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RADIATION INDUCED REPRODUCTIVE DEATH AS A
FUNCTION OF MAMMALIAN CELL PLOIDY

DONNER LABORATORY

David Alan Philbrick
(Ph. D. thesis)

September 1976

Prepared for the U. S. Energy Research and
Development Administration under Contract W-7405-ENG-48

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FUNCTION OF MAMMALIAN CELL PLOIDY

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David Alan Philbrick

RADIATION INDUCED REPRODUCTIVE DEATH AS A
FUNCTION OF MAMMALIAN CELL PLOIDY

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September, 1976

ABSTRACT

Mammalian cells containing different multiples of the diploid chromosome set were created through drug induction and cell fusion. In all cell strains used the chromosome number was determined from metaphase spreads, as well as from DNA content and cell size. The survival of cells as a function of radiation dose was determined for cell lines with differing chromosome complements at 37°C, 4°C, in hypertonic media, while frozen, and with increasing levels of incorporated IUdR. Survival of frozen diploid and hypotetraploid Chinese hamster cells was determined following varying numbers of decays of incorporated ^3H IUdR and ^{125}I IUdR.

The percent of reproductively viable cells following irradiation is a function of the cell ploidy, i.e. the number of haploid sets of chromosomes contained in the cell genome. At 37°C and in hypertonic media, the Chinese hamster cells of progressively higher ploidies are increasingly sensitive to irradiation. As the number of chromosomes per unit cell volume increases the radiosensitivity increases. Both trends suggest interaction between chromosomes as an important cause of cell death.

In the frozen-state hypotetraploid cells consistently are equal or less sensitive than diploid cells to irradiation. This suggests that under these conditions, relative to 37°C, the fraction of cell death resulting from deletions is enhanced. The frozen-state data also suggest that the number of "two break" chromosome aberrations is reduced because of decreased chromatin movement.

In contrast to the hamster cell results, mouse L5178Y hypotetraploid cells are more resistant to radiation than L5178Y diploid cells. The increased resistance of the higher ploidy cells suggests that chromosome deletions are important in causing cell death in this cell line.

When IUDR is incorporated into the DNA of Chinese hamster cells in increasing amounts the changes in radiation sensitivity resulting from a change in ploidy are greatly reduced. As the amount of incorporated IUDR increases, the radiosensitivity of hyperdiploid and hypotetraploid cells increases to a point at which they both have similar sensitivities to X-rays.

The percent survival for frozen V79 hyperdiploid and hypotetraploid cells is the same following irradiation from incorporated ^{125}I UDR when compared on the basis of equal dose. On a decay basis the hypotetraploid cells are markedly more resistant. Exposure from incorporated ^{125}I UDR is for both cell types 5.7 times more effective in causing cell death than an equal X-ray dose.

Compared on the basis of equal numbers of decays from incorporated ^3H UDR, frozen V79 hypotetraploid cells are more resistant

than the hyperdiploid cells. On a dose basis, the two ploidy levels are of similar sensitivity. For both cell types $^3\text{HTdR}$ decays are .75 times as effective in causing cell death as X-rays. These tritium data suggest that the degree of damage caused by decay of incorporated $^3\text{HTdR}$ is comparable to the damage from an equal dose of X-rays.

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ACKNOWLEDGMENTS

One does not work alone. For his enthusiasm, support, ideas, and constructive suggestions I am extremely grateful for the efforts of Dr. Jack Burki. During this project I have appreciated the intellectual stimulus and help of Dr. Dennis Ross, Dr. Robert Mortimer, and Dr. Bob Painter. I have enjoyed working with Mrs. Mildred Hughes and Mrs. Velma McNeal and I am sincerely thankful for their technical assistance. I am grateful for the emotional support and confidence of my wife, Cathey Lee, and of my parents. For their friendship and their help with the laboratory administrative tasks and equipment maintenance, I would like to thank Igor Blake, Dewey Dean, Sue Pohlman, Sue Hargis, Frank Upham, and Pete Dowling. Finally, I want to acknowledge my many friends in the laboratory who have helped make this work enjoyable.

This investigation was supported by NIH Training Grant #5 T01 GM 00829 and by the U.S. Energy Research and Development Administration.

INTRODUCTION

"Considering both the in vitro and in vivo results (pertaining to mammalian cells) discussed, it seems to us that none of the data supports a clearly apparent, major influence of chromosomal breakage on survival at least in terms of the approximate frame of reference used. The in vitro data either shows an inconsistent dependency or essentially no dependency.

Still, there is evidence from other systems (e.g., yeast, plant, and insect polyploid sets) which indicates that ploidy or at least nuclear properties has a strong influence on response. ... On the surface, this suggests that either the right measurements have not as yet been made with mammalian cells (e.g., a killing response analysis on an energy per chromosome basis), or that in mammalian cells chromosome damage is not closely related to lethality..." (Elkind and Whitmore, 1967)

This statement by Elkind and Whitmore summarizes the inconclusive nature of existing work on the relationship between ploidy and radiation sensitivity. Ploidy is defined as the number of complete haploid sets of chromosomes which a given cell possesses. It usually refers to the autosomal complement only (Drake, 1970; Elkind & Whitmore, 1967). How a cell's chromosome complement is related to its radiation sensitivity has been looked at by many different researchers in differing systems. The research reported here support the thesis that ploidy does influence radiation sensitivity in mammalian cells. The results also show that establishment of a clear relationship between ploidy and radiosensitivity is complicated by species dependencies and by differences between cells such as the cell size relative to its chromosome number.

Historical Perspective

The amount of energy deposited within an irradiated cell that results in cell death is very small. For a typical mammalian cell receiving enough radiation to account for an average of one lethal event per cell, the absorbed energy would only raise the temperature of the cell 10^{-4} degrees (Puck, 1960). At specific cellular sites, however, the absorbed energy must be leading to major disruptions which result in cell death. This fact was realized relatively early in the study of radiation biology (Blau et. al., 1922; Condon et. al., 1927; Curie, 1929). It has led to a search for where these sites are located and what is happening in them to cause cell death.

During the 1940's and 1950's researchers using microbeams were able to irradiate the nucleus and the cytoplasm separately. These experiments were conducted on *Drosophila* eggs (Ulrich, 1958) and Green algae (Petrova, 1942) and demonstrated that damage to the nucleus is the most likely to lead to cell death (for a review see Zirkle, 1957). Support for the nuclear hypothesis also came from experiments using low energy electrons. In such experiments no major increase in mammalian cell sensitivity is noticed until the electrons are raised to a kinetic energy great enough to penetrate to the cell nucleus (Cole, 1965). Using alpha particles, Cole (1976) has developed further evidence localizing the site of greatest cell sensitivity as being near the nuclear membrane. Experiments have been done using radioactively tagged precursors of cellular macromolecules to position radioactive isotopes in different

components of the cell. The damage resulting from their decay occurs within and near the molecules in which they have been incorporated (Stent and Fuerst, 1960). For cultured mammalian cells these experiments show that damage within the nucleus is most likely to cause reproductive death (Burki et. al., 1968).

There is significant evidence that the DNA of the nucleus is the location where damage resulting from radiation is most likely to cause cell death. In cells exposed to ultraviolet radiation thymine dimers within the DNA is one of the major causes of cell death (Settow et. al., 1962). Cells with demonstrated deficiencies in the repair of this form of damage are significantly more sensitive to exposure to U.V. (Cleaver, 1970). Halogenated pyrimidines like IudR and BUdR when incorporated in cellular DNA result in sensitization of the cells to ionizing radiation (Szybalski, 1974). Assays of cell killing following incorporation of tritium into different cellular macromolecules show that incorporation into DNA is the most lethal to the cell (Burki and Okada, 1968). Burki (1974) has proposed that there are critical sites within the DNA which once damaged are most likely to result in cell death. Using incorporated ^{125}I UdR he has shown that decay in late replicating DNA is the most lethal to the cell (Burki, 1976).

A number of researchers have argued that if damage to the nuclear DNA is the cause of cell death, a cell's radiosensitivity should be proportional to its DNA content (Terzi, 1961; Kaplan et. al., 1962). A representative study is that of Terzi (1961). With data from

thirty-two organisms he found, within four subgroups, a linear relationship between DNA content and radiosensitivity. The four subgroups are necessitated by the different complexities of the genetic organization of the organisms which range from the single stranded "naked" DNA of some viruses to the histone coated double stranded helices of the higher order mammalian cells.

In eukaryotic cells the nuclear DNA is organized into structural units called chromosomes. A number of recent studies have strongly implicated structural damage to chromosomes as the cause of cell reproductive death. It is assumed that DNA damage leads to chromosome damage and reproductive death. Carrano (1973 a & b) found that with V79 cells both asymmetrical chromosome exchange (dicentric, centric ring, or tricentric) and chromosome deletion are likely causes of cell death. The initial decrease in viability is caused predominately by the formation of anaphase bridges, while cell death from fragment loss becomes increasingly important in later generations. Dewey et. al. (1971 a) found an average of one aberration per cell at the D_{37} of Chinese hamster cells. This is what is expected from target theory if one such event resulted in and was the only significant cause of cell death. In the same study they found a linear proportionality between the number of chromosome aberrations and the log of cell survival up to an average of three aberrations per cell. Good correlations exist between the number of chromosome aberrations and radiosensitivity of cells during different periods of the cell cycle (Dewey, 1970), following BUdR

incorporation (Dewey, 1971 b), and following potentially lethal and split-dose recovery (Dewey, 1971 a). The BUdR correlation is the strongest evidence from the Dewey studies that the cause of cell death results from a chromosomal event and not proportional damage to another nuclear organelle. These experiments still leave open the possibility that there is some change in the chromosome, other than the observed aberration that leads to cell death. Sparrow (1967), has attempted to correlate chromosome number and radiation sensitivity from large numbers of cell types. He found the best correlation to be between the chromosome volume (the quotient of the interphase nuclear volume and the cell's chromosome number) and radiation sensitivity. Sparrow used data from seventy-nine cell types and is able to group them into eight radiological groupings. Within each group the same amount of energy must be deposited per individual chromosome to kill each organism. This would be equivalent to Terzi's conceptualization only if the DNA per chromosome is in direct proportion to chromosome volume. Sparrow concludes, however, that this probably doesn't hold since the comparison of larger numbers of different organisms shows many exceptions to a correlation between radiation sensitivity and DNA content.

Influence of Ploidy on Radiation Sensitivity

Haploid mammalian sperm and ovum cells do not divide until they have fused to form a diploid and thus are not suited for studies of reproductive death. Diploid cells represent the lowest ploidy

level available for study of reproductive death in a mammalian cell system. However, parts of a diploid's genome are or act as if they are monosomic. Such regions are associated with viable deletions, sex linked genes in male cells, and control mechanisms like the one causing inactivation of one of the x chromosomes in a female cell.

Many of the early ploidy studies (Zirkle et. al., 1953; Puck, 1960; Sparrow, 1958 and 1961) found increasing radioresistance with increasing ploidy. The explanation given for the observed increase in radioresistance was the increase in genetic redundancy of the higher ploidy cells. An alternate interpretation was given in a later paper by Sparrow (1964). He argued that much of this increased resistance is due to a decrease in the chromosome volume of the higher ploidy cells. He found that increases in the nuclear volume were often less than the ploidy increase. In a related observation Harris (1971 b) reported that variation of the ploidy from the normal diploid is accompanied by an equivalent change in the size of the cell.

Contrasting with this are the findings of Mortimer (1958) using a yeast system. He found that the haploid was the most sensitive, the diploid the most resistant, and that from diploid to hexaploid the yeast cells increased in sensitivity with the increase in ploidy. Utilizing micromanipulative techniques Mortimer (1955) was able to demonstrate that in haploid yeast approximately 5% of X-ray induced lethal damage was of the dominant lethal type. Dominant lethality was measured by pairing single irradiated cells with unirradiated

cells and determining the viability of the resultant zygote. Owen and Mortimer (1956), found that diploid cells required only half the dose to produce an equal frequency of dominant lethal damage as in the haploid cells. With increases in ploidy the probability increases that a dominant lethal lesion will result from a given radiation dose. Haploid yeast would be expected to be highly susceptible to killing by recessive damage. The results observed by Mortimer for the polyploid series in yeast are consistent with these findings. The high sensitivity of the haploid cells is due to killing by recessive lethal damage. The increase in radio-sensitivity observed as the yeast ploidy increased from diploid to hexaploid was due to enhanced formation of dominant lethal lesions in the higher ploidy cells.

Many researchers (Lea, 1955; Muller, H.J. et. al., 1942; Whiting, 1945) have proposed that dominant lethality resulted from dicentric chromosomes which in anaphase were pulled between the two daughter cells causing bridges. If this were true; then the presence of a cell wall in plant cells could possibly account for the difference between Sparrow's and Mortimer's results. During cytokinesis the cell wall would be expected to cleave any bridge that had formed between the daughter cells. This could result in a lessening of the impact such an aberration has on cell killing.

In mammalian cells a number of researchers have studied the relation between a cell's chromosome complement and its radiation sensitivity. This is of significant practical importance since

many tumor cells in human cancer are polyploid and have abnormal chromosome numbers. Puck (1960) analyzed results from Hela S3 cells showing an increased extrapolation number and a slight decrease in slope of the tetraploid cells relative to the diploid. Throughout the range of doses checked, the tetraploids are more resistant. Revesz and Norman (1960), studied growth of Ehrlich ascites tumor cells grown in diffusion chambers located in the peritoneal cavity of mice. Under normal conditions the diploid cells have a growth advantage. However, following X-ray doses of up to 4,000 rads the tetraploids grew better. Following doses above 4,000 rads neither the tetraploid nor the diploid cells grew. From this Revesz and Norman conclude that the tetraploids are more resistant to radiation. Silini and Hornsey (1962), using a dilution assay reported that Ehrlich ascites tumor cells in the presence of oxygen have a dose response curve with the same slope and a larger extrapolation number than the diploid. In the presence of nitrogen this is shifted slightly such that the tetraploid slope is less than the diploid value. In the anoxic state the extrapolation number of the tetraploids continues to be larger than its value for the diploid cells.

Till (1961), reported that in the L-cell line of mouse there is essentially no difference between the radiation sensitivities of cells differing in chromosome content by as much as a factor of two. In his data it appears that there is a very slight increase in the shoulder and in the final slope of the highest ploidy cells. Roger Berry (1963), gave results for two lymphocytic leukemias

growing in the same mouse subline, one diploid and the other tetraploid. Under both oxic and anoxic conditions he found that the tetraploids were slightly more resistant and have an extrapolation number equal to twice that of the diploid. Following repeated irradiations he found the population became increasingly resistant to radiation. Along with this change was an increase in the number of cells that were tetraploid and aneuploid. The average amount of DNA per cell also increased with the observed increase in cell resistance. Berry's study was carried out using an in vivo assay system. Nias and Ockey (1965), used tritiated water to irradiate Hela cells. This procedure is less precise since the exact temporal and spacial dose distribution is not known. They found a drop in the number of chromosomes in the irradiated cells relative to the non-irradiated controls. Bedford and Hall (1967), investigated the radiation characteristics of Chinese hamster cell lines having a preponderance of 23 and 46 chromosomes. Survival curves for their tetraploid cells have a larger extrapolation number and steeper final slope than for their diploids.

