

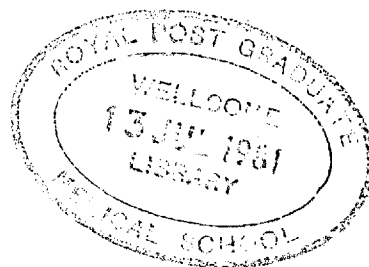
THE EARLY RESPONSE OF THE INTESTINE TO FRACTIONATED
DOSES OF X-RAYS AND NEUTRONS

by

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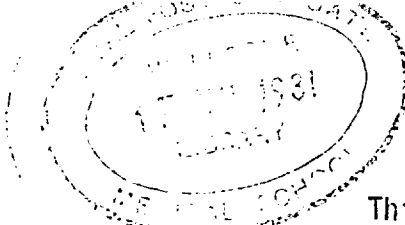
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ABSTRACT

This thesis describes investigations on the relative importance of a number of factors influencing the radiation sensitivity of the small intestine.

The effects of mitotic delay, premitotic death, cell movements and cell age distributions on intestinal crypt cell survival during fractionated X-ray and neutron treatments have been investigated. A comparison of the effects of X-rays and neutrons on synchronous and asynchronous intestinal crypt cells in the mouse indicates that the age distribution of cells is one of the most important factors affecting radiosensitivity within a species. The amount of radiation induced mitotic delay was determined after low doses of X-rays and neutrons. The importance of mitotic delay in fractionated radiation treatments is found to be primarily related to the partial synchronisation induced by the delay, while the importance of other factors such as premitotic death are found to be small due to the rapid homeostatic repair system operating in the intestine.

By comparing the radiosensitivity of mouse and Chinese hamster intestine it has been demonstrated that there are large interspecies differences in sensitivity and that at least in part this reflects differences in the Interphase Chromosome Volume together with differences in the age distribution of the stem cell population.

The effect of varying neutron dose-rates between 2.25 krad/min and 2 rad/min on intestinal crypt cell survival is described. A small but significant effect of dose rate has been observed in the jejunum for neutron dose rates within this range.

Recovery from sublethal damage during fractionated X-ray treatments is measured between doses/fraction of 200 rads and above. For doses/fraction below 200 rads all sublethal damage is recovered and there is very little additional effect by splitting the dose further.

The usefulness of using crypt survival data to predict the acute intestinal response in the therapy dose range is also discussed.

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SECTION 1.

1.0. Introduction

1.1. General

Treatments in radiotherapy are limited by the amount of damage to the normal tissues in the radiation field which can be tolerated by the patient. Treatments are usually given in fractionated doses which are relatively small, of the order of 200-300 rads over a period of approximately 4-6 weeks with total doses of up to 5-6000 rads of X-rays and 1500 rads of neutrons. The ability of normal tissues to recover between fractions from these low doses will be an important factor in determining the normal tissue tolerance.

The recovery between the dose fractions in radiotherapy will be influenced by a number of factors including the shape of the cell survival curve of the target cells, the cell kinetics of the target cells and the lengths of the differentiation pathway and lifetime of the physiologically mature cells.

An understanding of the contribution of these various factors to changes in tissue tolerance both to X-irradiation and to higher LET radiation during fractionation schedules may aid in predicting tissue responses to radiation schedules outside the normal experience. By allowing for the contributions from these different factors, a greater understanding of tissue cell radiosensitivity and of the pathological development of the tissue radiation damage may be obtained.

1.2. Shape of cell survival curve

There has been some controversy during the last few years about the exact shape of the initial part of the X-ray survival curve. The question raised is whether the shoulder is continually bending so that the initial slope is zero or whether there is a finite initial slope, i.e. at what point the lethal event changes from being caused by a one-hit to a multihit mechanism. It has been suggested by Wambersie and Dutreix from work on human skin (1973) and mouse intestine (1974) that

cellular recovery from sublethal damage is unimportant for doses per fraction of less than 300 rads. Their data was compatible with a two component model for cell killing in which a greater part of the cell killing is due to one-hit events. This does not agree with the micro-dosimetric determinations of Kellerer and Rossi (1972) which showed that for low LET radiations the single hit component of damage is important for doses of only up to a few millirads while for doses above this there is a finite probability of more than one transversal through the cell *target* (Rossi, 1976). When radiotherapy treatments are given it is generally assumed that recovery does take place between low dose fractions of X-rays and consequently the implications of the observations of Wambersie and Dutreix for radiotherapy are profound. The results obtained by Wambersie and Dutreix on mouse intestine were obtained using LD50 at 20 to 30 days as an end point. Since there are likely to be many factors which affect death at 30 days apart from the sensitivity of the intestinal epithelium a study was made here using crypt survival as a more reliable end point for assessing the damage to the intestine alone.

The aim of this study was to obtain more detailed data on the amount of recovery and its kinetics following an irradiation and also to obtain detailed information on some of the factors which effect recovery or its expression in the intestine such as mitotic delay, cell death, and cellular migration out of the crypts. These early effects were studied using the crypt squash technique which enabled individual crypts to be examined for the numbers of mitoses and the number of viable cells.

The study of the factors which influence recovery and cell sensitivity are of great significance in tissues in vivo because of their direct relevance to the use of fractionated treatments in radiotherapy. However, there are inherent difficulties in scoring damage quantitatively in tissues in vivo because of the relatively high doses

which must be used before assayable damage can be measured. These problems with tissues make them less convenient for studying the basic mechanisms of radiation damage in cells compared with cells grown in vitro in which many of these problems can be overcome. However, the amount of recovery observed after radiation with most tissues grown in vitro is much less than that observed in many tissues in vivo such as skin, gut, lung and spinal cord. Thus for cell sensitivity at low doses studies on tissues may give more satisfactory data than can be obtained in vitro, particularly if the tissue studied is one in which damage can be measured in a reasonably quantifiable way, such as in the intestinal crypt cells.

The studies were made using X-rays or neutrons because information on recovery in the intestine following neutrons are needed to assess the hazards to the intestine in using neutron therapy for abdominal tumours and also because differences between the response to X-rays and neutrons help to highlight the relative importance of the different factors in the overall physiological expression of damage.

1.3. Radiation effects on the Intestine - General

The epithelium of the small intestine is one of the most useful and important systems for quantitative and qualitative studies of radiation effect in vivo. Intestinal function encompasses secretion, digestion and the protection of its integrity is essential to the well-being of the host. Intestinal cells have been shown to possess a large capacity to repair damage deposited by X-irradiation (Hornsey and Alper, 1966) but the recovery after neutron irradiation is small (Hornsey, 1973; Hendry et al., 1975). Ionising radiations affect intestinal function primarily by preventing the normal cell renewal processes taking place but the earliest effect of ionising radiation is its effect on stomach clearance with resultant nausea and slowing down of the passage of food (Hulse, 1957). The cause of this effect which is observed in the first

12 hours following irradiation is uncertain but may be nervous in origin.

Early effects of reduction of cell turnover of the epithelial cells in the small intestine are the next systematic effect and are evident at 3 and 4 days, when, if the dose is sufficiently high, animals die from the loss of epithelial cells and collapse of the villi. This results in water and electrolyte loss together with attack by proteolytic enzymes (Quastler, 1956) and by 4-8 days bacteremia and protein loss play a more important role in the second phase of the intestinal syndrome. Deaths that occur at much later times of up to 90 days have been attributed to cell adhesion and restriction of the lumen of the intestine (Geraci^{et al}, 1974) and are probably not due to epithelial cell damage.

The first observations on the effects of radiation on the intestine were made by Walsh in 1897 who reported the first case of radiation injury in a co-worker who developed diarrhoea after abdominal X-ray exposure. Early animal experiments proved conclusively that the fundamental event in the "systemic intoxication" due to Roentgen rays was the primary injury to the epithelium of the small intestine (Warren and Whipple, 1922). It was found that after irradiation to the intestine alone compared with a thoracic exposure in dogs, diarrhoea and vomiting occurred on the 2nd day and death occurred on the 4th day, when there was almost total disappearance of the villi, whilst no death occurred after the thoracic irradiation. This finding was followed by more detailed studies which showed that radiation affected the mechanism of cellular replacement (Friendman, 1945). Friendman showed that in the first 12 hours after irradiation there was a profound effect on the crypts when radiation inhibits mitosis and killed cells, while the villi cells remained relatively unaffected. The movement of cells out of the crypts and onto the villi and eventually into the lumen of the intestine was later studied in detail and a fuller understanding of the intestinal system obtained (Leblond and Stevens, 1948; Leblond and

Messier, 1958; Lipkin et al., 1963). With the introduction of radioactive labelling of cells and contact autoradiography, particularly the use of tritium labelled thymidine as a marker of DNA synthesis, further information became available on the cell kinetics of the intestinal epithelium (Hughes et al., 1958). Cell cycle and turnover rates have since been studied in many different species. Information on the human intestine has been limited to those patients from whom biopsies could be readily obtained (Wiernik and Plant, 1970).

1.4. Radiation effects on the small intestine

The main intestinal response to radiation in experimental animals such as mice and rats is observed at 3-5 days post-irradiation. The early work on the sensitivity of the intestine to X-rays and neutrons was done using death at this time as the endpoint.

Assuming death at 3-5 days is related to loss of stem cells in the crypt of Lieberkuhn, parameters for cell survival curves were proposed from the animal survival data (Hornsey and Vatistas, 1963) which have been verified using more direct methods of cell survival assessment (Withers and Elkind, 1969, 1970). This verification and also the similarity of response of animal killing curves and cell survival curves (Hornsey, 1970b) to different modifying factors such as oxygen tension or radiation quality give a good support to the proposition that animal death at 3-5 days after irradiation of the intestine is due to the loss of epithelial cells in the small intestine. Other evidence came from the differences in the time of death and the cell kinetics in normal mice compared with germfree mice. The latter animals have a much slower epithelial cell turnover and migration in the small intestine and consequently a much later time of death following irradiation (Wilson, 1963).

Withers and Elkind described the first method for estimating crypt survival. They scored the number of colonies of crypt cells that

arose from the division of 1 or more surviving crypt cell after irradiation. These colonies could be scored from histological sections of intestine to give the number of surviving crypts per circumference (the microcolony method), or from whole test areas of intestine as large visible nodules scored at 13 days post-irradiation (the macrocolony method). The latter method involved giving a dose of 3000 rads of X-rays to the lateral segments of the test zone in order to locate it and prevent migration into it by neighbouring unirradiated cells. Hagemann et al., (1971) developed a method of labelling the proliferating cells in the crypt with tritiated thymidine and scoring the number of disintegrations per minute (dpm)/crypt and the number of dpm per mg in order to determine the number of crypts/mg. This method overcame the problems of scoring sections of crypt colonies which Cairnie (1977) suggested can lead to spurious results due to problems with branching crypts. Cairnie determined the number of crypts per mg by simply counting the numbers of crypt cell colonies on small weighed pieces of tissue through a dissecting microscope after the villi had been removed.

In this report the method used for cell survival studies was the microcolony technique described by Withers and Elkind (Figures 1.1.a&b). A comparison was also made between the microcolony method and the method described by Cairnie.

Intestinal stem cells in the crypt are rapidly dividing cells under homeostatic control and therefore their response to radiation will be primarily determined by their ability to recover from sublethal damage and their ability to repopulate. Coutard (1934) was the first to observe the importance of the time factor in normal tissue recovery during radiotherapy treatment and he recommended using the duration of treatment which did least damage to normal tissues.

The time factor is particularly important in rapidly dividing tissues such as the intestine during long fractionation treatments, as

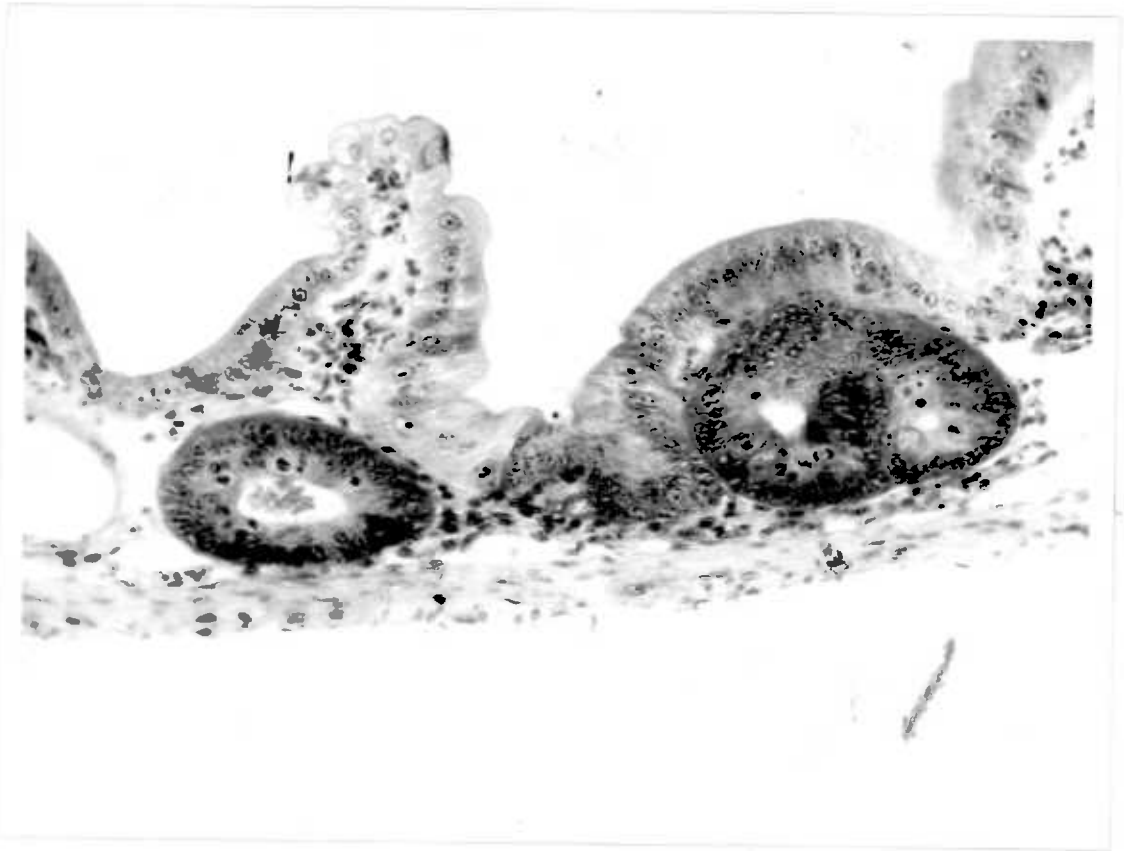


Figure 1.1. a) Crypt cell colony 4 days after irradiation.

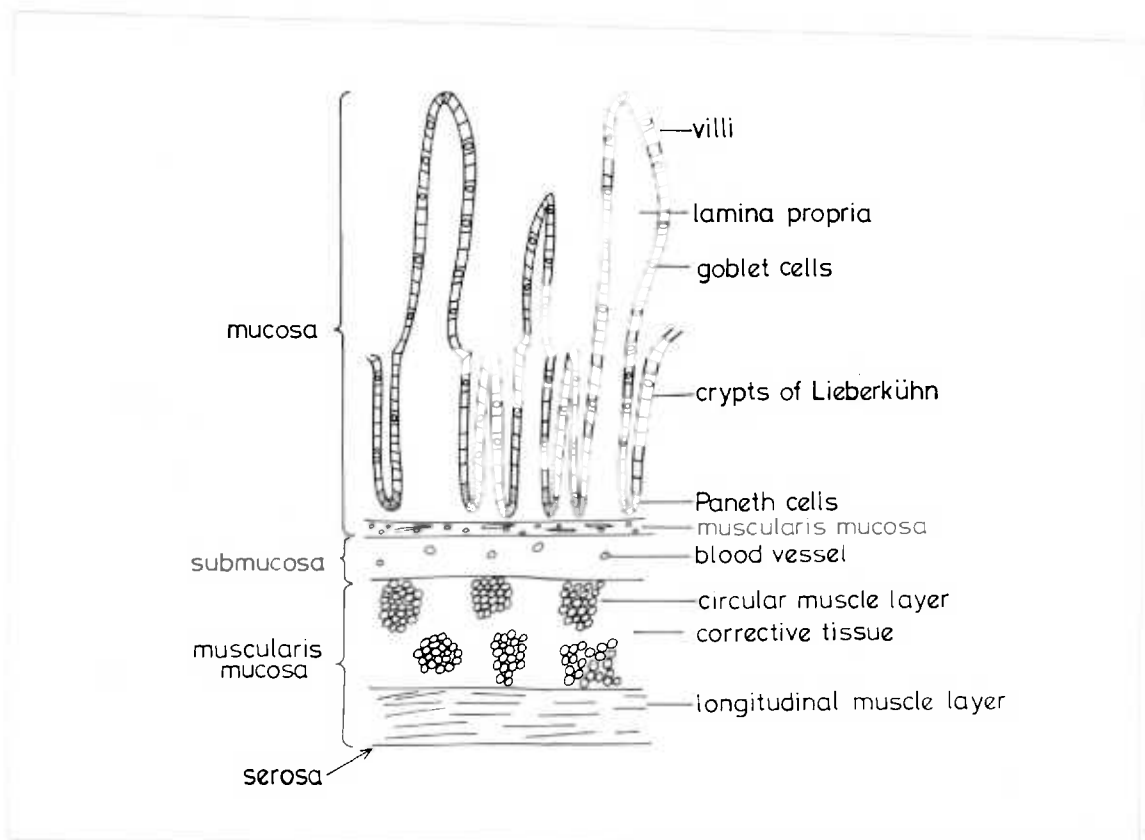


Figure 1.1. b) Diagrammatic section of normal small intestine.

repopulation can occur between treatments and enable the tissue to recover. It has also been observed that repopulation rates do not remain constant during a treatment but are stimulated by homeostatic control in response to the presence of dying cells or the products of cell lysis. An increase in proliferation has been detected within 2 or 3 days after irradiation in mouse duodenum epithelium (Leshner and Bauman, 1967) and in mouse jejunum (Withers and Elkind, 1969).

Repopulation may include recruitment of cells from a reserve population of proliferative cells either within the crypt or without and an increase in birth rate due to shortening of the cell cycle (Lamerton, 1966).

1.5. Recovery from sublethal damage and the subcellular target

Recovery from sublethal damage in the intestine is rapid and takes place within 3-4 hours (Hornsey and Vatistas, 1963; Withers and Elkind, 1969; Dutreix et al., 1973). The word sublethal was first used by Atwood and Norman (1949) when they studied the amount of cell killing by U.V. in the conidia of Neurospora. They observed that the initial effect on cell killing was not a simple exponential relationship but that there was an initial shoulder to the survival curve. The existence of a shoulder was explained by the hypothesis that cell death was a consequence of inactivating a certain number of targets so that the cell remained viable as long as any one of them escaped a hit, this was called the multitarget model of the target theory. The target theory was originally put forward by Lea (1938) who proposed a multihit model which was based on the assumption that cell death was a consequence of inactivating a single target that will survive $n-1$ hits but succumb to the n th one. The first survival curve for mammalian cells was obtained by Puck and Marcus (1956). From their work on HeLa cells they showed that mammalian cell survival curves have a shoulder followed by an exponential portion (linear on a semilog plot) and by extrapolating back up from the linear portion of the curve to the ordinate line they

obtained an extrapolation number of approximately 2, which they interpreted as indicating that 2 targets must be inactivated before a cell is killed. The equation describing this curve is:-

$$S = 1 - (1 - e^{-D/D_{37}})^N$$

where S = surviving fraction,

D = radiation dose

D_{37} = the dose increment that reduced the survival fraction by a factor of 0.37 in the exponential portion of the curve. D_{37} is sometimes designated D_0 or the mean lethal dose which would on the average inactivate one target.

$e = 2.71828$

N = the ordinate intercept of the back extrapolation of the straight line portion of the curves to zero dose, i.e. the extrapolation number or target number.

Alper introduced a further term for characterising the multi-target survival curve called the intercept or quasithreshold dose, for which the symbol D_q was adopted. D_q is the intercept which the extrapolated exponential portion of the curve makes with an abscissa through 100% survival and gives a good indication of the amount of wastage of dose due to recovery of sublethal damage. (1962). (Fig. 1.2).

A second 2 component model for cell killing has been put forward and contains a single hit as well as a multihit component. In this case the initial region is exponential with a finite slope and the equation describing this is:-

$$S = (e^{-D/D_1}) (1 - (1 - e^{-D/D_2})^N)$$

Over the last few years several repair models have been postulated and they propose that repair of some potentially lethal lesions occurs concurrently with their induction. The capacity for repair is however limited either because the rate at which lesions are induced is greater

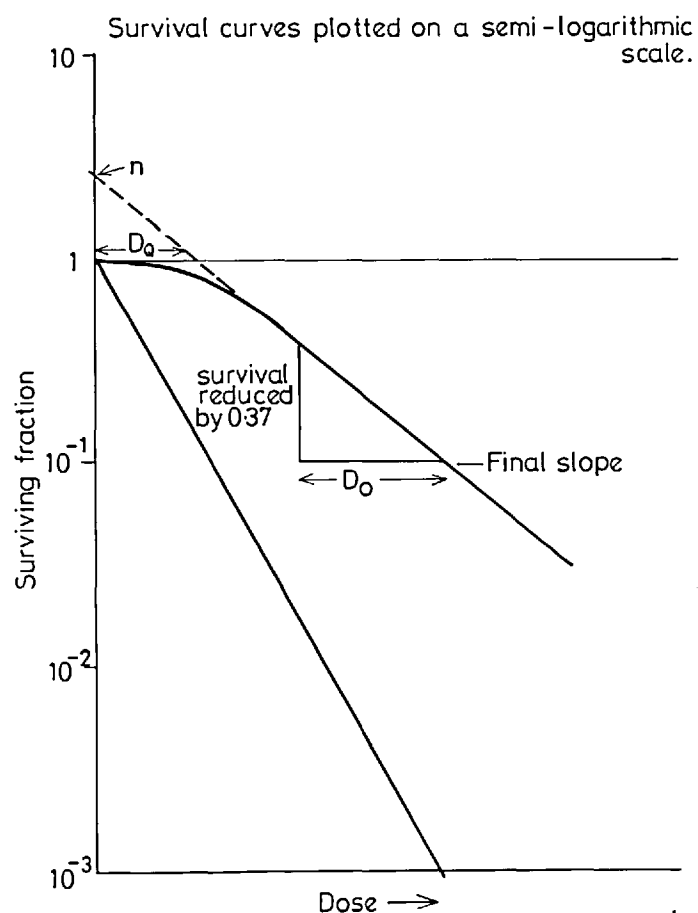


Figure 1.2. Cell Survival plotted on a logarithmic scale (ordinate) against radiation dose in rads on linear scale (abscissa), showing cellular parameter determination.

than that at which they are repaired or because the repair mechanism becomes saturated or is depleted by radiation (Haynes, 1966). The repair hypothesis suggests that all cell damage is potentially lethal and is exponentially dose related but is modified by repair processes such as the Q factor. Q factor is believed to be present in the cell in varying amounts through the cell cycle protecting the cell from damage (Sinclair, 1972), so that damage becomes exponential when all the Q factor is used up. As a result, the amount of Q present will influence the size of the shoulder on the survival curve. These potentially lethal lesions must be a different category from those subject to what is known as "repair of potentially lethal damage" or P repair (Phillips & Tolmach, 1966). That form of repair is associated with the observation of decreased terminal slopes of survival curves when there is a change in cell cultivation conditions after irradiation.

The repair theory has received added support recently from the studies of Goodhead and his co-workers with soft X-rays which suggest that there are two targets either of whose inactivation will be lethal, and the repair of which takes place by a repair process which saturates with increasing dose. (Goodhead et al., 1980).

Which of these various models of radiation action is correct is still unresolved because of the inherent difficulties of obtaining sufficiently accurate biological data to differentiate between them.

The first demonstration of recovery from sublethal damage in mammalian cells was reported by Elkind and Sutton in 1959. They proposed a series of experiments to test whether or not surviving cultured cells could repair sublethal damage. Using Chinese hamster cells they examined the variation in net survival when a fixed exposure was divided into two parts. They also examined the time dependence of the repair of this sublethal damage by exposing cells to two equal doses separated by varying time intervals. The resultant surviving fractions

rose immediately to a maximum at 2 hours dropped to a minimum at 4.5 hours and rose again at 6 hours. They concluded that damage received from an initial dose of radiation could be repaired and that repair was rapid and began immediately. Kallman (1963) subsequently suggested that the phasic changes observed following the maximum value was due to the movement of cells through the cell cycle.

The exact nature of the target for the lethal lesion and the sublethal lesion is still a matter of controversy. Kellerer and Rossi (1972) proposed that radiation of low LET achieves its effect mainly by the interaction of two sub-cellular lesions engendered by separate electron tracks - "the dual radiation action hypothesis". They postulate that the size of the sensitive target is of the order of $1\text{ }\mu\text{m}$ and that the probability of the formation of a primary lesion is a function of the distance of the sublesions.

Neary and his co-workers (Neary, Savage and Evans, 1964; Neary, Preston and Savage, 1967; Neary, 1967) used X-rays of energies down to 1.3 kV and deduced that chromatid aberrations in *Tradescantia* resulted from the interaction of two damaged structures of site radius of about $0.2\text{ }\mu\text{m}$. In developing a model to explain the action of ionizing radiation on eukaryotic cells Chadwick and Leenhouts (1973) have proposed that the critical lesion is a DNA double strand break and the sublethal lesion a single strand break and they provided a description for the formation of chromosomal aberrations from these lesions. (1978). This suggests that a chromosome aberration results from lesions in two chromatid arms, a radiation induced lesion in one chromosome arm combines with a repair enzymatically induced lesion in the chromatid arm of an undamaged homologous chromosome in close association. They postulate that rearrangements will only arise in regions of the chromosome which can associate with homologous DNA from other chromosomes or a different part of the same chromosome. These assumptions are based on information that

shows that a large proportion of the DNA in eukaryotic organisms is made up of rapidly reannealing and repetitive heterochromatic DNA (Yunis and Yasmirch, 1971) and that there is a high incidence of breaks in heterochromatic DNA. (Kihlman, 1966; Evans and Bigger, 1961; Natarajan and Ahnström, 1970). The size of the target proposed by Chadwick and Leenhouts is small and of the order of 1-10nm, much smaller than Kellerer and Rossi's target of 1 μm . Goodhead and Thacker (1977) have tried to discriminate between the two models by the use of aluminium ultrasoft X-rays on mammalian cells in culture. These X-rays give rise to electrons of ranges of $\approx 0.07\mu\text{m}$ which are small compared to the subcellular dimensions of interest. Results showed that the effectiveness of aluminium X-rays for inactivation of Chinese hamster cells and human diploid cells (Cox et al. 1977) is much greater than that for gamma rays which would suggest that the target size is less than 1 μm , and therefore is in contradiction to Kellerer and Rossi's model and favours Chadwick and Leenhouts. More recently, Goodhead and Thacker (1980) used accelerated heavy ions of 20 to 470 keV μm^{-1} to induce inactivation in cultured V79 Chinese hamster cells. Their results could be explained on the hypothesis that each lethal lesion is produced by a deposition of such energy in a distance of about 3 nm which is similar to the size of the DNA double helix.

There is much evidence in favour of regarding DNA as one of the primary targets of radiation damage. (Terasima, 1969). The possibility of DNA repair as a determinant of cellular radiation sensitivity is also supported by the observations that enzymes have been isolated from rat liver (Paynes, 1974) and calf thymus (Bacchetti and Benne, 1975) which have a high affinity for X- or gamma-ray irradiated DNA. Evidence supporting the hypothesis put forward by Alper (1971) that both DNA and membrane may form a critical site for radiation action in mammalian cells has come from studies of the sedimentation properties

of DNA and associated structures in sucrose (Elkind and Kamper, 1970). It has been suggested that small amounts of absorbed radiation may detach DNA from the nuclear membrane causing sublethal damage and that recovery from sublethal damage involves reattachment of DNA to the membrane (Ben Hur and Elkind, 1975). Bryant and Parker (1977) observed that recovery from sublethal damage in *Chlamydomonas* after X-rays was inhibited by a small dose of UV but that this block was removed by photoreactivation. UV light in the form of pyrimidine dimers presented a block to the repair of cells damaged sublethally by ionising radiations and since this block could be removed by photoreactivation it was suggested that the site of accumulation and repair of sublethal damage is in the DNA.

1.6 . Stem cell multiplicity

One major problem with studying low dose effects in tissues such as the gut is that each crypt contains a population of approximately 250 cells, 135 of which are rapidly dividing. Since the method of scoring involves scoring whole crypts rather than individual cells, in order to obtain a dose effect relationship it is necessary to reduce crypt cell number down to 1 and this necessitates the use of relatively high doses of radiation. Also in order to study the amount of recovery from sublethal damage it is necessary to eliminate the repair element which is due to repopulation of the crypts through cell division. Four methods have been described to study low dose effects on repair of sublethal damage on rapidly dividing tissues such as skin and gut:-

1. By comparing the effectiveness of a dose given in multiple fractions as opposed to a large single dose and correcting for repopulation (Field et al., 1975).
2. The method described by Wambersie and Dutreix (1974) which compares the effectiveness of fractionated doses with the effects of pairs of fractionated doses given in the same overall time so that

repopulation is the same for the two treatments and the difference can therefore be attributed to recovery from sublethal damage between the pairs of doses.

3. A technique used by Withers (1975) in which a single dose or pairs of fractions is followed by a large single dose of neutrons to reduce the crypt cellularity down to countable levels.

4. A technique used by Denekamp (1969) in which a large dose of X-rays (assay dose) is given to reduce the number of surviving cells to assayable levels when the split dose to give the same amount of damage as a single dose can be measured.

Observations on human and mouse skin using these techniques have shown that the amount of recovery from sublethal damage increases with increasing dose. Dutreix et al. (1973) who investigated repair in human skin cells after fractionated treatments, showed that after doses per fraction of less than 300 rads the amount of recovery from sublethal damage was very small but increased as the dose per fraction was increased. Field et al. (1975) investigated the response of mouse tail skin to multiple fractions and concluded that at low doses there is a significant component of irreparable damage, but it was not clear whether this was due to a high LET component of the X-irradiation or to a variable sensitivity of the cells of the skin as they move through the cell cycle. Field and Hornsey (1977) in multiple fractionation experiments on mouse lung and skin observed an increase in the total dose with increase in fractionation and after adjusting for repopulation this increase in the total dose was concluded to be due to recovery from sublethal damage and was similar in lung and skin. Denekamp et al. (1969) studied the recovery of mouse skin cells after fractionated doses of X rays with various time intervals. The variation in the amount of recovery depending on the time interval between fractions was attributed to movement of the cells into phases of different sensitivity in the cell cycle of cells which had been partly synchronised by the first dose.

Results on the effect of fractionated doses of X-rays on mouse intestine obtained by Wambersie and Dutreix agreed with their own observations on skin and indicated that the amount of recovery from sublethal damage was very small for doses of 300 rads and under. They used the LD50 of mice scored at 20 - 30 days after abdominal irradiation as an end point and compared the effectiveness of pairs of doses of 177 - 640 rads of X-rays separated by time intervals of 1 - 6 hours with single doses of 320 - 1004 rads.

Withers (1975) studied recovery in the intestine after low doses of X-rays using crypt survival as an end point. He compared the effectiveness of 3 dose fractions separated by 3 hours with a single dose both followed 4 hours later by a dose of 650 rads of neutrons to reduce cellular survival down to levels readily countable. He obtained evidence of recovery from sublethal injury down to doses of 75 rads, although the recovery from sublethal damage fell sharply after doses below 250 rads.

1.7. The effect of cell cycle distribution

A major problem in determining the shape of the survival curve at low doses in asynchronous tissues is that the survival curve is the production of a composite of many survival curves relating to cells in different stages of the mitotic cycle (Figure 1.3). Biological response to radiation varies dramatically through the cell cycle both in repair capabilities, mitotic delay and the dose required to produce a lethal event, i.e. the D_0 (Sinclair and Morton, 1964). So the assessment of recovery from fractionation experiments with different fractionation regimes using different assay procedures may vary because of differences in the degree of induced synchrony and its staging.

1.8. X-irradiation - Physical factors

An X-ray beam as it passes through matter interacts with orbital electrons or atomic nuclei and 3 kinds of interaction occur.

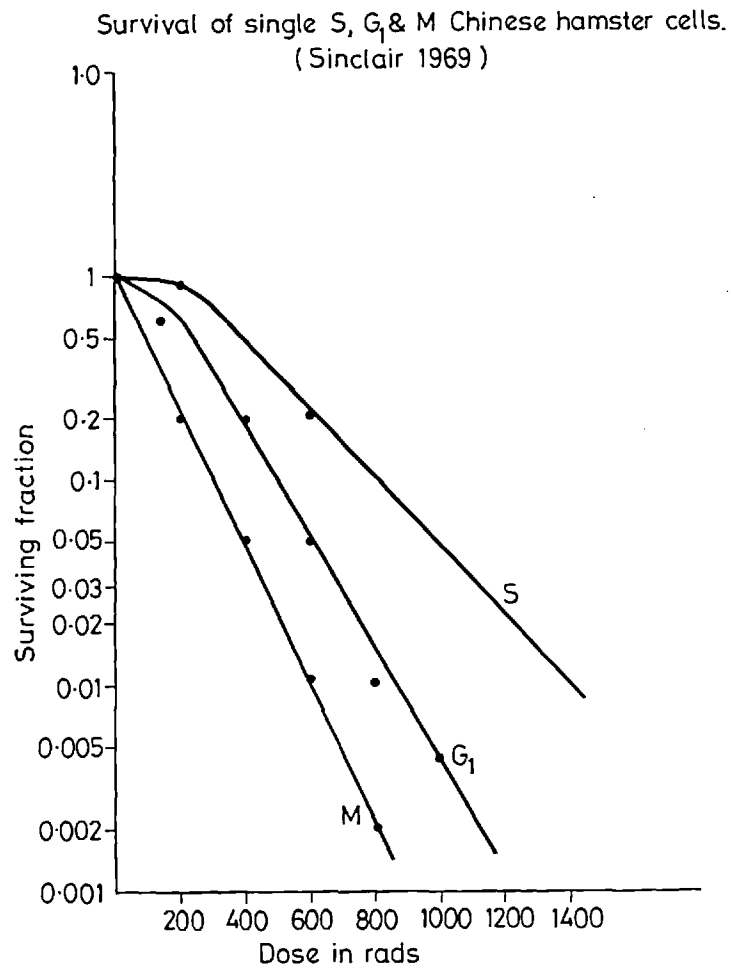


Figure 1.3. Cell survival curves plotted against X-ray dose in rads for Chinese Hamster cells in the G₁, S, and M phases (Sinclair 1969a).

1. Photoelectric absorption in which a photon gives up all of its energy to an orbital electron which is then ejected from the atom. This high velocity electron will then give rise to ionisations and excitations.
2. Compton scattering, a photon gives up only a portion of its energy to an orbital electron which is ejected from the atom at high velocity. The photon now of lower energy is deflected from its previous path and may become involved in further interactions.
3. Pair production, high energy photons interact with atomic nuclei to create an electron and a positron, any excess energy remaining after the creation of these particles being carried off as kinetic energy. The positron at the end of its track may combine and annihilate an electron producing gamma rays.

These interactions will occur to a greater extent in dense materials with a high atomic number such as bone, and less in less dense materials of low atomic number such as normal soft tissue. As a result of these interactions the emergent beam is less intense than the incident beam. There is also a certain amount of scattering of X-rays as the beam passes through a substance, the electrons are set in vibration and radiate X-rays in all directions.

1.9. Neutron irradiation

Neutrons are being tested increasingly in Radiotherapy. It is expected that a therapeutic gain will be obtained because the hypoxic cells in tumours should be killed to a greater extent than with a radiobiologically equivalent dose of X-rays. However, the neutron dose will be limited by the normal tissue tolerance to neutrons and this will be governed in part by the extent of recovery from sublethal damage deposited by neutrons during the fractionation of neutron dose.

1.9.1. Physical factors

Fast neutrons interact with biological material with the production

of protons and to a smaller extent heavy recoil nuclei of carbon, nitrogen and oxygen and products of nuclear reactions such as α particles. Figure 1.4. shows the energies of the different particles that are produced. When a neutron strikes a hydrogen nucleus which contains a single proton which has a mass almost identical to that of the neutron, anything up to the whole of its energy can be transferred to the proton. However, when it strikes a much heavier nuclei it is more likely to rebound without losing much energy. Since biological material is composed of largely hydrogenous matter about 85% of the energy absorption takes place via recoil protons. As a consequence of the greater loss by interactions with protons different tissues will have a different energy absorption depending on their hydrogen content. Consequently the energy absorption is greatest in subcutaneous fatty tissues and least in bone which is the reverse of conditions with 250 kVp X-rays (Bewley, 1963).

1.9.2. Recovery from sublethal damage after neutrons

The extent of sublethal damage after neutron irradiation has not been fully established. Some investigations with fission neutrons (Railton et al., 1975) and fast neutrons (Durand and Olive, 1976) have demonstrated that no repair of sublethal damage occurs between fractions. Repair after fission neutrons has however been observed in Chinese hamster cells (Ngo et al., 1977) and after fast neutrons in Ehrlich tumour cells (Hornsey and Silini, 1962). Densely ionising radiations such as neutrons contain a densely ionising component (the heavy recoil particle) which will lethally damage a cell whenever a track penetrates the nucleus (Giles, 1943). This component of dose will be increasingly important with fractionation of neutron dose and may account for the reduced dependence on fractionation of dose observed for response to neutrons (Hornsey et al., 1965). This component of dose will also be increasingly important with protracted low dose rate neutron doses and may be particularly important in comparisons between different neutron beams

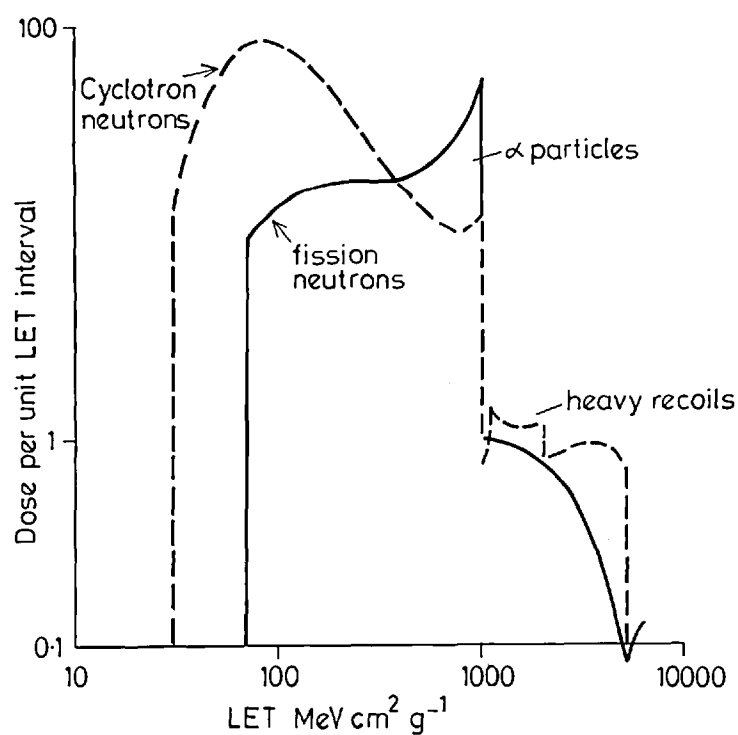


Figure 1.4. Distributions of LET of fast neutrons produced by bombarding a thick beryllium target with 16 MeV deuterons. (Bewley, 1968).

if dose rates vary considerably. Some information on the effect of dose rate is included in this thesis.

Despite the killing effect of the densely ionising secondary charged particles from neutron irradiation, sublethal damage will be deposited by the proton component of the dose and this sublethal damage will be observed in those cells that are not lethally damaged. This contribution to cell damage may be difficult to detect in an invitro cell system which generally do not accumulate as much sublethal damage as cells in vivo. It was considered that studies on this component of damage might be more easily undertaken in a quantitative tissue system. Further, the interaction of sublethal damage deposited by neutrons may be particularly important in interactions with X-rays when part of the therapy regimes are devised using both neutrons and X-rays. Studies on such interactions are included in this thesis.

1.10.. Interspecies comparison of intestinal radiosensitivity

The extrapolation of radiobiological data from mouse to man is a questionable procedure. By comparing the radiosensitivity of different species some idea of the uniformity among species response can be obtained. For studies on skin reaction the RBE response of four species, mouse, rat, pig and man was the same and give the radiobiologist confidence in extrapolating from mouse to man. Minor species differences have been observed in other tissues where the data species comparison is poor. In this thesis some data on the sensitivity to X-rays and neutrons on the hamster intestine is compared with mouse and with other data in the literature.

SECTION 2.

2. Materials and Methods

2.1. Experimental animals

Female CFLP mice aged 6 - 12 weeks and male Chinese hamsters aged 3 - 4 months were used. They were maintained on Diet PRD (Labsure Diets) and tap water ad libitum. Hamsters were given an additional diet of sunflower seeds. The animal rooms were maintained at a constant temperature and humidity and on an approximate 12 hour light and 12 hour dark cycle. Irradiations were carried out between 10.00 - 12.00 hours whenever possible to minimise any differences due to diurnal variation in sensitivity (Hendry, 1975).

2.2. Irradiation procedure

2.2.1. X-rays

Irradiations were carried out with X-rays produced from a Philips or Marconi set to give photons with a maximum energy of 250 KeV. A filtration of 0.5 mm of Copper and 1 mm of Aluminium was used, with a half value layer of 1.3 mm of Copper, to remove the lower energy photons and this gives a more uniform dose distribution by reducing the fall off in dose with depth of penetration.

When single and one pair of fractions were given the exposures were to the whole body of 4-6 unanaesthetised mice breathing air held in well ventilated Plexiglass boxes measuring 20 x 20cm. When multiple fractions were given the abdomen only was irradiated, the rest of the body being shielded with 3 mm of lead. The animals were held in position by a method first described by Wambersie and Dutreix (1974) which involves restraining the unanaesthetised mice by clamping the feet and paws with padded wooden clothes pegs. This method was found to be fairly satisfactory although after multiple fractions some of the animals suffered some injury to their front paws. Consequently for the 5 fractions in 5 days and 10 fractions in 12 days the animals were

anaesthetised with an intraperitoneal injection of 0.9 mg. of Sagital per gm. of mouse to avoid pegging.

X-ray dosimetry -

(a) Ionisation Chambers. The radiation dose emitted by the X-ray machine was measured by means of an ionisation chamber calibrated in Roentgens. The Roentgen is a unit of exposure in dry air and is converted to the absorbed dose in tissue (rad) by multiplying by 0.96 (ICRU handbook).

The normal distance of the mouse box was 30 cm from the source to give a dose rate of approximately 240 rads per minute. Differences in dose rate of 550 rads per min down to 4.91 rads per min were obtained by altering the tube current or the effective distance from the source. Dose rates of 5.0 and 2.54 rads per min were obtained from a Cobalt 60 source.

