steroid compound demonstrated to have an inhibitory effect upon the plasma membrane ATPase which is activated by Na\(^+\), K\(^+\), Mg\(^{++}\) (250). Thus active transport of K\(^+\), Na\(^+\) across the plasma membrane is mediated by this enzyme (Na/K ATPase). It follows that the explanation for resistance to ouabain in mutant cells is due to mutant transport sites with an altered response to ouabain.

In contrast to the previously discussed mutation assays, the mutation to ouabain resistance has been shown to be co-dominant. (250). This was demonstrated by cell hybridisation between the ouabain resistant mutant and the ouabain sensitive wild-type cells for CHO cells, where the hybrid of two wild-type cells showed ouabain sensitivity, the hybrid of wild-type with ouabain resistant cells gave intermediate resistance to ouabain, indicating the ouabain resistance marker to be co-dominant or incompletely dominant.
MATERIALS AND METHODS

Cell Line
The parent line L5178Y was used for this work and was maintained as described in Chapter 2, with subcultures to $10^3$ cells per ml once weekly to maintain an homogenous population with respect to spontaneous mutation frequencies for the resistance markers under study.

Logarithmically growing cells were used for irradiation, and maintained in this growth phase throughout the expression times.

Ouabain
Ouabain (molecular weight 728.8) was obtained from Sigma Chemical Co (Sigma grade), stored at room temperature and protected from light. The ouabain stock solution was prepared by dissolving 2.9152G of ouabain in distilled water, by stirring and making up to 200 ml in a volumetric flask. The solution was filter sterilised, and stored in 100 ml volumes in foil wrapped sterile glass bottles with plastic caps. The solution, which had a final concentration of $2 \times 10^{-2}$ M was stored at room temperature for up to one month.

Ouabain selective medium was prepared by adding 28.41 ml of the $2 \times 10^{-2}$ M solution to the FM20 medium, prior to making up to 500 ml with distilled water.

6-Thioguanine
6-thioguanine (molecular weight 167.2) was obtained from Sigma Chemical Co (Sigma grade) and stored dessicated at room temperature. The 6-thioguanine stock solution was prepared by dissolving 0.1672G of 6-thioguanine in 7.5% w/v sodium bicarbonate solution (Flow) and 100 mg sodium carbonate (A.R) were added to aid dissolution, with heating and stirring, and was made up the 200 ml in a volumetric flask. The solution was filter sterilised and stored in 100 ml sterile glass bottles with plastic caps, at 4°C until required, but not longer than 24 hours. Fresh solutions were prepared for subsequent experiments. The final solution had a concentration of $5 \times 10^{-3}$ M.
Six-thioguanine selective medium was prepared by the addition of 20.45 ml of the $5 \times 10^{-3}$ M 6-thioguanine solution to the FM20 medium omitting the sodium bicarbonate, prior to making up to 500 ml with distilled water.

Initial experiments with 6-thioguanine dissolved in 0.5% w/v sodium carbonate solution rendered the FM20 too alkaline for growth of the cells, and so sodium bicarbonate solution was used as the solvent. This then precludes the addition of further sodium bicarbonate to the medium as that from the 6-thioguanine solution maintains the concentration at the accepted 1-2 G per litre.

**Final Concentrations of Selective Agent**

In both cases, the volumes of solutions of selective agents added to the FM20 were adjusted to account for the addition of agar and the cell suspension to the FM20.

The final concentrations of selective agent in the plating mixture were as follows:

- **Ouabain** $10^{-3}$ M
- **6-thioguanine** $1.8 \times 10^{-4}$ M

These values had been determined as suitable inhibitory concentrations for the growth of the wild-type cells by Cole and Arlett (246), and were confirmed in this laboratory by the following experiment.

**Determination of the Toxicity of Ouabain and 6-Thioguanine**

In order that an agent may be used to select a particular mutant from a cell population, which is defined as resistant to that agent, a concentration of the selective agent must be used which inhibits growth of the sensitive wild-type cells.
The L5178Y cells were plated in FM2O agar, which was supplemented with ouabain or 6-thioguanine in graded concentrations.

The concentration range for ouabain was $10^{-6}$ M to $10^{-3}$ M, and for 6-thioguanine was $10^{-7}$ M to $10^{-4}$ M.

The cells were appropriately diluted in PBS prior to plating, as previously described, and incubated at 37°C for 10 days. The survival curves, plotting logarithm of surviving fraction against concentration of selective agent, also on a logarithmic scale are given in figure 4.4(a) for ouabain and 4.4(b) for 6-thioguanine. The survival data are given in the Appendix.

The Mutation Assay

The treatments applied to the cell suspensions were ultraviolet irradiation either at 254 nm or 313 nm.

Cells for irradiation were prepared as previously described in Chapter 3, at a cell concentration of approximately $10^5$ per ml. Mutation frequencies were determined for UV radiation fluences giving surviving fractions of 1 (control), 0.9, 0.5 and 0.1, as determined from the survival data presented in Chapter 3. The mutation frequency obtained for the control could then be used to calculate the induced mutation frequencies for UV irradiation.

Expression Times

According to Cole and Arlett (246) chemically induced ouabain resistant ($\text{oua}^R$) mutants appear immediately after mutagen treatment of a cell population and are present for at least 96 hours. Six-thioguanine resistant ($6\text{-TG}^R$) mutants appear at a peak at 144 - 192 hours after mutagen treatment. Thus for convenience, the expression times for these preliminary experiments were taken as 96 hours for mutation to ouabain resistance and 192 hours for mutation to 6-thioguanine resistance.
Figure 4.4a Inactivation of L5178Y cells by Ouabain.
Figure 4.4b  Inactivation of L5178Y cells by 6-Thioguanine.
The irradiation of mammalian cells in suspension, as described in Chapter 3, requires small volumes of cell suspension and low cell concentrations. However, to successfully carry out a mutation assay as described in this chapter, large numbers of cells are required. In order to obtain results, the longest expression times possible were employed, to allow sufficient cells to be available for the mutation assay. A single sample of cell suspension was taken after each radiation fluence, added to the culture medium and incubated for the four days expression, when the plating for oua$^R$ mutants was carried out. The culture was diluted into fresh medium at this time, and incubated for a further four days, then plated for mutation to 6-TG$^R$.

Sampling

The initial cell concentration at irradiation was approximately $10^5$ per ml. The sample volumes for each UV radiation fluence were as follows:

<table>
<thead>
<tr>
<th>For Surviving fraction</th>
<th>Sample volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>0.9</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>0.1</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

These volumes were added to FM10 to give a total of 50 ml, in an 80 cm$^2$ tissue culture flask and incubated in the dark at 37$^\circ$C for 4 days. The volumes chosen were such that, after four days incubation the cells were in logarithmic growth phase, and the concentration did not exceed $5 \times 10^5$ cells per ml.

Prior to plating for oua$^R$, the cultures were diluted, 0.05 ml volumes to 50 ml with FM10 and incubated at 37$^\circ$C for a further 4 days, giving a final cell concentration of between $5 \times 10^4$ and $10^5$ cells per ml, for plating for 6-TG$^R$. 
Plating

For each sample, 50 ml of cell suspension were transferred to 5 x 10 ml screw cap tissue culture tubes, and centrifuged at 1500 rpm for 5 minutes in an MSE minor centrifuge. The supernatant was decanted and the cells resuspended in 2 ml of PBS, pooling the cells using two separate 1 ml volumes. The final cell concentration was then $5 \times 10^6$ to $10^7$ per ml for the ouabain assay and $2.5 \times 10^6$ to $5 \times 10^6$ per ml for the 6-thioguanine assay.

To plate for survival, 0.2 ml of the cell suspension was diluted in PBS to give a final cell count per plate of 100 - 200. An 0.5 ml volume of the final dilution was added to 24.5 ml of FM20 agar and 4 x 5 ml plates prepared.

To plate for mutants, 3 x 0.5 ml volumes were added to each of 3 x 24.5 ml of FM20 agar containing the selective agent and a total of 12 x 5 ml plates prepared. All plates were boxed, gassed with carbon dioxide and incubated in the dark at 37°C for 10 days.

Determination of Mutation Frequencies

The colonies on all plates were scored by eye and the mean of four replicates determined for the survival plates.

For the mutation plates, a mean of the twelve replicates was determined.

All mean counts were corrected by multiplication by the dilution factor. The mutants per survivor value was found by dividing the mutant count by the survival count, for each sample.

The induced mutation frequency was determined by subtracting the mutants per survivor value for the control from the value for each irradiated sample.
RESULTS

1. 254 nm Radiation

The mutants per survivor and the induced mutation frequencies for 254 nm radiation induced mutation to oua$^R$ and 6-TG$^R$ are given in table 4.2(a) and (b). The induced mutation frequency on a logarithmic scale was plotted against the logarithm of UV radiation fluence, and given in figure 4.5.
Table 4.2a Mutants per Survivor and Induced Mutation Frequencies for 254 nm Radiation induced Resistance to Ouabain.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (Jm$^{-2}$)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>$9.87 \times 10^{-7}$</td>
<td>-</td>
</tr>
<tr>
<td>3.00</td>
<td>$9.00 \times 10^{-1}$</td>
<td>$9.14 \times 10^{-6}$</td>
<td>$8.16 \times 10^{-6}$</td>
</tr>
<tr>
<td>9.00</td>
<td>$5.00 \times 10^{-1}$</td>
<td>$3.44 \times 10^{-5}$</td>
<td>$3.34 \times 10^{-5}$</td>
</tr>
<tr>
<td>14.00</td>
<td>$1.00 \times 10^{-1}$</td>
<td>$5.61 \times 10^{-5}$</td>
<td>$5.51 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Table 4.2b Mutants per Survivor and Induced Mutation Frequencies for 254 nm Radiation induced Resistance to 6-Thioguanine.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (Jm$^{-2}$)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>$5.43 \times 10^{-6}$</td>
<td>-</td>
</tr>
<tr>
<td>3.00</td>
<td>$9.00 \times 10^{-1}$</td>
<td>$4.10 \times 10^{-5}$</td>
<td>$3.56 \times 10^{-5}$</td>
</tr>
<tr>
<td>9.00</td>
<td>$5.00 \times 10^{-1}$</td>
<td>$5.23 \times 10^{-5}$</td>
<td>$4.69 \times 10^{-5}$</td>
</tr>
<tr>
<td>14.00</td>
<td>$1.00 \times 10^{-1}$</td>
<td>$1.14 \times 10^{-4}$</td>
<td>$1.09 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Figure 4.5  254 nm Radiation Induced Mutagenesis in L5178Y cells:

(●) Ouabain Resistance.

(▲) 6-Thioguanine Resistance.
2. 313 nm Radiation

The mutants per survivor and the induced mutation frequencies for 313 nm radiation induced mutation to oua$^R$ and 6-TG$^R$ are given in table 4.3(a) and (b). The induced mutation frequency on a logarithmic scale was plotted against the logarithm of UV radiation fluence and given in figure 4.6.
### Table 4.3a Mutants per Survivor and Induced Mutation Frequencies for 313 nm Radiation induced Resistance to Ouabain.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJm⁻²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.05 x 10⁻⁶</td>
<td>-</td>
</tr>
<tr>
<td>1.00</td>
<td>9.00 x 10⁻¹</td>
<td>1.42 x 10⁻⁶</td>
<td>3.70 x 10⁻⁷</td>
</tr>
<tr>
<td>3.00</td>
<td>5.00 x 10⁻¹</td>
<td>5.25 x 10⁻⁶</td>
<td>4.20 x 10⁻⁶</td>
</tr>
<tr>
<td>5.50</td>
<td>1.00 x 10⁻¹</td>
<td>1.01 x 10⁻⁵</td>
<td>9.05 x 10⁻⁶</td>
</tr>
</tbody>
</table>

### Table 4.3b Mutants per Survivor and Induced Mutation Frequencies for 313 nm Radiation induced Resistance to 6-Thioguanine.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJm⁻²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>3.63 x 10⁻⁶</td>
<td>-</td>
</tr>
<tr>
<td>1.00</td>
<td>9.00 x 10⁻¹</td>
<td>6.34 x 10⁻⁶</td>
<td>2.71 x 10⁻⁶</td>
</tr>
<tr>
<td>3.00</td>
<td>5.00 x 10⁻¹</td>
<td>1.91 x 10⁻⁵</td>
<td>1.55 x 10⁻⁵</td>
</tr>
<tr>
<td>5.50</td>
<td>1.00 x 10⁻¹</td>
<td>3.40 x 10⁻⁵</td>
<td>3.04 x 10⁻⁵</td>
</tr>
</tbody>
</table>
Figure 4.6 313 nm Radiation Induced Mutagenesis in L5178Y cells:

- (●) Ouabain Resistance.
- (▲) 6- Thioguanine Resistance.
DISCUSSION

The experiments carried out to examine the induction of mutation by UV radiation were of a preliminary nature, and in most cases, the data presented are the results of single experiments. The conclusions that can be drawn from the data are limited, and the emphasis of the discussion will be upon the experiments required to clarify the effects seen in the preliminary results, rather than on interpretation of the results themselves.