Burki and Carrano (1973 a) extended the work of Bedford and Hall using diploid and colcemid induced hypotetraploid Chinese hamster cells. Their results show increases, relative to diploid values, in both extrapolation number and final slope for the hypotetraploid cells relative to diploid values. At doses below 600 rads their hypotetraploids are more resistant than their diploid V79 cells whereas above 600 rads the hypotetraploids are more sensitive.

Many of the early studies used either colcemid, a drug disrupting microtubules (Kleinfeld et. al., 1966; Borisy and Taylor, 1967), to induce higher ploidies or tetraploid cells which occur naturally in cell populations. Recently, techniques have been developed to produce higher ploidy mammalian cells by fusing mutants. Often a small proportion of mutant cells when placed together will spontaneously fuse. This rate can be increased by using inactivated sendai virus or physiochemical methods (Harris and Watkins, 1965; Barski et. al., 1961; Littlefield, 1964, 1966). The fused cells are selected in the resulting mixed population by using media in which only the fused cells will grow (Harris, 1972). Little (1972), spontaneously fused mouse fibroblasts and rat pituitary cells. The hybrid is markedly more resistant to radiation than the precursor cells. In the Little study, the extrapolation number of the fused cells is slightly larger than the two diploid lines. The major change is in the D_0 value which for the fused tetraploid is dramatically larger than for the two diploids. Limbosch et. al. (1974), reported results for a study in which they fused two mutant sublines of DON Chinese hamster lung cells. Inactivated sendai virus was used in this study. They found essentially no difference between the radiation characteristics of the fused cell line or of the two precursor lines.

One potential complication of the study by Limbosch et. al. (1974) is the wide variation in the doubling times of the two precursor lines: 24 and 14 hours. Cells in different periods of the cell

cycle have different radiation characteristics. For Chinese hamster cells the most resistant period is late S, followed by early S, G1 cells, and finally by the G2-M cells which are the most sensitive (Sinclair and Morton, 1966). With a different doubling time a population in exponential growth is likely to have different percentages of cells at different phases of the cell cycle. For example, if G1 is extended there may be an additional resistant stage as shown by Hahn and Bagshaw (1966).

Increases in extrapolation number with ploidy are observed in the studies of Puck (1960), Silini and Hornsey (1967), Berry (1963), Bedford and Hall (1967), Burki and Carrano (1973), and Little (1972). Till (1961), Nias and Ockey (1965), and Limbosch (1974) working with fused cells, all found no increase in extrapolation number with ploidy. Work with Chinese hamster V79 cells (Burki and Carrano, 1973 a; Bedford and Hall, 1967) showed an increase in the final slope of the survival curve with increasing ploidy. Puck (1960) and Little (1972) using Hela, rat and mouse fused cells, and mouse lymphocytic cells found decreases in the final slopes with increasing ploidy. Silini and Hornsey (1967), Till (1961), and Berry (1963), found essentially no change in the final slopes of the survival curves for cells of different ploidy levels.

Explanations for the Observed Survival Characteristics

Explanations for the observed shifts in radiosensitivity following ploidy changes fall within two general categories: repair capacity, and changes in the nature of the lesion causing reproductive death.

i.) Repair. Studies of the reproductive capacity of whole cells following exposure to ionizing radiation have identified two types of repair phenomena. The first was observed for mammalian cells by Elkind and Sutton, 1960. They showed that if the dose is split into parts separated by time, the cells are able to recover from some of the damage of the previous exposure before the next dose is received. The final loss of reproductive capacity from the total exposure is less than if it had been given all at once. This recovery accounts for dose rate dependencies of cell survival. Experiments testing the relative ability of tetraploid cells for split dose recovery have been made by Little (1972) and by Bedford and Hall (1967). Little (1972), testing his fused cell line did not find a difference large enough to account for the increased resistance of the hybrid. Following a split in the dose the tetraploids survive three times as well compared with a factor of two for the two diploid lines. Bedford and Hall (1967), found their tetraploids exhibited a capacity for split dose recovery twice that of the diploids.

A second repair phenomenon influencing a cell's radiosensitivity is recovery from potentially lethal damage. This is damage which ordinarily causes cell death, but whose expression can be prevented by appropriate postirradiation treatments. The amount of "recovery" is usually measured relative to the amount of killing that would occur under normal conditions at 37°C. Conditions which enhance cell survival include holding the cells at 20°C or 4°C for up to a few hours following irradiation (Belli and Shelton, 1969; Winans,

1972), holding the cells in balanced salt solution (Belli and Shelton, 1969), or keeping the cells in plateau phase (Little, 1969). The recovery rate typically has a half-time of 15 minutes (Winans, 1972) and reaches a maximum after about one hour. The ability of the cells to repair potentially lethal damage varies throughout the cell cycle (Belli and Shelton, 1969; Little and Hahn, 1973; Winans, 1972). Some conditions such as hypertonic media have been shown to eliminate the repair of potentially lethal damage (Winans, 1972). Storage for long periods at 0°C to -4°C has been shown to potentiate potentially lethal damage (Koch and Burki, In Press).

Two studies are of particular importance for assessing the potential importance of differential repair in the diploid-polyploid comparisons. Rommelaere and Errera (1972), studied the effect of U.V. on Chinese hamster DON cells. They exposed both diploid and fused tetraploid cells under normal conditions and with caffeine in the media. Under the normal conditions they found no difference between the cell responses. With caffeine in the media the tetraploid cells are more sensitive than the diploid cells, particularly in the low dose region. Caffeine was selected because of its suspected role in inhibiting an error prone repair system. The results seem to indicate that a more active repair capacity was keeping the resistance of the tetraploids as high as the diploids.

A second group of studies done on the rad52 yeast mutant substantiate the importance of repair on the radiosensitivities of higher ploidy cells. The rad52 mutant was first described by

Resnick (1969), and is characterized by increased sensitivity to ionizing radiations. Ho and Mortimer (1973), found that the ratios of the survival curve slopes for diploid and triploid cells homozygous for mating type and rad52 to the slope of the haploid dominant lethal curve¹ averaged 1.8 and 2.81 respectively. This suggests that nearly all the lethal damage in the diploid and triploid strains is of the dominant lethal type. Dominant lethal curves involving one wild type haploid all are much more radioresistant than the rad52 x rad52 cross. The simplest interpretation for these results is that rad52 cells are deficient in a repair system and that unrepaired damage can be expressed as dominant lethal damage. Ho (1975), using yeast tetraploids containing one to four copies of the rad52 mutation was able to show a gene dosage phenomenon. With increasing numbers of the rad52 mutation the survival curves became progressively steeper. This demonstrated that differing numbers of a repair gene can affect both the shoulder and slope of a survival curve.

1. A dominant lethal curve in this case is formed by irradiating one haploid yeast cell and mating it with a non-irradiated haploid. If the resulting diploid fails to grow the irradiated haploid is considered to have a dominant lethal.

ii.) Change in the nature of the lethal lesion. Differences between the radiosensitivities of cells with different ploidy levels have been explained by relating them to shifts in the predominate cause of lethality. Cells with single copies of genes would be killed by mutations at loci necessary for cell survival. This mechanism of killing is expected to be of major importance for killing of haploid organisms or of higher ploidy organisms with monosomic regions. For cells with multiple gene copies death could result from damage to each of the gene sites. This is expected to be very uncommon since the inactivation of a specific gene would require a dose of about 10^6 rads. For cells with multiple gene copies, it is more likely that death would result from chromosome lesions. Dicentric and ring aberrations involve the interaction of two damaged chromosome areas. Dicentrics are formed between different chromosomes, and rings usually between damaged areas on different arms of the same chromosome. If cell death results from dicentrics and rings; cells with increasing numbers of chromosomes would be expected to be increasingly sensitive to radiation. For a polyploid series the probability of a dicentric or ring forming would increase with ploidy level. A second form of chromosome damage that could cause cell death is chromosome deletion. For a haploid organism or a monosomic region of a higher ploidy cell its effect would be similar to what was previously discussed for mutations. As the ploidy of a cell increases it would be expected that death caused by deletion would occur only after a similar

section from each haploid chromosome set had been deleted. As a result higher ploidy cells would be more resistant to radiation. Results with yeast (Mortimer, 1958; Zirkle and Tobias, 1953) show that the predominate cause of cell death does not remain constant throughout a ploidy series. Haploid yeast are killed exponentially by single events, such as mutations or deletions, whereas ploidy levels of diploid and above are killed by a more complex mechanism.

Studies With ^3H and ^{125}I

The availability of cells of the same type but at different ploidy levels permit an analysis of the various hypotheses for radiation induced cell death. This is of interest both for cells in normal conditions and for cells irradiated under conditions known to affect radiation sensitivity.

Decay of ^{125}I UdR incorporated within cellular DNA is quite efficient in producing reproductive death. The D_{37} for human T-cells following the decay of ^{125}I UdR in cellular DNA is 31 rads, while the D_0 for similar cells following ^3H -UdR decays is 495 rads (Feinendegen et. al., 1974). Survival curves for V79 cells following decay of incorporated ^{125}I UdR have a D_0 of 50 rads, compared to 456 rads following incorporation of ^3H IUdR (Burki et. al., 1973 b). Hofer and Hughes (1971), report that ^{125}I UdR decays are four to five times as deadly as tritium and ^{131}I UdR. They conclude that the killing of ^{125}I , "is due to high local concentrations of radiant energy within radiosensitive volumes considerably smaller than the

cell nucleus." The results with ^{125}I are similar to those with external high-LET irradiation (Burki et. al., 1973 b). This seems not to be due to its low energy electrons, but to the high charge imbalance due to the Auger effect (Ertl et. al, 1970; Kalpern and Stocklin, 1974). Further support for the localized nature of the damage comes from results of Burki (1974). This study shows that decay of $^{125}\text{IUdR}$ in the late replicating regions of DNA is a more efficient cause of cell death than decay in regions which replicate early. Ahnstrom et. al. (1970), compares the killing of bacteria by ^{125}I , ^3H , and X-rays. The ^{125}I dose response curve is without a shoulder leading to speculation that its decay may cause non-repairable damage. Following decay of incorporated $^{125}\text{IUdR}$ only one-half as many of the single strand breaks are rejoined as after X-rays (Painter et. al., 1974). Krisch (1972), has argued that $^{125}\text{IUdR}$ causes double strand breaks in DNA. Burki et. al. (1975), has shown it to be an effective maker of dicentric and ring chromosome aberrations. These results all emphasize the high efficiency of lethal damage induction due to $^{125}\text{IUdR}$ decay in cell DNA. Burki et. al. (1973 b), found that survival curves for L5178Y cells have a D_{37} following $^{125}\text{IUdR}$ decays of 46 whereas the normally more resistant V79 cells had a D_0 of 39 decays. From this they proposed that 39-46 decays of ^{125}I in cellular DNA results in a dominant lethal like lesion. The hyperdiploid, hypotetraploid system is well suited for testing this hypothesis.

Damage to cells from tritium decays is very comparable to damage from X-rays (Bond and Feinendegen, 1966; Feinendegen et. al., 1971). D_0 values for survival curves of frozen V79 cells in which the dose was given by X-rays and incorporated tritium are 860 and 456 rads respectively (Burki et. al., 1973b). The number of single strand breaks formed after decay of $^3\text{HTdR}$ in frozen cells is equivalent to the number observed after a similar X-ray exposure (Cleaver et. al., 1972). The Kinetics and efficiency of their repair is comparable to strand break rejoining following X-rays (Cleaver et. al., 1972).

Incorporation of non-radioactive IUdR into cellular DNA sensitizes the cell to radiation. Up to one quarter the thymidine in a cell's DNA can be replaced by IUdR. With these high levels of substitution cells are up to three and a half times more sensitive to X-rays (Szybalski, 1974). The degree of sensitization is proportional to the amount of IUdR incorporated (Erikson et. al., 1963).

A possible mechanism for the sensitization is given by Szybalski (1974). He proposes that radiation removes the halogen from the halogenated analog, resulting in the formation of two very reactive radicals. Secondary reactions involving these radicals are the basis of the major radiochemical damage to the DNA. The importance of the secondary changes is demonstrated by the experiments of Hotz (1963). He showed that radical scavengers such as cysteamine can abolish the radiosensitizing effects of the five halodeoxyuridines.

The Current Study

The present study investigates the relation between mammalian cell radiosensitivity and ploidy in Chinese hamster and mouse cells in vitro.

- 1) Cells containing different multiples of the diploid chromosome set are created through drug induction and cell fusion. Survival curve parameters of these series are determined. Analysis of the extrapolation numbers and final slopes supports the thesis that changes in cell ploidy causes shifts in cell radiosensitivity. The exact nature of the impact of cell ploidy on a cell's radiation response is species specific; the shift in radiosensitivity as the ploidy level is raised is different between Chinese hamster and mouse cells.
- 2) The number of chromosomes in a given cell volume is an important parameter of radiosensitivity. However, Sparrow's relationship between interphase chromosome volume and radiosensitivity does not hold for the cells used in this study. For these cells, the radiosensitivity increases with decreasing chromosome volume.
- 3) Freezing Chinese hamster cells reverses the relationship between hyperdiploid and hypotetraploid survival curves. This suggests the importance of indirect action on the relative positions of the survival curves of the two cell lines irradiated at 37°C.

- 4) On the basis of decays the hypotetraploids are more resistant than the hyperdiploid cells to both $^{125}\text{IUdR}$ and $^3\text{HTdR}$. On the basis of dose to the nucleus the relationships between the diploid and tetraploid survival curves is the same following $^{125}\text{IUdR}$ and $^3\text{HTdR}$ decays as following X-irradiation. The average RBE observed in these studies for incorporated $^3\text{HTdR}$ and $^{125}\text{IUdR}$ are respectively .76 and 5.7.
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MATERIALS AND METHODS

Cell Lines and Growth Conditions

Three cell lines were used in these studies; two originated from Chinese hamster and one from mouse. Most studies were conducted with cells derived from Chinese hamster lung tissue by Yu and Sinclair (1964). In this study the cells designated V79, are the V79-S171 clone obtained from Warren Sinclair. In this laboratory, they have been stored frozen at -196°C for six years in media with 5% dimethyl sulfoxide (DMSO). The San Francisco Medical Center PPLO Laboratory tested these cells and found them free of PPLO. For fusion studies, a V79 clone was obtained from Paul Aebersold (1975) that was efficient in Hypoxanthine-Guanine phosphoribosyltransferase (HGPRT⁻). This cell line originated from the same cell stock as our normal V79 cells and was selected due to its resistance to 5ug/ml of 6-Thioguanine. The second Chinese hamster line used was a thymidine kinase mutant of B150 cells generously provided by Morgan Harris. These cells had been isolated from the peritoneal cavity by Humphrey and Hsu (1965). The thymidine kinase mutant had been selected through its resistance to high concentrations of bromodeoxyuridine by Morgan Harris (1972).

The Chinese hamster lines were grown attached to culture flasks and dishes in a 37°C humidified atmosphere containing 5% CO_2 . Both were grown in media composed of: 500ml Puck's Saline F; 5 ml BME amino acids (100X); 2.5ml BME vitamins (100X); 5ml L-glutamine (200mM); 5 ml penicillin (10,000 units/ml) and streptomycin (10,000 ug/ml); 0.15 ml $\text{CaCl}_2\cdot\text{H}_2\text{O}$ (33.3% w/V)-stock solution made from Worthington reagents and then filter sterilized; and 60ml fetal calf serum.

