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I am submitting herewith a dissertation written by Michael William Heartlein entitled "Mechanisms of Induction of SCE and Mutations by BrdU and CldU and the Use of Inhibitors of DNA Repair to Study Mechanisms of Radiation-Induced Chromosome Aberrations." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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# MECHANISMS OF INDUCTION OF SCE AND MUTATIONS BY BrdU AND CldU AND THE USE OF INHIBITORS OF DNA REPAIR TO STUDY MECHANISMS OF RADIATION-INDUCED CHROMOSOME ABERRATIONS

A Dissertation

Presented for the

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Degree

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Michael William Heartlein

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DEDICATION

TO MY PARENTS JANICE AND JACK, LISA,

MY BROTHER MARK, AND TIM

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## ABSTRACT

The induction of sister chromatid exchanges (SCE) and specific locus mutations was studied by utilizing incorporation into DNA of the nuceloside analogues 5-bromo- and 5-chlorodeoxyuridine (BrdU and CldU). CldU was found to induce SCE seven-times more efficiently than BrdU at equal extracellular concentrations. This induction was linearly associated with substitution for thymidine from 0.5-20 μM. In these experiments, specific locus mutations were not detected at concentrations less than 50 μM and were not correlated with SCE induction. At concentrations greater than 50 μM, the mutagenicity of CldU and BrdU was similar, although BrdU was slightly more mutagenic than CldU. In experiments designed to examine SCE and mutagenicity as a function of replication of BrdU or CldU-substituted DNA, it was shown that at low analogue concentrations, CldU caused a seven-fold greater rate of mutation and cytotoxicity than BrdU, and that replication of twice as much analogue-substituted DNA was associated with a two-fold increase in SCE for both CldU and BrdU. SCE induction, therefore, appears to be a consequence of replication on templates containing these analogues. Mutations, however, result from two different mechanisms; errors of incorporation where BrdU or CldU substitutes in DNA for deoxycytidine and replication errors which, similar to the mechanisms of SCE induction, require replication on templates substituted with BrdU or CldU.

In the examination of radiation-induced chromosome aberrations in mammalian lymphocytes, 3-aminobenzamide and cytosine arabinoside, which are excision repair inhibitors, were used to show that the induction of chromosome aberrations depends upon the ratio of base damage to directly-induced DNA strand breaks for a particular radiation quality. In addition, it was shown that sensitivity of various mammalian species to X ray-induced aberrations depends upon the rate of repair of base damage. This is in contrast to aberration induction by fission neutrons. Since neutrons produce mainly directly-induced double strand breaks and apparently rather little base damage, it was shown that aberration formation with neutrons is dependent upon mis-rejoining of directly-induced strand breaks, and was therefore, independent of excision repair rate. The relative biological effectiveness of a particular radiation quality therefore, can be accounted for by the ratio of misrepair of directly-induced double strand breaks to base damage and varies for different cell types and species. These results, furthermore, appear to provide an explanation for both the mechanism of and sensitivity to radiation-induced chromosome aberrations.

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# CHAPTER I

# INTRODUCTION

## Sister Chromatid Exchange

Sister chromatid exchanges (SCE) are the result of breakage and reunion of DNA strands within a single chromosome. SCE occur during the synthesis phase of the mammalian cell cycle and are influenced by a variety of agents and treatments (for review, see Latt, 1981). There are a number of proposed molecular mechanisms of SCE formation; most of which are heavily influenced by models of meiotic recombination. For example, the Meselson-Radding model for recombination (Radding, 1982) can explain SCE formation rather well in terms of the exchange of DNA strands. This model, however, may require specific enzymatic cleavage of DNA, which is probably not the case with SCE. Most of the data support a replication model proposed by Painter (Painter, 1980; Ishii and Bender, 1980; Figure 1). The replication model fits well with results suggesting that perturbations in the DNA replication machinery and DNA damage present at replication forks are instrumental in SCE formation.

The visualization of SCE is accomplished by labelling the chromatids with the nucleoside analogue, 5-bromodeoxyuridine (BrdU). After passage of the cells through two cycles of DNA replication in the presence of BrdU, incorporation of BrdU into the DNA allows

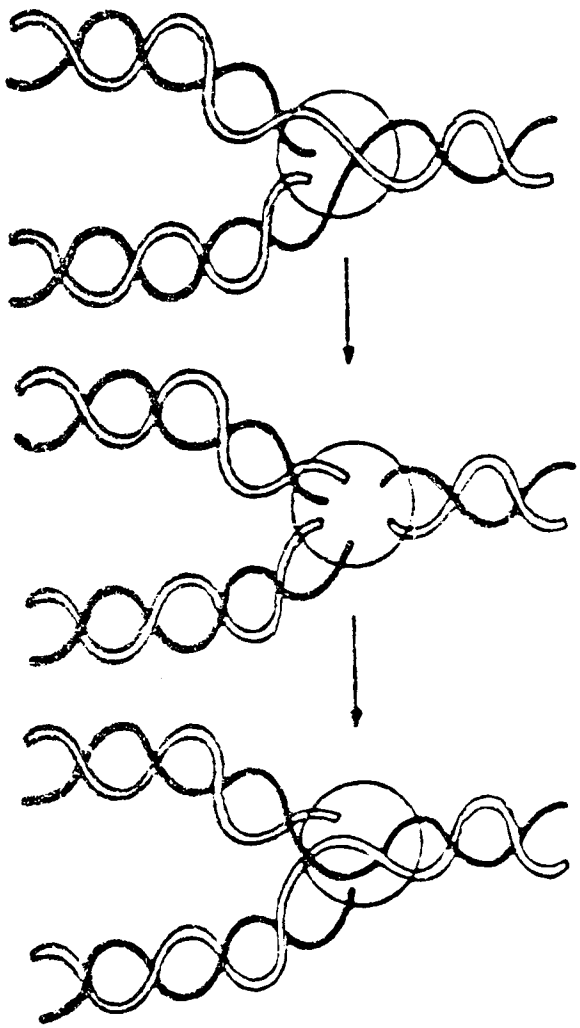


Figure 1. A replication model for SCE. (After Painter, 1980).

differential staining of chromatids (Figure 2). Treatment of cells which have incorporated BrdU with the bisbenzimidizole dye Hoechst 33258, black light, heat, and Giemsa stain, causes the chromatids to have a characteristic light or dark appearance (Figute 2). SCE are counted by recording the number of switches in the continuity of these darkly and lightly stained portions of the chromosome.

If there is any biological significance of SCE it is at present unknown. However, the utility of SCE lies in its high sensitivity to agents which cause mutations, although the mechanisms of SCE induction and mutation induction appear to be different in some cases. SCE also appears to be a sensitive indicator of exposure to potentially harmful agents, and may therefore be a useful technique for monitoring of human populations. Nonetheless, the study of SCE induction may shed some light on other genetic processes in mammalian cells, e.g., meiotic recombination, mutation, and DNA replication and repair.

## Mutagenesis in Cultured Mammalian Cells

Gene mutations can result from a number of different mechanisms (for review, see Fox, 1982). There are, for example, base-pair substitutions, shifts in the DNA reading frame caused by single nucleotide deletions or insertions, and more complete deletions of the gene; all of which can adversely affect gene product function.

Systems for the quantification of cellular mutagenesis rely on powerful selection techniques. That is, treatments which, in