(b) Lithium Fluoride. The use of Lithium Fluoride chips measuring $1/9 \times 1/4 \times 0.035$ " enables the radiation absorbed dose to be measured in situ so that the exact dose to the tissue under investigation may be determined. Lithium Fluoride (LiF) has a regular crystal structure but when impurities are added imperfections arise in the lattice. When LiF crystals are irradiated energy is absorbed from the radiation beam and some of the electrons in the LiF crystals are raised to higher energy levels. Most electrons immediately return to the ground state but a few remain trapped in the impurity levels. Subsequent heating elevates these electrons to still higher levels from which they can return to the ground state and emit light in so doing. The total light emitted is proportional to the energy absorbed from the radiation. LiF is approximately tissue equivalent, essentially independent of dose rate and capable of detecting a few mR to 10^5 R.

Dosimetry for whole body mouse box

1. A Farmer Baldwin dosimeter was placed in a midpoint position

in a bolus filled whole body mouse box. The number of Roentgens/min measured were 250.7 which were converted to absorbed dose or rads/min by multiplying by 0.96 to give $240.7 \pm 2\%$ tissue rads/min.

2. Lithium Fluoride chips were placed inside the jejunum and ileum of four animals under anaesthetic and the animals were allowed to recover before placing in the mouse box. The dose in tissue rads obtained in a 40 second exposure was used to determine the dose in rads/min. The mean dose received by the jejunum was $197 \pm 5\%$ rads/min while the dose to the ileum was $213 \pm 5\%$ rads/min.

Abdomen only jig

1. A Farmer Baldwin dosimeter was placed inside a bag of bolus and placed in each of the four positions on the jig. The number of Roentgens/min measured was 144.1 giving a dose in rads/min of $138.3 \pm 4\%$.

2. Lithium Fluoride dosimeter chips were placed in the jejunum and ileum of four mice which were pegged out on the jig with the abdomen uppermost. The dose received by the jejunum and the ileum was 146.1 rads/min. ($\pm 5\%$).

2.2.2. Neutrons

Fast neutrons were generated by bombarding a thick sheet of beryllium with 16 MeV deuterons from the MRC 45in cyclotron. The resultant beam radiates in all directions but is most intense in the forward direction, with the greatest intensity of the radiation at around 6 MeV. Mice were irradiated laterally in Plexiglass compartmentalised boxes, each compartment measuring 8 x 3.5 cm. and ventilated by holes in the side and lid.

Neutron dosimetry -

The radiation dose was determined for each neutron session by measuring the recoil protons produced in an air-filled polythene walled pencil chamber placed in the jig before each session. This chamber was regularly calibrated against a tissue equivalent gas filled ionisation

chamber in order to obtain the tissue absorbed dose. The mouse box was placed 117 cm from the target with no collimation, which is the normal distance used for therapy, to give a dose rate of 70 rads/min. Difference in dose rate of 2.25 K rad/min down to 2 rads/min were obtained by altering the distance from the target or by reducing the beam current from 100 amps to 12 amps (Table 2.1). The maximum distance from the target used was 150 cm and the minimum distance was 17 cm. The gamma contamination of the beam close to the target was approximately 10% due to gammas produced in the steel collimator. The fall off of dose across the animal which is approximately 10% at 117 cm, will be greater at 17 cm and will add to the variability in the absorbed dose.

2.3. Tissue Assay Techniques

The problem of studying low dose effects in normal tissue is that most measurable endpoints of tissue damage require large doses of radiation before a response is observed. In the small intestine, if crypt survival is used as the endpoint, doses greater than 1000 rads of X-rays and 500 rads of neutrons are needed to reduce the number of crypts to below normal levels. This is due to the presence of a population of stem cells, all of which must be killed if the crypt is not to repopulate. Hagemann et al. (1971) investigated the number of cells per crypt that would be required to repopulate a crypt and deduced that a crypt with a single surviving cell will regain its cellularity independent of the magnitude of the exposure. After a large enough dose of radiation a proportion of the crypts will be completely depopulated of stem cells and consequently fewer than the normal number of crypts per circumference will be observed at 4 days. The reduction in crypt number with increasing dose may be plotted to give a crypt survival curve and from this cell survival can be estimated.

TABLE 2.1.

Beam current and effective target distance for varying neutron dose rate

<u>NEUTRONS</u>		<u>Beam current</u>	<u>Effective target</u> <u>distance</u>	<u>Dose rate in rads/min</u> <u>at centre of mouse bo</u>
Cyclotron	6MeV	100 μ A	17 cm	2.2 Kr
	average	100 μ A	117 cm	70 rads
	"	35 μ A	117 cm	20
	"	17 μ A	117 cm	10
	"	25 μ A	150 cm	5
	"	12 μ A	150 cm	2

2.3.1. Crypt survival assay

Survival of crypts was measured here by counting the number of viable crypts per circumference in sections of intestine at 4 days as described by Withers and Elkind (1970). A crypt was assumed to be viable if it contained 10 or more basophilic cells. Mice were killed by cervical dislocation and 2 segments of gut approximately 2cm long were removed, one from the mid-jejunum and the other from the ileum approximately 5cm from the ileocaecal junction. When the gut sections were to be analysed for crypt survival they were fixed in Bouins fluid for 24 hours and then transferred to 70% alcohol, passed through an increasing concentration of alcohols to xylol and then into paraffin wax. The gut sections were then cut into 4 pieces which were set into blocks of wax from which sections of approximately 5 μ were cut. The sections were stained in Haematoxylin and Eosin and mounted in Euparal. The number of surviving crypts were scored from 4 gut sections per slide, each section being selected from a different piece of gut. The sections were selected on the basis of staining quality and completeness.

2.3.2. Cell survival assay

Due to the random nature of lethal events by ionizing radiations there will be a proportion of surviving crypts with 1, 2, 3 or more surviving cells after a lethal exposure. As a consequence a crypt survival curve will not be equivalent to a cell survival curve particularly at the lower dose range. A cell survival curve can be obtained from a crypt survival curve by making a correction for the likelihood of more than 1 cell surviving in a crypt. This calculation is based on a number of assumptions:- 1. that cells survive independently of one another (Elkind and Whitmore, 1967); 2. one cell is sufficient for regeneration of a crypt; 3. the number of survivors per crypt is distributed according to a Poisson distribution. Assuming these statements to be correct then the average cell survival per crypt = $-\ln \frac{135-x}{135}$

where x = the number of crypts surviving per circumference

n = natural logarithm

135 = the number of crypts per circumference in the jejunum of unirradiated mice used in this study. Surviving fractions were obtained by assuming there were 135 proliferating cells per crypt in the jejunum and in the ileum. ^(Hendry and Potten 1974) Recent work using split dose experiments have indicated that the cryptogenic stem cells are present in the crypts in numbers lower than those of proliferative cells, the errors are large on the estimated numbers of 28-268 (Potten and Hendry, 1975) when a mean number of 47 was estimated. Because of this uncertainty in stem cell number it was assumed here that the cryptogenic cells were the same as the proliferative cell population. Also, Hornsey (1973) found good agreement between D_q (Alper, 1962) of the cell survival curve estimated by using the proliferative cell number and $D2-D1$ indicating that the approximate stem cell number and the proliferative cell number are not very different.

Survival curves were fitted to the surviving fractions using least squares regression analysis in which the mean value was weighted according to the inverse of the square of its standard error.

Standard Errors (see also Appendix I).

In order to determine the correct formula for the calculation of standard errors a dispersion test was used to determine whether the number of crypts per circumference and also, for the assay of other end-points, the mitosis per crypt counts were distributed according to a Poisson distribution. The goodness of fit was tested by the Chi square statistic:-

$$\chi^2 = \sum_{i=1}^n \frac{(x_i - \bar{x})^2}{\bar{x}} \quad \text{on } n-1 \text{ degrees of freedom}$$

where χ^2 is very small the distribution of events is underdispersed and, if large, is overdispersed compared with a Poisson. The χ^2 for the crypts per circumference data was very large, while mitoses/crypt and

cells/crypt were a good fit to a Poisson distribution. In order to correct for the deviation of the crypts per circumference from a Poisson distribution the Poisson Standard error calculation where

$$S.E. = \frac{\text{mean no. of crypts/circumference}}{\text{no. of observations}}$$

was multiplied by a heterogeneity factor S_2 where

$$S_2 = \frac{\chi^2}{n-1} (\text{dispersion index})$$

to allow for over dispersion which was due to variation between animals. There was some variation in the numbers of crypts/circumference within animals but it was no greater than would be expected to occur by chance.

2.3.3. Crypts/mg assay - a comparison of methods

Difficulties are sometimes encountered when scoring surviving crypts from crypts from an histological section. Problems caused by branching and hypertrophy of crypts after irradiation have made some experimenters choose other methods for determining the number of crypts that have survived in the intestine.

Hagemann et al. (1971) determined the total proliferative cellularity of the intestine at 3 days after irradiation by measuring the amount of tritiated thymidine incorporated when total cellularity was found to be directly proportional to crypt survival. Cairnie (1977) developed a method which involved scoring the numbers of crypts/per mg of intestine at 3 days after irradiation. He found that this method overcame the difficulties found in scoring sectioned pieces of gut and more reliable estimates of surviving crypts could be obtained. In order to determine the most reliable method for determining crypt survival a comparison was made in this study of the proportion of surviving crypts after doses of 900-1800 rads of X rays using the method described by Cairnie and the microcolony technique of Withers and Elkind. After irradiation there is a large variation in the

size of regenerating crypts due partially to differences in the number of surviving cells in the crypts. One of the problems associated with the Withers method is that when crypts are sectioned, the larger the crypts the greater is the likelihood that they will be scored. This problem was allowed for by Potten and Hendry (1975) by introducing a correction factor, e.g. 0.840 at 4 days. No allowance was made here for changes in the crypt size after irradiation since it was found that crypt size at 4 days was dose dependent and no single factor could be applied.

Method

CFLP mice were irradiated with X-ray doses of 1200-2000 rads and killed at 3 and 4 days. Sections of gut to be analysed by the microcolony technique were fixed in Bouins and those sections of gut to be assayed for crypts/mg were fixed in 3:1 alcohol:acetic acid. After fixation of gut pieces in 3:1 alcohol:acetic acid the tissue was stained in Feulgen by the method described in Section 8. A piece of gut was cut out and weighed and then the villi were removed by scraping off with a needle. When the muscle layer was removed it was possible to observe crypts under a dissecting microscope. Counts were made of the number of surviving crypts per gut sample and the number per mg. was determined. Difficulty was encountered at 3 days in assessing whether a crypt was a survivor or not due to the very small size of some of the crypts. By day 4 the crypts had increased in size and could be scored unambiguously. Crypts/mg were converted to cell surviving fractions by applying a Poisson correction and are plotted in figure 2.1. together with the surviving fractions obtained from crypts per circumference data.

Results

Figs 2.2 & 2.3. show the surviving fraction of crypts per circumference in the jejunum and ileum at 3 and 4 days using the Withers microcolony method. A lower level of survival was observed at 3 compared with 4 days, using the Withers microcolony method, the greatest difference being observed

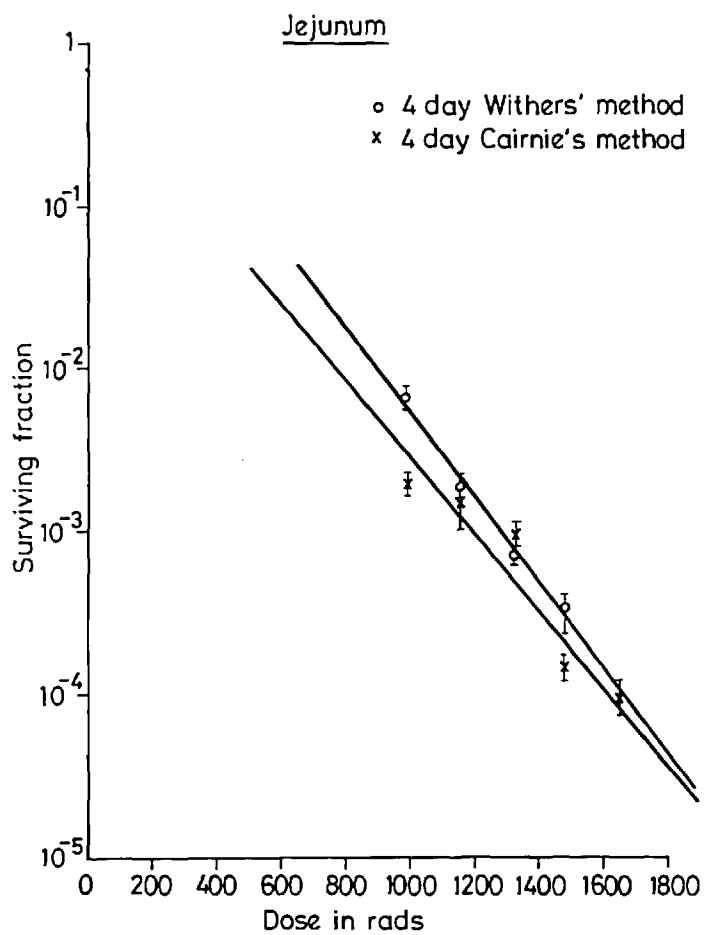


Figure 2.1. Cell survival curves for jejunal crypt cells determined from crypt/mg and crypts/circumference tissue assay methods 4 days after whole body irradiation with 250 kVp X-rays.

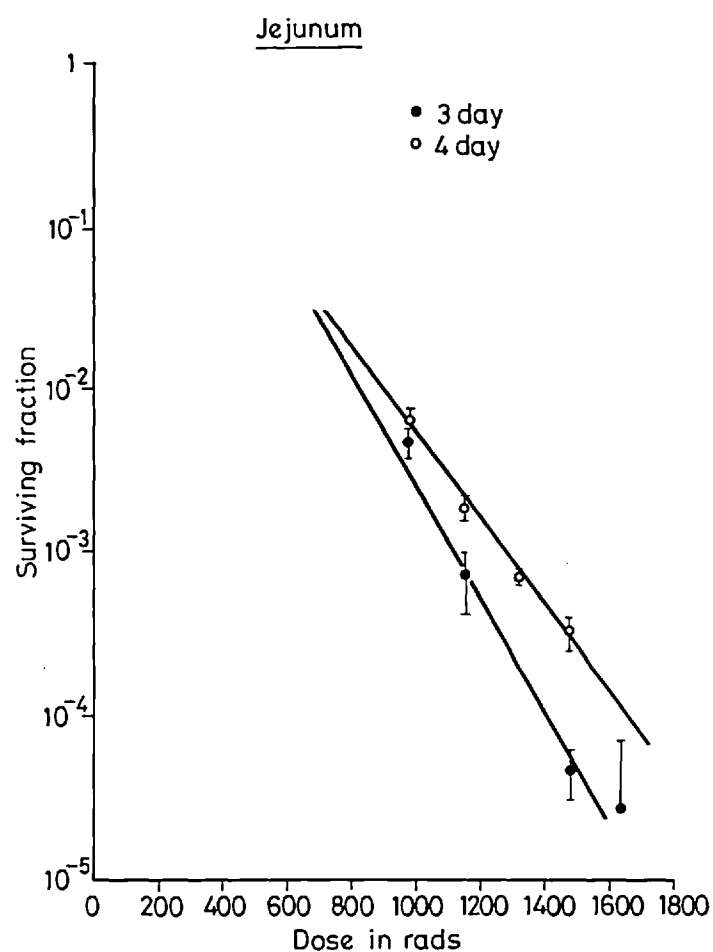


Figure 2.2. Cell survival curves for jejunal crypt cells 3 and 4 days after whole body irradiation with 250 kVp X-rays.

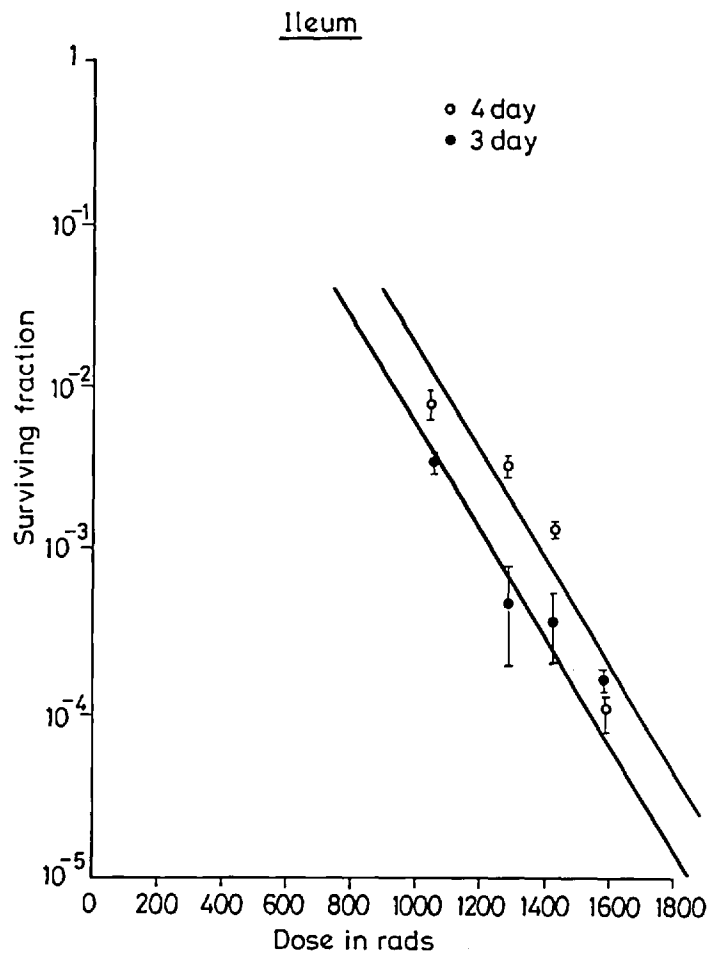


Figure 2.3. Cell survival curves for ileal crypt cells 3 and 4 days after whole body irradiation with 250 kVp X-rays.

at the highest dose in the jejunum. This is possibly due to the fact that at high doses the crypts take longer to repopulate due to a longer mitotic delay, than crypts that have received a lower dose. By day 4 the crypts had fully repopulated and were readily scorable. Figure 2.1. shows the surviving fractions at 4 days for the crypts per circumference and the crypts per mg technique. The main difference between the two methods is observed at the lower dose range and this may be due to the difficulty which is encountered in scoring the large numbers of crypts after low doses of X-rays from whole pieces of tissue. It was concluded that although the Cairnie method was a good method for obtaining a rapid crypt count it was not as reproducible as the Withers method, which was shown to be a reliable method for comparing the effects of different treatments provided one is aware of the inherent problems caused by branching crypts.

2.4. Comparison of whole body versus abdomen only irradiation procedures

Irradiations were normally administered to the whole animal as this was a more convenient and simpler procedure than irradiating the abdomen only. In order to determine if there was any significant differences between whole body and partial body X-irradiation a comparison was made of the number of surviving crypts per circumference using the two different irradiation procedures.

Method

Mice were irradiated with doses of 1200-2000 rads of X-rays and the dosimetry was determined using a Farmer Baldwin dosimeter placed in the midpoint of a tissue equivalent bolus filled mouse box for the whole body irradiations and in the midpoint of a mouse shaped phantom for the abdomen only irradiations. There was found to be more survival after whole body compared with abdominal only irradiation and the difference was significant at the 5% level. ($P = 0.001$). Since this result was in disagreement with the findings of Withers^{& Elkind} (1970), Hornsey (1973) and Hamilton (1977) a further investigation of the dosimetry was made.

Lithium Fluoride chips were placed inside the jejunum and ileum and after a 40 second exposure of X-rays it was found that a lower dose was measured when the animals were irradiated in the whole body mouse box than in the abdomen only jig. When mice are positioned in the jig for abdomen only irradiations they were irradiated ventro-dorsally and in this position the small intestine lies in a position approximately midpoint in the mouse and received a dose only slightly higher than that calculated from the Farmer Baldwin measurements. When the mice are irradiated in the whole body jig they are irradiated dorso-ventrally, here the ventral surface of the mouse is in direct contact with the base of the mouse box and the small intestine is situated just below this, well below the midpoint position of the mouse box. Consequently the lower dose measured using the Lithium Fluoride dosimeter chips in situ compared with the Farmer Baldwin ionization chamber in the whole body mouse box could be due to two effects. 1. A fall off in the intensity of the X-ray beam as it passes through the mouse. 2. A reduced amount of scattering of X-rays when 4-6 mice are irradiated compared with tissue equivalent bolus in the mouse box.

Results

After corrections in the dosimetry were made there was found to be more survival after abdominal only irradiation compared with whole body irradiation and the difference was significant ($P = 0.02$). Best fitting lines for the cell surviving fractions are shown in figure 2,4.

Discussion

Hamilton (1977) observed an increase in crypt survival in the mouse colon after irradiating the gut only as opposed to the whole animal and attributed this difference to the presence of a repair promoter in the blood which would be destroyed or not synthesized after whole body irradiations. It was suggested that the lack of any protective effect from partial body irradiation observed by Withers & Elkind (1970) and Hornsey (1973) using the macro-

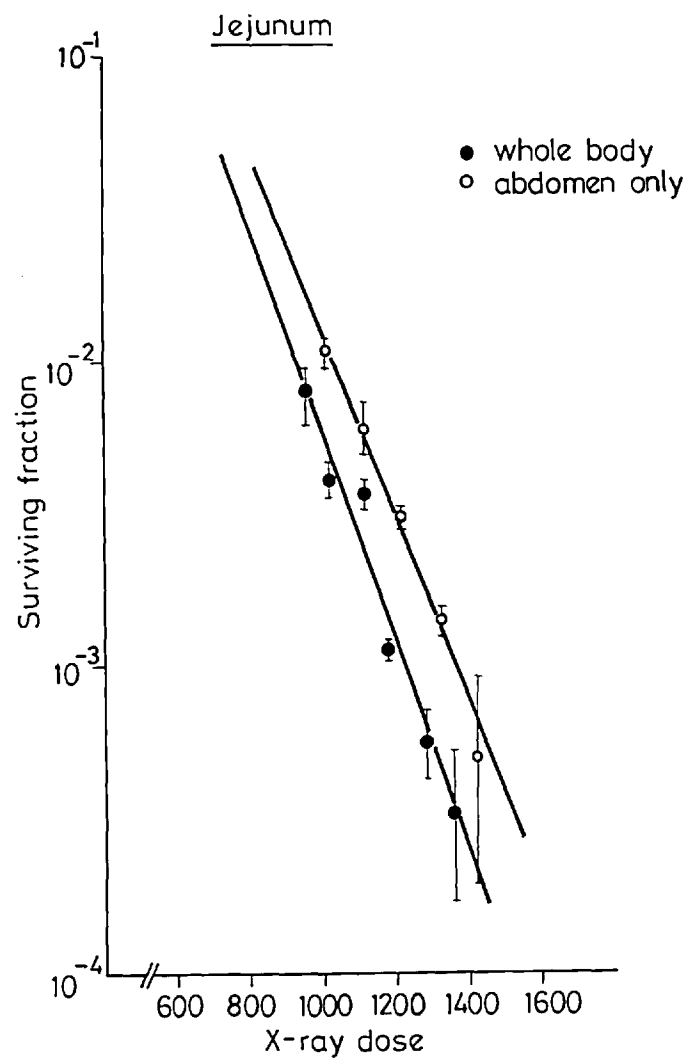


Figure 2.4. Cell survival curves for jejunal crypt cells
4 days after whole body and abdomen only irradiation.

colony (exteriorized gut only) and microcolony (whole body) assay techniques may have been due to the shock induced by the operation of gut exteriorization affecting circulation (Hamilton 1977).

2.5. Cell cycle time analysis

Since the S phase is known to be one of the most resistant phases in the cell cycle (Sinclair, 1969; Gillette et al., 1974), any variation in the length of this phase in different tissues could affect the response of that tissue to irradiation. The proportion of cells in the DNA synthesis phase can be readily determined by means of tritiated thymidine labelling and was determined here for jejunum and ileum cells of the mouse and hamster.

Method

Female CFLP mice and male Chinese hamsters were injected intraperitoneally with 30 μ Ci of tritiated thymidine (specific activity 5 Ci/mMole) and killed 20 minutes later. A second group of mice were given 2 injections 1 hour apart and killed 20 minutes after the second injection. The uptake of tritiated thymidine is rapid and within approximately 5 minutes of intraperitoneal injection, cells with labelled nuclei are observed and by 10-20 minutes all the cells synthesizing DNA are labelled. (Quastler and Sherman, 1959).

Sections were made according to the method described for crypt survival analysis except that the tissue was fixed in 3:1 alcohol:acetic acid since Bouins fluid causes fogging of photographic emulsion. Autoradiographs were prepared using Kodak K2 emulsion exposed in the dark for two weeks at 4°C and developed in Kodak D19 and fixed in Kodak F5 fixer. The slides were washed in running water for 20 minutes and then air dried before staining with haematoxylin and eosin and mounting in Euparal. The number of labelled crypt cells were scored on randomised slides.

Results

The percentage of cells in the ^{mouse} crypts after a single 20 minute pulse

label was $39\% \pm 4.4$ in the jejunum, and $30\% \pm 4.6$ in the ileum. When a second injection was given 1 hour later the percentage of labelled cells was $43\% \pm 4.9$ in the jejunum, and $36\% \pm 4.7$ in the ileum. In the Chinese hamster $28\% \pm 4.5$ and $23\% \pm 3.4$ of the crypt cells were labelled in the jejunum and ileum respectively after a single pulse label.

Discussion

Cell cycle analysis of mouse intestinal cells have been carried out by several investigators and a summary of their results is shown in Table 2,2. They all used the labelled mitosis method first described by Quastler and Sherman (1959), who obtained a difference of 20% between the jejunum and the ileum in the number of cells in the synthetic phase after a pulse label, with 40% labelling in the ileum and 60% labelling in the jejunum. Assuming that the length of the cell cycle was the same for the two tissues they concluded that the difference in the percentage of labelled cells in the jejunum and ileum was due to a shorter G1 phase in the jejunum.

The results obtained in this study for CFLP mice show that there are 9% fewer cells in the S phase in the ileum than in the jejunum at any one time, while the rate of entry of cells into the S phase is faster in the ileum than in the jejunum. In the jejunum 39% labelled cells were observed at 0 hours and 43% at 1 hour, therefore 4% of the cells move into the S phase per hour. With 39% labelled at 0 hours the length of S must therefore be $39/4$ hours = 9.75 hours. In the ileum an increase of 6% labelled cells/hour are observed so with a pulse label of 30%, $30/6 = 5$ hours is estimated for the length of the S phase in the ileum. In this CFLP strain of mice therefore the length of S in the proliferative cells of the ileum is about half that of S in the proliferative cells of the jejunum. Estimates of the length of S by other authors support the observation that the length of S in the ileum is less than in the jejunum. For example, Hagemann and Stragand (1977) determined the length of the cell phases in the ileum of

TABLE 2.2.

<u>Intestinal Cell Cycle Times</u>							
	Mice	Tc(hr)	Time of day	G ₁ (hr)	S(hr)	G ₂ + M(hr)	Author
Duodenum	BCF	13.1		4.6	6.9	1.6	Lesher (1967)
Jejunum	DBA-2	14-17			5-8		Potten, Kovacs & Hamilton (1974)
	C57B1	11.1 13.1	0300 1500	1.1 3.8	8.3 7.6	1.85 1.65	Sigdestad & Lesher (1972)
Ileum	C57	18.75		9.75	7.5	1.5	Quastler & Sherman (1959)
	Ha/ICR	12.6		5.3	5.9	1.4	Hagemann & Stragard (1977)
Colon		18.2		9.9	7.1	1.2	Hagemann & Stragard (1977)
		18-20			6-8		Potten et al. (1974)

<u>% of labelled crypt cells</u>			<u>After 1hr second injection</u>	
	<u>Mice</u>	<u>L</u>	<u>U</u>	
Jejunum	CFLP	39 ± 4.4	61 ± 4.4	43 ± 4.9 58 ± 4.9
	C57	60	40	(Quastler & Sherman 1959)
Ileum	CFLP	30 ± 4.5	70 ± 4.5	36 ± 4.7 62 ± 4.7
	C57	40	60	(Quastler & Sherman 1959)
<u>Chinese Hamster</u>				
Jejunum		28 ± 4.5	72 ± 4.5	
Ileum		23 ± 3.4	77 ± 3.4	

female HA/ICR mice and obtained a value of 5.9 hours for the length of the S phase and 5.3 hours for the G1 phase, while Signestad and Lesher (1972) obtained a G1 = 3.8 hours and an S phase = 7.6 hours at 1500 hours in the jejunum of female C57 B1 mice. They also observed a difference in the length of the G1 and S phases at different times of the day with the longest G1 phase and shortest S phase being observed at 1500 hours and the shortest G1 and longest S phase being observed at 0300 hours.

These results all indicate a shorter G1 and longer S phase in the jejunum compared with the ileum in mice.

Conclusions

Dosimetry corrections

In view of the different measurements obtained from Lithium Fluoride and Farmer Baldwin dosimeters, the measurements obtained using the latter were adjusted to allow for a reduction in scatter and a fall off in dose through the mouse.

Lithium Fluoride measurements were not carried out on the hamster. In view of the fact that these animals are considerably larger than the mouse and consequently filled the whole body mouse box their doses were only adjusted to allow for an 8% fall off through the animal. No significant difference between the jejunum and ileum was anticipated since the intestine in the hamster is very short compared with that of the mouse. Consequently it is unlikely that a significant positional difference between the various regions of the gut exists in the hamster.

SECTION 3.

3.0. Early effect of irradiation on the epithelium of the small intestine

3.1.0. Introduction

Most investigations into the causes of intestinal death after irradiation are based on cell survival studies, since the ability of an intestinal stem cell to divide and replenish the villi can be expected to give a good indication of the chance of survival of the animal. The retention of an effective barrier in the intestine will, however, depend not only on the production of new villi cells but also on the rate of cell loss off the tip of the villus by normal erosion or premature death. This was first shown by studies on germ-free mice. Matsuzara and Wilson (1965) showed that germ-free mice lived longer than conventional mice after a dose of 1-3 kilorads of 250 kVp X-rays. They attributed this to the longer life span of the villi cells in the germ-free mice. These mice have longer villi and the whole cycle of stem cell turnover and differentiation and migration is slower than in conventional animals.

Factors which will affect the intestinal surface after irradiation include:-

1. the life span of the villus cell,
2. the delay induced by irradiation on the crypt cell population,
3. the migration rate of cells out of the crypts, and
4. the premitotic and mitotic death of crypt cells.

Mitotic delay and cell death in the crypts can be readily investigated with the use of a crypt squash technique and the relevance to changes in the epithelial surface assessed. In this section I report on the effects of doses of X-rays and neutrons on the intestinal surface area and the contribution to these changes of mitotic delay and reduction in crypt cellularity.

3.1.1. Surface area

Relatively few comparative studies have been made on intestinal cellularity or absorptive surface. Shiragai et al. (1976) studied the

effects of 1000 rads of 200 kVp X-rays and 200 rads of 2 MeV neutrons on the total cells in the duodenum of female CRF mice. From sections they counted villi and crypt cells at 1-10 days post irradiation and observed a difference between the neutron and X-ray irradiated villi in that the maximum number of cells was observed on day 7 after neutrons compared with day 6 after X-rays. Meder and Michalowski (1980) have also measured intestinal absorptive surface in the jejunum after 5.5 MeV neutrons and X-rays. They observed very little difference in the response pattern after 1000 rads of X-rays and 300 rads of neutrons. A clear dose response relationship for absorptive surface was obtained which gave an RBE for reduction in intestinal absorptive area of approximately 3 at 3 days post irradiation when the nadir occurred.

The use of intestinal absorptive surface area measurements as a means of assessing damage to the intestine after ionising radiations enables some of the effects of low doses to be determined, while much higher doses are required in order to assess damage by crypt survival analysis.

3.1.2. Mitotic delay

The effect of radiation in causing mitotic delay in various cell systems in vivo and in vitro has been extensively studied (Neary et al. 1959; Alexandrov 1965; Scaife, 1970; Geard and Marino, 1977 and Lloyd et al. 1977). Early experiments on sea urchins eggs indicated that the X-ray induced delay is primarily caused by damage to the nucleoprotein, while some component of the cytoplasm is necessary for recovery (Failla, 1962). Mitchinson (1974) suggested that there were two events which caused cells to fail to enter mitosis, one was damage to DNA and the other damage to certain proteins required for division, such as microtubular protein. Inhibition of protein synthesis has been shown to cause delay in cells dividing in vitro (Bacchetti and Sinclair, 1970). In the cell cycle a point is reached after which there is no delay and this is called the G2

block or transition point (Mitchinson, 1974). With irradiation delay increases as the irradiation is given closer to the transition point and the explanation given is that there is a build up of division protein which is sensitive to radiation and if it is destroyed has to rebuild again. The radiation induced block occurs at about the end of the synthesis of protein required for mitosis. After the transition point is reached the division protein is stabilised and cells irradiated after this point divide without delay. Work on synchronised Chinese hamster cells in vitro have demonstrated retention of cells in the S phase after irradiations as well as in G2 and the amount of delay in S was about 30 minutes per 100 rad dose (Yu and Sinclair, 1967). The rate of DNA synthesis was also seen to be reduced in irradiated cells, although the total amount synthesised was about the same as in unirradiated cells (Sinclair, 1968a). Experiments by Froerse and Cormack (1968), also on Chinese hamster cells in vitro, suggested that the loss of colony forming ability and part of the delay in post irradiation division resulted from the same radiation induced lesion. They studied the relationship between division delay and colony forming ability in Chinese hamster (V79) cells using time lapse cinematography. The number of cells in each micro colony was examined daily until all the cells in it had disintegrated or until it reached a count of 50 when it was scored as a surviving colony. They found that the generation time for non colony formers was longer than that for colony formers. Trott (1972) also found a relationship between division delay and damage expressed in later generations in mouse cells. He studied cells irradiated in G1 with 200 rads of X rays and found that the generation times were shorter in cells, both of whose daughter cells continue to divide, than in cells one of whose daughters failed to divide again. Mitotic delay leads to two major misfunctions of the mitotic apparatus, the degree of non-disjunction is increased by either misalignment of chromosomes on the spindle or premature anaphase separation of some

chromatids (Stubblefield, 1971). If the mitotic delay is sufficient to allow the maturation of daughter procentrioles a multipolar spindle is formed and in all these cases aneuploid cells are produced which are usually non-viable.

However, not all reports show a relationship between mitotic delay and chromosome aberrations. Grot e and Revell (1972) found no relationship between the chromosome aberration content of a cell and its delay in entering mitosis in Syrian hamster cells; a relationship was however indicated between aberration content and survival.

In this section I report on the amount of mitotic delay and cell death that is induced in mouse intestinal stem cells by doses of 100-1000 rads of 250 kVp X-rays and 20-400 rads of neutrons. This study was made in order to determine the effect of radiation induced delay on crypt cellularity during fractionation intervals of 2-24 hours and also to explain the effect observed on the villus epithelium during the first few days post irradiation. Before mitotic delay and crypt cellularity could be determined after irradiation it was necessary to determine if there was any diurnal rhythm in the intestinal stem cells of these animals under normal laboratory conditions as changes in mitotic activity have been reported by many investigators (Brown and Berry, 1968; Sigdestad et al. 1969; Potten et al. 1977).

3.2.0. Methods

Mice were killed by cervical dislocation and a segment of gut approximately 2cm long was removed from mid-jejunum.

3.2.1. Surface Area

Transverse sections of jejunum and ileum stained with Haematoxylin and Eosin were projected onto a 10" in diameter flat screen of a Projectina microscope magnified to X200. Tracings were made of the inner circumference of the gut onto acetate paper, the drawings were then scanned by a Marconi television camera which recorded the X-ray co-ordinates and this information was fed directly into the computer where the data were converted into a

measurement in millimetres.

3.2.2. Mitotic delay and crypt cellularity

The gut sections were placed on pieces of card and cut open longitudinally with the villi side uppermost, before washing in phosphate buffered saline. They were immediately fixed in 3:1 alcohol and acetic acid, 24 hours later transferred to 70% alcohol and then stored at 4°C. The tissue was hydrolysed in 5 N HCl at 22°C for 30 minutes and stained in Feulgen for 1 hour (Fox 1969). The crypt squash technique first described by Wimber et al. (1960) was used. The stained tissue was placed on a microscope slide in a drop of 45% acetic acid and approximately 30 crypts were dissected out using fine needles. The individual crypts were cut off at their junction with the villi and approximately 15 crypts squashed on each slide with a coverslip. The coverslips were removed by freezing on dry ice (Conger et al., 1953) and the slides were passed through ascending alcohols and mounted in Euparal.

All phases of mitosis and the number of cells per crypt were scored on coded randomised slides. Only cells with intact nuclei were counted which were squashed so that there was very little overlap of cells. Fibrocytes, lymphocytes and mature villi cells were excluded, but it was not possible to differentiate between other non-proliferative cells such as goblet and Paneth cells. An unirradiated crypt squash is shown in Figure 3.1.

3.3.0. Results

3.3.1. Variation in mitosis/crypt

Assuming a Poisson distribution of mitosis per crypt an analysis of variance was used to test if there was any significant variation in the numbers of mitosis per crypt in different regions of the same piece of gut, between different mice and between fixation times in control and irradiated animals. Within the control animals there was no significant difference between the numbers of mitosis per crypt observed in different parts of the same piece of jejunum, and there was no overall difference

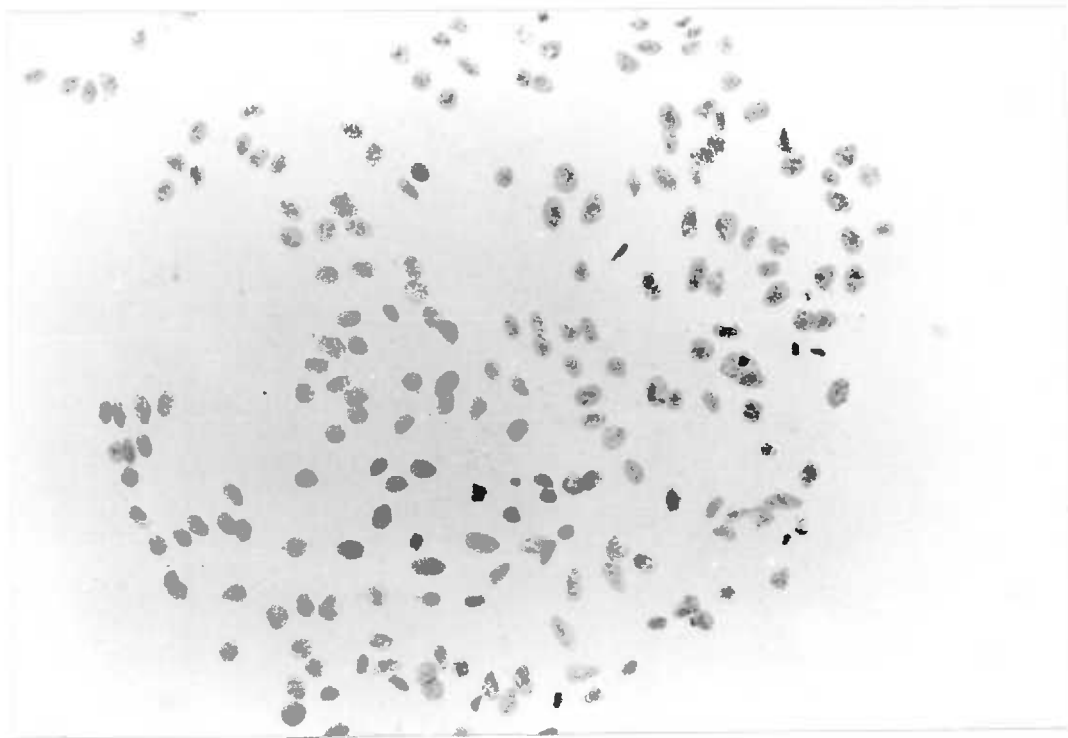


Figure 3.1.

Unirradiated crypt squash.

between mice. However, the differences observed in mitosis/crypt at different fixation times was highly significant (Table 3.1).

After X-irradiation the analysis of variance showed that with doses over 400 rad there was a significant difference within mice in the number of mitosis per crypt, while below 400 rad there was no difference within mice. After doses ranging from 100-1000 rad there was a significant difference between mice in the number of mitosis per crypt at every fixation time. After neutron irradiation with doses over 300 rad the analysis of variance showed that there was a significant difference within and between mice in the number of mitosis per crypt, while below 300 rads there was no significant difference within or between mice as was the case with the control mice. There was a significant difference between fixation times in the number of mitosis per crypt for all treatments.

3.3.2. Diurnal rhythm

Although no regular trend was evident, there was a slight indication of a biphasic rhythm with a minimum at 1800 - 2000 hours and 8-1000 hours and a maximum at 1200 and 2400-6000 hours. However, this does not show a clear diurnal rhythm (Figure 3.2).

3.3.3. Mitotic delay

Figure 3.3. shows the mean number of mitosis per crypt $\pm$ standard error after X-ray doses of 100-1000 rad from 1-96 hours after irradiation. The irradiations were given at 1000 hours. The trend was similar after all doses in that by 1 hour post irradiation there was a suppression of mitosis followed by an initial recovery probably caused by cells which had piled up in G2 coming through to divide (Mitchinson, 1974). By 24 hours the numbers of mitosis per crypt were almost at control levels, and by 4 days after 1000 rads they were twice that in control.

The number of mitosis per crypt from 1-96 hours after neutron doses of 20-400 rads are shown in Figure 3.4. The general trend was similar to that observed after X-rays although mitosis did not reach

Table 3.1. Analysis of variance on the number of mitoses/crypt

<u>Source of variation</u>	<u>Dose</u> (rads)	<u>Degrees of</u> <u>freedom</u>	<u>Sum of</u> <u>squares</u>	<u>Probability</u>
Between mice	control	9	14.09	0.12
Within mice		6	4.771	0.57
Between fixation times		8	39.0	0.005 *
<u>X-RAYS</u>				
Between mice	100	5	13.5	0.025
Between fixation times		4	61.5	0.0005*
Between mice	200	2	99.9	0.0005*
Within mice		4	5.22	0.25
Between fixation times		11	142.8	0.0005*
Between mice	400	10	79.3	0.0005*
Within mice		8	21.1	0.010
Between fixation times		10	286.1	0.0005*
Between mice	600	9	25.7	0.005*
Within mice		3	13.2	0.005*
Between fixation times		12	471.2	0.0005*
Between mice	1000	8	26.8	0.001*
Within mice		7	15.4	0.04*
Between fixation times		10	300	0.0005*
<u>NEUTRONS</u>				
Between mice	20	5	4.43	0.
Between fixation times		4	13.3	0.01*
Between mice	50	6	8.62	0.20
Within mice		1	0.07	0.79
Between fixation times		6	57.4	0.0005*
Between mice	100	6	11.91	0.09
Within mice		2	0.97	0.65
Between fixation times		5	54.2	0.005
Between mice	200	7	11.897	0.10
Between fixation times		6	31.42	0.0005*
Between mice	300	10	25.3	0.0005*
Within mice		5	30.3	0.0005*
Between fixation times		8	174	0.0005*
Between mice	400	1	5.1	0.02*
Within mice		1	5.1	0.02*
Between fixation times		5	62.4	0.0005*

* Significant at 5%.