Arlett and Harcourt (251) have demonstrated that the expression time required for mutant induction to be optimal is dependent upon the fluence of UV radiation. This would be expected, as UV radiation induces growth delay. Further, Arlett and Harcourt have shown that the optimal expression time is the same as that for the control, for high UV radiation fluences, once the cell concentration has reached that of the control, i.e. when the growth delay induced by UV irradiation had been overcome. However, the overall effect was to prolong the expression time for high UV radiation fluences, by which time the control mutant frequencies had passed the peak. Thus the use of a single expression time, such as that by Bridges and Huckle (245) for UV radiation induced mutation to azg^R would underestimate the mutation frequency at low UV radiation fluences, and would overestimate it at high UV radiation fluences. Therefore, any mutation assay using UV radiation as the mutagen must include a range of expression times for mutation frequency, allowing the method of Arlett and Harcourt (251) to be employed, by calculating the induced mutation frequency from the optimal mutant frequencies for both the treated and control cell populations. This method to-date is the best available, taking into account, to some extent, the UV induced lethality and growth delay. Obviously a method which allows quantification of the latter would be ideal, but is difficult to obtain in practice.

Subject to the above limitations, a number of conclusions may be drawn from the results.
Firstly, both 254 nm and 313 nm UV radiation induce mutation in L5178Y cells to both ouabain and 6-thioguanine resistance. The 254 nm radiation induced mutagenesis has been reported for a number of cell lines and assay systems, for example, mutation to 8-azaguanine resistance in Chinese hamster V79 cells by Bridges and Huckle (245), Arlett and Harcourt (251) and mutation to thymidine resistance in P388 mouse cells by Anderson and Fox (252). Jacobson et al (253) have demonstrated the mutagenic nature of both germicidal wavelengths of UV radiation, 250 - 260 nm, and of longer UV radiation wavelengths from a number of broad-spectrum sources, in mouse L5178Y cells, using the TK locus, assaying for resistance to BuDR. The mutagenic nature of sunlight has been demonstrated in CHO cells by Hsie and co-workers (254).

The second observation made from the results is that the induction of mutants resistant to ouabain was generally less than that for resistance to 6-thioguanine, for a given radiation treatment. This is as would be expected, since the Na/K ATPase system, affected by ouabain, is an essential function of the cell, and therefore deletion of this activity would be lethal to the cell. It follows that the ouabain assay system would only detect point mutations, and in the case of gamma radiation, the majority of mutagenic lesions being deletions, induction to ouabain resistance was not detectable in Chinese hamster cells (246).

The HGPRT enzyme, selected against by mutation to 6-thioguanine resistance (HGPRT\(^{-}\)) however, is not essential to cell metabolism and thus, the survival of variants at this locus can be brought about in a number of ways, which give rise to different structural defects of the HGPRT enzyme (255). Thus it would be expected that the numbers of mutants induced to 6-thioguanine resistance would be greater than those induced to ouabain resistance, and would reflect a wider range of lesions.

Finally a comparison of the relative levels of mutation induction between wavelengths cannot be made, using the data available. It is well established in bacteria that the induction of growth delay is
wavelength dependent (256) and so, the optimal expression times of UV-radiation induced mutation at 313 nm would not be expected to be the same as that at 254 nm.

The induction of mutants by gamma or by UV radiation has been attributed to the events of error-prone repair. The use of this mouse lymphoma cell line for routine mutagen testing has been criticised, because of the high mutability of the line due to a deficiency in excision repair capabilities. However, work by Clive et al (234) comparing this line with other cell lines competent in excision repair, falls in favour of the L5178Y cell line as a useful system for mutation assay.

The results generally suggest that the two mutation assay systems, used with the mouse lymphoma cell line L5178Y, would give good indications of the mutagenic nature of UV radiations including those of longer wavelength, provided careful attention be paid to the phenomena associated with UV radiations and their effects upon the expression times for mutation to ouabain or 6-thioguanine resistance.
CHAPTER FIVE

THE SENSITISATION OF 313 nm ULTRAVIOLET RADIATION INDUCED INACTIVATION AND MUTAGENESIS IN L5178Y CELLS BY THE SUNSCREEN AGENT PARA AMINO BENZOIC ACID
INTRODUCTION

Although radiation emitted from the sun is of wavelengths throughout the electromagnetic spectrum, that which reaches the earth's surface is usually of wavelengths greater than 290 nm. The shorter wavelengths of the UVC region are absorbed by atmospheric oxygen and ozone. Ozone is present in the upper atmosphere and the thickness of the ozone layer determines the intensity of the shorter wavelength UVB radiation reaching the earth's surface. Figure 5.1 indicates the relative intensities of solar radiation together with that at the earth's surface.

Sunlight, for centuries, was considered essential for good health, and many curative properties were attributed to it. While sunlight is necessary for the production of vitamin D, it is now generally accepted that sunlight has harmful effects, both for acute and chronic exposure.

The acute effects of sunlight on previously unexposed human skin are erythema and induction of melanin production, or tanning.

Erythema may be produced by UV radiation of all wavelengths, as illustrated in figure 5.2. The peak at 310 nm would account for the efficiency of sunlight in bringing about the erythemal response. The characteristic reddening of the skin occurs 1 - 2 hours after exposure, and lasts for 48 - 72 hours. It is usually accompanied by oedema, and over-exposure may lead to desquamation.

Some natural protection against sunlight is provided by the pigment melanin, found in most human skin. Exposure to sunlight will bring about two types of tanning, the increased pigmentation of the skin. Most skin contains a residual amount of melanin precursors and immediate tanning or pigment darkening is due to oxidation of this residue. The wavelengths which bring about this response are in the UVA and visible light regions. As the amount of precursors in the skin is small, the degree of pigment darkening is minimal.
Figure 5.1 Solar Ultraviolet Radiation:

A. Extraterrestrial.

B. At the Earth's Surface.
Figure 5.2 Erythema Action Spectrum for Human Skin (257)
Delayed tanning, due to the production of new melanin begins about ten hours after sunlight exposure. The time courses for the acute responses to sunlight are given in figure 5.3. The action spectrum for delayed tanning was thought to be the same as that for erythema (1) but has now been shown to extend into the UVA and visible light regions (258).

In summary, the UVB region of the electromagnetic spectrum, 290 - 320 nm is capable of producing the erythemal response, and delayed tanning in normal human skin, while UVA, 320 - 380 nm and to some extent visible light are capable of producing a delayed tanning response as well as pigment darkening.

While the acute effects of sunlight exposure have received much attention, until recent years the results of chronic exposure to sunlight were not recognised. It has now been established that the alterations in the skin structure characteristic of old age may be accelerated by chronic exposure to sunlight (259).

The premature ageing of skin is observed in those people whose lifestyle demands excessive exposure to sunlight, on the exposed areas of the body, generally the face, hands and back of the neck. In most cases, unexposed skin resembles that of a younger person. The acceleration of the ageing process does not constitute a hazard to the human individual, but is considered unsightly.

Sunlight induced Skin Cancer

A report in the Lancet as early as 1899 (250) attributed multiple growths on the face of an individual to chronic sunlight exposure, and as far as knowledge of that time would allow, these growths were considered to be malignant.

Urbach (261) in 1969 implicated sunlight in the formation of some cancers of the skin and it is now accepted that sunlight is important in the formation of basal cell carcinoma, is the main carcinogen in the formation of squamous cell carcinoma of exposed skin, and may
have some role (but is not the primary causative factor) in the formation of melanoma (262).

It follows that chronic exposure to sunlight may result in increased incidence of skin cancer, and the groups most at risk are those whose work keeps them out-of-doors for long periods of time.

The health risks associated with squamous cell carcinoma and basal cell carcinoma are not great, mainly because diagnosis is simple and surgical removal can be carried out at early stages of development. However, the prevention of the occurrence of sunlight induced skin cancer is desirable.

Sunscreen Agents

Sunscreen agents were originally used to protect against the acute effects of sunlight, and it is now accepted that they may also give protection against the chronic effects of sunlight exposure.

In addition to this, sunscreens may also be used to reduce the extent of abnormal response to sunlight, including the prevention of phototoxic and photoallergic responses to some drugs.

Sunscreens are usually divided into two main groups having either a physical or chemical action.

Physical Sunscreens

The simplest barrier to light of all types is clothing, although some fabrics, such as nylon do allow the transmittance of UV light. It also follows that clothing does not offer protection for the hands, head or neck.

Some agents act as an opaque barrier, for example titanium dioxide, and have some use in the prevention of light reactions by photosensitising chemicals and in porphyria (263).
Chemical Sunscreens

The agents in this category act by absorbing UV radiation usually in the 290 - 340 nm range, thus aiming to prevent the erythemal response while permitting the tanning action by UVA radiation. Most of these agents are used purely for cosmetic purposes, although they may afford some protection against the chronic effects of UV radiation, and for those individuals with abnormal responses to sunlight.

Agents in this group include the cinnamates, salicylates, benzophenone derivatives, mexenone and para-amino benzoic acid and its derivatives.

Evaluation of Sunscreen Agents

The primary test for determining the efficiency of a sunscreen agent is the reduction in minimum erythemal dose (MED) of artificial UV light (264, 265) or of sunlight (265, 266) on intact human skin. However, a number of other criteria are important, including a neutral pH, non-toxicity and non-irritancy, water insolubility, non-volatility and non-absorbtivity by the skin (267).

Most sunscreens fulfill these requirements except that experiments with individuals using sunscreen preparations and undertaking bathing or rigorous exercise to induce sweating showed removal of effective amounts of the sunscreen by these activities (265, 266). One exception to this was para-amino benzoic acid, PABA.

Properties of PABA

PABA (NH₂ C₆H₄ COOH) is a simple molecule with the following structure:

![PABA structure]

The absorption spectrum for PABA is given in figure 5.4. Although the absorption peak at 280 nm is outside the UVB radiation range, the broad nature of the absorption band gives substantial absorption up to approximately 315 nm wavelength.
Figure 5.4 The Absorbance of 0.1mM Solution of PABA in Ethanol (268)
The superiority of PABA in protecting against the acute effects of sunlight may be attributed to its ability to remain on the skin during bathing and exercise, although the duration of the activity and the time allowed for PABA to be in contact with the skin prior to exercising or swimming greatly affected the efficiency of PABA as a sunscreen (269). The prolonged action of PABA has been thought to be due to its ability to bind to the horny layer of the epidermis (270).

The structure of PABA, like most of the chemical sunscreens is such that the absorption of UV radiation or visible light raises the electrons in the molecule from the ground state to an excited, higher energy state. The wavelengths of energy absorbed and the degree of excitation that the electrons undergo are characteristic of the electronic configuration of the particular molecule (267). The energy absorbed which raises the electrons to the higher energy states, is usually dissipated as heat, but may be transferred to adjacent molecules (271).

**Sunscreen Agents and Skin Cancer**

As previously mentioned, the test most often used for sunscreens is the reduction in M.E.D., and this takes into account the acute effects of sunlight.

There are no routine tests used at present to determine the effect, if any of sunscreens on the chronic effects of sunlight. The sunlight induced ageing of skin would appear to be a phenomenon that would benefit from the use of a sunscreen agent, although the wavelengths responsible for this effect would need to be elucidated.

A major concern recently has been with the implication of sunscreen agents in sunlight induced skin cancer.

Recent work with a model bacterial system has demonstrated the sensitisation by PABA of mid UV radiation induced inactivation (272) and mutagenesis (273). The relevance of a bacterial system to the human situation is questionable, and so in order to further the work in this field, PABA sensitisation of mid UV radiation induced inactivation and mutagenesis was studied in a mammalian cell system.
MATERIALS AND METHODS

The Cell Line

The parent line L5178Y was used for these experiments. Culture procedures were as described in Chapter 2.

PABA solutions

Two stock solutions of PABA were prepared:

(i) 0.25% w/v PABA in PBS.
(ii) 0.50% w/v PABA, 5.0% v/v DMSO in PBS.

The 5% v/v DMSO was added to the second stock solution to increase PABA solubility.

(i) 0.25 G of PABA (Sigma grade, Sigma Chemical Co) were dissolved in 95 ml of PBS by warming at 37°C.
(ii) 0.50 G of PABA were dissolved in 90 ml PBS plus 5 ml DMSO, by warming at 37°C.

