RESPIRATION INDUCED OXYGEN GRADIENTS
IN CULTURED MAMMALIAN CELLS

by

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ABSTRACT

Oxygen is known to sensitize X-irradiated cells to lethal radiation damage. At low ambient oxygen tensions, however, the molecular mechanisms of the sensitization process and the metabolic requirements of the cell may be forced to compete for the cellular oxygen supply. The effect of cell respiration on the availability of intracellular oxygen during irradiation was consequently investigated by comparing the radiosensitivities of respiring and non-respiring cells. Cultured mammalian cells were irradiated in single cell suspensions and thin film monolayers at respiration inhibiting (4°C) and at normal cell culturing (37°C) temperatures. Due to oxygen equilibration and radiolytic depletion problems, the results of the suspension culture experiments were inconclusive. By subsequently analyzing the diffusive mass transfer of oxygen in the suspension medium, the stirrer flask was determined to be an inappropriate culture vessel in which to irradiate cells at constant low oxygen concentrations. A thin film cell culture system in which the oxygen concentrations to which the cells were exposed during irradiation could be more accurately controlled was then developed. A comparison of the oxygen enhanced radiosensitivities of the respiring and non-respiring cells in thin film monolayers suggested that the metabolic depletion of oxygen at low oxygen tensions has a significant effect on the local and intracellular oxygen distribution. These effects are representative of those that would be produced if respiration induced oxygen gradients existed inside and immediately around respiring cells. The magnitude of the differential radiosensitivities was found to be dependent on cell shape and to have

values that agreed very well with theoretical predictions based on the existence of such gradients.

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1. INTRODUCTION:

1.1 An Overview

A cancer cell is considered to be effectively dead if it is unable to produce viable offspring. In the treatment of cancer, the induction of reproductive cell death is typically the result of molecular interactions involving the cell's genetic material, the DNA, and therapeutic agents such as radiation, chemotoxins, or a combination thereof. Although it is difficult to optimize or even accurately evaluate the effectiveness of anti-tumor treatments if the molecular mechanisms by which they function are not understood, it is often even more difficult to extract information about such mechanisms from the complex biological systems in which they operate. Because the administration of a therapeutic agent to even the simplest of these systems will rarely produce an isolated or easily interpreted experimental response, the identification of subcellular cause-effect relationships and the accuracy with which they can be quantified is almost always biologically limited. It is, therefore, not surprising that such limitations are also encountered in attempts to determine the therapeutic role of molecular oxygen.

Although not a therapeutic agent per se, oxygen is instrumental in the function of various cancer treatments. Oxygen is, of course, also an integral and essential component of cellular energy metabolism. The importance of this metabolic process in normal cell function restricts the manner in which cellular oxygen can be experimentally manipulated. The nature of such investigations is further restricted by the technical

difficulties associated with making non-disruptive measurements at the molecular level.

An equally fundamental consequence of oxidative metabolism is the interdependent relationship that is implied to exist between the respiratory and therapeutic demands on the cellular oxygen supply. Oxygen consumed by respiration is not available to act as a therapeutic co-factor and, given the already limited oxygen supply to the poorly vascularized regions of tumor tissue, the effectiveness of oxygen-enhanced cancer treatments may be additionally affected by intracellular and localized metabolic depletion.

The objective pursued in this thesis was to determine the consequences of this concurrent demand on the cellular oxygen supply. The means by which the relationship between the respiratory and therapeutic utilization of cellular oxygen could be examined, however, were limited by biological and technical restrictions similar to those that would be encountered in attempting to ascertain oxygen's role as cancer therapy co-factor. An innovative approach was required to accommodate these restrictions in two ways. Firstly, the experimental rationale was formulated such that it incorporated and used the cellular metabolism of oxygen to its own advantage. Secondly, an experimental system was developed in which the cell environment could be carefully and accurately controlled. On this basis, the effect of cell respiration on the availability and distribution of intracellular oxygen could be investigated from an informative and previously unexamined perspective.

1.2 The Role of Oxygen in Cancer Therapy

1.2.1 Oxygen as a radiosensitizer

Oxygen is known to play a variety of roles in different cancer treatment modalities. It is, however, predominantly known for its ability to sensitize cells to the effects of X-rays and other forms of ionizing radiation. A brief historical outline highlighting some early research into the radiobiological role of oxygen is given in Table I. Although the influence of oxygen on cellular and tissue radiosensitivity predates most current knowledge of radiobiology, initial theories regarding the cause of this effect were largely speculative. Oxygen's role as radiosensitizer was not definitively established until 1941, when Anderson and Turkowitz demonstrated that the absence of oxygen and not the biochemical state of anaerobiosis was responsible for a subsequently observed decrease in the radiosensitivity of yeast cells (Alper, 1979).

In the history of radiobiological research, two developments in the mid 1950's dramatically increased awareness regarding the nature of radiosensitization by oxygen and its importance to radiotherapy. First, Thomlinson and Gray (1955) discovered that human lung cancer cells were most resistant to radiotherapy if they lay just beyond the calculated range of oxygenation by tumor blood flow. Shortly thereafter, Alper and Howard-Flanders (1956) developed an empirically based, mathematical model that established a quantitative relationship between oxygen concentration and the radiosensitivity of cultured bacteria. (Fig. 1) These successive discoveries emphasized the link between the research

Table I

A Summary of Early Studies of Oxygen in Radiobiology

- 1912.....SWARTZ notes skin reaction to radium is reduced by pressing applicator tightly onto skin.
- 1921.....HOLTHAUSEN shows *Ascaris* eggs are resistant to radiation in absence of oxygen.
- 1923.....PETRY finds correlation between presence of oxygen and radiosensitivity of vegetable seeds.
- 1934.....CRABTREE and CRAMER study radiation survival of human cancer cells in the presence and absence of oxygen.
- 1941.....ANDERSON and TURKOWITZ use yeast cells to show that decrease in radiosensitivity is due to lack of oxygen not the state of anaerobiosis.
- 1952.....READ measures growth inhibition of broad bean root by irradiation as a function of oxygen concentration.
- 1955.....THOMLINSON and GRAY discover relationship between tumor blood flow and radiosensitivity in human lung cancers.
- 1956.....ALPER and HOWARD-FLANDERS develop mathematical model of oxygen mediated radiosensitization as a function of concentration.

(Source: Alper, 1979; Hall, 1988)

Figure 1

Alper and Howard-Flanders' model of radiosensitization by oxygen. The relative radiosensitivity of cells is plotted against a logarithmically scaled oxygen concentration and is defined by the parameters m and K .

laboratory and the cancer therapy clinic and precipitated an unprecedented interest in oxygen-related radiobiological research.

1.2.2 The oxygen effect mechanism

Despite several decades of research, much of what is presently known about the mechanism by which oxygen-mediated radiosensitization occurs remains theoretical (Quintiliani, 1979; Hall, 1988). The most widely accepted theory of the sensitization mechanism postulates the following process (Fig. 2): A direct radiation-cell interaction or an encounter with a free radical, such as those produced by the radiolysis of water, causes a lesion in the cell target, the DNA. This lesion may also be in the form of a radical which will then react readily with any nearby oxygen molecule to form a peroxy radical. The peroxy radical, in turn, combines with hydrogen to form a stable hydroperoxide. The final step renders the lesion virtually irreparable and, in permanently damaging the DNA, may have an adverse affect on the reproductive ability of the cell.

This mechanism is consistent with the fact that oxygen's sensitizing ability decreases as the linear energy transfer (LET) of the radiation increases. If the amount of oxygen available to fix radiolytically induced lesions increases, cellular radiosensitivity is observed to increase in accordance with the Alper and Howard-Flanders model. The oxygen enhancement ratio, or OER, is the factor by which the radiation dose that produces a particular biological endpoint under aerobic conditions must be multiplied to achieve the same endpoint in anoxia. The value of the OER for cell killing by X-irradiation varies

Figure 2

The fixation of DNA radicals by oxygen. Lesions in DNA caused directly by secondary electrons or by radiolytically induced free radicals may combine irreversibly with molecular oxygen.

between 2 and 4 for all classes of cells, but is reduced to 1 for high LET radiations such as α -particles (Barendsen et al., 1966; Alper, 1979). The severity of high LET radiation damage and the high number of lesions this radiation induces are alone sufficient to kill the cell and make lesion fixation by oxygen redundant.

The lesion fixation hypothesis is also compatible with the results of time-resolved rapid-mixing and gas explosion experiments (Shenoy et al., 1975; Watts et al., 1978). These have confirmed that since the radiolytically induced radicals in the DNA and in solution have half-lives of the order of milliseconds and will recombine with other molecules in the absence of oxygen, the level of oxygen enhanced radiosensitization is solely dependent upon the amount of oxygen present during the time of irradiation.

Suggested alternative or additional mechanisms, such as the electron abstraction hypothesis (Adams, 1972), have also been put forward but their significance to the oxygen effect are less clear. Multiple oxygen sensitization mechanisms and/or multiple sites of radiation damage are occasionally invoked to explain unusual experimental results (Millar et al., 1979), but these have been difficult to repeat (Ling et al., 1981) and their proposed mechanisms remain unsubstantiated. It is generally accepted, therefore, that oxygen mediated radiosensitivity is quantitative rather than qualitative, and that cell death is caused by the same mechanism(s) at all oxygen tensions. Whether or not radiosensitization by oxygen is solely due to lesion fixation as described above, however, remains to be confirmed.

1.2.3 Oxygen and radioprotectors

The functional opposites of radiosensitizers are radioprotectors. As their name indicates, radioprotectors protect cells from radiation damage and can provide an additional method of increasing tissue selectivity in the radiotherapeutic treatment of tumors. The most prominently studied radioprotective agents are thiols (Biaglow et al. 1983a). Thiols occur naturally in the intracellular environment in the form of the non-protein sulphhydryls glutathione, cysteine, cysteamine and coenzyme A. They can also be administered in the form of drugs such as the phosphorothioate, WR-2721 (Yuhás and Storer, 1969; Purdie, 1979).

A proposed explanation for the radioprotective properties of thiols is that they and other reducing species compete with oxygen for the free radicals induced by irradiation and thereby reduce the indirect production of DNA lesions (Fig. 3). In addition, whereas the consequence of binding with oxygen is postulated to be lesion fixation, thiols bound to the radicals in damaged DNA are believed to promote repair by hydrogen donation and to restore the cell's reproductive capacity (Biaglow et al., 1983a; Hall, 1988).

Evidence obtained from experiments with WR-2721, however, supports a hypothesis which claims that these radioprotectors also act by oxygen depletion mechanisms (Durand, 1983a; Purdie et al., 1983; Durand, 1984). Thiols are known to oxidize spontaneously and to consume intracellular oxygen in the process (Gray, 1956). An oxygen differential could, consequently, be created between the intra and extracellular environments. Since target oxygen levels at the time of irradiation determine the degree of oxygen enhanced radiosensitivity, the

Figure 3

Radical scavenging by thiols. Thiols are believed to protect cells from radiation damage by combining with free radicals before they are able to induce DNA lesions.

"radioprotection" attributed to the presence of thiols may, in large part, actually be a decrease in oxygen-mediated radiosensitization of the radiation target due to increased intracellular hypoxia. This would also explain the observed correlation between increases in the intracellular thiol content and decreases in the oxygen enhanced radiosensitivity of cells at low oxygen tensions (Cullen and Walker, 1980; Cullen et al., 1980;).