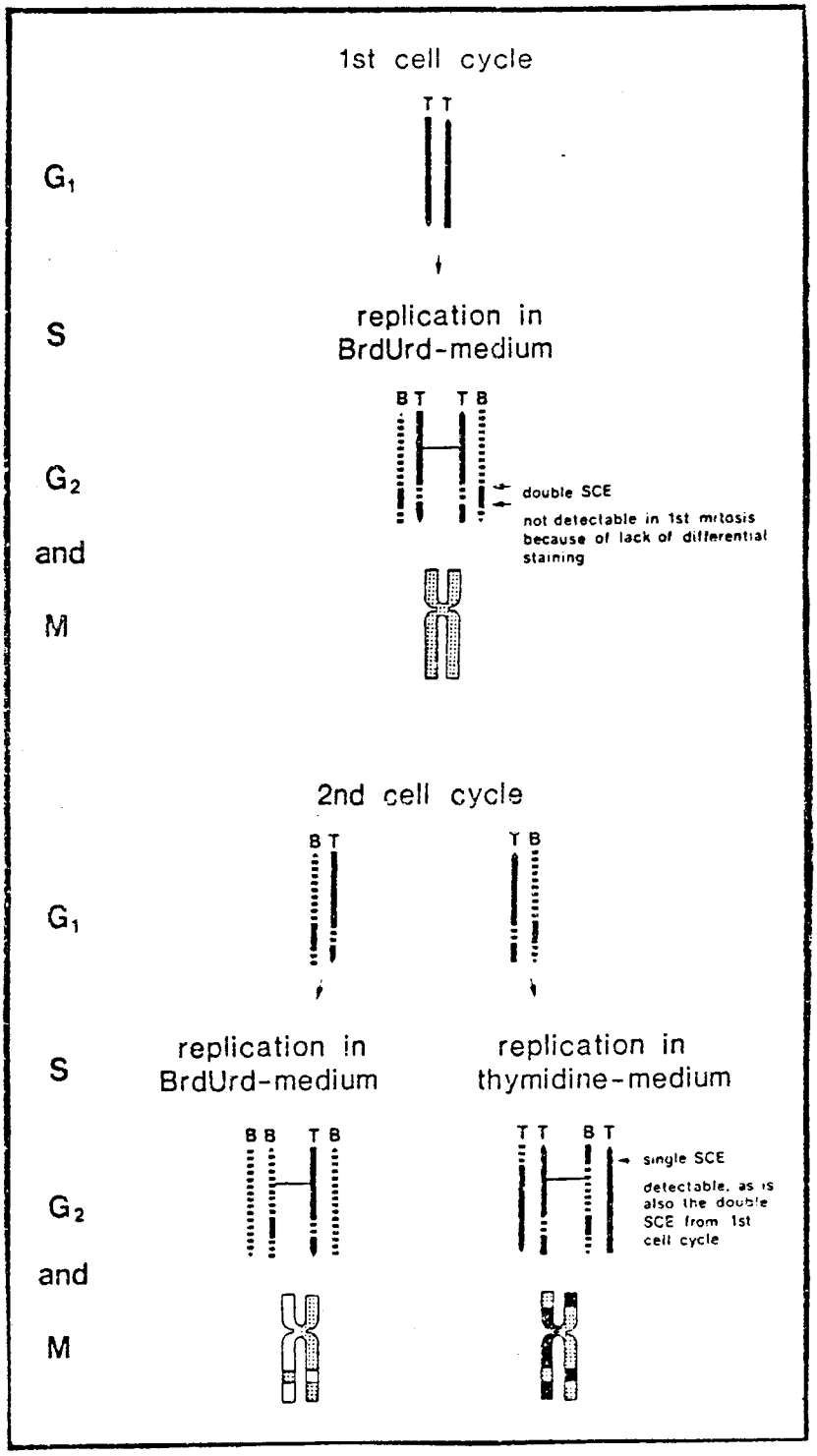



Figure 2. Staining methodology for visualization of SCE. (After Khilman, 1982)

theory, allow growth of mutant cells exclusively. Mutations are fixed in cells by a combination of processes involving dilution of wild-type enzyme activity via cell division and loss of activity due to spontaneous degradation of the enzyme (dependent upon turn-over rate). This process occurs during phenotypic expression. Therefore, selection can be accomplished only after residual wild-type enzyme activity is lost. These experiments rely on selection due to the cellular incorporation of 6-thioguanine (TG). Thioguanine is incorporated either into DNA or RNA only when functional hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is present. Presumably, 6-thioguanylic acid incorporation into DNA or RNA is lethal to cells at the concentrations used in selection. In the case of defective or absent HGPRT, TG is not incorporated, thereby allowing TG resistant mutant cells to proliferate. The mechanism of TG killing however, is incompletely understood at present (i.e., whether or not TG is incorporated into DNA).

Mutation induction primarily depends upon the type(s) and repair of damage induced in the DNA; alkylation, intercalation, depurination/depyrimidination, for example. Whether or not there is an inducible and/or "error-prone" repair (as in bacteria) is presently an unresolved question, although there is a recent report of inducible UV damage repair in human fibroblasts (Tyrell, 1984). It has also become clear that there is in some cases a cell cycle specificity for induction of mutations by certain chemicals. For

example, some types of DNA damage may cause mutations more efficiently providing that the damage remains unrepaired, and is present during the DNA synthesis phase. Mutations affecting the fidelity of replication may result from a mechanism similar to that by which SCE are induced.

## Chromosome Aberrations

Various chemicals and radiations can induce direct breakage of or repairable DNA damage to chromosomes. The production of aberrations is dependent both on the type of treatment (chemical or radiation) and the time of treatment during the cell cycle ($G_0$, $G_1$, S, or $G_2$). This is primarily a result of the replication state of the DNA; i.e. whether the chromosome consists of a single DNA double helix ($G_0$ and $G_1$) or two DNA double helices (S and $G_2$). For example, cells treated with X rays in S or $G_2$ produce primarily chromatid-type aberrations while cells treated in $G_0$ or $G_1$ primarily produce chromosome-type aberrations (Figure 3). Aberrations can be visualized with the light microscope at metaphase of the cell cycle since the chromosomes are highly compacted and amenable to the detection of deletions and rearrangements. The type of aberration produced is dependent also on the repair of DNA damage such that chromatid-type aberrations may be produced, for example, if a particular lesion is unrepaired at the time of DNA replication.

Unlike SCE, the biological consequences of most aberrations are known. It can be concluded that most chromosome-type

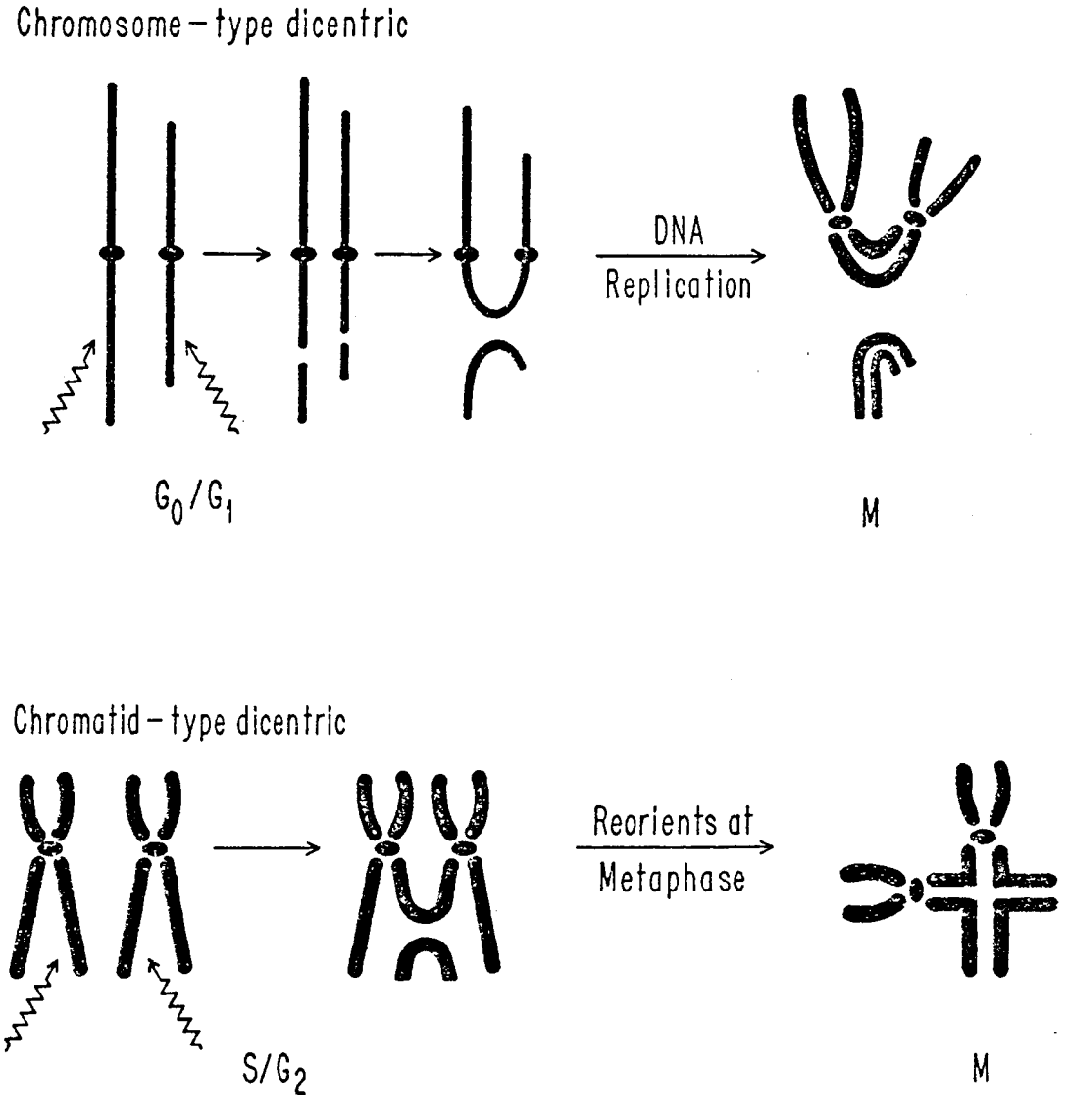



Figure 3. Examples of chromosome- and chromatid-type aberrations.

aberrations which involve the production of acentric fragments are lethal events, since at the time of cell division this sometimes large piece of DNA is usually lost from one of the daughter cells. Chromosome-type exchanges (translocations), however, are probably not deleterious in that no genetic material is lost and the mitotic behavior of the chromosome is presumably unaffected. This type of aberration is probably most relevant to humans, however, since it is transmissable and, when germ cells are affected, it can lead to chromosomally unbalanced offspring due to duplications and deficiencies, a decrease in fertility, and/or other congenital abnormalities.