In order that PABA effects on a biological system may be studied, the PABA was dissolved in PBS. However, as shown in figure 5.5, the pH of the final solution varied with PABA concentration. This in turn would be expected to affect ionisation and therefore radiation absorption of PABA. The solutions of PABA were therefore adjusted to the buffer pH of 7.4 prior to making up to volume, by dropwise addition of 10 N NaOH (VoluconM&B) up to pH 7.0, then by addition of 1N NaOH (1 in 10 dilution of 10 N solution) to pH 7.4. The pH readings were taken using a Pye Unicam 292 mark II pH meter.

The solutions were then made up to 100 ml volumes in volumetric flasks with PBS, filter sterilised into sterile 100 ml glass bottles with plastic caps and stored, foil wrapped for up to one month at 4°C. The solutions were allowed to equilibrate to room temperature before use.
Figure 5.5 pH - Concentration Profile for PABA in PBS.
Irradiation Procedures

Irradiation was at 313 nm wavelength using the apparatus described in Chapter 3.

Preparation of cell suspensions for irradiation was as described in Chapter 3, with cells finally resuspended in PABA solutions where specified.

Survival determination was as described in Chapter 2.
EXPERIMENTAL

This section will be divided into two main subsections.

1. The effect of PABA on 313 nm radiation inactivation of L5178Y cells.

2. The effect of PABA on 313 nm radiation induced mutagenesis in L5178Y cells.

1. The Effect of PABA on 313 nm Radiation Induced Inactivation

Control Experiments

Before the effect of PABA on 313 nm radiation induced cell killing could be studied, it was necessary to determine whether PABA itself or its photoproducts are toxic to either irradiated or unirradiated L5178Y cells. Therefore, four control experiments were carried out.

1. Unirradiated PABA with unirradiated cells.

2. Unirradiated PABA with irradiated cells.

3. Irradiated PABA with unirradiated cells.

4. Irradiated PABA with irradiated cells.

A single culture of L5178Y cells was used for the four experiments. The cells were centrifuged, resuspended, recentrifuged and finally resuspended in PBS at a concentration of approximately $2 \times 10^5$ per ml. The stock PABA solution at 0.25% w/v was diluted to 0.20% w/v with PBS. A 3 ml volume of the 0.20% w/v PABA solution and 3 ml of the cell suspension in PBS were individually irradiated with 2.5 KJm$^{-2}$ of 313 nm radiation. This fluence had been previously shown to reduce the surviving fraction of an L5178Y population to approximately 0.5.

One ml volumes of unirradiated cell suspension were added to 1 ml volumes of either irradiated or unirradiated PABA solution, giving a final cell concentration of $10^5$ per ml and PABA concentration of 0.1% w/v.
One ml volumes of irradiated cell suspension were added to 1 ml volumes of either irradiated or unirradiated PABA solution, giving a predicted viable cell concentration of $5 \times 10^4$ per ml and PABA concentration of 0.1% w/v.

The suspensions of L5178Y cells in 0.1% PABA solution were held at 0°C for one hour, with samples taken at 0, 15, 30, 45 and 60 minutes, and assessed for survival.

The results expressed as surviving fraction of the survival at time zero, are given in table 5.1 and indicate that up to 30 minutes holding time, the presence of PABA does not significantly affect survival over that for holding at 0°C in PBS alone (see table 3.1).

Since exposure times for 313 nm radiation in the presence of PABA, including the 10 minutes preparation time, did not exceed 30 minutes it was considered that the toxicity of PABA or its photoproducts was negligible over the 30 minute period.
<table>
<thead>
<tr>
<th>Holding Time (Minutes)</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>$8.78 \times 10^{-1}$</td>
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<td>30</td>
<td>$8.45 \times 10^{-1}$</td>
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<td>45</td>
<td>$7.42 \times 10^{-1}$</td>
</tr>
<tr>
<td>60</td>
<td>$5.59 \times 10^{-1}$</td>
</tr>
</tbody>
</table>
Irradiation of L5178Y cells in the presence of PABA

Cultures of L5178Y cells were taken in logarithmic growth phase, and prepared for irradiation as previously described.

Cells were suspended at $10^5$ per ml in PBS, or PABA solutions in PBS at concentrations of 0.01, 0.02, 0.05, 0.1, 0.2 % w/v, or in PABA solutions, 5% DMSO in PBS at concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 % w/v.

The total of 10 minutes preparation time prior to irradiation was allowed to standardise experimental conditions. This time had also been shown to be adequate to allow absorption of PABA into the cells, since a holding time of 20 minutes did not increase the sensitisation by PABA over that shown after 10 minutes holding time.

Cell suspensions were irradiated at 313 nm and 0.1 ml samples taken at time 0 (control) and at regular intervals during irradiation. The samples were diluted and assessed for survival as described in Chapter 2.

RESULTS

Survival curves were obtained for 313 nm UV radiation in the presence of each concentration of PABA, with or without DMSO, as stated. Since the DMSO was used at higher PABA concentrations to increase solubility, it was considered necessary to determine the effect, if any, of DMSO on PABA sensitisation. Hence parallel experiments were performed, with and without DMSO at PABA concentrations up to 0.20% w/v.

The survival curves for the 313 nm irradiation in the presence of PABA, with and without DMSO are given in figures 5.6(a) to (c). The survival data are given in the Appendix.
Figure 5.6a  Inactivation of L5178Y cells by 313 nm Radiation at 0°C:

○ Alone.

▲ With 0.01% PABA.

▼ With 0.02% PABA.

○ With 5% DMSO.

▲ With 0.01% PABA + 5% DMSO.

▼ With 0.02% PABA + 5% DMSO.
Figure 5.6b Inactivation of L5178Y cells by 313nm Radiation at 0°C:

- (●) With 0.05% PABA.
- (▲) With 0.1% PABA.
- (■) With 0.2% PABA.
- (○) With 0.05% PABA + 5% DMSO.
- (▲) With 0.1% PABA + 5% DMSO.
- (■) With 0.2% PABA + 5% DMSO.
Figure 5.6c  Inactivation of L5178Y cells by 313 nm radiation at 0°C:

(O) With 0.3% PABA + 5% DMSO.  (A) With 0.4% PABA + 5% DMSO.  (O) With 0.5% PABA + 5% DMSO.
DISCUSSION

The conclusions to be drawn from the survival curves presented are:

1. The presence of DMSO at a concentration of 5% has no effect on 313 nm radiation inactivation of L5178Y cells.

2. For all concentrations of PABA studied, with the exception of 0.01%, there is an increase in sensitivity to 313 nm radiation in the presence of PABA. For 0.01% PABA, the low concentration may have a sufficiently small sensitisation effect which is not detectable by this experimental procedure.

3. For the concentrations of PABA up to and including 0.2%, where parallel experiments with and without DMSO were carried out, the sensitisation to 313 nm radiation in the presence of PABA with DMSO, in all cases exceeded that produced by PABA alone.

However, if any attempt is made to correlate the degree of sensitisation to the PABA concentration from the survival data presented a complex pattern arises, with no obvious trends. The \( D_{10} \) value (see Chapter 3) was determined for each survival curve and was plotted against PABA concentration. The resulting plots are given in figure 5.7(a) for PABA and 5.7(b) for PABA in the presence of 5% DMSO.

The initial observation that can be made from these curves is that the effect of PABA on 313 nm radiation inactivation, is one of sensitisation.

If the events taking place during irradiation are considered, it becomes apparent that the response of the cells depends upon two separate phenomena:

1. The sensitivity of the cells to the UV radiation in the presence of PABA.

2. The effect of the absorption of UV radiation by the PABA during irradiation, which would reduce the effective UV radiation fluence.
Figure 5.7 The Fluences for 10% survival ($D_{10}$) in KJm$^{-2}$ for 313 nm Radiation Inactivation of L5178Y cells at 0°C:

A. In the presence of PABA.

B. In the presence of PABA + 5% DMSO.
reaching the cells. Therefore, in order to determine the true effect of PABA on 313 nm radiation inactivation, the effect of PABA absorption of UV radiation must be accounted for.

Absorbance of UV radiation at 313 nm by PABA solutions in PBS

Due to the nature of the light emitted from the 313 nm radiation source, it was considered more relevant to use this optical arrangement for absorption determinations than a conventional spectrophotometer.

The PABA solutions in PBS were placed in a 1 cm$^2$ Quartz spectrophotometer cuvette, in the path of the focussed beam from the monochromator at 313 nm wavelength, with the Mylar filter in place, so that the entire front surface of the cuvette was within the beam. The thermopile was placed immediately behind the cuvette and readings in microvolts taken for each concentration of PABA both with and without DMSO, in addition to those for PBS alone and PBS with 5% v/v DMSO.

Absorbance values for each PABA concentration, with and without DMSO, and for DMSO alone were calculated according to the following formula:

\[
\text{Absorbance} = \log_{10} \left( \frac{I_0}{I} \right)
\]  

(5.1)

Where $I_0$ and $I$ are the thermopile readings for PBS and PABA solutions respectively.

The absorbance values were plotted against PABA concentrations and are given in figure 5.8. The plots give a linear relationship up to 0.1% after which the lines tend to plateau. Therefore, at concentrations above 0.1% PABA, an absorbance value was taken at each concentration as a mean of three determinations.

Using a modified Morowitz equation for the absorption by a stirred suspension (210) a fluence correction factor could be calculated for each PABA concentration.

\[
\text{Fluence Correction Factor (F.C.F.)} = \int_{0}^{1} 10^{-AL} \, dl
\]  

(5.2)
Figure 5.8  Absorption of 313 nm Radiation at pH 7.4:

(O) By PABA.

(●) By PABA + 5% DMSO.
where \( A \) = absorbance
\( L \) = pathlength in cm

\[
= \frac{-2.303 \, A \, L}{2.303 \, A}
\]  

(5.3)

\[
= \frac{1 - e^{-2.303 \, A}}{2.303 \, A}
\]  

(5.4)

The values for absorbance and fluence correction factors are given in table 5.2.

The UV radiation fluences incident upon the irradiation cuvette, that is the fluences calculated from exposure times and fluence rate, which were used to construct the survival curves in figure 5.6 may be multiplied by the correction factor to give the average fluences received by the cell suspension during irradiation.

The survival curves replotted using the average UV radiation fluences are given in figures 5.9(a) for PABA and 5.9(b) and (c) for PABA with 5% DMSO.

Examination of these composite plots now indicates a trend in the increase in sensitivity with increase in PABA concentration.

In order to quantify the effect of PABA on 313 nm radiation sensitivity in terms of the effect upon the cells themselves, the \( D_{10} \) values were taken, for the average radiation fluences, and from these a sensitisation ratio of the \( D_{10} \) value in the absence of PABA to that in its presence, was calculated for each concentration. The plots of the sensitisation ratio against PABA concentration are given in figure 5.10(a) for PABA and 5.10(b) for PABA with 5% DMSO.

It is apparent that the sensitisation ratios increase linearly with PABA concentration up to 0.2%, after which, in the case of PABA with DMSO, the curve tends to plateau. It is also apparent that in the presence of DMSO, sensitisation is greater than for PABA alone, reflected also in the plots for the incident \( D_{10} \) values (figure 5.7).
### Table 5.2a
Absorbance Values and Fluence Correction Factors for PABA Solutions in PBS at pH 7.4

<table>
<thead>
<tr>
<th>PABA Concentration (%)</th>
<th>Absorbance</th>
<th>Fluence Correction Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.01</td>
<td>0.11</td>
<td>0.88</td>
</tr>
<tr>
<td>0.02</td>
<td>0.20</td>
<td>0.80</td>
</tr>
<tr>
<td>0.05</td>
<td>0.45</td>
<td>0.62</td>
</tr>
<tr>
<td>0.10</td>
<td>0.79</td>
<td>0.46</td>
</tr>
<tr>
<td>0.20</td>
<td>1.24</td>
<td>0.33</td>
</tr>
</tbody>
</table>

### Table 5.2b
Absorbance Values and Fluence Correction Factors for PABA Solutions with 5% DMSO in PBS at pH 7.4.

<table>
<thead>
<tr>
<th>PABA Concentration (%)</th>
<th>Absorbance</th>
<th>Fluence Correction Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.06</td>
<td>0.98</td>
</tr>
<tr>
<td>0.01</td>
<td>0.16</td>
<td>0.84</td>
</tr>
<tr>
<td>0.02</td>
<td>0.25</td>
<td>0.76</td>
</tr>
<tr>
<td>0.05</td>
<td>0.50</td>
<td>0.59</td>
</tr>
<tr>
<td>0.10</td>
<td>0.85</td>
<td>0.44</td>
</tr>
<tr>
<td>0.20</td>
<td>1.39</td>
<td>0.30</td>
</tr>
<tr>
<td>0.30</td>
<td>1.66</td>
<td>0.26</td>
</tr>
<tr>
<td>0.40</td>
<td>1.77</td>
<td>0.24</td>
</tr>
<tr>
<td>0.50</td>
<td>1.87</td>
<td>0.23</td>
</tr>
</tbody>
</table>
Figure 5.9a  Inactivation of L5178Y cells by 313 nm Radiation at 0°C after Correction of Fluences for absorption by PABA:

- (●) Control.
- (○) 0.01%
- (▲) 0.02%
- (▲) 0.05%
- (■) 0.1%
- (□) 0.2%
Figure 5.9b  Inactivation of L5178Y cells by 313 nm Radiation at 0°C after Correction of Fluences for absorption by PABA + 5% DMSO:

- (●) Control.
- (O) 0.01%
- (▲) 0.02%
- (▲) 0.05%
- (■) 0.1%
- (□) 0.2%
Figure 5.9c  Inactivation of L5178Y cells by 313 nm Radiation at 0°C after Correction of Fluences for absorption by PABA + 5% DMSO:

(O) 0.3%
(△) 0.4%
(□) 0.5%
Figure 5.10 Sensitisation of 313 nm Radiation
Inactivation of L5178Y cells:
The Sensitisation Ratios.