1.2.4 Oxygen and chemotherapeutic drugs

Recently, it has been discovered that oxygen also plays a critical role in the function of some chemotherapeutic drugs (Kennedy et al., 1980; Taylor and Rauth, 1982; Koch et al., 1984; Rauth et al., 1984). Chemotoxins with a high specificity for hypoxic cells have been found to be strongly inhibited from binding to their tumour target cells by very low concentrations of oxygen. Because of their selective toxicity to hypoxic cells, both nitroheterocycles, such as misonidazole, and bioreductive alkylating agents, such as mitomycin C, were once considered promising candidates for mixed modality chemo-radiotherapy. The rationale for such therapies is that hypoxic tumor cells, normally radiobiologically protected by the lack of oxygen, could be specifically targeted by these chemotoxic compounds. However, since a large proportion of tumor cells are neither continuously well oxygenated, nor chronically hypoxic, and the effective toxicity of these drugs is dramatically reduced by the presence of less than a micromolar of oxygen, treatments of this type may be less promising than initially anticipated.

1.3 Modelling Cell Respiration

A direct consequence of oxygen's influence on cellular radio- and chemo-sensitivity has been to focus research interest on factors affecting the oxygen tension in and around cells, in particular on cell respiration and on the transport of oxygen to cells. Cells in culture and in tissue exist in essentially aqueous environments, and the transport of oxygen from the gas overlying the culture medium, or from the blood supply to the tissue, occurs primarily by diffusion. The rate at which oxygen is supplied to cells is, therefore, determined by the factors that govern the ability of oxygen molecules to diffuse through the medium or extracellular fluid.

The mechanics of the oxygen supply and cell respiration relationship have been thoroughly analyzed by Boag (1969; 1970). Using the single cell suspended in a liquid growth medium as an idealized model of the cell in tissue, Boag hypothesizes that cells act as oxygen "sinks", depleting the oxygen in their immediate environment as they respire. As a result a diffusion gradient is created around each sink, which in turn provides the motivating force for the transport of more oxygen to the cell. A steady state of oxygen supply and consumption is achieved when the diffusion gradient becomes large enough to sustain a normal level of cell respiration or when the gradient size is limited by the oxygen available from the source.

In his model, Boag assumes a uniform distribution of oxygen-consuming mitochondria throughout the cell. Cultured mammalian cells are commonly used in radiobiological research and have respiration rates of the order of 10^{-17} moles cell⁻¹ sec⁻¹ at 37°C (Boag, 1970, Biaglow,

1983). Using this value for the cell respiration rate and known values for cell size and the diffusion coefficient of oxygen in aqueous solutions, an evaluation of the effects of cell respiration on the oxygen distribution around the cell predicts the creation of a steady state diffusion gradient with a concentration differential of the order of $0.5 \mu\text{M}$. According to Boag's model, approximately two thirds of this gradient would lie outside of the cell (Fig. 4). Since the actual distribution of mitochondria in respiring cells is often localized around the cell nuclei, however, the intracellular portion of the gradient probably exceeds this first approximation.

Cellular respiration rates are known to remain independent of the ambient oxygen tension to relatively low (5 to $20 \mu\text{M}$) concentrations (Froese, 1962; Wilson et al., 1979; Marshall et al., 1986). Recent advances in oxygen sensor technology have allowed accurate measurements of cellular oxygen consumption below these concentrations and have revealed a commensurate decrease in the respiration rate as surrounding oxygen tensions approached zero. Expanding on the Boag model, Marshall et al. (1986) propose that, at very low oxygen tensions, the diffusion gradient becomes too small to provide oxygen to all areas of the cell (Fig. 5) and forces the cell's respiration rate to decrease. If the oxygen tension in the cell environment decreases even further, the essentially anoxic cell is hypothesized to act similarly to a polarized electrode, instantly consuming any oxygen molecule that it encounters.

Figure 4

Boag's model of respiration induced oxygen gradients inside and immediately surrounding single cells in suspension. Approximately one third of the gradient is predicted to lie within the cell ($r < a$).

a = cell radius

K = O_2 consumption rate per unit cell volume

D = diffusion constant for O_2 in water

Figure 5

Gradient limited cell respiration at low oxygen tensions. Reduced cell respiration rates are attributed to diffusion gradients that are too small to supply oxygen to all areas within the respiring cell.

1.4 Intracellular Oxygen Gradients

The oxygen gradient mechanism is aesthetically appealing because it is able to quantify the complex biophysical process of cellular respiration with conceptual and mathematical simplicity. From the perspective of cancer research, however, localized and intracellular oxygen gradients not only form the basis of an elegant model, but may also provide explanations for experimental observations involving oxygen and the radio- and chemo-sensitivity of cells.

As noted previously, the radioprotective properties of thiols may be largely attributed to the existence of such gradients. The consumption of intracellular oxygen by thiols at low oxygen tensions may result in a more hypoxic cell nucleus and "protect" the irradiated cell from the irreparable damage that might otherwise be caused by oxygen's lesion fixation properties. The existence of intracellular oxygen gradients may also be critical in evaluating the performance of, and ascertaining the mechanisms by which oxygen-sensitive chemotherapeutic drugs function. Such gradients would call into question the accuracy with which oxygen tensions at the cell target are being predicted and, consequently, the degree to which oxygen actually inhibits drug binding. Intracellular oxygen gradients may even disguise the nature and the mechanism of the oxygen effect itself. Variations in cellular radiosensitivity at low oxygen tensions, caused by factors affecting these gradients, may be mistakenly attributed to a cellular repair process or multiple radiation targets and oxygen sensitization mechanisms.

It is notable that intracellular oxygen diffusion gradients are not purely theoretical; evidence supporting their existence has been documented. A comparison of the oxygen-dependent mitochondrial activity of intact cells with that of isolated mitochondria suggests that oxygen gradients exist in both isolated rat cardiac cells and rat hepatocytes (Jones and Mason, 1978; Wittenberg and Wittenberg, 1981; Gayeski et al., 1986). Fluorescent probes have been used to demonstrate the existence of similar gradients in mouse liver cells (Benson et al., 1980; Podgorski et al., 1981). Indirectly, the respiration measurements made by Marshall et al. (1986) also imply the existence of intracellular gradients.

Cancer therapies frequently involve one or more oxygen-dependent mechanisms. If respiration induced intracellular oxygen gradients, such as those predicted by Boag and supported by the preliminary empirical evidence exist, they could play a significant role in the function of these mechanisms. An experimental analysis which would either disprove or confirm the existence and nature of these gradients would also provide a clearer understanding of the relationship between the therapeutic and respiratory utilization of cellular oxygen.

1.5 Experimental Rationale and Thesis Objectives

The radiosensitizing properties of oxygen are well established even if they are not fully understood. The oxygen enhancement ratio for the X-ray induced killing of cultured mammalian cells requires approximately three times the dose received under aerobic conditions to be delivered under hypoxic conditions if the same fraction of cells are

to be killed. Furthermore, oxygen mediated radiosensitization, as modelled by the Alper and Howard-Flanders equation, is very sensitive to changes in oxygen concentration at low oxygen tensions. This raised the possibility of using radiosensitivity as a gauge of the oxygenation status of the cell target. The existence of an oxygen-depleted zone around the cell nucleus could be confirmed by exposing both respiring and non-respiring cells to the same low extracellular oxygen tension during irradiation. According to Boag's theory (1970), the respiring cells should then deplete their intracellular oxygen, create an oxygen gradient and, consequently, display less radiosensitivity than non-respiring cells, for which the intracellular and distant extracellular oxygen tensions remain the same. The magnitude of these respiration induced gradients could then be approximated from the observed radiosensitivity differential and compared with theoretical predictions.

Cell shape was likely to be an important factor in the expression of these gradients, the average distance that oxygen molecules must diffuse through the cytoplasm to reach uniformly distributed mitochondria being larger for spherical cells than for cells growing in a flat monolayer. If the oxygen concentrations at the surface of both flat and spherical cells are the same, the rounder cells should have larger intracellular oxygen gradients and, hence, allow correspondingly less oxygen to penetrate to the cell center.

The challenge and the specific goal addressed in this thesis, therefore, was to develop an experimental protocol that was capable of detecting these respiration induced intracellular gradients. It was hoped that during the course of the experimental investigations, the following questions would be answered:

1. Is radiosensitivity a sufficiently specific and resolvable indicator of cell oxygenation?
2. Can respiration induced intracellular oxygen gradients of the magnitude predicted by theory be shown to exist?
3. What is the role of cell shape in determining the nature and magnitude of such gradients?

2. CELL CULTURE

The controlled manipulation of cell respiration and cell oxygenation required in the proposed radiation survival experiments could best be carried out in a simple cultured cell system. Although *in vitro* research is subject to the criticism that it includes the implicit, and at times, questionable assumption that information obtained from cultured cells reasonably reflects the behavior or properties of cells *in vivo*, for the purposes of this thesis, the use of a cultured cell system was considered justified by the intrinsic nature of the cell properties being investigated and the belief that the importance of these properties to normal cell function was likely to produce the same response regardless of the growth environment.

All experimental results in this thesis were, subsequently, obtained with cultured V79-171 Chinese hamster lung fibroblasts. These cells are a sub-line of non-malignant fibroblasts that were first isolated and cultured by Ford and Yerganian (1958). A number of sub-

lines have since been cloned and are being used extensively in radiobiological research. A major reason for the popularity of these cells among radiobiologists is their ability to serve as *in vitro* tumor models (Sutherland et al., 1970; Durand, 1972; Sutherland and Durand, 1976). Fibroblasts have also been dubbed "the weeds of the tissue culturist's garden" (Freshney, 1987), however, and their primary appeal is probably due to this more basic property.

Cultured fibroblasts are relatively easily maintained and proliferate rapidly with minimal nutritional and environmental requirements. The V79 cells used in the following experiments were grown in Eagle's minimal essential medium, MEM, supplemented with 10% fetal calf serum (Gibco). A 100 mm x 20 mm plastic Petri dish (Falcon) containing 10 ml of growth medium provided sufficient nutrients and space for the development of a confluent monolayer of the order of 10^7 cells. A bicarbonate/carbon dioxide buffer maintained a pH of 7.2-7.4 in the medium when the cells were incubated in 5% CO₂. A 37°C incubation temperature was used to mimic the optimal *in vivo* conditions for mammalian cell growth. Unfortunately, these temperature and pH conditions also favor the growth of bacteria and fungi. To prevent contamination, therefore, the cells, medium and the culturing equipment with which they came into contact were always handled under strictly sterile conditions.

The cell line was propagated by means of routine bi-weekly sub-culturing. Since monolayer fibroblasts anchor themselves firmly to the bottom of the culture dish, a proteinase enzyme was required for the transfer procedure. After aspirating the medium from the dish, all traces of serum protein were removed by rinsing the exposed cells with 2

ml of a citrate buffer solution containing 0.1% trypsin (Gibco). Following an 8 minute incubation with a second 2 ml volume of enzyme solution, the trypsin was inactivated and the loosened cells suspended by adding 10 ml of medium containing 10% FCS. Any remnants of the intact monolayer were disaggregated to single cells by gently pipetting the suspension. Petri dishes containing fresh medium were inoculated with aliquots of approximately 10^4 cells and were then incubated under the appropriate temperature and pH conditions.

A typical V79 growth curve is seen in Fig. 6a. After being transferred to a Petri dish containing fresh medium, the cells pass through a short lag period before beginning to divide at an exponential rate. The average cell cycle time during exponential growth is approximately 11 hours (Fig. 6b). If sufficient nutrients and favorable growth conditions are supplied, the cells will continue to divide until they form a confluent monolayer that covers the base of the Petri dish. At this point, population growth is inhibited and the cells move out of cycle. If renewed growth conditions are not provided, the depletion of nutrients and accumulation of cell waste will result in the eventual death of the cells.