Mouse leukaemic cells L5178Y, originally provided by Dr. G.A. Fischer (Fischer and Sartorelli, 1964) were used. They have been stored frozen in our laboratory since 1964 at -196°C in media containing 5% DMSO. These were grown in suspension in a 37°C humidified 5% CO_2 incubator. Their media contained: 500ml Fischer's media for leukaemic mice (made from powder); 55ml horse serum; 5ml penicillin (10,000 units/ml) and streptomycin (10,000 ug/ml); and 5ml L-Glutamine (200mM).

Cell populations were kept in exponential growth by subculturing late log phase cultures 1:10 every other day or 1:4 every day. Chinese hamster cells were loosened from their growth surface by rinsing with Puck's Saline A followed by cold .03% W/V lyophilized trypsin (Worthington) in Saline A. The dish was then transferred to a 37°C atmosphere for 1.5 minutes, after which the trypsin was immediately removed, and regular media at 37°C placed on the dishes. Cells were then detached by pipeting this media vigorously over the growing surface. The mouse cell populations were kept between 1×10^4 and 5×10^5 cells/cc to maintain them in exponential growth.

All plastic culture dishes were supplied by Falcon and Corning. No differences were detected between the two product lines in their ability to support cell growth. All biochemicals, unless specifically noted, were purchased from Grand Island Biologicals.

Colony Formation

The endpoint for these studies was to score the ability of a single cell to divide giving reproductively viable progeny for a minimum of six generations, minimum of 50 cells (Puck and Marcus, 1956).

To assay this individual cells were plated under conditions which limit cell movement. Following eight days, about sixteen doubling times for the cell types used, visible cell clusters were scored as having originated from a reproductively viable cell.¹ The Chinese hamster cells, with their attachment to the growth surface, were assayed by plating at low concentrations of viable cells under normal culturing conditions. Following eight days the dishes were scored by adding 5% of a 1%W/V methylene blue solution to the media in the dishes. This dye solution was poured off after 40 minutes at which time the colonies could be counted, by eye and with the help of a 10X dissecting microscope on questionable colonies.

The "agar" colony method of Fischer as modified by Pang (1971) was used for the L5178Y cells. Cell movement was restricted by using soft agar to suspend them three dimensionally in culture tubes. Four percent W/V Nobel Agar (Difco) was diluted 1:10 with 45° media and 4cc of this solution was mixed with 6cc of media containing the

1. If there was any question the number of cells was counted and scored as a colony if it contained 50 or more cells. This is the same criterion used by Puck and Marcus (1956). Elkind and Sutton (1960) showed that counting essentially all stained areas rather than those containing more than 150 cells, produced only a small shift in the survival curve suggesting that the lower limit of the acceptable colony size is not too important.

desired number of cells in a culture tube. Following a thorough mixing this was placed in an ice bath for eight minutes and then into the CO₂ incubator at 37°C. After eight days the colonies are scored, unstrained, with the help of a low powered lens.

Induction of Higher Ploidies

i.) Cloning. Before use all near diploid populations were cloned, a population grown, and many vials frozen. At later times, when it was suspected that the properties of cells in use had changed, they were discarded and a new population started from the frozen stock. The cloning method used for the Chinese hamster cells was the plastic punch method of Todd et. al. (1966). Cloning the L5178Y cells was accomplished by growing colonies, as previously described in soft agar. Individual colonies were then picked by sucking them gently into a sterile lcc plastic pipet and transferring to a new growth flask.

ii.) Colcemid induction. Procedures similar to those of Carrano and Burki (1973) and Harris (1971 b) were followed. Freshly reconstituted lypholized colcemid in concentrations of .03, .06, and .1 micro-gram/ml was added to diploid cell cultures in exponential growth. Following 12 to 36 hours the media was removed either by pouring or sucking from the Chinese hamster cells and by centrifugation from the mouse populations. The remaining cells were rinsed with 37°C media and cloned using the previously mentioned cloning procedures. To obtain clones with similar growth kinetics, only the larger colonies were picked and grown. Growth curves for each of the cell clones were

made to verify that the selection yielded cells with growth rates similar to the lower ploidy cells. This induction procedure was later repeated on hypotetraploid populations to obtain cells of even higher chromosome number.

iii.) Cell fusion. The V79 HGPRT⁻ cells and the B150 TK⁻ hyperdiploid cells were fused by placing 10^4 TK⁻ and 10^6 HGPRT⁻ cells into 60mm dishes containing 5cc of media. After seven hours the media was replaced with 5cc "THAG" media. THAG media consists of 4×10^{-7} molar Aminopterin, 10^{-4} molar Hypoxanthine, 1.6×10^{-5} molar Thymidine, and 10^{-4} molar Glycine added to normal media. It needs to be made up fairly fresh as particularly the Aminopterin is unstable in such dilute solution. This selection procedure and its conceptual basis is described by Harris, 1972.

The THAG media on the dishes was changed every four days. After eight days growth, visible colonies appeared. The colonies were allowed to grow in the THAG media for six more days at which time they were "picked" using the punch technique. Once cloned, they were grown on normal media and karyotypes made to check to see if they were really a fused population. This method and the cell types used are the same as reported by Harris (1972).

Cell Freezing

Cells are frozen at a concentration of approximately 10^6 cells/ml in media containing 10% DMSO using a Linde Biological Freezer (Union Carbide Cryogenic Products). This method uses liquid nitrogen vapor to freeze the samples. The vapor is blown

at a regulated rate around the cell ampules. The freezing rate is 1.5°C/minute as determined by a temperature probe in a sample vial. The cells were frozen with 1ml of cell sample in 2.5ml plastic provials supplied by Microbiological Associates. All frozen cells were stored in Cryenco-1000-X Biostat liquid nitrogen storage tanks until needed. The thawing procedure consisted of removing the vials from the storage tank, holding them at room temperature for two minutes enabling trapped nitrogen to escape, and then warming as quickly as possible in a 37° waterbath.

Cell Spreads

The most direct measure of a cell's chromosome complement is the microscopic counting of chromosome number from preparations of stained mitotic spreads. To obtain mitotic spreads, enough colcemid to give a final concentration of 0.6 micro-gram/ml was added to the media of cells in exponential growth. Following two to three hours in the colcemid solution, the media was pipetted vigorously over the growing surface. The rounded mitotic cells are less tightly attached to the surface than cells in other phases of the cell cycle, so this results in a suspended population with a high percentage of mitotic cells. The preferential selection of mitotic cells, following the colcemid treatment is not possible with L5178Y cells since they are already growing in suspension. This solution was then centrifuged in a counter-top centrifuge for 10 minutes and all but .5cc of the supernatant decanted. The pellet

was mixed with a pasteur pipet into the remaining .5cc, and 4.5cc of 0.075 molar KCl added and mixed well with the cell solution. After sitting for four minutes each sample was centrifuged for five minutes and the supernatant discarded. Four milliliters of fresh 3:1 methanol-acetic acid fixative was added, a pasteur pipet used to mix the pellet and fixative, and then the suspension centrifuged for five minutes. This last step was conducted three times with the supernatant being discarded after each centrifugation. Approximately eight drops of fresh 3:1 methanol-acetic acid was finally added to each tube, the pellet mixed, and one drop per slide was dropped from three inches above the slide onto the slide. The slide was set aside for at least half an hour for drying and was then stained using Giemsa stain (Gurr's Improved R66) diluted 1:20 in McIlvans buffer for four minutes. The stained slide was rinsed under running water and set aside to dry. Chromosomes were counted under phase contrast microscopy at 500× magnification or under oil immersion at 1250× using a Zeiss Photomicroscope II. Where the chromosome count was uncertain, the spreads were photographed using the built in 35 mm camera and the chromosomes counted from an enlargement of the resulting negative. All the chromosomes from a picture of a representative spread were cut out and organized on a page in groups according to size and general morphology. These were used to compare the karyotypes of the different cell lines.

Cell and Nuclear Volume

Cell volume measurements were made using the computerized cell volume apparatus of Mel and Yee (1975), Ross (1972) and a model F Coulter Counter. Cells flowing through the apertures of these machines result in a signal roughly proportional to cell volume. The number of voltage peaks within a given range are measured using a single or multi-channel analyzer and the size distribution of the input population determined. Ross (1972), found the mean and the mode for these distributions to be nearly identical. The peak volume, the mode, is considered to be the better parameter since it is less sensitive to noise and shape factors. As a result, the mode, the channel proportional to the most common cell size, was used in this study as the measure of cell volume.

With L5178Y cells the initial screening for colcemid induced clones of higher ploidy was done on the basis of size. Only those clones were grown whose predominate cell size was at least twice the diploid volume.

Relative nuclear volumes were determined by photographing cells in suspension and growing on cover slips. The nuclear and cell diameters were then measured. From the average of these values the volume and nuclear-cytoplasmic ratio relative to V79 hyperdiploid cells was calculated.

DNA Determination

A diphenylamine procedure adapted from Davidson and Waymouth (1944) was used to determine DNA contents. The DNA from a known number of cells was prepared by the following procedure:

- a) suspend cells by trypsinization if necessary and then centrifuge and decant supernatant
- b) wash with 2.5 ml 10% TCA, centrifuge and decant supernatant
- c) wash twice with 5 ml 95% ethanol centrifuging and decanting supernatant after each time
- d) add 1.3 ml H_2O , 1.3 ml 19% TCA, heat to $90^{\circ}C$ for 15 minutes, centrifuge and save supernatant
- e) wash with 2.5 ml 5% TCA centrifuge and combine the supernatant with that from step d.

All centrifugations are at 1000g for 10 minutes. 1 ml of the final supernatant and known DNA standards were then mixed with 2 ml of diphenylamine reagent (1gm/diphenylamine, 100 ml glacial acetic acid, and 2.75 ml of concentrated sulfuric acid) and cooked at $90^{\circ}C$ for 10 minutes. These were then cooled and allowed to set at least 24 hours before reading optical density at 600nm with a Gilford spectrophotometer.

X-Ray Techniques

Two X-ray machines were used. One is a Norelco MG150 machine operated at 145kv, 12 ma with 1 mm aluminium added filtration.

Dosimetry was measured using a Victoreen dosimeter and Fricke ferrous

sulfate Dosimetry (Weiss, Allen, and Schwarz, 1956). Under the conditions most frequently used, this machine gave a dose rate between 180 and 130 rads/minute.

The second X-ray machine has a beryllium window tube (Machlett OEG60) operated at 50 kv, and 20 ma. A .5 mm aluminium filter was added. Dose measurements were made using the Victoreen Dosimeter, Fricke ferrous sulfate dosimetry and a beryllium window ionization chamber. Only cells attached to a plastic surface were irradiated with this source. These cells were irradiated in inverted flasks such that the beam always penetrated a 1.5 mm layer of plastic and then the cells. Under these conditions and the geometry used, the dose rate was 540 rads per minute.

Radiation Temperature Conditions

Temperature was carefully controlled during irradiation. When using the more penetrating 145 kv X-rays at 37°C, the cells, in closed flasks, were positioned on a holder such that the bottom part of the flask was submerged in 37°C water. In the 50 kv machine, the flasks were inverted and placed in the stream of an air curtain incubator at 37°C during exposure. 4°C exposures were made with the cells in closed flasks positioned on a Thermoelectric TCD-2 cold plate (Thermoelectrics Unlimited, Inc.) at 0°C. When in the X-ray machine theses flasks were kept on a layer of ice, or in a refrigerator. For irradiation of frozen cells the desired ampules were transferred from their liquid nitrogen storage into an insulated tray filled with liquid nitrogen under the beam. They were held vertical by a small

wire holder. At completion of the desired dose, the ampules were returned to the liquid nitrogen storage tanks until being thawed and plated.

IUdR Incorporation

Non-radioactive IUdR was incorporated unifilarly into the cells. This was accomplished through 15 or 16 hours of growth in media containing 1×10^{-6} IUdR with and without 10^{-7} FUdR, and in media 2.5×10^{-6} molar IUdR + 10^{-7} molar FUdR. Under these conditions the number of cells would double in 15 to 16 hours. After the period for incorporation the media was poured off and the cells rinsed twice with 37°C Puck's Saline A. Normal media was then put on the cells. The cells were irradiated almost immediately after this second media change. The degree of incorporation of IUdR under each of the growth conditions was measured. $^{125}\text{IUdR}$ was added such that the same proportion of the total IUdR was $^{125}\text{IUdR}$. Under these conditions the amount of $^{125}\text{IUdR}$ incorporated within the cell is proportional to the amount of IUdR incorporated. The resulting activity in the cell's DNA was assayed and from it the relative amount of IUdR taken up determined. The toxicity of the IUdR was determined by growing cells in media containing 0 to 5×10^{-4} molar IUdR. Following 15 hours of growth the cells were plated in numbers suitable for determination of plating efficiency.

Hypertonic Conditions

X-ray survival curves were made in hypertonic media based on the work of Dettor et. al. (1972). The media was made hypertonic by addition of 5% W/V myoinositol to normal media which was then sterilized by filtering. The tonicity of this media, 525 milli-osmolar, and of normal media 287 milli-osmolar, was checked using a Fricke osmometer. The volume change in cells was followed using the previously described coulter counter technique. In these experiments all cell sizing and irradiations were done with cells in solution. The normal trypsinization procedure was followed except that, following the removal of the trypsin, media 5% in myoinositol was added to the dishes. The cells were then loosened from the dish and suspended in this media through vigorous pipetting. For irradiation experiments all of the suspended cells were pooled and dispersed to 25cm² falcon flasks. Within 30 minutes of being suspended in the hypertonic media, the cells were taken to the X-ray machine and irradiated at 37°. Following irradiation, the cells were diluted and plated at the desired concentration for assaying survival.

Studies With ³H and ¹²⁵I

¹²⁵IUdR, 200 milli-curies/ml, was purchased from New England Nuclear and used within one half-life, 60 days, or discarded. Cells were unifilarly labelled through 15 hours growth in media containing .002 to .008 micro-curies ¹²⁵IUdR per ml plus 10⁻⁶ molar IUdR. The cells were then rinsed three times with Saline A. Most of the cells were trypsinized and frozen in 10% DMSO and stored at -196°C.

this enables decay of the incorporated radioactive isotopes while the cells are in the metabolically inert, frozen state. Control cultures were trypsinized, the cells collected, and rinsed three times in cold 5% Perchloric Acid. The ^{125}I activity in the acid insoluble DNA was counted on a Nuclear Chicago gamma counter. The counting efficiency was determined using a ^{129}I standard. At desired intervals the frozen ampoules were thawed and assayed for survival. The exact number of decays was determined according to the equation $N = N_0 (1 - e^{-0.0116t})$. Where t equals the time in days from when the activity was actually measured and N_0 is the number of atoms of ^{125}I in the DNA at that time: $N_0 = (t \frac{1}{2} / 0.693) dN/dt = (60 \text{ days} / 0.693) dN/dt$. dN/dt is the measured activity. At the time the activity in the cells was measured, ampoules were thawed and plated as controls. All subsequent survival values were determined relative to these. As a result the number of decays per cell included only those decays occurring while the cells are frozen, and any lethality caused by decays during the labelling period is corrected for. A control experiment was conducted in which exponentially growing cells incorporated 1×10^{-6} molar non-radioactive IUdR for 16 hours and then frozen. Using 145 kv X-rays, these cells were irradiated while frozen. They were then thawed and their viability assayed.