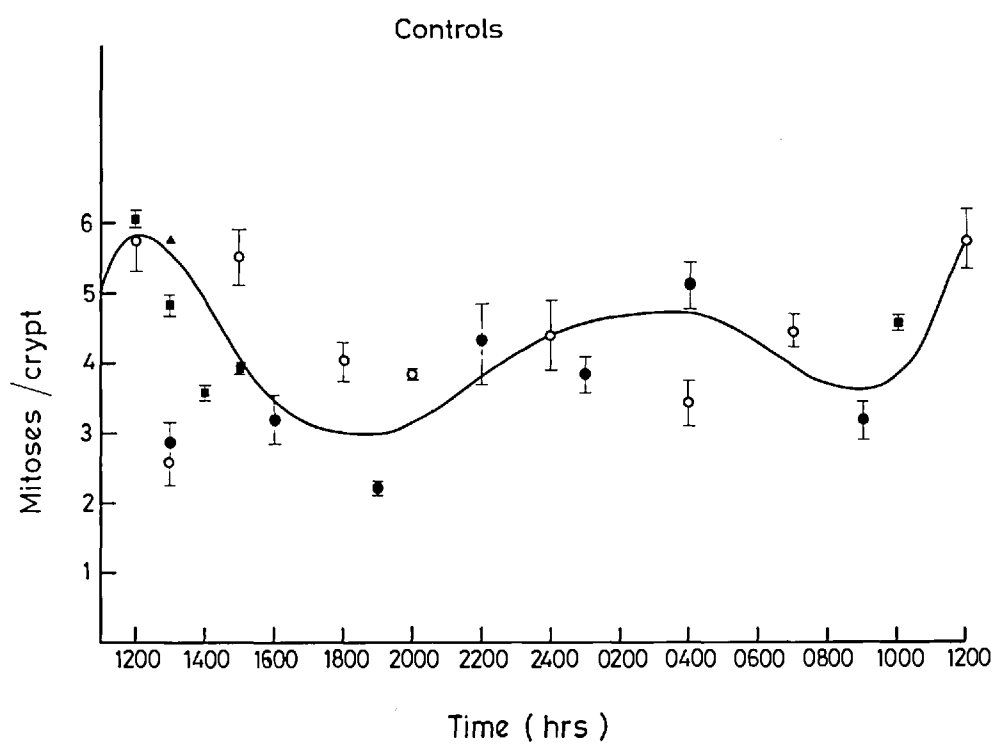


Figure 3.2. 24 hour mitotic rhythm in normal, unirradiated jejunal crypts.

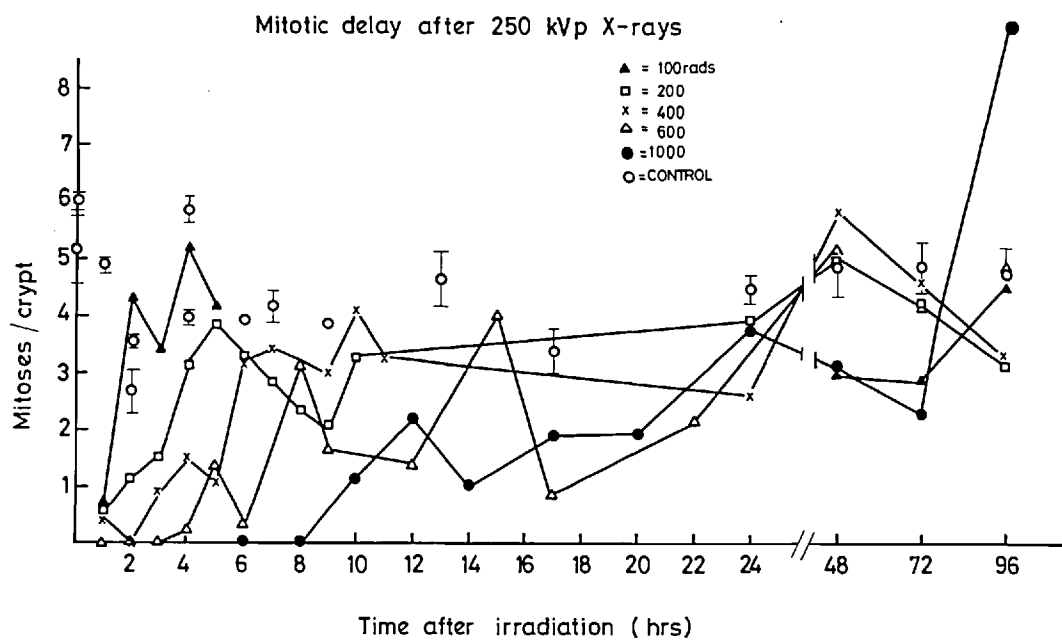


Figure 3.3. Mitosis/crypt 1 - 96 hours after irradiation with 100 - 1000 rads of 250 kVp X-rays.

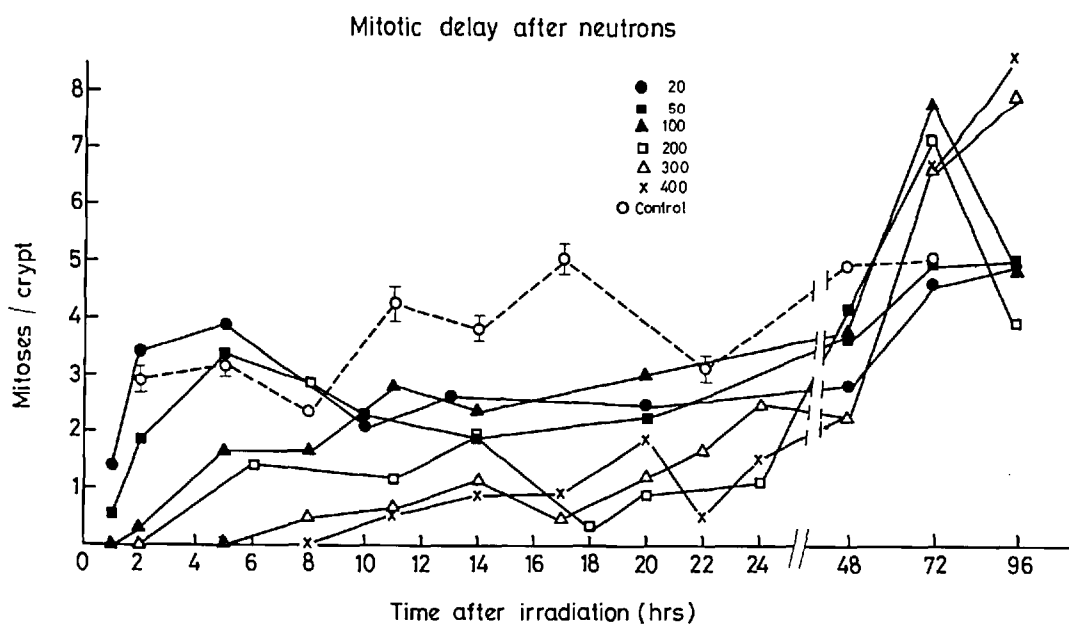


Figure 3.4. Mitosis/crypt 1 - 96 hours after irradiation with 20 - 400 rads of 6 MeV neutrons.

control levels until 48 hours. By 3 days after 300 and 400 rads there was a substantial increase in mitosis which continued to increase at day 4, while after lower doses of 20 and 50 rads mitosis had reached control levels at day 3.

The mitotic delay, defined as the time in hours after irradiation to reappearance of one mitosis per crypt, is plotted against X-ray and neutron dose in rads in figure 3.5. The relationship was approximately linear with slopes of 1.3/100 rads after X rays and 3.8/100 rads after neutrons. The RBE is therefore constant with dose and is 2.92 ± 0.15 .

3.3.4. Chromosome and nuclear abnormalities

Necrotic nuclei were first observed 6 hours after irradiation and the first mitotic figures that appeared were sticky. Anaphase bridges were observed after all doses above 600 rads of X-rays and 200 rads of neutrons (Figure 3.6). Giant nuclei caused by cells doubling their DNA but failing to divide were first seen on day 3 after 600-1000 rads of X-rays and 300-400 rads of neutrons and at all doses on day 4. (Figure 3.7).

3.3.5. Crypt cellularity

The number of cells per crypt scored from 1-96 hours after X-rays are shown in figure 3.8. The cellularity in irradiated animals showed considerable variation but at doses of 200 rads and above there was a persistent tendency for values below control levels from 1-24 hours after irradiation. By 3 days the crypt had reached near normal size and appearance except after the two highest doses (600 and 1000 rads). By day 4 very large crypts were observed after doses of 1000 rads (Figure 3.9).

The numbers of cells per crypt scored from 1-96 hours after neutrons are shown in Figure 3.10. Crypt cellularity was below normal at 12 hours after all doses but by 2 days had returned to near normal. By day 3 the crypts looked normal in appearance except after 300 and 400 rads when there was a variation in crypt size. (Figure 3.11).

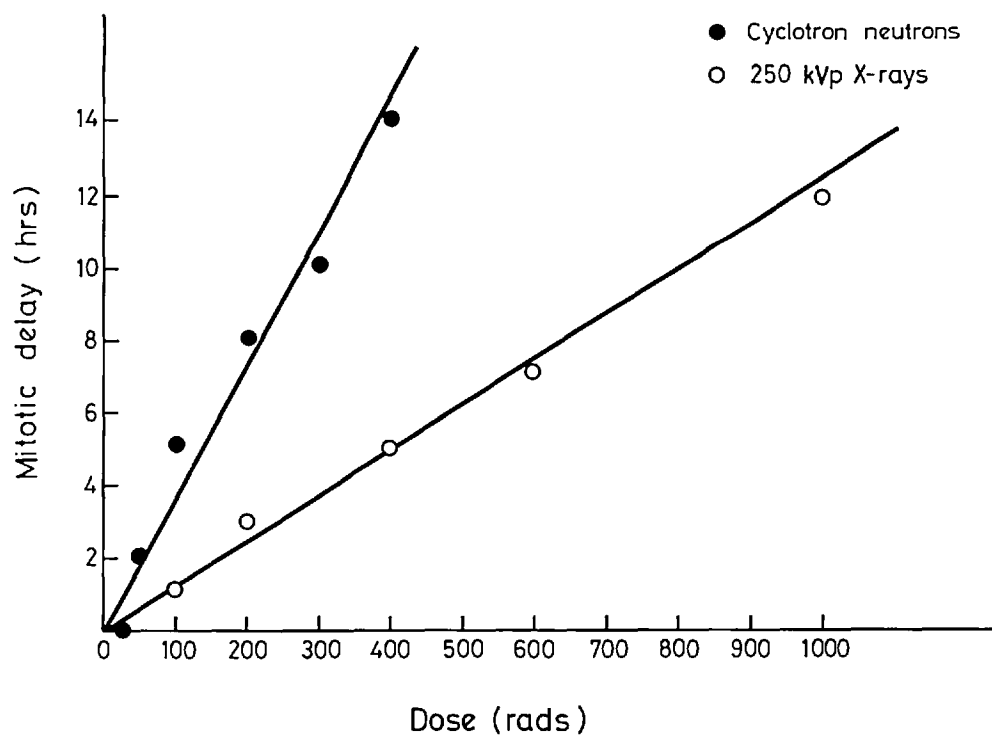


Figure 3.5. Mitotic delay (hrs) in jejunal crypt cells after 250 kVp X-rays and 6 MeV neutrons.

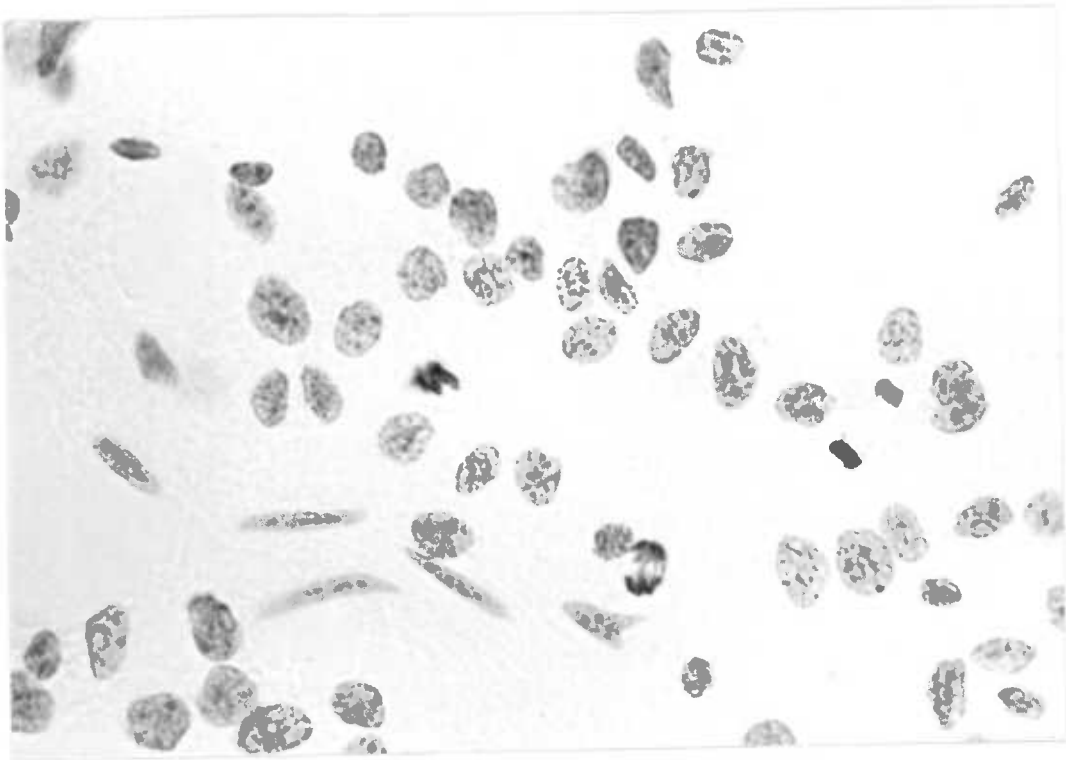


Figure 3.6. Anaphase bridges observed at 8 hours after 1000 rads pf 250 kVp X-rays in jejunal crypt squash.

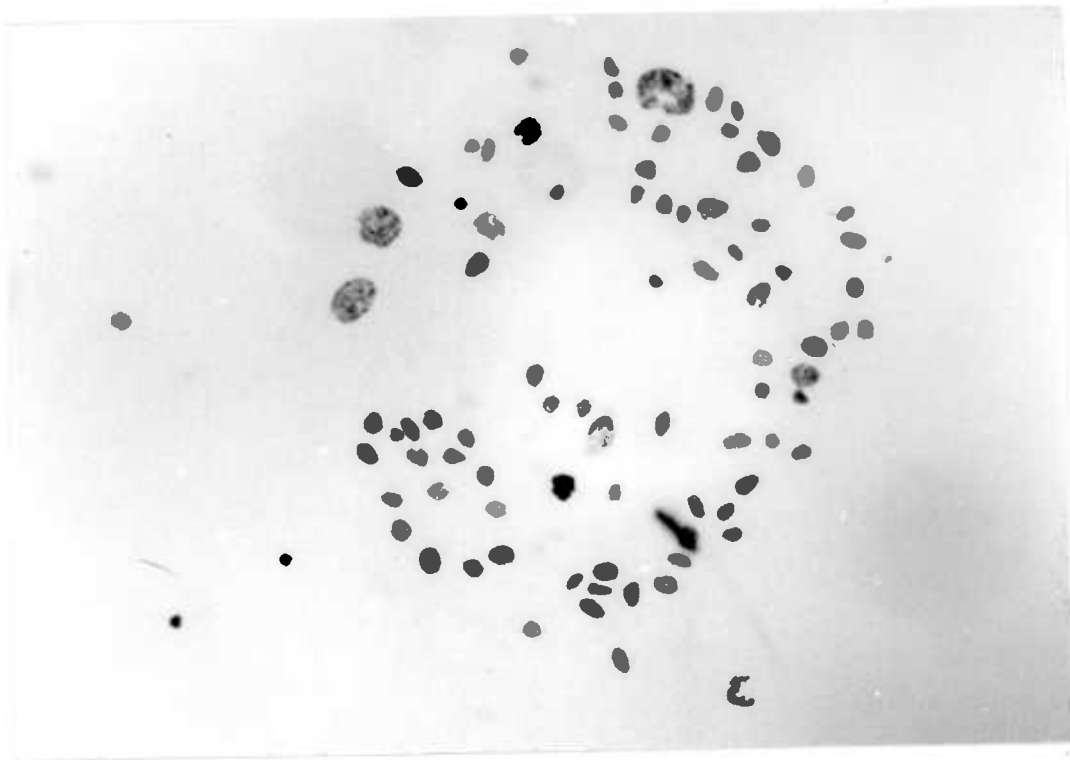


Figure 3.7. Giant nuclei observed at 4 days after 1000 rads of 250 kVp X-rays.

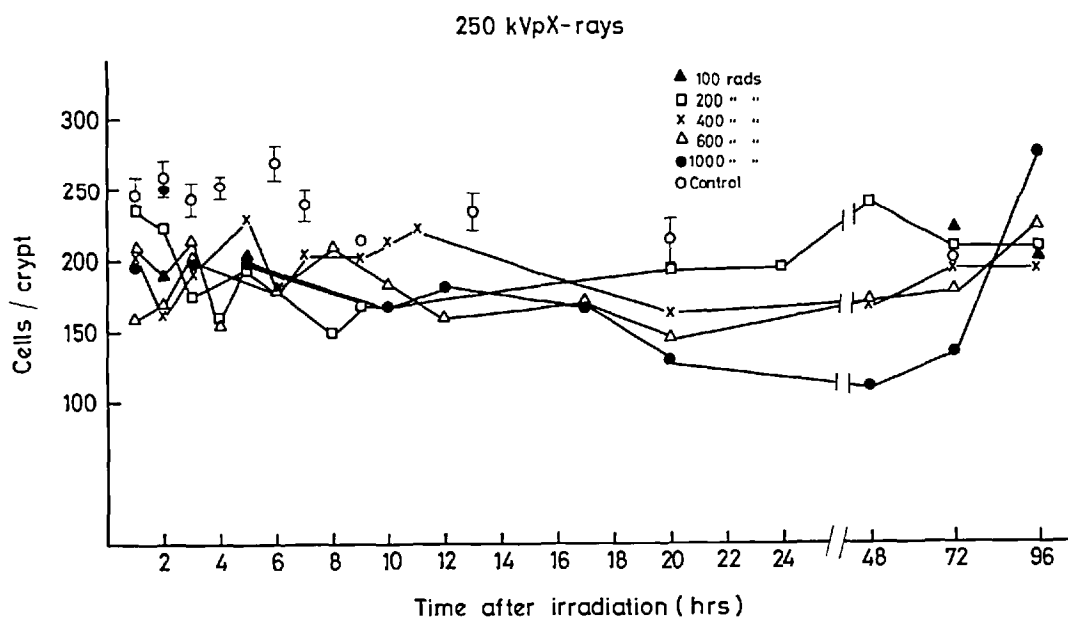


Figure 3.8. Cells/crypt 1 - 96 hours after irradiation with 100 - 1000 rads of 250 kVp X-rays.

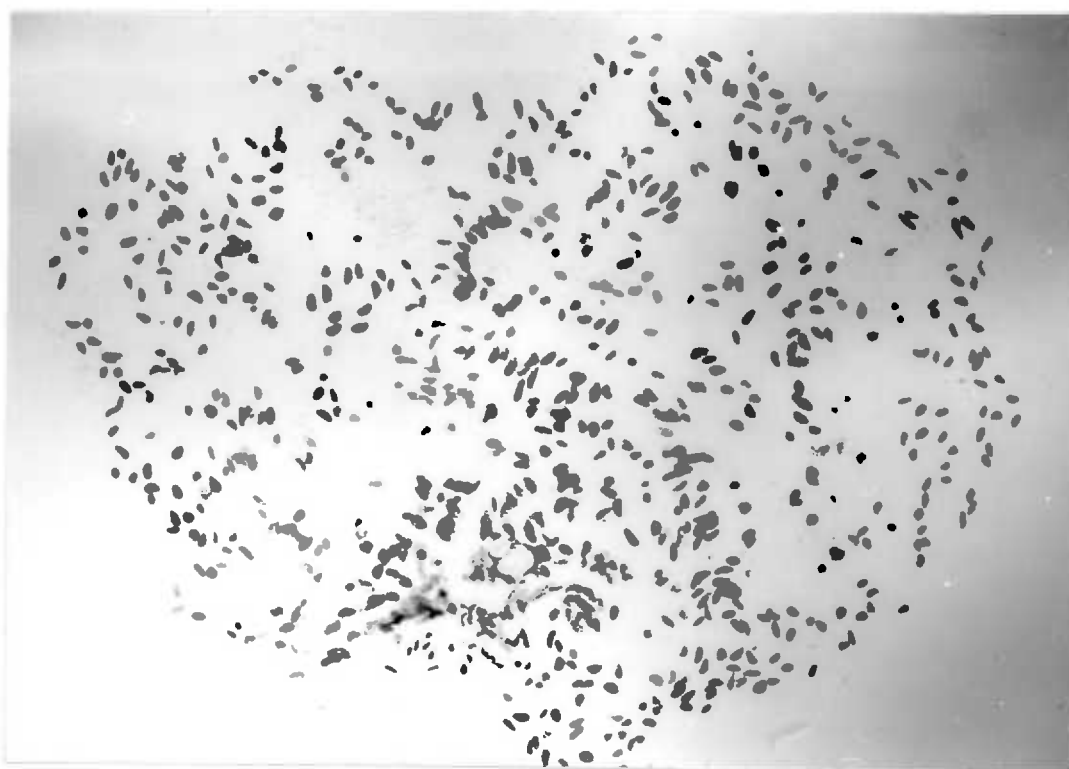


Figure 3.9. Large crypt observed at 4 days after irradiation with 1000 rads of 250 kVp X-rays.

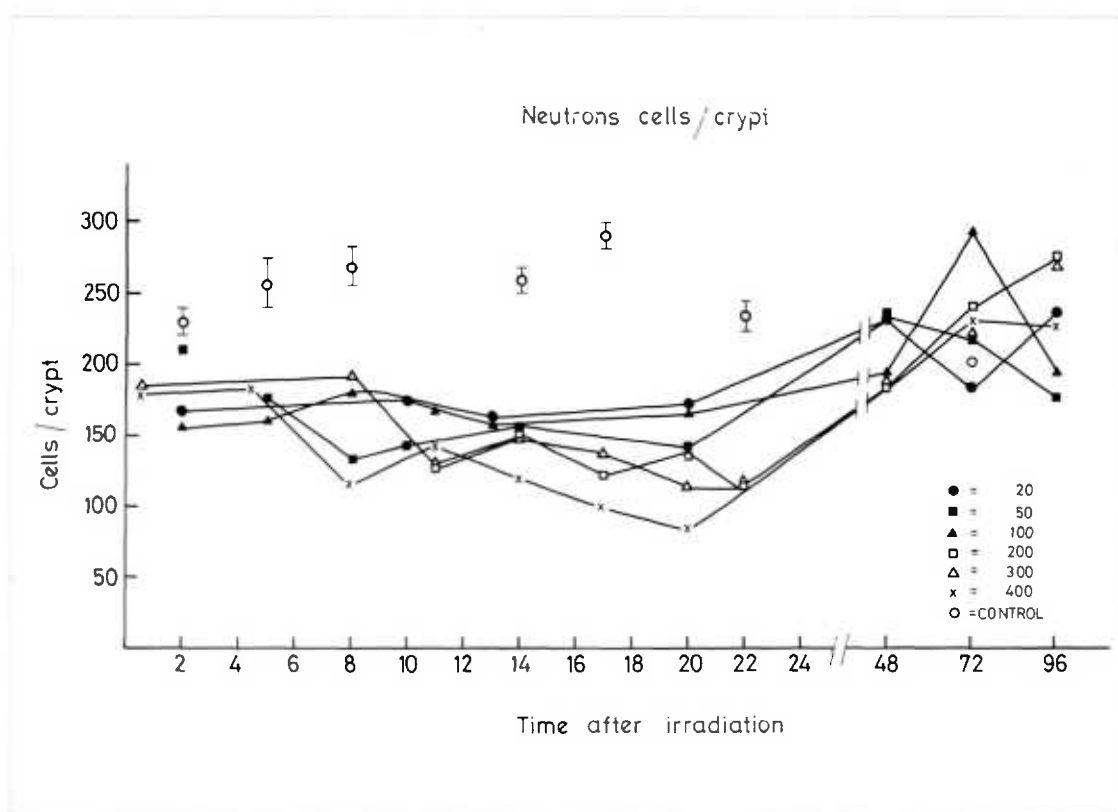


Figure 3.10. Cells/crypt 1 - 96 hours after irradiation with 20 - 400 rads of cyclotron neutrons.

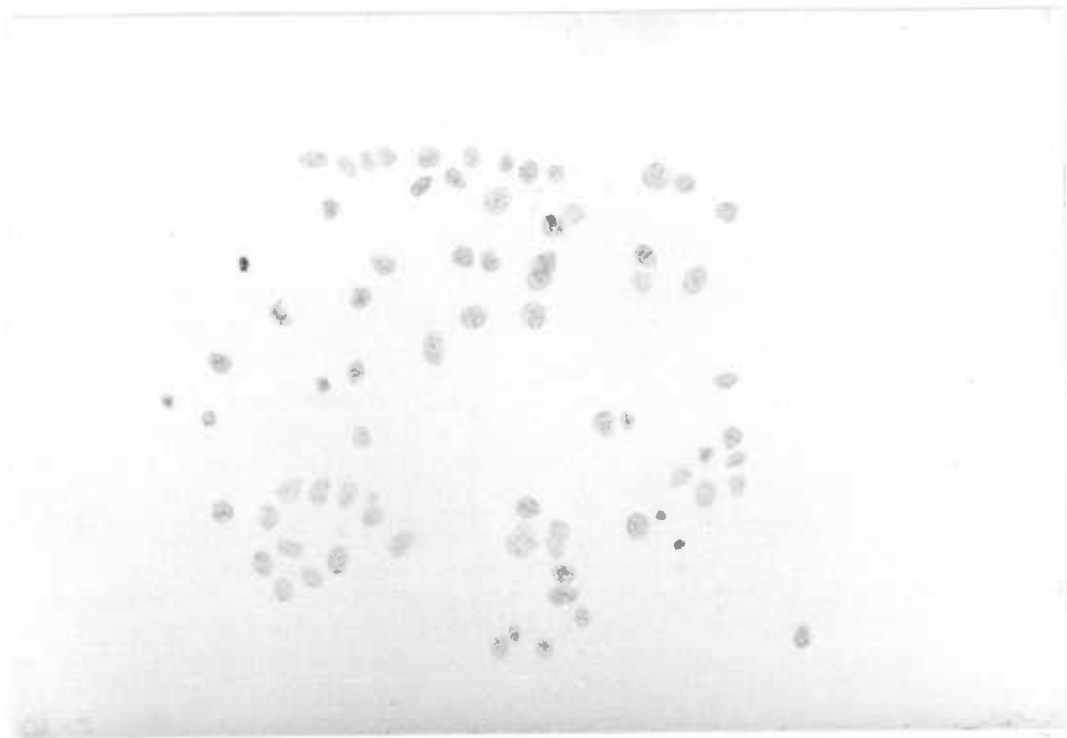


Figure 3.11. Different sized crypts observed at 3 days after irradiation with 400 rads of cyclotron neutrons.

The proportion of mitotic cells or the mitotic index (MI) at 3 and 4 days after X-rays and neutrons are shown in figures 3.12 and 3.13. At 3 days the mitotic index was higher after neutrons than after X-irradiation. At 4 days the MI after neutron irradiation was found to vary considerably with dose with the lowest value recorded after 100 rads while high MI values were obtained after 20 and 400 rads. The MI after X-irradiation at 4 days was found to be directly proportional to dose with the maximum value observed after 1000 rads.

Figure 3.14. shows the variation in crypt sizes at 3 and 4 days after X-rays and neutrons and shows a broadening of crypt size with increasing dose. There was also a slightly greater variation in crypt size after neutrons than after X-rays.

In Figure 3.15. the number of cells per crypt are plotted as a percentage of the control at the time of onset of mitosis in order to determine the proportion of cells that had died premitotically, i.e. before reaching mitosis. There is a fall in the crypt cell number after doses of 200 rads and below, while after doses greater than 200 rads of X-rays and neutrons there is no further reduction in the crypt cell number.

3.3.6. Surface area

The circumference measurements presented as a percentage of the control will be referred to as the Intestinal Absorptive surface or IAS. In figure 3.15 the IAS from 1-10 days post irradiation after doses of 200-1000 rads of X-rays is plotted. It shows that 24 hours after irradiation with doses of 200-1000 rads of X-rays the IAS was greater than control levels. The IAS then falls reaching a minimum by 4 days post irradiation. Recovery was not observed until 8 and 9 days following irradiation when an overshoot was observed after 800-1000 rads of X-rays. By day 10 the IAS approached normal levels.

Figure 3.17 shows the IAS measurements in the jejunum 1-7 days post irradiation after doses of 50, 200 and 400 rads of neutrons. At day 1

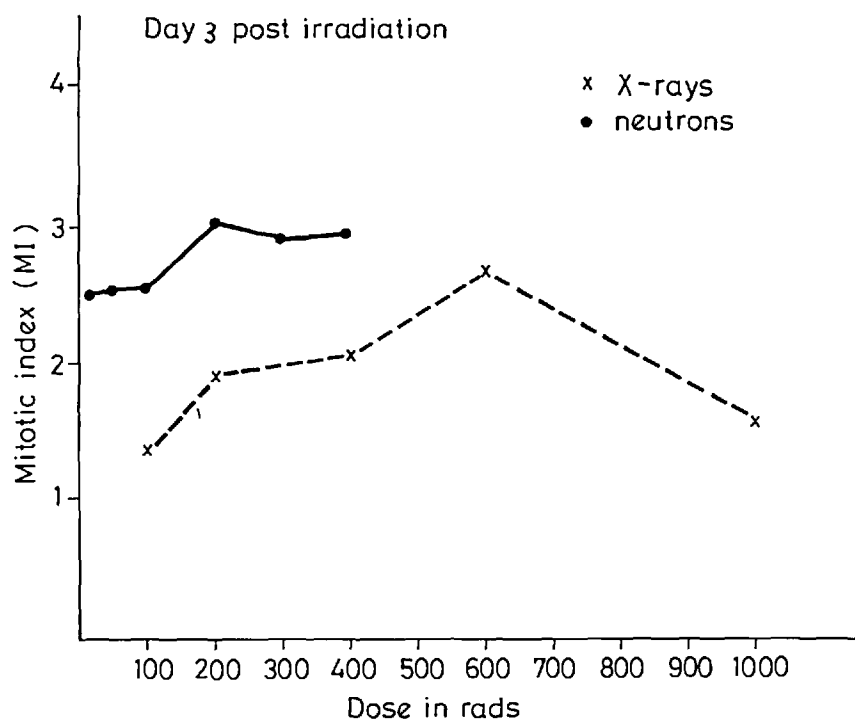


Figure 3.12. Mitotic Index at 3 days(after 250 kVp X-rays and cyclotron neutrons in jejunal crypts).

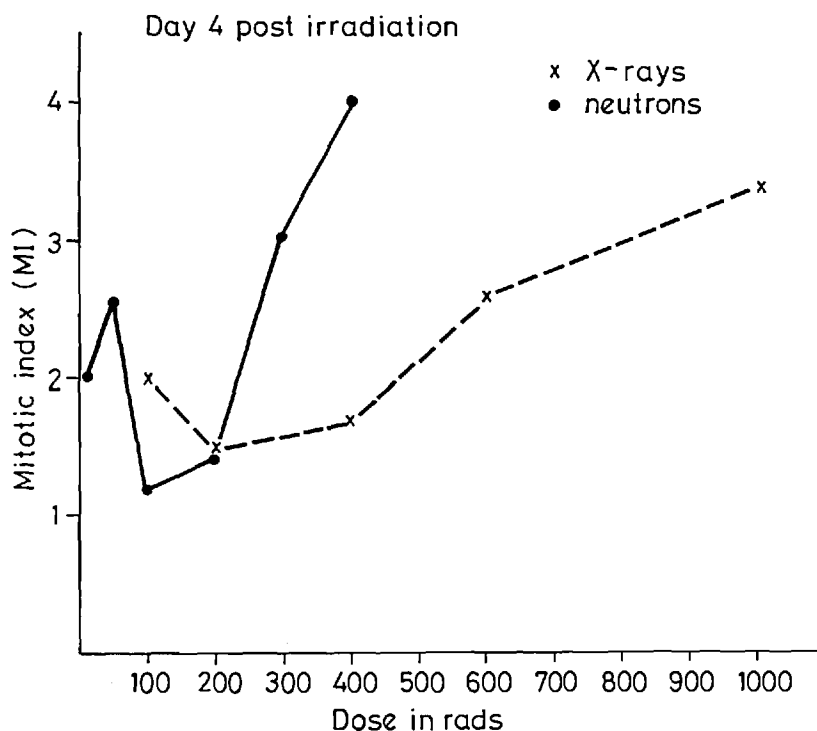


Figure 3.13. Mitotic Index at 4 days (after 250 kVp X rays and cyclotron neutrons in jejunal crypts).

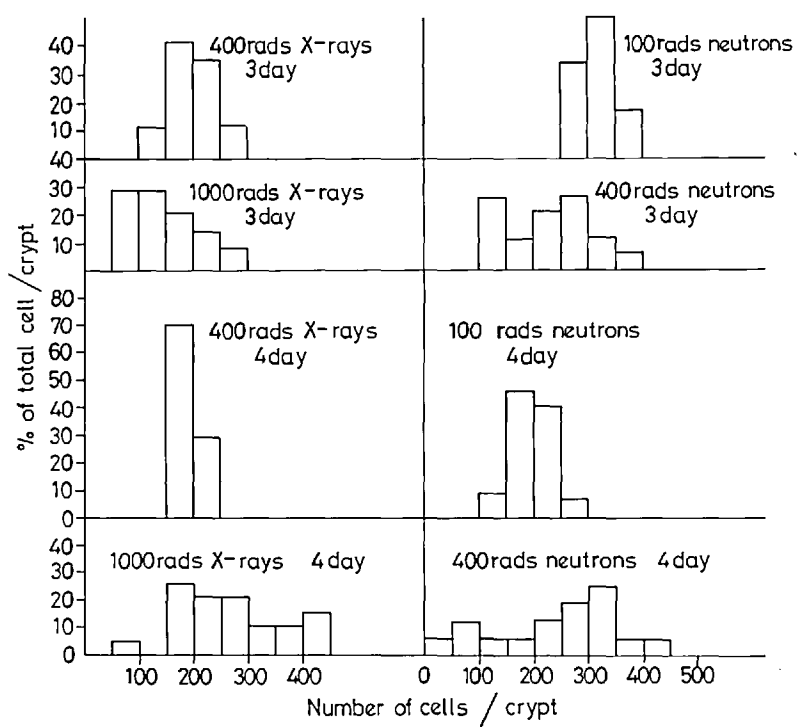


Fig. 3.14. Histogram showing variation in crypt size at 3 and 4 days after 250 kVp X-rays and cyclotron neutrons.

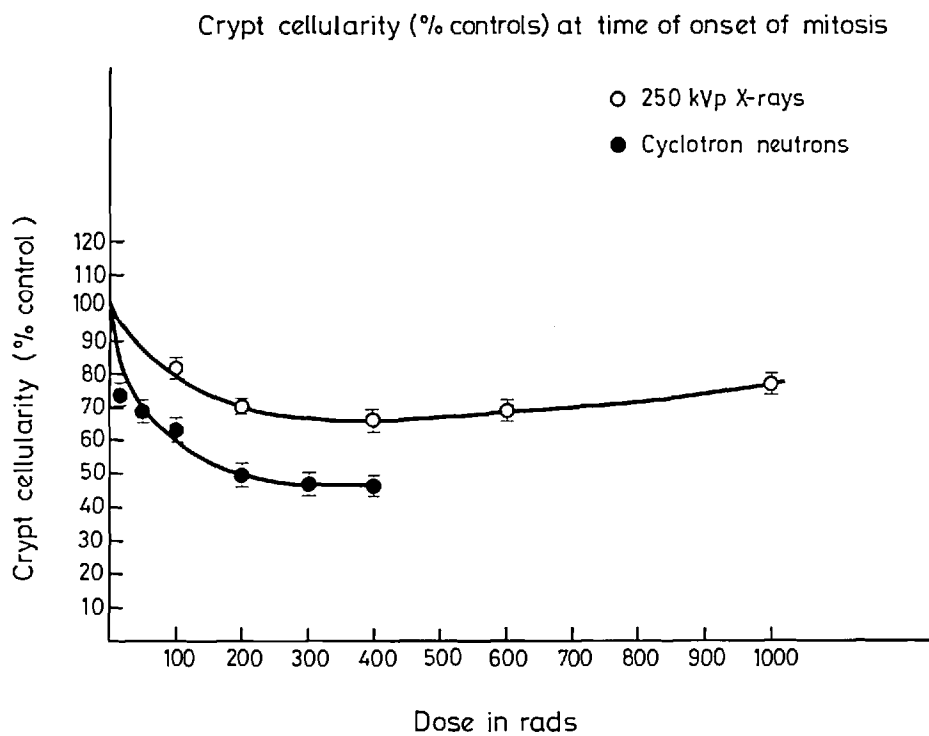


Figure 3.15. Cells/crypt plotted as a percentage of control at time of onset of mitosis, after irradiation with 250 kVp X-rays and cyclotron neutrons.

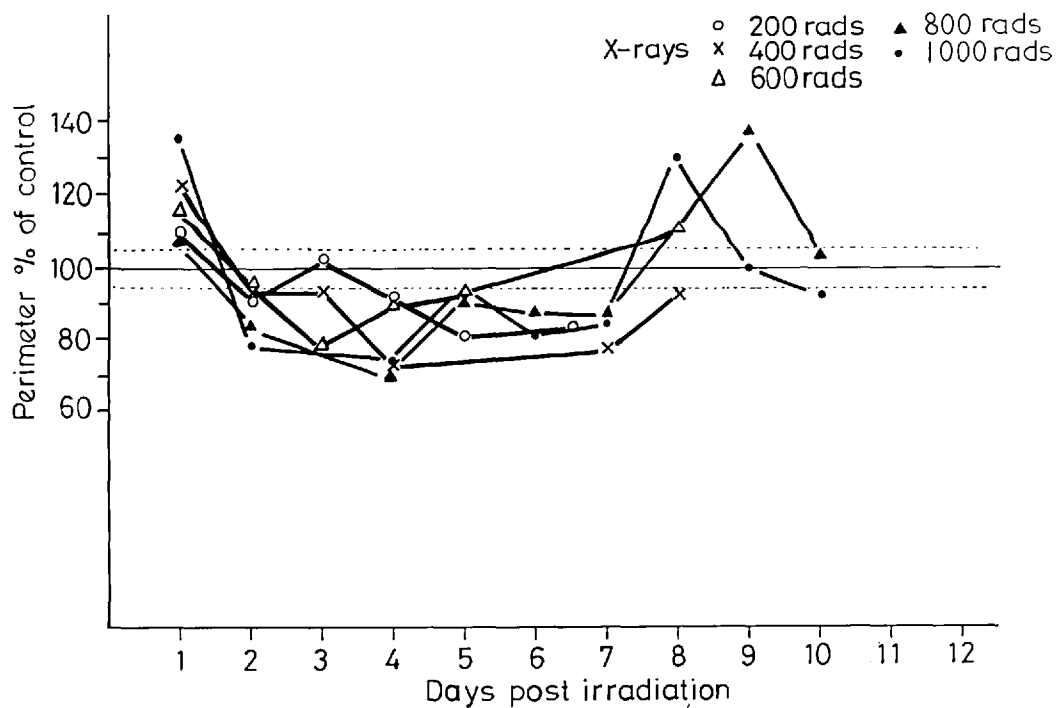


Figure 3.16. Intestinal absorptive surface plotted as a percentage of control after irradiation with 250 kVp X-rays.

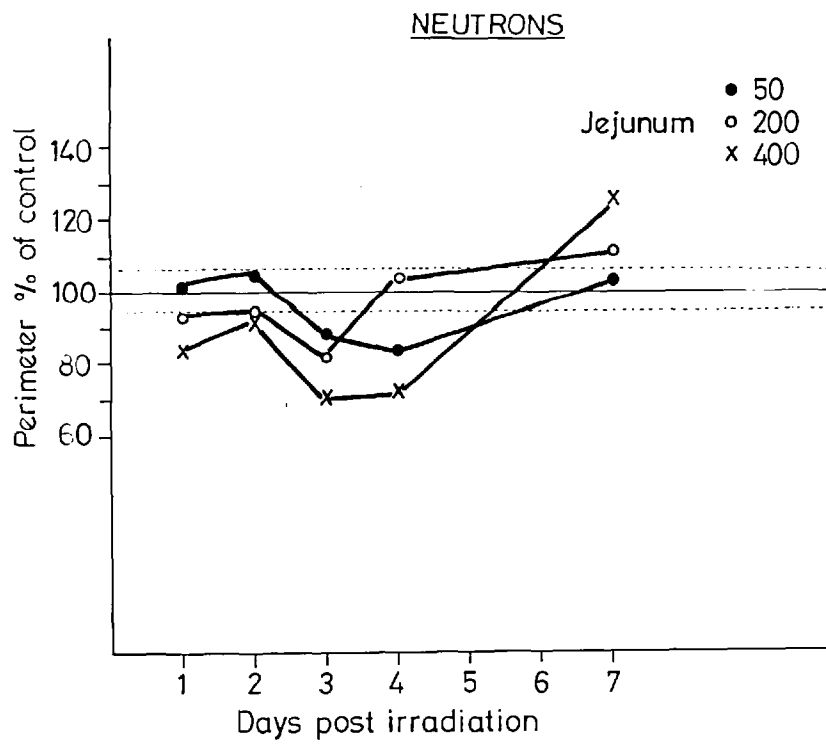


Figure 3.17. Intestinal absorptive surface plotted as a percentage of control after irradiation with cyclotron neutrons.

the IAS, unlike the values obtained after X-irradiation, was below normal after 50-400 rads. The minimum values were reached at, or by, day 3 and 4, and by day 7 recovery had occurred when the IAS was above control levels.

3.4. Discussion

3.4.1. Diurnal rhythm

Fluctuations in mitotic activity in tissues with relatively rapid cell turnover, e.g. intestinal epithelium, buccal cavity, have been attributed to the stimulus of feeding (Loran and Carbone, 1968) the suppressing activity of work and stress (Bullough, 1952;1962: Bullough and Lawrence, 1961; Alov, 1963; Chiakulas et al. 1966) and partial synchronisation of cells in the S phase (Pilgrim, 1963). Signestad et al. (1969, 1972) and Brown and Berry (1968) have demonstrated a distinct diurnal rhythm in mouse intestinal epithelium and hamster cheek pouch respectively. Signestad and Leshner (1970) using the crypt squash technique observed a peak in the number of cells in mitosis and in the number of cells synthesising DNA at 0300 hours while the nadir was noted at 1500 hours. They later found that the peak could be shifted 12 hours by reversing the light cycle (1971). Since mice are nocturnal animals they concluded that loss of villus cells that would occur during feeding could signal the proliferative compartment in the crypts. Potten et al. (1977) however, showed that the same pattern of response was observed in the skin and tongue epithelium of mice and feeding activity would be unlikely to cause a rhythm elsewhere in the body, they suggested a possible humoral influence as suggested by Bullough and Lawrence (1961). Hendry (1975) observed a peak of sensitivity in the mouse crypt cells at 0300 hours which corresponded with the peak in the number of mitoses observed by Signestad and Leshner. The lack of any distinct diurnal rhythm in the mouse crypt cells studied here may be due to the continual disturbance of the animals that occurs during the day in our laboratory,

and our observations are that the mice feed at any time of the day when they are disturbed. However, other workers who have failed to observe a rhythm in the mitotic activity in the gut include Pilgrim et al. (1963) who studied diurnal fluctuations in the number of mitosis and DNA synthesising nuclei in various tissues. They observed a diurnal variation in 3 tissues studied but none was seen in the mitotic index in the jejunal crypt epithelium or in the number of grains per nucleus after tritiated thymidine labelling. Williams et al. (1968) studied the rate of cell division in relation to time and showed that there was no variation in the number of mitosis in the crypts of rat jejunum, duodenum and ileum. Both workers used fixed sections for counting mitoses.