Figure 6

Growth of V79 Chinese hamster cells in 100 mm x 20 mm plastic Petri dishes. Panel (a) plots the growth of the cell population per Petri dish with time and panel (b) shows the calculated average cell cycle time during the exponential growth phase.

3. GENERAL EXPERIMENTAL METHODS

3.1 Clonogenic Survival and Data Analysis

The experiments in this thesis were based on the premise that the existence of intracellular oxygen gradients at low oxygen tensions will cause respiring cells to be measurably less radiosensitive than non-respiring cells. The foremost requirement in the development of an experimental protocol was, therefore, to choose an appropriate cellular radiosensitivity assay.

Because reproductive cell death is an easily determined experimental endpoint, the radiosensitivity of the cells was most suitably and efficiently measured using the clonogenic survival assay (Puck and Marcus, 1956). The assay derives its name from the ability of viable single cells in culture to divide and produce "colonies" or aggregates of genetic clones (Fig. 7). With this assay, the effect of a given treatment on the proliferative ability of cells is measured by the ratio of the number of cell colonies produced to the number of treated cells initially cultured. According to the Puck criterion, a treated cell is considered a survivor if it is capable of producing a colony containing 50 or more cells (Puck and Marcus, 1956). The measured ratio is, hence, termed the surviving fraction.

The data obtained from the survival assay is usually displayed in a semi-logarithmic plot of the surviving fraction of a treated cell population versus the treatment dose. Typical radiation survival data for an X-irradiated mammalian cell population are shown in Fig. 8a. In practice, the calculation of a surviving fraction also includes a

Figure 7

V79 cell colony formation. These colonies were formed from single cells after one week of incubation. The colonies formed by the irradiated cells (left) are notably smaller and more poorly defined than those of the unirradiated population (right).

Figure 8

Typical radiation survival data for V79 cells irradiated under anoxic conditions. Panel (a) shows the raw data obtained with the clonogenic survival assay and panel (b) shows the same data normalized and fitted with the Linear Quadratic survival model.

"plating efficiency" correction to account for the cells that fail to form colonies for reasons other than exposure to the specified treatment. An experimental control or zero dose data point will provide an approximate value for this correction factor, but in order to minimize the uncertainty associated with a single data point, it is usually preferable to fit a curve to the survival data and then to normalize the data with respect to the curve's zero intercept (Fig. 8b).

Fitting a curve to the survival data requires a mathematical expression that can relate cell survival to the treatment dose. Several models describing the effect of radiation on cell viability have been developed and, within the bounds of experimental and biological uncertainty, fit the radiation survival data equally well. The Linear Quadratic model used to fit the data in this thesis (Fig. 9) postulates that there are two components of cell killing, one that is proportional to the radiation dose and dominates in the initial or shoulder region of the survival curve, and one that is proportional to the square of the dose and becomes significant in the latter portion of the curve (Sinclair, 1966; Kellerer and Rossi, 1972; Chadwick and Leenhouts, 1973; 1981). Like other multiparameter survival models, the LQ model generally fits radiation survival data well, particularly in the first few decades of the survival curve, but also has the additional advantage of requiring the optimization of only two variable parameters. A more detailed discussion of the LQ model and other survival models can be found in the appendix (Sec. 8.2).

Figure 9

Relative contributions of the two components of cell killing described by the Linear Quadratic model of radiation survival.

3.2 Respiratory Inhibition

To obtain an accurate measurement of the effect of a treatment on cell viability using the clonogenic survival assay, a statistically significant fraction of treated cells must remain viable, and all factors affecting the colony forming ability of the cells must be quantifiable. The use of clonogenic survival as a measure of the radiosensitivity of respiration inhibited cells, therefore, requires the toxicity of the respiratory inhibition to be sufficiently small not to obscure or interfere with the radiation survival characteristics of the inhibited cells. Biochemical inhibitors, such as rotenone and cyanide, have been used to induce varying degrees of respiratory inhibition in cultured cells. These are toxic at the dose levels required to completely inhibit cell respiration (Durand and Biaglow, 1974; Durand and Biaglow, 1976), however, and may introduce additional complications to the radiosensitivity assay. Under certain conditions, respiratory inhibitors have even been found to protect cells against radiation damage (Biaglow et al., 1983b). An alternative to using these respiratory toxins was to cool the cells and slow their metabolism naturally. The exposure of cells to low temperatures is an effective and easily reversible method of inhibiting cell respiration and is relatively non-toxic if used for short periods (see Sec. 5.1.8). The temperature induced inhibition of respiration was, consequently, adopted as the basis upon which radiosensitivity comparisons were made.

3.3 Choice of Irradiation Vessels

Because the most appropriate manner in which to irradiate the cells was not initially obvious, two different methods were employed and evaluated. The first series of experiments was performed with cells in a stirred single cell suspension culture. This was followed by a more extensive series of experiments using a modified thin film culture technique. In the interests of clarity and the logical development of the respective methods, a description of the rationale, the experimental protocol, and a discussion and analysis of the results will be presented separately for each of these techniques.

4. THE SINGLE CELL SUSPENSION

4.1 Materials and Methods

In designing an experimental protocol, an attempt was made to optimize factors that would create the maximum likelihood for a positive result. Cell shape was hypothesized to be such a factor. According to Boag's (1969) respiring cell model, cell shape can determine the magnitude of the respiration induced intracellular oxygen gradient by increasing or decreasing the distance that oxygen molecules must penetrate to reach the mitochondria. From the perspective of maintaining a geometrical simplicity in modelling the diffusion process, and also to create the maximum potential for "radioprotection" by oxygen depletion, it was, therefore, desirable to use cells that were spherical in shape. The first series of radiation survival experiments were

consequently performed with cells in a stirred suspension similar to that used by Boag (1970). A mitigating factor in the choice of this experimental apparatus was the relatively easy access to the necessary suspension culture equipment, eliminating the need for expensive purchases or time-consuming custom construction.

A modified Bellco spinner flask was used as the suspension culture and irradiation vessel (Fig. 10). The flask was water-jacketed, which allowed the temperature of the cell suspension to be controlled by means of a recirculating water bath (Haake). The oxygen content of the gas overlying the stirred suspension was regulated by flowmeters and a mixing chamber (Matheson), which combined oxygen and nitrogen from compressed gas cylinders (Canadian Liquid Air) in the desired proportions and metered the mixture at a flow rate of 10 l hr^{-1} . To prevent excessive condensation or evaporation within the spinner flask, the mixed gas was humidified by bubbling it through a temperature controlled gas washing bottle before it passed into the flask. With the exception of two silicon stoppers and a teflon coated stir bar, the entire gassing pathway was constructed of either glass or stainless steel. This was designed to minimize the unregulated diffusion of oxygen into the system. As a final measure of the system's atmospheric integrity, the effluent gas from the flask was monitored by an oxygen analyzer (Applied Electrochemistry) and a record of the oxygen tension in the flask produced with the use a strip chart recorder.

To maintain the flask as an isolated system throughout the experiment, a sampling technique employing a stainless steel cannula and valve was devised. The cannula tip was held below the surface of the stirred medium by a silicon stopper secured in one of the ports at the

Figure 10

Experimental apparatus for the suspension culture irradiations.

top of the flask. This allowed samples to be drawn up into a disposable syringe without compromising the oxygen content of the gas overlying the suspension. During the sampling procedure, two samples were taken, the first of which was discarded to remove any cells that might have been inside the cannula during the irradiation.

The cell suspension was irradiated through the side of the flask by a Picker 270 kVp X-ray machine. To ensure that subsequent cell suspensions would be exposed to the same 4.0 Gy min^{-1} dose rate, a plexiglas jig was used to hold the spinner flask in place. (The dose rate was measured in a mock experimental irradiation using Victoreen 500 electrometer with 0.6 cc ion chamber probe.) A magnetic stirrer beneath the jig kept the cells in suspension at a non-vortexing 180 rpm. This stirring speed caused no measurable damage to the cells and allowed the gas exchange process to be mathematically modelled.

4.2 Experimental Protocol

The experimental protocol for a typical suspension culture irradiation can be summarized as follows (Fig. 11):

Seed plates were prepared approximately 2.5 days prior to the experiment. Twenty-four plastic Petri dishes containing 10 ml medium (MEM + 10% FCS) were inoculated with 10^4 V79 Chinese hamster lung fibroblast cells and incubated at 37°C in 5% CO_2 . Since variations in the radiosensitivity of the different cell populations can be caused by differences in their cell cycle distributions (Sinclair and Morton, 1963), attempts were made to minimize such effects by choosing the inoculate number so that the seed cell population would always be in

Figure 11

Protocol flowchart for the suspension culture experiments:

2.5 days prior to experiment:

prepare 24 seed plates: 2×10^4 V79 cells in
10 ml MEM + 10% FCS

day of experiment: (repeat three times)

trypsinize 6 plates $\rightarrow 10^6$ cells plate⁻¹ and
suspend in MEM + 10% FCS @ 10^5 cells ml⁻¹

↓

place 50 ml of suspension in modified Bellco
spinner flask

↓

equilibrate for 1 hour @ given oxygen
concentration and 37°C or 4°C

gassing rate: 10 l hr⁻¹

stir speed: 180 rpm

↓

take sample and place
in previously prepared
dilution tube containing
MEM + 10% FCS @ 20°C

→

irradiate flask
@ 4.0 Gray min⁻¹

←

↓

dilute samples if required
and plate in 3 Petri dishes
to produce 300 - 500 colonies

→

count remainder of
sample w/ Coulter
counter

↓

incubate for 6-7 days before
staining and counting colonies

an exponential growth phase on the day of the experiment.

The experimental procedure was started by routinely trypsinizing six of the dishes, each of which contained monolayers of approximately 10^6 cells, and suspending the cells in MEM + 10% FCS. The suspended cells were then pooled at a density of 10^5 cells ml^{-1} . A 50 ml aliquot of this suspension was pipetted into the modified Bellco spinner flask, which was then placed into the plexiglas jig attached to the X-ray machine and equilibrated at the desired oxygen concentration and at either 4°C or 37°C for one hour prior to irradiation. The gas flow rate was set at 10 l hr^{-1} and the stir speed of the magnetic stirrer was 180 rpm.

Before the irradiation was started, a control sample was taken and placed in a previously prepared dilution tube containing sufficient medium to make up a total volume of 10 ml. The cell suspension was irradiated in cumulative 2.0 - 6.0 Gy increments with samples being taken after each increment. Sample sizes were minimized so that the volume of the suspension culture, and, hence, the cross-sectional area being irradiated changed by less than 7% throughout the experiment. The sample aliquots varied from 0.25 - 5.0 ml and were held in their respective dilution tubes at room temperature until the irradiation procedure was completed.

If required according to predicted survival rates, each sample of cells was further diluted with medium before a 0.5 ml volume (sufficient to produce 300 - 500 colonies) was pipetted into each of three Petri dishes containing 10 ml of growth medium. In order to obtain an accurate estimate of the number of cells pipetted into the dishes, the cell densities of the diluted samples from which the cells were plated

were measured using a Coulter cell counter. The culture dishes were then incubated at 37°C in 5% CO₂ and the irradiation procedure was repeated for the next six seed plates at a different oxygen tension. When completed, this protocol produced radiation survival data at four oxygen tensions.