The mechanism of aberration formation at a fundamental level has been the subject of controversy for several decades. Essentially, there are two main theories of chromosome aberration (for review, see Savage, 1975). The first, which is sometimes called the breakage and reunion or classical theory, was proposed by Sax in 1940. This hypothesis proposed that the primary event caused by radiation was the deposition of its energy in the DNA to produce a break. Following the severance of the chromosome, there were three possible routes; restitution to give the original configuration, to rejoin assuming that the chromosomes are in close enough proximity to thereby produce an exchange aberration, and finally, to fail to restitute or rejoin, which would leave a break. With this theory, the break is primary; everything else is derived from it.

The second theory is called the exchange hypothesis (Revell, 1959). Revell suggested that the primary effect of radiation which leads to aberration formation is what he called an "unstable lesion." This lesion tended to "decay" to normal. If, however, two pairs of lesions are brought together either through repair or some other process, these interact to exchange. If the exchange is not completed, the result is a simple break. An exchange event, however, leads to some type of chromosome aberration. All breaks are secondary with this theory, in contrast to the classical theory where breaks are primary events.

Neither theory is probably entirely correct, although the recent body of evidence tends to support the exchange hypothesis of Revell (e.g., Duncan and Evans, 1983). The exchange hypothesis is also consistent with mechanisms which incorporate the repair process as an integral role in aberration formation. Depending upon the type of lesion induced in the DNA, however, elements of both theories are probably taking place during the repair of DNA damage and subsequent aberration formation.

The enzymology of aberration formation is, at present, unknown. This assumes, that aberration production is dependent on actual enzyme-mediated breakage and/or reunion of DNA strands. It is entirely possible, however, that some aberrations form simply as a result of the hydrodynamic and hybridisation strengths of a particular stretch of DNA, in addition to changes in physical proximity of chromosomes during the cell cycle following DNA damage and repair.

Regardless of the precise mechanism, providing hypotheses to explain aberration production is of considerable importance in interpreting data bearing on radiosensitivity and risk to human populations.

# PART I

## MECHANISMS OF INDUCTION OF MUTATION AND SISTER CHROMATID EXCHANGES BY BrdU AND CldU

CHAPTER II

# THE INDUCTION OF SPECIFIC LOCUS MUTATIONS AND SISTER CHROMATID EXCHANGES BY 5-BROMO- AND 5-CHLORODEOXYURIDINE

## Summary

The induction of 6-thioguanine resistant ($TG^r$) mutants and sister chromatid exchanges by 5-bromo- and 5-chlorodeoxyuridine (BrdU and CldU) was determined with Chinese hamster ovary cells in culture. Over a concentration range of 0.05-200 μM, CldU treatment caused 3-5 times the number of SCE found with BrdU; for example, with 10 μM CldU, 60.0 ± 14.2 SCE/metaphase (mean ± standard deviation) were induced, while with 10 μM BrdU, only 14.6 ± 4.1 SCE/metaphase were found. Despite this difference in SCE induction, both compounds were mutagenic only at concentrations greater than 50 μM, and BrdU was more mutagenic than CldU. These results indicate that SCE and gene mutations are induced by these thymidine analogues through two different mechanisms.

## Introduction

The thymidine analogue 5-bromodeoxyuridine (BrdU) is widely used in mammalian cell culture systems as an integral part of methodologies that allow the differential staining of sister chromatids and thus the study of sister chromatid exchanges (SCE) (reviewed

in Latt et al., 1981). However, BrdU itself has been shown to induce SCE and the increased frequency appears to be due to the incorporation of the analogue into cellular DNA (Mazrimas and Stetka, 1978). In addition, BrdU induces gene mutation in mammalian cells, but this mutagenic activity does not appear to correlate with DNA incorporation (Abersold, 1976; Davidson and Kaufman, 1978). The relationship between the induction of SCE and gene mutations by BrdU is not clear, but several studies suggest that two different mechanisms may be involved (Davidson and Kaufman, 1978; San Sebastian et al., 1980; Davidson et al., 1980). In an effort to define further the basis for these two genetic effects, we have determined the activity of another thymidine analogue, 5-chlorodeoxyuridine (CldU), in inducing SCE and mutations in Chinese hamster ovary (CHO) cells. This analogue was chosen because preliminary studies showed that CldU induces 3-4 fold more SCE than an equal concentration of BrdU (DuFrain and Garrand, 1980; Pal et al., 1981).

## Materials and Methods

These studies employed the CHO-$K_1$ cell strain originally described by Kao and Puck (1967). The cultures were maintained in Hams medium F12 containing 5% heat inactivated (56°C, 30 min.) fetal bovine serum (FBS) under standard growth conditions as described previously (O'Neill et al., 1977). In all experiments, exponential phase cultures were detached with 0.05% trypsin and plated in medium F12 containing 5% heat inactivated dialyzed FBS (F12/5) at a density

of 3 x $10^5$ cells per 100 mm dish for the SCE determination and at 5 x $10^5$ cells per 75 $cm^2$ flask for the mutation induction studies. After a 20-24 hour growth period, fresh medium containing BrdU or CldU was added. Medium F12 contains 3 μM thymidine.

SCE Determination

Following the addition of BrdU or CldU, the cultures were then incubated for 25-32 hours, 10 μM colchicine added for 2 hours, and the mitotic cells collected. Slides were prepared as described previously (San Sebastian et al., 1980) and differential staining of the chromatids accomplished by the method of Goto et al. (1978). The method was modified slightly in that the black light exposure at 50°C was increased to 60 min. The frequency of SCE was determined in 25 cells, each of which contained 20 well differentially stained chromosomes, and is expressed as mean number of SCE per metaphase.

Mutation Induction

The cultures were incubated in the presence of BrdU or CldU for 25 hours, washed and fresh medium added. After an additional 20-24 hour growth period, the cultures were subcultured at 48 hour intervals to allow phenotypic expression and plated for selection of 6-thioguanine resistant ($TG^r$) mutants after 9 days of expression (O'Neill et al., 1977; San Sebastian et al., 1980). For mutant selection, .2 x $10^5$ cells/100 mm dish were plated in quintuplicate in hypoxanthanine-free F12/5 containing 10 μM TG. For cloning efficiency, 200 cells/60 mm dish were plated in triplicate in the same

medium without TG. The mutant frequency is expressed as mutants per $10^6$ cells, corrected for the cloning efficiency at the time of selection.

## Results and Discussion

Cultures were incubated with BrdU or CldU for a time sufficient for two replications, that is 25-32 hours, and the frequency of SCE determined in differentially stained cells (second division) containing 20 chromosomes. The results are shown in Figure 4. With both compounds, differential staining was not obtained with concentrations less than 0.5 μM. As reported previously (Pal et al., 1981), CldU induces a three to five-fold greater number of SCE than an equimolar concentration of BrdU (Figure 4). Over the concentration range of 0.5-5 μM, where the increase in SCE frequency is proportional to the concentration of BrdU and its incorporation into DNA (Mazrimas and Stetka, 1978), the SCE frequency with CldU is two to five-fold greater than that found with BrdU (Figure 4B). This greater induction of SCE by CldU appears to result from the nature of the halogen substitution and not due to a large difference in incorporation into DNA since preliminary studies show that incubation with either BrdU or CldU at 1 μM causes 17-20% substitution for thymidine. With 50 μM BrdU or CldU, 75-90% substitution for thymidine is observed, but at this concentration, CldU induces a five-fold more SCE than BrdU (Figure 4A). At concentrations greater than 100 μM, the scoring of SCE is complicated by the high incidence of chromosome

Figure 4. Induction of SCE by BrdU and CldU.

CHO cells were incubated for 25-32 hours in medium F12/5 containing 0.5-200 μM BrdU (○) or CldU (●) and scored for SCE as described. The symbols represent the mean number of SCE/metaphase (25 cells scored) and the vertical lines represent the standard deviation of the mean. In A, the results for the entire concentration range is shown, and in B the results with 0.5-10 μM is shown on an expanded scale.