3.4.2. Mitotic Delay

A linear relationship between dose and mitotic delay observed here (Fig.3.5) was similar to that obtained by Kember and Sadik (1970) in rat duodenum. They also obtained a delay of 1.3/100 rads over a similar dose range of 200-1000 rads of X-rays. A slight fall off in the slope of the curve at high doses was, however, indicated here and could be due to changes in the cohort of cells reaching mitosis with increase in dose. At higher doses cells from early G1 which are delayed less than cells in late G1 will overtake the latter cells and reduce the delay per 100 rads. A similar effect was observed by Whitmore et al. (1967) in mouse L cells after 200 kVp X-rays.

The RBE of 2.92 obtained for mitotic delay in jejunal crypt cells for the $E_d = 16$ MeV Be neutron irradiation in this study compares with an RBE of 2.8 ± 0.3 and 3.8 ± 0.7 for mitotic delay in Chinese hamster cells and mouse L cells respectively with neutrons. (Schneider and Whitmore, 1963). While RBE values for mitotic delay in Vicia faba root tip cells ranged from 2.4-8.0 with 0.32 MeV neutrons (Geard and Marino, 1977) and 10.1 for fission neutrons relative to Cobalt 60 gamma rays (Neary et al., 1959).

The variation that was observed in the number of mitosis per crypt (Section 3.3.1 and Table 3.1) after irradiation was greater after X-rays than neutrons. A significant variation introduced by irradiation appeared after the lowest dose of X-rays (100 rads) and after high doses of neutrons (300 rads). This variation was not observed at every fixation time however and therefore could be related to cell cycle perturbations. The variations observed could also be due to variations in oxygen tension along the gut caused by peristalsis and since low LET radiations such as X-rays are more dependent on oxygen tension this would affect cells irradiated with X-rays more than neutrons. (Bewley and Hornsey, 1964). Neary et al. (1959) observed an oxygen enhancement factor of 2 for mitotic delay by gamma irradiation in *Vicia faba* roots. Also the lack of variation between and within mice after low doses of neutrons and X-rays may be due to the fact that a large proportion of the damage received is unreparable single hit damage (Hornsey et al., 1977).

3.4.3. Crypt cellularity

Distributions of clone sizes have been studied with X-irradiated Chinese fibroblasts by Sinclair (1964). X-irradiated HeLa cells by Nias et al. (1965) and X- or α -irradiated human kidney cells by Westra and Barendsen (1966). The data presented here for 250 kVp X-rays and 6 MeV neutrons (Figure 3.4) are in agreement with these other studies showing a broadening of the spectrum of clone sizes with increasing dose. Westra and Barendsen found a similar RBE for α -irradiation, for the effect on colony size and the value obtained for impairment of the capacity of clone formation. They suggested that this result ruled out a correlation of the radiation induced broadening of clone size spectra with the LET spectra of the radiation employed. 250 kVp X-rays exhibit a wide spectrum and it was suggested that this caused the damage induced in individual cells to range from very slight to very severe. However, if this were the case the broadening of clone size spectrum after high LET radiation

which has a relatively narrow LET distribution should be less than ^{with} λ X-rays. Sinclair (1964) suggested, on the basis of his results with X-rays, that the damage expressed by the broadening of clone size spectra occurs at sites other than those involved in lethality.

A loss of cells from the crypts was observed within the first few hours after irradiation and reached a maximum at about 20 hours. At least two factors will affect crypt cellularity at this time and these are:-

1. The cells continuing to migrate up the villus during mitotic delay.
2. Premitotic death of cells which will be removed by phagocytosis by neighbouring stem cells and macrophages (Leblond and Cheng, 1976).

Cell migration

The effect of radiation on the rate of migration from crypt to villus and off the villus tip is uncertain. Quastler (1959) showed no effect of irradiation on cell progression while Lieb et al. (1977) found that cells were not shed from the villi after doses of 250-1000 rads of X-rays in rat jejunum and ileum during the first 24 hours post-irradiation. This may be because food is retained in the stomach after X-irradiation so that the abrasive action of food going down the intestine will be temporarily reduced.

In a normal controlled state it takes approximately 12 hours for a cell to move from the proliferative zone in the crypts onto the villus (Rijke et al. 1974). The number of cells remaining in the crypts after a mitotic delay of time t was calculated as follows:

$$n = \frac{250 - 250t}{12}$$

where n = number of cells remaining

t = time of mitotic delay

250 = normal cells per crypt

Table 3.2. shows the estimated numbers of cells per crypt assuming continued migration after irradiation together with the observed cells per crypt at the time immediately prior to the onset of mitosis. There

Table 3.2.

Dose (rads)		Mitotic delay (hrs)	cells/crypt* (calculated)	cells/crypt* (observed)
Control		-	-	249 $\pm$ 3.44
Neutrons	20	0		
	50	2	208	212
	100	5	146	160
	200	8	83	140
	300	10	42	127
	400	14	0	120
X-rays	100	1	225	207
	200	3	188	186
	400	5	146	183
	600	7	105	192
	1000	12	0	181

* At the time of onset of mitosis

is a large difference between calculated and observed cell numbers after X-rays and neutrons particularly at the higher doses indicating that cells are not migrating at normal rates after irradiation (Figure 3.18),

Premitotic Death

Premitotic death has been observed in the first 6 hours after X-irradiation in the mouse intestine (Devik, 1968) and is generally believed to be caused by damage to lysosomal membranes which cause lysis of the cell and breakage of DNA (Allison and Paton, 1972). Work on lymphocytes suggests that the targets and mechanism leading to premitotic death are not related to those leading to chromosome damage. No difference was found between stimulated and unstimulated lymphocytes in their chromosome aberration content (Evans, 1967) but a large difference in X-ray induced premitotic death in unstimulated lymphocytes compared with stimulated lymphocytes has been observed (Hedges and Hornsey, 1978).

Crypt cells are less sensitive to premitotic death after irradiation than lymphocytes. The difference in sensitivity to premitotic cell killing may be attributed to the abundant amount of cytoplasm in crypt cells compared with the scarcity in lymphocytes. Previous studies on interphase death (Allison and Paton, 1972) have indicated that sensitive cells have less cytoplasm and fewer mitochondria than do resistant ones.

Potten (1977) observed an extremely radiosensitive population of cells at the base of the crypt which died within 3 hours after very low doses of gamma irradiation. One possible explanation put forward to explain the hyper-sensitivity of these cells was that damaged crypt stem cells may pass on their damaged DNA to one of its daughter cells that subsequently dies.

Premitotic death was investigated here by determining the number of cells/crypt at the time of onset of mitosis when there would be no repopulation, and the number of cells/crypt were plotted against dose. (Figure 3.15). However, any fall in crypt cell number will be due to

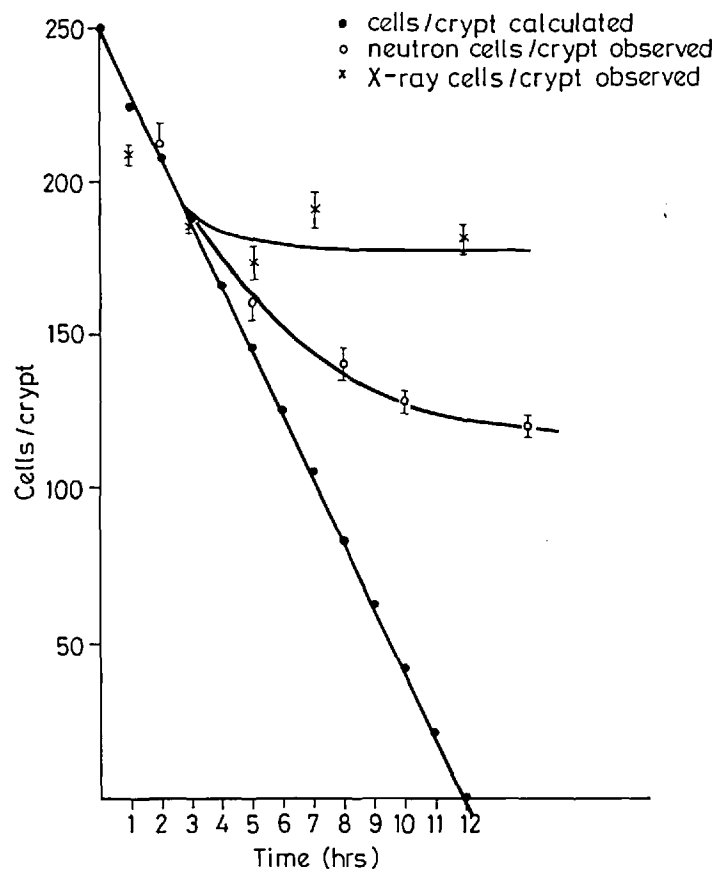


Figure 3.18. Observed and expected (calculated) migration rate of cells out of jejunal crypt onto villus.

cell migration out of the crypts during the mitotic block as well as pre-mitotic death.

After doses greater than 200 rads of X-rays and neutrons no further fall in crypt cellularity was observed. The absence of any dose effect relationship above 200 rads was observed by Devik (1968). He found no difference in the number of pyknotic nuclei after irradiation with doses of 200-700 rads of X-rays. In stimulated lymphocytes Hedges and Hornsey (1977) also observed no dose effect relationship for cell death (pyknosis) at high doses higher than 200 rads of X-rays and 200 rads of neutrons and obtained an RBE of 1 for radiation damage of unstimulated lymphocytes which leads to cell death. Their results also suggested a mixed cell population consisting of a very sensitive fraction of approximately 80% and a smaller rather insensitive fraction forming about 20% of the population. The data here for crypt cell sensitivity to early cell death suggest an X-ray sensitive population of 30% compared with a neutron sensitive population of 50%.

3.4.4. Surface Area

Differences were observed in the surface area measurements after X-rays and neutrons (Figs. 3.16 & 3.17). An increase in surface area at day 1 after X-rays was not observed after neutrons. After X-irradiation of large abdominal areas in radiotherapy, patients often develop nausea and vomiting within a few hours, whereas patients treated for abdominal tumours with fast neutrons have showed a marked absence of vomiting (Catterall, 1974). In mice, vomiting does not occur but food is retained in the stomach, this effect is sensitive to radiation and occurs after small doses (Turner and Fowler, 1963). The absence of this initial increase in surface area after neutrons may reflect the small effect of neutrons on stomach retention. Retention of the cells on the villi for a period that exceeds the normal life of the cell was indicated by work done by Lieb et al. (1977) on the fine structure of X-irradiation rat epithelium cells.

The increase in the villus surface area of the jejunum after X-irradiation may be due to the reduced erosion from the passage of food because of the retention in the stomach.

The decrease in surface area observed at 3 and 4 days after X-rays and neutrons will be due to mitotic delay and cell death in the crypts preventing cell production, so that migration out of the crypts will be slowed down. The increase in surface area at day 8 after X-rays and day 7 after neutrons is possibly due to the large numbers of cells migrating onto the villi from the enlarged crypts.

The more rapid recovery after neutrons than X-rays reflects the more rapid recovery in crypt cellularity after neutrons. This may be the direct consequence of the greater demand for cells on the villi due to the greater villus cellular erosion after neutrons immediately post-irradiation compared with X-rays and to a greater stimulus for cell division in the crypts caused by more dying cells. Mitotic delay is ^{however} greater after neutrons than X-rays so should delay the repopulation after neutrons. The earlier repopulation and increase in surface area with neutrons than after X-rays suggests therefore that repopulation may be faster after neutrons than X-rays. This requires further investigation.

Hamlet et al. (1976) also observed a difference in the appearance of the villi of the jejunum after neutrons compared with X-rays, neutron irradiated villi appearing more conical. Meder and Michalowski (1980) measured the internal surface area of mouse jejunum after 250 kVp X-rays and 6 MeV neutrons and observed a nadir at 3 days when an RBE of 3 for intestinal absorptive area was recorded. They also observed a clear dose effect relationship and no difference was observed between neutrons and X-rays in the pattern of response. The lack of any clear dose relationship in this study may be due to the intrinsic problems associated with the technique used, e.g. standardization of fixation procedures. Studies by Carr et al. (1979) have suggested that observations on villus structure

after irradiation is a more reliable indication of tissue damage than conventional crypt counting. They observed a marked difference in 2 out of 3 schedules between the damage expressed as crypt counts and that described by quantitative techniques using a scanning electron microscope to study damage to the villi. No correlation was observed with schedules when irradiations were to the whole animal while when partial body irradiations were administered there was a good correlation between crypt count and the structure of the villus. This would suggest that after whole body irradiation there may be an indirect effect on the villus, i.e. toxic substances produced in the blood, or an effect on the connective tissue which maintains villus structure.

3.5.0. Conclusions

The measurement of intestinal surface area is a useful means of studying the direct effects of radiation on the intestinal epithelium. However, the main disadvantages of the method found in this study were the difficulties inherent in the technique, together with the fact that it is not possible to exclude dead or dying cells from the measurement, while in crypt survival assay only viable cells are counted.

There are many factors which affect villus appearance after X- and neutron-irradiation. These include mitotic delay, premitotic death and cellular progression and crypt cell survival, all of which will determine the ability of an animal to survive and recover from a dose of radiation. The relative effect of X-rays and neutrons is different on these different factors.

1. The dose effect relationship for mitotic delay was linear for both X-rays and neutrons and an RBE of 2.92 ± 0.15 was obtained.
2. The dose effect relationship for crypt cellularity was complex with a sensitive and insensitive component. The insensitive component to X-rays was about 30% of the population and to neutrons about 50% of the population.
3. A combination of the effects of premitotic death and cell migration

during the period of mitotic delay was greater after neutrons than X-rays. However, since these two factors were not determined independently it is not possible to determine the extent that cellular reduction is due to each.

All these factors will contribute to changes in the structure and surface area of the villus epithelium. It is therefore not surprising that considerable structural differences in the topography of the villus surface have been observed after X-rays and neutrons using the scanning electron microscope (Hamlet et al. 1976) for a qualitative effect which is dependent on multiple factors which are affected differently by the two irradiations.

SECTION 4.

4.0. Repair of sublethal damage after low doses of X-rays

4.1. Introduction

The most important factors which will govern the response of rapidly dividing tissues such as the intestine to multiple fractionation treatments with radiation will be repair of sublethal damage and repopulation between fractions. Repopulation of the crypts may take place by recruitment of cells from a reserve population of proliferative cells, either from within the crypt or from without, or by an increase in birth rate due to a shortening of the cell cycle (Lamerton et al. 1968). Sublethal damage deposited by low LET radiations is repaired rapidly (Wambersie and Dutreix, 1974; Hornsey, 1970b) and maximum repair has occurred by 2 - 3.5 hours in the mouse intestine. (Withers and Elkind, 1969).

It is difficult to study the amount of repair of sublethal damage after low doses of X-rays in the intestinal crypts because of the high radiation doses needed to reduce crypt cellularity. Methods used to overcome this problem include the use of multifractionation, or the use of single large radiation doses given immediately before or after the low investigative doses.

Wambersie and Dutreix (1974) devised a method of investigating the repair of sublethal damage in the crypts of the mouse intestine using a multifraction technique which was independent of cellular repopulation that occurred in the crypts during treatment. They compared the relative effectiveness of multiple single fractions and multiple pairs of fractions given in the same overall time assuming that the repopulation would be the same in both fractionations. They used the technique to look at recovery in both skin and the intestine. From their results on intestine measured at 9 - 20 days they stated that no additional recovery between X-ray fractions occurred when doses per fraction of less than 300 rads were administered. When radiotherapy treatments are given

it is generally assumed that recovery takes place between low dose fractionation treatments and therefore the results of Wambersie and Dutreix suggesting that no such recovery takes place could have an important effect on future radiotherapy rationale.

Withers (1975) studied recovery in mouse intestinal crypts at low doses by giving a large dose of neutrons to reduce the crypt cellularity after the investigative doses. He used neutrons because it kills cells more uniformly through the cell cycle than do X-rays. Withers compared the effectiveness of 3 dose fractions of X-rays separated by 3 hours with a single dose, both followed 4 hours later by a dose of 650 rads of neutrons to reduce cellularity in the crypts. He obtained evidence of recovery from sublethal damage down to doses of 75 rads of X-rays; however the recovery from sublethal damage fell sharply after doses below 250 rads.

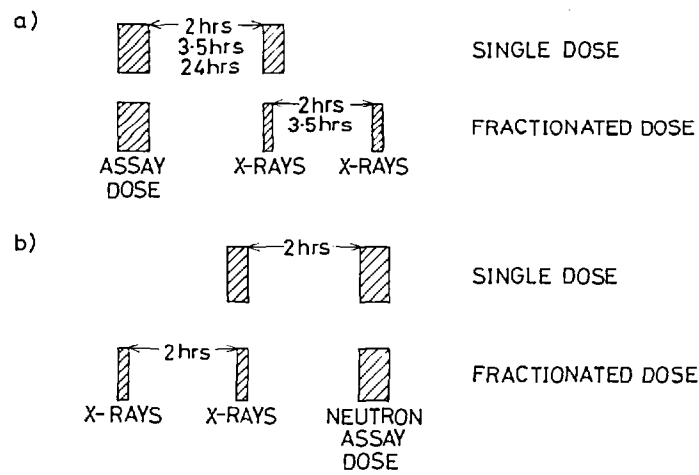
In this section I report on the effects of low doses of X-rays on cell survival in the crypts of mouse intestine measured at 4 days using two different experimental techniques designed to compare the results obtained by Wambersie and Dutreix and Withers but on the same strain of mice, and also to obtain further information into the recovery of the small intestine from low doses X-ray fractions (Figure 4.1).

1. The first method involved giving a large assay dose of X-rays or neutrons to reduce cellularity before or after the investigative or test doses. The X-ray test doses were given either as a single dose or as two doses split into equal fractions and separated by 2, 3.5 or 24 hours. By the use of neutrons as well as X-rays to reduce cellularity in the crypts a comparison could be made of the effect of high and low LET radiations on cell cycle perturbations..

2. The second method compared the effectiveness of pairs of doses with single doses by giving multiple fractions sufficient to reduce survival to countable levels.

SCHEDULE FOR FRACTIONATION EXPERIMENT

- 1) ASSAY DOSE OF 540RADS NEUTRONS OR 1250RADS X-RAYS FOLLOWED 2,3-5,24 HOURS LATER BY TEST DOSES.



- 2) MULTIPLE FRACTIONATION. (Wambersie & Dutreix 1974)

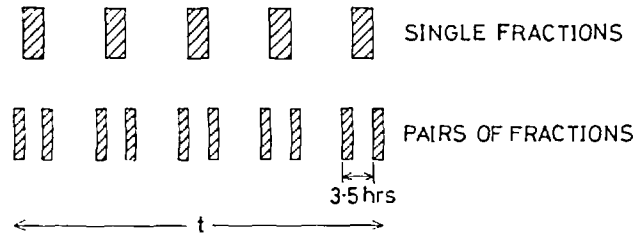


Figure 4.1. Experimental procedures for estimating the effects of low doses of X-rays on crypt cell populations.

4.2. Methods

1. Unanaesthetised animals were given whole body doses of 250 kVp X-rays or 7 MeV neutrons. Assay doses of 1025 rads of X-rays and 540 rads of neutrons were followed by X-ray doses of 125-500 rads given 2, 3.5 or 24 hours after the assay dose either as a single dose or as equal split doses. In addition single and split doses of 62.5 - 500 rads of X-rays were given 2 hours before a neutron assay dose.

2. Unanaesthetised animals were given abdominal only X-irradiation (Section 2) but when multiple fractions were given over 5-12 days the animals were anaesthetised with Sagital. Single X-ray doses of 125-500 rads and split doses of 62.5-250 rads were given every 12-48 hours over periods from 48 hours to 2 weeks duration.

Crypt survival was assayed in all cases at 4 days after the last dose fraction in both the jejunum and the ileum.

4.3. Results

4.3.1. Neutron assay dose before or after test dose

Crypt survival after single or split doses (2 hours) of X-rays are compared in Figure 4.2. when test doses were given after or before an assay dose of 540 rads of neutrons. When the test doses were given 2 hours after the neutron assay dose (Figure 4.2. a and b) some additional recovery between split X-ray doses compared to that after a single dose is demonstrated by the separation of the single dose and the split dose X-ray curves. Results show that recovery is taking place after doses/fraction of 120 rads (240 total dose) and may be taking place at doses/fraction of 60 rads X-rays and below. (Figure 4.2b). The crypt survival curves have a very small initial slope which further indicates that most of the radiation damage is being recovered at low doses.

When the test X-ray doses are given before the neutron assay dose there is no separation of the single dose and the split dose X-ray

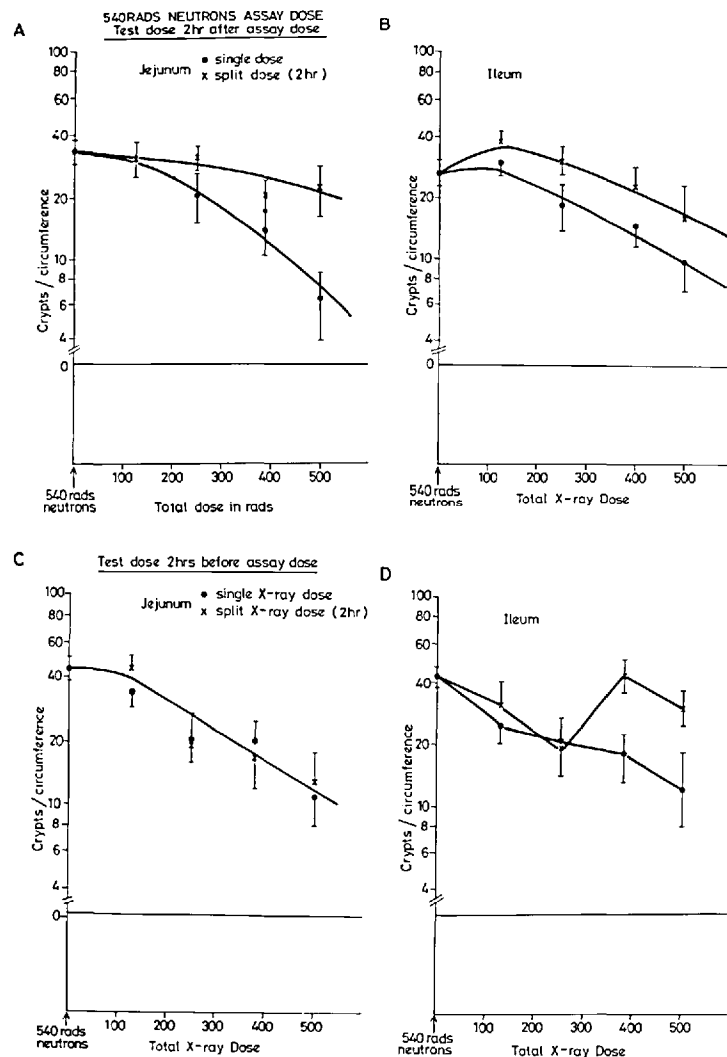


Figure 4.2. A and B show the number of crypts/circumference in the mouse jejunum (A) and ileum (B) when X-ray test doses were given 2 hours after a 540 rad neutron assay dose. C and D show the number of crypts/circumference in the jejunum (C) and in the ileum (D) when X-ray test doses were given 2 hours before a 540 rad neutron assay dose.

curves in the jejunum (Figure 4.2c). In the ileum the results are less clear but indicate that little additional recovery is taking place at low doses. (Figure 4.2d).

4.3.2. Time-dependent recovery after neutron assay dose

In Figure 4.2a and b and in Fig. 4.3 crypt survival is shown in the jejunum and ileum when the X-rays test doses were given at 2 hours (Figure 4.2a and b), 3.5 hours (Figure 4.3a and b) and 24 hours (Figure 4.3 c and d) after the neutron assay dose.

In the jejunum most of the recovery between split doses is observed when the doses are separated by 2 hours and given 2 hours after the neutron assay dose (Figure 4.2a). When the separation of the X-ray split doses is 3.5 hours, given 3.5 hours after the assay dose, the additional recovery is small (Figure 4.3a) and when the test doses are given 24 hours after the assay dose no additional recovery is observed in the jejunum (Figure 4.3c). This pattern of response is similar to that obtained when the unperturbed intestine is irradiated (Figure 4.2c).

4.3.3. Time-dependent recovery after X-ray assay dose

Crypt survival after single or split X-ray doses given 2 hours, 3.5 hours or 24 hours after an assay dose of 1025 rads of X-rays are shown in figure 4.4. In figure 4.4a and b the X-ray test doses were split by a 2 hour interval; in figure 4.4c and d by a 3.5 hour interval and in figure 4.4e and f by a 24 hour interval. When the test doses follow 2 hours after the assay dose no additional recovery is seen in the X-ray split dose data until the dose/fraction is above 120 rads (240 total dose) in the jejunum and above 240 rads (500 rads total dose) in the ileum. (Figures 4.4. and a. and b.). When the test doses are given 3.5 hours and 24 hours after the assay dose additional recovery is observed to occur between the split dose fractions.

4.3.4. Multiple fractionation

Crypt survival curves after multiple fractions of X-rays given

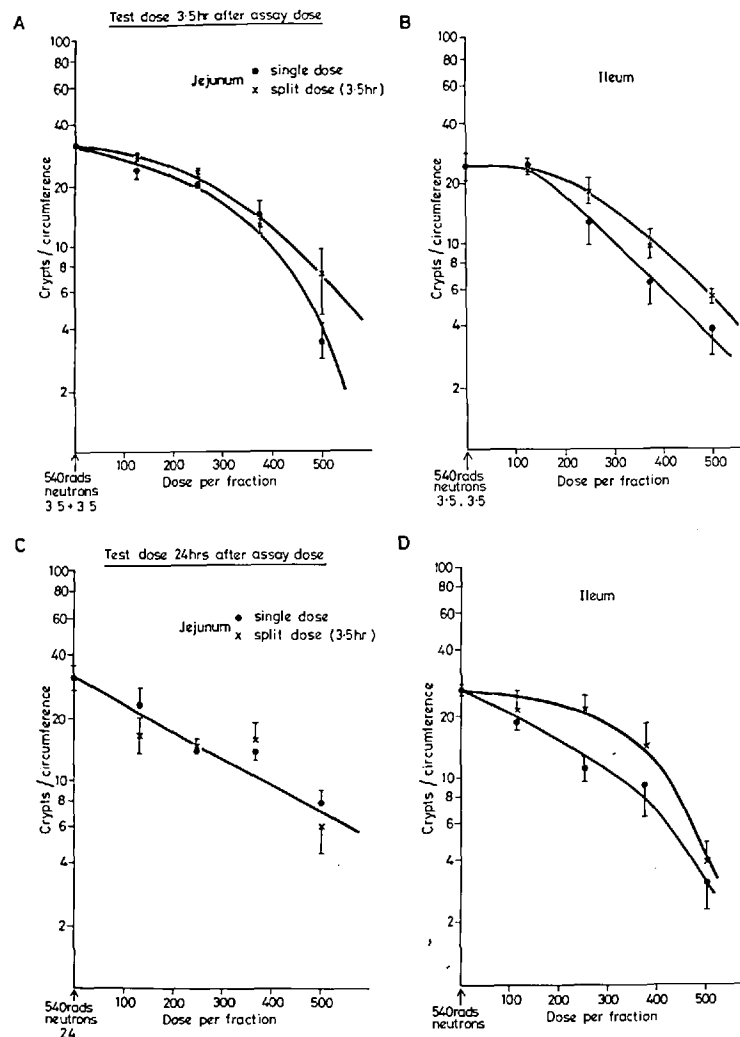


Figure 4.3. A and B show the number of crypts/circumference in the jejunum (A) and in the ileum (B) when X-ray test doses were given 3.5 hours after a 540 rad neutron assay dose. C and D show the number of crypts in the jejunum (C) and in the ileum (D) when X-ray test doses were given 24 hours after a 540 rad neutron assay dose.

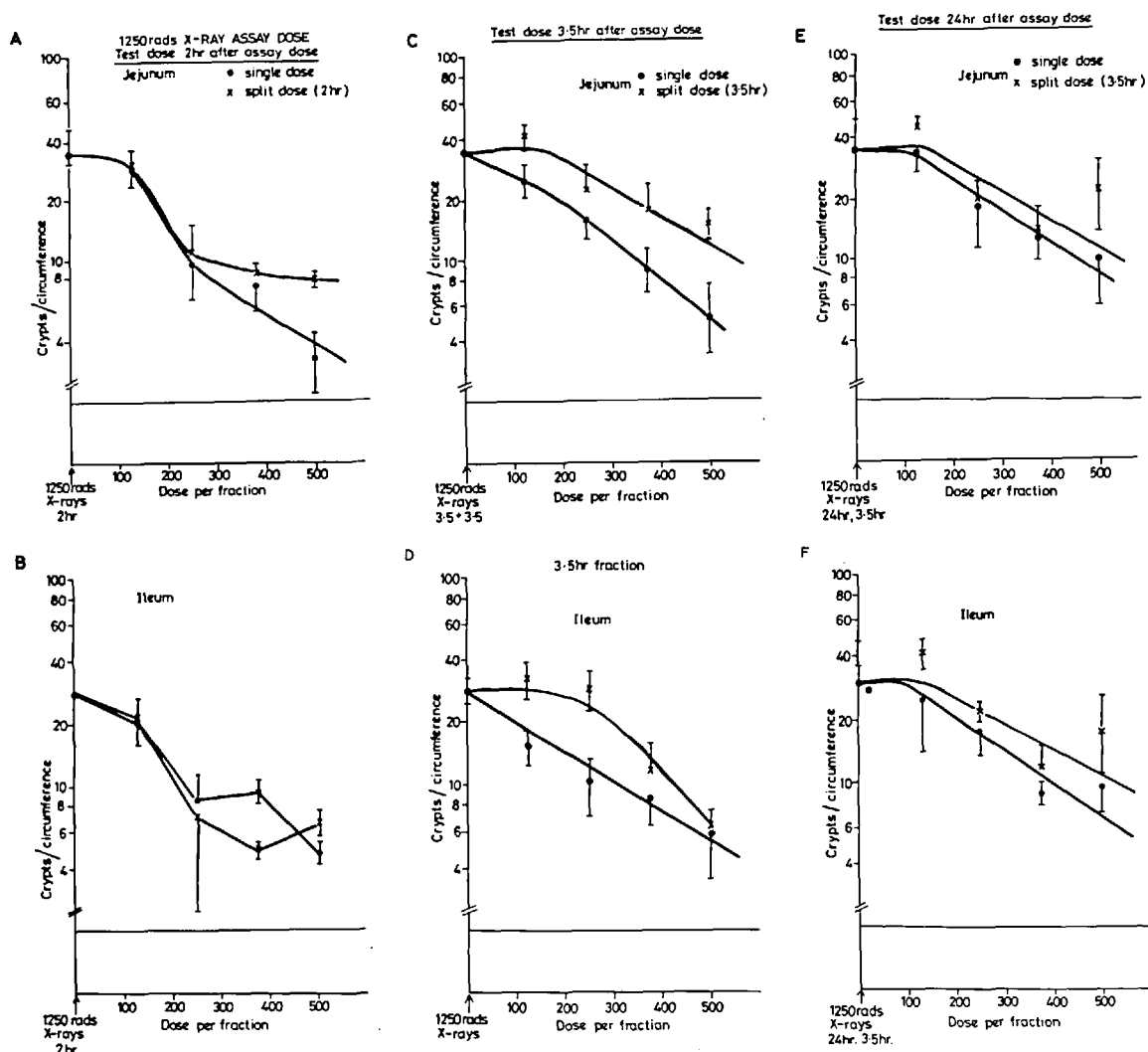


Figure 4.4.

A and B show the number of crypts/circumference in the jejunum (A) and in the ileum (B) when X-ray test doses were given 2 hours after an X-ray assay dose.

C and D show the number of crypts in the jejunum (C) and in the ileum (D) when X-ray test doses were given 3.5 hours after an X-ray assay dose.

E and F show the number of crypts in the jejunum (E) and in the ileum (F) when X-ray test doses were given 24 hours after an X-ray assay dose.

over periods of 2-12 days are shown in figures 4.5 - 4.8. When fractions were given at 12 hourly intervals (Fig.4.5 a&b) there was complete recovery from sublethal damage after doses per fraction of 160 rads and under (320 total dose). Additional recovery between the X-ray split dose compared with that of the single dose was observed after doses per fraction of 200 rads (400 rads total dose). When fractions were given at 24 hourly intervals (fig 4.6a&b) there was complete recovery between fractions of 475 rads (total dose) and below but separation of the single dose and split dose curves occurred after doses/fraction of 250 rads (500 rads total dose). There was very little effect on crypt survival when 5 fractions and 5 pairs of fractions were given in 12 days (Figure 4.7) and also when 10 fractions and 10 pairs of fractions were given in 12 days due to repopulation between fractions and recovery from sublethal damage. (Fig. 4.8).

4.4. Discussion

4.4.1. Neutron assay dose before or after test dose

The use of neutrons as well as X-rays for the assay dose was chosen since neutrons cause fewer perturbations in the survival pattern, killing cells more uniformly through the cell cycle compared with X-rays (Withers et al., 1974). By comparing the effect of a neutron assay dose given after the test doses the influence of any perturbations produced by the neutron assay dose could be assessed. It was evident that the effect of giving a neutron assay dose two hours before the test dose is not the same as giving it two hours after the test dose and indicate that neutrons might be perturbing the system sufficiently to change the pattern of crypt survival. When the X-ray doses were given two hours before the neutron assay dose there was no difference in crypt survival whether the X-ray dose was administered as a single dose, or split into two equal fractions separated by a two hour interval in the jejunum.

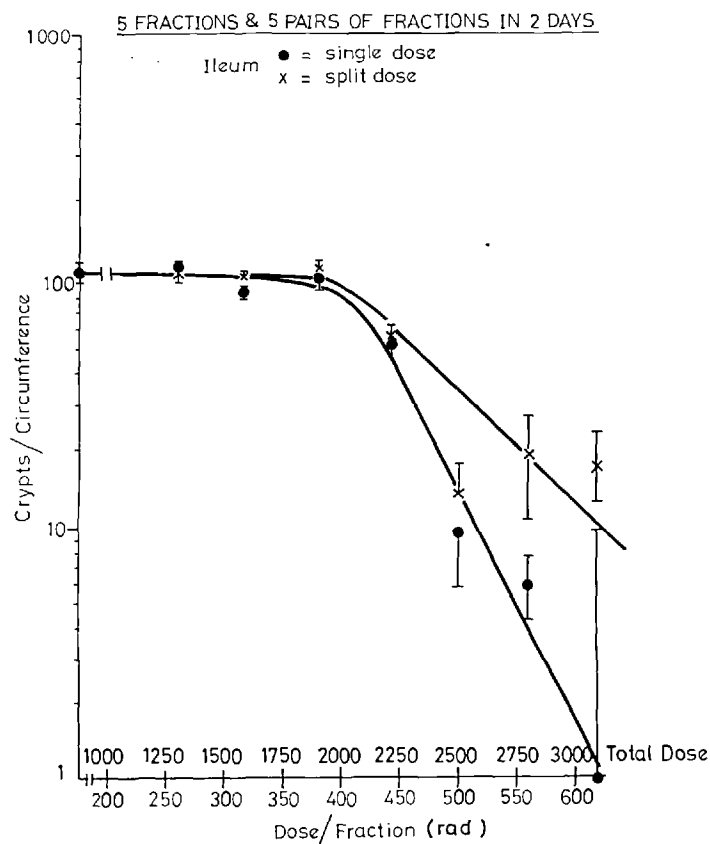
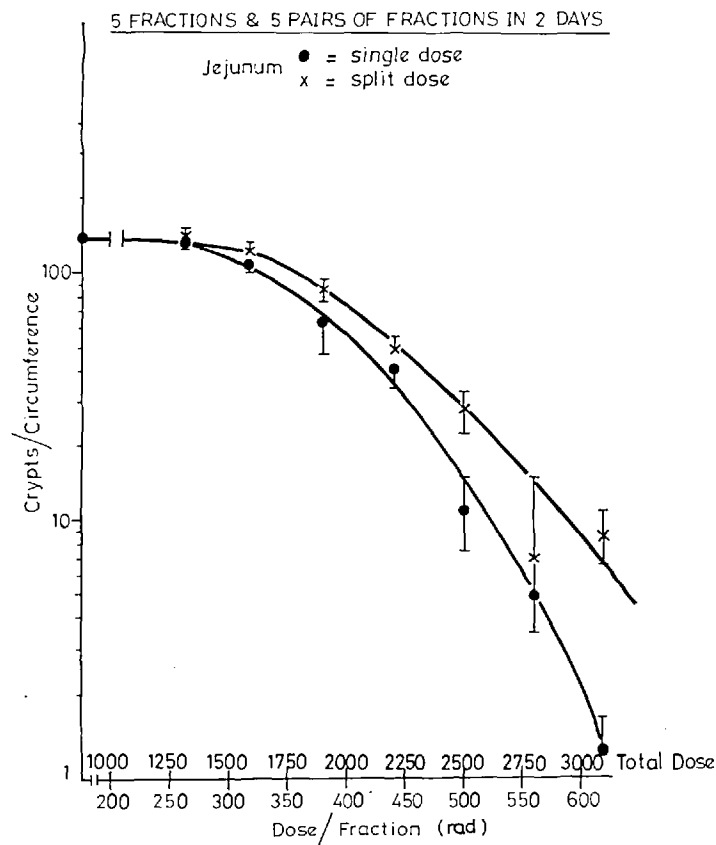


Figure 4.5. Relationship between total dose and crypt/circumference in the jejunum (a) and the ileum (b) after exposure to 5 single 12 hourly X-ray dose fractions or 5 pairs of 12 hourly dose fractions separated by 3.5 hours.

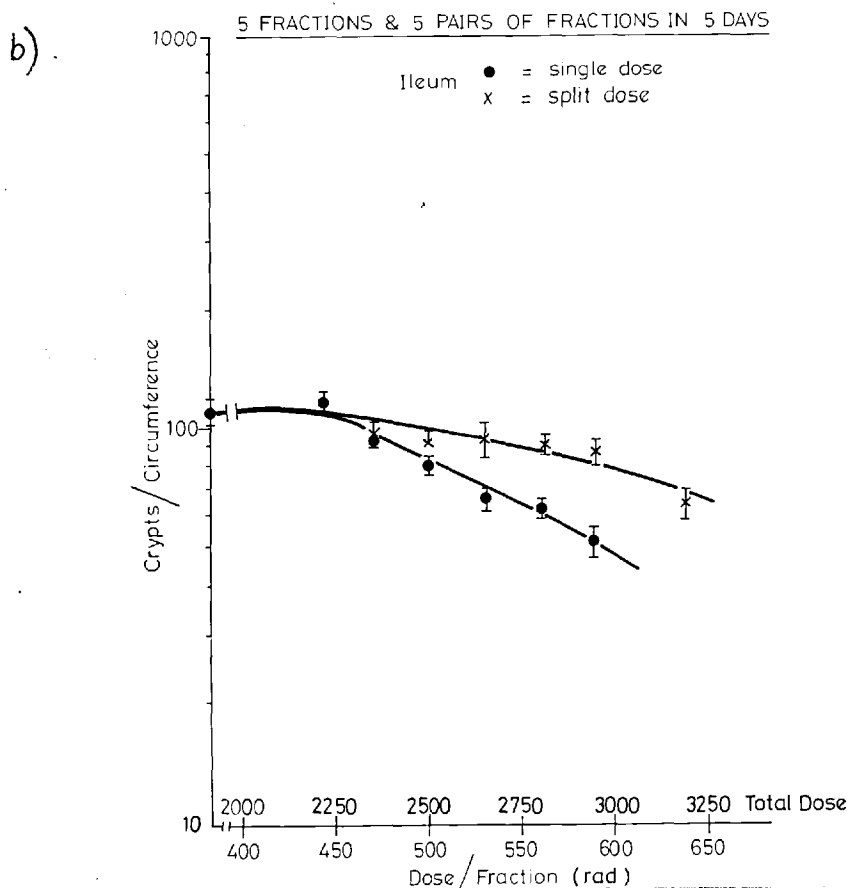
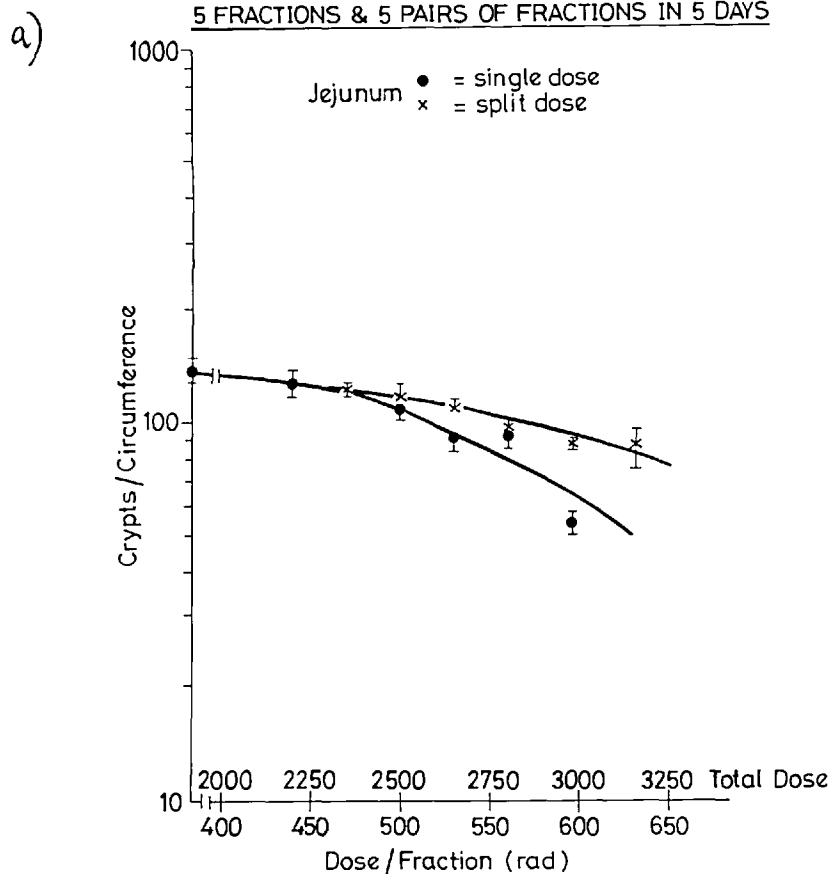


Figure 4.6. Relationship between total dose and crypts/circumference in the jejunum (a) and the ileum (b) after exposure to 5 single daily fractions or 5 pairs of daily fractions separated by 3.5 hours.