After a one week incubation period, the medium was removed from all of the dishes and the cell colonies were stained with a malachite green staining solution. After rinsing and drip drying the dishes, colonies containing 50 or more cells were counted and recorded.

4.3 Results

The radiation survival results for the suspension culture experiments are displayed in sets of four, corresponding to the four flasks used for each pooled cell population. For three of the flasks in each set, the gas above the cell suspension contained a low level of oxygen. The fourth flask was irradiated under aerobic conditions (95% air 5% CO₂) and was used to characterize the general radiation survival properties of the seed cell population. All calculations of experimental uncertainty were made in accordance with the method proposed by Boag (1975). Using Poisson sampling statistics, the variance in cell survival was based on the total number of colonies counted at a given dose rather than on the variation in the number of colonies on each dish.

4.3.1 Radioresponse of respiring cells

The raw cell survival data for irradiations performed at 37°C are plotted in Fig. 12. The data are displayed in this manner because their biphasic nature prevents the usual curve-fitting and normalization procedure. Biphasic characteristics are particularly noticable in the data from flasks in which the gas contained 1% oxygen or less. These plots typically have a steep slope over the first few dose points in the shoulder region before breaking and decreasing more gently at higher doses.

Multiphasic survival data is usually indicative of heterogeneously sensitive cell populations. The rigid configuration of cells in tumors or tumor models, for example, limits the ability of oxygen to diffuse to cells that are distant from the oxygen supply (Durand, 1983b). Spatially stratified cell subpopulations with varying degrees of oxygen enhanced radiosensitivity will develop under such conditions. If a representative crosssection of tumor or tumor model cells produces a biphasic radiation survival curve, the approximate proportions of hypoxic and oxic cells can be deduced from the zero dose intercepts of the two phases (Fig. 13).

A biphasic response in a stirred single cell suspension must be interpreted differently, however. Although degrees of spatially conferred oxygen-mediated radiosensitivity may also exist in stirred suspensions (Whillans and Rauth, 1980), the method by which samples at the various dose points were obtained makes it very unlikely that these results represent the existence of spatially stratified subpopulations of cells. A more probable explanation is that the amount of dissolved

Figure 12

Non-normalized suspension culture radiation survival data for respiring (37°C) cells. A biphasic radiation response prevented use of conventional curve fitting and normalization procedures. Each panel shows the results of a four flask experiment.

Figure 13

Hypothetical biphasic radiation response of a heterogeneously sensitive cell population in which 30% of the cells are hypoxic. Spatial variations in radiosensitivity producing this type of response are commonly seen in tumor models such as spheroids.

oxygen in the suspension medium and, hence, the radiosensitivity of the entire cell population changed over the course of the experiments (Koch et al.; 1973). In other words, the biphasic nature of the response is due to a temporal, rather than spatial variation in cell oxygenation. The shape of the survival data implies that the oxygen concentration of the suspension medium was not in equilibrium with that of the gas above it at the end of the allowed one hour equilibration period. If an equilibrium condition was achieved, it was only after radiolytically produced free radicals depleted the oxygen surplus during the initial stages of the irradiation procedure. This is supported by the steeper than expected dose response obtained over the first few dose points in all but the highest oxygen content flasks.

Since radiation survival models do not account for variations in the conditions of irradiation, the biphasic nature of the survival data pre-empted the use of the usual curve-fitting methods for the calculation of plating efficiency values. Therefore, because the suspension medium only attained the desired oxygen tension after a dose of several Gray, the first two or three data points in each of the data sets were ignored and the initial portion of the desired survival curve approximated by extrapolating back from the higher dose data. The intercept survival values were then used to normalize the curves fitted to the high dose data (Fig 14).

4.3.2 Radioresponse of the non-respiring cells

With the exception of the flask in which the gas mixture contained only 200 ppm oxygen, the cells irradiated at 4°C do not display the

Figure 14

Normalized and fitted suspension culture radiation survival data for respiring (37°C) cells. Data were fitted with the LQ model of radiation survival by ignoring the steeply decreasing initial survival points for each curve.

Figure 15

Normalized and fitted suspension culture radiation survival data for non-respiring (4°C) cells. Steeply falling survival curves imply a lack of diffusive equilibrium between the oxygen concentrations of gas and liquid phases of the suspension culture. At very low oxygen tensions in the gas, differences in the radioresponse become difficult to distinguish.

biphasic survival characteristics of those irradiated at 37°C (Fig. 15). The suspension cultures irradiated at 4°C are consistently and more highly radiosensitive than those irradiated at 37°C with the same oxygen concentrations in the gas. The dose responses are also considerably steeper than would be expected if a diffusion equilibrium had actually existed for the various oxygen tensions in the gas. This is not surprising when it is considered that if a diffusive equilibrium was not achieved at temperatures at which cells were able to respire and, therefore, aid in the removal of excess oxygen from the suspension medium, that it was also not attained when such respiration was inhibited! The radiolytic depletion of oxygen was by itself insufficient to cause the survival curves to break.

A factor that undoubtedly contributed to the greater radiosensitivity of the cells irradiated at 4°C is the increased solubility of oxygen in aqueous solutions (Boag, 1970). Oxygen is almost twice as soluble in water at 4°C as it is at 37°C (Fig. 16). The solubility of oxygen in the gas phase also increases over this temperature range, but by a considerably smaller degree. Low temperatures will, therefore, not only decrease the rate at which oxygen diffuses out of solution, but will also result in a suspension medium with a significantly higher equilibrium oxygen concentration.

The information salvaged from the results of these radiation survival experiments was, hence, not directly useful to the stated objectives of this thesis. Although the survival curves did show a qualitative increase in radiosensitivity with increasing oxygen tension, the data was frequently quite scattered and the resolution of differences in radioresponses at low oxygen tensions was poor. The need

Figure 16

Variation of the oxygen concentration in air and in air-equilibrated water with temperature at standard pressure (1 atm). The fractional change in the oxygen concentration over the range of 4°C to 37°C is notably smaller in air than in water.

[O₂] in air-equilibrated water:
 $\{439 - 12.2T + 0.239T^2 - 0.0019T^3\}$
 (Boag, 1969)

[O₂] in air at 1 atm:
 $\{P + (a^2 / (1/[O_2] + b)^2)\} 0.2095/RT$

$R = 0.08206 \text{ atm } ^\circ\text{K}^{-1} \text{ M}^{-1}$
 $a = 1.36 \text{ atm } \text{M}^{-2} \quad b = 0.03183 \text{ M}^{-1}$

to process the respiring cell data in the manner described above, also added uncertainty to the results and reduced the confidence with which differences between low oxygen tension survival curves could be claimed. Unfortunately, although differences in radiosensitivity due to the hypothesized intracellular oxygen gradients would be most evident at low oxygen tensions, the poor resolution of the data did not allow any relevant conclusions to be drawn.

4.3.3 Sources of error

The suspension culture experiments were subject to several sources of error that contributed to preventing the detection of respiration induced, localized differences in cell radiosensitivity. The alternating irradiation and sampling procedure required the repeated opening and closing of the shutter on an unpredictable 30 year old X-ray machine. It is suspected that this may have resulted in the accumulation of errors in dose measurement. Dilution and colony counting errors incurred through the use of the clonogenic assay, were magnified by the inexperience of the researcher and contributed to the random scatter of the data points. Most significantly, however, the combined suspension culture results indicate that any variation in cellular radiosensitivity that might have been present due to the existence of respiration induced intracellular oxygen gradients was obscured by the much greater and uncontrolled variability in the oxygen content of the suspension medium. This was not believed to be due to oxygen contamination of the gassing system, but to inherent equilibration problems of the suspension culture system. A more

detailed analysis of the mass transfer characteristics of the stirrer flask and suspension culture was required to determine whether this major source of experimental uncertainty could be eliminated.

4.4 Analysis of Suspension Culture Method

The all-glass water-jacketed stirrer flask provides a convenient means of manipulating biological systems in an isolated environment. In view of its general desirability as a vessel in which to perform environmentally controlled *in vitro* experiments, the stirrer flask was re-examined to determine whether or not the oxygen equilibration problems it posed could be overcome.

4.4.1 Diffusion rate constant

A comprehensive review of the diffusion of oxygen in stirred cell suspensions has been conducted by Whillans and Rauth (1980). They envisioned conditions inside a stirrer flask to exist as depicted in Fig. 17. A gas of a given oxygen concentration lies above suspension medium having a certain depth and surface area, and time-dependent oxygen concentration. The medium is well stirred, minimizing any gradients in the bulk of the liquid, but leaving a thin non-turbulent layer of medium at the gas-liquid interface. The concentration gradient between the gas and liquid phases in the flask is postulated to exist across, and completely within this layer of liquid. The gradient is described by Fick's Law (the time rate of change in the number of molecules diffusing in a given direction is directly proportional to the

Figure 17

Analysis of mass transfer in a suspension culture spinner flask. The mathematical model shown below was derived by assuming that a gas of oxygen concentration C_g lies above medium of depth d , surface area A and time-dependent oxygen concentration $C(t)$. The depth of the non-turbulent layer of medium at the gas-liquid interface is x and the rate of oxygen consumption in the medium is R .

from Fick's Law:

$$\begin{aligned} dC(t)/dt &= AD/xV (C_g - C(t)) \\ &= k (C_g - C(t)) \end{aligned}$$

where $k = AD/xV = D/xd$

account for oxygen consumed:

$$dC(t)/dt = k (C_g - C(t)) - R$$

ODE with i.c. $C(0) = C_0$

solution:

$$C(t) = C_s + (C_0 - C_s) e^{-kt}$$

where $C_s = C_g - R/k$
is the steady state

negative concentration gradient in that direction; Setlow and Pollard, 1962) and is expressed mathematically in the form of an easily solved ordinary differential equation. The solution to the ODE reveals that the rate constant governing the exponential approach to diffusive equilibrium between the oxygen concentrations of the gas and liquid phases is determined by three parameters of the system, specifically the coefficient of diffusion for oxygen in the medium, the medium depth and the thickness of the non-turbulent layer at the gas-liquid interface.

By adapting this analysis to the conditions existing during the suspension culture irradiations in the previous section, an approximate value for the oxygen concentration in the cell suspension at the end of the one hour equilibration period can be calculated. The layer of liquid at the gas-liquid interface has a thickness of approximately 50 μm for aqueous media being stirred at 200 rpm at 37°C (Davies and Rideal, 1963). The rate constant for the Bellco stirring flask containing 50 ml of suspension medium has a calculated value of 0.09 min^{-1} under these conditions. When this rate constant value is used to calculate the oxygen concentrations that would exist in the same suspension medium after a one hour equilibration period, the results confirm that the diffusion process is still far from equilibrium (Table II).

Because the theoretical calculations involve a significant degree of approximation, however, the value of the rate constant was also experimentally determined. The experimental apparatus, assembled as shown in Fig. 18, was identical to that used in the irradiation experiments with the exception that an oxygen probe was inserted into the stirrer flask in place of the sampling cannula. The medium in the

Table II

Calculated oxygen concentrations in suspension culture medium after a one hour equilibration period.

C_s	C_o	t for $C_s+5\%$	C (60)
0.5%	20.95%	75 min	0.60%
0.05%	20.95%	100 min	0.15%

Figure 18

Apparatus used to measure the diffusion rate constant, k , of the spinner flask.

flask (MEM + 10% FCS) also did not contain any cells. The probe (Controls Katharobic) was used to measure the change in the oxygen concentration of the suspension medium with time while it was being stirred at 200 rpm. Nitrogen gas was metered to flow through the flask at 10 l hr^{-1} and oxygen content of the effluent was measured by an oxygen gas analyser (Applied Electrochemistry). The oxygen content of both the effluent gas and the medium was recorded from the digital displays on the respective measuring devices and with a strip chart recorder.