$^3\text{HTdR}$ was purchased from New England Nuclear. Cells were labelled during 15 hours of growth in media with 1 to 12 micro-curies/ml $^3\text{HTdR}$ plus 10^{-4} molar TdR and 10^{-5} molar CdR. The treatment of the cells for assay of survival was the same as with

the $^{125}\text{IUdR}$. Cells to be assayed for $^3\text{HTdR}$ content were prepared by 4° centrifugation for $\frac{1}{2}$ hour at 1000 g, the pellet was suspended in 10% cold TCA, and re-centrifuged. The resulting pellet was suspended in 1cc of 5% TCA, heated in boiling water for half an hour, and cooled. The 1cc solution of cell DNA in 5% TCA was added to 10cc of PCS solubilizer (Amersham/Searle) in a scintillation vial. This was counted in a Nuclear Chicago liquid scintillation counter. The counting efficiency was determined using the channels ratio method with quenched ^3H standards of known activity. The 12.5 year half-life of the tritium is long relative to the duration of the experiment making no correction necessary for decays during storage of the cells. The number of decays in the cell was $t(dN/dt)$; where t is the time following the plating of the initial frozen samples and dN/dt is the measured activity.

Repair of Potentially Lethal and Sublethal Damage

Recovery from potentially lethal damage was assayed using a procedure adapted from Winans et. al. (1972). V79 hyperdiploid and hypotetraploid cells were plated in small 25 cm^2 flasks in numbers appropriate for assaying survival following the experimental treatment. To facilitate cell attachment, the flasks were transferred for three hours to a 37°C incubator. Following this period the media was replaced with regular media at 20°C . The cells were then held in 20°C waterbaths during irradiation, and for up to four hours post irradiation. After the desired time at 20°C the flasks were placed in a 45°C waterbath for $1\frac{1}{2}$ minutes. This warms them quickly to 37°C ,

following which they were placed in a 37°C incubator. In control cultures the media was replaced with 37°C media, the cells irradiated at 37°C, and then returned immediately to a 37°C incubator. As a measure of the magnitude of recovery the ratio of the survival following post-irradiation storage at 20°C and the survival at 37°C was computed.

Repair of sublethal damage was determined using a procedure similar to the split dose experiments of Elkind and Sutton (1960). V79 hyperdiploid and hypotetraploid cells were plated in large plastic flasks and irradiated at 37°C. Following an initial dose of 540 rads, the flasks were placed in a 37°C incubator for varying periods of time before exposure to a second dose of 405 rads. One half hour was waited following this second dose before the cells were trypsinized and plated in numbers appropriate for determining viability. The recovery ratio defined as the ratio of survival following the split dose and following a single acute dose of 945 rads was determined.

Curve Fitting

Fitting of the data to a particular single hit-multitarget curve was done with the help of a computer. The program is a non-linear, least squares procedure using variable metric minimization (Davidson, 1959). Data was input as a dose and the log of the survival for that dose. The computer gave the "best" value for the extrapolation number and slope of the survival curve, and these were used for drawing the presented curve. The details of this procedure are given in Appendix 2.

RESULTS

Characteristics of Higher Ploidy Cells

Hyperdiploid Chinese hamster cell lines were treated with colcemid as described in materials and methods to obtain hypotetraploid lines containing an average of 42 chromosomes. Hypohexaploid cells with an average chromosome number of 64.4 were obtained by repeating the colcemid procedure on the hypotetraploid cells. Variability of the chromosome number within cells increased with the ploidy. In the B150 series the hyperdiploid cells average 23.6 ± 3.27 , the hypotetraploids average 42.5 ± 7.2 , and the hypohexaploid 64.4 ± 19.2 chromosomes. A similar trend occurred with the V79 cells; the hyperdiploid cells averaged 22.7 ± 3.3 and the hypotetraploid cells averaged 41.8 ± 6.5 chromosomes.

In preparation for the development of higher ploidy cells using cell fusion, thymidine kinase deficiency of the clones derived from B150 cells was verified. The cloned cells were grown in media containing .06 micro curies/ml ^{125}I UdR and 10^{-6} molar UdR. After 15 hours the labelling media was removed from the cells and the cellular DNA assayed for presence of ^{125}I . B150 TK⁻ cells incorporated enough ^{125}I UdR into cellular DNA to give 0.24 decays per cell day. Normal V79 cells following a similar protocol incorporated an amount giving 290 decays per cell day. The V79 hyperdiploid cells incorporated over 10^3 times as much UdR as the B150 TK⁻ hyperdiploid. In a further check, the B150 TK⁻ cells continued to grow well in media containing 10^{-2} molar thymidine,

a level of thymidine that blocks replication of nonmutant cells. HGPRT⁻ V79 cells were verified as being 6-Thioguanine resistant by placing them in media with five micro-grams/ml of 6-Thioguanine. The V79 HGPRT⁻ hyperdiploid cells continued to grow well whereas normal V79 cells were killed.

Following the procedure described in the materials and methods section, the B150 TK⁻ hyperdiploid cells were fused with the V79 HGPRT⁻ hyperdiploid cells. The efficiency of fusion was approximately one per 5×10^3 B150 cells. Hypotetraploid cells with an average of 44.7 ± 10.7 chromosomes were obtained. In a similar manner B150 TK⁻ hypotetraploid cells were fused with V79 HGPRT⁻ hyperdiploid cells to derive a hypertetraploid population with 54.9 ± 9.3 chromosomes.

The chromosome number of Chinese hamster diploid cells is quite stable, cell lines grown for ten years or more have had the same chromosome number. In our studies reported here the average chromosome number of both B150 and V79 hyperdiploid cells remained around 23 regardless of how long they were grown in culture. However, as the ploidy increased the chromosome number became more unstable. Following a month in culture, the V79 hypotetraploids had fallen from an average of 41.8 to 37.6 chromosomes. Burki and Carrano (1973) also report instability in V79 octaploid lines that they produced through colcemid induction. During each experiment the average pulse height generated by the coulter counter for cells being counted was scanned. This gave a measure of their size and an indication of their ploidy level. Chromosome spreads were made each week to insure that the

average chromosome number of a cell population had not declined. The chromosome content of hypertetraploid and hypohexaploid cells was verified by making cell spreads the same day as their use in an experiment. When the average chromosome number had decreased the cells were discarded and a new population started from the frozen stock.

Diploid mouse L5178Y cells with an average chromosome number of 39.2 ± 4.0 were treated with colcemid as described in the methods section. A hypotetraploid population having an average chromosome complement of 72.9 ± 11.7 was obtained. The chromosome number of the mouse hypotetraploid population is unstable. Following a month in culture the number decreased to an average of 57.6 chromosomes. A single attempt to develop an octaploid L5178Y population using colcemid was unsuccessful.

L5178Y cells possibly deficient in HGPRT⁻ were selected by adding five micro-grams/ml of 6-Thioguanine to a log phase culture. Initially the population number decreased, however, after a week it started to enlarge. This new population was resistant to 6-Thioguanine. An unsuccessful attempt was then made to spontaneously fuse these cells with the B150 TK⁻ cells.

Harris (1971 b) working with colcemid induced polyploid series of V79 and pig cells found a linear relationship between cell ploidy and cell volume. Measurements of cell volume and DNA content were made as verification of the validity of the counted chromosome numbers. Table 1 contains the doubling time, number of chromosomes,

size, and DNA content relative to V79 hyperdiploid cells for each of the cell types. Within related series there is good agreement between these parameters. For example, V79 hypotetraploids have 1.84 as many chromosomes, are 1.8 times as large, and have 1.86 times as much DNA as the V79 hyperdiploids. The B150 ploidy series has a larger change in measured size and DNA content than in chromosome counts (Table 1). For example, the hypoheptaploid have over four times as much DNA, are almost four times larger, but have only 2.8 times as many chromosomes as the hyperdiploid B150 cells. For the two fused lines the values agree with the average values for the two precursor lines corrected for the "quality"¹ of the union of the two original lines. The size of the fused hypotetraploids is the only value not closely agreeing with this relationship. These cells would be expected to have a size equivalent to 1.5 times the size of the V79 hyperdiploid cells. Their measured value is larger; equal to 1.89 times the size of the V79 hyperdiploid cell. The L5178Y hypotetraploid has 1.84 times the number of chromosomes, is 1.9 times as large, and has 2.4 times the DNA of the L5178Y diploid.

Measurements were made on V79 hyperdiploid and hypotetraploid cells to determine if the nuclear volume changed in the same

1.) This correction is equivalent to multiplying by the average number of chromosomes for the fused cells divided by the sum of the average number in each of the precursor cells.

proportion as the cell volume. For cells in suspension the average diameter of the nuclei was 1.2 times larger for the V79 hypotetraploid cells than for the hyperdiploid line. As a verification of the technique, similar measurements were made on the cell diameter. Again the hypotetraploid diameter was 1.2 times the hyperdiploid value. V79 hyperdiploid and hypotetraploid cells attached to cover slips were photographed and the nuclear diameters measured from the photographs. These measurements further confirm that the average nuclear diameter of the hypotetraploid cells is 1.2 times the hyperdiploid value. All of these measurements are consistent with the hypotetraploid cell volume and nuclear volume being 1.72 times the size of the hyperdiploid cells. This agrees well with the average cell volume as measured using the coulter counter showing that the hypotetraploid average was 1.8 times larger than the hyperdiploid.

Comparisons of the data in Table 1 between cell types are important. For example, it should be noted that the L5178Y diploid cells are the smallest and have more chromosomes than the Chinese hamster hyperdiploids. The B150 hyperdiploids have the same chromosome number as the V79 hyperdiploid cells but are only two-thirds the size.

Chromosome spreads are important both for verification of ploidy level and cell origin. Sample karyotypes for all the cell types are given in Figures 1 through 9. Each set of chromosomes represents the complete contents of a single cell spread. These are representative of the mitotic spreads counted to obtain the average

Table 1: Cell Properties

Cell Type	Doubling Time (Hour)	Number of Chromosomes	Relative Chromosome Number	Size	Relative Size	Relative DNA Content
V79 hyperdiploid	13	22.7 ± 3.3	1	21.8 ± 1.5	1.0	1.0
V79 hypotetraploid	12.5	41.8 ± 6.5	1.84	39.3 ± 3.8	1.8	$1.86 \pm .37$
B150 hyperdiploid	11.5	23.6 ± 3.27	1.04	14.1 ± 1.1	0.65	$.88 \pm .27$
B150 hypotetraploid	13.8	42.5 ± 7.2	1.87	31.5 ± 1.2	1.44	2.33 ± 1.36
B150 hypoheptaploid	18.5	64.4 ± 19.2	2.84	52 ± 5.7	2.4	$4.11 \pm .74$
V79 HGPRT ⁻	11.25	22.8 ± 4.1	1.0	19.75 ± 1.71	0.9	$1.0 \pm .136$
Fused hypotetraploid B150 $\times$ V79	11.75	44.7 ± 10.7	1.97	41.25 ± 2.5	1.89	$1.57 \pm .237$
Fused hypertetraploid B150 Tp $\times$ V79	16	54.9 ± 9.3	2.4	43 ± 7.1	1.97	2.57 ± 1.09
L5178Y	9.0	39.2 ± 4.0	1.74	13 ± 1.0	0.59	$.7 \pm .15$
L5178Y hypotetraploid	9.06	72.9 ± 11.7	3.21	$24.5 \pm .71$	1.12	1.65 ± 1.14

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values given in Table 1. For each cell type a minimum of 40 cells was counted. The morphology of the chromosomes from the Chinese hamster hyperdiploid cells, Figure 1 and 2, agree with published karyotypes (Harris, 1972). Fifty percent of these chromosomes are metacentric. Most of the remaining chromosomes are acrocentric with only three or four teleocentric. In contrast, the karyotype of the mouse L5178Y diploid, Figure 8, has 38 teleocentric chromosomes and one metacentric. This differs from the mouse karyotype published by Hsu and Benirschke (1967), which consists of 40 teleocentric chromosomes. The L5178Y hypotetraploid karyotype (Figure 9) has two or three metacentric chromosomes. As expected, no visible chromosomal changes occurred with the induction of an HGPRT⁻ mutant population (Figure 2) from V79 hyperdiploid cells (Figure 1). The karyotype of the B150 TK⁻ hyperdiploid cells (Figure 2) has five small metacentric chromosomes. The small chromosomes in a V79 HGPRT⁻ hyperdiploid cell consist of one metacentric and three acrocentric chromosomes (Figure 2). The karyotype of the fused hypotetraploid cells (Figure 6) possesses both small metacentric and acrocentric chromosomes. This is good evidence that the fused cells are, in fact, a union of the B150 TK⁻ and V79 HGPRT⁻ precursor cells.

X-Ray Survival Curves for Chinese Hamster Cells

Figure 10 shows the X-ray responses of hyperdiploid and hypotetraploid V79 clones. Both 150kv and 50kv X-ray sources were used. The average control plating efficiencies for the hyperdiploid and hypotetraploid cells were 91% and 100% respectively. The hypotetraploid survival curve has a slightly larger shoulder and steeper

V79 HYPERDIPLOID

Figure: 1

Figure: 2

B150 HYPERDIPLOID

V79 HGPRT- HYPERDIPLOID

Figure: 3

V79 HYPOTETRAPLOID

Figure: 4 B150 HYPOTETRAPLOID

B150 HYPOHEXAPLOID

Figure: 5

Figure: 6 B150xV79 HYPOTETRAPLOID

HYPERTETRAPLOID

Figure: 7

Figure: 8 L5178Y DIPLOID

L5178Y HYPOTETRAPLOID

Figure: 9

final slope than the hyperdiploid curve. A computer fit of the data to a single hit multi-target model derived $N=2.4$, $D_0=209.2$ rads for the hyperdiploid; and $N=13.7$, $D_0=137.1$ rads for the hypotetraploid. This is in general agreement with the findings of the Burki and Carrano study (1973).

Hypotetraploid Chinese hamster cells have a slope steeper than the hyperdiploid cells. This is the same type of trend observed by Mortimer (1958) with yeast. If this similarity were to continue, Chinese hamster cells with chromosome complements higher than tetraploid would show an even greater response. To test this a series of colcemid induced B150 TK⁻ hyperdiploid, hypotetraploid, and hypohexaploid cells was developed. Control plating efficiencies for the hyperdiploid and hypotetraploid was 85 and 87 percent respectively. The average control plating efficiency for the hypohexaploid cells was 67%. The population doubling time for the hypohexaploid cells was 18.5 hours compared with 13.8 hours for the hypotetraploid and 11.5 hours for the hyperdiploid. Dose response curves for these cells are shown in Fig. 11. A computer fit of the data to the single hit multi-target model gave $N=19.5$, $D_0=142.8$ rads for the hyperdiploid; $N=47.3$, $D_0=114.3$ rads for the hypotetraploid; and $N=145$, $D_0=98.5$ rads for the hypohexaploid. The hyperdiploid and hypotetraploid B150 cells have a similar shoulder on their survival curves. The final slope of the hypotetraploid cells is steeper than the final slope of the hyperdiploid cells which accounts for their larger extrapolation number. The survival curve of the B150 hypohexaploid has

Figure 10: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells irradiated at 37°C. The dashed line gives the hyperdiploid response. The error bars represent standard errors of the mean from three experiments.