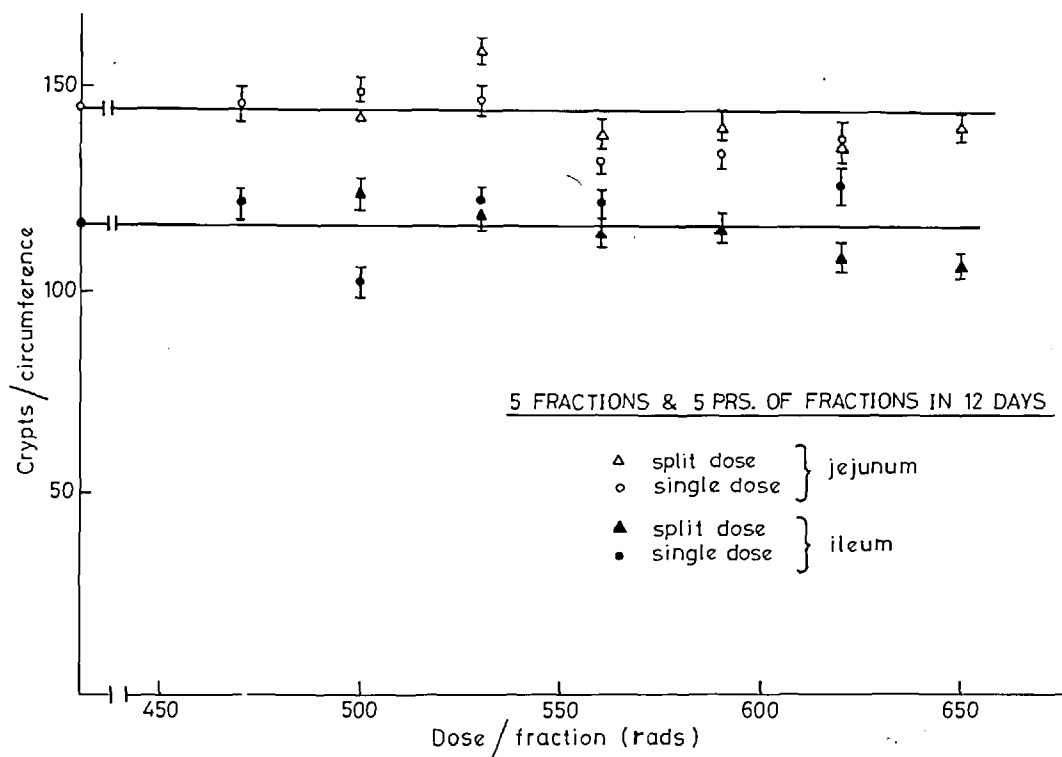


Figure 4.7. Relationship between total dose and crypts/circumference in the jejunum and in the ileum after exposure to 5 single dose fractions given at 2 day intervals and 5 pairs of dose fractions separated by 3.5 hours given at 2 day intervals.

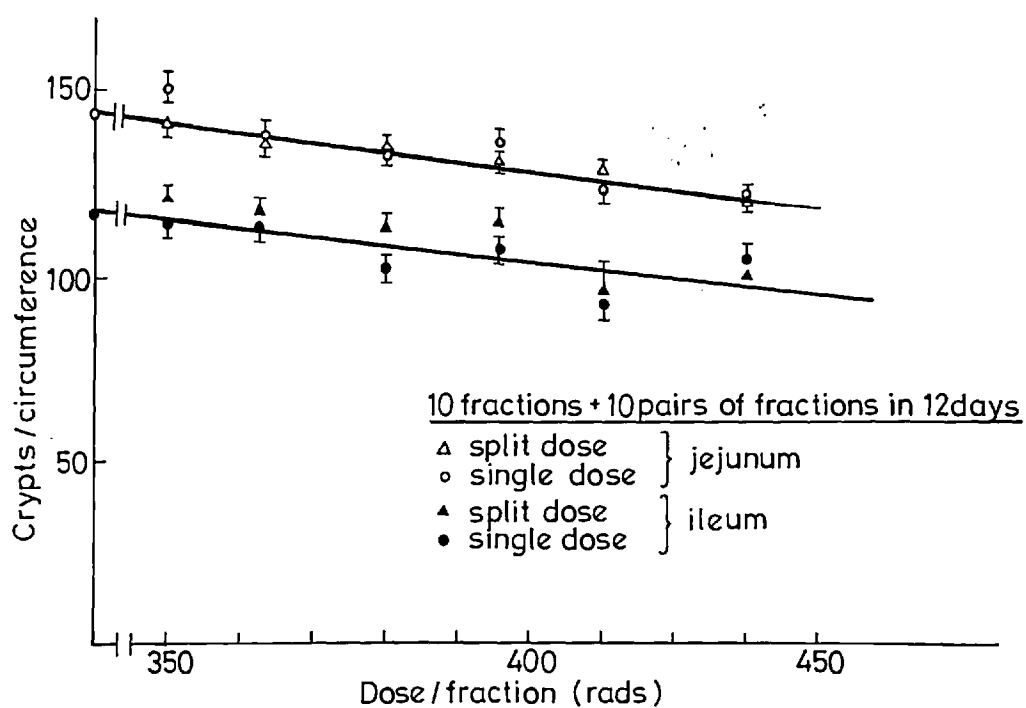


Figure 4.8. Relationship between total dose and crypts/circumference in the jejunum and in the ileum after exposure to 10 pairs and 10 single daily dose fractions separated by 3.5 hours.

This lack of any observed additional recovery from sublethal damage when the test dose is given before the assay dose may be due to differences in the cell cycle perturbations caused by the different sizes of the initial X-ray fraction. When doses are given as 2 split doses of 62.5 - 250 rads there will be little effect on cell cycle progression and the cells will have recovered from any mitotic delay after 2 hours. Consequently when the second X-ray dose of 75-250 rads is administered, and 2 hours later the neutron assay dose, the cells will be dispersed throughout the cell cycle and only a small additional amount of recovery from sublethal damage will be observed. However when a single unsplit dose of 250-500 rads is given there will be a mitotic delay of from 2 - 6 hours with delay being greatest for cells in the S phase (Yu and Sinclair, 1967). As a result when the neutron assay dose is given 2 hours later, there will be more cells in the resistant S phase and fewer cells will be killed than when the dose was split into two equal fractions.

In the ileum some recovery was observed after doses per fraction greater than 125 rads. This difference between the jejunum and the ileum in the amount of recovery from sublethal damage may be due to differences in their cell profile.

The additional recovery observed when test doses are given after an assay dose of neutrons may however be due to stimulation of repair by radiation. It has been shown by several workers, that survivors of an exposure to a conditioning dose followed by a radiation free interval had an increased capacity for repair in experiments with HeLa cells, algae (Bryant, 1975; Horsley and Laszlo, 1971) and Chinese hamster cells (Berry, 1966; Elkind, Moses and Sutton-Gilbert, 1967). Pollard and his co-workers (1975) had shown that ionising radiations could induce a protein which controlled the extent of DNA degradation. Additional capacity for repair was manifested by decreased terminal slopes to the survival curves and to increased extrapolation numbers (Alper, 1979).

4.4.2. Time dependent recovery after neutron assay dose

Following a 540 rad neutron assay dose the largest amount of recovery from sublethal damage observed between X-ray fractions was observed when the fractions were given 2 hours after the neutron assay dose and the interval between the fractions was 2 hours. Very small differences in stage sensitivity of cells after neutrons have been observed by Sinclair in Chinese hamster cells in vitro after fission neutrons (1968b). Withers, however (1974) observed considerable cyclic changes after 16 MeV neutrons in mouse jejunum crypt cells synchronised with hydroxyurea, although the variation was less marked than after X-rays. The S phase was found to be the most radiosensitive, while G_1 and G_2 were found to be equally sensitive.

In the jejunum and in the ileum less recovery was observed between fractions when the interval between fractions was 3.5 hours after an assay dose of neutrons. This may be due to some cell progression into the more sensitive phases or represent a true fall in the cellular recovery. Fractionation experiments using chromosome aberrations in human lymphocytes as an end point have demonstrated a rise in yield at a 4 hour fraction interval (Lane, 1953; Evans, 1966; Sasaki, 1978). Evans attributed this result to a re-opening of breaks at 4 hours when they would be available for interaction with the breaks induced by the 2nd dose fraction. Sasaki found that the second rise in chromosome aberration yield was suppressed by cyclohexamide and correlated this with repair of protein components of the chromosome. However a fall in recovery from sublethal damage between fractions was not observed here at 3.5 hours after X-rays. When the X-ray test doses are given 24 hours after the assay dose no recovery between fractions is observed in the jejunum. This may be due to a mitotic delay of approximately 17 hours which will be induced by 540 rads of neutrons so that the cells will be mainly in the G_1 , M and G_2 phases after 24 hours after irradiation, and these phases

are known to have a small ability to recover from sublethal damage. However recovery between doses was observed at 24 hours in the ileum.

4.4.3. Time dependent recovery after X-ray assay dose

The recovery that was observed between fractions in the jejunum after a large assay dose of X-rays was greatest when the interval between fractions was 3.5 hours and the fractions were given 3.5 hours after the assay dose. After a large dose of X-rays the majority of the surviving cells will be in the S phase, and the recovery observed between fractions 3.5 hours after the assay dose will be mainly due to the recovery of S phase cells, which are known to have a large capacity for repair (Sinclair and Morton, 1964). When the test dose is given 24 hours after the assay dose the observed recovery between fractions is small. This may be due to the movement of surviving S phase cells into the G₂, M and G₁ phases of the cycle by 24 hours after being released from a mitotic block of approximately 15 hours (see Section 3). Cells in these phases of the cell cycle have been found to show a small ability to recover from sublethal damage (Sinclair and Morton, 1964).

In the ileum no recovery was observed between pairs of X-ray fractions when the interval between fractions was 2 hours and the fractionated doses were given 2 hours after the X-ray assay dose. Intestinal cells in the ileum have a longer G₁ and a shorter S phase than cells in the jejunum (Quastler and Sherman, 1959; Hagemann and Stragand, 1977, Section 2), and as a consequence there will be more cells in the G₁ phase and fewer in the S phase at the time of the first irradiation. This may result in less recovery from sublethal damage being observed after 2 hours in the ileum compared with the jejunum. There is, however, also evidence that cells with a long G₁ phase such as the ileum show a resistance to X-rays in the early G₁ phase (Sinclair, 1968a). Two hours after an assay dose of 1025 rads of X-rays any G₁ survivors will have moved into the G₁/S border which has been shown to be a radiosensitive

stage in Chinese hamster cells in vitro. (Sinclair, 1968a.) 3.5 hours after the X-ray assay dose these G₁ survivors will have progressed into the S phase and some recovery is observed between fractions.

4.4.4. Multiple fractions

There was complete recovery from sublethal damage after doses/fraction of 160 rads given at 12 hourly intervals, 220 rads at 24 hourly intervals and 450-650 rads at 48 hourly intervals.

Wambersie and Dutreix using similar fractionation schedules to unanaesthetised mice obtained a single dose LD 50/20 of 489 rads and a split dose LD 50/20 of 537 rads after 5 fractions in 2 days intervals. So that using the same fractionation schedule they obtained some cell killing which was not observed here. Wambersie and Dutreix did not anaesthetise their animals, while in the experiments here the animals were anaesthetised when fractions were given in 5 and 12 days. It has been shown by Keizer (1976) and Hornsey (1970c) that the use of anaesthetic can cause an increase in survival due to hypoxia induced by the alteration in blood flow caused by the anaesthetic. It is also possible that this discrepancy in results using crypt survival as an assay and LD50 may indicate that at these doses per fraction of 400-600 rads there is a profound effect on villus function but no effect on crypt survival. This observation has also been made by Carr et al. (1979) and may be due to a failure of crypt cells to migrate onto the villus during long fractionation treatments. Hagemann (1976) however found that for doses of 1000 rads followed 12 and 24 hours later by 800 rads and pulse labelled with ³H thymidine many labelled cells were on the villi, showing that cells were migrating at the normal rate. Gut death after fractions of this size may therefore be caused by an inefficiency of these cells in serving as an effective barrier to the gut rather than a lack of cells. Lieb et al. (1977) found that the cells that covered the villi 3 days after irradiation were large undifferentiated cells which would not be

able to function as a normal differentiated villus cell in fluid and electrolyte transport.

4.4.5. Recovered Dose

The amount of recovery between split doses was measured from crypt survival curves as the additional dose necessary to reach a given biological effect with a single dose compared with the dose split into two sub-fractions separated by varying time intervals. The amount of recovery between doses or the recovered dose was measured for each experiment at 4 dose levels and the results are listed in Tables 4.1. 4.2. & 4.3. This measured recovery is the additional recovery which takes place between split doses above that which takes place after the single dose. The results show a considerable variation in the amount of recovery depending on the time interval between the assay dose and the X-ray dose and the time interval between pairs of X-ray fractions. The lack of any observed recovery between split doses indicates that there is no additional recovery either because all the recoverable damage has already been repaired after the single dose, or because there is no recovery taking place. The largest amount of recovery was observed when X-ray fractions were delivered after a large assay dose of X-rays or neutrons since this method selects the resistant S phase cells which have a large capacity to repair sublethal damage. When cells are uniformly distributed throughout the cell cycle, i.e. when the test dose is given before the assay dose, at 24 hours after the assay dose and in multiple fractionation experiments, very little if any additional recovery is observed between fractions. This suggests that all the recovery has taken place after the single dose so that no additional repair can take place by splitting the dose further.

4.4.6. Variation of D_r as a function of D_s

Figure 4.9. shows the variation of the recovered dose D_r as a function of dose per fraction D_s . D_r is shown to increase as the dose

TABLE 4.1.

Recovered dose1 fraction or 2 subfractions given after an assay dose of 540 rads neutrons.

<u>Interval between assay dose and X-ray fraction</u>	<u>Interval between pairs of fractions</u>	<u>Single dose</u>	<u>Total dose in 2 fractions</u>		<u>Recovered dos</u>	
			<u>jejunum</u>	<u>ileum</u>	<u>jejunum</u>	<u>ileum</u>
2	2	150	280	300	130	150
		200	400	300	200	160
		300	530	475	230	175
3.5	3.5	150	200	170	50	20
		200	240	260	40	60
		300	330	390	30	90
		400	440	480	40	80
24	24	150	150	275	0	125
		200	200	380	0	180
		300	300	420	0	120
		400	400	475	0	75

TABLE 4.2.

Recovered dose1 fraction or 2 subfractions given after an assay dose of 1250 rads of X-rays

<u>Interval between assay dose and X-ray fraction</u>	<u>Interval between pairs of fractions</u>	<u>Single dose</u>	<u>Total dose in 2 fractions</u>		<u>Recovered dose</u>	
			<u>jejunum</u>	<u>ileum</u>	<u>jejunum</u>	<u>ileum</u>
2	2	150	160	150	10	0
		200	210	215	10	0
		300	500	300	200	0
3.5	3.5	150	290	380	140	230
		200	350	400	150	200
		300	500	430	200	130
24	3.5	150	220	320	70	110
		200	280	360	80	160
		300	380	420	80	120

TABLE 4.3.

Recovered dose after multiple fraction schedule5 fractions and 5 pairs of fractions in 2 days

<u>Interval between subfractions</u>	<u>Single dose</u>	<u>Total dose in 2 fractions</u>		<u>Recovered dose</u>	
		<u>jejunum</u>	<u>ileum</u>	<u>jejunum</u>	<u>ileum</u>
3.5	350	390	380	40	30
	400	440	430	40	30
	450	490	510	40	60
	500	560	580	60	80

5 fractions and 5 pairs of fractions in 5 days

<u>Interval between subfractions</u>	<u>Single dose</u>	<u>Total dose in 2 fractions</u>		<u>Recovered dose</u>	
		<u>jejunum</u>	<u>ileum</u>	<u>jejunum</u>	<u>ileum</u>
3.5	450				
	475	494	540	20	65
	500	560	600	60	100
	550	640	660	90	110

5 fractions and 5 pairs of fractions in 12 days

<u>Interval between subfractions</u>	<u>Single dose range</u>	<u>Split dose range</u>	<u>Recovered dose</u>
3.5	470 - 620	250 - 325	-

10 fractions and 10 pairs of fractions in 12 days

<u>Interval between subfractions</u>	<u>Single dose range</u>	<u>Split dose range</u>	<u>Recovered dose</u>
3.5	350 - 430	175 - 215	-

SUB-LETHAL DAMAGE AFTER X-RAYS

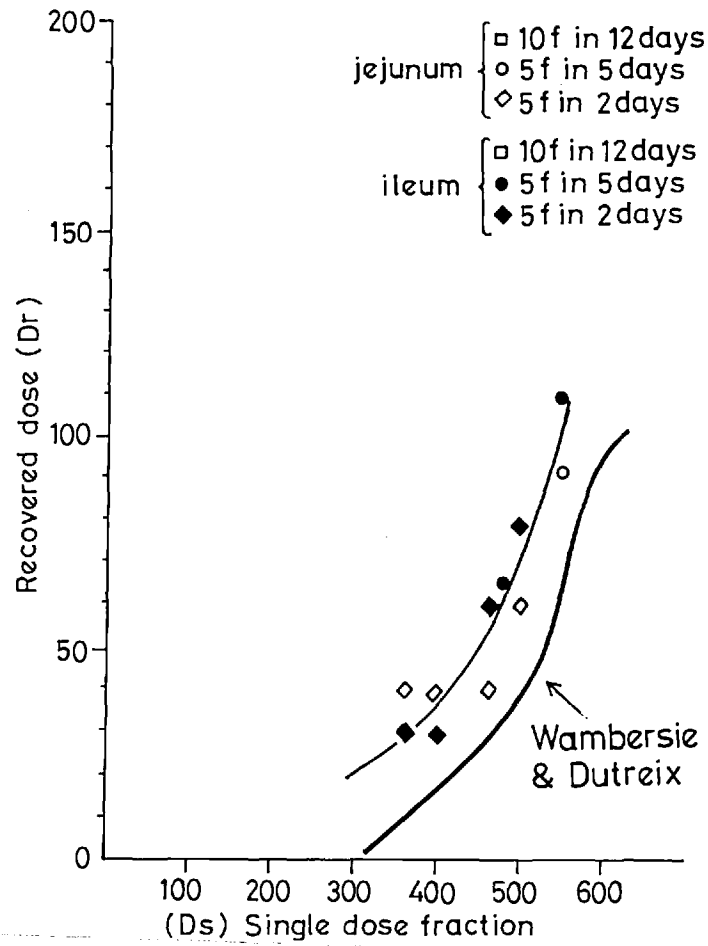


Figure 4.9. Recovered dose (D_r) is plotted against dose per fraction (D_s).

per fraction increases. Values of D_r obtained by Wambersie and Dutreix for similar fractionation schedules assayed for intestinal death at 9-20 days are also shown. The intercept of the recovered dose D_r with the D_s abscissa is the dose below which all sublethal injury is repaired and all cell killing results from single events (non-repairable injury). Thus dividing doses into fractions smaller than the D_s intercept of the D_r curve does not increase the sparing effect of dose fractionation associated with repair of sublethal injury. Results obtained here indicate that this occurs at approximately 200 rads in the intestine but it is not possible to state if damage below this dose is due to single hit damage from this data. The results in this study differ from Wambersie and Dutreix's data at the low dose levels for intestinal death although there is agreement at high doses fraction levels.

4.4.7. Isoeffect curves

The relationship between total dose for a certain effect (10 crypts/circumference) and the number of dose fractions is plotted against log co-ordinates in figure 4.10 to give an isoeffect curve with a slope of 0.318. The data are derived from experiments in which fractionation intervals of 3.5 and 12 hours were used. Withers (1974) obtained a similar slope ratio of 0.32 for intestinal crypt cells from experiments in which 3 hour fractionation intervals were used. When 12 and 24 hours were used Withers observed more cell survival and the data did not correspond with the data from the 3 hour fractionation schedule.

The slope ratio obtained in this study and also that obtained by Withers for the intestinal crypt cells is slightly lower than the value of 0.39 obtained for lung (Field and Hornsey, 1974) and the value of 0.37 for spinal cord (Hornsey and White, 1980), but is greater than the exponent of 0.25 in the Ellis formula derived from data on skin. The size of the slope in the intestinal crypt cells is however small considering the large capacity these cells have for repair.

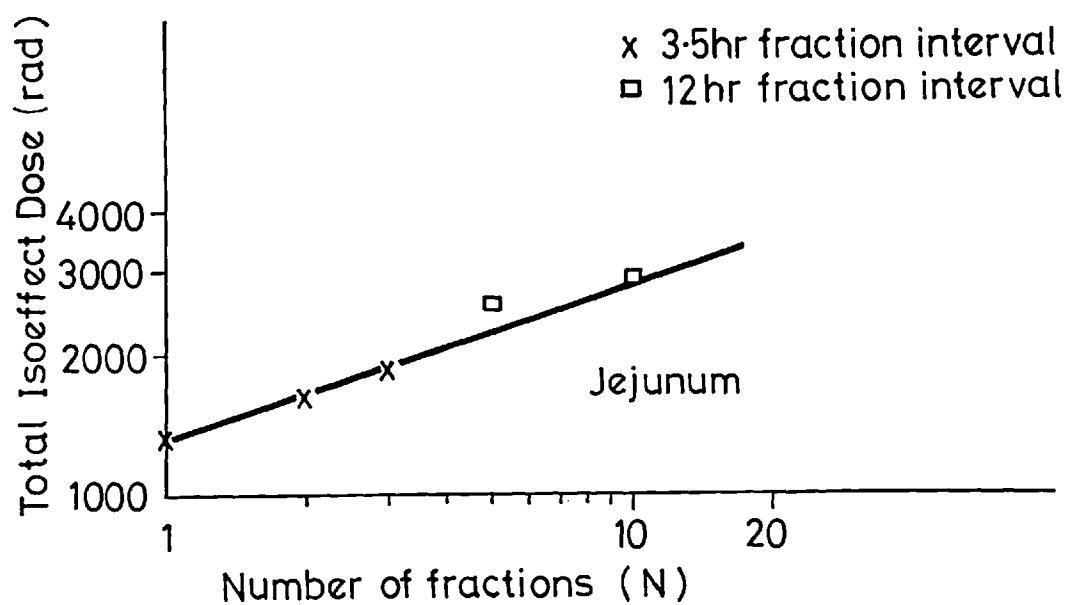


Figure 4.10. Isoeffect curve plotted on logarithmic co-ordinates.

The slope is 0.32.

4.4.8. Repair mechanism

The ability of cells to recover from radiation inflicted damage could be due to 1) the DNA synthetic activity of cells in the S phase (Sinclair, 1968), 2) the presence of a factor, the Q factor, which prevents the damage being expressed and may be particularly important in G_1 phase cells (Sinclair, 1972) or 3) the distribution of chromatin in the nucleus. Dewey proposed that the resistance of S phase cells could be attributed to the chromatin being spaced far apart so that there would be less chance for interaction between single sublethal lesions to form a lethal lesion than would be the case in a mitotic cell where the chromatin is tightly packed. On the other hand it is postulated in the repair model that when the repair system is fully saturated, damaged or misrepaired lesions will remain and the damage will be lethal (Elkind, 1980).

Whatever the method of recovery from radiation injury the proportion of cells in the S and G_1 phases will determine the size of the shoulder on the cell survival curve and hence the recovery between fractions.

4.5. Conclusions

1. The results reported here suggest that after doses/fraction of 200 rads of X-rays and below all sublethal damage is recovered and that no further recovery can take place by further splitting the dose indicating that all lethal damage at these doses could be due to single event killing.

2. The amount of additional recovery observed when a dose fraction is split into equal fractions is strongly dependent on the stage of the cell cycle of the population under investigation; the greatest amount of recovery from sublethal damage occurring in cells in the S and G_1 phases while very little recovery is observed in G_2 and M cells.

SECTION 5.

5.0. Repair of sublethal damage after neutrons when combined with X-rays

5.1. Introduction

An understanding of the radiobiological mechanisms involved in mixed irradiations of X-rays and neutrons is of considerable importance from a therapeutic as well as a radiobiological point of view. Experiments performed in vitro with fission neutrons demonstrated effects on chromosomes to be additive with no synergism in the formation of breaks. (Wolff et al., 1958; Savage, 1967; Preston, 1970). Savage (1967) working with *Tradescantia* microspores showed that X-rays and neutrons can act on the same exchange sites (places in the nucleus where the chromosomes come close enough together for an exchange to take place). Also he showed that the saturation of the dose response curve observed at high doses of X-rays and neutrons occurs at the same frequency of exchanges per cell. Survival curves obtained for *Tradescantia* interphase pollen by Preston (1970) irradiated with fission neutrons alone or with an X-ray dose followed rapidly by different neutron doses were found to be parallel, indicating that no interaction between the X-ray and neutron doses took place. This result was in agreement with that found from chromosome aberration scores in *Tradescantia*.

Similar findings were obtained by Barendsen et al. (1960) on cell survival of human cells in vitro with α -particles and gamma rays, when gamma ray irradiation was found to act on cells surviving α -irradiation as if they had not been irradiated at all. Recent work in vitro on Chinese hamster V79 cell survival by Ngo et al. (1977) has shown that fast neutrons deposited less sublethal damage than a dose of X-rays giving equivalent survival. When fission neutrons from the Janus reactor were followed immediately by X-rays the resultant X-ray survival curve exhibited a small shoulder, and when X-rays were given 2 hours after a dose of neutrons the shoulder of the fractionation survival curve increased substantially

while the D_0 remained unchanged, demonstrating that repair of sublethal damage deposited by the neutron dose had taken place.

These results disagree with the findings of Railton et al. (1975) and Durand and Olive (1976) on Chinese hamster cells in vitro which suggested that there was some synergistic damage when neutrons and X-rays were combined. Their results suggested that there was very little repair from sublethal damage deposited by neutrons after fractionation intervals of 1 - 3 hours. Railton et al. found that the resultant surviving fraction when gamma rays and 14 MeV neutrons were mixed was less than the product of the survivors for the individual doses though the differences were not significant.

Hornsey et al. (1977) showed that sublethal damage incurred by mouse intestinal crypt cells after 6 MeV neutrons was reparable and that reduced recovery observed after fractionating a dose of neutrons was due to a smaller deposition of sublethal damage. No difference was observed in the X-ray survival curve after a survival equivalent dose of neutrons. Also the rate at which the sublethal injury was repaired was the same regardless of the initial radiation.

I report here on experiments which were designed to obtain further information on the effect of combining treatments of X-rays and 6 MeV cyclotron neutrons. In these experiments the neutrons were followed immediately with doses of X-rays and the results are compared with those from X-ray fractionation experiments described in Section 4 where single X-ray doses were given at 2, 3.5 or 24 hours after an assay dose of X-rays or neutrons.

5.2. Methods

Female CFLP mice were irradiated with either 160 or 540 rads of neutrons or 160 or 1025 rads of X-rays, these doses were followed immediately (1- 2 mins) by a second dose of X-rays of 164-656 rads and the animals were killed at 4 days for crypt survival analysis.

5.3. Results

The results were plotted as crypt survival per circumference in figure 5.1. or as cell surviving fraction in figure 5.2.a and b against dose (semilog plot) and best fitting lines were obtained by a least squares regression analysis for the crypt data and from a weighted linear regression for the surviving fraction data. Single dose effect curves for X-rays and for neutrons are also plotted for comparison. D_0 values calculated from the cell surviving fraction data are listed in Table 5.1.

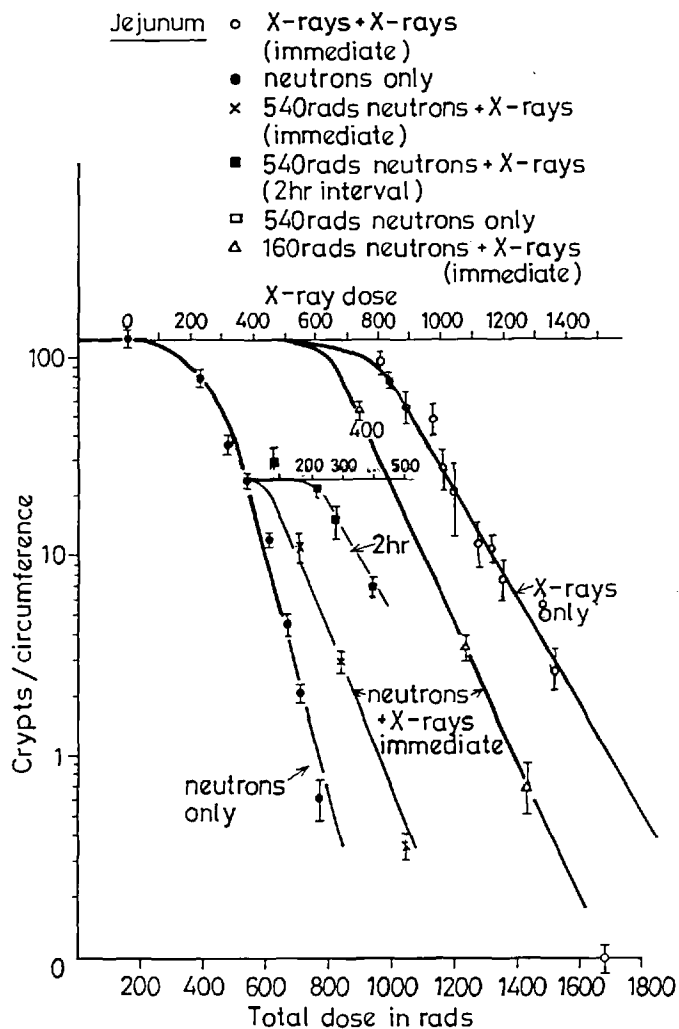
When neutrons are followed immediately by doses of X-rays the resultant X-ray curve had a small shoulder in the jejunum and no shoulder in the ileum while the slope of the curves was steeper than with X-rays alone (Figures 5.1. and 5.2). D_0 values of 98.2 ± 0.85 and 109 ± 1.29 were obtained for the jejunum and ileum respectively compared with 138 ± 1.77 , 128 ± 1.8 for X-rays alone. The presence of a shoulder in the jejunum indicates that there is still some more sublethal damage to be accumulated after 540 rads of neutrons. In the ileum however there has been full deposition of sublethal damage after 540 rads of neutrons.

When a dose of 160 rads of neutrons was followed immediately by X-ray doses of 200-800 rads the resultant X-ray curve exhibited a large shoulder showing a large accumulation of sublethal damage. There will only be a small amount of sublethal damage deposited after 160 rads of neutrons, most of the damage being caused by the high LET component of the beam (Hornsey ^{et al.} 1977) so that when X-rays are given immediately afterwards there is a large accumulation of sublethal damage. The slope of the X-ray curve after the small neutron dose was not significantly steeper than the slope for X-rays alone with a D_0 of 131 ± 1.1 and 138 ± 1.9 for the jejunum and ileum respectively.

5.4. Discussion

The small shoulder on the X-ray survival curve in the jejunum after an initial dose of 540 rads of neutrons indicates that there is

a).



b).

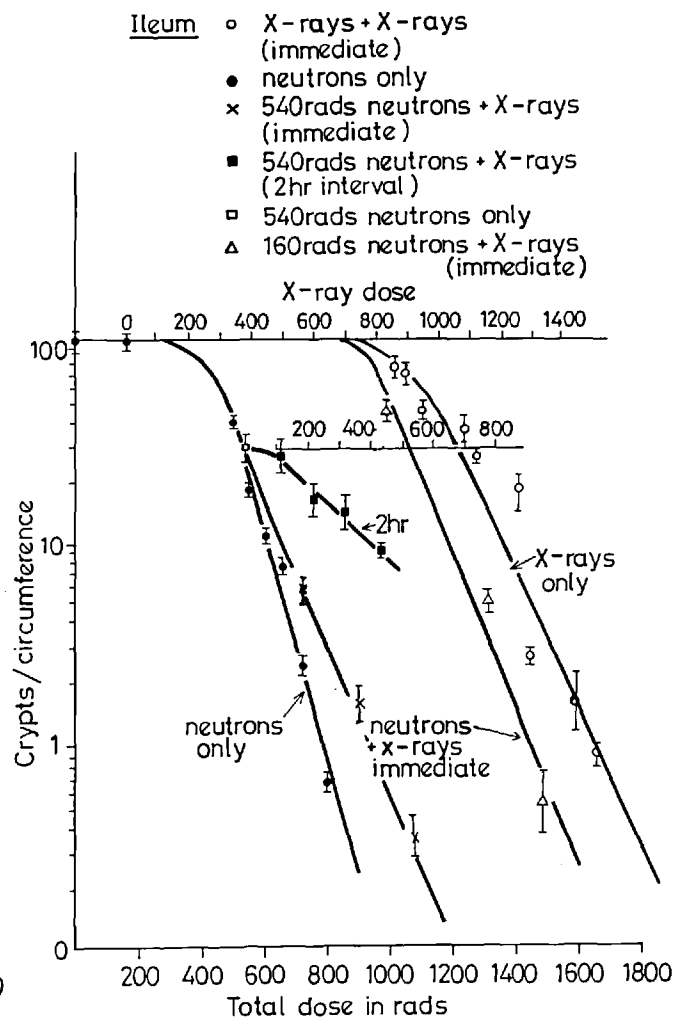


Figure 5.1. (a and b) Shows the number of crypts/circumference in the mouse jejunum (a) and in the ileum (b) when X-rays were given immediately after a first dose of neutrons. Single dose crypt survival curves after X-rays and neutrons are also shown.

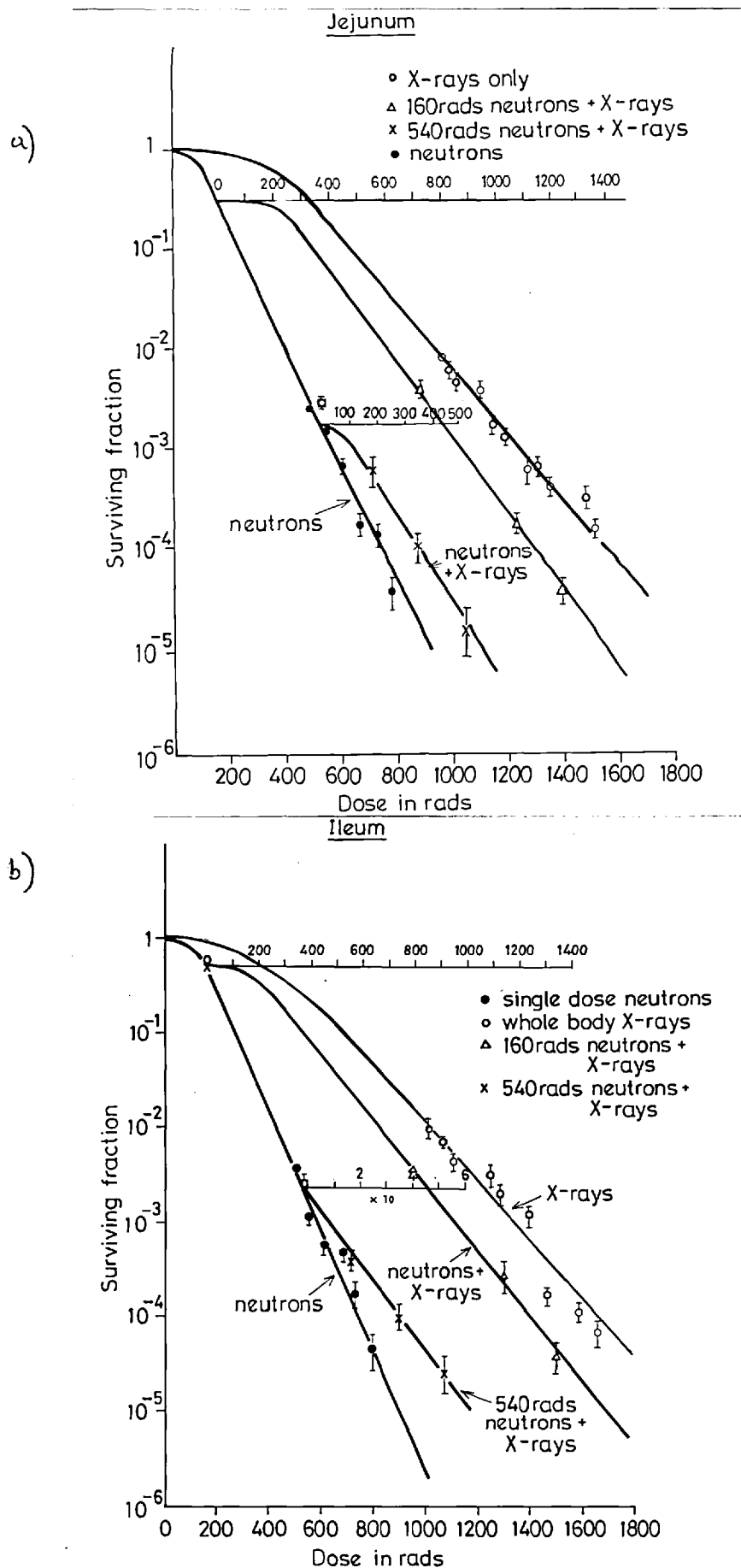


Figure 5.2. (a and b). Cell survival curves for jejunal (a) and ileal (b) cells after a 2nd dose of X-rays given immediately after a 1st dose of neutrons. Single dose survival curves after X-rays and neutrons are also shown.

Cell survival wave parameters after combined neutron and X-ray treatments

Table 5.1.

Treatment	<u>Jejunum</u>			<u>Ileum</u>		
	D_0	D_q	n	D_0	D_q	n
540 rads neutrons plus X-rays	98 ± 0.8	-31 ± 5.9	0.7 ± 0.3	109 ± 1.3	-134 ± 9.1	0.294 ± 0.1
160 rads neutrons plus X-rays	131 ± 1.1	210 ± 7.9	5.1 ± 0.1	138 ± 1.9	178 ± 5.4	3.6 ± 0.1
X-rays only	138 ± 1.7	305 ± 21	7.1 ± 0.1	128 ± 1.8	376 ± 27	9.4 ± 0.2
Neutrons only	83 ± 1.6	80 ± 9	3.5 ± 0.5	99 ± 3.5	35 ± 2.4	1.9 ± 0.4
540 rads neutrons plus X-rays 2 hrs	229 ± 9.9			378 ± 1.5		
" 3.5 hrs	220 ± 10.4			171 ± 5.4		
" 24 hrs	295 ± 11.5			212 ± 9.1		
1025 rads X-rays plus X-rays 2 hrs	157 ± 6.7			259 ± 10.1		
" 3.5 hrs	253 ± 12.8			309 ± 9.6		
" 24 hrs	236 ± 9.1			137 ± 24.5		

The cell survival curve parameters listed above were all based on a value of 135 proliferative cells/crypt as determined by Potten and Hendry 1975. An assumption was made that the proliferative cells are all cryptogenic cells.

still some sublethal damage to be accumulated after 540 rads of neutrons. The lack of any shoulder on the X-ray survival curve after 540 rads of neutrons in the ileum indicates that there has been full deposition of sublethal damage. Ngo et al. using fission neutrons on Chinese hamster cells in vitro obtained a small shoulder on the survival curve when 275 rads of neutrons were followed immediately by doses of 250 kVp X-rays. They concluded that the cells surviving the neutron dose would have fewer sublethal lesions than would be the case after a survival equivalent X-ray dose. Hornsey et al. (1977) obtained a shouldered X-ray survival curve in the jejunum when X-rays were given 15 minutes after 540 rads of neutrons. It was suggested that this could be due to recovery from sublethal damage during this 15 minute interval since recovery had been demonstrated to be very fast in the intestinal cells (Hornsey and Alper, 1966). The data in this study would suggest that this interpretation was correct since when cells were irradiated immediately with X-rays after neutrons the X-ray curve shows a greater sensitivity to X-rays compared with cells irradiated with X-rays 15 minutes after a neutron dose by Hornsey et al. (1977).

When a smaller assay dose of 160 rads of neutrons was given before the X-ray doses the shoulder of the X-ray survival curve was reduced for both jejunum and ileum (Figure 5.2.). This can be seen in the reduction of D_q from 305 rads for X-rays alone to 210 rads for the jejunum and 376 rads for X-rays alone to 178 rads for the ileum. This is still however a substantial shoulder to the X-ray survival curve following neutrons and confirms the suggestion that the amount of sublethal damage after neutrons shown up by subsequent X-irradiation will depend on the neutron dose used (Ngo et al., 1977).

5.4.1. Slope of X-ray survival curve following neutron assay dose

The slope of the X-ray survival curve obtained when X-rays are given immediately after 540 rads of neutrons for mouse intestinal cells

was steeper than that obtained with single doses of X-rays. This may be due to differences in the cell profile of survivors when survival was reduced by neutrons compared with X-rays. A larger number of S phase cells may survive 1025 rads of X-rays than 540 rads of neutrons. As cells in the S phase are the least sensitive, the X-ray curve immediately following X-rays will have a larger D_0 . The steeper slope after a neutron assay dose may also reflect some enhancement of X-ray damage. Ngo et al. (1980) observed some enhancement of effect using Chinese hamster cells when Neon ions were combined with X-rays.

When a smaller neutron dose (160 rads) was followed immediately by X-rays the slope of the X-ray curve was not significantly steeper than that obtained for X-rays alone. 160 rads of neutrons would not give much differential cell killing and therefore the slope of the following X-ray survival curve would be similar to that of the unperturbed system. However, although the D_0 of the X-ray survival curve following 160 rads of neutrons is not significantly different from the X-rays alone curve it is tending to be less. The slope of the X-ray survival curve after 540 rads of neutrons is, however, significantly steeper which suggests that with increasing neutron assay doses the X-ray survival curve steepens compared with the curve for X-rays alone.

5.4.2. Rate of recovery after neutrons

In order to study the single dose X-ray survival curves after large assay doses and since equivalent survival was not obtained after 1025 rads of X-rays and 540 rads of neutrons in every experiment, the crypts per circumference data was normalised to the value obtained from the single dose survival curve. Thus a clearer comparison could be made of the effects of varying fraction intervals on the resultant X-ray survival curve. In Figures 5.3. and 5.4. the surviving fractions after a neutron and X-ray assay dose are shown for the jejunum (a) and the ileum (b) when doses are followed 2, 3.5 and 24 hours after the assay dose.

a).