By rearranging the Whillans and Rauth equation, the data collected in this manner can be used to produce a straight line semi-logarithmic plot (Fig. 19). The negative slope of this plot corresponds to the value of the rate constant. As can be seen from the plot, the experimental value for the rate constant at 37°C is in good agreement with the theoretically calculated value (0.09 min^{-1} , p.47). The experimental data also confirms that the value of the rate constant decreases with temperature. This might have been expected in view of the slow deoxygenation of the suspension culture that was implied by the radiation survival data obtained at 4°C .

4.4.2 Oxygen consumption and radiolytic depletion

The time requirements to reach a static equilibrium between the oxygen concentrations of the gas and liquid phases in a stirrer flask could conceivably be fulfilled by extending the pre-irradiation equilibration period. To determine the value of the oxygen concentration to which the cells in a suspension are actually exposed,

Figure 19

Experimental confirmation of diffusion rate constant value. By rearranging the Whillans and Rauth expression, the diffusion rate constant can be determined from the slope of a semi-log plot of the measured oxygen concentration ratio versus time.

$$C_S = C_g \text{ when } R=0$$

$$(C(t) - C_S) / (C_0 - C_S) = e^{-kt}$$

the consequences of oxygen consumption must also be considered, however. According to the Whillans and Rauth model, the respiratory depletion of oxygen in the suspension medium will establish a dynamic equilibrium in which the oxygen concentration of the suspension medium is lower than that of the gas. The perpetual gradient between the suspension medium and the gas maintains the transport of oxygen to the cells across the gas-liquid interface. If all of the hypothesized oxygen diffusion gradients for fully respiring cells in a stirred suspension are added (Fig. 20), a stirrer flask containing a 50 ml single cell suspension with a cell density of 10^5 cells ml^{-1} at 37°C would require a minimum oxygen concentration of $2.3 \mu\text{M}$ in the gas above the suspension. This factor must be subtracted from the oxygen concentration of the gas, if the oxygen concentration to which the intracellular radiation targets are exposed to is actually desired.

A potentially more difficult problem to circumvent, however, is that of the radiolytic depletion of oxygen. An incremental exposure and sampling procedure such as that used in the suspension culture experiments, results in a cyclic depletion and reoxygenation of the suspension medium. The rate at which oxygen is consumed is linearly dependent upon the dose rate and for culture medium at 37°C the proportionality constant has been determined to be approximately $0.04 \mu\text{M Gy}^{-1}$ (Whillans and Rauth, 1980; Palcic and Skarsgard, 1984). Recalling that the dose rate used in the experiments was 4 Gy min^{-1} and again making use of the mathematical model by Whillans and Rauth, the change in the oxygen concentration of the suspension medium for an incremental one minute irradiation can be charted as shown in Fig. 21. Since the sampling time is also only one or two minutes in duration, at this dose

Figure 20

Respiration induced oxygen gradients between the gas and the intracellular environment in a single cell suspension culture. The magnitude of the gradient at the gas-liquid interface is cell density dependent.

around cell:

$$C_s - C_i = Ka^2/2D$$

@ gas-liquid interface:

$$C_g - C_s = R/k$$

for full respiration,

$$C_g > Ka^2/2D + R/k$$

Figure 21

Calculated cycle of radiolytic depletion of oxygen in an irradiated suspension culture. The plot below shows a 1 minute irradiation followed by a 9 minute recovery period assuming a dose rate of 4 Gray min^{-1} and a depletion constant of $0.04 \mu\text{M Gray}^{-1}$.

rate it is obvious that even if an equilibrium at some low oxygen tension has been achieved, it would be impossible to maintain once the irradiation process is started.

The theoretical predictions were again tested with the polarographic oxygen probe and the apparatus used to determine the stirrer flask rate constant. The strip chart recordings in Fig. 22 show the approach to equilibrium for medium in contact with a gas mixture containing 0.5% oxygen. The plots confirm the predictions of the model and, after the initial one hour equilibration period, show the rapid decrease in the dissolved oxygen content when the X-ray machine is turned on and the more gradual recovery after the exposure is completed. (The vertical jumps in the plots are due to an increased probe background current when the X-ray machine is turned on.) It is interesting to note that, judging from the shape of the plots, the oxygen concentration in the suspension medium is considerably further from equilibrium prior to the start of the intermittent radiation exposures at the lower temperature.

In view of the results of this analysis, it was concluded that a suspension culture was inappropriate for irradiating cells at constant low oxygen concentrations. The suspension culture experiments demonstrated that a different cell culture technique was required if the oxygen concentration to which cells were exposed during irradiation was to be accurately controlled. The methods of analysis used to investigate the mass transfer characteristics of the stirrer flask were subsequently utilized to develop such a technique.

Figure 22

Strip chart recordings of radiolytic depletion of oxygen in a suspension culture. Traces for one minute irradiations followed by two minute recovery periods at dose rate of $4.0 \text{ Gray min}^{-1}$ were obtained at both 4°C and 37°C following a one hour equilibration period with gas containing 0.5% oxygen. The vertical jumps in the recording patterns are due to an increased background current in the irradiated oxygen probe.

5. THE THIN FILM MONOLAYER

5.1 Materials and Method

Possible solutions to the problems encountered in equilibrating and maintaining low oxygen concentrations in irradiated single cell suspension cultures were examined with the aid of the Whillans and Rauth equation (Fig. 23). One possibility was to reduce the rate of radiolytic oxygen depletion by reducing the irradiation dose rate. The amount by which the dose rate could be reduced, however, was limited by the time constraints within which the irradiation procedure had to be completed. This limitation did not allow a large enough reduction to solve the equilibration problems caused by cyclic radiolytic oxygen depletion.

By this process of elimination, it was decided that a solution would most likely be found by increasing the equilibration time constant of the irradiation vessel. This can be accomplished, to some degree, by increasing the stirring speed and using a less viscous medium to decrease the thickness of the layer across which the diffusion gradient is developed. A more effective method of increasing the rate constant, however, is to increase the surface area to volume ratio of the suspension medium (Whillans and Rauth, 1980). Bubbling gas of the desired oxygen concentration through the medium is an effective, although, impractical example of this, since suspended mammalian cells are easily damaged by the resulting turbulence. If the increased area to volume solution is extrapolated to the ideal case, however, the gas-liquid equilibration time can be minimized if the suspension medium is

Figure 23

Possible solutions to oxygen equilibration problems using the Whillans and Rauth equation.

from Whillans and Rauth (1980):

$$C(t) = C_s + (C_0 - C_s) e^{-kt}$$

$$C_s = C_g - R/k$$

$$k = AD/xV = D/xd$$

possible solutions:

- reduce R by reducing dose rate
- increase k by decreasing x
- increase k by decreasing d

removed entirely and the cells are irradiated while covered by only a thin layer of liquid. This is the basis of thin film cell culture.

Several versions of the thin film culture technique have already been used to study the radiosensitization of cells by oxygen at low oxygen tensions. To avoid oxygen depletion at ultrahigh dose rates, Michaels et al. (1978) and Ling et al. (1981) have successfully made use of a method pioneered by Epp et al. (1972) in which cells are irradiated on a coverslip made of a special oxygen impermeable plastic. The cells were plated onto the coverslip and then held in a medium filled culture dish. The radioresponse of the cells at low oxygen concentrations was determined by completely aspirating the medium from the dish, removing the coverslip and irradiating the exposed cells in a gas-filled chamber. Due to the extremely short (fractions of a second) equilibration time required for the diffusion of oxygen between the cells and the gas, the radiolytic depletion of oxygen within the thin film of medium covering the cells during the irradiation was determined to be insignificant using this technique, even at dose rates of 20 Gy min^{-1} (Michaels et al, 1978).

In a modified version of the thin film culture technique, cells are allowed to attach to the surface of glass Petri dishes (Koch and Painter, 1975; Koch, 1984). With this technique, the growth medium is aspirated from the dish and the cell monolayers are irradiated at various concentrations of oxygen after a small volume of medium (1 ml) is returned to the dish. This method has the advantage that it enables cells to be maintained in thin film culture for extended periods (hours to days) without incurring cell drying or toxicity problems. It is also simpler to implement in that it utilizes readily available labware.

Because medium is returned to the dish before irradiation, however, the radiolytic and metabolic depletion of oxygen must still be considered in determining the oxygen concentration at the cell surface with this method (Koch, 1984).

A hybrid of these techniques seemed ideally suited to the purposes of this thesis. A thin film culture system was consequently developed using a Petri dish cell culture system similar to that used by Koch, but in which the problems of radiolytic oxygen depletion are eliminated by completely aspirating the growth medium before irradiation.

5.1.1 Choice of irradiation vessel

A primary consideration in the use of thin film culture was the nature of the culture vessel surface. The cells in thin film culture must be firmly attached to a culture vessel surface so that they are not removed during the aspiration of the growth medium. Under certain conditions, this could compromise the required control over the various low oxygen concentrations to which the cells were exposed during the irradiation procedure. At sufficiently low oxygen tensions, cells attached to the surface of commonly used plastic disposable tissue culture dishes and flasks can thwart attempts at controlling the cellular oxygen supply by absorbing the oxygen molecules that diffuse through and out of the plastic (Chapman et al. 1970). The surface to which the cells were attached in the following thin film experiments, therefore, had to have a low oxygen diffusivity.

Glass culture dishes have the desired oxygen impermeability and were the most readily accessible substitutes for the ubiquitous plastic.

A thin film culture system was consequently developed based on the use of 50 mm x 10 mm glass Petri dishes similar to those used by Koch. Cells attached to these dishes were irradiated in the apparatus shown in Fig. 24. A dish containing cells and growth medium was sealed using a tightly fitted pure gum rubber stopper (Fisher). Stainless steel tubing served as both gas inlet and outlet and was connected, by means of two short pieces of thick walled rubber tubing, to the same gas metering and oxygen monitoring system used in the previous suspension culture experiments. Constant monitoring ensured that neither the tubing nor the stopper compromised the oxygen content of the gas above the plated cells. Prior to irradiation, a vacuum pump was used to carefully, and as completely as possible, aspirate the growth medium from the dish through a stainless steel cannula that passed through the rubber stopper. The metabolic activity of the cells was controlled by means of a specially adapted recirculating water bath that maintained the dish temperature at 40°C or 37°C. The cells were irradiated from below by a Philips 250 kVp X-ray machine fitted with a 0.5 mm Cu filter.

5.1.2 Dosimetry considerations

The decision to use glass culture dishes provided an oxygen controlled environment, but also introduced dosimetry complications. Due to the high atomic density of the glass, X-irradiation scatters low energy electrons that can travel beyond the surface of the dish (Dutreix and Bernard, 1966; Sinclair, 1969). These electrons significantly increase the dose to cells attached to the glass. Complications arise from the fact that the increased dose is difficult to measure using

Figure 24

Experimental apparatus for thin film culture irradiations.

conventional dosimetry techniques. A biological dosimetry measurement was, therefore, made by irradiating aerobic cells attached to both plastic and glass culture dishes. By comparing the two sets of radiation survival data, it was determined that the effective dose received by cells attached to the glass surface was 1.5 times that of the dose to cells attached to the plastic for the same X-ray exposure (Fig. 25). This value compares favorably with those found in the literature (Blakely et al., 1979; Elkind and Whitmore, 1967; Chapman et al., 1970). An electrometer (Victoreen 500) with a 0.6 cc ion chamber probe had been used to measure the photon dose at the dish surface (5 Gy min^{-1}) for the apparatus assembled as shown in Fig. 24. The radiation dose actually received by cells attached to the glass could then be calculated by multiplying this measured X-ray dose by the biologically determined factor.