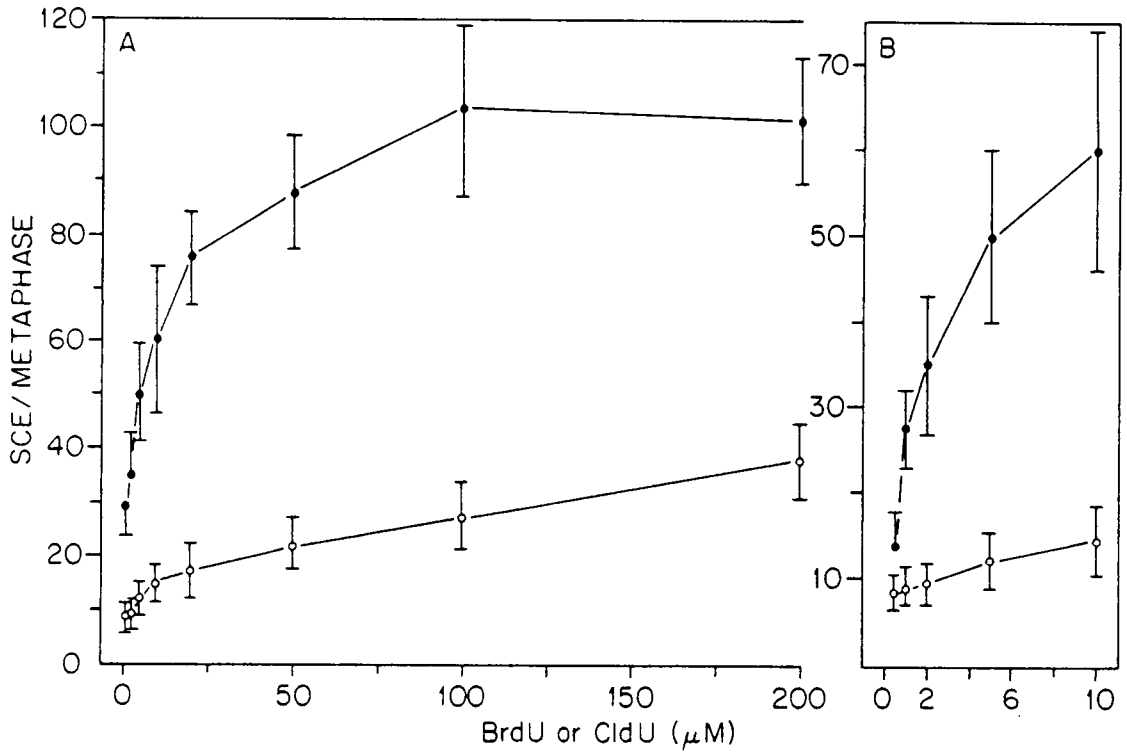

A
B
SCE / METAPHASE
BrdU or CldU (μM)
0
20
40
60
80
100
120
10
30
50
70
0
50
100
150
200
0
2
6
10

Figure 4.

aberrations, especially with CldU. These results show that the presence of CldU in replicating DNA greatly increases the incidence of SCE, with a five-fold greater activity than that found with BrdU. This suggests that CldU induces more replication errors that result in SCE formation (Painter, 1980; Ishii and Bender, 1980).

BrdU has been shown to induce gene mutations, but this mutagenic activity does not appear to be related specifically to its incorporation into DNA in place of thymidine (Abersold, 1976; Davidson and Kaufman, 1978). It has been proposed that mutations and SCE are induced by similar mechanisms, since both are suppressed by deoxycytidine (Davidson et al., 1980). However, the fact that thymidine increases BrdU mutagenesis while having no effect on SCE induction suggests that two different mechanisms are involved (San Sebastian et al., 1980). In view of the differences in SCE induction found with BrdU and CldU, determination of mutation induction with these compounds might provide insight into the relationship between these two genetic effects. Cultures were incubated with the analogues for 25 hours, and plated for selection of $TG^r$ mutants after 9 days expression. In agreement with results reported previously with CHO cells (San Sebastian et al., 1980), BrdU induces mutations only at concentrations of 50 μM or greater (Figure 5). CldU shows a similar pattern of concentration dependent mutation induction, although it consistently induces a lower mutant frequency than found with BrdU. In addition, treatment of cultures with both BrdU and CldU in equal concentrations results in mutant frequencies intermediate between

Figure 5. Mutation induction by BrdU and CldU.

Cultures of CHO cells were incubated in medium F12/5 containing BrdU (○) or CldU (●) for 25 hours, subcultured for phenotypic expression and plated for selection of $TG^r$ mutants as described. Two independent determinations are presented in A and B. In B, cultures were also treated with both BrdU and CldU at 50 and 75 μM each (△). The spontaneous frequency was 4.2 and 5.6 in A and B, respectively.

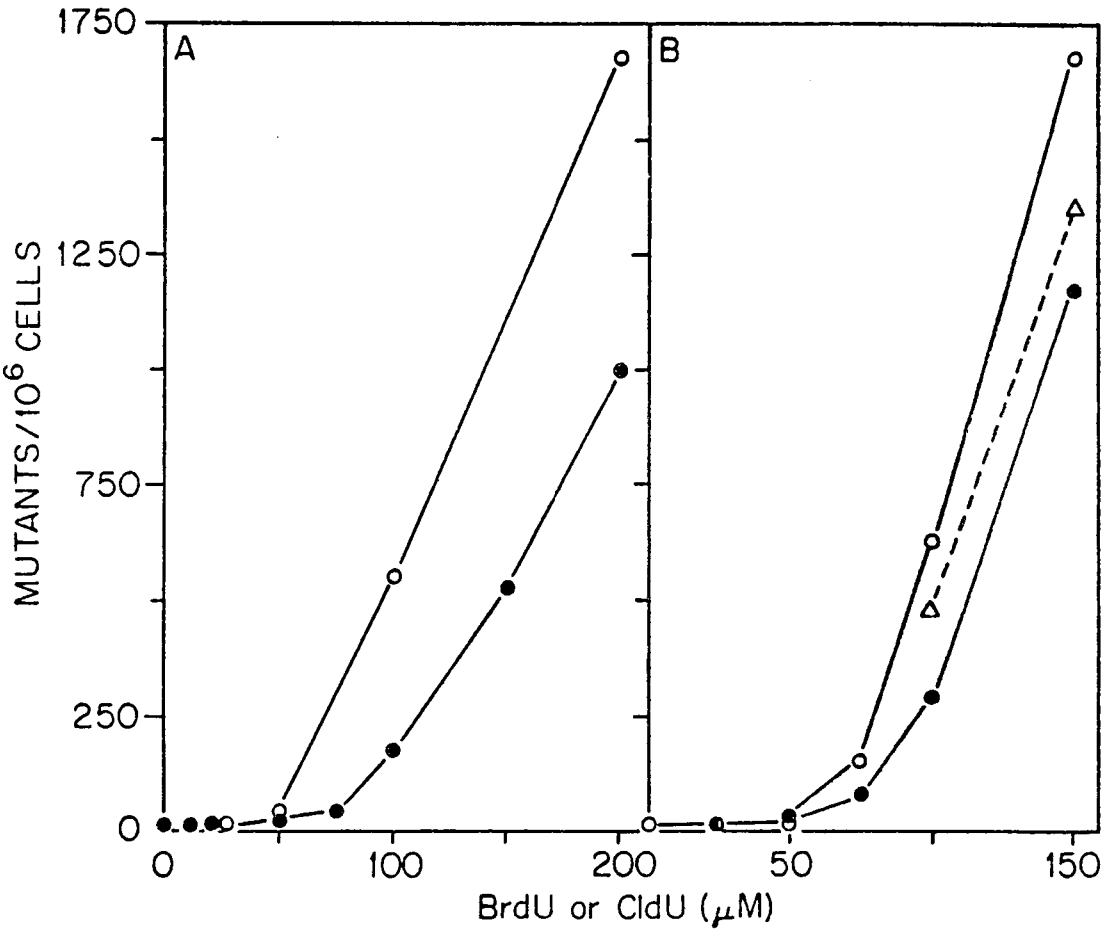

1750
1250
750
250
0
MUTANTS/10^6 CELLS
A
B
0
100
200
50
150
BrdU or CldU (μM)

Figure 5.

those found with each alone at the same total concentration (Figure 5B). These results suggest that BrdU and CldU exert mutagenic activity through the same mechanism, but that BrdU is more potent. The difference in mutation induction is not due to differential cytotoxicity as the cellular lethality of both compounds is similar (Table 1).