Figure: 10

XBL766-5582

a steeper final slope and a larger shoulder than either the hyperdiploid or the hypotetraploid responses.

Since higher ploidies also can be produced without drugs using the technique of somatic cell hybridization, this method was used to generate higher ploidy cells. B150 TK⁻ hyperdiploid cells have a broader shoulder and steeper final slope on their survival curve than the V79 HGPRT⁻ hyperdiploid cells (Figure 12). Following fusion the resulting hypotetraploid cell has a shoulder similar to the B150 TK⁻ hyperdiploid's and a final slope between the two hyperdiploid lines. A non-linear least squares computer fit of the data to a single hit-multitarget model gives: $N=20.8$, $D_0=157.2$ rads for fused hypotetraploids, $N=3.7$, $D_0=185.9$ rads for V79 HGPRT⁻ hyperdiploids, $N=19.5$, $D_0=142.8$ rads for B150 TK⁻ hyperdiploids. During these experiments the plating efficiencies of the controls averaged 74% for the fused cells, 67% for the HGPRT⁻ mutant, and 85% for the TK⁻ mutant. The population doubling times were 11.25 hours, 11.5 hours, and 11.75 hours for the HGPRT⁻, TK⁻ hyperdiploid and fused hypotetraploid respectively.

A second ploidy series using fused cells was made from B150 TK⁻ hypotetraploid cells and V79 HGPRT⁻ hyperdiploid cells. These were fused to make a hypertetraploid line with a doubling time of 16 hours and an average control plating efficiency of 82%. The X-ray dose-response curves for these cells are given in Figure 13. The shoulder for the hypertetraploids is greater than for the two precursor lines. The final slope of the hypertetraploids is steeper than the final

Figure 11: X-ray survival curves for B150 hyperdiploid and hypohexaploid cells irradiated at 37°C. The error bars represent standard errors of the mean from four or five experiments. The dashed line represents the survival of B150 hypotetraploid cells.

Figure: 11

XBL766-5577

Figure 12: X-ray survival curves for V79 HGPRT⁻ hyperdiploid cells and hypotetraploid cells resulting from their fusion with B150 TK⁻ hyperdiploid cells. The dashed curve represents the survival of B150 TK⁻ hyperdiploid cells. The error bars represent standard errors of the mean from four or five experiments.

Figure: 12

XBL766-5575

slope of the HGPRT⁻ hyperdiploids, but not as steep as for the B150 TK⁻ hypotetraploids. A non-linear least squares computer fit of the data to a single hit-multitarget model gives: fused hyper-tetraploid N=26.1, D₀=145.6 rads, V79 HGPRT⁻ hyperdiploid N=3.7, D₀=185.9 rads, B150 TK⁻ hypotetraploid N=47.3, D₀=114.28.

A consequence of the broad shoulders on hypotetraploid survival curves is that relative differences in their survival compared with diploids are not markedly different until doses of 1200 rads and above. Experiments were designed to maximize the effects of ploidy on radiosensitivity. If the shoulders of the survival curves could be removed, the differences in the final slopes of the curves would be apparent immediately. Increasing amounts of IUDR incorporated into cellular DNA is known to remove the shoulder of the resulting survival curve (Szybalski, 1974, Elkind and Whitmore, 1967). IUDR was incorporated into V79 hyperdiploid cells. Figure 14 graphically shows the toxicity of increasing amounts of IUDR present in the media of exponentially growing cells for 15 hours. No toxicity is noticed until the IUDR concentration surpasses 1×10^{-6} molar. At higher concentrations the viability of the V79 hyperdiploid cells decreases more rapidly than the viability of the hypotetraploid cells. IUDR in cell media blocks the de novo synthesis of DNA precursors (Cohen et. al., 1958) so incorporation of IUDR from the precursor pool is increased. Cells were grown for 15 or 16 hours in media containing different amounts of IUDR and FUDR. ¹²⁵IUDR was added such that the same proportion of the total IUDR was ¹²⁵IUDR.

Figure 13: X-ray survival curves of B150 TK⁻ hypotetraploid cells and hypertetraploids resulting from their fusion with V79 HGPRT⁻ hyperdiploid cells. The dashed line gives the dose-response curve of V79 HGPRT⁻ cells. The error bars represent standard errors of the mean from four experiments.

Figure: 13

XBL766-5573

Figure 14: Survival of V79 hyperdiploid and hypotetraploid cells as a function of concentration of cold IUdR in their growth media. Cells were grown for 15 hours in media containing the specified IUdR concentration before being assayed for reproductive viability.

- V79 hyperdiploid
- + V79 hypotetraploid

Figure : 14

XBL767-9093

$^{125}\text{IUdR}$ incorporated within the cell is proportional to the amount of IUdR incorporated. Table 2 gives the results for IUdR incorporation under conditions used in the X-ray studies. The incorporation of IUdR with FUdR present is twice the value for incorporation without it. With 1×10^{-6} M IUdR in the media the hypotetraploid cells incorporated approximately 1.7 times as much IUdR as the hyperdiploid cells. This is as expected if the hypotetraploids have about 1.7 times the DNA content of the hyperdiploids. With FUdR in the media the hypotetraploids incorporate proportionately more IUdR than the hyperdiploids. This explains the differential toxicity seen in Figure 14 for concentrations of IUdR in excess of 1×10^{-6} moles/liter.

Survival curves for V79 hyperdiploid and hypotetraploid cells with increasing amounts of unifilarly incorporated IUdR are given in Figures 15, 16, and 17. The control plating efficiency for the hyperdiploid cells dropped from 97% following growth in media with 1×10^{-6} M IUdR, to 48% for growth in similar media with 1×10^{-7} M FUdR, and to 38% following growth in media with 2.5×10^{-6} M IUdR and 1×10^{-7} M FUdR. Control plating efficiencies for hypotetraploid cells following growth under similar conditions were 92%, 78% and 55% respectively. These values are all consistent with the toxicity results seen in Figure 14. Computer generated survival curve parameters for survival following growth in media 1×10^{-6} M IUdR (Figure 15) are $N=4.4$, $D_0=115.2$ rads for the hyperdiploid, $N=7.44$, $D_0=98$ rads for the hypotetraploid cells. Following growth in media 1×10^{-6} M IUdR and 1×10^{-7} M FUdR (Figure 16) the computer fitted

Table 2: Uptake of IUdR

Amount of IUdR & FUDR in media	Counts/min. cell V79 hyperdiploid	Counts/ min. cell V79 hypotetraploid
0	0	0
1×10^{-6} M IUdR	0.0436	0.0736
1×10^{-6} M IUdR + 1×10^{-7} M FUDR	0.0827	0.1508
2.5×10^{-6} M IUdR + 1×10^{-7} M FUDR	0.0773	0.1492

parameters are $N=1.5$, $D_0=103.8$ for the hyperdiploid cells, and $N=2.5$, $D_0=98.7$ rads for the hypotetraploids. Computer fitted parameters for survival following growth in media 2.5×10^{-6} M IUdR and 1×10^{-7} M FUdR (Figure 17) are: $N=1.0$, $D_0=117.6$ rads for the hyperdiploids, and $N=1.45$, $D_0=108$ rads for the hypotetraploids. As the amount of IUdR incorporated increased the shoulder decreased as has previously been reported (Szybalski, 1974). After 16 hours of growth in media with 2.5×10^{-6} M IUdR and 1×10^{-7} M FUdR the V79 hyperdiploid cells have an exponential survival curve with no shoulder. The average amount of IUdR incorporated following growth in media 2.5×10^{-6} M IUdR and 10^{-7} M FUdR relative to growth in similar media with 1.0×10^{-6} M IUdR and 10^{-7} M FUdR (Table 2) decreases. In the survival curves this accounts for the decrease in the final slopes of cells grown in media 2.5×10^{-6} M IUdR and 10^{-7} M FUdR (Figure 17) compared with the cells grown in media 1×10^{-6} M IUdR and 10^{-7} M FUdR (Figure 16).

As developed in the introduction, Szybalski proposes that the sensitization caused by incorporated IUdR involves indirect action. In the frozen state indirect action is almost eliminated (Dertinger and Jung, 1969). Studying the response of cells with IUdR when frozen provides a procedure in which to test the proposed mechanism. The response to X-rays of V79 hyperdiploid and hypotetraploid cells while frozen in 10% DMSO was determined. These studies were conducted on cells grown normally and on cells whose DNA was unifilarly labelled with IUdR. The dose response curves for V79 hyperdiploid and hypotetraploid cells irradiated while frozen at -196°C in media

Figure 15: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells with IUdR incorporated unifilarly in cell DNA. The cells were grown for 15 hours in media containing 1×10^{-6} molar IUdR prior to irradiation at 37°C. The error bars represent the standard error of the mean from three experiments. The dashed line gives the hyperdiploid response.

Figure: 15

XBL766-5572

Figure 16: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells with increasing amounts of IudR incorporated unifilarly. Prior to irradiation at 37°C these cells were grown for 16 hours in media containing 1×10^{-6} M IudR and 10^{-7} M FudR. The error bars represent the standard error of the mean from three or four experiments. The dashed line gives the hyperdiploid response.

Figure: 16

XBL766-5578

Figure 17: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells with the highest unifilarly substitution of IUdR. Prior to irradiation at 37°C these cells were grown for 16 hours in media containing 2.5×10^{-6} M IUdR and 1×10^{-7} M FUdR. The error bars represent the standard error of the mean from three or four experiments.

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Figure: 17

XBL766-5583

with 10% DMSO is given in Figure 18. Computer fitted survival curve parameters for these curves are: $N=8.17$, $D_0=541$ for hyperdiploid, $N=7.1$, $D_0=584$ for the hypotetraploid. Figure 19 presents the results for similar cells which were grown for 15 hours in media with 1×10^{-6} M IUdR prior to freezing. Computer fitted survival curve parameters for these curves are: $N=10.4$, $D_0=363.6$ for the hyperdiploid, $N=9.16$, $D_0=425.53$ for the hypotetraploid. A result of having no indirect effect in the frozen state is the observed increase in D_0 to around 500 rads from between 130 and 200 rads for cells at 37°C.

The results of Rommelaere and Errera (1972) suggested that if repair could be reduced, the differences between the dose-response curves of different ploidy cells could be enhanced. Winans and Dewey (1972) showed that repair of potentially lethal damage was inhibited when cells are in hypertonic media. Using protocols similar to the Winan's study the media was made hypertonic by adding 5% myoinsitol. The change in cell size as V79 hyperdiploids and hypotetraploids are placed in this media is presented in Figure 20. The reduction in cell size occurs within the first three minutes and the cells do not start to increase noticeably in size for at least the first one and a half hours. Within this one and a half hour period all experimental manipulations were completed. The method of cell sizing described in the materials and methods section was used to determine cell size under these conditions. The volume of V79 hyperdiploid cells in hypertonic media is $.62 \pm .08$ of their volume in

Figure 18: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells irradiated while frozen at -196°C in media containing 10% DMSO. The error bars on each curve represent standard error of the mean from seven or eight experiments.

Figure: 18

XBL766-5576

Figure 19: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells containing IUdR unifilarly in their DNA. The cells were irradiated while frozen at -196°C in media containing 10% DMSO. Before freezing the cells were grown for 15 hours in media containing 1×10^{-6} M IUdR. The error bars represent standard errors of the mean for two experiments. The dashed curve gives the hyperdiploid response.

Figure: 19

XBL767-9095

normal media. In hypertonic media V79 hypotetraploid cells are reduced in size by a factor of $.56 \pm .02$. B150 hyperdiploid and hypotetraploid cells are reduced to .7 of their normal size. X-ray survival curves for V79 hyperdiploid and hypotetraploid cells irradiated while suspended in hypertonic media are given in Figure 21. The average control plating efficiencies for the hyperdiploids was 100% and for the hypotetraploids was 77%. Similar curves for B150 hyperdiploid and hypotetraploid cells with control plating efficiencies between 80 and 100 percent are given in Figure 22. Computer generated survival curve parameters for these curves are: $N=6.7$, $D_0=152.7$ rads for V79 hyperdiploids, $N=189$, $D_0=103.7$ for V79 hypotetraploids, $N=10.6$, $D_0=109.2$ rads for the B150 hyperdiploid, and $N=29.5$, $D_0=86.96$ rads for the B150 hypotetraploid. Under these hypertonic conditions the proportional change of the D_0 values from their value in normal media is very similar for each cell type: D_0 normal conditions/ D_0 hypertonic = 1.29 for V79 hyperdiploid, 1.32 for V79 hypotetraploid, 1.31 for B150 hyperdiploid, 1.31 for B150 hypotetraploid.

Survival of V79 hyperdiploid and hypotetraploid cells was studied under a second set of conditions minimizing repair of potentially lethal damage. Koch and Burki (In Press) found that prolonged storage at 4°C "potentiates" potentially lethal damage. The results of experiments in which V79 hyperdiploid and hypotetraploid cells were irradiated and stored for four days at 4°C are given in Figure 23. During these experiments the control plating efficiency of the hyperdiploid cells averaged 41%. The average control plating efficiency

Figure 20: Three graphs showing the change of cell size in hypertonic media:

- A. This is a representative curve of the cell size distribution obtained using a model F Coulter Counter. The data shown was obtained from V79 hyperdiploid cells immediately following their suspension in hypertonic media.
- B. The change in size of V79 hyperdiploid cells with time in hypertonic media.
- C. The change in size of V79 hypotetraploid cells with time in hypertonic media.

-81-

Figure: 20

XBL767-9094

Figure 21: X-ray survival curves for V79 hyperdiploid and hypotetraploid cells in hypertonic media at 37°C. The error bars represent the standard error of the mean for data from three or four experiments. The dashed line gives the V79 hyperdiploid response.

Figure: 21

XBL766-5581

Figure 22: X-ray survival curves for B150 hyperdiploid and hypotetraploid cells in hypertonic media at 37°C. The dashed line shows the survival of the hyperdiploid cells. The error bars represent the standard error of the mean for results from two independent experiments.

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Figure: 22

XBL766-5580

for the hypotetraploids was 38%. Computer fitted survival curve parameters are $N=5.6$, $D_0=138.9$ rads for the hyperdiploid and $N=2.2$, $D_0=162.5$ for the hypotetraploid. Chinese hamster cells irradiated under these conditions and while frozen are the only times when it was observed that the final slope of Chinese hamster hypotetraploids was less than the final slope for Chinese hamster hyperdiploids. Koch and Burki (In Press) found for all the cell types they tested prolonged storage at 4°C led to sensitization. The hyperdiploid cells in this study followed the same pattern with the D_0 falling from above 200 rads to around 140 rads. A similar decrease was not observed for the hypotetraploid cells. The measured D_0 following the 4°C protocol was 162 rads compared to 137 rads observed under normal conditions for the hypotetraploids.