5.1.3 Thin film toxicity

Another consideration in using thin film culture techniques concerned the toxicity of the gassing procedure and the likelihood of cells drying out during extended thin film exposure. In a typical irradiation procedure the rubber stopper was inserted into the Petri dish and the air above the growth medium was purged from the dish by increasing the flow of the humidified gas mixture. When the gas analyzer indicated that oxygen content of the effluent gas was sufficiently low, the medium was completely aspirated from the dish through the cannula. This was followed by an immediate reduction in the gas flow rate from 30 to 10 l hr^{-1} . This flow rate was maintained

Figure 25

Biological dosimetry used to measure the additional dose to cells attached to glass surfaces. Low energy electrons increased the dose to cells irradiated in the glass culture dishes by a factor of 1.5 over those irradiated in the plastic dishes.

Figure 26

Toxicity of thin film culture for exposure of cells to gas flowing at 10 l hr^{-1} . No measurable effect on the plating efficiency was noticed for exposure times of up to eight minutes.

during the remainder of the irradiation procedure. Measurements of the plating efficiencies for mock-irradiated cells exposed to increasing thin film exposure times are displayed in Fig. 26. These show no increase in toxicity for exposure periods of up to eight minutes. Since the maximum expected irradiation time in the planned radiation survival experiments was four minutes in length, cell drying or toxicity was not regarded as a factor in cell survival.

5.1.4 Spot inoculation

In using Petri dishes as thin film culture vessels, it was noticed that the small amount of growth medium that remained in the dish (< 0.1 ml) after the aspiration procedure tended to accumulate in the outer rim of the bottom of the dish. This accumulation could significantly affect the oxygenation of the cells attached in this area, the diffusion equilibration time constant for an unstirred layer of medium being related to the square of the medium depth (Boag, 1969). Since a similar effect occurs with larger volumes of medium (the meniscus created by the walls of dish leaving a thicker layer of medium above the outer perimeter of the dish surface) Koch (1984) avoided this problem by restricting the attachment of cells to the center of the dish. The Petri dishes were prepared using a "spot" inoculation procedure in which a small amount of cell suspension is carefully micropipetted into the center of a dish already containing the required growth medium. The dish was then allowed to sit undisturbed while the cells attached themselves to the glass surface in a central, two to three centimetre spot (Fig. 27). By Koch's measurement, less than 3% of the cells were

Figure 27

Spot inoculation of glass Petri dishes. Cells were only allowed to attach to a 1-2 cm² area in the center of the glass dish, thereby avoiding the variation in depth of the thin film layer that occurs at the outer rim of the dish. The stained spot inoculated and normally plated cells below demonstrate the difference in monolayer configuration.

attached outside of a clearly defined central area using this method.

5.1.5 Cell attachment time

In order to establish a time frame for an experimental protocol, an investigation of the time required for cells to attach to the glass surface was carried out. Glass Petri dishes containing growth medium were spot inoculated with a known number of cells and left to sit undisturbed at room temperature. At defined time intervals the medium from one of the dishes would be aspirated and the remaining cells trypsinized, suspended and counted. The results shown in Fig. 28 indicate that maximum cell recovery is achieved after approximately forty minutes. A minimum one hour attachment period was consequently adopted as the experimental standard.

5.1.6 Cell shape in thin film monolayer

The anticipated importance of cell shape in establishing radiobiologically significant intracellular oxygen gradients was a key factor in the decision to perform the initial suspension culture experiments. Thin film culture, however, presented an opportunity to determine directly whether or not cell shape plays a significant role in the formation of such gradients. This was investigated by performing two separate series of experiments using the thin film culture technique. In the first series, cells were allowed to attach to the glass Petri dishes and were incubated for 24 hours prior to irradiation. This allowed cells to spread out and form a flat monolayer in the dish

Figure 28

Flat and round cell monolayers in glass Petri dishes. The cells shown below were photographed (scale 65:1) after overnight incubation (upper) and after a 2 hour attachment period at room temperature (lower). Both were plated in 100 μ l aliquots at densities of 10^7 cells ml^{-1} .

Figure 29

Time required for cells to attach to a glass surface was measured by the ability to recover cells at various times after plating in Petri dishes. Maximum cell recovery was achieved approximately one hour after plating.

(Fig. 29a). The experiments then went on to determine the survival characteristics of cells exposed to gas mixtures containing various low levels of oxygen and irradiated while in this configuration.

The change in the area of a V79 cell as it spreads out after attaching to a surface is plotted in Fig. 30. The change in shape of attached cells with time was determined with the aid of an imaging microscope and the data shows that, at room temperature, a cell will maintain a minimal cross-section or rounded shape for several hours after plating. A second series of radiation survival experiments was subsequently performed, but differed from the first in that the cells were only allowed a one hour room temperature attachment period before being irradiated. These cells were firmly attached to the surface of the Petri dish, but also retained an essentially round shape throughout the remainder of the experiment (Fig. 29b). The oxygen mediated radiation survival of cells with this round shape was then also determined.

5.1.7 Equilibration time measurements

Attempts were made to measure the gas-liquid equilibration time for cells in both the round and flat configurations. For these measurements, the medium was removed from dishes and the cells exposed to a 95% N_2 / 5% CO_2 atmosphere for increasing time intervals prior to the onset of irradiation. The cells were irradiated with an X-ray dose that produced a known level of survival for hypoxic cells. Oxygen enhanced cell killing was expected if a gas-liquid equilibrium had not been achieved prior to the start of the irradiation. After an

Figure 30

Time dependent change in cell cross-sectional area after plating.

Average change in cell area with time for 40 V79 cells was measured with the aid of a computer controlled microscopic imaging technique and shows that cells remain relatively round for 2-3 hours after plating. These measurements were made at 37°C and represent the most rapid possible flattening of the cells.

equivalent exposure to flowing air, a control was irradiated at a dose that would produce a comparable level of survival. Judging from the results of previous similar thin film experiments (Michaels et al., 1978; Ling et al., 1981), it was expected that oxygen levels would be extremely rapidly equilibrated. Since the shortest possible pre-irradiation time interval was fifteen seconds, these expectations were confirmed by the results in Fig. 31 which show no enhanced radiosensitivity for either cell configuration.

5.1.8 Low temperature toxicity

The spot inoculated Petri dishes were pre-cooled on ice before the start of the irradiation procedure to ensure the respiratory inhibition of the cells. Because the Petri dishes were irradiated individually and sequentially, the total time required from the beginning of the first irradiation to the completion of the last could extend for as long as one hour. An experimental investigation was, therefore, carried out to determine the effect of extended exposure to low temperatures on cell survival. Glass Petri dishes containing cells in spot monolayers were placed on ice and then irradiated in fifteen minute intervals at 4°C following the irradiation protocol in Sec. 5.2.2. The cells were exposed to a low concentration of oxygen during the irradiation, which produced a known level of radiation survival. The irradiated cells and an unirradiated control were immediately trypsinized, plated and incubated for one week in the manner also described in the following protocol section. The resulting clonogenic survival data is plotted in Fig. 32 and shows no measurable decrease in plating efficiency and no

Figure 31

Upper limit measurement of thin film culture equilibration time. Cells were irradiated in nitrogen (21 Gray) at various times after aspiration of the medium and were expected to show an increased radiosensitivity if any oxygen remained in the dish at the onset of irradiation. No increase in sensitivity was noted for the minimum possible time of fifteen seconds. Air control data (irradiated with 7 Gray) revealed no thin film toxicity.

Figure 32

Toxicity of low temperatures. Plating efficiencies of control and irradiated (17 Gray in 200 ppm O_2 at $4^\circ C$) cells were measured after various times on ice prior to irradiation. Toxic effects were noted after approximately forty-five minutes in both the irradiated and the control cells.

time dependent synergism between the radiation and the low temperature for the first forty-five minutes of the cells' time on ice. To ensure a consistent plating efficiency for all of the cells being irradiated at 4°C, therefore, the Petri dishes were pre-cooled for a maximum of thirty minutes prior to irradiation.

5.2 Experimental Protocol

5.2.1 Preparation of cell culture

The flat cell monolayer was used in the first series of thin film culture experiments and was prepared as follows (Fig. 33): Seed plates were made up 3 days before an experiment by adding 10^4 V79 cells to the 10 ml of MEM & 10% FCS (Gibco) growth medium contained in each of six 100 mm x 20 mm plastic Petri dishes (Falcon). The seed plates were then incubated at 37°C in 5% CO₂.

On the day prior to the experiment, all six seed plates were trypsinized in the usual manner and the cells suspended in 50 ml of growth medium. The cell suspension was transferred to a 50 ml conical culture tube (Falcon) and spun down by centrifuging at 300 rpm for 5 minutes. The medium supernatant was carefully aspirated and 2-3 ml of fresh medium was added to the cell pellet. The pellet was then disaggregated and the cells suspended by gentle vortexing. The cell density of this suspension was measured and diluted to 10^7 cells ml⁻¹.

Depending on the number of data points desired, 20-25 small 50 mm x 10 mm glass Petri dishes were prepared on an incubator tray. Five millilitres of pre-warmed growth medium were added to each dish. With

Figure 33

Protocol flowchart for thin film culture experiments:

3 days prior to experiment:

prepare 6 seed plates: 10^4 V79 cells in
10 ml MEM + 10% FCS

day before experiment (flat cell configuration):

- trypsinize seed plates and suspend cells in medium
- centrifuge @ 300 rpm for 5 minutes
- aspirate supernatant and resuspend cells in medium
@ 10^7 cells ml^{-1}

↓

prepare 20-25 small glass Petri dishes on incubator
tray and add 5 ml MEM + 10% FCS

↓

pipette 100 μl aliquot of cell suspension into each
dish and allow cells to attach @ 20°C for 2 hours

day of experiment:

4°C irradiations: place 10-12 Petri dishes on ice 15-30
minutes prior to irradiation

37°C irradiations: irradiated immediately upon removal
from incubator

irradiation procedure (repeat for each Petri dish):

seal Petri dish with rubber stopper and place in water
bath

↓

increase gas flow from 10 to 30 l hr^{-1}

↓

aspirate medium after air has been purged and reduce
gas flow rate to 10 l hr^{-1}

↓

irradiate cells @ 5 Gray min^{-1}

↓

remove Petri dish and add 5 ml of medium at 20°C

↓

trypsinize Petri dishes (without rinsing)

↓

dilute and plate cells in 3 dishes to produce 300-500
colonies per Petri dish

↓

stain and count colonies after one week incubation

the tray on a vibration free surface, a 100 μ l aliquot of the cell suspension was very carefully micropipetted into the center of each glass dish and the cells allowed to settle and attach to the surface. After a 2 hour room temperature attachment period, the Petri dishes were incubated at 37°C in 5% CO₂ for an additional 24 hours.

The procedure for preparing the glass Petri dishes with round cell monolayers was the same with two exceptions. Since an overnight incubation of the glass dishes was not required, the seed plates were prepared only two days prior to an experiment. Secondly, in order to take advantage of the maximum roundness of the cells, the irradiation procedure was started immediately following the minimum one hour room temperature attachment period.