A. PABA.

B. PABA +5% DMSO.
2. **The Effect of PABA on 313 nm Radiation Induced Mutagenesis**

Since PABA caused sensitisation of 313 nm radiation induced cell killing, the next step was to investigate the effect, if any, of PABA on 313 nm radiation induced mutagenesis in the mouse L5178Y cells.

The background to the mutation assays and the experimental procedures were as described in Chapter 4.

**EXPERIMENTAL**

A single PABA concentration was used for these experiments, 0.2% w/v, both with and without 5% DMSO. This concentration was chosen because it was the highest used in the survival experiments both in the presence and absence of 5% DMSO.

**Irradiation**

Cell cultures were prepared and irradiated as described in the first section of this chapter. The preparation time prior to irradiation was 10 minutes.

The cell suspensions, in PBS, or PABA 0.2% in PBS, with or without DMSO were irradiated with 313 nm radiation fluences to give predicted survival levels of 1 (control) 0.9, 0.5 and 0.1 as determined from the survival data presented in this chapter.

Cell samples were taken after the appropriate exposure times, and subjected to the mutation assay procedures as described in Chapter 4.

The induced mutation frequencies were calculated for mutation to oua^R for an expression time of 96 hours, and for mutation to 6-TG^R for an expression time of 192 hours.
RESULTS

The induced mutation frequencies were plotted on a logarithmic scale against the UV radiation fluence on a logarithmic scale. The UV radiation fluences were corrected for absorption of UV radiation by the PABA.

The resulting plots are given in figure 5.11(a) for mutation to oua\(^R\) and in figure 5.11(b) for mutation to 6-TG\(^R\).

The UV radiation fluences used in the experimental, together with the variants per survivor and induced variant frequencies are given in table 5.3, (a) and (b).

DISCUSSION

The results indicate a parallel effect of PABA on 313 nm radiation induced mutation as upon inactivation at this wavelength, one of sensitisation. This effect is again enhanced by the presence of DMSO.

DMSO alone appears to have no sensitising effect on 313 nm radiation induced mutation to oua\(^R\), in fact the induced mutation frequency was less in the presence of DMSO. However, DMSO appears to sensitise 313 nm radiation induced mutation to 6-TG\(^R\), and which may on further investigation prove to be significant.

Plotting the induced mutation frequency against surviving fraction yielded no significant sensitising effect. That is to say, the number of mutants per lethal event appeared to be the same for 313 nm irradiation both with and without PABA, for both mutation assay systems.

The results presented are for a single expression time, and for a single PABA concentration. For reasons explained in Chapter 4, a number of expression times need to be used in order to obtain a clear picture of UV radiation induced mutagenesis. It would also be of use to obtain
Figure 5.11a Mutation to Ouabain Resistance by 313 nm Radiation in L5178Y cells:

(●) Alone.
(○) With 5% DMSO.
(▲) With 0.2% PABA.
(■) With 0.2% PABA + 5% DMSO.
Figure 5.11b: Mutation to 6-Thioguanine Resistance by 313 nm Radiation in L5178Y cells:

- (●) Alone.
- (○) With 5% DMSO.
- (▲) With 0.2% PABA.
- (■) With 0.2% PABA + 5% DMSO.
Table 5.3a Mutants per Survivor and Induced Mutation Frequencies for 313 nm Radiation induced Resistance to Ouabain.

1. In the Presence of 5% DMSO.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJ/m²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>5.38 x 10⁻⁷</td>
<td>-</td>
</tr>
<tr>
<td>1.00</td>
<td>9.00 x 10⁻¹</td>
<td>8.33 x 10⁻⁷</td>
<td>2.95 x 10⁻⁷</td>
</tr>
<tr>
<td>3.00</td>
<td>5.00 x 10⁻¹</td>
<td>3.02 x 10⁻⁶</td>
<td>2.48 x 10⁻⁶</td>
</tr>
<tr>
<td>5.50</td>
<td>1.00 x 10⁻¹</td>
<td>8.15 x 10⁻⁶</td>
<td>7.16 x 10⁻⁶</td>
</tr>
</tbody>
</table>

2. In the Presence of 0.2% PABA.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJ/m²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.17 x 10⁻⁶</td>
<td>-</td>
</tr>
<tr>
<td>0.50</td>
<td>9.00 x 10⁻¹</td>
<td>1.80 x 10⁻⁶</td>
<td>6.30 x 10⁻⁷</td>
</tr>
<tr>
<td>1.50</td>
<td>5.00 x 10⁻¹</td>
<td>5.40 x 10⁻⁶</td>
<td>4.23 x 10⁻⁶</td>
</tr>
<tr>
<td>2.75</td>
<td>1.00 x 10⁻¹</td>
<td>2.00 x 10⁻⁵</td>
<td>1.88 x 10⁻⁵</td>
</tr>
</tbody>
</table>

3. In the Presence of 0.2% PABA + 5% DMSO.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJ/m²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>4.67 x 10⁻⁷</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>9.00 x 10⁻¹</td>
<td>1.24 x 10⁻⁶</td>
<td>7.73 x 10⁻⁷</td>
</tr>
<tr>
<td>1.00</td>
<td>5.00 x 10⁻¹</td>
<td>6.34 x 10⁻⁶</td>
<td>5.81 x 10⁻⁶</td>
</tr>
<tr>
<td>2.00</td>
<td>1.00 x 10⁻¹</td>
<td>9.49 x 10⁻⁶</td>
<td>9.02 x 10⁻⁶</td>
</tr>
</tbody>
</table>
Table 5.3b Mutants per Survivor and Induced Mutation Frequencies for 313 nm Radiation induced Resistance to 6-Thioguanine.

1. In the Presence of 5% DMSO.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJ/m²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>4.07 x 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>9.00 x 10⁻¹</td>
<td>1.60 x 10⁻⁵</td>
<td>1.19 x 10⁻⁵</td>
</tr>
<tr>
<td>3.00</td>
<td>5.00 x 10⁻¹</td>
<td>8.39 x 10⁻⁵</td>
<td>7.99 x 10⁻⁵</td>
</tr>
<tr>
<td>5.50</td>
<td>1.00 x 10⁻¹</td>
<td>9.72 x 10⁻⁵</td>
<td>9.31 x 10⁻⁵</td>
</tr>
</tbody>
</table>

2. In the Presence of 0.2% PABA.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJ/m²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>4.15 x 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>9.00 x 10⁻¹</td>
<td>1.73 x 10⁻⁵</td>
<td>1.32 x 10⁻⁵</td>
</tr>
<tr>
<td>1.50</td>
<td>5.00 x 10⁻¹</td>
<td>2.61 x 10⁻⁵</td>
<td>2.20 x 10⁻⁵</td>
</tr>
<tr>
<td>2.75</td>
<td>1.00 x 10⁻¹</td>
<td>7.60 x 10⁻⁵</td>
<td>7.19 x 10⁻⁵</td>
</tr>
</tbody>
</table>

3. In the Presence of 0.2% PABA + 5% DMSO.

<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJ/m²)</th>
<th>Predicted Surviving Fraction</th>
<th>Mutants per Survivor</th>
<th>Induced Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>7.03 x 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>9.00 x 10⁻¹</td>
<td>1.99 x 10⁻⁵</td>
<td>1.29 x 10⁻⁵</td>
</tr>
<tr>
<td>1.00</td>
<td>5.00 x 10⁻¹</td>
<td>1.38 x 10⁻⁴</td>
<td>1.31 x 10⁻⁴</td>
</tr>
<tr>
<td>2.00</td>
<td>1.00 x 10⁻¹</td>
<td>2.08 x 10⁻⁴</td>
<td>2.01 x 10⁻⁴</td>
</tr>
</tbody>
</table>
mutation frequencies for a range of PABA concentrations parallel to those for the survival experiments.

However it would be reasonable to suggest that PABA does have a sensitising effect on UV radiation induced mutation in L5178Y cells, both to oua\(^R\) and 6-TG\(^R\) and that a further, more comprehensive study would be justified.

The Mechanism of Action of PABA

The PABA molecule is a small, flat structure and as such can easily penetrate the DNA helix to exert its effect. It is typical of molecules such as benzophenone, which are known biological sensitisers. The benzophenones are thought to act by triplet energy transfer from the sensitisier to the DNA molecule (274). The same mechanism would be feasible for PABA, since the triplet energy of PABA, at 26,000 wave numbers is higher than those for all four of the nucleotide bases (275) and thus triplet energy transfer to the bases would increase the probability of reaction. It has been demonstrated using a model bacterial system that 313 nm irradiation in the presence of PABA increases the proportion of lesions susceptible to the pyrimidine dimer specific repair process of photoreactivation (272). Recently, evidence to support the hypothesis of triplet energy transfer has been produced by chromatographic analysis of irradiated DNA, where a significant increase in pyrimidine dimer formation was observed when PABA was present during near UV irradiation (275).

Quantitatively, there is a similarity in the sensitisation ratios obtained with 0.1\% PABA in L5178Y cells of about 5, compared with a value of approximately 6 for \textit{E. coli} (272).

The increased sensitisation observed when irradiation was carried out with PABA in the presence of DMSO, over that for PABA alone, together with the observation that DMSO alone did not affect 313 nm radiation induced inactivation nor probably mutagenesis suggests that DMSO may be exerting an effect by increasing the permeability of the cell membrane to PABA.
The sensitisation of 313 nm UV radiation induced mutation by PABA has been demonstrated in *E. coli* K12 AB1886 *uvr A* assaying for mutation to histidine independence (273).

The evidence presented indicates that the sensitisation process by PABA may be a fundamental one acting on DNA, for the bacterial systems studied. To-date, no direct evidence is available for mammalian cells. However, the quantitative similarities between the bacterial and mammalian cell results, together with the indication of induced mutation by PABA above that for 313 nm radiation alone, strongly suggests that PABA in mammalian cells is exerting an effect on the cells genetic material, probably by the triplet energy transfer mechanism.

**The Use of PABA as a Sunscreen**

When considering the possible consequences of the sensitisation process in the series of experiments, it is pertinent to discuss the net effect of the PABA sensitisation together with the absorption by PABA of the incident radiation fluence. This is more easily done by using the survival data, where the plots of $D^{10}$ values indicate that above a concentration of PABA of 0.1% w/v the sensitisation effect tends to level off, this being reflected in the sensitisation ratio plots, where a plateau region is formed. This suggests that at higher PABA concentrations an equilibrium is reached where the absorbance of the PABA solution counterbalances the sensitisation of mid UV radiation inactivation. Nevertheless, even in the plateau region, a sensitisation of at least two-fold is observed. It would be interesting to determine at even higher concentrations, whether the plateau region is maintained or whether the absorption effect overcomes the sensitisation effect. The commercial formulations containing PABA usually employ a concentration of 2.5 to 5% w/v, up to ten-fold greater than the highest value studied with the mouse lymphoma cells, and the absorption at this concentration approaches 100% (276). However, if the situation of a layer of PABA on the skin is considered, the thickness of the layer will be at least 100 fold less than that of the experimental situation which employed a 1 cm pathlength for absorption measurement and irradiation.
With respect to the prevention of erythema, PABA is an efficient sunscreen, but the apparent reactivity of the molecule after absorption of UV radiation is cause for concern. One argument in support of PABA as a sunscreen is that it has been demonstrated to absorb singlet oxygen, produced by UV radiation and implicated in UV radiation damage (277). However, in doing so, the PABA assumes an excited energy state. No suggestion was given as to the fate of the energy absorbed by the PABA molecule, after singlet oxygen quenching.