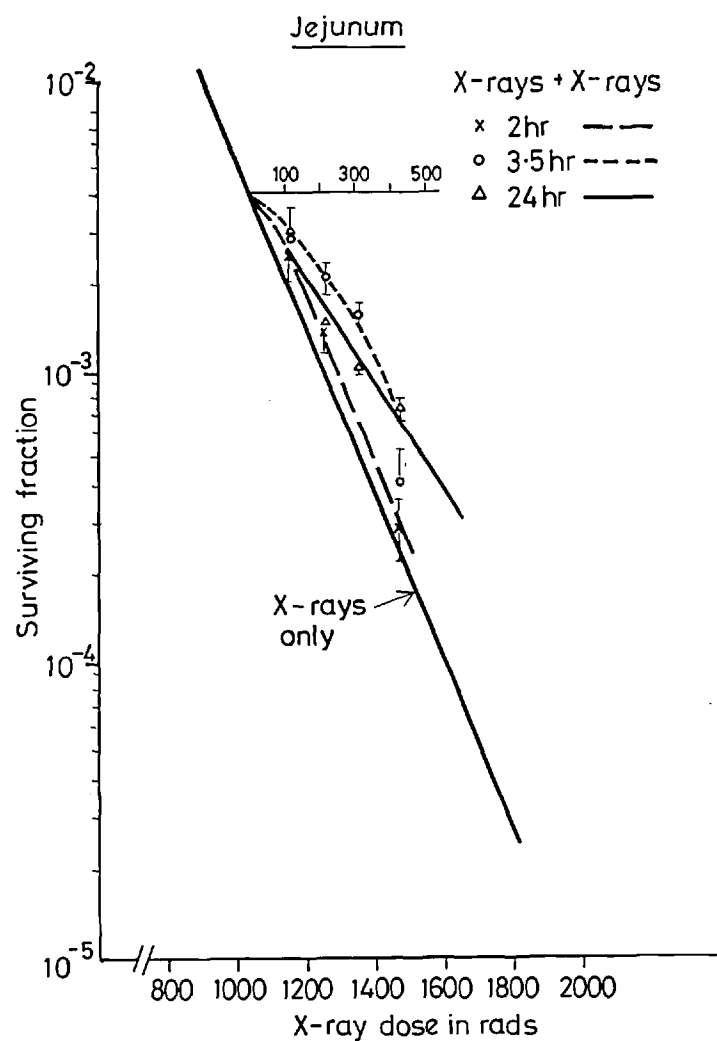


Figure 5.3. (a and b) Cell survival curves for jejunum (a) and ileum (b) after = 2nd dose of X-rays given 2, 3.5 and 24 hours after an assay dose of X-rays.

b).

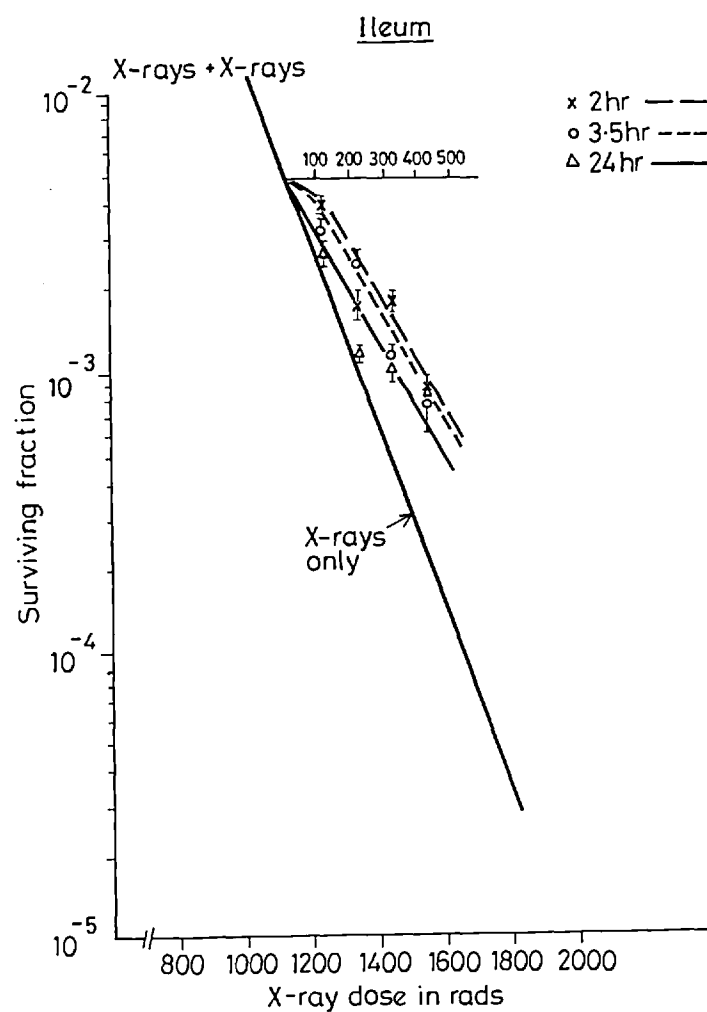


Figure 5.3. (a and b) Cell survival curves for jejunum (a) and ileum (b) after = 2nd dose of X-rays given 2, 3.5 and 24 hours after an assay dose of X-rays.

a).

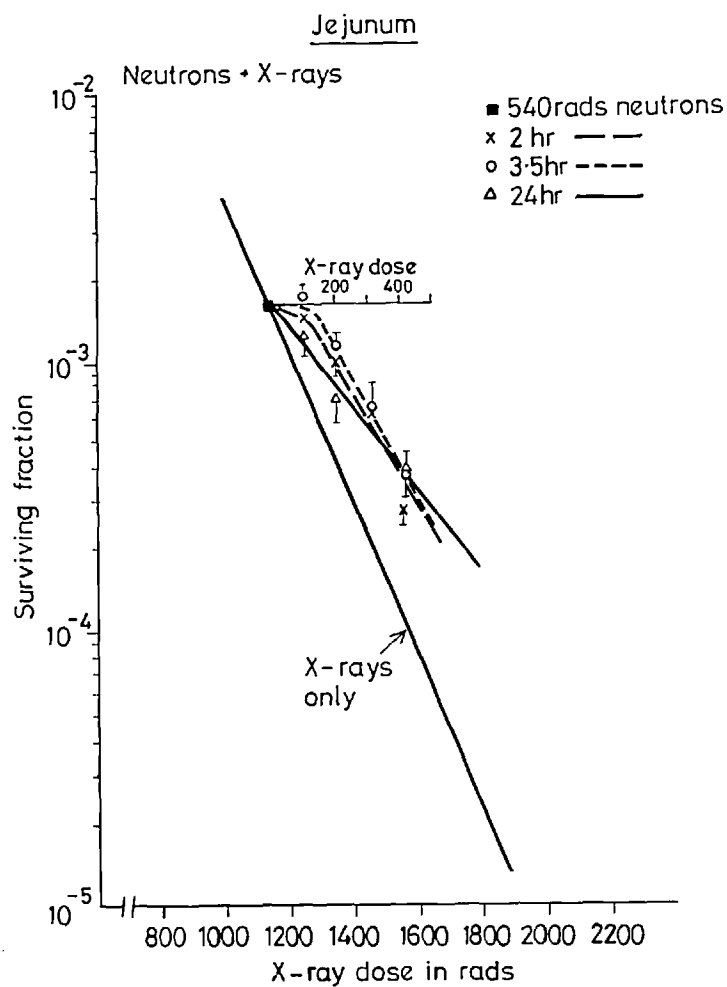


Figure 5.4. (a and b). Cell survival curves for jejunal (a) and ileal (b) crypt cells after a 2nd dose of X-rays given 2, 3.5 and 24 hours after an initial assay dose of neutrons.

b).

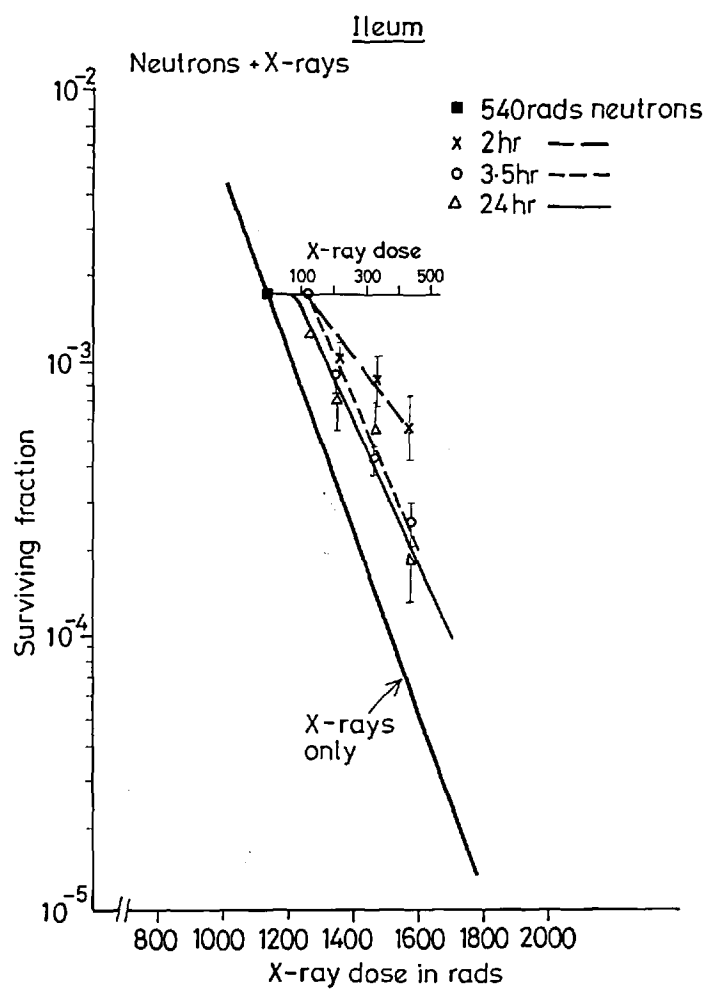


Figure 5.4. (a and b). Cell survival curves for jejunal (a) and ileal (b) crypt cells after a 2nd dose of X-rays given 2, 3.5 and 24 hours after an initial assay dose of neutrons.

The results show that the rate of recovery from sublethal damage after neutrons is similar to that after X-rays. Within the errors of the data, the recovery rate is similar after neutrons and X-rays. There is slightly more cell survival with X-rays following a neutron assay dose than following an X-ray assay dose and there is less variability after neutrons. This may be due to the different perturbations of the cell profile by neutrons compared with X-rays.

5.5. Conclusions

1. There is very little further sublethal damage to be accumulated when X-rays follow within 2 minutes of a large neutron dose (540 rads) suggesting that there is almost full deposition of sublethal damage.

2. Results show that recovery from sublethal damage is as rapid after neutron irradiation as after X-irradiation.

3. The X-ray survival curve immediately following a large neutron dose is steeper than for X-rays alone because of a difference in the killing cell profile of neutrons relative to X-rays. This results in a different population of cells surviving the 1025 rad X-ray assay dose compared with the 540 rad neutron assay dose.

SECTION 6.

6.0. The variation in response of crypt cells to neutron and X-ray irradiation through the cell cycle

6.1. Introduction

Studies on cell killing and repair of sublethal damage in mouse intestinal crypt cells after fractionated treatments have indicated that different amounts of cell killing and repair take place depending on the time interval between fractions. (Withers and Elkind, 1969; Field and Hornsey, 1977; this thesis Section 4). In addition when an assay dose is used to reduce crypt survival down to measurable levels the amount of recovery observed between X-ray dose fractions which follow depends on the interval of time allowed between assay dose and fractionation treatment. Previous results on both Chinese hamster cells in vitro (Sinclair 1968) and mouse cells in-vivo (et al. Gillette et al 1974) (Withers, 1974) have shown that the amount of cell killing and repair after X-rays and neutrons is dependent on the age distribution of the population under investigation. So that some of the variation in repair observed with different time intervals between split doses may be due to differences in the cell profile caused by partial synchronisation after the first dose fraction. Differences may be expected between X-rays and neutrons because neutrons kill cells less selectively than X-rays, so that a different population of cells will survive a dose of X-rays compared with an equivalent dose of neutrons killing the same proportion of the total cell population.

In this section I report on the response of partially synchronised intestinal crypt cells to radiobiologically equivalent doses of 250 kVp X-rays or 6 MeV neutrons as they progress through the cell cycle.

Before a study can be made of the sensitivity of the cells in the different phases of the cycle the cells must be synchronised so that they move together through the cycle for as long as possible. Synchronising agents which have been used in vitro include Hydroxyurea (HU), high

(Sinclair, 1968a)

specific activity tritiated thymidine³ and mitotic selection (Sinclair and Morton, 1966). Only the former two methods can be used in in vivo studies. Hydroxyurea was employed in this study since it has been shown to both kill in excess of 90% of S phase cells and cause a block in the G₁ - S transition for 2-3 hours (Phillips et al., 1967). By giving two injections separated by 2 hours it is possible to accumulate cells at the G₁/S border so that 2-3 hours later these cells will begin to move into the S phase together.

6.2. Methods

Hydroxyurea (1.5mg/g body weight) was administered to female CFLP mice by intraperitoneal injection in 0.1 ml of physiological saline. This was followed 2 hours later by a second injection of 0.6mg/g body weight. At various times after HU treatment from 10 minutes to 7 hours 6 animals per group were irradiated with either 1025 rads of X-rays or 540 rads of neutrons, crypt survival was determined at 4 days post irradiation.

6.3. Results

When crypt cells are irradiated with X-rays immediately after administration of HU there is less crypt survival than with irradiation alone due to the killing of S phase cells by the hydroxyurea treatment (HU) as shown in Figure 6.1. The initial fall is followed by a slight rise in crypt survival at 1 and 2 hours in the jejunum, by 3 hours crypt survival is at its lowest level before rising to a maximum at 6 hours.

In the ileum where there is less variation in the response to X-rays there is a small initial fall in crypt survival immediately after HU treatment and then the level of survival remains fairly constant until 6 hours when it peaks before falling again at 7 hours as in the jejunum.

The same general phasic changes observed after X rays were also

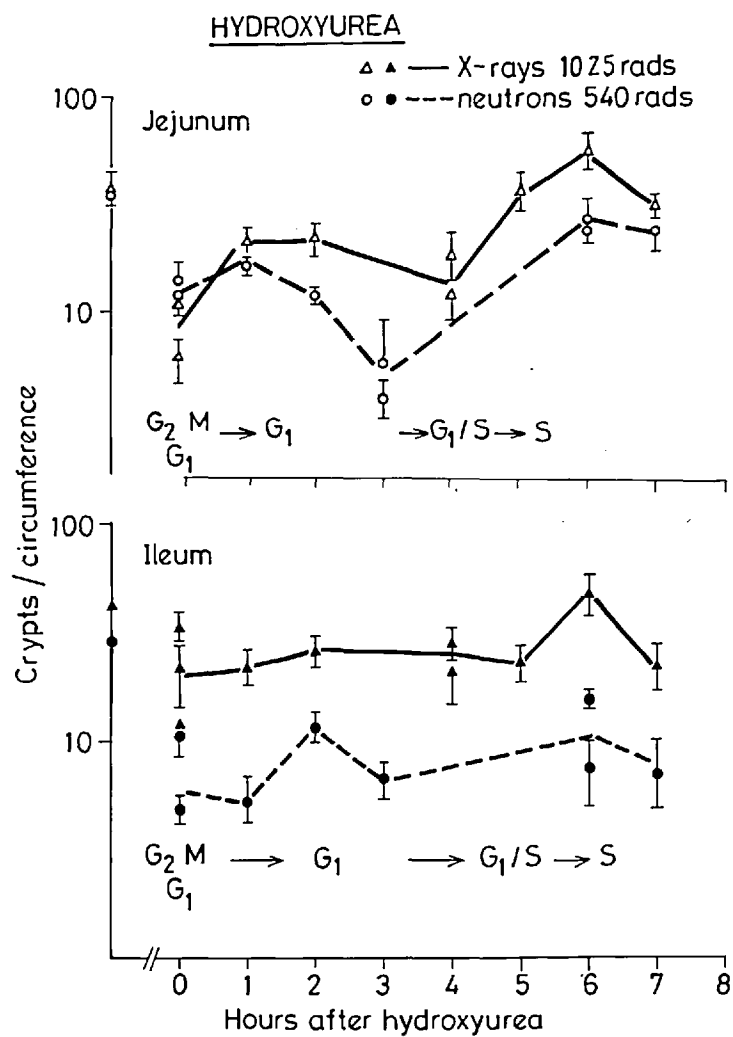


Figure 6.1. Crypts/circumference for jejunum and ileum plotted as a function of time after injection of hydroxyurea after X-rays and neutrons.

observed after neutron irradiation with the exception that in the jejunum crypt survival observed immediately after HU treatment was greater than after X-irradiation. The survival level then remained fairly constant at 1 and 2 hours falling to a minimum value at 3 hours. There was a peak in crypt survival at 6 hours as observed with X-rays. In the ileum there was a small increase in crypt survival at 3 hours after which the level remained fairly constant with no significant peak at 6 hours.

6.4. Discussion

General

Comparative studies on age response of cells to X-rays and neutrons have shown a more marked cyclic dependence of sensitivity to X-rays than to neutrons (Sinclair, 1968b; Withers et al., 1974). Most of the studies on cell cycle response to X-rays have been made on synchronised Chinese hamster cells in vitro. In general, results have shown that cells are least sensitive during the latter part of the S period. In addition, in cells with a long G_1 , there is usually a resistant phase early in G_1 followed by a sensitive stage near the end of G_1 (Sinclair, 1968a). Sinclair also studied the effects of fission neutrons and X-rays on the age response of synchronous and asynchronous Chinese hamster cells in vitro. He observed a large cyclic variation after X-rays but a very small variation after fission neutrons. A similar result was obtained by Masuda (1971) with 14 MeV neutrons on synchronised mouse L cells.

Hall et al. (1972) studied the age response of Chinese hamster cells in vitro, synchronised with hydroxyurea, when exposed to X rays and α -particles. They obtained a similar pattern of cyclic response after X-rays and α -particles although the variation between the most sensitive and most resistant phases of the cell cycle was less with α -particles than with X-rays.

Studies of age response of cells in vivo have demonstrated similar phasic changes in sensitivity to X-rays after synchronising with HU as have been observed in vitro. (Hagemann & Lesker 1971; Withers et al. 1974). Withers (1974) compared the effectiveness of 16 MeV and 50 MeV cyclotron neutrons and observed a variation in the radiation response of jejunal crypt cells as they progressed through the cell cycle although the changes were less marked than after X-rays.

6.4.1. Initial effects of HU

In the present study doses of 1025 rads of X-rays and 540 rads of neutrons were selected to study age response since these doses were known to produce a similar amount of cell survival in the jejunum. These were the assay doses used in the studies described in sections 4 and 5. The doses do not, however, give an equivalent response in the ileum, the neutron irradiation being relatively more effective (Figure 6.1). Cells in the ileum have been shown to possess a longer G_1 phase and a shorter S phase than cells in the jejunum, so that this difference in response may be due to a population of early G_1 cells being resistant to X-rays but not to neutrons.

After a 2 hour accumulation period with HU, referred to as 0 hours post hydroxyurea, surviving crypt cells will be mainly in the M and G_2 phases and radiation given at this time will therefore primarily reflect the survival of M and G_2 phase cells. In the jejunum where the proportion of cells in the S phase is greater than in the ileum the fall in survival after X-irradiation is more dramatic. The fall in survival is also greater after X-irradiation than after neutrons in the jejunum and this may be due to the greater dependence of the X-ray survivors on the resistant S phase cells. In contrast, in the ileum with the smaller proportion of cells in the S phase, greater survival is observed after X-rays than after neutrons immediately after synchronisation.

6.4.2. Radiation survival as cells progress through the cell cycle

As the cells surviving HU move into early and mid G_1 the sensitivity to X-rays decreases (shown by an increase in crypt survival) and then increases in sensitivity as the cells approach the G_1/S border (Figure 6.1). By 4 hours cells will be released from the HU block and pass into the S phase when the sensitivity to X-rays sharply declines. The changing pattern of radiosensitivity after HU is similar for neutron irradiation as for X-irradiation. There is, however, little variation in sensitivity through G_1 until sensitivity increases as the G_1/S border is approached; there is then a sharp decline in sensitivity as cells enter S.

The small cyclic variation observed in the ileum after both X-rays and neutrons compared with the jejunum probably reflects the smaller proportion of S phase cells and the larger proportion of G_1 cells so that much less cell synchrony will be induced by the hydroxyurea compared with the jejunum.

6.4.3. Comparable studies on intestinal cell sensitivity to X-rays and neutrons after HU

The overall pattern of response after X-irradiation observed in the jejunum is in general agreement with the findings of Hagemann and Leshner (1971). They measured total proliferative cellularity in mouse jejunum at 3 days post irradiation and obtained a peak resistance at 6 hours after synchronising with HU, as was observed in this study. Withers et al. (1974) who measured cell survival per circumference in mice after X-rays and cyclotron neutrons, observed a much lower survival of crypts after irradiation 1-3 hours after HU treatment than that observed here. They gave a series of 5 hourly injections of HU to accumulate cells at the G_1/S border before irradiation and therefore may have achieved tighter synchronisation.

6.4.4. RBE considerations

Since equivalent survival was not observed here after X or neutron

irradiation at the start of synchronisation, a clearer indication of the relative sensitivities of cells through the different phases of the cell cycle to X or neutron irradiation was obtained by normalising crypt survival at the time 0 after the 2nd injection of HU to a value of 1 and plotting other values as a ratio of this. (Figure 6.2). This normalisation procedure indicates that in the jejunum the greatest difference in the relative sensitivities of X or neutron irradiation (the RBE) is observed for S phase cells, when at the peak, approximately 3 times as many survivors will be observed after 1025 rads of X-rays compared with 540 rads of neutrons (Figure 6.2). The smallest difference in effect is observed for G_1 cells.

In the ileum the relative changes in sensitivity to X or neutron irradiation are fairly constant with time after HU synchronisation and there is only a small difference in the response to the two irradiations. The method of synchronisation involved killing S phase cells only, and in the ileum the early G_1 cells may be important in determining the survival after X-rays. These G_1 cells will increase the survival after X-rays compared with that after neutrons, while the smaller proportion of S phase cells present will lead to a poorer synchronisation in the ileum.

In section 5 it was shown that the slope of the X-ray survival curve when X-rays immediately (2 min) followed a dose of neutrons for jejunal cells was steeper than the slope of the normal X-ray curve. It was suggested that this was due to perturbations in the profile of the cell population so that the final slope of the X-ray survival curve would reflect the sensitivity of the S phase cells whilst the X-ray survival curve following a neutron assay dose would be determined by a more mixed population of G_2 , G_1 and S phase cells. The observations reported in this section showing that the assay dose of neutrons would kill about 3 times more S phase cells for the same total cell killing as the assay X-ray dose, gives support to this interpretation.

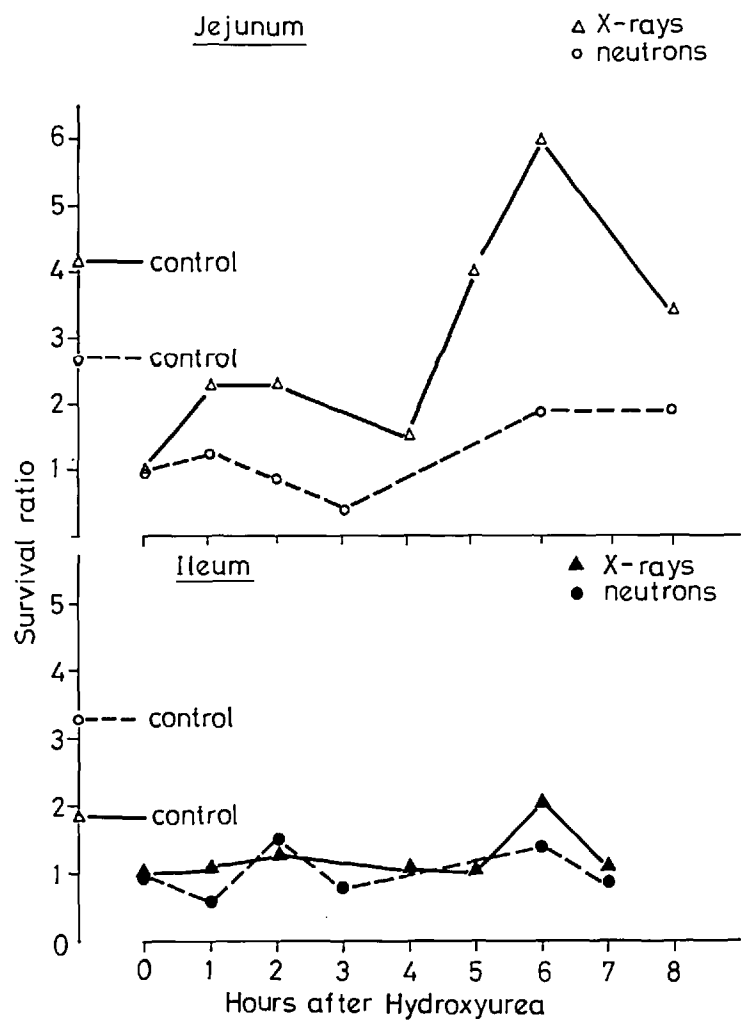


Figure 6.2. Crypts/circumference for jejunum and ileum normalised and plotted as a function of time after injection of hydroxyurea after X-rays and neutrons.

Withers observed a large fluctuation in survival as a function of position of crypt cells within the division cycle. The separation of the gamma and neutron curves varied through the cycle and was greatest after the peak when the cells would be in G_2 and late S. Sinclair (1968b) studied the effects of fission spectrum neutrons on the variation in the cycle of Chinese hamster V79 cells and observed an RBE for the S phase cells of 6 at a neutron dose of 250 rads and 3 at 1000 rads, while the RBE of G_1 and G_2 cells was 4 at 150 rads and 2.5 at 1000 rads of neutrons.

6.5. Conclusions

1. Cyclic fluctuations in radiation responses were observed after large doses of X-rays and neutrons, the variation being greater after X-rays than after neutrons.

2. The cyclic response was more marked in the jejunum with a long S phase compared with the ileum with a shorter S phase due to the method used for synchronisation.

3. The RBE varied throughout the cycle being greatest for S phase cells. This leads to considerable variation in the RBE in the jejunum with time following synchronisation of the cell cycle. The variation in the RBE with time after synchronising was small in the ileum because of the smaller proportion of S cells in the ileum which resulted in a poorer synchronisation.

4. Differences demonstrated on the proportionate cell killing particularly of S phase cells by a neutron compared with an X-ray assay dose support the interpretation given in section 5 that the steeper X-ray survival curve observed after a neutron assay dose is due to a smaller proportion of S phase cells in the post neutron irradiation population.

7.0. The effect of varying neutron dose rate on crypt cell survival

7.1. Introduction

Early studies on growth inhibition in Vicia faba bean roots at dose exposure times of 8, 12 and 24 hours, with sparsely ionising radiations such as X-rays and gamma rays, have shown a diminished inhibition of growth with protraction of exposure. In contrast, after densely ionising particles such as α -rays the dose required to give a 75% reduction in growth rate was not affected by altering the exposure time from 8 minutes to 24 hours (Gray and Scoles, 1951). The diminished effectiveness of low LET radiations as the exposure time was increased was attributed to recovery from sublethal damage which had an estimated half time of 1.5 hours during the protracted exposure (Lajtha and Oliver, 1961).

Lamerton and Courtenay (1968) in a review article on effects of continuous irradiation drew attention to three principal factors which could determine the response of dividing tissues to continuous irradiation.

1. The radiosensitivity of the stem cells. Cells which exhibit a broad shoulder to their survival curve after acute exposure would be expected to be less susceptible to low dose rate irradiation since the shoulder is continuously reconstructed during a protracted exposure.
2. The length of the cell cycle. When an exposure is continuous over a number of cell generations the sensitivity will be directly dependent on the length of the cell cycle. Thus a given dose rate of continuous irradiation should be more damaging to cells of long cell cycle than to cells of short cell cycle.
3. Homeostatic induced increase in cell turnover. Some tissues adapt to the effects of continuous irradiation by increased repopulation rate. Cairnie (1967) has shown how rapidly adaptation occurs in the small intestine of the rat when

exposed to 350 rads/day. He found that the cell cycle lengthened within 6 hours of the start of the irradiation but this trend was reversed after 24 hours and the cell cycle rapidly became shorter than normal.

A large effect of dose rate has been observed using 250 kVp X-rays and 8 MeV electrons for intestinal mortality of T0 mice measured 4 days after irradiation (Hornsey and Alper, 1966). Hornsey and Alper used dose rates of 20 to 50,000 rads/minute and observed an increased survival after dose rates of 100 rads/minute compared with 6,000 rads/min which had not previously been observed in other systems (Lindop and Rotblat, 1963). It was postulated that some repair of sublethal damage must have occurred during the 10-15 minute exposure at the lower dose rate and this explanation was confirmed by giving a range of doses at 5000 rads/minute each split into 2 fractions separated by 10 minutes. The resulting survival showed that significant recovery did occur within the 10-15 minutes following the first irradiation. When LD50/4 was plotted against dose rate an inverse relationship was obtained up to dose rates of approximately 10,000 rad/min. At higher dose rates oxygen consumption by reactions with the radiolysis products of irradiation reduced the tissue oxygen tension and therefore reduced the effectiveness of the irradiation (Hornsey and Bewley, 1971). Similar effects of hypoxia induced by high dose rate irradiation has been observed in skin (Field and Bewley, 1974). Since the early work on dose-rate using the LD50/4 endpoint Hornsey (1970) has found a dose-rate effect over the same range of dose rates scoring crypt survival at 4 days as a more reliable endpoint than LD50.

Catcheside et al. (1946) studied the yield of chromosome aberrations in *Tradescantia bracteata* after different exposure times of X-rays. They found that the yield of chromatid breaks (regarded as 1 hit events) was proportional to dose but independent of dose rate, while the yield of interchanges (2 hit events) increased slightly less rapidly than the square of the dose when the doses were given in a constant time and

were dependent on dose rate. Sax (1939) had suggested from fractionation experiments that the yield of interchanges produced by a given dose diminished with increase of exposure time, since 2 hits which are favourably located in space to form an exchange may fail to exchange if produced several minutes apart in time, as the first break may reconstitute before the second break is produced.

Densely ionising radiations such as α -particles and to some extent neutrons would not be expected to show a dose rate effect since damage is predominantly of the lethal 1 hit type. A small dose-rate effect with fission neutrons was observed by Scott et al. (1967) for the induction of chromosome exchange aberrations in non-stimulated human lymphocytes after dose rates of 3.4 rad/min compared with 6.75 rads/min. This difference was not however observed in stimulated lymphocytes.

Investigations on the effect of dose-rate on animal survival after fission neutrons have shown that mortality in mice measured at 30 days is independent of dose rate between 0.15 rads/min and 500 rads/min (Vogel et al. 1961). Spalding et al. (1964) found, however, that after fractionated sublethal doses of fission neutrons given at 2 hourly intervals recovery took place between exposures in mice and that it proceeded at approximately the same rate as after gamma irradiation.

Studies on the effects of combining fast neutrons and X-rays suggested that a small dose-rate effect with neutrons should occur, as recovery from sublethal damage deposited by the neutrons occurs at a similar rate to that observed after X-rays (Hornsey et al. 1977). However, Hornsey (pers. comm.) using the LD50/4 endpoint had detected no effect of dose-rate with neutrons at dose-rates between 10 rad/min and 1000 rad/min. It was decided therefore to re-examine the question of an effect of neutron dose rate on the intestine but using the crypt assay technique, rather than LD 50/4. Crypt assay measured over a wide dose range is more accurate and therefore a more sensitive technique than the LD50/4 assay and therefore smaller differences can be observed.

7.2. Methods

Doses of 500-800 rad of neutrons were delivered at dose-rates of 2.25 krad/s, 70, 20, 10, 5 and 2 rads/min to unanaesthetised mice breathing air. The dose-rate was varied by altering the distance of the mouse box from the target applying the inverse square law. To obtain the highest dose-rate of 2.25 krad/s/min the mouse box was placed inside the steel collimator 17cm from the beryllium target by means of a remotely controlled arm. A higher gamma contamination of 10.5% is measured at this distance from the target compared with 6% at the normal therapy distance of 170cm from the target. This higher gamma contribution to the dose is due to a neutron reaction with the steel collimator producing scattered gamma rays. The distances from the target used to achieve the variation in dose-rate together with the various beam currents are listed in Table 2.1. Section 2.

7.3. Results

Jejunum Cell surviving fractions calculated from the crypt survival (a and b) observations are plotted against dose in Figure 7.1. for all dose-rates. The surviving fractions have been calculated using a value of 135 stem cells/crypt as described in Section 2. The lines of best fit were drawn for the 2.25 krad/min, 70 rad/min and 5 rad/min data. The surviving fraction after 2 rad/min were similar to those after 5 rad/min, while the values after 10 rad/min fall between the 70 rads and 5 rad/min lines of best fit.

There was a significant difference between the numbers of surviving crypts for irradiations of 2.25 krad/min compared with 70 rad/min ($P = 0.03$) and between 20 rad/min and 5 rad/min ($P = 0.001$. Table 7.1). The D_0 and D_q values calculated from the survival curves at all dose rates are listed in Table 7.2. The errors on D_0 and D_q are too great to specify whether the change in sensitivity is in either or both of these values. The data show a continuous decrease in sensitivity as dose-rate is decreased from 2.25 krad down to at least 5 rad/min.

a).

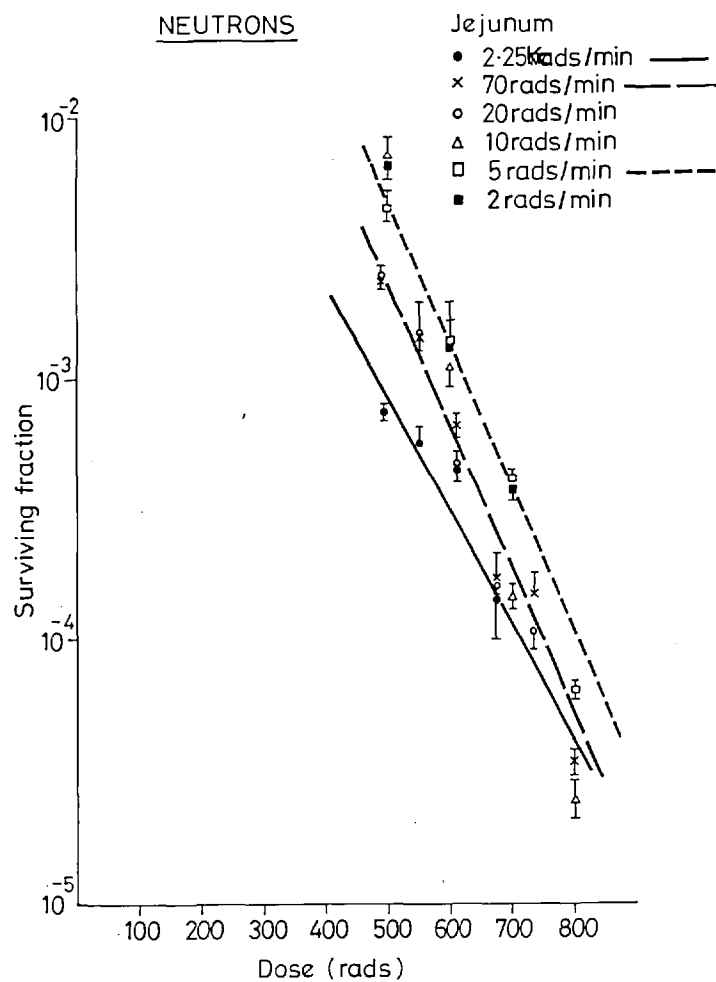


Figure 7.1. (a and b). Cell survival curves for jejunal (a) and ileum (b) crypt cells plotted against total neutron dose.

b).

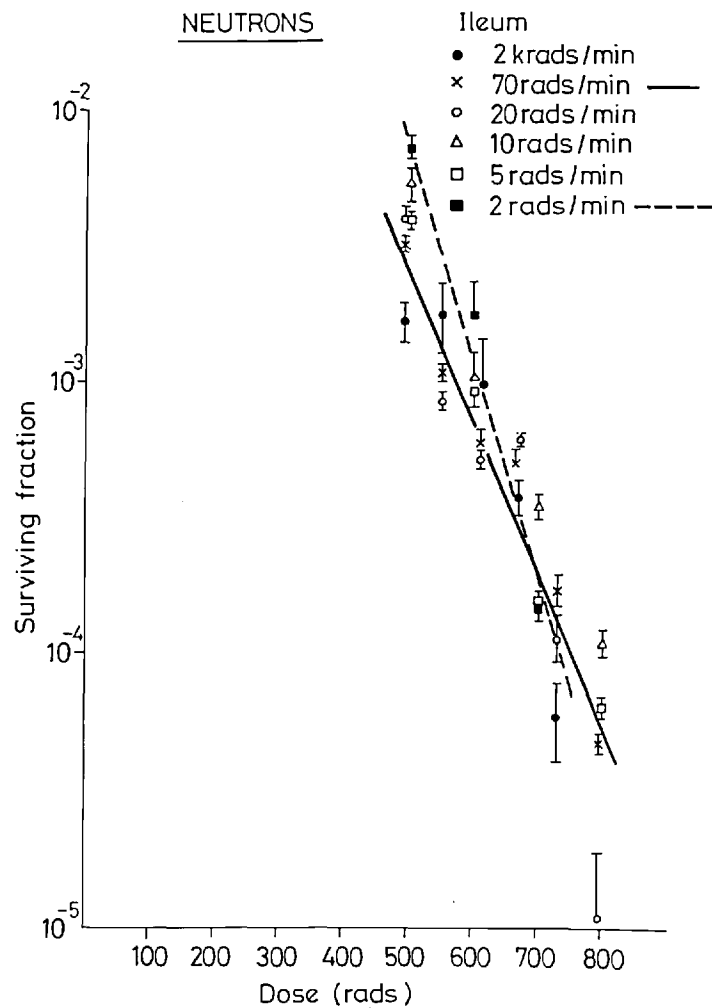


Figure 7.1. (a and b). Cell survival curves for jejunal (a) and ileum (b) crypt cells plotted against total neutron dose.

Table 7.1.

Significance Tests

Neutrons

2 krads versus 70 rad/min

	t	P
jejunum	- 2.4171	0.03*
ileum	- 0.7383	0.45

* significant at 5% level

70 rad/min versus 20 rad/min

jejunum	- 0.1322	0.90
ileum	0.0952	0.90

20 rad/min versus 5 rad/min

jejunum	- 5.9237	0.001*
ileum	- 1.1330	0.30

5 rad/min versus 2 rad/min

jejunum	0.3583	0.75
ileum	- 2.5583	0.03*

Table 7.2.

Neutron dose rate survival curve
parameters

Jejunum

<u>Dose rate</u>	$D_0 \pm SE$	$D_q \pm SE$	$n \pm SE$
2.25 krad/min	95.2 ± 2.15	- 107.89	0.19 ± 0.91
70 rad/min	83.6 ± 1.6	80.1 ± 9.0	3.5 ± 0.5
20 rad/min	68.6 ± 1.3	83.3 ± 4.3	3.3 ± 1.3
10 rad/min	52.3 ± 0.2	243.0 ± 21.9	104.0 ± 0.49
5 rad/min	78.8 ± 0.76	80.1 ± 6.2	2.77 ± 0.88
2 rad/min	69.13 ± 0.7	152 ± 11.77	9.07 ± 0.08

Ileum

Dose rate

2.25 krad/min	174.8 ± 19.6	- 602.6 $\pm$ 79	0.3186 ± 45
70 rad/min	99.1 ± 3.5	35.3 ± 3.4	1.9 ± 0.43
20 rad/min	101.1 ± 5.1	- 77 $\pm$ 46	0.46 ± 11.1
10 rad/min	70.1 ± 0.45	137.7 ± 3.2	7.13 ± 0.7
5 rad/min	71.1 ± 0.3	108.0 ± 4.2	4.56 ± 0.04
2 rad/min	55.51 ± 1.7	237.8 ± 7.2	72.5 ± 0.13

Ileum Cell surviving fractions plotted against dose are shown in Figure 7.3. for all dose rates and the lines of best fit are drawn for the 70 rad/min and 2 rad/min data. There was a significant difference in the number of surviving crypts observed after a 5 rad/min compared with 2 rad/min exposure ($P = 0.03$). There was no significant difference in the numbers of surviving crypts after exposures of 2.25 krad/min and 70 rad/min, 70 rad/min and 20 rad/min and 20 rad/min and 5 rad/min. The difference observed between the 5 rad/min and the 2 rad/min surviving fractions suggest that there is a small decrease in sensitivity with a decrease in dose rate in the ileum but that it is smaller than that observed in the jejunum.

7.4. Discussion

A small but significant effect of dose-rate on irradiation with neutrons was observed in the jejunum. This may be due largely to recovery from the sublethal damage deposited by the lower LET components of dose during the irradiation. The small effect of dose-rate reflects the small but positive slope to the isoeffect curve for fractionated neutron irradiations that have been observed by Hendry et al. 1975, Withers et al. 1974 and Geraci et al. 1977. When fractions were given every 24 hours (Hendry et al., 1975) some repopulation occurred directly increasing the slope of the isoeffect curve. With daily fractions the slope was 0.34 but when fractions were given at 3 hourly intervals the slope was much less (Withers et al., 1974) particularly when the dose/fraction was 300 rad or less. The small increase in total dose to produce a standard amount of damage observed in the jejunum with increased fractionation of neutron dose and concomitant reduction in neutron dose/fraction is due to the increasing proportion of damage from the high LET components of dose. Damage from the high LET component of dose are simply additive with each fraction whilst damage from the lower LET components will show some recovery between fraction and will therefore decrease proportionately (Hornsey et al. 1977). Similarly, with decrease in dose rate there will

be an increasing proportion of damage from the higher LET component of dose and this will account for the smaller dose rate effect on the jejunum observed with neutrons compared with that observed with X-rays.

The dose-rate effect observed on the ileum with neutron irradiations was smaller than that seen in the jejunum so that a significant decrease in sensitivity was not seen until the dose rate was reduced from 70 rads/min down to 10 and 2 rads/min. This smaller effect of dose-rate on the ileum compared with the jejunum may be due to the smaller proportion of cells in the S phase in the ileum than in the jejunum. The S phase is the most resistant to radiation damage and this is largely due to the ability to accumulate and recovery from sublethal damage (see Section 4). The sparing effects of fractionation and reduced dose-rate will therefore be greatly influenced by the size of the S phase cohort in the population and the larger this cohort the greater the sparing. It has been shown in Section 4 that differences in the proportion of S phase cells in the jejunum and ileum govern the detailed differences in response to fractionation and recovery between these two adjacent sections of the intestine.

The small effect of neutron dose-rate reported here is unlikely to be of any therapeutic importance. However, the jejunum is increasingly used in comparison of different neutron beams. Cyclotron neutrons are of a uniformly high dose rate (30 - 70 rads/min) and the small differences in dose rate from one beam to another will not introduce errors of any significance. However, neutrons from DT generators are often at a low dose-rate (2 - 10 rads/min) and a correction should be made for dose-rate when comparing results of these neutrons with cyclotron-produced neutrons used at much higher dose-rates.

8.0. Metaphase Chromosome aberrations in Chinese Hamster intestinal epithelial cells after a single and split X-ray dose

8.1. Introduction

When a dose of X-rays is given in two fractions the observed yield of chromosome exchanges per cell at the first post-irradiation mitosis falls as the time interval between the fractions increases until a level is reached which approximates to the sum of the 2 fractions given independently (Sax, 1939, 1940, 1941). The classical explanation for the fall in yield is that radiation induces breaks in the chromosome threads immediately following the absorption of energy and that these broken ends remain open or available for rejoining with other broken ends for only a limited time period. When breaks do join up the majority restore the chromosome to its original structure. However, when breaks are close together in space and time they can interact to give an aberration. With increasing time between fractions, breaks induced in the first exposure would have rejoined amongst themselves and would not interact with breaks produced during the second exposure.