5.2.2 Irradiation procedure

Irradiations were carried out sequentially, first at 4°C and then at 37°C. Prior to irradiation, the cells were either pre-cooled on ice or, as required in the case of the round cells being held at room temperature, pre-warmed by incubating at 37°C. For the low temperature irradiations, the required number of Petri dishes were either removed from the incubator in the flat cell monolayer experiments, or from the room temperature tray in the case of the round cell experiments, and placed on ice approximately 15 minutes before the first irradiation. For the 37°C irradiations, the remaining Petri dishes in the round cell experiments were incubated a maximum of 30 minutes before irradiation in order to minimize any changes in cell shape.

The gassing system was assembled and allowed to equilibrate at the

desired oxygen concentration prior to the start of the low temperature irradiations. (The 4°C irradiation procedure was usually carried out first because refrigeration unit of the water bath required a relatively long time to equilibrate.) The irradiation of the cells then followed a repeated sequence of steps. With the apparatus prepared as shown in Fig. 24, a Petri dish would be removed from the ice, sealed with the rubber stopper and placed in the 4°C water bath. The gas flow would then be increased to 30 l hr⁻¹ and the effluent monitored using the oxygen analyzer. When the oxygen level indicated that the air had been purged from the stoppered Petri dish, the growth medium was carefully aspirated through the cannula by tilting the dish. Due to surface tension effects and the slightly convex shape of the glass, almost all residual medium was drawn to the periphery of the dish. The gas flow was immediately reduced to 10 l hr⁻¹ and the desired radiation dose was delivered to the cells. After the irradiation was completed, the stopper was removed from the dish and 5 ml of fresh room temperature growth medium added. The covered Petri dish was then held at room temperature until the remainder of the irradiations at that temperature had been completed. The same procedure was followed for the irradiations at 37°C with the exception that the Petri dishes were removed from the incubator immediately prior to irradiation.

5.2.3 Assaying clonogenic survival

A set of irradiated spot monolayers were trypsinized by carefully aspirating the medium covering the cells, adding 1 ml of pre-warmed 0.1% trypsin in citrate buffer and incubating the dishes at 37°C for 5

minutes. The dishes were not rinsed with trypsin after the aspiration of the medium because this resulted in unacceptably high cell losses, particularly when the cells were in the round configuration. The trypsinized monolayers were suspended and diluted in previously prepared dilution tubes containing the appropriate amounts of medium. Aliquots from the diluted cell suspensions were added to 100 mm x 20 mm plastic Petri dishes (Falcon) containing 10 ml of growth medium. Samples of the diluted cell suspensions obtained from each irradiated monolayer were pipetted into three plastic dishes in volumes sufficient to produce 300-500 cell colonies. A portion of the diluted cell suspension was also centrifuged, the medium supernatant aspirated, and the cells resuspended in an ethidium bromide nuclear stain. After vigorous vortexing to lyse the cells and ensure the penetration of the stain into the cell nuclei, fluorescent flow cytometry was used to characterize the cell cycle distribution of the experimental population (Vindelov, 1977). The cell density of the remainder of the diluted suspension was measured using a Coulter cell counter to determine the number of cells plated in each plastic Petri dish.

After a one week incubation, the medium was removed from the plastic dishes and the cell colonies stained using a malachite green solution. The colonies were then scored manually and the number of colonies in each dish was recorded.

5.3 Results

5.3.1 Flat cell monolayer

The survival data obtained with thin film culture cells irradiated in the flat configuration are shown in Fig. 34. The two survival curves in each panel represent the radiation responses of respiring and non-respiring V79 cells that were irradiated while being exposed to a gas of the stated oxygen tension. The results for all irradiations at oxygen tensions below 1000 ppm in the gas above the monolayer were confirmed by one or more repeated experiments and the survival data were pooled to reduce random error. Each curve was produced by a computer calculated least squares fit of the Linear Quadratic cell survival model to the data obtained with the clonogenic assay. All variances in the survival data were again calculated according to the method proposed by Boag (1975).

The plotted survival curves display the established characteristics of oxygen mediated radiation survival, showing the expected increase in cellular radiosensitivity with increasing oxygen tension. The data corroborate the findings reported by Ling et al. (1981) by showing that oxygen mediated radiosensitization is evident even at very low oxygen tensions (100 ppm in gas for the non-respiring cells).

Irradiating the cells at the two different temperatures did not always produce anticipated results, however. Most surprising perhaps, is the marked difference in the hypoxic radioresponses of the cells at 4°C and 37°C. These curves represent the data pooled from several

Figure 34(e-g)

experiments in which the repeat experiments displayed identical results. This ruled out the possibility that the observed difference in radiosensitivity is the result of random artefacts. The radiosensitivity differential is also not due to the presence of oxygen. In addition to having closely monitored the oxygen content of the gas to ensure that the cells were truly hypoxic during the irradiation procedures at both temperatures, it is not likely that a respiring cell culture would retain a greater amount of residual oxygen than a non-respiring one. Possible explanations for this result are examined in greater detail in a discussion of the thin film results.

Manipulating cell respiration by means of temperature produced a more predictable effect at higher oxygen tensions. A notable feature of the data is that as the partial pressure of oxygen in the gas increases from near zero to a few hundred parts per million, the radiosensitivity of the non-respiring cells increases more rapidly than that of the respiring cells. The difference in the dose responses of the respiring and non-respiring cells reaches a maximum and then becomes less marked as the oxygen content of the gas increases even further. This pattern is consistent with the creation and maintenance of small oxygen gradients in respiring cells that eventually become less radiobiologically significant as the oxygen concentration is increased.

The difference in radioresponses at 4°C and 37°C for a given oxygen tension in the gas are not solely due to the existence of oxygen gradients, however, and the implicit oxygen solubility differences at these two temperatures must also be considered. (As the temperature increases from 4°C to 37°C, the proportional change in solubility of oxygen is greater in aqueous solutions than it is in air; Fig. 16)

Because the same partial pressure of oxygen in the gas was maintained throughout the irradiation procedures at both temperatures, the oxygen concentration was commensurately higher in the aqueous cell cytoplasm at 4°C. Although adjusting the oxygen content of the gas between irradiations at the two temperatures to compensate for such solubility differences was attractive in theory, it was judged too difficult to implement accurately using the available oxygen monitoring and gas mixing equipment.

The true radioprotective effect of respiration in the flat cell configuration can be seen more clearly in a semilogarithmic plot of the OER versus oxygen concentration (Fig. 35). The enhancement ratio values were calculated at doses required to kill 99% of the cells for each oxygen tension and plotted against the corresponding temperature corrected oxygen concentrations. The non-respiring cell data were fitted analytically (Alper, 1979) with the Alper and Howard-Flanders model ($m = 3.1$ and $K = 3.0 \mu\text{M}$) and display results typical of the oxygen enhanced killing of mammalian cells. The small shift to the right in the 37°C OER data corresponds to a $0.15 \mu\text{M}$ differential between the oxygen concentrations of the respiring and non-respiring cells. The respiring cell data were fitted with a curve defined by the same parameters as the curve fitted to the non-respiring cells, but in which the oxygen concentration was adjusted by this factor. The presence of this shift is precisely the result that might be expected if small, localized, respiration-induced oxygen gradients existed within and immediately around the respiring cells.

Figure 35

Oxygen enhancement ratio for flat cells in thin film culture. Data fitted analytically with the Alper and Howard-Flanders equation were determined to be defined by the curve parameters $m = 3.1$ and $K = 3.0 \mu\text{M}$. Enhancement of respiring cells (open circles) was found to lag that of non-respiring cells by $0.15 \mu\text{M}$.

5.3.2 Round cell monolayer

The radiation response for the cells in the round configuration was similar to that of the flat cells (Fig.36). The results for all irradiations at oxygen tensions below 1000 ppm in the gas were again confirmed by one or more repeated experiments and the survival data pooled. In a similar gradient invoking pattern, the radiosensitivity of the non-respiring cells again increased more rapidly with increasing oxygen tensions than that of the respiring cells. However, although quantitative comparisons at any low partial pressures of oxygen are again difficult to make given the implicit solubility differences at 4°C and 37°C, the differences in cellular radiosensitivity at the two temperatures are qualitatively more dramatic for the round cells than for flat cells irradiated under identical conditions.

The quantitative effect of cell respiration on the oxygen mediated radiosensitivity of round cells in thin film culture is most clearly displayed in a *K* curve plot (Fig. 37) The OER values were again calculated for doses that give a 1% cell survival and plotted against the corrected oxygen concentrations. Cell shape did not affect the oxygen enhancement of the non-respiring cells and the analytically fitted curve was defined by identical curve parameters ($m = 3.1$ and $K = 3.0 \mu\text{M}$). The similarity of the oxygen enhanced radiosensitivity of flat and round non-respiring cells implies that the radiolytic depletion of intracellular oxygen is insignificant in the creation of oxygen gradients within and around the thin film cultured cells. Conversely, the oxygen enhanced radiosensitivity of respiring round cells is shifted considerably further to the right than that for the flat cells, implying

Figure 36(e-f)

Figure 37

Oxygen enhancement ratio for round cells in thin film culture. Data fitted analytically with the Alper and Howard-Flanders equation were again defined by the curve parameters $m = 3.1$ and $K = 3.0 \mu\text{M}$.

Enhancement of respiring cells (open circles) was found to lag that of non-respiring cells by $0.35 \mu\text{M}$.

the existence of larger respiration induced intracellular gradients in cells in this thin film configuration. The 37°C data were also fitted with the same K curve, but at oxygen concentrations adjusted to lag those of the non-respiring cells by 0.35 μM . Since the differential clearly demonstrates the dependence of gradient size on cell shape and is in excellent agreement with the cell respiration predictions made by Boag (1969; 1970), these results are very compatible with theories that propose oxygen gradients not only exist in the medium immediately surrounding respiring cells, but extend into the cells themselves.

5.4 Discussion and Analysis of Thin Film Method

5.4.1 Temperature mediated radiosensitivity

In order to demonstrate the existence of intracellular oxygen gradients, low temperatures were employed to inhibit cell respiration during the thin film irradiations. It was anticipated that irradiated cells would display a temperature dependent radiosensitivity due to the influence of cell respiration on the local and intracellular oxygen distribution. Further investigation was prompted, however, by the discovery of an unexpected temperature mediated increase in the radiation survival of hypoxic cells irradiated at 4°C.

As previously noted, the possibility that the low temperature increase in cell survival was a random artefact was easily eliminated by repeating the experiments several times. The hypoxic radiation survival data for cells in the round and flat configurations, for example, were

pooled from five and seven experiments, respectively, all of which displayed similar responses.

Since position in the cell cycle is known to affect cellular radiosensitivity (Sinclair and Morton, 1963), a second possibility was that their exposure to low temperatures selected a subpopulation of the 4°C cells or simply arrested the cells in a less sensitive portion of the cell cycle. Investigating the effects of low temperatures on cell survival, as described in the Methods section 5.1.8, however, showed no significant decrease in cell viability for cells that had been placed on ice for up to forty five minutes (Fig. 32). Because the maximum "ice time" for the thin film irradiations was thirty minutes, the 4°C radiation survival data does not represent the response of a selected, radioresistant subpopulation of cells. Additionally, samples of cells that were being removed from the ice and trypsinized in fifteen minute intervals, during the same low temperature experiment, were stained with an ethidium bromide nuclear stain and analyzed using fluorescent flow cytometry techniques (Vindelov, 1977). A plot of the cell cycle distribution displays no detectable changes in the population samples over the entire two hours that the cells were held at or below 4°C (Fig. 38). No differences were seen in similar plots obtained from cells irradiated at 4°C and 37°C during the series of thin film experiments. The lower sensitivity of the non-metabolising cells is, therefore, also not attributable to temperature induced shifts in the cell cycle distribution.