In terms of the effects of PABA on UV radiation induced skin cancer, experiments with hairless mice have demonstrated the protection by PABA of UV radiation induced tumours in the presence of 7, 12, dimethyl benzanthracene (278). The relevance of these findings can be questioned from two viewpoints. Firstly, the thickness of the layer of PABA on the mouse skin is not known, and may not be representative of that attained by using commercial products. Secondly the appearance of UV radiation induced tumours was accelerated by using 7,12, dimethyl benzanthracene, and it may be likely that the mechanism of tumour induction by UV radiation alone may differ to that in the presence of the accelerating agent. It follows that the involvement of PABA whether as a sensitiser or a protector, may be different for the two cases.

Recently, protection against UV radiation induced cancer in mouse skin has been demonstrated, with the PABA analogue octyl, dimethyl PABA, and with oxybenzone (279). Both these agents differ from PABA in that they are insoluble in water, and oxybenzone has been shown not to sensitise pyrimidine dimer formation in DNA (276).

Although a number of lines of evidence now support the sensitisation by PABA of UV radiation induced inactivation and mutagenesis, it must be emphasised that no analogy may be made between such effects in a mouse cell system and the effects on human skin. The main criticism would be that the responses seen in the mouse cells would be enhanced due to the deficiencies in repair capabilities. However, the L5178Y mouse lymphoma system has been used extensively for mutation assays,
involving chemical agents and provides a relatively simple system for manipulation and is inexpensive.

Perhaps one of the most important points to emphasise is the need for the inclusion of an assay in the presence of UV radiation or visible light as a routine test, when the mutagenic properties of new chemicals are being assessed. This is of particular importance when screening pharmaceutical and cosmetic preparations for topical application, as otherwise innocuous chemicals may prove to have hazardous effects when used in the presence of sunlight.
CHAPTER SIX

THE ISOLATION OF MUTANTS OF L5178Y CELLS SENSITIVE TO
254 nm UV RADIATION
INTRODUCTION

Methods of Mutant Isolation

The incorporation of 5BudR into DNA, followed by exposure to visible light has been used to select UV radiation sensitive lines from HeLa cells (280), and Chinese hamster V79 mutants have been selected using $^3$H-thymidine (281). Other methods of mutant production involve cell hybridisation (282, 283) the selection of nutritional auxotrophs, as described in Chapter 4 (243) and temperature sensitive mutants (284).

In recent years, replica plating methods have been developed for mammalian cells either using transfer of cells from the wells of microtitre plates (245), by modifications of the Lederberg replica plating method (285) using pile fabrics (286) glass rods or similar devices (286-288) or by combinations of these methods (289). Finally, techniques involving differential staining and microscopic examination of mutagenised clones have been employed by some workers (290).

One method, developed by Kuroki (286) has successfully employed the Lederberg replica plating method for cell lines such as L5178Y, from colonies grown on the surface of solid agar medium.

The ability of mammalian cells to grow on the surface of solid agar is not widely adopted, and so the investigation was carried out in two parts.

A. To establish the optimal conditions for the growth of L5178Y colonies on the surface of solid agar medium, and to determine the plating efficiency by this method.

B. To isolate 254 nm radiation sensitive mutants of L5178Y cells by replica plating.

A. To Establish the Plating Efficiency of L5178Y Cells on the Surface of Solid Agar Medium

According to Kuroki (286), 0.50% w/v agar gave a sufficiently rigid medium to withstand manipulation, while allowing adequate diffusion of nutrients to the cells on the surface.
**Preparation of Plates**

Double strength (D/S) FM20 was prepared by making up the medium in the usual manner, but to 250 ml volume instead of 500 ml. The D/S FM20 was stored at 4°C for up to one month. Prior to use for plate preparation, 50 ml volumes were distributed into 100 ml glass bottles (Flow) and held at 50°C in the water bath for a minimum period of time.

Five hundred mg quantities of Noble Agar were placed in each of 5 x 100 ml glass bottles (Flow), made up to 50 ml with glass distilled water and autoclaved at 121°C for 15 minutes. The bottles were allowed to cool and held at 50°C.

Fifty ml of agar were gently poured into 50 ml of D/S FM20, mixed by slow rotation of the bottle to avoid frothing, and distributed equally between 5 x 9 cm diameter bacterial grade Petri dishes, giving approximately 20 ml per dish. The medium was allowed to cover the surface of the dish by gentle movement, and air bubbles were removed from the medium by rapid flaming of the surface with a microbunsen flame. The plates were left undisturbed for at least 15 minutes to allow the agar to set, then stored at 4°C in a sealed plastic box for up to one week.

**Overdrying of Plates**

The plates were placed at the back of the laminar flow cabinet, and the lids were removed. The plates were left in this manner for 30 minutes, after which time the lids were replaced.

**Inoculation of Plates**

The storage and overdrying of plates results in loss of CO₂ from the medium. Therefore plates were placed in a plastic box, the box gassed with an excess of CO₂, and the box sealed. The plates were allowed to equilibrate for at least 30 minutes.

Cells were serially diluted in PBS and 0.05 ml volumes of the final dilution, were placed at the centre of each plate using a Gilson P200 replicating pipette plus sterile tip. The suspension was spread over the surface of the plate using a small sterile glass spreader, care
being taken to avoid the extreme edge of the plate. The plates were allowed to stand at room temperature for 15 minutes, under laminar flow, to allow the moisture to be absorbed by the agar, then inverted, boxed and gassed, as for the soft agar plating procedure, and incubated at 37°C for 14 days.

Discussion of Methodology

The plating efficiency of L5178Y cells by the surface plating method was found to be variable, at best approaching 60%, which is comparable with the value given by Kuroki, of 69.8% (286). Therefore, the procedure was investigated in order to determine the particular steps which may cause the variation.

The 30 minutes exposure of the medium surface, under laminar flow had been previously determined by weight loss measurement, to be sufficient to remove at least 0.2 ml of moisture from the medium. It would therefore be expected than any volume less than 0.2 ml on the surface of the medium would be adequately absorbed. This method of overdrying, also had the advantage of minimal airborne mould contamination.

Agar Concentration

Although 0.5% w/v Noble Agar in the medium produced a rigid structure, the slightest excessive pressure applied to the surface caused disruption. The agar at this concentration also tended to produce a 'wet' plate. However increasing the agar concentration to 0.6% w/v prevented the growth of colonies altogether.

Inoculum Size

The 0.05 ml inoculum size was considered to be the smallest acceptable volume. Any volume larger than this increased the time required for the liquid to be absorbed into the medium. Since the pH of the medium would be increasing throughout the holding time, it is reasonable to keep the holding time to a minimum.
Incubation of Plates

1.  Position of Plates

Plates incubated upright produced colonies which were uncountable, due to merging of colonies in the moisture layer which appeared on the agar surface, after 2 - 3 days incubation. Thus to obtain discrete colonies, the plates were incubated in an inverted position.

2.  Humidification

As with the incubation of soft agar suspension plates, the surface plated cells required additional humidification of the incubation box for clonal growth to occur, since no growth was evident when plates were incubated in a non-humidified atmosphere.

A layer of moisture appeared in the lids of the inverted plates, 2 - 3 days after incubation, whether the boxes were humidified or not. Thus it was suggested that the variation in plating efficiency by the surface plating method may be attributed to the physical removal of the cells from the medium surface, when the moisture comes out of the medium.
B. Replica Plating

Although the plating efficiency of L5178Y cells on the surface of solid agar is not critical, the replica plating efficiency should be as high as possible if an isolation experiment is to be valid. Two methods of replica plating were attempted:

1. Pile Fabric

2. Glass Rod

1. Pile Fabric

The Lederberg style of replica plating was attempted initially, using sterilised squares of cotton velvet over an aluminium replicating block designed to fit a 9 cm Petri dish. The velvet was held in place by an aluminium band.

Replica plating by this method was possible, however, the moisture present on the surface and around the edges of the plates tended to cause colonies to merge.

2. Glass Rod

The transfer of cells on the tip of a glass rod, while being a more tedious procedure than using pile fabric, proved to be more efficient in the accurate transfer of cells to replica plates, and was thus employed as the replica plating method.

Plates for replica plating were prepared as for surface plating but with a numbered grid marked on the undersurface of the Petri dish. The grid used was as shown in figure 6.1. The outer area of the plate was left unmarked, to avoid shadowed areas when plates were irradiated.

Plates were pre-gassed with CO$_2$ and placed in sets of four in the laminar flow cabinet.
Figure 6.1  Grid Arrangement for Replica Plating of L5178Y cells.
Using a 1 mm diameter glass rod, cells from colonies on the master plate were transferred to each of the four replica plates in turn, ensuring that the same numbered square on the grid was used each time. The glass rod was washed in 95% alcohol, flamed in a microbunsen flame to burn off the alcohol and allowed to cool before re-use.

The small diameter of the glass rod employed for this procedure ensured adequate penetration of the colony on the master plate. Care was taken to ensure that the cells transferred in this way were not in sufficiently large numbers to be visible on the replica plate, which could lead to misinterpretation of results at a later stage. This was also best achieved by use of the small tip glass rod.

**Mutant Isolation Procedure**

A chemical mutagen is employed to produce 'mutant' cells, and UV radiation is used as the selective agent.

This procedure for mutant isolation, after Kuroki (291), may be considered under the following headings:

1. Treatment of cells with the mutagen.
2. Expression of the mutant cells.
3. Plating of the cells.
4. Replica plating.
5. Irradiation.
6. Analysis of results.

1. **Treatment of Cells with the Mutagen**

The mutagen employed was N-methyl, N', nitro, N,nitrosoguanidine (MNNG).

The MNNG solution was prepared, with strict adherence to the standard procedures for handling toxic chemicals.

The MNNG (Sigma grade, Sigma Chemical Co.) was stored dessicated, foil wrapped at -20°C. A small quantity of the compound was placed in a small, disposable, tared screw-cap plastic container, and the
weight determined. This was then dissolved and made up to 100 ml volume with sterile distilled water, in a volumetric flask. The volume of solution finally added to the cells and the toxicity of the MNNG were such that the solution was not filter sterilised. The solution was transferred to 25 ml sterile screw cap glass bottles, and stored, foil wrapped, upright in 50 ml glass beakers at -20°C for up to one month.

The solution used for the experiments in this section had a concentration of 438 µg per ml. The solution was thawed at 37°C prior to use.

A culture of log phase L5178Y cells was centrifuged at 1500 rpm in an MSE minor centrifuge, the supernatant decanted, and the cells resuspended in FM10 at a concentration of 10^6 per ml. The mutagen was required at a concentration of 0.2 µg per ml per 10^6 cells and so the stock MNNG solution was diluted 0.1 ml + 0.9 ml PBS, then 4.6 µl of the diluted solution were added to 1 ml of the cell suspension, equivalent to 10^6 cells. This was incubated at 37°C for 1 hour. The volume of MNNG solution added was considered small enough to have a negligible effect upon the cell suspension volume.

2. Expression of Mutant Cells

The cell suspension after treatment was centrifuged, the supernatant decanted, and the cells resuspended in 1 ml PBS. The suspension was recentrifuged, and the cells finally resuspended in 1 ml FM10. This was added to 9 ml of FM10 in a 25 cm^2 tissue culture flask, gassed with 5% CO₂ in air and incubated at 37°C for two days, to allow the expression of mutants induced by the MNNG.

The MNNG treatment, 0.2 µg per ml per 10^6 cells for 1 hour was estimated to kill approximately 70 - 90% of the cells.
3. **Plating of the Cells**

Cells after the two-day expression period were counted, appropriately diluted and plated. 1. In soft agar at a concentration of 100 cells per plate, in 5 cm diameter Petri dishes: 30 plates were prepared. 2. On the surface of solid agar in 9 cm diameter Petri dishes, at a density of 200 cells per plate: 25 plates were prepared.

It was considered necessary to take the precaution of plating cells in soft agar, in case insufficient numbers of colonies were obtained on the surface plates. Using the small-tip rods, transfer of cells from colonies within soft agar had previously been shown to be possible.

Both sets of plates were incubated at 37°C for 14 days. This ensured that the clones in soft agar were large enough to be used for replica plating.

4. **Replica Plating**

In both experiments carried out, the surface plates produced an insufficient number of colonies, and so the colonies in soft agar suspension were used.

As previously described, colonies were transferred to each of four replica plates. The entire number of colonies on each master plate was replica plated, to ensure no selection of a particular type of colony, eg. large colonies, was made.