The breakage and reunion theory proposed by Sax is still widely accepted although in 1959 Revell proposed another mechanism for radiation induced chromatid aberration formation. Revell's hypothesis states that all chromatid lesions are derived from exchanges and that chromatid breaks are failed or incomplete exchanges. The apparent high yield of chromatid breaks could be accounted for by the difficulty in distinguishing them from gaps or discontinuities. It is now widely agreed that some of the observed breaks at metaphase are derived from exchanges but probably not all of them (Savage, 1976).

When cells are examined at the first mitosis post-irradiation two types of exchange can be seen, chromosome type and chromatid type aberrations (Sax, 1939; Mather, 1934). Chromosome aberrations affect both sister chromatids of a chromosome at the same location and are

produced in G1 before replication of DNA when the chromosome is unsplit. Chromatid type changes involve only one chromatid with the exception of isochromatid breaks, and are formed when DNA replication is or has taken place and the cells are in the S, G2 and M phases.

The formation and types of chromosome aberrations encountered are shown in Fig. 8.1. Many types of lesion are found by irradiation of duplicated chromosomes in S, G2 and M than irradiation before duplication and the most frequently observed intra-arm interchanges are shown in Fig. 8.2. Some common aberration types are shown in Figs. 8.3. and 8.4.

Many studies have been made on the chromosome aberration yield of synchronised and asynchronous cells in-vitro after ionizing radiations. (Evans, 1962; Evans, 1967; Bender 1960; Brooks et al. 1971). Cell lines are chosen from species with a few large metacentric chromosomes in which chromosome and chromatid aberrations are readily scorable. Chromosome aberration studies in cells in-vivo are limited to tissues with a rapid cell turnover which can be readily sampled.

The mouse has 44 small acrocentric chromosomes, and the identification of chromosome aberrations in such chromosomes is extremely difficult without the use of recently developed banding techniques. The hamster with 22 large metacentric chromosomes was therefore selected for the study of the effects of radiation on the chromosomes in the intestinal crypt cells (Fig. 8.5) in-vivo. Intestinal cells have been shown to have a large capacity for repair of sublethal damage, and previous investigations of in-vivo fractionation effects in chromosomes have not been well documented. A study was therefore made on the induction of chromosome and chromatid aberrations in the Chinese hamster crypt cells after single and split doses of X rays in an attempt to determine to what extent the repair of chromosome damage affects recovery of sublethal damage.

8.2. Materials and Methods

Male Chinese hamsters aged between 3-4 months were irradiated

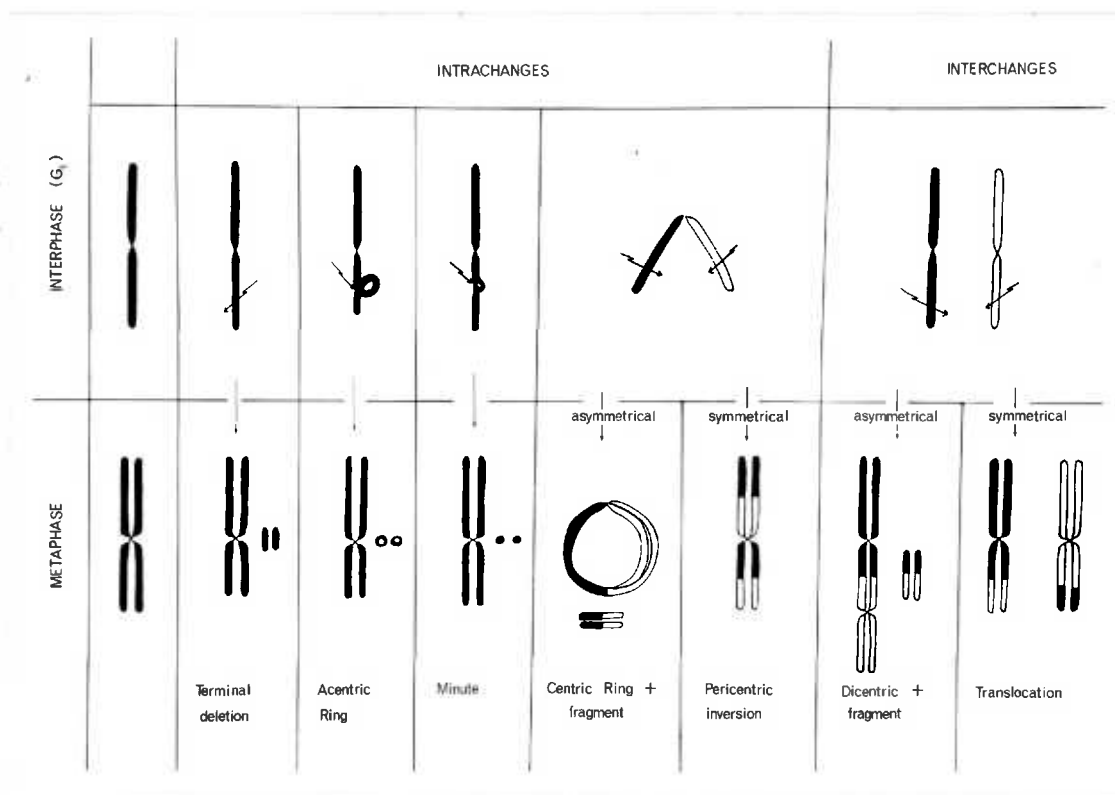


Figure 8.1. Types of chromosome aberrations observed at metaphase.

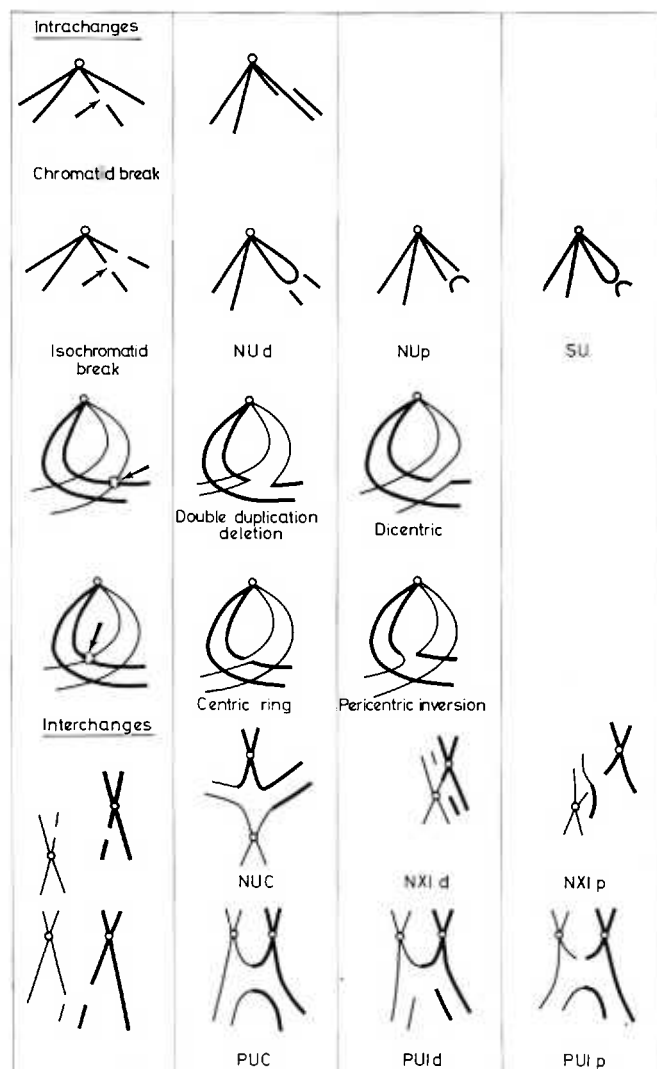


Figure 8.2. Types of frequently observed chromatid aberrations observed at metaphase.



Figure 8.3. Chromosome and chromatid fragments and gaps.



Figure 8.4. Centric and acentric ring aberrations.

CRICETULUS GRISEUS (Chinese hamster)
 $2n = 22$

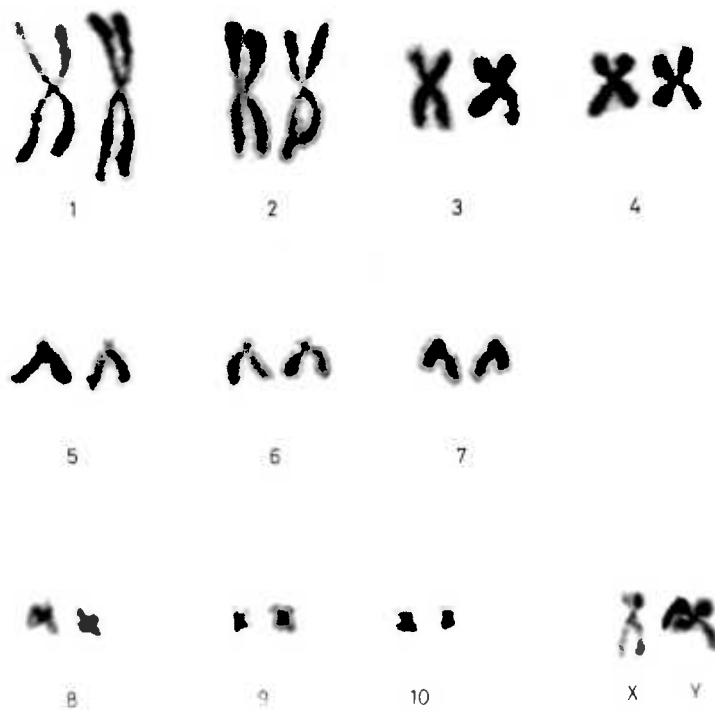


Figure 8.5. Karyotype of male Cricetulus griseus.

with 250 kVp X rays either as a single dose of 200 rads or as a split dose consisting of 100 rads followed 3.5 hours later by a further dose of 100 rads. [^3H] thymidine (5 $\mu\text{Ci}/10\text{ gm}$ Specific Activity 5 Ci/mMole) was injected intraperitoneally (i.p.) into animals 30 minutes before they received 200 rads of X rays or 30 minutes before the second 100 rad X-ray dose. This was followed immediately by an i.p. injection of 40 μg of colchicine to accumulate proliferating cells in metaphase. Animals were killed at 4, 8, 12, 16 and 24 hours after irradiation together with unirradiated controls with and without thymidine.

A section of the mid-jejunum approximately 4 cm long was removed and placed onto a piece of card, it was cut longitudinally and washed in sterile P.B.S. A portion of this tissue was then cut off and after the villi had been removed by scraping with a needle it was placed in a hypotonic solution of 1% sodium citrate at 37°C for 10 minutes. The tissue was transferred to 60% acetic acid where it was chopped into very small pieces and a cell suspension was obtained by macerating the tissue fragments with needles. After 15 minutes in acetic acid the suspension was dispersed onto hot clean coverslips and air dried (Evans, pers. comm).

In order to overcome the difficulty which is encountered when scoring chromosome aberrations in cells labelled with [^3H] thymidine and therefore covered with silver grains, cells dried onto coverslips were autoradiographed and the coverslip preparations were inverted and mounted onto a slide so that the chromosome would be at a different level from the silver grains and could be focussed onto separately. However, this method did not prove successful with the liquid emulsion used and it was found to be impossible to focus on the chromosome without interference from overlying silver grains. As a consequence of this, undeveloped preparations had to be used for chromosome aberration scoring, while the labelled mitoses were scored off the developed autoradiograph preparations.

8.3. Results

8.3.1. [³H] thymidine labelling

The number of labelled and unlabelled pro, meta and anaphase cells scored at 4-24 hours after irradiation and the proportion of labelled mitosis (LI) after each 4 hour accumulation with colchicine are shown in Fig.8.6 . Unlabelled (U) cells undergoing mitosis immediately following [³H] thymidine treatment and before the time when the LI reaches a maximum value are usually considered to have been in G2 at the time of irradiation (Quastler and Sherman, 1959; Evans and Scott, 1964). Very few unlabelled cells were observed in this study 0-4 hours after irradiation and therefore the lengths of G2 could not be determined and indicate that it must be short. The proportion of labelled mitosis is at a peak 0-8 hours and then falls to a trough at 10-12 hours. S phase cells can be defined as all those labelled mitoses from their first appearance to the fixation time just prior to the trough between the 1st and 2nd mitoses of labelled cells. The length of the S phase varies among the cellular population and therefore the average length of S is defined as the length of time that 50% of the cells spent in the S phase and the other phases are similarly defined. (Quastler and Sherman, 1959). Unirradiated control animals treated with [³H] thymidine were fixed at 8 hours, 20 and 24 hours and their LI are shown in Fig. 8.7. together with the LI previously recorded by Lipkin et al. (1968) on newborn hamsters intestinal crypt cells. Lipkin's results show a 16 hour cell cycle with S=8 hours, G1 and M=6 hours and G2 = 2 hours. Results here in 3 month old hamsters indicate a slightly shorter time of approximately 14-15 hours.

The length of the S phase was approximately 10 hours after 200 rads of X rays and approximately 8 hours after 100 + 100 rads. An increase in the length of time spent in the S phase after X irradiation has been observed by Sinclair in Chinese hamster cells in-vitro (1968a).

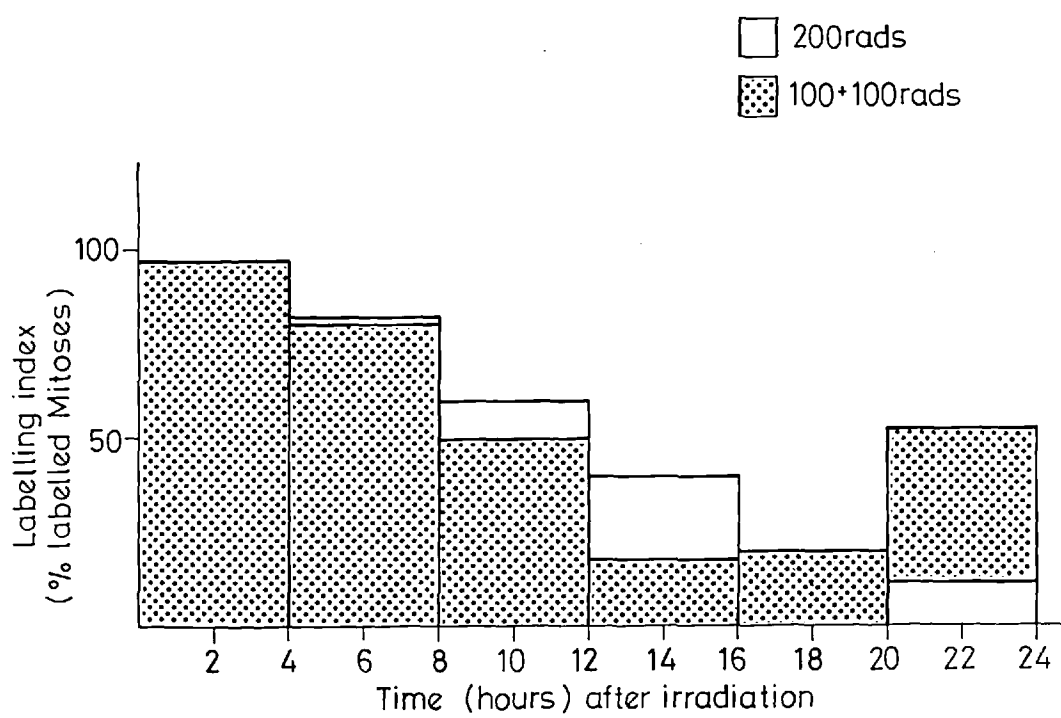


Figure 8.6. Histogram showing % labelled mitoses against time (hr) after $[^3\text{H}]$ thymidine treatment.

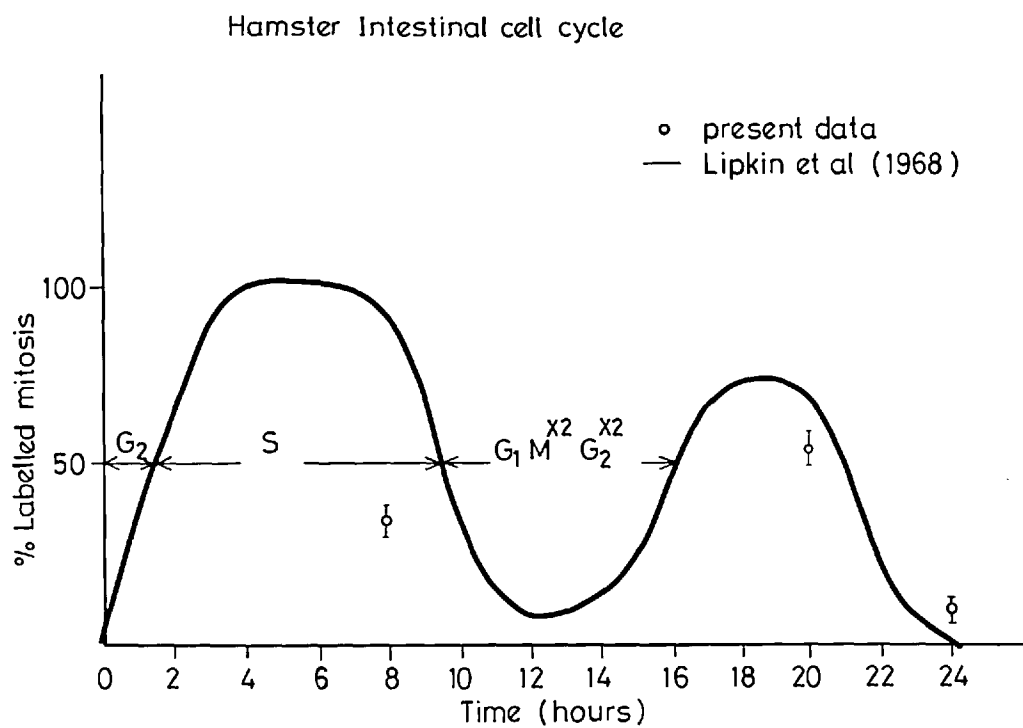


Figure 8.7. Metaphase Labelling Index curve for cells treated with $[^3\text{H}]$ thymidine.

8.3.2. Chromosome aberrations

All chromosome aberrations scored at metaphase were classified into the categories described by Savage (1976) and are listed in Table 8.1. The aberration yields after each 4 hour colchicine accumulation are plotted against time in Fig. 8.8.

8.3.3. Overall pattern of response

There is a peak of chromatid aberrations in cells reaching mitosis 0-4 hours after irradiation when the cells were in the S phase at the time of irradiation (98% LI). Chromosome aberrations were observed at 8-12 hours in cells that would have been in the pre-duplication G1 phase at the time of irradiation when the chromosomes are known to behave as single structures. The yield of chromatid aberrations falls off for cells irradiated in G1 and mitosis and remains low in subsequent mitosis. Very few chromosome or chromatid aberrations were observed from 16-24 hours when cells would be going into their second mitosis after irradiation.

8.3.4. Fractionation effect

The difference in yield observed between fractionated and unfractionated treatments is greatest at 0-4, 4-8 and 12-16 hours when the cells were in S, G2 and early G1 respectively, at the time of irradiation. The difference was significant at 12-16 hours ($P = 0.001$). At 8-12 hours when the cells were in early S and late G1 at the time of irradiation there is very little difference between the fractionated and unfractionated yield. By 16-20 and 20-24 hours cells will be coming through to their 2nd division after irradiation and here there is also a higher yield after the split dose compared with the single dose. There was a significant difference between the total aberration yield of the fractionated compared with the unfractionated treatments ($P = 0.001$).

8.4. Discussion

8.4.1. Fractionation effect

Due to different perturbations imposed on the progression of

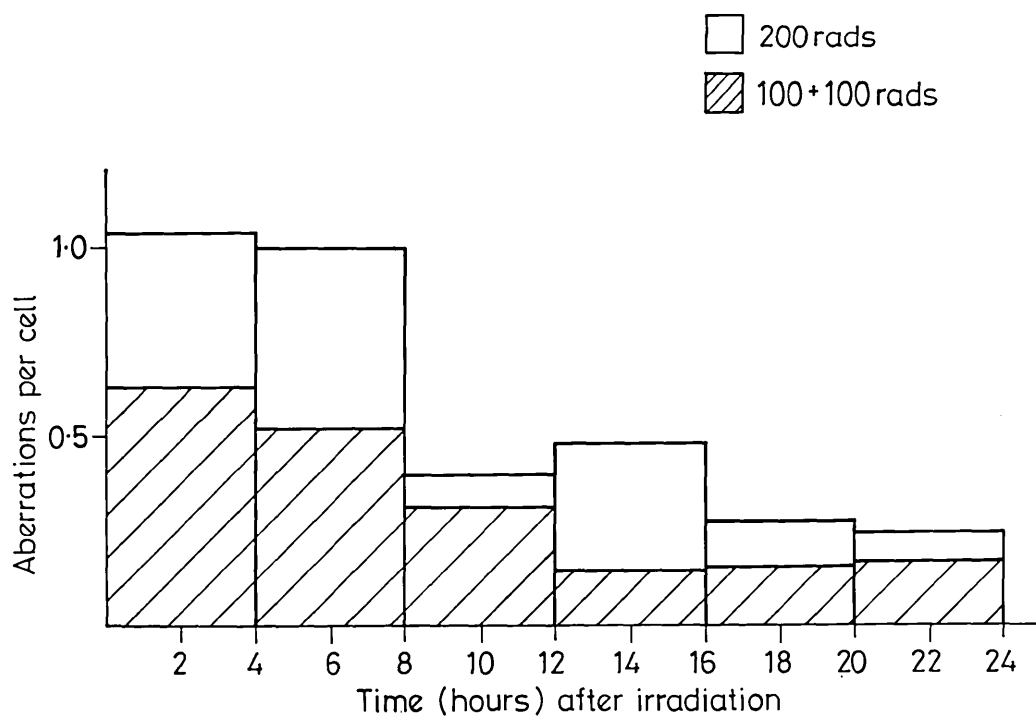


Figure 8.8. Histogram showing total aberration content/cell against time (hrs) after $[^3\text{H}]$ thymidine treatment.

Chinese hamster chromosome aberration yields and means

[illegible]

cells through the cell cycle by a dose of 200 rads of X rays compared with 100 + 100 rads, a direct comparison of yields at different stages is not entirely meaningful. Also when the animals were given a fractionated dose cells could not be accumulated with colchicine during the 3.5 hour recovery period. Cells that were in G2 and late S at the time of the first 100 rad dose of X rays would have passed through mitosis before the second dose of X rays was given and before colchicine was injected into the animals for the 0-4 hour fixation time. Cells in S and G2 after a single dose of irradiation carry a high aberration yield compared with cells in other phases (Table 8.2.) and the loss of some of these cells will immediately reduce the overall yield of aberrations at 0-4 hours after the fractionated dose compared with the single dose. This may account for some of the reduction in yield seen in this study after fractionated doses compared with a single dose at the early fixation times. The low yield of unlabelled mitosis (cells in G2 at the time of irradiation) at 0-4 hour accumulation time after irradiation with both fractionated and unfractionated treatments may be due to G2 cells passing through mitosis during the time interval between the injection of ^3H thymidine and colchicine acting on the spindle. Another possibility is that G2 cells are delayed to such an extent that they do not divide for many hours. Whatever the reason no aberration yield could be obtained for G2 cells.

S phase cells are known to be the most radioresistant cells in the cell cycle to cell killing (Sinclair, 1968a; Dewey et al. 1970a) and the high yield of chromatid aberrations/cell observed here in S phase cells indicates that the ability of a cell to divide does not depend on its total break content per cell. Cell killing may be more dependent on the number of chromosomal aberrations involving both chromatids as shown by Dewey et al. (1972) and these aberrations are not seen in S phase cells.

A high chromatid aberration content per cell for S phase cells was also observed in Vicia faba bean roots by Scott and Evans (1967).

The reduced yield after the fractionated dose that was observed here for S phase cells (0-8 hours fixation time) may be due to repair between fractions or to differences in the cell populations analysed due to differential mitotic delay after the two different treatments.

The fractionation effect seen at 8-16 hours may be due entirely to the presence of more S phase cells in the sample after the single dose of 200 rads compared with the fractionated dose due to a greater mitotic delay induced after the single dose compared with the split dose (see LI histogram). Since S phase cells carry a higher aberration yield than G1 cells their greater incidence after a single 200 rad dose at 8-16 hours will increase the observed yield. However, the fall in aberration yield observed after fractionating the dose may be due to repair of breaks in the fractionation interval before the G1 cells enter synthesis. After duplication in S they will carry chromosome aberrations at the first mitosis after irradiation. Aberrations that involve both chromatids are most likely to prevent division of the cell by the formation of anaphase bridges (Conger, 1965) and may also lead to chromatid loss in one or both daughter cells which may lead to cell death (Preston, 1970; Revell, 1977).

At 20-24 hours there are a larger proportion of labelled cells after the fractionated dose compared with the unfractionated dose. These are cells which were in S at the time of irradiation and have now come through to their second mitosis after irradiation. This may have the effect of increasing the aberration yield of the fractionated sample relative to the unfractionated sample due to the high yield of chromatid aberrations observed in the S phase cells so that only a small overall difference is observed between the two treatment schedules.

The general fall off in yield of aberrations at 16-24 hours is probably due to cells carrying aberrations being unable to proceed further through another cell cycle so that there will be more undamaged cells coming through into their second mitosis.

The overall yield of chromatid deletions is not significantly different after 200 rads and 100 + 100 rads ($P = 0.10$). These are regarded as one hit events and therefore would not be expected to show a fractionated effect.

While some of the reduction in overall yield of aberrations observed after fractionated irradiations will be due to more cells in late S and G2 moving through mitosis prior to the colchicine acting on these cells compared with the single radiation dose it seems improbable that this could account for all the difference. The cells irradiated with a split dose have about half the overall yield of aberrations that cells with the single dose show (Table 8.1.). Therefore the cells escaping the colchicine block after split dose irradiations would have to contain 50% more aberrations to account for the reduction in yield. There are no grounds on which to expect such an aberration yield so it may be concluded that repair of breaks due to fractionation of dose has reduced the overall aberration yield.

8.4.2. Chromosome aberrations versus cell killing

Many studies have been made on Chinese hamster cells in-vitro both for cell survival and chromosome aberration formation and attempts have been made to relate the two events. Dewey et al. (1970a) analysed synchronised Chinese hamster lung cells both for lethality assessed as loss of colony forming ability and chromosome aberrations during different phases of the cell cycle after X irradiation. All aberrations were scored and interchanges were counted as two aberrations. A positive correlation was found between lethality and total chromosomal aberrations. Cells in G1 that are sensitive in terms of lethality sustained a high aberration yield after X irradiation, while cells engaged in DNA synthesis (S phase cells) that are resistant to cell killing sustained a low aberration yield. Dewey et al. (1972) attributed the differences in yield to changes in the distribution of chromatin in the nucleus during the cell

cycle. They noted that during the radioresistant S phase chromatin was spaced further apart than in the radiosensitive G1 and M phases and suggested that there would be less chance for interaction between single sublethal lesions to form a lethal lesion in S phase cells than would be the case in a mitotic cell where the chromatin is tightly packed.

Brooks and Lengemann (1968) determined the repair rate of radiation induced chromatid deletions in Chinese hamster testis and bone marrow cells in-vivo and put forward the hypothesis that chromosomes have the same sensitivity to radiation induced chromatid breaks in the G2 and S portions of the cell cycle, but that differences in aberration yield observed in different phases were due to the different lengths of time available for repair before the cells were analysed at metaphase.

8.4.3. Chromosome aberration studies in-vivo - phase differences

Studies of aberration frequencies of cells in-vivo are limited to tissues which can be stimulated to divide or have a constant cell turnover. Comparison of aberration yields obtained by different workers on different tissues is, however, difficult due to the differences in the scoring methods used in different laboratories and because some workers scored only certain categories of aberration.

Brooks et al. (1968) scored chromatid and isochromatid breaks in Chinese hamster testis and bone marrow 2-12 hours after 100 roentgens of Co60 gamma rays and they obtained a maximum yield of 0.9 aberrations/cell for the testis and 0.58 aberrations/cell for the bone marrow after two hours colchicine accumulation when the cells would probably be in G2 or late S at the time of irradiation, although no accompanying labelling experiment was performed. The yield fell at each successive 2 hour accumulation to 0.1 breaks/cell at 12 hours in the testis and bone marrow. The total aberration yield for intestinal cells in late S per 100 rads of X rays was 0.50 in this investigation which is lower than the Chinese hamster testis result but similar to the result for bone marrow.

The yield of chromosomal aberrations (dicentrics and rings) were scored in Chinese hamster liver cells after 200 roentgens of Co60 gamma irradiation by Brooks et al. (1971), the majority of the cells in the liver are normally in G_0 but can be stimulated to go into the mitotic cycle by a partial hepatectomy. Brooks looked at the aberration yield at 1 hour and 7 days post irradiation. No difference in aberration yield was found at 1 hour and 7 days post irradiation indicating that no additional repair had taken place in the cells during that time while the cells were in the G_0 phase and the chromosome exchange yield/cell after 200 roentgens of Co60 gamma was 0.16/cell. A yield of 0.09 total aberrations/cell after 200 rads of X rays was obtained for Chinese hamster intestinal cells here for cells in the G_1 phase.

8.5. Conclusions

Aberration yields in Chinese hamster intestinal cells after 200 rads and 100 + 100 rads of 250 kVp X rays were found to vary throughout the cell cycle in agreement with previous studies (Dewey et al. 1968; Scott and Evans, 1967). Although differences due to different perturbations introduced into the cell cycle by the different treatments may account for some of the differences, the results suggest that fractionation of dose does cause a reduction in total aberration yield in vivo.

SECTION 9.

9.0. Chinese hamster cell survival

9.1. Introduction

Radiation mortality in the Chinese hamster has been studied at 6 and 30 days after 250 kVp X-rays (Ward et al. 1972; Lappenbusch et al. 1973; Bond and Robertson, 1957, 1964; Bond et al., 1965), while the mean survival time has been estimated after large doses of cobalt gamma irradiation (Fry et al., 1969). Animals which die at 3-6 days after a dose of radiation show damage to their intestine with reduced villi and loss of proliferative cells from the crypts and are stated to have died from intestinal death. An LD50 determination at 6 days is therefore a measure of the sensitivity of the intestine. Deaths that occur at 7-30 days post irradiation show evidence of haemopoietic damage with numerous haemorrhages, anaemia and sepsis occurring and an LD50/30 is therefore a measure of the sensitivity of the haemopoietic system. Ward et al. (1972) obtained an LD50/6 for the Chinese hamster of 700-800 rads and an LD50/30 of 635-800. While Lappenbusch obtained an LD50/30 of 821^{rads}. These results indicate that the Chinese hamster is more radiosensitive than the mouse, for which LD50/5 values ranging from 1000-1350 rads and LD50/30 values ranging from 400-1000 rads have been estimated depending on strain and X-ray dose rate. (Hornsey and Vatistas, 1963; Hagemann et al., 1969; Nelson, 1971; Wambersie and Dutreix, 1974).

A direct relationship between survival time after exposures of doses of irradiation large enough to result in intestinal death and the intestinal cell renewal rate was suggested by Quastler (1956) and shown experimentally by Wilson (1963), who found that germfree mice with a slow renewal rate had a longer survival time than ordinary mice. Fry et al. (1969) measured intestinal cell movement in mice, Chinese hamsters and Syrian hamsters in an attempt to relate cell movement with the mean survival time after 4000 rads of gamma rays. Survival time was plotted as a function of transit time t , estimated as the time from the injection

of $^3\text{HTdR}$ label to the time labelled cells to reach the tips of the villi. It was found that animals with a longer survival time generally had a longer intestinal cell transit time. However, the results were not conclusive for Chinese hamster or mouse since similar mean survival times were recorded, but the transit times of the hamster were longer than in the mouse. The importance of factors other than transit time was also suggested by the results of an experiment with young Syrian hamsters. It was found that 30 day old Syrian hamsters had a shorter mean survival time compared with adult hamsters although a difference of 26 hours, which was observed in the mean survival time, was not reflected in any difference in the transit time.

In this section the relative sensitivities of the Chinese hamster to X-rays and neutrons is reported using the intestinal crypt micro-colony technique of Withers and Elkind (1970). The ability of hamster cells to recover from sublethal damage deposited by X-rays was determined by comparing the effectiveness of single and split doses in their ability to reduce crypt survival down to the same level. The radiosensitivity of the hamster is compared with that of the mouse, and factors which may account for the differences in sensitivity are discussed. Apart from zoological differences some factors which would probably affect radiosensitivity include chromosome number, body size and the associated difference in the numbers of relevant cells and generation times with consequent differences in length of time spent by cells in various stages.

A direct correlation between the radiosensitivity of a species as indicated by growth inhibition and the average volume of interphase nuclei has been demonstrated in a large number of plant species by Sparrow (1965) who studied plants with different numbers of chromosomes, DNA content per nucleus and nuclear volumes. There is a two-fold difference in the number of chromosomes in the mouse compared with the hamster and therefore an attempt is made to determine the importance of

Interphase Chromosome Volume in relation to their relative radiosensitivities.

The RBE values obtained after single doses of X rays and neutrons in the hamster have been compared with those obtained for mice. RBE data for tissues other than skin and including the intestine have been obtained for relatively few species (Bond, 1964; Edmundsen and Batchelor, 1971; Batchelor et al., 1973) so that this study was undertaken to widen the data.

9.2. Methods

2-3 month old male Chinese hamsters were given whole body doses of 400-1400 rads of X rays and 400-800 rads of 7 MeV neutrons. In split dose experiments with X rays a dose of 900 rads was followed 3.5 hours later by a further 200-800 rads. Two animals per dose were killed at 4 days for crypt survival assay in the jejunum and the ileum as described in Section 2.

Cells/crypt and mitosis/crypt were determined from isolated crypt squashes in the jejunum and ileum of unirradiated animals. Nuclear volume measurements were made on fresh cell suspensions of hamster and mouse intestinal cells by means of a micrometer eyepiece graticule in order that Interphase Chromosome Volume estimations could be made.

9.3. Results

9.3.1. Cells/crypt and Mitosis/crypt

The number of cells/crypt and the numbers of mitoses/crypt in the hamster and in the mouse are listed in Table 9.1. There is no significant difference between the jejunum and ileum cell counts either in the mouse or the hamster. There is however a significantly greater number of cells/crypt in both jejunum and ileum of the hamster than in the mouse (Fig.9.1). The number of mitoses/100 cells or Mitotic Index was similar in both the hamster and the mouse.

The number of proliferative cells per crypt in the mouse determined by autoradiographic means is 135 cells/crypt (Kovacs and Potten, 1973). Using the figure of 135 cells/crypt for the mouse together with the data

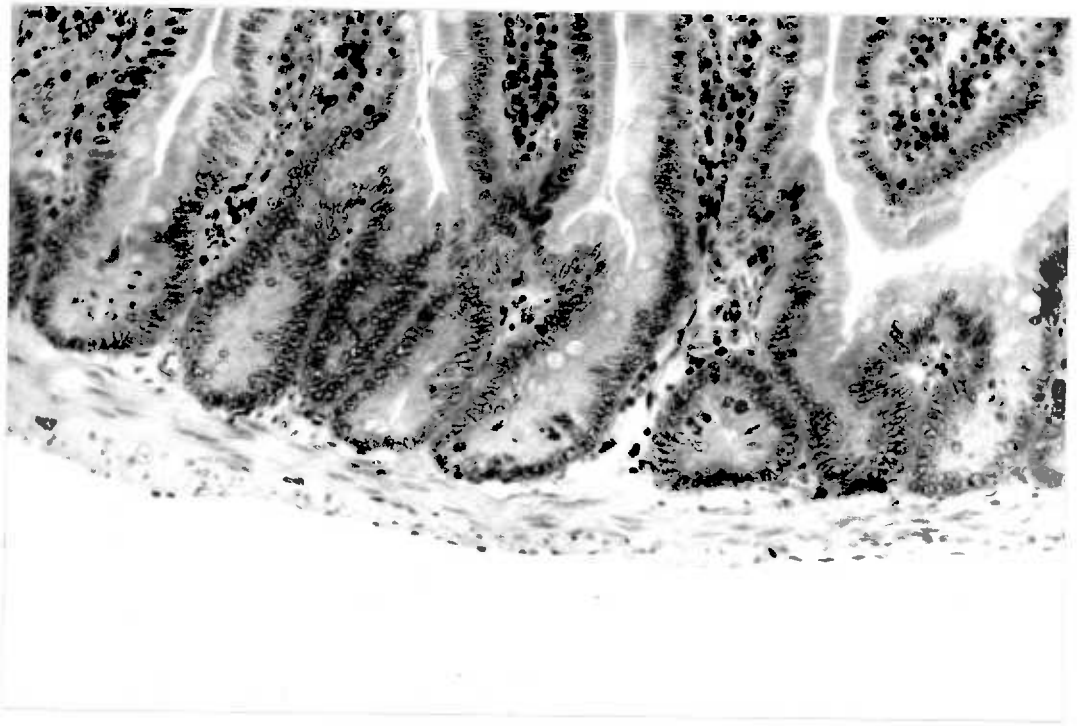


Figure 9.1 a) Histological transverse section through mouse crypts

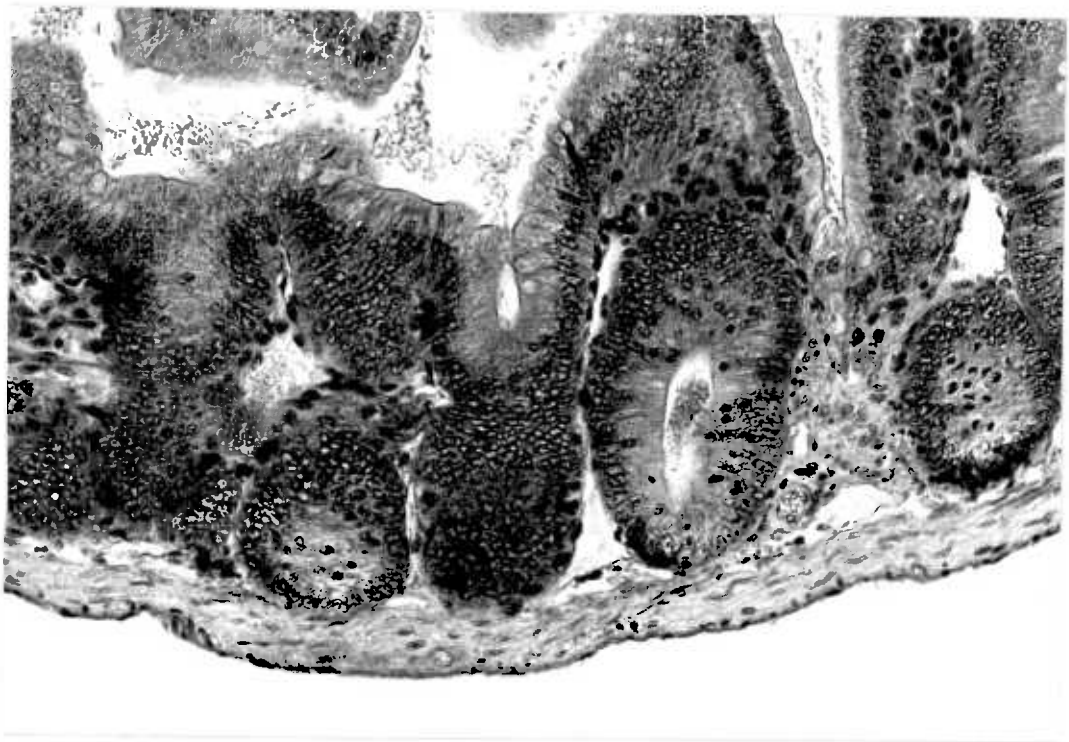


Figure 9.1. b) Histological section through hamster crypts.

TABLE 9.1.

Hamster and mouse cells/crypt and mitoses/crypt

	cells/crypt	mitosis/crypt	MI
<u>Hamster</u>			
jejunum	434 $\pm$ 6.5	11.25 $\pm$ 0.68	2.59
ileum	395 $\pm$ 6.2	9.50 $\pm$ 0.98	2.40
<u>Mouse</u>			
jejunum	249 $\pm$ 2.8	5.0 $\pm$ 0.71	2.0
ileum	234 $\pm$ 4.4	5.50 $\pm$ 0.83	2.3

obtained here for cells/crypt and mitosis/crypt and assuming the process is statistically random a figure of 268 proliferating cells/crypt was obtained by simple calculation.

9.3.2. Crypt survival curves

Hamster crypt survival curves after single and split doses of X rays and single doses of neutrons are shown in figures 9.2.a and b for the jejunum and for the ileum together with mouse crypt survival data after single doses of X rays. Cells surviving fractions derived from these data are shown in figs. 9.3. & 9.4. Survival curve parameters for hamster jejunum and ileum were calculated and D_0 values of 119 ± 25 and 144 ± 33 and D_q values of 167 ± 97 and 150 ± 66 respectively were obtained after X irradiation. A D_0 of 63 ± 2.11 and 52 ± 0.7 and D_q values of 29 ± 4.9 and 52 ± 7.5 were obtained after neutron irradiation for the jejunum and ileum respectively. These results were compared with the parameters calculated from mouse survival data (see Sections 4 and 7) and are listed in Table 9.2.

9.3.3. Cellular recovery

From the split dose experiments a measure of recovery from sublethal damage after X rays during the time interval of 3.5 hours between fractions was obtained. The amount of recovery (D_2-D_1) was estimated as that dose increment required to produce the same amount of cell killing when the dose is split into two equal fractions (D_2) as when the dose is administered as a single exposure (D_1). From the crypt survival curves in Figure 9.1. a recovered dose of 360 and 380 was measured in the jejunum and ileum respectively.

9.3.4. RBE

The RBE values for neutrons relative to X rays are plotted against neutron dose in Figure 9.5. and ranged from 2.05 - 2.21 in the jejunum and 2.36 - 2.4 in the ileum. Data obtained for CFLP mice were also included for comparison.