A review of the literature reveals that a similar low temperature radioprotective effect was noted by Belli and Bonte (1963) in experiments with cultured Hela cells. Although no evidence for a

Figure 38

Cell cycle distribution versus time on ice prior to irradiation
Ethidium-bromide labeled DNA profiles of cell populations from Petri dishes exposed to low temperatures for increasing periods of time prior to irradiation reveal no changes in cell cycle distribution over the observed 2.5 hour period.

particular molecular mechanism was found, it was suggested that exposing cells to low temperatures during irradiation may result in slowed rate processes and the molecular recombination of radicals before deleterious reactions can occur. Ben-Hur et al. (1974) have, however, documented results that seem to contradict these, observing that low temperatures potentiated lethal radiation damage in a V79-753B-3M subline when the cells were irradiated in a buffer solution at 0°C. A third result is claimed by Koch and Burki (1977) who found no measurable difference in the radiation survival characteristics of yet another V79 subline when these cells were irradiated at 4°C and 37°C. Although it is possible that the variability of these results reflects real differences between cell lines and sublines, they are most likely due to differences in experimental protocol (the length of time for which the cells were held at low temperatures and the nature of the irradiation and post-irradiation conditions).

5.4.2 Oxygen gradients in thin film culture

The role of cell shape in determining the cellular radiosensitivity in thin film culture can be envisioned as depicted in Fig. 39. Glass Petri dishes containing typical spot inoculated cell monolayers were carefully weighed before and after removing the culture medium. It was determined that less than 0.1 ml of liquid remained once the medium was aspirated. Hypothetically, this residium would wet the dish surface with a thin film of medium an average of a few tens of microns in depth. In practice, however, surface tension effects will reduce the depth of the film above the cells considerably, perhaps to a

Figure 39

Gradients in thin film cultured cells of different shapes. A proposed mechanism by which the variation in oxygen enhanced radiosensitivity of flat and round thin film cultured cells can be explained. Approximate values for differences in diffusion distances and intracellular oxygen concentrations are given for the flat and round cells.

thickness the order of a spherical cell radius ($<10\ \mu\text{m}$). According to Ling et al. (1981), the film is thin enough to allow an immediate exchange of diffusing molecules between the gas and the surface of the cell. Oxygen diffuses through the film and into the cells down a concentration gradient created by cell respiration. In thin film culture, round cells significantly increase the diffusion distance from the gas above the film of medium to the oxygen consuming mitochondria within the cell and, hence, also to the critical radiation target, the cell nucleus. The magnitude of the oxygen gradient must increase commensurately to maintain full cell respiration. Consequently, if the oxygen concentration immediately above the thin film is the same for cells of both shapes, the nuclei of round cells are likely to be exposed to a lower oxygen concentration than those of flat cells. This results in the observed lag in oxygen-enhanced cell killing of the round cells. Furthermore, when the difference in the magnitude of the oxygen gradients for the two cell shapes is of the same order as the oxygen concentration of the gas (a few hundred parts per million at 37°C) the differences in the radioresponses are most acute. As the oxygen concentration of the gas increases, the difference in oxygen concentration at the cell nucleus for the two cell shapes becomes less radiobiologically significant and the oxygen enhancement ratios become increasingly similar.

5.4.3 Evaluation of the thin film technique

The thin film cell culture technique has been shown to be ideally suited for experiments in which control of the oxygen concentration to which cells are exposed at low oxygen tensions is required. The stated objectives were met successfully using this technique and the questions posed in the statement of the hypothesis have been answered decisively. Differences in the radiation responses of thin film cultured cells could be accurately resolved at very low oxygen tensions and the technique did not display the oxygen equilibration or depletion problems associated with the suspension culture method. Random experimental error was relatively low for these experiments and the greatest variability in the results seemed to be biological in origin. The results of the experiments were easily repeated within variations that can be attributed to slightly differing cell cycle distributions in the experimental populations.

The irradiation of cells in thin film culture, as it is presented in this thesis, is somewhat longer and more cumbersome than others (Michaels et al., 1978; Ling et al., 1981) in that it requires two trypsinization procedures, once prior to and once after irradiation. This did not seem to adversely affect the quality of the experimental results, however, and allowed the use of statistically superior survival assays. No toxicity or contamination problems were encountered despite the complete removal of the medium covering the cells prior to irradiation and the various other cell handling procedures.

Finally, although it was not a factor in these experiments, the dosimetry problems associated with cells attached to glass surfaces

would present difficulties if absolute dose responses are desired. For such applications, a plastic culture surface with a low oxygen permeability such as that used by Michaels et al.(1978) may be a more suitable alternative.

6. SUMMARY

The contents of this thesis can be clearly summarized by a brief review of its most salient points. These are as follows:

1. The need to understand the effect of cell respiration on the availability of intracellular oxygen at low ambient oxygen concentrations was reviewed by citing established examples of the role of oxygen in the radiosensitization, radioprotection and chemosensitization of cells.
2. A hypothesis was formulated based on the use of radiosensitivity as an indicator of intracellular oxygen concentration and, hence, as a method of determining the existence of theoretically predicted, respiration induced intracellular oxygen gradients.
3. The equilibration and the radiolytic and metabolic depletion of oxygen in stirred suspension cultures were examined and showed that this culturing method is inappropriate for applications in which accurately controlled low, but non-zero cellular oxygen

concentrations are desired.

4. A very rapidly equilibrating, non-toxic thin film culturing technique was developed to solve the problems incurred during the suspension culture experiments.
5. The differential radiosensitivity of respiring and non-respiring thin film cultured cells was established and interpreted to be a consequence of respiration induced intracellular oxygen gradients. The magnitude of the implied difference in cell oxygenation was shown to be dependent upon cell shape and was in good agreement with theoretical predictions regarding such gradients.

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8. APPENDIX

8.1 The Interaction of Ionizing Radiation with Cells

Ionizing radiation is defined as a radiation having sufficient energy to remove an orbital electron from an atom or molecule with which it interacts. The ionization of an atom can occur as a result of two modes of radiation action. Charged particle radiations (electrons, protons, α -particles, etc.) act by directly removing orbital electrons from the atoms through which they pass. Electromagnetic radiations (X and γ -rays) act indirectly, first producing fast moving secondary electrons, which then proceed to ionize other atoms and molecules. Neutrons are also a form of indirectly ionizing radiation, interacting with atomic nuclei to produce charged particles (fast moving protons, α -particles and other nuclear fragments) through which ionization processes occur.

An ionization event releases approximately 33 eV and is sufficient to break strong chemical bonds. (A C=C bond has an associated energy of 4.9 eV.) The density with which these ionizing events occur in irradiated matter is dependent upon the linear energy transfer (LET) of the radiation. Particulate radiation has a much higher LET than electromagnetic radiation and the nature of particulate radiation-matter interactions is also much more complex. The interactions of X and γ -rays with matter have, by comparison, been well defined. For electromagnetic radiation with energies below 10 MeV, the production of the secondary electrons is known to occur primarily as a result of elastic collisions between photons and free electrons (Compton effect)

and the release of bound orbital electrons by photon annihilation (photoelectric effect).

The most critical target of radiation action in cells is the DNA. The interaction of a DNA strand with a fast moving electron or photon can cause a rupturing of the phosphate sugar back bone or the formation of a reactive DNA free radical (atom with unpaired electron in its outer orbital shell). Both of these types of DNA damage could adversely affect the reproductive ability of the cell. In addition to direct radiation-DNA interactions, damage may also be caused by free radicals that are radiolytically induced in liquid environment close to the DNA. The radiolytic products of water (cells are composed of 80% water) includes the H_2O^+ ion radical which rapidly combines with another water molecule to produce highly reactive hydroxyl free radical. Approximately two thirds of radiation damage incurred by cells during the X-irradiation is due to interactions with such free radicals.

As discussed in the main body of the thesis, radiation damage to cells can be modified by both repair and fixation. The radioprotective effect of thiols in cells has been partially attributed to the chemical repair of radicals by hydrogen donation. Conversely, radiolytically induced lesions in DNA can also be made permanent by combining with oxygen.

An approximate time scale of events after a radiation-DNA interaction is depicted in Fig. 40. The physical stage, during which the charged particle passes through the atom or molecule and ionization occurs, is completed in less than 10^{-16} seconds. Ion radicals continue to exist for approximately 10^{-10} seconds and free radicals for

Figure 40

An approximate time scale of events after a radiation-DNA interaction.

approximately 10^{-5} seconds. All radiochemical events are completed one second after irradiation and the various biological expressions of the damage become evident hours, days or months after the initial interaction.

8.2 Modelling Radiation Survival

Radiation survival curves are commonly utilized as tools of quantitative analysis in *in vitro* radiobiology. However, in addition to simply measuring the effect of radiation on cell survival, the shape of these curves has given rise to speculation about the relationship between the mechanism and the biological expression of radiation action. A common feature of survival curves for cultured mammalian cells irradiated with low LET radiations is the existence of a "shoulder" in the initial portion of the curve. This feature has been the source of several theories regarding the response of cells to radiation damage. Some of these theories interpret the shoulder to be a consequence of a lesion repair mechanism. The repair mechanism is hypothesized to ameliorate radiation induced lesions by biochemically restoring the damaged DNA. The steeper decline in survival at higher doses is attributed to the eventual saturation or inactivation of the mechanism. An alternative theory proposes that the existence of the shoulder is a reflection of the innate ability of most irradiated cells to withstand a certain amount of radiation damage before succumbing to more lethal doses.

It has been proposed that the mathematical models used to fit curves to radiation survival data also reveal clues that connect the

molecular mechanisms of radiation action with their biological consequences. The exponential nature of radiation survival has led to the development of theories which attempt to explain the induction of lethal radiation damage in terms of Poisson distributed "hits" on specific cellular targets. The simplest such theory is the "single hit, single target" theory which postulates that a single critical event at a radiation target is sufficient to inactivate the cell. The most basic mathematical representation of this hypothesis is a simple negative exponential curve (Fig. 41) which has been found to closely model the inactivation of irradiated molecules and some bacterial cells.

A more elaborate radiation survival model, the Linear Quadratic model, was used to fit the mammalian cell survival data in this thesis and is based upon the theory that the primary mode of radiation action on cells is the breaking of DNA strands. The lethal radiation event is postulated to be an unrepaired DNA double strand break. In an irradiated cell, a double strand break, or "hit", can occur as a result of a single ionization event that breaks both strands, or by two separate ionization events, each breaking a single strand. Using the single hit, single target hypothesis, the probability that either such lethal event will occur is proportional to the dose and to the square of the dose, respectively. The net probability of radiation survival is the product of these two individual probabilities (Fig. 42).

Although its continuous curving does not match *in vitro* data as closely at lower levels of survival, the LQ model fits data in the first two decades of survival extremely well. Other more complex models of radiation survival possess a multitude of adjustable parameters (as opposed to two for the LQ model) and generally offer no significant

Figure 41

Single hit, single target model of radiation survival produces a straight exponential survival curve.

Figure 42

Proposed molecular basis for the Linear Quadratic model of radiation survival. The model can be derived as the product of the individual survival probabilities resulting from two modes of DNA double strand break production.

Total survival:

$$S = e^{-(aD + bD^2)}$$

where $a = k_1$ and $b = k_2^2$

improvement in the accuracy with which a curve is fitted to radiation survival data over this range of cell survival.