Studies With ^3H and ^{125}I in Chinese Hamster Cells

The likelihood that a single decay of $^{125}\text{IUdR}$ could result in dominant lethal damage was investigated using Chinese hamster V79 hyperdiploid and hypotetraploid cells. Cells were grown for one doubling time, 15 hours, in media containing $^{125}\text{IUdR}$ and 10^{-6} M cold IUdR. Under these conditions the specific activity of the labelling isotope is very low. Figure 24a shows the incorporation of $^{125}\text{IUdR}$ under the experimental conditions as a function of the amount of radioactive isotope added to the labelling media. Over the range tested the response is linear. Hypotetraploid cells incorporate 1.56 times as much $^{125}\text{IUdR}$ from similar growth conditions as the hyperdiploid cells. This is consistent with the combined effects

Figure 23: X-ray survival curves for cells irradiated at 4°C.

Following irradiation, the cells were stored for four days at 4°C before being placed in a 37°C incubator. The error bars represent the standard error of the mean for three and four independent experiments. The dashed line gives the dose response of the V79 hyperdiploid cells.

Figure: 23

XBL766-5574

of the hypotetraploids having more DNA and a similar doubling time as the V79 hyperdiploid cells (see Table 1). Survival curves for V79 hyperdiploid and hypotetraploid cells plotted on the basis of an equal number of decays are given in Figure 25. A non-linear least squares computer fit of the data to a single hit-multitarget model gives $N=2.9$, $D_0=50.8$ decays for the hyperdiploid cells and $N=2.1$, $D_0=102.4$ decays for the hypotetraploids. The extrapolation numbers are about the same and the slope of the diploids on a decay basis is a little over twice as large. The conversion between the number of decays and dose in rads is given in Appendix 1. Plotting the survival curves given in Figure 25 on an equal dose basis the curves in Figure 26 are obtained. Computer derived survival curve parameters for these curves are: $N=2.9$, $D_0=64.47$ rads for the hyperdiploid, $N=2.1$, $D_0=76.7$ rads for the hypotetraploid.

Tritium decays are thought to interact with the cell similarly to X-rays (Burki et.al., 1973 b). Cells were labelled unifilarly through 15 hours of growth in media containing tritiated thymidine, 10^{-4} molar thymidine and 10^{-5} molar deoxycytidine. The deoxycytidine is added to overcome any block in DNA synthesis resulting from the excess thymidine. Figure 24b shows the incorporation of $^3\text{HTdR}$ under the experimental conditions as a function of the amount of radioactive isotope added to the labelling media. Over the range tested the response is linear. Hypotetraploid cells incorporate 1.71 times as much $^{125}\text{IUdR}$ from similar growth conditions as the hyperdiploid cells. Survival curves for V79 hyperdiploid and hypotetraploid cells plotted

Figure 24:

A. Incorporation of $^{125}\text{IUdR}$ in cellular DNA as a function of $^{125}\text{IUdR}$ in the labelling media. Cells were grown for 15 hours in media 1×10^{-6} M IUdR with varying amounts of $^{125}\text{IUdR}$. V79 hypotetraploids (•) have a slope 1.56 times as steep as the V79 hyperdiploids (+).

B. Incorporation of $^3\text{HTdR}$ in cellular DNA as a function of $^3\text{HTdR}$ in the labelling media. Cells were grown for 15 hours in media 1×10^{-4} M TdR and 1×10^{-5} M CdR with varying amounts of $^3\text{HTdR}$. V79 hypotetraploids (•) have a slope 1.71 times as steep as the V79 diploid (+).

-91-

Figure: 24

XBL767-9092

Figure 25: Survival of V79 hyperdiploid and hypotetraploid cells following decay of incorporated $^{125}\text{IUdR}$. The cells were labelled during 15 hours of growth in media containing 1×10^{-6} M IUdR and .002-.006 micro-curies $^{125}\text{IUdR/ml}$.. Survival was determined for decays occurring while the cells were frozen in media containing 10% DMSO at -196°C . The error bars for each point represent the standard deviation from four replicate plates.

- ° --- V79 Hypotetraploid
- — V79 Hyperdiploid

Figure: 25

XBL766-5569

Figure 26: Survival of V79 hyperdiploid and hypotetraploid cells following decay of incorporated $^{125}\text{IUdR}$ compared on the basis of equal dose. The dashed line represents the dose-response curve for hypotetraploids. The solid line is the response of V79 hyperdiploids. The experimental data appears in Figure 25.

Figure: 26

XBL766-5570

on the basis of an equal number of decays are shown in Figure 27. Computer fitted survival curve parameters for the data are: $N=1.6$, $D_0=1544.4$ decays for the hyperdiploid, $N=2.85$, $D_0=2711$ decays for the hypotetraploids. The conversion between the number of tritium decays and the dose in rads is given in Appendix 1. The curves in Figure 28 are obtained by converting to an equal dose basis the data in Figure 27. Computer derived survival curve parameters for the dose-response curves are: $N=1.6$, $D_0=725.9$ rads for hyperdiploid cells, $N=2.85$, $D_0=755.7$ rads for the hypotetraploids.

X-Ray Survival Curves for Mouse Cells

It is of interest to know whether the relative dose response relationship between mammalian cells of different ploidy remains constant for different species. This was tested by studying the X-ray responses of mouse L5178Y diploid and colcemid induced hypotetraploid cells. Both cell types had an average control plating efficiency of 55% and a population doubling time of nine hours. The results for X-ray survival experiments with these cells are presented in Figure 29. As with the Chinese hamster results, the hypotetraploid cells have a larger shoulder and extrapolation number than the diploids. However, unlike the hamster 37°C results, the final slope of the L5178Y hypotetraploid survival curve is less than or equal to the slope of the diploid L5178Y curve. Computer generated survival parameters for these curves are: $N=2.6$, $D_0=83.33$ rads for the diploids and $N=5.65$, $D_0=88.73$ rads for the hypotetraploid cells. The diploid response is very similar to values published by Shinohara and Okada (1972).

Figure 27: Survival of V79 hyperdiploid and hypotetraploid cells following decay of incorporated $^3\text{HTdR}$. The cells were labelled during 15 hours of growth in media containing 2.0-5.0 micro-curies/ml. of tritiated thymidine, 10^{-4} molar cold thymidine, and 10^{-5} molar deoxycytidine. Survival was determined for decays occurring while the cells were frozen in media containing 10% DMSO at -196°C . The error bars for each point represent the standard deviation from four replicate plates.

- — V79 Hyperdiploid
- ° --- V79 Hypotetraploid

XBL766-5568

Figure: 27

Figure 28: Survival of V79 hyperdiploid and hypotetraploid cells following decay of incorporated $^3\text{HTdR}$ compared on the basis of equal dose. The dashed line represents the response of the hypotetraploid cells. The solid line is the response of the V79 hyperdiploids. The experimental data appears in Figure 27.

Figure: 28

XBL766-5571

Figure 29: X-ray survival curves of L5178Y diploid and hypotetraploid cells irradiated at 37°C. The error bars each represent standard errors of the mean for three experiments. The dashed line gives the survival of the L5178Y diploid.

Figure: 29

XBL766-5584

Repair Capacity of Chinese Hamster Cells

The ability of V79 hyperdiploid and hypotetraploid cells to repair potentially lethal damage was determined using the method described in the materials and methods. Results averaged from five experiments are given in Figure 30. Survival of V79 hyperdiploids reaches a maximum of 1.77 times the survival at 37°C after twenty minutes at 20°C. The V79 hypotetraploids recover within 10 minutes at 20°C to a survival equal to 1.48 times their survival at 37°C. After one hour at 20°C the V79 hypotetraploid recovery curve goes through a maximum at 1.54. Figure 30c shows the relative ability of the two cell types to recover from potentially lethal damage. Within the first hour there is no clear trend. After one hour, however, the hyperdiploids have, relative to the hypotetraploids, an enhanced ability to recover from potentially lethal damage. The observed behavior could be due to a decrease in the ability of the hypotetraploid cells to survive the storage at 20°C. A determination of the percent survival of control cultures held at 20°C for varying periods of time showed that this was not occurring. The plating efficiency of the hypotetraploids increased from an average of 75.7% with zero time at 20°C to 82.5% after four hours.

Repair of sub-lethal damage was determined as described in the materials and methods. In a split dose experiment the hypotetraploids show a higher amount of repair of sublethal damage than hyperdiploid cells, Figure 31. The hyperdiploid cells have a maximum survival enhancement of 1.54 following a separation of the X-ray

Figure 30: Recovery from potentially lethal damage. Cell survival for V79 hyperdiploid and hypotetraploid cells held at 20°C for varying times following exposure to a fixed X-ray dose. A. and B. values on the vertical axis represent the survival following storage at 20°C divided by the survival of similar cells which are irradiated and kept at 37°C.

- A. Recovery from potentially lethal damage by V79 hyperdiploid cells.
- B. Recovery from potentially lethal damage by V79 hypotetraploid cells.
- C. The ordinate is the ability of V79 hyperdiploid cells to recover from potentially lethal damage relative to the hypotetraploid cells for various periods of time at 20°C. The value at which the magnitude of repair is the same is represented as a dashed line.

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XBL767-9096

Figure: 30

exposures by one hour. The maximum enhancement in survival of the hypotetraploids occurs with a separation of three hours and is equivalent to 2.36. These results are in agreement with values published by Bedford and Hall (1967) and Little (1972). Wan studied Elkind-Sutton repair as a function of ploidy. He used these same cells and observed similar results (Wan, 1975).

Figure 31: Repair of Elkind-Sutton Damage

- A. Survival of V79 hyperdiploid cells following a dose given in two parts separated by different periods of time relative to survival from an equal dose given all at once.
- B. Survival of V79 hypotetraploid cells following a dose given in two parts separated by different periods of time relative to survival from an equal dose given all at once.
- C. Survival of V79 hyperdiploid cells following a split dose exposure relative to survival of V79 hypotetraploid cells treated in a similar manner. The value at which the magnitude of repair is the same is represented by a dotted line.

Figure: 31

XBL767-9097

DISCUSSION

The Influence of Ploidy

As developed in the introduction, ploidy has been used in many biological systems as a tool to study the action of ionizing radiation on living cells. For mammalian cells the published results and the findings of this study show no simple trend between radiosensitivity and a cell's ploidy level. The results reported here are consistent with the classical understanding that genetic redundancy reduces the impact of deletions on higher ploidy cells. Similarly, this work supports the classical idea that for cells which differ in chromosome number only, the probability of a "two break" chromosome aberration varies directly with the chromosome number.

Chromosome deletions can result from a single interaction of ionizing with a chromosome. As a result, at low doses of X-rays the production of deletions predominates over the production of two-break chromosome aberrations which require two interactions. The probability of a two-break chromosome aberration increases as a function of the dose squared. At high doses formation of these aberrations is favored over deletions, the probability of which is linearly related to dose. It is expected that with increasing ploidy the fraction of cells killed by deletions would decrease and the fraction killed by two break chromosome aberrations increase. The changes in the survival curve parameters observed in our studies with Chinese hamster cells at 37°C (Figure 32) are consistent with these expectations. As the ploidy increases the low dose shoulder

increases due to the reduced impact of deletions and the final slope becomes steeper, the cells more sensitive, due to the increased likelihood at higher doses of formation of two break chromosome aberrations. Both the increase in shoulder and final slope leads to increases in the extrapolation number as seen in Figures 32C and 32D.

During these studies, ploidy series were made from combinations of two different Chinese hamster cell lines. It was found that the magnitude of the change in survival characteristics resulting from a ploidy shift depended on the origin of the cells used. The relative rate at which the survival curve parameters changed for the different Chinese hamster cell types is presented in Figures 32B and 32D.

V79 cells originating from Chinese hamster lung material gave the greatest change in survival curve parameters with ploidy, B150 cells from the Chinese hamster peritoneal cavity gave less of a change with ploidy, and cells of higher ploidy derived from fusion of these two cell types exhibit the smallest degree of change.

One of the reasons for testing the radiosensitivity of the fused Chinese hamster cells was to check the response of an intraspecific hybrid with the increased resistance observed by Little et. al. (1972), for an interspecific hybrid. Our results are similar to those of Limbosch et. al. (1974), who found in an intraspecific hybrid no change in the radiosensitivity of the fused cell.

The impact of ploidy on radiation induced reproductive death varies with the species from which the cells originate. The L5178Y mouse survival curve presented in Figure 29 is similar to the Chinese

Figure 32: Computer fitted values for cell sensitivity and extrapolation number for Chinese hamster cells of different ploidy. Cells originating from B150 hyperdiploid cells are designated by "B", cells originating from V79 are represented by "V", B150 $\times$ V79 hypotetraploid cells are denoted by an "F", and the fused hypertetraploid values are marked by an "H". Figure 32A shows the change in sensitivity with chromosome number. In 32B the ratio of the sensitivity of a given cell type divided by the sensitivity of the diploid from which it originated is plotted against the chromosome number. The "diploid" value for the two fused lines is taken as the average of the B150 and V79 HGPRT⁻ hyperdiploid values. Figure 32C presents the change in extrapolation number with chromosome number. Figure 32D is a plot of the extrapolation number divided by the extrapolation number of the diploid precursor. Again, "F" and "H" are plotted relative to the average of the values for the B150 and V79 HGPRT⁻ hyperdiploid cells.

Figure: 32

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hamster response in that the extrapolation number increases with higher ploidy. However, the final slope of the hypotetraploid does not show an increase in sensitivity. A possible explanation is that for the doses used the fraction of these cells killed by two break chromosome aberrations is very small. This same species dependency is evident in previously published results. Bedford and Hall (1967) and Burki and Carrano (1973) working with Chinese hamster cells published results similar to our Chinese hamster results. Work by Berry (1963) on mouse cells agrees with our L5178Y data.

Ploidy and Chromosome Volume

As the cell size decreases and chromosome number increases the sensitivity of the cell to radiation increases (Figure 33). If the nuclear volume of all the cells changed proportionately with cell size as it does for the V79 hyperdiploid-hypotetraploid cells, then cell volume per chromosome would be proportional to volume per chromosome or inversely proportional to the number of chromosomes per volume. The increase in cell sensitivity with increasing chromosomes per volume strongly suggests that at 37°C chromosome interactions are involved in the mechanism of cell killing. Consistent with this interpretation are the findings of Dettor et. al. (1972) which showed a correlation between the condensation of chromatin and radiosensitivity. A similar finding was found in an older study by Sax (1943) who observed that when *Tradescantia* was centrifuged following irradiation, to compact the chromosomes, the number of aberrations increased.

In addition to the increase in D_0 with increasing chromosome volume, Figure 33 shows that, for a given chromosome volume, cells of different ploidy levels have different radiosensitivities. All the hypotetraploid Chinese hamster cells represented in Figure 33 are more sensitive (have smaller D_0) than the hyperdiploid cells of similar volume per chromosome. It is of interest that the hypotetraploid cells seem to fall along a line of similar slope to the one through the hyperdiploid cell values. This suggests that for a given ploidy level the relation between sensitivity and chromosome volume is similar. The increase in radiosensitivity with ploidy at a given chromosome volume is further evidence that chromosome interactions are involved in a mechanism of cell killing.