Two platings were carried out:

- 7 sets of 4 plates 308 colonies
- 4 sets of 4 plates 176 colonies
- Total = 484 colonies

5. **Irradiation of Cells**

Before a suitable UV fluence for isolation of sensitive cells could be determined, the sensitivity of the cells to 254 nm radiation on replica plates was established.
Cells from a culture of L5178Y, without mutagen treatment, were plated on the surface of solid agar and incubated at 37°C for 14 days. Colonies from the master plates were replica-plated onto 5 replica plates. One plate was retained as control, and the remaining four were exposed to UV radiation fluences of 4.5, 9.0, 13.5 and 18.0 Jm⁻². The plates were incubated at 37°C for 7 days, after which time the number of colonies on each plate were scored. The number of colonies on each irradiated plate was expressed as a percentage of the number of colonies on the control plate, and a survival curve constructed from these values, given in figure 6.2. The plate counts obtained are given in table 6.1.

It was determined that a UV radiation fluence of 4.5 Jm⁻² prevented the growth of one colony in 10, and it was argued therefore that if a colony failed to grow after mutagen treatment and selection with UV radiation, there is only a 1 in 10 chance of that colony having normal sensitivity to 254 nm radiation.

The sets of replica plates prepared from the mutagenised colonies were randomly labelled A to D. Plates A and C were kept as controls, plates B and D were exposed to 4.5 J m² of 254 nm radiation using the apparatus described in Chapter 3.

All plates were incubated at 37°C for 7 days, after which time the colonies were recorded in the following manner. No colony growth scored 0, colony growth scored 1. The formation of a large, rounded colony was scored as 1, any other intermediates as 0.

<table>
<thead>
<tr>
<th>Table 6.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony Counts for L5178Y cells after 254 nm irradiation on the surface of solid agar medium</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>UV fluence Jm⁻²</th>
<th>Colony Counts (44 per plate total)</th>
<th>Percent Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>42</td>
<td>100</td>
</tr>
<tr>
<td>4.5</td>
<td>38</td>
<td>90.48</td>
</tr>
<tr>
<td>9.0</td>
<td>28</td>
<td>66.67</td>
</tr>
<tr>
<td>13.5</td>
<td>21</td>
<td>50.00</td>
</tr>
<tr>
<td>18.0</td>
<td>9</td>
<td>21.43</td>
</tr>
</tbody>
</table>
Figure 6.2 Inactivation of L5178Y cells by 254 nm Radiation: Irradiation of Cells on the Surface of Solid Medium.
Thus, any colonies where growth occurred on plates A and C but not on B and D ie. scoring $\begin{align*} A & \quad B & \quad C & \quad D \\ 1 & \quad 0 & \quad 1 & \quad 0 \end{align*}$ were regarded as potential UV-sensitive mutants.

The colonies fulfilling this requirement were picked off the surface of the agar using a pasteur pipette and transferred to 10 ml of FM10, gassed with 5% CO$_2$ in air and incubated at 37°C.

The cultures were maintained in this way for a period of time, and the sensitivity to 254 nm radiation was determined at 1 week and 2 months after culturing from the colony.

RESULTS AND DISCUSSION

Three potential UV radiation sensitive colonies were isolated from the 484 colonies tested, giving a frequency of $6.2 \times 10^{-3}$. This was approximately half that obtained by Kuroki (291) which was $1.6 \times 10^{-2}$.

The three sublines in culture were designated PJ1, PJ2, PJ3.

254 nm Radiation Sensitivity at 1 week

Although the cultures of the three sublines showed normal growth in suspension culture, there appeared to be a great variation in plating efficiency in soft agar, when the survival experiments were carried out, at one week after the start of culturing.

PJ1 gave a plating efficiency of less than 1%, with no growth on plates with irradiated cells.

PJ2 and PJ3 gave plating efficiencies of 19% and 26% respectively. The survival curves obtained for these sublines after 254 nm irradiation are given in figure 6.3. The survival data are given in the appendix.
Figure 6.3a  Inactivation of the PJ2 subline by 254 nm Radiation: Culture at 1 Week.
(A) PJ2.
(---) Control.
Figure 6.3b  Inactivation of the PJ3 subline by 254 nm Radiation: Culture at 1 Week.

(■) PJ3.

(--) Control.
It was apparent that the sensitivity of the two sublines to 254 nm radiation was greater than that for the parent line. The $F_{10}$ value for PJ2 was 9.0 Jm$^{-2}$ and for PJ3, was 8.5 Jm$^{-2}$, compared with the parent line of 14.2 Jm$^{-2}$.

The low plating efficiencies obtained for these cells may be due to the short period of time the cells had been in culture. Thus the cultures were maintained in the usual manner for a period of two months, after which time the 254 nm radiation sensitivity was determined.

**254 nm Radiation Sensitivity after 2 months**

Survival curves for 254 nm radiation were successfully obtained for all three sublines. PJ1 gave a persistently low plating efficiency, of the order of 10%, but a survival curve was still obtained.

PJ2 gave a plating efficiency of 81% and PJ3, of 75%.

The survival curves after 254 nm irradiation at 2 months are given in figures 6.4 (a) to (c). The survival data are given in the Appendix.

The $F_{10}$ values obtained for the sublines are as follows:

<table>
<thead>
<tr>
<th>Subline</th>
<th>$F_{10}$ Jm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJ1</td>
<td>11.0</td>
</tr>
<tr>
<td>PJ2</td>
<td>10.6</td>
</tr>
<tr>
<td>PJ3</td>
<td>12.6</td>
</tr>
<tr>
<td>(Control)</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Thus the sublines appear to be less sensitive after two months culture than after 1 week.

It would be reasonable to suppose that the sensitivity shown by the cultures of PJ2 and PJ3 after 1 week reflected the low plating efficiencies of the cells, and that the sensitivity of the sublines at two months is more representative.
Figure 6.4a  Inactivation of the PJ1 subline by 254 nm 
Radiation: Culture at 2 Months.
(O)  PJ1.
(---) Control.
Figure 6.4b  Inactivation of the PJ2 subline by 254 nm Radiation: Culture at 2 Months.

(▲) PJ2.

(---) Control.
Figure 6.4c  Inactivation of the PJ3 subline by 254 nm Radiation: Culture at 2 Months.

(■) PJ3.

(--) Control.
Alternatively, the change in sensitivity could be explained by the loss of phenotypic stability, as demonstrated by Kuroki, attributed to the leaky nature of the mutants produced by MNNG.

However, the technique has been demonstrated as a feasible method for the selection of 254 nm radiation sensitive mutants, and so an attempt was made to increase the sensitivity by subjecting the PJ2 subline to the mutagen treatment and selection procedure.

Isolation of 254 nm Radiation Sensitive Mutants from Subline PJ2

After the sensitivity of the sublines to 254 nm radiation had been determined at 2 months, PJ2 was chosen for the repeat procedure, because it gave a high plating efficiency in soft agar suspension, and the change in sensitivity to 254 nm radiation over the 2 month period was not as great as that of the PJ3 subline.

The entire procedure was repeated except that the replica plates were subjected to 2 Jm" of 254 nm radiation as the selective fluence.

Results and Discussion

A total of 308 colonies from the mutagen treated PJ2 cells were replica plated, which gave rise to one potentially 254 nm radiation sensitive isolate. This was a frequency of 3.25 x 10\(^{-3}\), approximately half that for the initial procedure.

The colony isolated was grown up in FM10 for one week and subjected to a normal 254 nm radiation survival determination. The survival curve is given in figure 6.5. The survival data is given in the Appendix.

The subline was designated PJ4.

The \(D_{10}\) value for PJ4 after 254 nm irradiation was 12.0 Jm\(^{-2}\), thus it is apparent that the procedure has failed to increase the sensitivity of the PJ2 subline.
Figure 6.5  Inactivation of the PJ4 subline by 254 nm Radiation: Culture at 1 Week.

(△) PJ4.

(---) Control.
GENERAL DISCUSSION

The results presented suggest that the procedure for the isolation of mutants sensitive to 254 nm radiation described may have a definite use in the production of mutants of L5178Y with a range of sensitivities to UV radiation. It is felt that alterations to the different stages of the procedure may lead to the isolation of more stable, and more specifically sensitive mutants.

1. The Mutagen

The use of MNNG by Kuroki has resulted in large numbers of mutant cells with high survival, a property well known to be exhibited by MNNG. However, MNNG also produces unstable mutants. Therefore, it would seem reasonable to suggest an alternative mutagen, eg. EMS or MMS which would produce less mutants, but those which were induced may be more phenotypically stable.

The Plating Procedure

The only drawback with the glass rod method of replica plating is the time required. Transfer of 1 set of 44 colonies takes approximately 20 minutes.

This could be improved upon by using pile fabrics, but the problem of moisture on the plates would be required to be overcome before this method were feasible.

The Selective Agent

The selective agent for the work described in this chapter was 254 nm radiation. This appears to be the agent of choice, as sources tend to emit a reproducible fluence rate at this wavelength. However, the results presented in chapter 3, together with the knowledge to date of relative sensitivities of cell systems to different radiation wavelengths, indicate no reason to assume that mutants with 254 nm radiation sensitivity will show any degree of sensitivity to other UV radiation wavelengths. Thus it may be possible using the same system, but varying the selective UV radiation wavelength, to produce a range of mutants with sensitivities to the different agents.
Selection Procedure

The number of potential UV radiation sensitive mutants produced by the method under discussion appears to be high compared with a number of other techniques. For example, the microscopic examination of CHO cell colonies by Thompson et al (290) yielded mutant frequencies of $-10^{-4}$, and those by Busch et al (289) at a frequency of $2 \times 10^{-4}$ using the 'Cyclops' photographic technique. Of the 150 potentially UV radiation sensitive colonies selected by the latter technique, 52 were shown to be UV radiation sensitive, with a sensitivity of up to five-fold greater than the parent line.

The degree of sensitivity increase shown by the isolates from the replica plating procedure was not as great as that shown by other isolation procedures. This is demonstrated by Busch et al (289) who isolated two UV radiation sensitive mutants from CHO cells by replica plating, having less sensitivity to UV radiation than the mutants isolated by the Cyclops method.

It is arguable as to which system is the more efficient: the production of many mutants by replica plating, with time-consuming screening for radiation sensitivity after isolation, or the isolation of fewer mutants, with definite UV-radiation sensitivity by painstaking microscopic or photographic techniques.

Clearly one advantage of the replica plating technique is its use for cells which grow in suspension, such as L5178Y. The relatively simple culturing and plating techniques which may be applied to this cell line, make it a good candidate for the selection of a range of UV radiation sensitive mutants. This is supported by the knowledge of the genetic makeup of the cell line, and the deficiencies in excision repair capabilities shown by this cell line would aid in the study of other repair mechanisms acting upon damage to genetic material in mammalian cells.
CHAPTER SEVEN

CONCLUSIONS
The aims of the work presented in this thesis were three-fold:

1. To establish a mammalian cell system in culture, optimising growth conditions for this laboratory.

2. To use the mammalian cell system to study radiation induced inactivation and mutagenesis under a range of conditions.

3. To study the sensitisation of mid UV radiation induced inactivation and mutagenesis in the mammalian cell system, by the sunscreen agent para-amino benzoic acid. This has been discussed in full in Chapter 5.

The mouse lymphoma cell line L5178Y was established in culture and conditions for growth and plating in soft agar suspension optimised for this laboratory. Three further mouse lymphoma cell lines were also cultured. The JL line was used to compare effects, if any, of culture history and growth medium on the response to radiation. The radiosensitive line L5178YS and radioresistant line AIV were used to study the relative responses to UV radiation as well as that of ionising radiation.

The responses of the four cell lines to the four radiation treatments, γ radiation, 254 nm, 313 nm and 365 nm radiation revealed a general trend in that L5178YS appeared to be more sensitive to 254 nm and 313 nm radiation, and the AIV line appeared consistently more resistant to all treatments, with respect to the parent line. This is in contrast to data published (292) where the radioresistant L5178Y cell line was more sensitive to 254 nm radiation than the radiosensitive line.

Whatever the true response of the different sublines to radiation, it is reasonable to suppose that the sensitivity is a function of the repair capability of the cells. Dimer excision in L5178Y cells as in most rodent cell lines, is low (293) but postreplication repair has been demonstrated (294) after UV irradiation. Therefore the difference in sensitivity to radiation may be due to differences in post-
replication repair capabilities between the sublines of L5178Y. This is supported by Walicka et al (295) who have demonstrated that treatment with caffeine after 254 nm irradiation enhanced the lethal effect of the radiation in the radiosensitive line but not in the radioresistant line. This effect was interpreted as there being two types of post-replication repair, which proceed at different rates, the fast rate process occurring in the radioresistant line, the slow rate process in the radiosensitive line. The reduced molecular weight of DNA in UV-irradiated cells of the radioresistant line, in the presence of caffeine, may be due to inhibition of the fast rate repair process (293).

The origin of the so-called sensitive and resistant L5178Y cell lines as defined by Walicka et al leads to the conclusion that their L5178Y-R line is in fact the parent line L5178Y, from which the radiosensitive L5178YS line was isolated (200). Therefore, the increased sensitivity to ionising radiation exhibited by the L5178YS line may be explained by a further defect in repair capabilities.