The results presented here suggest that the incorporation of CldU into DNA is a more potent inducer of SCE than is BrdU. This observation should allow future studies to elucidate the mechanism responsible for SCE formation. The data further suggest that the induction of gene mutations by these compounds occurs through a mode of action different from that which induces SCE. CldU induces five-fold more SCE than BrdU, and yet is slightly less mutagenic. In addition, highly mutagenic concentrations of BrdU result in far fewer SCE than are induced by non-mutagenic concentrations of CldU. These results further indicate that it is presumptious to make predictions on mutation induction from studies designed to measure SCE--a practice that is often followed.

TABLE 1

CYTOTOXICITY OF BrdU AND CldU

| Conc. (μM) | | Relative Cloning Efficiency[a] (%) |
|---|---|---|
| A. BrdU | | |
| 0 | | 100 |
| 25 | | 98 |
| 50 | | 109 |
| 75 | | 71 |
| 100 | | 66 |
| 150 | | 21 |
| 200 | | 7 |
| B. CldU | | |
| 0 | | 100 |
| 25 | | 99 |
| 50 | | 97 |
| 75 | | 70 |
| 100 | | 51 |
| 150 | | 15 |
| 200 | | 7 |
| C. BrdU + CldU | | |
| 0 | 0 | 100 |
| 25 | 25 | 96 |
| 50 | 50 | 52 |
| 75 | 75 | 12 |
| 100 | 100 | 5 |

[a]Cultures were incubated with BrdU or CldU for 25 h, washed and fresh medium added. Cloning efficiency was determined 24 h later and is expressed relative to a control culture (absolute cloning efficiency = 76%).

# CHAPTER III

## SCE INDUCTION IS PROPORTIONAL TO SUBSTITUTION IN DNA FOR THYMIDINE BY CldU AND BrdU


### Summary

The incorporation of 5-bromo and 5-chlorodeoxyuridine into cellular DNA and the induction of sister chromatid exchanges (SCE) was examined with Chinese hamster ovary cells in culture. Although CldU caused three to five times more SCE than BrdU at equal extracellular concentrations, the two analogues were incorporated into DNA in place of thymidine similarly over a concentration range of 0.5-20 μM. The results show that SCE induction is linearly proportional to substitution for thymidine by both BrdU and CldU. Finally, based on linear extrapolation, a spontaneous level of about six SCE per cell is estimated.


### Introduction

The pyrimidine nucleosides 5-chlorodeoxyuridine (CldU) and 5-bromodeoxyuridine (BrdU) are thymidine analogues that contain halogen substitutions at the five position on the six-membered pyrimidine ring. BrdU has been extensively studied for its effects on mutation (Ashman and Davidson, 1981; Ashman et al., 1981; Davidson and Kaufman, 1978; Davidson et al., 1980), virus induction (Sugar and Kaufman, 1973) and sister chromatid exchange (Davidson

et al., 1980; Heartlein et al., 1982; Mazrimas and Stetka, 1978; San Sebastian et al., 1980), and is commonly used for labelling chromatids in staining for SCE. Mazrimas and Stetka (1978) showed that the frequency of SCE is proportional to BrdU substitution for thymidine. Data such as these provided evidence for spontaneous SCE in Chinese hamster M3-1 cells. Furthermore, SCE caused by BrdU incorporation or replication has been related to mutation induction (Davidson et al., 1980; Heartlein et al., 1982; San Sebastian et al., 1980). However, two different mechanisms for SCE and mutation induction may be involved (Davidson et al., 1980; Heartlein et al., 1982).

In order to study further whether or not there is a true spontaneous frequency of SCE, and whether the mechanisms of induction of SCE and specific locus mutations are similar, we have made use of the observation that the incorporation of CldU can be used to differentially stain chromosomes, but that the frequency of SCE is 3-5 times higher with CldU than with BrdU at the same extracellular concentrations (DuFrain and Garrand, 1981; Heartlein et al., 1982; Pal et al., 1981). In addition, it has been shown that the frequencies of thioguanine resistant mutants induced by CldU and BrdU are very similar (Heartlein et al., 1982).

The results reported here show that SCE induction is linearly proportional to CldU and BrdU substitution for thymidine in DNA and also suggest that a relatively high level of spontaneous SCE (six/cell) occurs in CHO cells in culture.

# Materials and Methods

## Cell Culture

These studies employed the CHO-$K_1$ cell strain. The cultures were maintained in Ham's F12 medium (K. C. Biological) containing 5% heat inactivated (56°C, 30 m in.), dialyzed fetal bovine serum (Sterile Systems). Standard culture conditions of 5% $CO_2$ in air, 37°C and 95-99% humidity were routinely used. Cells were detached with 0.05% trypsin and plated at a density of $1.5 \times 10^6$ cells per 150 mm dish for incorporation studies and at $3 \times 10^5$ cells per 100 mm dish for SCE studies. After 24 hours of growth, fresh medium was added containing either BrdU or CldU (Calbiochem) at the different concentrations shown in the results. (Medium F12 contains 3 μM thymidine.) To provide time for equilibration of nucleotide pools, cell cultures used in incorporation studies were allowed 4 hours of BrdU and CldU exposure before 2 μCi/ml $^{32}PO_4$ (New England Nuclear) was added.

## DNA Base Composition Analysis

In order to provide time for approximately two rounds of DNA replication in the presence of BrdU or CldU, the cells were harvested 24 hours following addition of $^{32}PO_4$. The DNA was extracted and purified by previously described methods (Kaufman and Davidson, 1977). Hydrolysis of the DNA to the nucleotide level was achieved by sequential incubation at 37°C for one hour with DNAase I (Millipore Corp., 0.5 mg/ml, 0.02M Tris-HCl pH 7.6, 0.01M MgCl) and snake

venom phophodiesterase (Millipore Corp., 0.05 mg/ml). Nucleoside 5'-monophosphates were then separated by high performance liquid chromatography (HPLC) using a μBondapak C-18 column, length 30 cm, internal diameter 3.9 mm (Water Associates, Milford, MA 01757). The mobile phase consisted of 0.125M ammonium phosphate at a flow rate of 2 ml per minute and column pressure of 3,000 psi. Absorbance at 260 nm was continuously monitored using an UV absorbance spectrometer (Schoeffel Instrument Corp., Model SF 770). Chart speed was set at 2 cm per minute. For separating chlorodeoxyuridine 5'-monophosphate, the mobile phase was adjusted to pH 7.7 (Figure 6). Whereas, a pH of 6.3 was required to separate bromodeoxyuridine 5'-monophosphate (Figure 7). Fractions of 0.05 ml were collected and radioactivity determined by scintillation counting.

SCE Determination

Following the addition of BrdU or CldU, the cultures were incubated for 25 hours, 10 μM colchicine added for 2 hours and the mitotic cells collected. Slides were prepared as previously described (San Sebastian et al., 1980) and differential staining of the chromatids accomplished by the method of Goto et al. (1978). The method was slightly modified by extending the length of black light exposure at 50°C to 60 minutes. The frequency of SCE was determined in 25 cells, each of which contained 20 well differentiated chromosomes, and is expressed as a mean number of SCE per metaphase.

Figure 6. Chromatography of a CHO CldU substituted DNA hydrolysate.

A 20 µl sample was injected into a µBondapak C-18 column and eluted with 0.125 M ammonium phosphate, pH 7.70 at 2 ml/min. (about 3,000 psi). Absorbance peaks correspond to the deoxynucleoside monophosphates of cytosine (pdC), thymine (pdT), guanine (pdG), adenine (pdA) and halogen substituted uracil (p5-CldU or p5-BrdU).