The increase in D_0 with cell volume per chromosome is the "opposite" of what is expected from the work of Sparrow (1967). Sparrow argues that the chromosome volume is directly proportional to cell sensitivity. Sparrow's result is what would be expected if cells were killed by deletions. A possible explanation for the difference between Sparrow's results and those in this study and of Mortimer (1958) is the increased importance in the latter two studies of two break chromosome aberrations as a mode of cell death. Deletions could be of greater importance in the Sparrow study due to properties specific to the plant cells such as the cell wall (discussed in the introduction) or because of the low dose rate of the chronic exposure used by Sparrow (1958).

Figure 33: Cell sensitivity as a function of ploidy and

"compactness" of chromosomes. The abscissa is a relative measure of the cell volume divided by the chromosome number.

- cells near diploid
- ▲ cells near tetraploid
- + hypertetraploid and hypohexaploid

The origin of the cell type is also listed. The data presented in this graph was obtained for cells at 37°C in normal and hypertonic media.

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Figure: 33

The correlation between number of chromosomes per volume and radiosensitivity suggests the importance of chromatin interaction in cell killing. An alternate explanation would be that the more closely packed chromosomes decrease the accessibility of damaged regions to repair enzymes. Such an explanation would not, however, account for the increase in sensitivity with increasing ploidy, whereas the chromatin interaction hypothesis would explain both.

Freezing Effects

The radiosensitivity of V79 hypotetraploid cells is less than V79 hyperdiploid cells when irradiated while frozen (Figures 18 and 19). This differs from the increase in sensitivity seen with ploidy at 37°C (Figure 32). Possible reasons for the change in the relation between ploidy and radiosensitivity is the dehydration which occurs during freezing (Mazur, 1970), the elimination of indirect effects, or of a reduction in chromatin movement which occurs as a result of freezing. For irradiation of these cell types under hypertonic conditions (Figures 21 and 22), the hypotetraploid cells are more sensitive than the hyperdiploids. This is the same relation between ploidy and sensitivity seen in normal media at 37°C (Figures 10 and 32). Assuming the hypertonic conditions are analogous to what occurs during dehydration, the change in the relation between ploidy and radiosensitivity in the frozen state is not a result of the dehydration.

In the frozen state the indirect effects of radiation are minimized. The ratio of the D_0 for V79 cells irradiated frozen and at 37°C is $541/209=2.59$ for hyperdiploid cells and $584/137=4.25$ for the

hypotetraploid cells. This would indicate that the role played by indirect action under normal conditions is greater for the hypotetraploids. In this regard the results of Silini and Hornsey (1962) showing an enhanced protection of tetraploid cells under anoxic conditions relative to the hypodiploid cells under similar conditions could be related to a similar effect.

When cells were irradiated and stored for four days at 4°C (Figure 23) the hypotetraploids were less sensitive than the hyperdiploids. Relative to the relationship between the cell types at 37°C, this is the same type of shift seen in the frozen state. The results at 4°C, conditions in which indirect effects are present, argues that the increased resistance of the hypotetraploid relative to hyperdiploid cells does not result from an increased importance of indirect action in the hypotetraploid cells.

Sax (1939) in a study of chromosome structural changes in *Tradescantia* as a function of temperature found that at lower temperature the amount of structural changes was greater than at higher temperatures. If the temperature was raised for a short period of time the number of chromosome aberrations was reduced. Koch and Burki (In Press) studied cell lethality as a function of time at 4°C and observed a similar result. If the cells were warmed to 37°C and then re-cooled, no further enhancement of lethality occurred. These findings are consistent with the view that reduced temperature and the frozen state affects the rejoining of broken chromatin. Reduced recombination would be expected to result in a

reduction in the formation of two-break chromosome aberrations and an increase in cell killing due to deletions. Such a shift would result in the hypotetraploids being more resistant than the hyperdiploids as seen at both 4°C and while frozen.

Split Dose Recovery and Repair of Potentially Lethal Damage

Following storage for more than an hour at 20°C fewer of the hypotetraploid cells are able to recover from potentially lethal damage than hyperdiploid cells (Figure 30). This is consistent with the proposal that the steeper final slope of the hypotetraploid cells is caused by an increase in the amount of damage, relative to the hyperdiploid, that is comparable to dominant lethal damage. It suggests that a number of the two hit chromosome aberrations are not repairable once they are formed.

In order to reduce damage to normal cells, radiotherapy protocols for treatment of cancer use highly fractionated doses. However many tumors have large numbers of polyploid cells which makes the amount of split dose recovery of higher ploidy cells clinically very important. During the time between exposures of a split dose experiment, the hypotetraploid cells recover more completely than the hyperdiploid cells (Figure 31). A similar observation was published by Bedford and Hall (1967). The increased shoulder of the V79 hypotetraploid cells at 37°C could be due both to increased repair and to decreased susceptibility to deletions. Clinically the enhanced split dose recovery of the higher ploidy cells reduces the desirability of a split dose procedure since it would result in a larger degree of protection to the tumor cells than for the normal cells.

As discussed in the introduction, Rommelaere and Errera (1972) found that tetraploid cells had a larger decrease in survival when irradiated in the presence of caffeine than the diploid cells. This could be due to the increased relative importance of repair in the tetraploid cells.

Effect of IUdR

As the amount of incorporated IUdR increased the radiosensitivities of the V79 diploid and hypotetraploid cells become more similar. With increasing amounts of incorporated IUdR, both the hyperdiploid and hypotetraploid V79 cells approach a highly sensitized state with a D_0 value between 100 and 110 rads (Figure 34, $D_0=1/\text{sensitivity}$). The extrapolation numbers for both cell types approach 1.0 as the amount of IUdR in the cell increases (Figures 34C and 34D). Instead of increasing the difference between the radiosensitivities of cells of different ploidy levels, IUdR in the DNA reduces it. The two cell types progress to a single highly sensitized state. For the V79 hyperdiploid this involves a larger decrease in final slope, and less of a decrease in extrapolation number than the hypotetraploid. This suggests that the cells of different ploidy levels are approaching a state in which they are both killed by the same mechanism.

As previously mentioned, in the frozen state the indirect effects of radiation are minimized. The ratio of the D_0 for V79 cells irradiated frozen without IUdR to those irradiated frozen with IUdR is $541/364 = 1.49$ for the hyperdiploid cells and $584/426 = 1.37$ for the hypotetraploid cells. Similar ratios for these cells irradiated at 37°C

Figure 34: Changes in survival curve parameters for V79 hyperdiploid and hypotetraploid cells containing different amounts of IUdR. The abscissa of each graph is the relative amount of IUdR incorporated for 15 hours of growth in media 1×10^{-6} M IUdR. Figures A and B give the change in sensitivity with changes in the amount of unifilar incorporation of IUdR for V79 hyperdiploid and hypotetraploid cells. Figures C and D show the relation between extrapolation number for V79 hyperdiploid and hypertetraploid cells and amount of incorporated IUdR.

Figure: 34

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with and without IUdR are $209/115 = 1.82$ and $137/98 = 1.4$ for the hyperdiploid and hypotetraploid respectively. The sensitization of the hyperdiploid cells is less for cells irradiated frozen than for cells irradiated at 37°C . This supports Szybalski's proposal (1974) that indirect effects do play a role in the sensitization by IUdR. However, for the hyperdiploid cells one half of the sensitization observed at 37°C remains in the frozen state where there are no indirect effects. As a consequence there must be a second mechanism that is dependent on direct action which also leads to sensitization of cells by IUdR. The amount of sensitization by IUdR of the hypotetraploid cells at 37°C is only slightly more than with frozen cells. This indicates that in the hypotetraploid cells at 37°C little of the sensitization is due to increased susceptibility to indirect action. The values for sensitization of frozen hyperdiploid and hypotetraploid cells and for the sensitization of the hypotetraploid line at 37°C are very similar: 1.49, 1.37, and 1.4 respectively. If the average of these is taken, the amount of sensitization due to the direct mechanism would be around $1.42 \pm .08$ for this level of incorporation.

Survival Following $^{125}\text{IUdR}$ and $^3\text{HTdR}$ Decays

The high killing efficiency of decays from $^{125}\text{IUdR}$ incorporated within cells was observed in our hyperdiploid and hypotetraploid lines. D_0 values for cells with incorporated IUdR irradiated with X-rays while frozen are 363 and 425 rads for V79 hyperdiploid and hypotetraploid cells respectively. The comparative values for cells

irradiated by $^{125}\text{IUdR}$ are 64 rads (50 decays) and 75 rads (102 decays) (see Appendix 1 for dose calculations). The ratio of D_0 for X-ray to D_0 for $^{125}\text{IUdR}$ is 5.7 for both the hyperdiploid and hypotetraploid results. This value is a measure of the degree to which cell killing is enhanced following decays of $^{125}\text{IUdR}$ compared with 145 kv X-rays. The enhancement ratios for the hypotetraploids and hyperdiploids were calculated independently but agree. This serves as some verification of the dose estimates calculated in Appendix 1. However, with the evidence of the large amount of local damage caused by the Auger Effect which results from the decay of ^{125}I (Hofer and Hughes, 1971; Burki, 1974) the similarities of the enhancement ratios may be simply fortuitous.

The generalization that 39-46 disintegrations of $^{125}\text{IUdR}$ in cell DNA leads to a lethal lesion (Burki et. al., 1973) is not supported by this study. The D_0 for V79 hypotetraploid cells is 102 decays, twice the value for the hyperdiploid V79 cells.

The observed dose response curves for tritium decays in hyperdiploid and hypotetraploid cells supports the idea that the amount of damage from incorporated tritium decays is very similar to external X-rays. The ratios for the D_0 of cells irradiated by external X-rays and by decay of incorporated tritium are $541/726 = .75$ and $584/756 = .77$ for the hyperdiploid and hypotetraploid cells respectively. Again, this agreement to within 2.5% gives confidence in the calculated dose conversion.

On the basis of decays the hypotetraploids are more resistant than the hyperdiploid cells to both $^{125}\text{IUdR}$ and $^3\text{HTdR}$. As discussed above, when these are changed to a dose basis the relationship between the hyperdiploid and hypotetraploid curves becomes very comparable to what happens when exposed to X-rays under similar conditions. In each case the hypotetraploids are slightly more resistant to irradiation than the hyperdiploids. The "dose modification factor" resulting from a change in ploidy is the same for all three sources of ionizing radiation. This similarity suggests that the fraction of cells killed by deletions and two-break chromosome aberrations is the same following decay of incorporated $^3\text{HTdR}$, $^{125}\text{IUdR}$ and X-rays for cells in the frozen state.

Proposed Mechanism for Shifts in Cell Sensitivity with Ploidy

To provide a quantitative base for the ideas presented in the discussion, results from these studies were entered into a computer and fitted using a non-linear least squares routine to four different theoretical models. The results of the fit showed no single model which "best" described the data. Furthermore, the behavior of the models was very predictable; those curves with the largest shoulders had the highest target number, largest fraction of two hit phenomenon, or largest repair parameters depending on the theoretical constructs upon which the model was based. As a result, all models tested had inconsistencies and did not really help to develop new insights. However, the data in this thesis has been used to evaluate the changes and magnitudes of the various model parameters. The fitted parameters have been included as an appendix, Appendix 3.

The increase in radiosensitivity observed at 37°C for Chinese hamster cells with higher ploidies and with increasing chromosomes per volume is evidence that lethality results from interaction between chromosome lesions. Such interaction is necessary if a two-break chromosome aberration is to be formed. Hypotetraploid V79 cells were shown to have an enhanced capacity to repair sublethal damage. The extent that damaged areas are repaired before they can join with a second lesion decreases the formation of two-break chromosome aberrations. Relative to diploid cells, the decreased ability of the V79 hypotetraploid cells to recover from potentially lethal damage seems to indicate that once such a "two-break" lesion is formed, it is not repairable.

Increases in radiation resistance with increases in ploidy level is evidence that lethality also results from deletions. In response to ionizing radiation, higher ploidy L5178Y and frozen Chinese hamster cells are more resistant throughout their survival curves. For V79 cells at 37°C, increased resistance of the higher ploidy cells is observed only at low doses. It is expected that some form of recombination is necessary for formation of a two-break chromosome aberration. In the absence of any recombination, the number of deletions would be expected to be high. Lack of repair in the more radiation sensitive L5178Y cell line may lead to the observed increased involvement of deletions in L5178Y cell lethality.

The results of this study support the hypothesis that both deletions and "two-break" chromosome aberrations cause mammalian

cell reproductive death. At any given exposure the relative participation of these two modes of cell death changes with environmental factors, number of chromosomes per volume, capacity for repair, cell type, and ploidy level. The difficulty in isolating ploidy from the rest of these factors explains why there has been so much difficulty in establishing a clear relationship between ploidy and radiosensitivity in mammalian cells.

APPENDICES

Appendix 1: Dose Calculations

Dose to the nucleus assuming uniform random distribution of the DNA within the nucleus.

Rads/decay for ^{125}I = $18.9 \times 10^{-3} \text{ Mev/decay}^{\dagger} \times 1/\text{nuclear mass (grams)}$
 $\times 1.602 \times 10^{-6} \text{ erg/Mev} \times 10^{-2} \text{ rad/erg} \times \text{edge effect}^{\ddagger} = 1.27 \text{ rads/decay}$
for V79 hyperdiploids and .75 rads/decay for the hypotetraploid cells.

Rads/decay for ^3H = $5.7 \times 10^{-3} \text{ Mev/decay}^{\dagger} \times 1/\text{nuclear mass (grams)}$
 $\times 1.602 \times 10^{-6} \text{ erg/Mev} \times 10^{-2} \text{ rads/erg} \times \text{edge effect}^{\ddagger} = .47 \text{ rads/decay}$
for V79 hyperdiploid cells and .28 rads/decay for hypotetraploid cells.

For V79 hyperdiploid cells the nuclear mass is 1.57×10^{-10} grams (3.6 μ nuclear radius); the hypotetraploid is 1.7 times larger with a nuclear mass of 2.67×10^{-10} grams.

[†] The dose per decay comes from Ert1 (1970).

[‡] Compensation for the edge effect (loss of radiation from the periphery of the nucleus). For ^{125}I this is .66 in V79 hyperdiploid cells and .68 in V79 hypotetraploid cells. For ^3H this is .81 for V79 hyperdiploid cells and .83 for V79 hypotetraploid cells (Ert1, 1970).

Reference - the method used is the same as published by Burki et. al., 1973.

Appendix 2: Computer Fits

Computer fits of the experimental data to different survival curve models were made using the program LSQVMT from the Lawrence Berkeley Laboratory's Computer Library. This program uses a non-linear least squares procedure to minimize the distance between the experimentally determined survival values at a particular dose and the value predicted by the theoretical model. The method of minimization is that of W.C. Davidson (1959). To give equal weight to all points, the fit was made to the log of the survival. Had this not been done, low survival points would have had very little impact on the fit since the magnitude of the function at these points is very small. Documentation for this program is available through the LBL Computer Library.