However, when considering the AIV line with increased radiosensitivity above that of the parent line, an explanation is less easy to find. Courtenay (222) in her work with isolating radioresistant mutants of L5178Y, has indicated two types of mutant, with respect to survival characteristics. Type A are characterised by an increase in the width of the shoulder, thought to be due to effective repair of damage at low radiation doses, whereas type B mutants are characterised by an increase in the D$_{37}$ value, interpreted as being due to a further type of repair process, which does not become saturated at higher radiation doses. The AIV cell line would appear to be a type B mutant.

Cell hybridisation experiments, using crosses between radioresistant and radiosensitive L5178Y lines, yield hybrids with D$_{37}$ values below those of the resistant line, illustrated by Dale (224). Since the tetraploid wild-type hybrids yield the same sensitivities as the normal cells, the alteration in sensitivity can only be explained in terms of an interaction between genes, and that the radioresistant
genome is behaving in a dominant fashion, restoring the repair capab-
ilities of the radiosensitive line. In this way a complementation process
could be occurring between the two sets of genetic material.

An alternative suggestion for the increased radioresistance expressed
in some somatic cell hybrids (296) has been the re-expression of
repressed repair functions.

The sensitivity of a cell line to radiation treatment is usually
accepted to be related to the repair of genetic damage. However, it
cannot be ruled out that other factors, including mitotic delay effects
may play an important part in radiation induced lethality. In the
case of the L5178YS line, the radiosensitivity may be due to the long
mitotic delay, correlation between these two phenomena being made by
Rosenberg et al (297).

The responses of the four cell lines to mid and near UV radiation were
less clear cut than those for ionising and 254 nm radiation, and work
with the two longer wavelengths would need to be continued, before
conclusions could be drawn. It is evident at this stage that the AIV
line is more resistant to the longer wavelengths of UV radiation than
the parent line, which may be expected from the results for the other
radiation treatments.

The increased resistance of the JL line at 365 nm may illustrate a
general phenomenon at this wavelength, that of a strong effect of pre-
and post-irradiation growth conditions on UV radiation induced
lethality.

The next logical step in the study of UV radiation damage and repair,
is UV radiation induced mutagenesis.

The preliminary results indicate that both 254 nm and 313 nm radiation
induce mutation to oua$^R$ and 6-TG$^R$ although not to the same extent. It
would be interesting therefore, to establish the mutagenic responses
of the four cell lines, using the four radiation treatments. With the
knowledge available for the survival responses for the cell lines,
further insight may be gained into the mechanisms of repair of radiation damage.

An alternative approach to the problem of elucidating repair mechanisms in mammalian cells has been by the isolation of mutants with increased sensitivity to UV-radiation. This type of approach is still in its infancy, and until the specific structure of mammalian genetic material is determined, no logical approach, as has been applied to bacteria, can be made. However, a method, such as that described in Chapter 6 provides relatively large number of sublines with different sensitivities to the parent line. The biochemical techniques which are now becoming available may then be employed to determine the deficiencies, if any, in the repair mechanisms.
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APPENDIX 1.

Gamma Radiation Dosimetry.

The ferrous sulphate dosimetry system was used (298).

The reactions involved are as follows:

\[
\begin{align*}
\text{Fe}^{2+} + \text{OH}^{-} &= \text{Fe}^{3+} + \text{OH}^- \\
\text{Fe}^{2+} + \text{HO}_2^- &= \text{Fe}^{3+} + \text{HO}_2^- \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 &= \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\end{align*}
\]

The dosimeter solution consists of the following:

\[
\begin{align*}
10^{-3} \text{ M } \text{Fe} (\text{NH}_4)^2 (\text{SO}_4)^2 \cdot 6\text{H}_2\text{O} \\
10^{-3} \text{ M } \text{NaCl} \\
0.8 \text{ N } \text{H}_2\text{SO}_4
\end{align*}
\]

Stock Solutions

5.84G of sodium chloride (BDH) were dissolved and made up to 1L with distilled water.

213ml of H\textsubscript{2}SO\textsubscript{4} (concentrated) were made up to 1L with distilled water.

Fresh Dosimeter Solution

Twenty five ml of each of the stock solutions, together with 0.985G of Fe (NH\textsubscript{4})\textsubscript{2} (SO\textsubscript{4})\textsubscript{2} \cdot 6H\textsubscript{2}O were made up to 250ml with distilled water.

The ferric ions show strong absorption in the ultraviolet region, whereas the ferrous ions show negligible absorption in this region. Therefore optical density readings after exposure of the dosimeter solution to the gamma radiation were taken at 305 nm.
The dose limits for this system were determined to be 4,000 Rads (40 Gy) to 40,000 Rads (400 Gy).

Therefore 2 ml volumes of dosimeter solution were sealed into the glass irradiation tubes as described in Chapter 3, and exposed to the $^{60}$Co source for graded periods of time to give acceptable optical density readings at 305 nm.

Calculation of Absorbed Radiation Dose.

If 1 cm pathlength cuvettes are used for optical density measurements, then:

$$O.D = eC$$  (Beers Law)

Where $e$ = Molar extinction Coefficient

$C = \text{Concentration in moles/litre.}$

Thus:

$$C_{Fe^{+++}} \text{ (moles/litre)} = \frac{O.D.}{e}$$

$$C_{Fe^{+++}} \text{ (moles/ml)} = \frac{O.D.}{10^3e}$$

$$C_{Fe^{+++}} \text{ (moles/g)} = \frac{O.D.}{10^3 e p}$$

Where $p = \text{density of the solution.}$

The number of $Fe^{+++}$ ions per g of solution

$$= \frac{O.D. \times 6.023 \times 10^{23}}{10^3 e p}$$

If $G = \text{the number of ions formed per 100 eV absorbed:}$

Then the energy absorbed

$$E_{abs} \text{ (eV/g)} = \frac{O.D. \times 6.023 \times 10^{23}}{e p \times G \times 10^{-2}}$$
\[
1 \text{ eV} = 1.602 \times 10^{-12} \text{ ergs}
\]

\[
E_{\text{abs}} \text{ (ergs/g)} = \frac{O.D. \times 6.023 \times 10^{23} \times 1.602 \times 10^{-12}}{e \times p \times G \times 10^{-2}}
\]

One Rad = 100 ergs/g

Dose in Rads
\[
= \frac{O.D. \times 6.023 \times 10^{8} \times 1.602}{e \times p \times G \times 10^{-2} \times 10^{2}}
\]

\[
= \frac{O.D. \times 9.65 \times 10^{8}}{e \times p \times G}
\]

e at 305 nm and 25°C = 2196 (+ 0.06% per °C)

G = 15.3
APPENDIX 2.

Ultraviolet Radiation Dosimetry.

The potassium ferrioxalate actinometer system was used (299) Preparation of Potassium Ferrioxalate.

The equation for the reaction to produce potassium ferrioxalate is as follows:

\[
\text{FeCl}_3 \cdot 6\text{H}_2\text{O} + 3\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O} \quad \rightarrow \quad \text{K}_3\text{Fe} (\text{C}_2\text{O}_4)_3 \cdot 3\text{H}_2\text{O} + 3\text{KCl} + 6\text{H}_2\text{O}
\]

Thus the ferric chloride and potassium oxalate were mixed in the proportions of 1:3.

Molecular weight of \(\text{FeCl}_3 \cdot 6\text{H}_2\text{O}\) 240.20
Molecular weight of \(\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}\) 184.22

1.5M solutions were prepared. All manipulation were carried out under red light.

One hundred ml of 1.5M \(\text{FeCl}_3\) solution were mixed with 300ml of 1.5M \(\text{K}_2\text{C}_2\text{O}_4\) solution in a beaker. The beaker was left to stand at room temperature overnight, after which time a crystalline solid had developed. The supernatant was decanted and the crystals were redissolved in 150ml of distilled water in a 70°C water bath. The solution was filtered through a no.1 Whatman filter paper using negative pressure and collected in a clean beaker. The beaker was placed on ice for approximately three hours, after which time the solid had recrystallised. The supernatant was decanted and the crystals were dried at room temperature. On examination in daylight, a small portion of the crystals appeared bright green.
in colour. The remainder of the crystals were stored in an amber, foil wrapped glass bottle.

In order to determine whether the crystals were of the desired compound, a dilute, acidic solution was prepared, divided into two portions, one kept in the dark and the other exposed to 10 minutes of 254 nm radiation. Both samples were developed using the procedure described (298). The unirradiated solution appeared green in colour whereas the irradiated solution had become pale yellow. Thus it was assumes that the crystals were of potassium ferrioxalate, and had undergone photo-oxidation during UV irradiation.

Preparation of Solutions for Actinometry.

1. Actinometer Solution.

2.947G of potassium ferrioxalate were dissolved in 800ml of distilled water, 100ml of N.H_2SO_4 (Volucon, M&B) were added and the solution was made up to 1L with distilled water. This gave an 0.006M solution of potassium ferrioxalate.

2. Calibration Solution.

2.7802G of ferrous sulphate.7H_2O (A.R) were dissolved and made up to 100ml with distilled water, giving an 0.1M solution of ferrous sulphate. One ml of this solution was diluted to 250ml with 0.1N.H_2SO_4 (Volucon,M&B ), giving the calibration solution containing 4 x 10^-7 moles of Fe^{++} ions per ml.

3. Phenathroline Solution

1.00G of 1:10 phenanthroline monohydrate (BDH) was dissolved in hot distilled water, cooled and made up to 1L with distilled water.
4. Buffer.

600ml of N.sodium acetate solution (A.R. solid) were added to 350ml of N.H₂SO₄ (Volucon, M&B) and made up to 1L with distilled water.

Calibration of the Actinometer Solution.

The actinometer solution was calibrated using the ferrous sulphate solution as follows:

Eleven x 25ml stoppered volumetric flasks were used: volumes of calibration solution were added from 0 to 5.0ml in 0.5ml steps. The necessary volume of 0.1N.H₂SO₄ was added to 12.5ml, followed by 2.0ml of 1% phenanthroline solution, and made up to 25ml with buffer. The flasks were shaken and allowed to stand for 30 minutes to develop the colour, after which time an optical density reading for each solution was taken at 510 nm, using the solution with no calibration solution as blank.

A plot of optical density at 510 nm against moles of Fe²⁺ ions was prepared and the slope of the line calculated by regression analysis.

Dosimetry for Ultraviolet Sources.

The actinometer solution was prepared as previously described. Four ml volumes of the solution were placed in a 1 cm pathlength Quartz spectrophotometer cuvette and exposed to 254 nm or 313 nm radiation at a known distance from the source for a time which would give an acceptable optical density reading after development.

After exposure, 3ml of solution were removed from the cuvette and developed as previously described: The optical density at 510 nm and the slope of the calibration curve were used to calculate the fluence rate.
Calculation of Fluence Rate.

For All Wavelengths.

Slope of calibration curve = $0.120 \times 10^8$
Volume irradiated = 4.0ml
Irradiation area = $4.0\text{cm}^2$
Volume sampled and developed = 3.0ml

An exposure time of $n$ seconds gave an optical density at 510 nm.

$$\frac{0.510 \text{ nm}}{\text{Slope of calibration curve}} = \text{Moles per ml of Fe}^{++} \text{ ions.}$$

This Fe$^{++}$ ion concentration per ml was in 25ml of developed solution originally from 3ml sampled from a total of 4ml of solution irradiated. Thus the above concentration of Fe$^{++}$ ions multiplied by 25 and by $4/3$ gives the total Fe$^{++}$ concentration in moles, in the 4ml of irradiated solution.

If the total moles of Fe$^{++}$ ions in 4ml of irradiated solution is $Y$, then the fluence rate for each wavelength may be calculated as follows:

1. 254 nm Radiation.

The quantum yield at 254 nm is 1.26 moles per Einstein (See table at end of section)
Thus $Y$ moles of Fe$^{++}$ ions = 1.26$Y$ Einsteins

At 254 nm, ergs per Einstein = $4.71 \times 10^{12}$ (See table)
Thus 1.26$Y$ einsteins = $4.71 \times 10^{12} \times 1.26Y$ ergs.

This is for $400\text{mm}^2$ irradiation area:
Thus fluence = $\frac{4.71 \times 10^{12} \times 1.26Y}{400}$ ergs per $\text{mm}^2$. 
Therefore the fluence rate = \( \frac{4.71 \times 10^{12} \times 1.26Y}{400n} \) ergs per mm\(^2\) per sec.

\[
= \frac{4.71 \times 10^{12} \times 1.26Y}{400n \times 10} \text{ J per m}^2\text{ per sec.}
\]

313 nm Radiation.