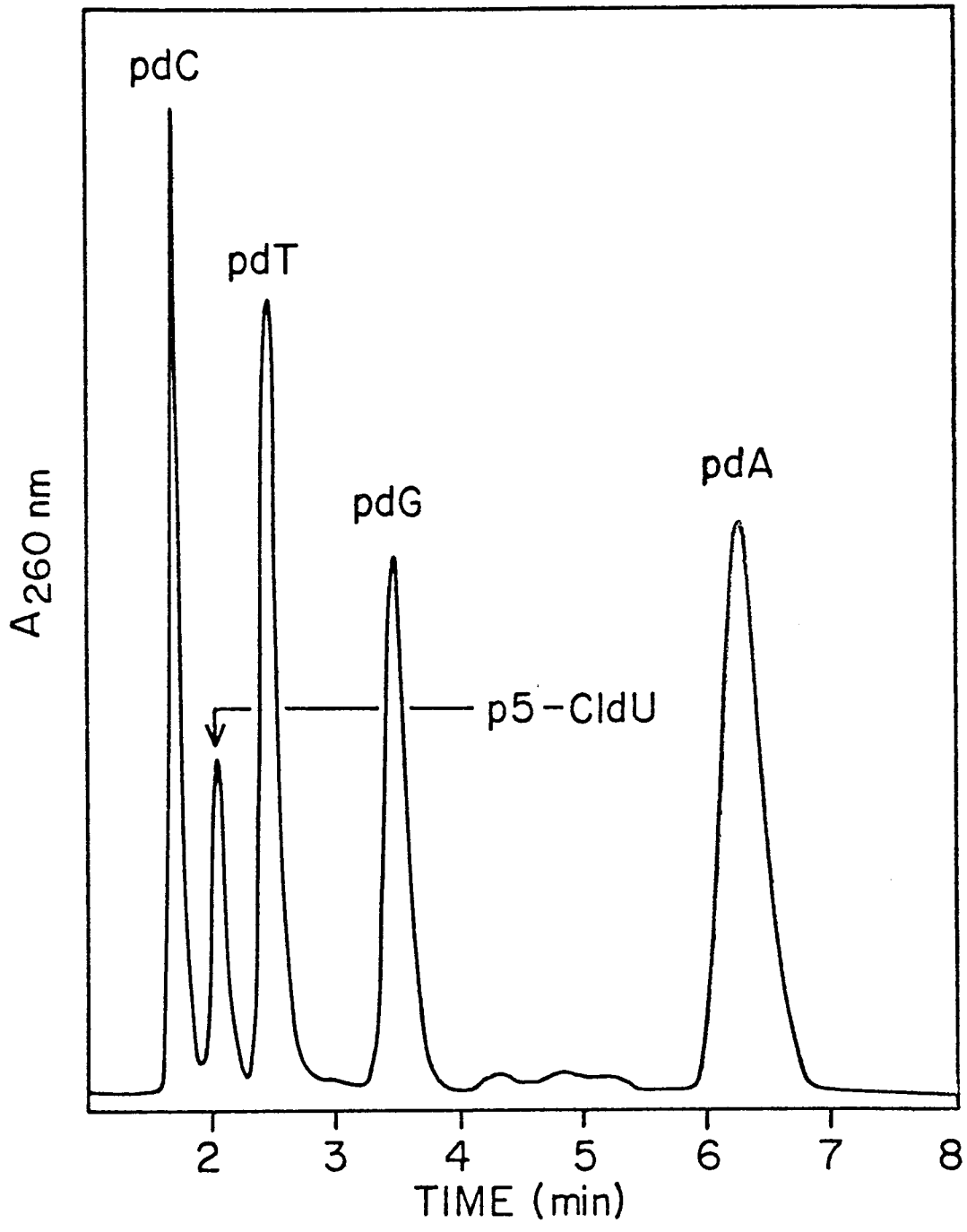



Figure 6.

Figure 7. Chromatography of a CHO BrdU substituted DNA hydrolysate.

A 20 µl sample was injected into a µBondapak C-18 column and eluted with 0.125 M ammonium phosphate pH 6.3 at 2 ml/min.

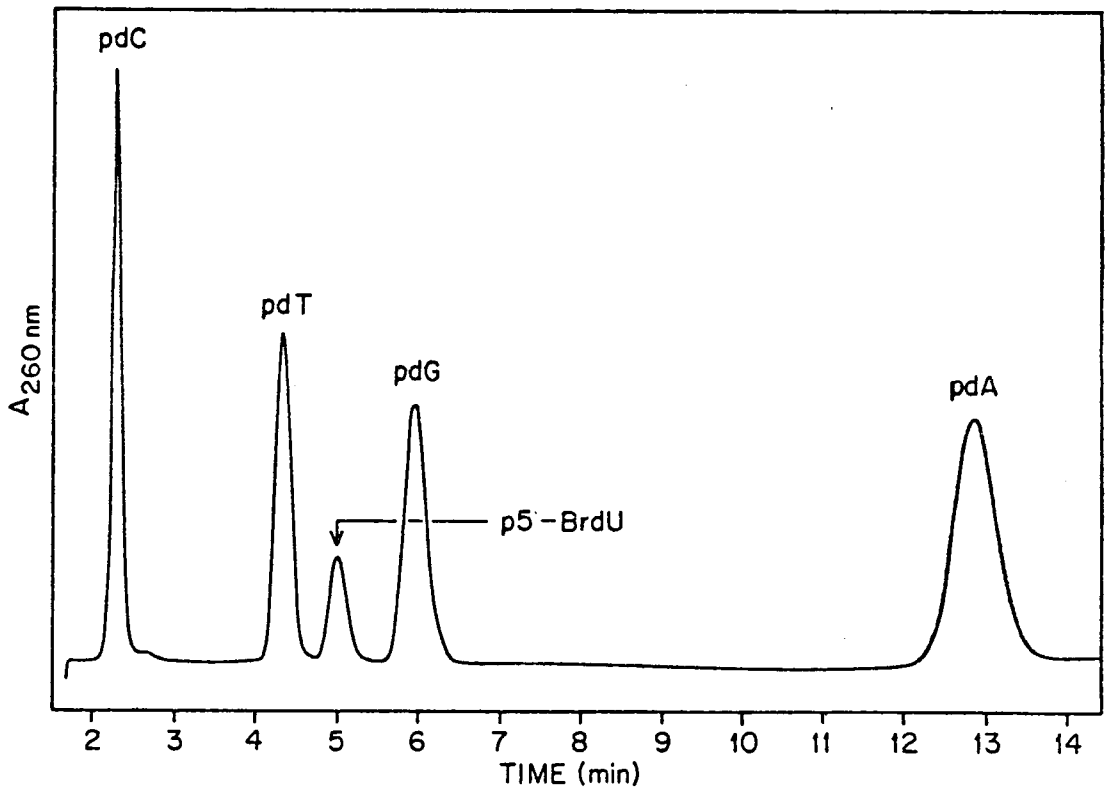



Figure 7.

Hydrolyzed DNA was injected into the HPLC and the amount of radioactivity in approximately 200 fractions was measured. Those fractions corresponding to the nucleoside 5'-monophosphates were totalled and the percentage of each determined. Figure 8 shows the results obtained for both BrdU and CldU incorporation into CHO cellular DNA. These results demonstrate that guanosine, adenosine, and cytidine levels remain essentially constant irrespective of BrdU or CldU incorporation (Figure 8a and 8b). Furthermore, as previously shown with BrdU (Mazrimas and Stetka, 1978), the incorporation of CldU and BrdU increases with increasing extracellular concentration (Figure 8c and 8d) and the amount of thymidine in DNA decreases proportionately. Thus, when BrdU or CldU incorporation is expressed in terms of thymidine substitution, it is readily apparent that levels of BrdU and CldU incorporation into DNA are equivalent (Figure 8e and 8f). Thus, for example, analogue concentrations of 0.5 μM and 20 μM yield 13-19% and 68-71% substitution for thymidine, respectively.

It has been previously demonstrated that at any particular concentration, CldU causes three to five times more SCE than BrdU (DuFrain and Garrand, 1980; Heartlein et al., 1982; Pal et al., 1981; Table 2). In addition, these SCE frequencies are consistent from experiment to experiment. Furthermore, the present studies indicate that DNA incorporation of BrdU and CldU is essentially equal at the concentrations examined. Thus, the relationship between either CldU or BrdU incorporation and SCE can be tested. Figure 9 demonstrates

Figure 8. Nucleotide analysis of BrdU- and CldU-Substituted DNA.

Cultures of CHO cells were incubated for 24 hours in the presence of $^{32}PO_4$, and BrdU or CldU. The DNA composition was obtained as described in Materials and Methods and Results. Panels A and B show levels of adenosine (○), guanosine (▲), and cytidine (△) in the DNA. The composition in DNA of thymidine (●) and nucleotide analogue (□) is shown in panels C and D. Expression of BrdU and CldU incorporation as a percent of thymidine composition in DNA is presented in panels E and F.