Appendix 3: Survival Models

Results from these studies were entered into a computer and fitted using a non-linear least squares routine to four different theoretical models. A more specific description of the method is given in Appendix 2. The four models studied are ones proposed by Haynes (1975), Green and Burki (1974), single hit-multitarget, and Chadwick and Leenhouts (1973). The Haynes and Green-Burki models explain the sigmoidal shape of typical survival curves through mechanisms involving repair. The single hit-multitarget and Chadwick-Leenhouts models explain the sigmoidal shape on the basis of physical differences in the type of lesion leading to the lethal event.

i.) The Single Hit-Multitarget Model. The single hit-multitarget model describes the shape of the dose response curve on the basis of the probability of a lethal lesion occurring. It assumes that there is no repair, that energy is absorbed in discrete units called hits, that the probability of interactions within different areas or targets is independent of each other, and that the likelihood of an interaction occurring follows a Poisson distribution. Using these assumptions, the target theory model can be mathematically described by the formula:

$$\text{Survival} = 1 - \left(e^{-vD} \sum_{k=0}^{n-1} \frac{(vD)^k}{k!} \right)^m$$

in which m is the number of targets, n is the number of hits, D is the dose, and v is the target sensitivity (for a more comprehensive treatment see Zimmer, 1961). The simplification has been made that all the targets require the same number of hits and have the same sensitivities.

This is in general not true, but the extension of the formula to cover these cases is fairly straight forward. This model requires that all of the targets are inactivated to kill the cell. In normal usage the simplification is made to assume a single hit, multi-target model. The above equation then reduces to $S = 1 - (1 - e^{-vD})^m = 1 - (1 - me^{-vD} + \dots \pm e^{-mvD})$. Graphed as the log of the survival verses dose, this equation describes a curve with an initial shoulder. In the high dose region it becomes a straight line approximated by the equation $\ln S = -vD + \ln m$. The slope of this final linear portion is the cell sensitivity = v and its y intercept is the extrapolation number = $\ln m$.

In their simplest forms, if a two hit chromosome aberration leads to a dominant lethal, then regardless of ploidy level the cell survival would be described by $S = 1 - (1 - e^{-v_1 D})(1 - e^{-v_2 D})$; where v_1 and v_2 are the probabilities that the first and second "hits" will occur. Any differences between the survival curves of the different ploidy cells will be due to changes in v_1 and v_2 . A certain proportion of "two hit" chromosome aberrations are caused by a single interaction. As a consequence, in its most general form, the survival anticipated if cells are killed by multi-centrics and rings is given by $S = 1 - e^{-v_3 D} (1 - (1 - e^{-v_2 D})(1 - e^{-v_1 D}))$; where v_3 would be the probability of both "hits" being caused by a single interaction (Wolff, 1961).

If cell death results from deletions, then it would be expected that $S = 1 - (1 - e^{-vD})^m$; where v is the probability for a given deletion and m is the ploidy level. For this formulation, as the ploidy level of the cell increased so would the size of its shoulder and

extrapolation number. The final slope of the survival curve would be expected to be the same for all ploidy levels.

The survival curve parameters fitted to the single hit-multitarget model have already been discussed in the main text.

ii.) The Green-Burki model. The Green and Burki model (1974) is a two parameter non-linear differential equation of the form $S = (p+1)/(p+e^{\alpha D})$. S is the surviving factor, p is a recovery parameter, and α is a measure of radiation sensitivity. This model relates the existence of the shoulder of radiobiological survival curves to the saturation of repair mechanisms. For $p > 0$ the equation describes a curve with a shoulder in the low dose region but which falls off exponentially for large doses. The "extrapolation number" of a survival curve corresponds to $p+1$. The fitted values of p and α follow the same trends as the extrapolation number and sensitivity of the single hit-multitarget model. With increases in ploidy p increases. The sensitivity increases with ploidy except for with the L5178Y cells. As the amount of incorporated IUDR increases the values of p and α both decrease.

iii.) Haynes model. The Haynes model (1975) for radiobiological survival curves is based on the hypothesis that the initial shoulder is due to the inactivation of repair as a cell absorbs larger doses of radiation. Inactivation of a cell's repair capacity is itself considered to follow a single hit-multitarget model. The mathematical formulation of the Haynes model used is $\ln S = -vD(1-\exp(-BD))^t$. The term $(1-\exp(-BD))^t$ represents the inactivation of the cell's repair

capacity as a function of dose. B is the sensitivity of the repair genes, t is a measure of their multiplicity.

The gene dosage experiments of Ho (1975) leads one to expect that the value of t would increase with higher ploidies. In general the fitted values of t do increase with ploidy. An example is the B150 hyperdiploid, hypotetraploid, hypoheptaploid series in which the values of t are 1.02, 1.57, and 2.4 respectively. One experimental set where this does not occur is in the experiments with incorporated IUDR. With increasing amounts of incorporated IUDR the value of t for the hyperdiploid cell is greater than for the hypotetraploid. There is no clear trends with the B and v values. For example, in the B150 ploidy series the B values increase and v values decrease with higher ploidies, but for V79 hyperdiploid and hypotetraploid cells the opposite occurs.

iv.) Chadwick-Leenhouts model. The Chadwick-Leenhouts model (1973) assumes that cell death is the result of double strand breaks in cell DNA. Such a break can result from either a single interaction breaking both strands or from two independent interactions each breaking one strand. The mathematical formulation of the model reduces to $S = \exp(-AD)\exp(-BD^2)$ or that $\ln S = -A \times D - B \times D^2$. "A" is a measure of the likelihood that death will be caused by a single interaction, a one-hit event. "B" is a measure of the likelihood that it will be caused by two independent interactions, a two-hit event. The extent to which repair takes place eliminates the damage as a source of cell death and reduces the value of A and B.

Trends occurring within the Chadwick-Leenhouts parameters are more easily visualized by comparing the value for the ratio of A/B, Table #4. As the ploidy increases this ratio decreases indicating a larger amount of killing due to a two hit mechanism. With freezing, the amount due to a single hit mechanism has a large increase except for with ¹²⁵IUdR in which a two hit mechanism is still pronounced. As the incorporated IUdR increases the amount of killing due to a single hit mechanism increases.

Parameters for these models which give the "best" computer fit of the model to the experimental data are presented in Table #3.

Table 3

Cell Type	Single hit-Multitarget			Chadwick-Leenhouts			Green - Burki			Haynes			
	N	D ₀	X ²	A $\times 10^{-3}$	B $\times 10^{-6}$	X ²	P	$\alpha \times 10^{-3}$	X ²	V $\times 10^{-3}$	B $\times 10^{-3}$	t	X ²
1. V79-hyperdiploid	2.4	209.2	1.42	3.0	.832	1.77	1.72	4.88	1.4	4.3	2.6	1.0	1.4
2. V79 hypotetraploid	13.7	137.1	1.69	1.2	3.15	.192	18.7	7.61	1.1	8.5	.809	1.1	4.4
3. B150 hyperdiploid	19.5	142.8	.89	.59	3.08	.03	27.5	7.31	.57	12.1	.377	1.0	.109
4. B150 hypotetraploid	47.3	114.2	.59	.80	3.73	.27	89.	9.24	.38	9.3	1.02	1.57	.075
5. B150 hypohexaploid	145	98.5	1.94	.0046	4.822	1.2	306.	10.9	1.47	10.1	1.37	2.4	.87
6. V79-HGPRT ⁻ hyperdiploid	3.7	185.9	.62	2.5	1.3	.12	3.56	5.53	.47	4.9	1.64	1.0	.56
7. B150 $\times$ V79 hypotetraploid	20.8	157.2	.77	.5	2.45	.2	28.6	6.59	.579	11.9	.292	1.0	.18
8. B150 $\times$ V79 hypertetraploid	26.1	145.6	.315	.32	3.03	.128	36.3	7.14	.13	7.3	.97	1.6	.0048
9. V79 hyperdiploid + 1×10^{-6} M IuDR	4.42	115.2	1.1	4.0	3.09	.67	4.5	8.9	.88	8.6	1.85	1.0	1.01
10. V79 hypotetraploid + IuDR	7.4	97.9	4.6	4.9	3.2	6.9	7.8	10.4	4.5	10.7	1.69	1.5	8.43

Table 3 Cont.

Cell Type	Single hit-Multitarget			Chadwick-Leenhouts			Green - Burki			Haynes			
	N	D ₀	X ²	A×10 ⁻³	B×10 ⁻⁶	X ²	P	α×10 ⁻³	X ²	v×10 ⁻³	B×10 ⁻³	t	X ²
11. V79 hyperdiploid + IUdR & FudR	1.5	103.8	.298	8.95	.67	.94	.345	9.53	.305	9.2	10.7	2.16	.139
12. V79 hypotetraploid +IUdR & FudR	2.5	98.7	1.33	8.0	1.095	1.97	1.61	10.2	1.4	9.6	2.65	1.0	2.99
13. V79 hyperdiploid +2.5×IUdR & FudR	1.0	117.6	.77	8.31	.203	.91	0	8.5	.77	8.5	24.7	10.	.73
14. V79 hypotetraploid+2.5IUdR& FudR	1.45	108	.97	8.4	.384	1.15	.447	9.2	.996	8.96	2.8	1.0	4.36
15. V79 hyperdiploid -196°C	8.2	541	.556	.54	.182	.23	9.1	1.89	.315	1.99	.26	1.0	.293
16. V79 hypotetraploid -196°C	7.1	584	.029	.47	.174	.815	8.56	1.78	.29	1.4	.63	1.9	.258
17. V79 hyperdiploid +IUdR at -196°C	10.4	364	.8	.79	.363	.45	12.8	2.8	.652	2.9	.34	1.0	.49
18. V79 hypotetraploid + IUdR at -196°C	9.16	425	.95	.56	.328	.649	11.25	2.4	.85	2.3	.412	1.14	.76
19. V79 hyperdiploid hypertonic	6.7	152.7	.252	1.97	2.42	.102	7.94	6.8	.17	6.8	1.08	1.0	.135
20. V79 hypotetraploid hypertonic	189	103.7	2.77	0	4.26	2.65	268.2	9.9	2.6	9.77	1.2	2.4	2.45

Table 3 Cont.

Cell Type	Single hit-Multitarget			Chadwick-Leenhouts			Green - Burki			Haynes			
	N	D ₀	X ²	A×10 ⁻³	B×10 ⁻⁶	X ²	P	α×10 ⁻³	X ²	v×10 ⁻³	B×10 ⁻³	t	X ²
21. B150 hyperdiploid hypertonic	10.6	109.1	.143	2.2	4.5	.051	13.56	9.5	.07	9.5	1.25	1.0	.012
22. B150 hypotetraploid-hypertonic	29.5	86.96	1.85	1.95	6.1	2.78	36.8	11.8	1.8	8.98	3.8	4.1	1.7
23. V79 hyperdiploid 4°C	5.63	138.9	.135	2.23	2.99	.515	6.56	7.5	.1	22.8	.3	1.13	2.7
24. V79 hypotetraploid 4°C	2.16	162.5	2.8	3.5	1.8	2.3	1.57	6.33	2.7	6.3	2.22	1.6	4.9
25. V79 hyperdiploid ¹²⁵ IuDR	2.93	50.76	2.63	8.87	22.9	2.0	2.85	20.6	2.3	17.9	7.84	1.0	2.6
26. V79 hypotetraploid ¹²⁵ IuDR	2.12	102.4	.722	4.68	7.76	.8	1.93	10.55	.69	8.38	6.37	1.0	.823
27. V79 hyperdiploid ¹²⁵ IuDR = dose	2.93	64.47	---	6.99	14.2	2.0	---	---	---	---	---	---	---
28. V79 hypotetraploid ¹²⁵ IuDR = Dose	2.12	76.7	---	6.2	.138	.8	---	---	---	---	---	---	---
29. V79 hyperdiploid ³ HTdR	1.63	1544.4	5.3	.535	.0059	6.6	.66	.6487	5.5	.61	.731	2.9	4.28

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Table 3 Cont.

Cell Type	Single hit-Multitarget			Chadwick-Leenhouts			Green - Burki			Haynes			
	N	D ₀	X ²	A ^x -3 10 ⁻³	B ^x -6 10 ⁻⁶	X ²	P	α^x -3 10 ⁻³	X ²	v^x -3 10 ⁻³	B^x -3 10 ⁻³	t	X ²
30. V79 hypotetraploid ³ HTdR	2.85	2711.5	2.16	.125	.013	2.4	3.34	.405	2.2	.333	.185	1.6	2.77
31. V79 hyperdiploid ³ HTdR = Dose	1.63	725.86	---	1.14	.0262	6.5	---	---	---	---	---	---	---
32. V79 hypotetraploid = Dose	2.85	755.7	---	.45	.167	2.4	---	---	---	---	---	---	---
33. L5178Y diploid	2.6	83.3	1.7	6.4	5.8	1.3	2.37	12.4	1.6	11.0	4.56	1.0	1.86
34. L5178Y hypotetraploid	5.65	88.73	.13	3.8	6.4	.2	7.166	11.75	.03	10.6	2.46	1.02	.014

Table 4: Chadwick-Leenhouts

Cell Type	A/B
1. V79 hyperdiploid	3567.99
2. V79 hypotetraploid	367.78
3. B150 hyperdiploid	191.6
4. B150 hypotetraploid	215.79
5. B150 hypohexaploid	9.58
6. V79 HGPRT ⁻ hyperdiploid	1911.15
7. B150 × V79 hypotetraploid	203.77
8. B150 × V79 hypertetraploid	104.5
9. V79 hyperdiploid + IuDR	1296.6
10. V79 hypotetraploid + IuDR	1539.77
11. V79 hyperdiploid + IuDR & FudR	13,343.
12. V79 hypotetraploid + IuDR & FudR	7332.8
13. V79 hyperdiploid + 2.5 × IuDR & FudR	40,915.8
14. V79 hypotetraploid + 2.5 × IuDR & FudR	22,044
15. V79 hyperdiploid -196°C	2984.6
16. V79 hypotetraploid -196°C	2720.6
17. V79 hyperdiploid + IuDR -196°C	2180
18. V79 hypotetraploid + IuDR -196°C	1710
19. V79 hyperdiploid-hypertonic	810.978
20. V79 hypotetraploid-hypertonic	0.00
21. B150 hyperdiploid-hypertonic	480.9
22. B150 hypotetraploid-hypertonic	318.76
23. V79 hyperdiploid 4°C	746.9

Table 4: Chadwick-Leenhouts, Continued

Cell Type	A/B
24. V79 hypotetraploid	1927.6
25. V79 hyperdiploid $^{125}\text{IUdR}$	387.6
26. V79 hypotetraploid $^{125}\text{IUdR}$	602.59
27. V79 hyperdiploid $^{125}\text{IUdR} = \text{Dose}$	492.35
28. V79 hypotetraploid $^{125}\text{IUdR} = \text{Dose}$	451.45
29. V79 hyperdiploid $^3\text{HTdR}$	90,671
30. V79 hypotetraploid $^3\text{HTdR}$	9665.9
31. V79 hyperdiploid $^3\text{HTdR} = \text{Dose}$	43,703
32. V79 hypotetraploid $^3\text{HTdR} = \text{Dose}$	2685.5
33. L5178Y diploid	1094.7
34. L5178Y hypotetraploid	589.44

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This report was done with support from the United States Energy Research and Development Administration. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the United States Energy Research and Development Administration.

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