At 313 nm ergs per Einstein = \(3.82 \times 10^{12}\)

Therefore the fluence rate = \(\frac{3.82 \times 10^{12} \times 1.26Y}{400n \times 10}\) J per m\(^2\) per sec.

Thus a simple conversion can be made from the optical density of the developed solution after irradiation to the fluence rate of the ultraviolet source.

Calibration of the Thermopile.

The potassium ferrioxalate actinometer system is of particular use for calibration of fluence rate measuring equipment, such as the thermopile.

The Hilger Watt FT17 thermopile in conjunction with a microvolt ammeter (Keithley Instruments) was used for routine fluence rate measurement.

To calibrate the thermopile, a reading was taken immediately after an actinometer solution sample was exposed to the UV radiation source. Care was taken to ensure that the position of the two measuring devices coincided exactly.

The fluence rate values calculated using actinometry and the readings of the thermopile were determined for a range of distances from the radiation source.
The thermopile readings in microvolts were plotted against the fluence rate calculated using the actinometry, and a conversion factor for the thermopile determined from the slope of the plot.

The conversion factor was used for subsequent routine determination of fluence rate and for the Hilger Watt FT17 thermopile the conversion factor was 0.0113, for microvolts to J m\(^{-2}\) per second.

Table A1  **Quantum Yield and Erg per Einstein Values (299)**

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<th>Wavelength (nm)</th>
<th>Erg per Einstein</th>
<th>Quantum Yield (Moles per Einstein)</th>
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</thead>
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</tr>
<tr>
<td>435</td>
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<tr>
<td>405</td>
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<td>1.16</td>
</tr>
<tr>
<td>366</td>
<td>(3.27 \times 10^{12})</td>
<td>1.26</td>
</tr>
<tr>
<td>334</td>
<td>(3.58 \times 10^{12})</td>
<td>1.26</td>
</tr>
<tr>
<td>313</td>
<td>(3.82 \times 10^{12})</td>
<td>1.26</td>
</tr>
<tr>
<td>300</td>
<td>(3.99 \times 10^{12})</td>
<td>1.26</td>
</tr>
<tr>
<td>254</td>
<td>(4.71 \times 10^{12})</td>
<td>1.26</td>
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### Table A2: Survival Data for Gamma Radiation Inactivation

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<td>Cell Line</td>
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<td>Surviving Fraction</td>
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<td>----------------</td>
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</tr>
<tr>
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<td>Logarithmic Phase</td>
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<td>9.48 x 10^-1</td>
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|           | 4.50           | 8.15 x 10^-1      | 7.41 x 10^-1  
|           | 9.00           | 4.70 x 10^-1      | 3.38 x 10^-1  
|           | 11.25          | 2.72 x 10^-1      | -             
|           | 13.50          | 1.64 x 10^-1      | 6.19 x 10^-2  
|           | 18.00          | 2.13 x 10^-2      | 4.22 x 10^-2  
|           | 22.50          | 5.25 x 10^-3      | 9.52 x 10^-4  
|           | 27.00          | 2.67 x 10^-3      | -             
| JL        | 4.50           | 6.60 x 10^-1      | 9.60 x 10^-1  
|           | 9.00           | 3.48 x 10^-1      | 2.48 x 10^-1  
|           | 13.50          | 1.77 x 10^-1      | 8.27 x 10^-2  
|           | 18.00          | 7.08 x 10^-2      | 2.08 x 10^-2  
|           | 22.50          | 3.54 x 10^-2      | 2.26 x 10^-3  
|           | 27.00          | 2.12 x 10^-3      | 3.76 x 10^-4  
| L5178YS   | 4.50           | 5.73 x 10^-1      | 9.48 x 10^-1  
|           | 9.00           | 2.99 x 10^-1      | 3.59 x 10^-1  
|           | 13.50          | 8.91 x 10^-2      | 8.89 x 10^-2  
|           | 18.00          | 1.77 x 10^-2      | 2.83 x 10^-2  
|           | 22.50          | 6.00 x 10^-3      | 2.43 x 10^-3  
|           | 27.00          | 3.10 x 10^-3      | 8.08 x 10^-3  
| AIV       | 4.50           | 7.86 x 10^-1      | 9.77 x 10^-1  
|           | 9.00           | 7.50 x 10^-1      | 6.21 x 10^-1  
|           | 13.50          | 3.54 x 10^-1      | 4.00 x 10^-1  
|           | 18.00          | 1.89 x 10^-1      | 1.78 x 10^-1  
|           | 22.50          | 7.14 x 10^-2      | 7.43 x 10^-2  
|           | 27.00          | 3.14 x 10^-2      | 3.56 x 10^-2  

Table A3a: Survival Data for 254 nm Radiation Inactivation
Table A3b  Survival Data for 254 nm Radiation Inactivation of L5178Y Cells, Starting Cell Concentration $5 \times 10^5$/ml.

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<td>9.00</td>
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<td>13.50</td>
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<td>18.00</td>
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Table A4a Survival Data for 313 nm Radiation Inactivation, 0°C

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<th>Stationary Phase</th>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>4</td>
<td>2.67 x 10⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.72 x 10⁻¹</td>
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<td>5.14 x 10⁻¹</td>
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<td>3.61 x 10⁻¹</td>
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<td>10</td>
<td>-</td>
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<tr>
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<td>12</td>
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<tr>
<td>JL</td>
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<td>9.16 x 10⁻¹</td>
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<td>7.97 x 10⁻¹</td>
<td>5.66 x 10⁻¹</td>
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<td>7.13 x 10⁻¹</td>
<td>-</td>
</tr>
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<td>2.53 x 10⁻¹</td>
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<td>7.79 x 10⁻¹</td>
<td>3.40 x 10⁻¹</td>
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<td></td>
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### Table A4a  Survival Data for 313 nm Radiation Inactivation, 0°C

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<th>Fluence (KJ/m²)</th>
<th>Surviving Fraction</th>
<th>Logarithmic Phase</th>
<th>Stationary Phase</th>
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<tbody>
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### Table A4b  Survival Data for 313 nm Radiation Inactivation, 20°C

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<td>4</td>
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<td>$1.30 \times 10^{-1}$</td>
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<td>Logarithmic Phase</td>
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<td>5.18 x 10(^{-1})</td>
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<td>4.64 x 10(^{-1})</td>
<td>2.98 x 10(^{-1})</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2.15 x 10(^{-1})</td>
<td>1.56 x 10(^{-1})</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.37 x 10(^{-1})</td>
<td>9.46 x 10(^{-2})</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>-</td>
<td>5.21 x 10(^{-2})</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Fluence (KJ/m²)</td>
<td>Surviving Fraction</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------</td>
<td>--------------------</td>
<td>---</td>
</tr>
<tr>
<td>AIV</td>
<td>20</td>
<td>9.02 x 10⁻¹</td>
<td>6.72 x 10⁻¹</td>
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<tr>
<td></td>
<td>40</td>
<td>8.83 x 10⁻¹</td>
<td>6.09 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.82 x 10⁻¹</td>
<td>3.51 x 10⁻¹</td>
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<td></td>
<td>80</td>
<td>6.82 x 10⁻¹</td>
<td>1.70 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6.34 x 10⁻¹</td>
<td>1.35 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>-</td>
<td>1.03 x 10⁻¹</td>
</tr>
<tr>
<td>UV Radiation Fluence (KJm (^{-2}))</td>
<td>SURVIVING FRACTION</td>
<td>PABA Concentration</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>---------------------</td>
<td>--------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01%</td>
<td>0.02%</td>
<td>0.05%</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>9.36 \times 10^{-1}</td>
</tr>
<tr>
<td>1.0</td>
<td>8.02 \times 10^{-1}</td>
<td>8.06 \times 10^{-1}</td>
<td>8.94 \times 10^{-1}</td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>6.18 \times 10^{-1}</td>
<td>4.48 \times 10^{-1}</td>
<td>6.32 \times 10^{-1}</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>3.11 \times 10^{-1}</td>
<td>2.75 \times 10^{-1}</td>
</tr>
<tr>
<td>4.0</td>
<td>3.14 \times 10^{-1}</td>
<td>1.26 \times 10^{-1}</td>
<td>3.27 \times 10^{-2}</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
<td>6.44 \times 10^{-2}</td>
<td>6.00 \times 10^{-3}</td>
</tr>
<tr>
<td>6.0</td>
<td>3.33 \times 10^{-2}</td>
<td>3.50 \times 10^{-2}</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A6a  Survival Data for 313 nm Radiation Inactivation of L5178Y cells in the Presence of PABA.
<table>
<thead>
<tr>
<th>UV Radiation Fluence (KJm⁻²)</th>
<th>SURVIVING FRACTION</th>
<th>PABA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01%</td>
<td>0.02%</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>8.04 x 10⁻¹</td>
<td>8.55 x 10⁻¹</td>
</tr>
<tr>
<td>1.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>5.28 x 10⁻¹</td>
<td>5.59 x 10⁻¹</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>2.11 x 10⁻¹</td>
</tr>
<tr>
<td>4.0</td>
<td>2.79 x 10⁻¹</td>
<td>1.09 x 10⁻¹</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
<td>3.52 x 10⁻²</td>
</tr>
<tr>
<td>6.0</td>
<td>1.01 x 10⁻¹</td>
<td>1.32 x 10⁻²</td>
</tr>
</tbody>
</table>

Table A6b  Survival Data for 313 nm Radiation Inactivation of L5178Y cells in the Presence of PABA with 5% DMSO.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>UV Radiation Fluence (Jm$^{-2}$)</th>
<th>Surviving Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJ2</td>
<td>4.50</td>
<td>$3.08 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>$1.15 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>$9.62 \times 10^{-3}$</td>
</tr>
<tr>
<td>PJ3</td>
<td>4.50</td>
<td>$5.03 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>$3.96 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>$1.65 \times 10^{-1}$</td>
</tr>
<tr>
<td>PJ4</td>
<td>2.25</td>
<td>$8.20 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>4.50</td>
<td>$4.87 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>$2.52 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>$6.13 \times 10^{-2}$</td>
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<tr>
<td></td>
<td>18.00</td>
<td>$1.42 \times 10^{-2}$</td>
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<td></td>
<td>22.50</td>
<td>$6.29 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
## Table A7b
Survival Data for 254 nm Radiation Inactivation of the UV Radiation Sensitive Isolates at 2 Months.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>UV Radiation Fluence (Jm⁻²)</th>
<th>Surviving Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJ1</td>
<td>2.25</td>
<td>6.57 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>4.50</td>
<td>4.86 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>6.75</td>
<td>1.71 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>1.71 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>11.25</td>
<td>1.00 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>4.28 x 10⁻²</td>
</tr>
<tr>
<td></td>
<td>15.75</td>
<td>2.86 x 10⁻³</td>
</tr>
<tr>
<td>PJ2</td>
<td>2.25</td>
<td>7.63 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>4.50</td>
<td>6.47 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>6.75</td>
<td>3.36 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>1.03 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>11.25</td>
<td>8.99 x 10⁻²</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>2.56 x 10⁻²</td>
</tr>
<tr>
<td></td>
<td>15.75</td>
<td>9.70 x 10⁻³</td>
</tr>
<tr>
<td>PJ3</td>
<td>4.50</td>
<td>7.00 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>6.75</td>
<td>4.86 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>9.00</td>
<td>3.80 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>11.25</td>
<td>9.26 x 10⁻¹</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
<td>7.01 x 10⁻²</td>
</tr>
<tr>
<td></td>
<td>15.75</td>
<td>4.49 x 10⁻²</td>
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</tbody>
</table>
### Table A8a  Survival Data for L5178Y cells plated in Medium containing Ouabain.

<table>
<thead>
<tr>
<th>Ouabain Concentration (M)</th>
<th>Surviving Fraction</th>
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</thead>
<tbody>
<tr>
<td>$10^{-6}$</td>
<td>$9.90 \times 10^{-1}$</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>$9.18 \times 10^{-1}$</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$</td>
<td>$5.56 \times 10^{-1}$</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>$1.70 \times 10^{-3}$</td>
</tr>
<tr>
<td>$2.5 \times 10^{-4}$</td>
<td>$1.70 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

### Table A8b  Survival Data for L5178Y cells plated in Medium containing 6-Thioguanine.

<table>
<thead>
<tr>
<th>6-Thioguanine Concentration (M)</th>
<th>Surviving Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5 \times 10^{-7}$</td>
<td>$2.33 \times 10^{-1}$</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>$1.05 \times 10^{-2}$</td>
</tr>
<tr>
<td>$2.5 \times 10^{-6}$</td>
<td>$5.26 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5 \times 10^{-6}$</td>
<td>$4.20 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>$1.74 \times 10^{-4}$</td>
</tr>
</tbody>
</table>


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