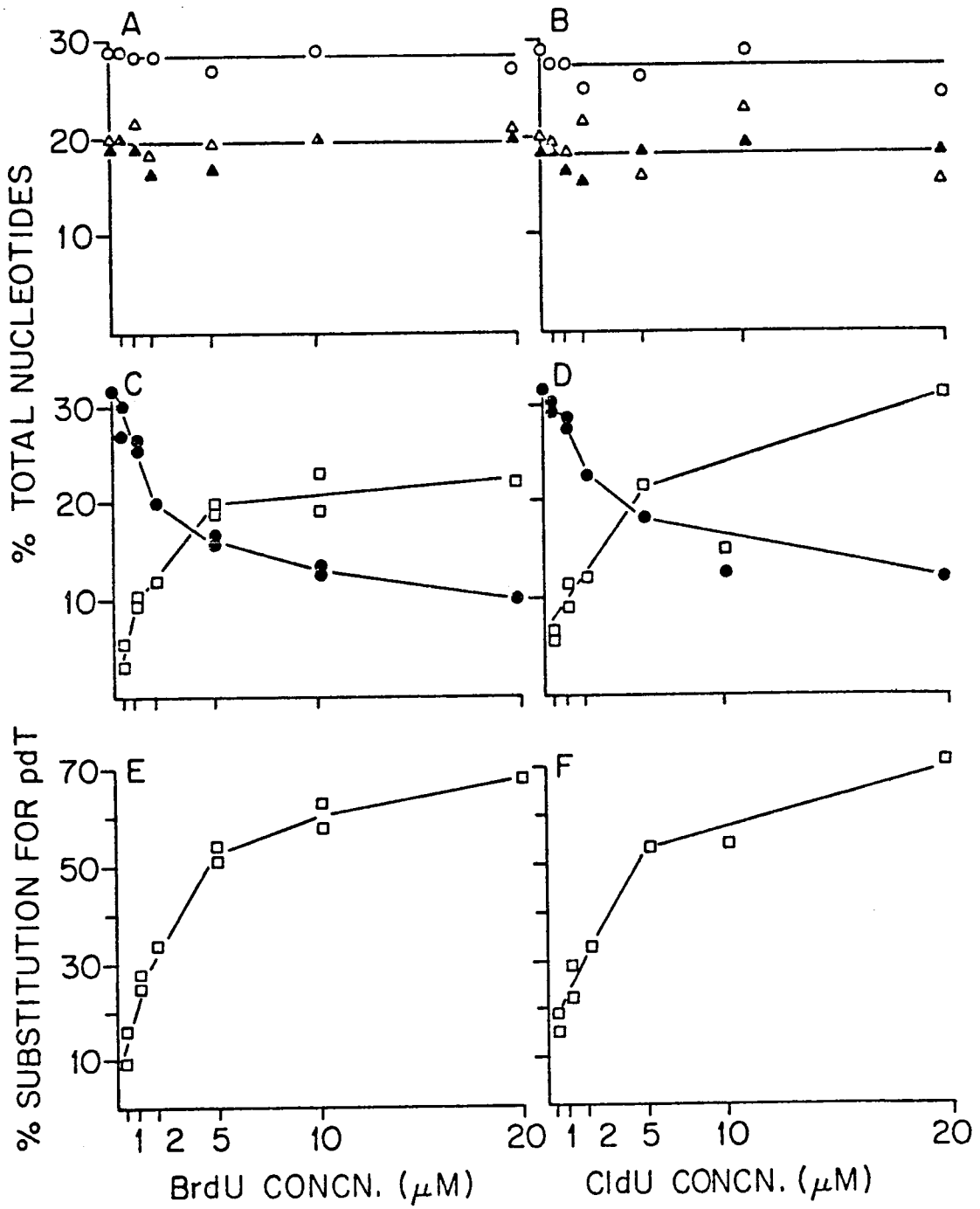

A
B
C
D
E
F
30
20
10
70
50
30
10
% TOTAL NUCLEOTIDES
% SUBSTITUTION FOR pdT
1 2 5 10 20
BrdU CONCN. (μM)
1 2 5 10 20
CldU CONCN. (μM)

Figure 8.

TABLE 2

SCE INDUCTION BY BrdU AND CldU

| | Concentration (μM) | | | | | |
|---|---|---|---|---|---|---|
| | 0.5 | 1.0 | 2.0 | 5.0 | 10.0 | 20.0 |
| BrdU | 8.5 ± 2.1[a] | 9.3 ± 2.6 | 10.5 ± 3.0 | 11.0 ± 4.7 | 14.6 ± 4.1 | 16.7 ± 5.2 |
| CldU | 21.0 ± 5.1 | 33.8 ± 6.9 | 35.0 ± 9.3 | 53.5 ± 10.7 | 56.1 ± 12.3 | 75.6 ± 8.6 |

[a]Mean SCE/metaphase ± standard deviation, from 1-4 experiments.

Figure 9. SCE induction as a function of substitution of BrdU or CldU for thymidine.

SCE values from Table 2 were plotted against percent substitution of BrdU (○) or CldU (●) for thymidine which was obtained from Figure 3, page 7, panels E and F. Lines represent linear fit ($y = a + bx$) to data. For CldU, $b = 0.9665$, $a = 4.112$, $r = 0.9842$. For BrdU, $b = 0.1381$, $a = 5.893$, $r = 0.8541$.

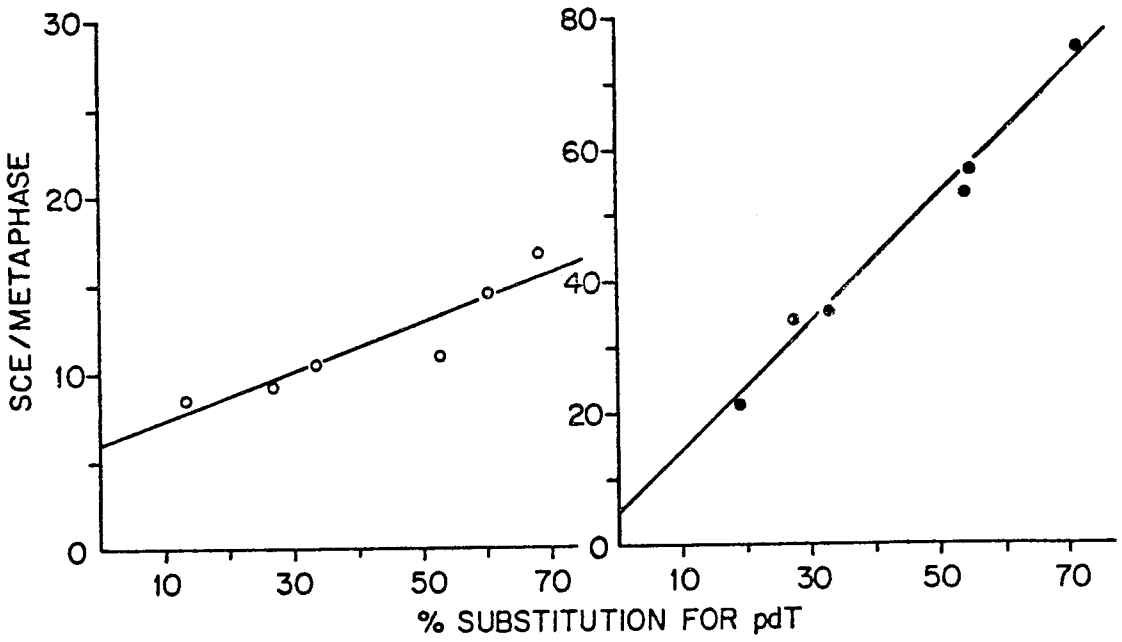

SCE/METAPHASE
30
20
10
0
10
30
50
70
80
60
40
20
0
10
30
50
70
% SUBSTITUTION FOR pdT

Figure 9.

that SCE induction is linearly proportional to substitution of BrdU or CldU for thymidine throughout a concentration range of 0.5-20 μM. Moreover, least squares analysis of the data provide best fit straight lines which, when extrapolated to zero substitution yield spontaneous SCE values of 6.00 and 5.33 SCE per metaphase for BrdU and CldU, respectively.

## Discussion

The present study was initiated primarily to further define possible mechanisms of induction of SCE by CldU and BrdU, since the possibility existed that higher levels of CldU incorporation into DNA could be responsible for the three to five-fold greater frequency of SCE. This does not appear to be the explanation, however, since it is demonstrated here that at equivalent extracellular concentrations, CldU and BrdU were incorporated into the DNA in similar amounts (Figure 8). These experiments focused on low concentrations of BrdU and CldU because it has been suggested that SCE induction at concentrations which yield 100% substitution for thymidine ($\geq$50 μM CldU or BrdU) operates by a different mechanism from that at lower substitution levels (Davidson and Kaufman, 1978; Heartlein et al., 1982). This is supported by the observations that throughout the entire range of 0.5-20.0 μM CldU or BrdU, no mutation induction was observed while at these same concentrations, SCE were induced in a concentration-dependent manner (Heartlein et al., 1982). Furthermore, at low analogue concentrations, SCE appears to be proportional to

substitution for thymidine (Figure 9, page 36) with no detectable substitution for any other of the nucleotides. Therefore, extrapolation of curves of SCE plotted against substitution for thymidine, to zero substitution yield similar values for "spontaneous" SCE with CldU and BrdU. These numbers are a good indication of the occurrence of true spontaneous SCE.