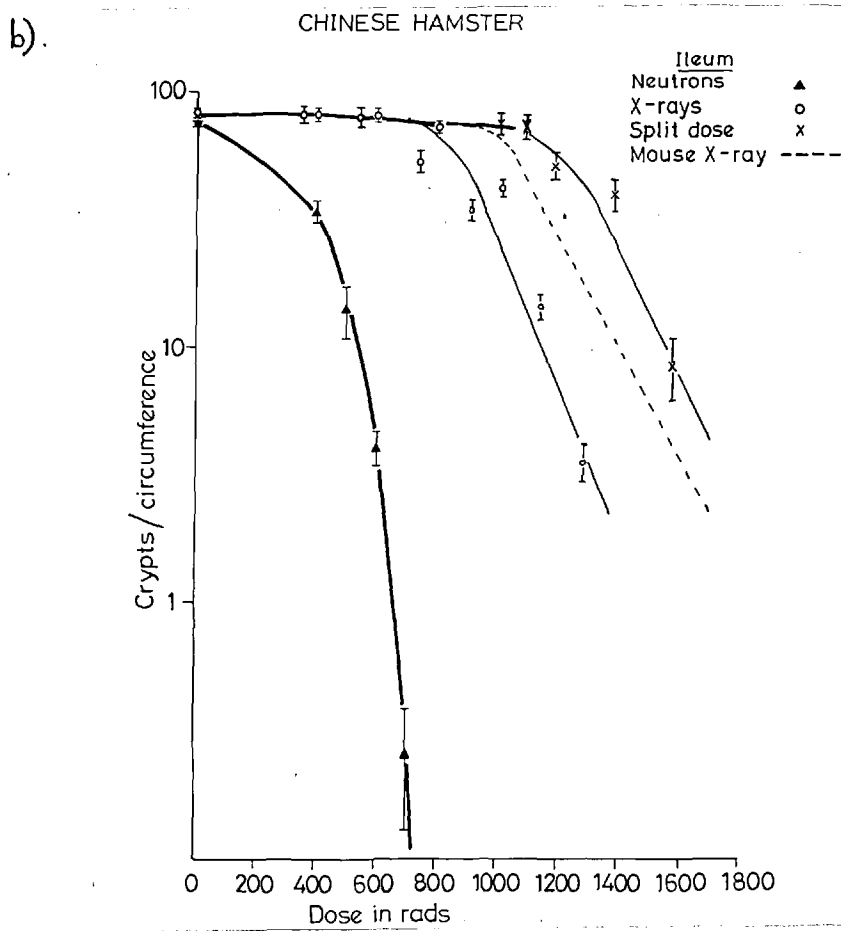
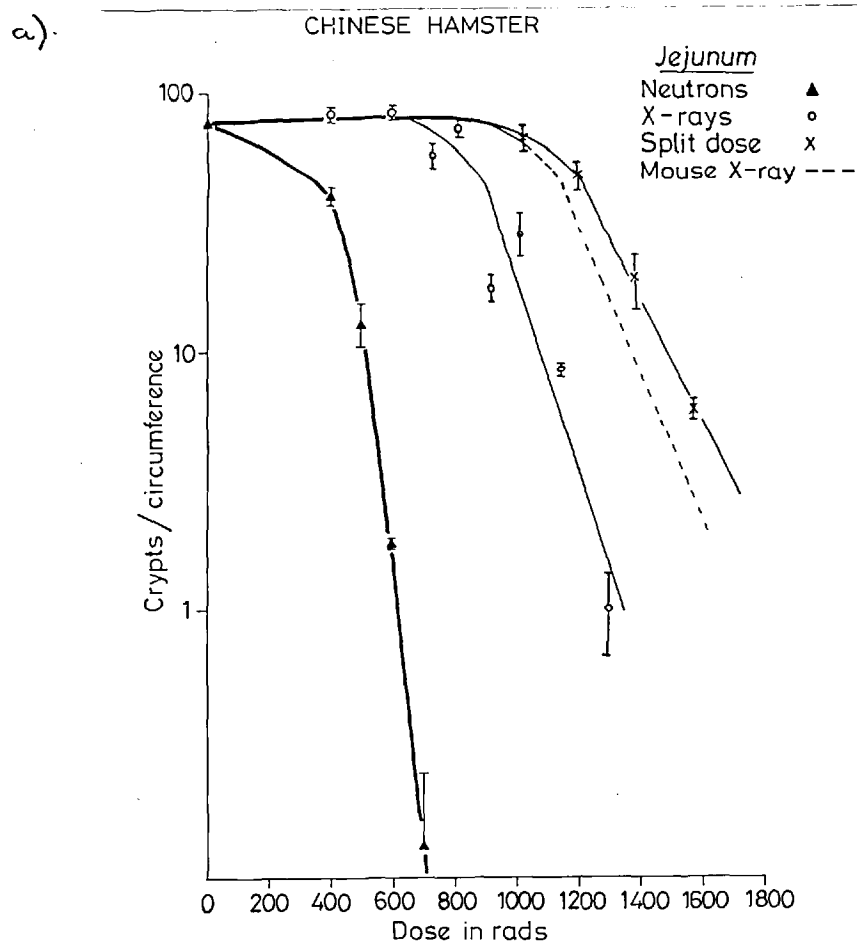


Figure 9.2. (a and b). Crypt/circumference for mouse and hamster jejunum (a) and ileum (b) after single doses of 250 kVp X-rays or cyclotron neutrons.

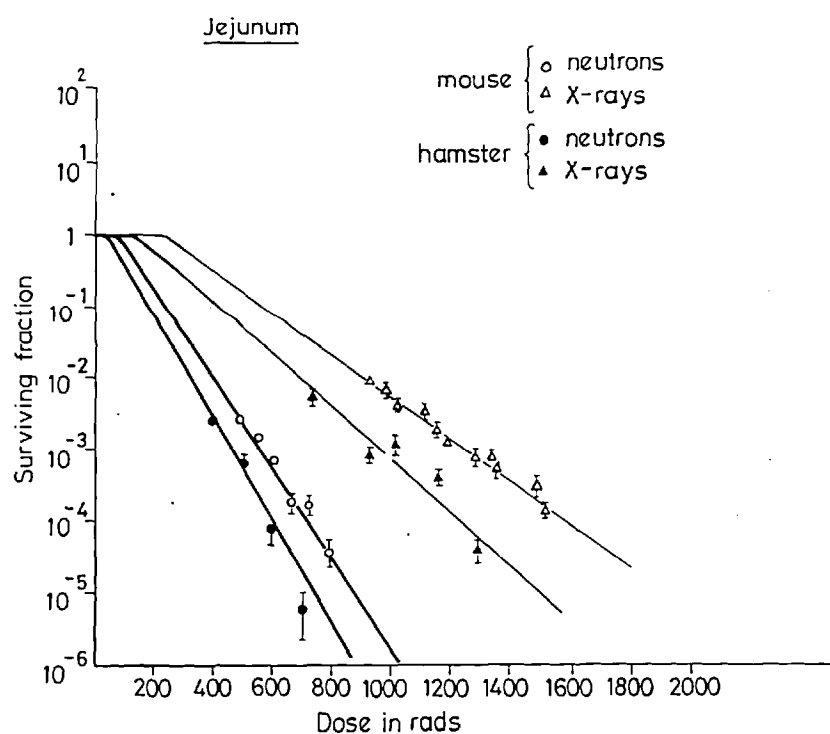


Figure 9.3. Cell survival curves for mouse and hamster jejunal crypt cells after single doses of 250 kVp X-rays or cyclotron neutrons.

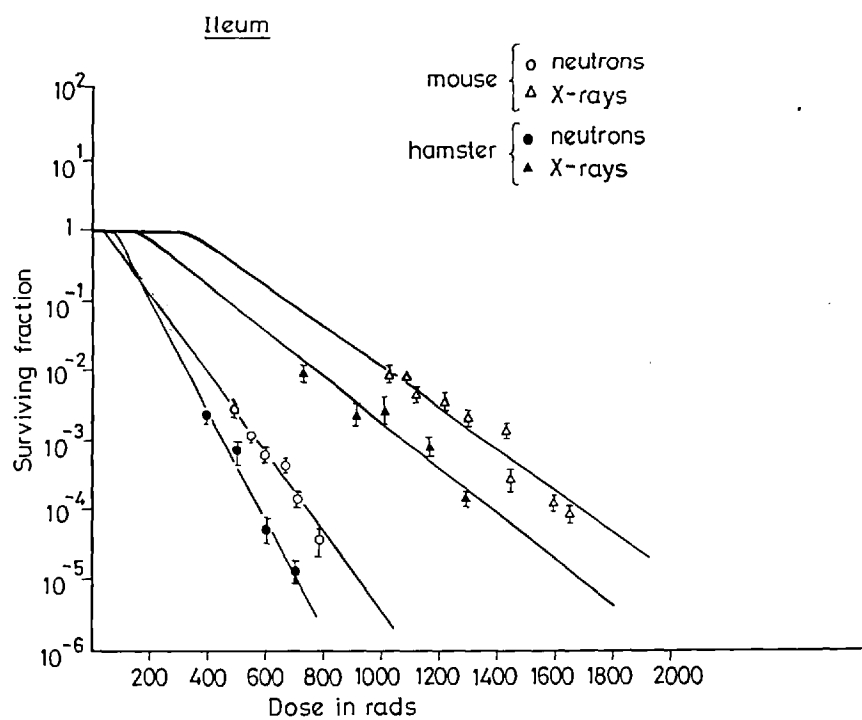


Figure 9.4. Cell survival curves for mouse and hamster ileal crypt cells after single doses of 250 kVp X-rays or cyclotron neutrons.

TABLE 9.2.

Chinese hamster and mouse cell survival curve parameters

		Hamster		Mouse	
		jejunum	ileum	jejunum	ileum
X-rays	D_0	119 $\pm$ 25	144 $\pm$ 33	138 $\pm$ 1.77	128 $\pm$ 1.81
	D_q	167 $\pm$ 97	150 $\pm$ 66	306 $\pm$ 21	376 $\pm$ 27.7
	n	4.07 $\pm$ 0.28	3.09 $\pm$ 0.17	7.1 $\pm$ 0.12	9.4 $\pm$ 0.16
	D_2-D_1	360	380	370	-
Neutrons	D_0	62.6 $\pm$ 2.11	51.5 $\pm$ 0.70	83.6 $\pm$ 1.61	9.1 $\pm$ 3.5
	D_q	29.0 $\pm$ 4.9	51.6 $\pm$ 7.5	80.2 $\pm$ 9.01	35.3 $\pm$ 3.41
	n	2.1 $\pm$ 0.057	3.1 $\pm$ 0.091	3.5 $\pm$ 0.46	1.9 $\pm$ 0.43

The cell survival curve parameters for the hamster were based on an estimated number of 270 cryptogenic cells/crypt calculated on a pro rata basis from the 135 value given for the mouse.

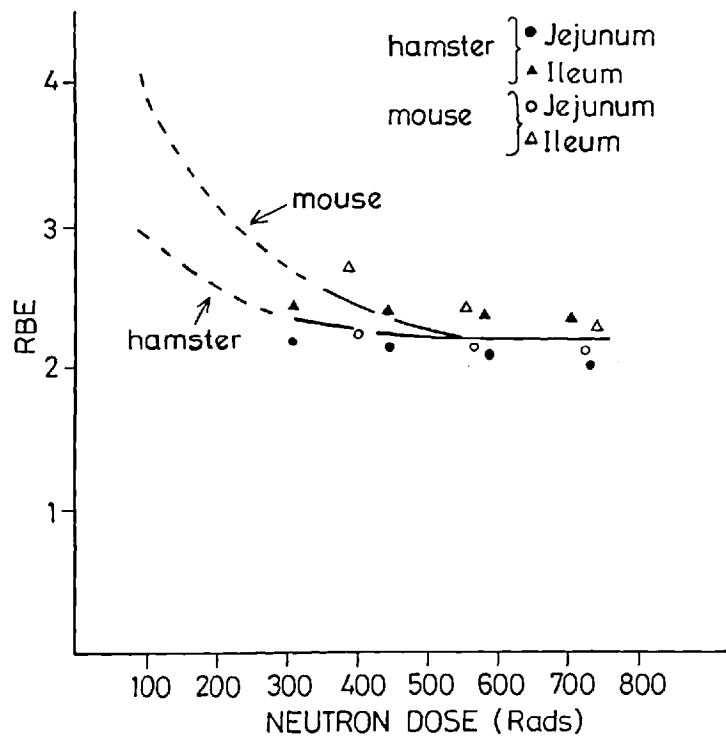


Figure 9.5. RBE values for hamster and mouse intestinal crypt cell survival data plotted as a function of neutron dose.

9.3.5. Nuclear and Chromosome Volume

Nuclear volume measurements obtained from mouse and hamster intestinal crypt cells together with Interphase Chromosome Volume (ICV) determinations are listed in Table 9.3. The ICVs were calculated by dividing the nuclear volume by the chromosome number.

9.4. Discussion

The greater sensitivity of the hamster compared with the mouse previously observed in LD50/5 data have been verified in the crypt survival data presented here. The shoulder of the hamster crypt survival curve is much smaller than the shoulder on the mouse curve (Figure 9.2). and this could be due to a number of factors:-

1. A smaller number of stem cells in the crypts of the hamster.
2. Less accumulation of sublethal damage.
3. A smaller number of targets in the nucleus.

9.4.1. Crypt stem cell size

There are nearly twice the number of cells in hamster crypts as there are in mouse crypts (Table 9.1). If a similar proportion of these cells are stem cells in the two species, then the hamster would be expected to be more resistant to intestinal death after radiation than the mouse. However this does not agree with the present findings, or those of others, for the hamster is observed to be more sensitive than the mouse. This may be due to the fact that there are 54 fewer crypts per circumference in the jejunum of the hamster compared with the mouse and 27 fewer crypts in the ileum. Consequently, in spite of the larger crypt size in the hamster the number of cells per circumference is similar in the two species. The number of proliferating cells per circumference in the hamster and in the mouse can be calculated by multiplying the number of crypts per circumference by the number of proliferating cells per crypt to give a figure of 21708 (268×81) and 21976 (268×82) cells per circumference in the

TABLE 9.3.

Nuclear Parameters

<u>Species</u>	<u>Neutrons</u>	<u>Nuclear Volume μm^3</u>	<u>Interphase Chromosome Volume</u>	<u>DNApg nucleus</u>	<u>RBE</u>	<u>References</u>
V. faba	fission 0.3MeV	490	40.8	38.4	7.0 (average)	Neary et al. 1959 Marshak, 1939 Geard & Marino, 1977 Sparrow, 1964
T. paludosa	fission	630	106.6	59.4	12.7	Sparrow, 1964
V79 Chinese hamster cells	7 MeV	1436 $\pm$ 8.5		18	4.0	D'Arville unpublished Harris, pers. comm.
Chinese hamster crypt cells	7 MeV	173 $\pm$ 2.9	7.86			
CFLP mouse crypt cells	7 MeV	121 $\pm$ 2.5	2.75	6.46	3.0	Handbook of Biological data.

hamster jejunum and ileum and 18223 (135 x 135) and 14715 (135 x 109) cells per circumference in the mouse jejunum and ileum. When crypt survival is plotted as cell surviving fraction of the number of proliferating cells in the control animals the hamster is still shown to be more sensitive than the mouse in spite of possessing a similar number of proliferating stem cells per circumference. This indicates that stem cell number is not one of the factors determining the relative sensitivities of the mouse and hamster.

9.4.2. Sublethal damage

The ability of hamster intestinal cells to recover from sublethal damage was determined by a split dose experiment. The recovery after an initial dose of 900 rads of X rays was determined when followed 3.5 hours later by a smaller X-ray dose (200 - 800). The amount of recovery (D2-D1) recorded for the jejunum was approximately 360 rads which is similar to the D2-D1 of 370 rads obtained for CFLP mice by Hornsey et al. (1977). This result would indicate that the hamster cells have a similar recovery ability to that of the mouse cells. If the shoulder of the cell survival curve which is shown in figure 9.2. represents repair of sublethal damage then the size of the shoulder measured by D_q which is the extrapolation of the dose back to a surviving fraction of 1, should equal the recovered dose (D2-D1). However, the recovery observed between fractions was found to be greater than D_q . (Table 9.2). This discrepancy in the recovery of the cells could be attributed to changes in the cell age density distributions. In determining the recovered dose (D2-D1) it is necessary to give a large initial dose of 828 rads to the hamster and 1025 rads to the mouse to overcome the multiplicity in the crypts and to deposit the maximum amount of sublethal damage. The majority of survivors of the first dose will be in the most resistant phases when they will occur a smaller amount of damage after a second dose and therefore D2-D1 will be larger than D_q (Withers, 1969). However, Hornsey (1973)

obtained a larger D_q than $D2-D1$ for T0 mice after 7 MeV X-rays. This difference was attributed to the size of the first dose used in the split dose experiment which lay in the shoulder region of the survival curve so that the dose was insufficient to deposit the maximum amount of sublethal damage. Also errors intrinsic in the calculation of the cell surviving fraction make it difficult to compare the two parameters. Evidently the measurement of the parameters D_q and $D2-D1$ are subject to a large number of errors and make any analyses difficult.

The size of the single dose curve shoulder will depend upon the age density distribution and this will differ from one species to another (see Section 2). The cell cycle time of the hamster intestinal cells is approximately 15 hours while in the mouse it is 11 hours, consequently at any one time there will be about 28% cells in the more resistant S phase in the hamster and about 40% in the mouse. As a result of this a smaller shoulder will be expected on the survival curve for hamster cells due to a smaller capacity for accumulation of sublethal damage in other phases of the cell cycle. (Sinclair and Morton, 1964).

9.4.3. Target volume or number

There is considerable evidence that the sensitive target for the radiation induced lethality is in the nucleus. For example, localised irradiations carried out with α -particles or with tritiated compounds incorporated into specific regions of the cell, point to the predominance of the nucleus in radiation induced cell lethality (Whiting, 1955). Additional evidence for the involvement of nuclear material is provided by studies of chromosome aberrations induced by ionising radiations. Failure of structurally abnormal chromosomes to separate at post-irradiation division or loss of parts of chromosomes after division are likely to result in cell death and hence nucleoprotein is considered a primary target for cell killing (Grote and Revell, 1972). Sparrow (1965) found that the sensitivity of a plant species was correlated with the average

volume of interphase nuclei and with chromosome number, indicating that the number and size of the chromosomes in the nucleus are the major factor determining radiosensitivity. A simple index of radiosensitivity was obtained by dividing the average nuclear volume by the number of chromosomes. This was called the Interphase Chromosome Volume or ICV. Cellular radiosensitivity measured by D_0 values was found to be inversely related to the ICV for a large group of organisms ranging from simple organisms such as viruses to the more complex higher plants, amphibians and mammals. (Sparrow et al., 1967; Underbrink et al., 1968; Sparrow et al. 1970).

The intestinal nuclear volumes and chromosome volumes of mouse and hamster cells are listed in Table 9.3. together with previously recorded data on other tissues from several other species. An ICV of 7.86 was measured for the hamster and 2.75 for the mouse, and this difference in the size of the sensitive target could partially explain the difference in sensitivity to both X-rays and neutrons observed between mouse and hamster. However, the interrelationships between the cell survival curve parameters for mouse and hamster and the factors which determine them are evidently complex. There is only a slight difference in the size of D_0 after X-irradiation but a large difference in the size of n and D_q . After neutron irradiation there is a significant difference in the D_0 value but no significant difference in the value of n or D_q for hamster and mouse. The larger difference in the shoulder size (D_q) after X-irradiation could in part be due to differences in the number of chromosomes in the nucleus.

Till (1961) who studied mouse L cells found no relation between chromosome numbers of 53 to 109 and the ability of the cells to form macroscopic colonies. It is well established that aneuploid cells are more radioresistant than euploid cells and that they will tolerate considerable chromatin loss (Richards and Atkin, 1959; Revell, 1963)

and therefore are not a useful model for study. Silini and Hornsey (1962) studied cell survival of Ehrlich ascites tumour cells and compared the survival curve obtained with a diploid and a tetraploid strain. They obtained no difference in D_0 but a difference in the extrapolation number with $n = 10$ for the tetraploid and $n = 2.4$ for the diploid cell line. Berry (1963) demonstrated a difference in response to X-rays and Co 60 gamma rays in vivo between two closely related mouse lymphocytic leukaemias, one diploid and the other tetraploid. He obtained similar survival curves, i.e. the sensitivity of the individual targets was the same but the extrapolation number of the $4n$ population was twice that of the $2n$ population. There was also a two-fold difference in the DNA content of the lymphoma cells. These results suggest that an increase in chromosome number increases the resistance of a species due to genetic duplication of redundancy. However, Ichikawa and Sparrow produced evidence that this was not the case in his work on Tradescantia (1967). Sparrow selected a polyploid series of Tradescantia and studied cellular survival of the terminal cells of stamen hairs. He showed that D_0 values were inversely proportional to ICV but there was no difference in the value of n . This polyploid series may be a more relevant model to the normal euploids of cells in animal tissues. The Tradescantia species are capable of independent growth and capable of all the normal processes of differentiation. The so-called diploid and tetraploid Ehrlich ascites and lymphoma cells are all aneuploids and exist in highly nurtured conditions and never differentiate.

There is therefore no firm evidence to suggest that an increase in chromosome number or ICV will influence the size of the shoulder on the X-ray survival curve or the value of the extrapolation number n . This suggests that the difference in shoulder size observed between mouse and hamster after X-irradiation is due almost entirely to a difference in the proportion of S phase cells in the two species (approximately 40% in

the mouse and 28% in the hamster) and therefore to differences in the accumulation of sublethal damage. The lack of any difference in D_0 after X-irradiation may be due to the paucity of the hamster data due to a shortage of animals.

The D_0 values recorded after neutron irradiation are inversely proportional to IVC measurements in the mouse and hamster and agree with the findings of Sparrow after X-irradiation. Neutron irradiation is less affected by phase differences and large differences in shoulder size would not be expected. However, there is now evidence that nuclear volume alone may significantly influence the cell sensitivity to neutrons. A single heavy recoil particle produced by neutron interaction within a nucleus has been shown to be enough to inactivate that nucleus and the probability of producing such a particle is dependent on nuclear volume (Lunec, Cramp and Hornsey, 1980). Although the heavy recoil particle contributes to only 20% of the absorbed dose, because of the high RBE for these particles they account for about 50% of the biological damage. The larger nuclear volume of the hamster intestinal cells compared with the mouse cells (Table 9.3) will therefore contribute to the greater sensitivity of these cells to neutrons.

9.4.4. RBE for Intestinal Damage

RBE values for fast and fission neutrons compared with Co 60 gamma or 250 kVp X rays have been determined in a number of species (Batchelor et al., 1966; Edmundsen and Batchelor, 1971; Batchelor et al., 1973; Jacobson et al., 1977) including mouse, rat, dog and gerbil. The RBE's for LD50/5 range from 2-3 for all species investigated, while the RBE for LD50/30 days range from 0.9-3 with the lowest value recorded for the dog (Table 9.4). An inverse relationship between RBE and body size was indicated in the results on LD50/30 obtained by Batchelor et al. for fission neutrons. It was suggested by Bond (1964) that the low RBE for the larger species might be due to changes in neutron energy with the

RBE values in relation to animal body weight

TABLE 9.4.

<u>Species</u>	<u>Weight range</u> (kg)	<u>Neutrons</u>	<u>RBE</u>	<u>Reference</u>
* Goats	60 - 95	Fission	0.75 ± 0.07	Edmundsen & Batchelor, 1971
* Sheep	15 - 30	"	0.79 ± 0.12	Edmundsen & Batchelor, 1971
* Rabbit	2 - 4	"	2.43 ± 0.12	Batchelor et al. 1966
* Guinea pig	0.5 - 1.0	"	2.00 ± 0.13	Batchelor et al. 1973
+ Chinese hamster	0.04	7 MeV	2.05 - 2.4	
* Gerbils	0.04 - 0.05	14 MeV	1.33	Jacobson et al., 1977
* Syrian hamster	0.02 - 0.03	7 MeV	2.84 ± 0.10	Corp et al. 1974
+ Mice CFLP	0.02 - 0.03	7 MeV	2.11 - 2.7	
+ C ₃ HF/Bu	0.02 - 0.03	16 MeV	2.3 - 3.74	Withers et al. 1971

* LD50/30

+ crypt survival 4 days

larger body mass due to a sizeable gamma component either from the neutron source, from gamma build up following thermalisation or from different depth dose distributions with small versus large animals. Experimental evidence did not, however, support any of these explanations and data produced by Theus et al. (1974) indicate that RBE values should be essentially independent of tissue depth. A number of studies suggest that the gerbil is relatively resistant to ionizing radiations (Flynn, 1957; Chang et al., 1964; Nelson, 1971). Jacobson et al. (1977) found no deaths before 9 days after irradiation of gerbils with neutrons or 250 kVp X-rays. The RBE values for fast neutrons compared with cobalt 60 gamma are 1.45 for 50% mortality and 1.33 for 27% haematocrit depression. There was no clear distinction between intestinal death and haemopoietic death, and the apparent radioresistance of the gerbil was attributed in part to exogenous serotonin and also the fact that gerbils suffer only a 20% loss of granulocytes after 1000 rads while rats and mice lose 90% of their granulocytes after lower doses (Bond et al., 1965). Similarly the regeneration of red blood cells was more rapid than in rats or dogs.

In the mouse there is a clear distinction between intestinal death which occurs at 3.5 - 5 days post-irradiation in most strains due to crypt cell loss, and haemopoietic death which is not observed until 10-21 days post-irradiation and is due to stem cell loss in the bone marrow. After X-irradiation there is a large difference in the dose required to produce the two types of death. Haemopoietic death usually occurring after 500-900 rads depending on strain, and intestinal death occurring after doses above 1000 rads. After neutron irradiation, however, the dose separation is much less because the RBE for intestinal damage is much higher than for damage to the bone marrow (Hornsey, 1970a). There were insufficient Chinese hamsters available to make any observations on intestinal death or on the haemopoietic syndrome in this study.

Observations on the fall in the crypt count only could be made (Figure 9.2).

The RBE values for cyclotron neutrons compared with 250 kVp X-rays in reducing crypt survival in the jejunum and ileum in both hamster and mouse were measured at a number of survival levels from the curves in Figs. 9.3 & 9.4, and are shown as a function of neutron dose in Figure 9.5. Over the dose range observed there is little effect of dose on RBE for the hamster, the RBE for the ileum being slightly greater than that for the jejunum. The RBE values for the mouse intestine show a small increase with decreasing dose over the dose range used, with the RBE's for the ileum again being the higher and changing most with decrease in dose. It is only if RBE values are derived from the survival curves projected to lower doses that marked differences in RBE appear, and such values will depend upon the validity of the projection. Projection down to neutron doses of about 200 rads (survival levels of 10^{-1}) may not be in gross error and the RBE curves are dotted in over this range of neutron dose. (Figure 9.5). From these projections it appears that the RBE's for the intestinal damage in mice increase with decreasing dose much more rapidly than for the hamster and this tendency is more evident at the lowest dose levels due to the larger shoulder on the X-ray survival curve on the mouse.

Sparrow (1964) has shown a relationship between the nuclear DNA content and the RBE of fission neutrons relative to X-rays. He compared RBE values of species showing a considerable range of DNA content and found that RBE for growth inhibition in plants with fission neutrons increases as DNA content per nucleus and also nuclear volume increases, indicating that RBE is influenced by the size of the genetic target.

RBE data obtained for mitotic delay, and growth inhibition in *Tradescantia*, are shown in relation to DNA content per nucleus. Results indicate that there is a reasonable correlation between the RBE of various neutrons relative to X-rays and the size of the genetic target for mitotic delay and growth inhibition (*Tradescantia paludosa*) (Table 9.3).

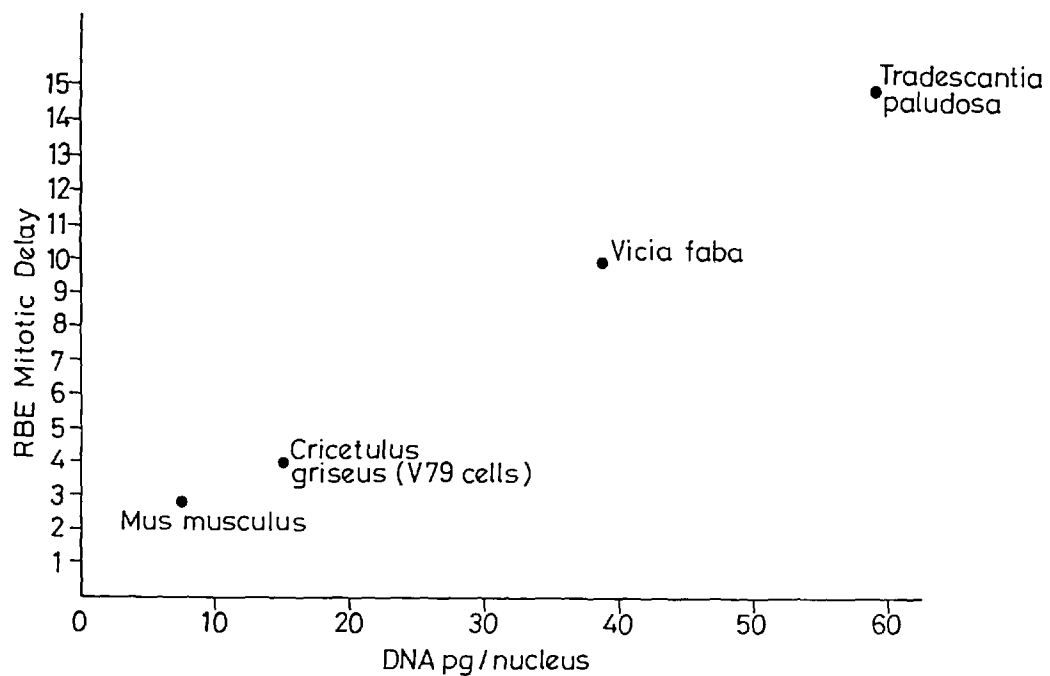


Figure 9.6. RBE for mitotic delay and growth inhibition
(*T. paludosa*) plotted against DNA pg/nucleus.

9.5. Conclusions

After adjustments have been made for differences in crypt cellularity it is clear that the stem cells of the hamster jejunum and ileum are more sensitive to irradiation than the mouse jejunum and ileum. These differences in sensitivity are due to a number of factors but the relative importance of each and the interrelationships between them have not been determined. The various factors which contribute to the greater sensitivity of the hamster intestinal stem cells compared with the mouse cells are listed below:-

1. The larger nuclear volume of the hamster cells compared with the mouse will contribute to a greater sensitivity particularly to neutrons.
2. The larger interphase chromosome volume ICV (nuclear volume/chromosome number) of the hamster intestinal cells will contribute to the greater sensitivity to X-rays compared with mouse cells and possibly to the greater sensitivity to neutrons.
3. The large shoulder on the mouse intestinal stem cell survival curve compared with the hamster is a major factor contributing to the differences in sensitivity to X-rays and to differences in RBE.
4. The higher proportion of cells in the S phase of the cell cycle in the mouse intestine contribute to the larger size of the shoulder of the cell survival curve of the mouse compared to that of the hamster.

10.0. SUMMARY AND CONCLUSIONS

10.1.0. Factors affecting recovery between fractions

10.1.1. Repair of sublethal damage

Results on intestinal crypt cells in vivo after low doses of X-rays suggest that cells in the G2 and M phases of the cell cycle have only a small ability to accumulate and repair sublethal damage, and cell killing is likely to be the result of a single lethal event, while cells in the S and G1 phases are able to fully repair all the sub-lethal damage deposited. The amount of recovery that will occur between fractions will therefore depend upon the cell profile of the population being irradiated.

10.1.2. Mitotic delay

The delay of cells in entering mitosis will delay the replacement of the crypt cell population immediately after irradiation resulting in the depletion of the crypt cell population while cells continue to migrate up the villi. The mitotic delay induced by neutron irradiation was greater than with X-irradiation and the RBE of 2.9 obtained is within the range of values obtained for cell killing in the intestine.

10.1.3. Premitotic death

Results suggest that the crypt cell compartment consists of a mixed population of cells consisting of a sensitive and a resistant population. An RBE of 4 for cell death before mitosis is resumed was obtained for the sensitive population. This result will however be partially attributed to some cell movement out of the crypts.

10.1.4. Cell movement out of the crypts

Results indicate that the cells are not moving out of the crypt at a normal rate after neutron and X-irradiation which may be due to the reduction in output of cells from the crypts, due to a reduction in demand from the villi due to cells being retained on the villi for longer than in the normal unirradiated intestine.

10.1.5. Changes in villus surface area

There are very many factors which affect villus surface area after irradiation including mitotic delay, premitotic death and cellular movements. No clear dose response relationship was observed after X or neutron irradiation. Differences in the relative response of the villus surface area to X-rays or neutrons were observed at day 1 with an increase in surface area being observed after X-rays but not after neutron irradiation. Also a more rapid recovery was observed after neutron compared with X-irradiation.

10.1.6. Cell age distribution

The effects of X-rays and neutrons on cells in different stages of the cell cycle was determined by synchronising intestinal crypt cells with hydroxyurea. Results showed that there was a more marked cyclic response with X-irradiation than with neutron irradiation with the S and G1 phases being markedly less sensitive to X-rays than to neutrons.

The reduced cycle dependent response with neutrons is due to the single event killing by the high LET particles produced by the neutrons. This type of killing will predominate increasingly with fractionation of dose so that cycle dependent sensitivity will be much less important in fractionated course of treatment for neutron irradiation than for X-irradiation.

Differences in response to fractions of X-rays and neutrons due to the movement of cells into more sensitive or resistant phases after the previous fraction will be observed when the interval between fractions is less than 24 hours after which time the cells will be once again dispersed throughout the cycle.

10.2. Combinations of X-rays and neutrons

Differences in the cohort of cells killed with equivalent doses of X-rays and neutrons (i.e. doses killing the same proportion of total cells) will result in differences in the quality of the survivors.

^{S phase}
More [^] cells are killed by a neutron than an X-ray assay dose resulting in differences in the slope of X-ray survival curves produced in tissue previously irradiated with neutrons or X-rays. The surviving population following neutron irradiation will be more sensitive to X-rays than that following a dose of X-rays. These effects will have to be considered when X-rays and neutrons are given in combined radiotherapy treatments.

10.3. Dose rate effect

A small but significant dose rate effect was observed in the jejunum after neutron irradiation. This effect of neutron dose rate must be allowed for when RBE measurements are made of different neutron beams. The small intestine has been used as the tissue for the most comprehensive comparisons of RBE between different neutron sources and no allowance has been made for differences in dose rate as far as is known.

10.4. Cell profile of jejunum and ileum

The proportion of cells in the S phase was determined for cells in the jejunum and ileum of mouse and hamster crypt cells. Results show the proportion of cells in the S phase to be greater in the jejunum than in the ileum in the mouse, while the proportion of S phase cells in the hamster was significantly less in both the jejunum and in the ileum compared with the mouse.

10.5. Comparative radiosensitivities of mouse and hamster intestinal cells

The relative radiosensitivities of mouse and hamster were compared and an attempt made to relate the differences in sensitivity observed to differences in nuclear parameters and to differences in the cellular profile of the stem cells in the two different species. Results with the mouse, shown by a smaller D_0 value could be due to the larger Interphase Chromosome Volume and nuclear volume of the hamster cells compared with the mouse cells. The larger shoulder on the survival curve of mouse compared with hamster intestinal cells may be due to the presence of a larger

proportion of S phase cells in the mouse crypts compared with the hamster.

10.6. Chromosome aberration studies in hamster intestinal cells

The total aberration yield in Chinese hamster cells after 200 rads was compared with the total aberration yield after a split dose of 100 + 100 rads with a 3.5 hour interval for recovery. Results showed that repair of breaks occurs in the recovery period. The number of aberrations/cell was found to vary throughout the cell cycle, the greatest number of breaks being observed in S phase cells. Results on the formation and repair of chromosome aberrations in the intestinal crypt cells demonstrates that repair of breaks occurs in a similar time scale to the repair of sublethal damage. However, the relationship between the formation of chromosome aberrations and lethal and sublethal damage is not yet clear.

11.0. GENERAL DISCUSSION

This thesis describes the investigations made on a number of factors which affect the jejunal and ileal crypt stem cell populations and which will influence the radiosensitivity of the intestine as a whole.

11.1. Chromosome number

By comparing the sensitivity of the mouse and the Chinese hamster it has been demonstrated that there are large interspecies differences in sensitivity and that in part this reflects the differences in chromosome number per cell and more specifically differences in chromosome and nuclear volume.

11.2. Cell age distribution between species

An additional factor contributing to the interspecies difference in sensitivity is differences in the age distribution of cells in the Chinese hamster and the mouse. The larger shoulder on the mouse jejunal stem cell survival curve than the hamster reflects a greater proportion of S phase cells in the mouse jejunum and the greater insensitivity of the mouse intestine reflects in part the greater ability of the S phase cells to repair sublethal damage.

11.3. Chromosome aberrations in relation to cellular damage

The use of chromosome aberrations as a reliable indication of lethal and sublethal damage is still a matter of controversy. The S phase which is the most resistant phase to cell killing has been shown to be the most sensitive to aberration formation (chromatid type). No information is at present available for the immediate effects of loss of chromatid fragments and it is possible that aberrations involving only 1 chromatid may not be lethal to both daughters and that it is only when both chromatids are involved in an aberration that the mother cell or both daughter cells are lethally damaged. Examples of such chromosome aberrations are the formation of asymmetrical exchanges (dicentric or

ring chromosomes) of the loss of a chromosome fragment (figure 8.1).

Information on the effects of chromosome fragments resulting from asymmetrical exchanges have been obtained by Grotle and Revell (1973), and Preston (1970). Preston found that loss of any major chromosome fragment is sufficient to stop germination of *Tradescantia paludosa*. Similar experiments to those performed in *Tradescantia* were carried out with cultured Chinese hamster cells and the results were not so clear as those with *Tradescantia* and the surviving fraction was lower than expected if chromosome aberrations were the sole source of killing. Preston also found that the survival curves for cells irradiated with protons having an LET of 20 keV/ μ and Al X-rays having the same LET but a shorter track length were very similar. However, many more chromosome aberrations were induced by the protons than the Al X-rays. These results could indicate that if more than one target must be damaged for cell killing then these targets must either be closer together than are the pairs of chromosome targets in aberration production, or else far enough apart for a single proton track to be unlikely to damage both. Neary (1967) proposed that there were two targets for cell killing, one independent of LET, the primary target, and one repair system target sensitive to low LET. This interpretation is similar to the findings of Goodhead et al. (1980).

11.4. Survival curves - recovery at low doses

The cell survival data presented in this thesis has been interpreted in terms of the accumulation and recovery from sublethal damage primarily of cells in the S and G₁ phases. This data could also be interpreted in terms of the repair hypothesis (Haynes 1966) which postulates that irradiation damage is caused by a single event which is lethal and is not repaired. Shoulders on survival curves according to this hypothesis are a measure of the activities of a repair factor Q (Sinclair, 1972) which is used up or possibly inactivated by irradiation.

and exponential killing only occurs when this Q factor is depleted. RBE effects could be explained on the basis of an increased sensitivity of this repair system or Q factor to densely ionising radiations (Alper, 1977) or to the presence of a large target in the cell, sensitive to high LET radiations (Goodhead et al., 1980) such as the anchor points of the DNA onto the membrane (Elkind, 1980).

11.5. Cell age distribution within a species

The contribution of the age distribution in the stem cell population to differences in sensitivity of the intestine as well as being observed between species is also seen in differences in sensitivity between different sections of the small intestine within the same animal. The ileum is more sensitive than the jejunum and this reflects a smaller proportion of cells in the S phase in the ileum than in the jejunum.

11.6. RBE effects

When considering the relative sensitivity of the intestine to different types of radiation, e.g. X-rays and neutrons, it must first be recognised that the effects of these different irradiations vary for many of the different factors which affect radiosensitivity. The relative sensitivity of X-rays and neutrons is not easy to predict therefore, particularly when the radiations are given in many fractions given over variable periods of time.

These differences between X-rays and neutrons can be considered at two different levels:

- a) Effects of X-rays and neutrons at high dose levels required to produce a measurable reduction in crypt survival.
- b) Effects at dose levels well below that which produces a reduction in crypt survival.
- a) (i) Age distribution effects

The predominant influence at high dose levels is the age distribution effects of the stem cell population. The age distribution

affects the sensitivity to X-rays much more than to neutrons and the major factors are the proportion of S phase and early G₁ cells which are particularly able to repair sublethal damage. These cells are killed in greater proportion by a neutron than by an equivalent X-ray dose (an equivalent X-ray and neutron dose being a dose killing the same proportion of cells in the total cell population). This results in a higher RBE for X-ray resistant cells which may account for the RBE for jejunal damage in the Chinese hamster being lower than for the jejunal damage in the mouse.

A large X-ray dose will therefore produce some synchrony in the stem cell population with the result that the quality of the surviving stem cell population after equivalent cell killing doses of X-rays or neutrons will be different. The X-ray survivors will have a higher proportion of S phase cells than the neutron survivors. This will affect the subsequent X-ray dose or to concomitant treatment with cycle dependant chemotherapy drugs as illustrated by the effects with hydroxyurea. The effect of hydroxyurea, an S phase specific blocking and killing agent, was much greater on X-ray treated mice than on mice given an equivalent "cell killing" dose of neutrons.

b) (i) Age distribution effects

At the dose levels used in X-ray therapy (about 200 rads/fraction) it is unlikely that much synchrony will be produced, particularly once the cell turnover rate has been increased under the homeostatic stimulus. There will be no synchrony induced by neutrons if the therapy fractionation presently used (130 rads/fraction) are used in abdominal irradiations in the future, because of the more even killing through all stages of the cell cycle observed with neutrons.

(ii) Other factors

Factors affecting intestinal sensitivity at doses below those producing a reduction in crypt survival include mitotic delay, premitotic death and migration of maturing crypt cells onto the villus.

The relative effect of X-rays and neutrons for all of these effects

are different from those observed for cell killing. The RBE for mitotic delay is 2.92 over a wide range of doses and is independent of dose. This differs from the RBE for cell killing which is dependent on dose particularly at the low dose level where recovery from sublethal damage greatly influences RBE.

(iii) The RBE for premitotic death is difficult to determine due to the movement of cells out of the crypts during mitotic delay. Results suggest an RBE of approximately 4.

(iv) All the cell population studies in this thesis indicate a more rapid recovery after neutrons than after X-irradiation presumably as a direct consequence of the higher proportion of premitotic cell death by neutrons compared with X-rays.

(v) The importance of villus surface area in the acute intestinal syndrome in fractionated treatments has been demonstrated (Carr et al., 1979). Results here on crypt survival after long term fractionated treatments indicate that the acute syndrome may not be adequately predicted from crypt survival counts. Similarly the relative effects of fractionated treatments of X-rays and neutrons cannot be completely predicted from the crypt counts. The measurement of the circumference may give some additional information, but the technique I used evidently needs further development to give sufficient accuracy to be used.

The response of the intestine to fractionation treatments with different radiations and combinations of drug and irradiation treatments will be complex, depending on the intricate interdependence of the many factors which have been discussed here. The relative response evidently cannot be entirely predicted from crypt assay alone, since this assay depends on many other parameters which are more difficult to assay. At the doses/fraction normally used in therapy these additional parameters may be the ones responsible for the acute intestinal response though

crypt survival may have importance in longer term effects concerning ultimately the restoration of a structured and normal functioning villus epithelium.

Appendix

Calculation of standard errors of surviving fractions = SFE

$$SFE = \sqrt{(SFEI)^2 + (SFE2)^2}$$

where

$$SFEI = \frac{(\text{Standard error of a crypt/circumference value})}{(\text{The number of cells/crypt}) \times (\text{control number of cells/crypt} - X_i)}$$

$$X_i = \text{crypt/circumference}$$

$$SFE_2 = X_i \times \frac{(\text{Standard error of control crypts/circumference})}{(\text{control crypts/circumference}) \times (\text{no of cells/crypt})}$$

Calculation of standard errors of survival curve parameters

The equation of the line is

$$1 \text{ (SF)} = A + B \times \text{Dose}$$

$$D_0 = \frac{1}{B} \quad D_q = A \times D_0$$

$$SE(D_0) = \frac{1}{B^2} SE(B)$$

S.E. of D_q

$$\text{Variance of } D_q = \text{variance } (A \times D_0) = A^2 \text{ var } (D_0) + D_0^2 \text{ var } A$$

$$SE(D_q) = \sqrt{\frac{\text{var } D_q}{n}} = \sqrt{\frac{A^2 \text{ var } (D_0) + D_0^2 \text{ var } A}{n}}$$

$$\text{S.E. of } N = \sqrt{\frac{\text{var intercept}}{n}}$$

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