A measure of the relative efficiency of BrdU and CldU to induce SCE can be obtained from the ratio of the slopes of the best fit lines in Figure 9, page 36, (SCE vs. substitution). The ratio obtained is about seven, indicating that CldU induces seven times as many SCE as BrdU at any given substitution level. Therefore, because CldU induces about seven times more SCE than BrdU and is incorporated into DNA similarly, experiments bearing on the mechanism of SCE induction by these analogues can be visualized. That is, it could be the nature of replication of the halogen substituted DNA which results in SCE induction; by some error of DNA replication. Whereas, at high concentrations, incorporation of the analogue in place of cytidine may cause mispairing, mutation and possibly SCE. BrdU mutagenesis has been shown to be concomitant with a precipitous decrease in cellular dCTP pools (Ashman and Davidson, 1981; Ashman et al., 1981) and that mutation caused by BrdU is reversed by deoxycytidine (Davidson and Kaufman, 1978). Undoubtedly, this error of incorporation is more applicable to a mechanism for mutation than SCE, in light of the facts that SCE induction is linearly proportional to substitution for thymidine and large increases in SCE are

seen at concentrations which are non-mutagenic (Heartlein et al., 1982). Therefore, SCE may result from a mechanism quite different from that of mutation. Experiments from this laboratory have further defined this SCE mechanism in terms of replication of the analogue substituted DNA (O'Neill et al., 1983).

Thus, these studies have shown that incorporation of BrdU and CldU into CHO DNA are equivalent at equal extracellular concentrations. Furthermore, SCE increase proportionately with increasing BrdU or CldU substitution for thymidine. Finally, in CHO cells, evidence is provided for a spontaneous level of SCE of about six per cell.

# CHAPTER IV

## SISTER CHROMATID EXCHANGES AND GENE MUTATIONS ARE INDUCED BY THE REPLICATION OF 5-BROMO- AND 5-CHLORODEOXYURIDINE SUBSTITUTED DNA


### Summary

The thymidine (dT) analogue 5-chlorodeoxyuridine (CldU) induces seven to eight-fold more sister chromatid exchanges (SCE) than does 5-bromodeoxyuridine (BrdU) at equal substitution for dT in Chinese hamster ovary cells in culture. This difference facilitates study of the mechanism of induction of SCE by these analogues. Cultures were incubated with either BrdU or CldU for one cell cycle, followed by incubation in the presence of dT alone or BrdU or CldU for the second cell cycle and the SCE frequency determined in M2 cells. The results suggest that the induction of SCE is dependent only on the replication of the analogue substituted DNA during the second cell cycle. Additional studies employed cultures grown in the presence of BrdU or CldU for seven days to obtain mainly bifilarly substituted DNA, followed by two rounds of replication in the presence of dT alone. The SCE frequencies were approximately twice those found in cultures which had undergone the usual two rounds in the presence of the analogue; this is consistent with the replication of twice the amount of analogue substituted DNA. Furthermore, such long term growth in the presence of BrdU or CldU also results in concentration dependent

increases in the frequency of 6-thioguanine resistant mutants, suggesting that gene mutations also result from the replication of analogue substituted DNA.

## Introduction

The thymidine analogue 5-bromodeoxyuridine (BrdU) has been widely used in studies of the induction of sister chromatid exchanges (SCE) as part of the FPG method to achieve differential staining of the chromatids (reviewed in Wolff, 1977). BrdU itself, however, has been shown to induce SCE and the induction appears to be proportional to the amount of BrdU incorporated into DNA (Maxrimas and Stetka, 1978). Another analogue of dT, 5-chlorodeoxyuridine (CldU), also allows differential staining of the chromatids and has been shown to cause increases in SCE frequencies in Chinese hamster ovary (CHO) cells (Heartlein et al., 1982a). This SCE induction is also linearly proportional to incorporation into DNA in place of dT (Heartlein et al., 1982b). Comparison of the activity of BrdU with CldU shows that, at equal substitution, CldU induced seven times more SCE than does BrdU, suggesting that the probability of an SCE event is dependent both on the amount as well as the nature of the analogue in the DNA. In light of these results, this study was initiated to define the mechanism by which SCE are induced by these two analogues. It is also possible to examine the relationship between SCE and mutation induction. Previous studies have shown that both SCE and gene mutation are induced by BrdU and that this induction is suppressed

by deoxycytidine (Davidson and Kaufman, 1978; Davidson et al., 1980). These effects do not appear to be related to incorporation of the analogue into DNA in place of dT (Abersold, 1976; Davidson and Kaufman, 1978), but to deoxyribonucleotide pool imbalance which results in incorporation in place of deoxycytidine (Ashman and Davidson, 1981). CldU also induces mutations over a concentration range similar to that of BrdU (i.e., 50-200 μM), but this does not correlate with the SCE induction (Heartlein et al., 1982a).

## Materials and Methods

### Cell Culture

These studies employed the CHO-$K_1$ cell strain maintained in monolayer culture in Ham's medium F12 (K. C. Biological) containing 5% heat inactivated (50°C, 30 min.) fetal bovine serum (FBS; Sterile Systems) at 37°C in an incubator humidified to 95-100% in an atmosphere of 5% $CO_2$ in air. For subculture, cells were detached by use of 0.05% trypsin (1:300; Gibco) and the cell number determined with a Coulter Counter (Coulter Electronics, model B).

All experiments employed hypoxanthine free medium F12 containing antibiotics and 5% heat inactivated, dialyzed FBS (O'Neill and Hsie, 1979). Medium F12 contains 3 μM dT.

### Determination of SCE Frequency

Cultures were plated at a density of 3 x $10^5$ cells per 100 mm dish and incubated for 20-24 hours. Fresh medium containing the

designated concentration of BrdU or CldU (Calbiochem) as described below was then added. After 25-27 hours incubation, colchicine was added at a final concentration of 10 μM, and the mitotic cells collected 2 hours later. Slide preparation and differential staining was performed as described previously (Heartlein et al., 1982a) using the method of Goto et al. (1978) with the black light exposure time increased to 60 min. In each experiment the frequency of SCE in 25 cells, each containing 20 well differentially stained chromosomes, was determined. The values are given as the mean ± the standard deviation of the mean.

Two types of incubation were employed to obtain differentially stained chromosomes. The usual methodology employs incubation for two cell cycles in the presence of BrdU or CldU and results in M2 chromosomes containing one unifilarly and one bifilarly substituted chromatid. The modifications employed to obtain the data of Table 3 included one cell cycle in the presence of BrdU or CldU, followed by the second cycle also with BrdU or CldU, or in the presence of only dT. This last incubation yields M2 chromosomes containing one unsubstituted and one unifilarly substituted chromatid. In order to obtain the data shown in Figure 10, cultures were grown in the presence of BrdU or CldU for seven days. These cultures were plated at a density of $1 \times 10^5$ cells per 100 mm dish in analogue containing medium and subcultured at the same density after three days growth. After a second three-day growth period, they were subcultured at $3 \times 10^5$ cells per 100 mm dish in analogue containing medium. After

TABLE 3

INDUCTION OF SCE BY REPLICATION OF BrdU OR CldU SUBSTITUTED DNA

| Nucleoside Incorporating During | | SCE/Metaphase[b] | | |
|---|---|---|---|---|
| 1st Replication | 2nd Replication[a] | Exp. 1 | Exp. 2 | Average |
| BrdU | BrdU | 12.4 ± 3.7 | 9.8 ± 3.1 | 11.1 |
| CldU | CldU | 59.6 ± 12.9 | 50.8 ± 11.0 | 55.2 |
| BrdU | dT | 9.8 ± 2.1 | 10.7 ± 2.3 | 10.2 |
| CldU | dT | 51.8 ± 10.5 | 48.7 ± 8.8 | 50.2 |
| BrdU | CldU | 10.7 ± 3.4 | 12.4 ± 2.7 | 11.6 |
| CldU | BrdU | 52.6 ± 12.4 | 49.6 ± 10.2 | 51.1 |

[a]CHO cell cultures were incubated in medium containing 5 μM BrdU or CldU for 12.5 h (1st replication), washed and fresh medium containing no addition (dT) or 5 μM BrdU or CldU added. After 12.5 h incubation (2nd replication), the mitotic cells were collected and stained for SCE determination.

[b]The mean of 25 cells ± the standard deviation. The results of two independent determinations are given.

Figure 10. Induction of SCE by the replication of BrdU or CldU substituted DNA.

Cells were grown for seven days with subculture in medium containing BrdU (panel A, triangles), CldU (panel B, triangles) or no addition (panel A and B, circles). The cultures were then shifted to medium containing no additions (panel A and B, triangles), BrdU (panel A, circles) or CldU (panel B, circles) and incubated for 25 hours. Mitotic cells were collected and SCE frequency determined. The symbols represent the mean number of SCE/metaphase for 25 cells and the vertical lines represent the standard deviation. The dashed line represents the extrapolated spontaneous SCE frequency of approximately six SCE/cell. The open and closed symbols represent two independent determinations.

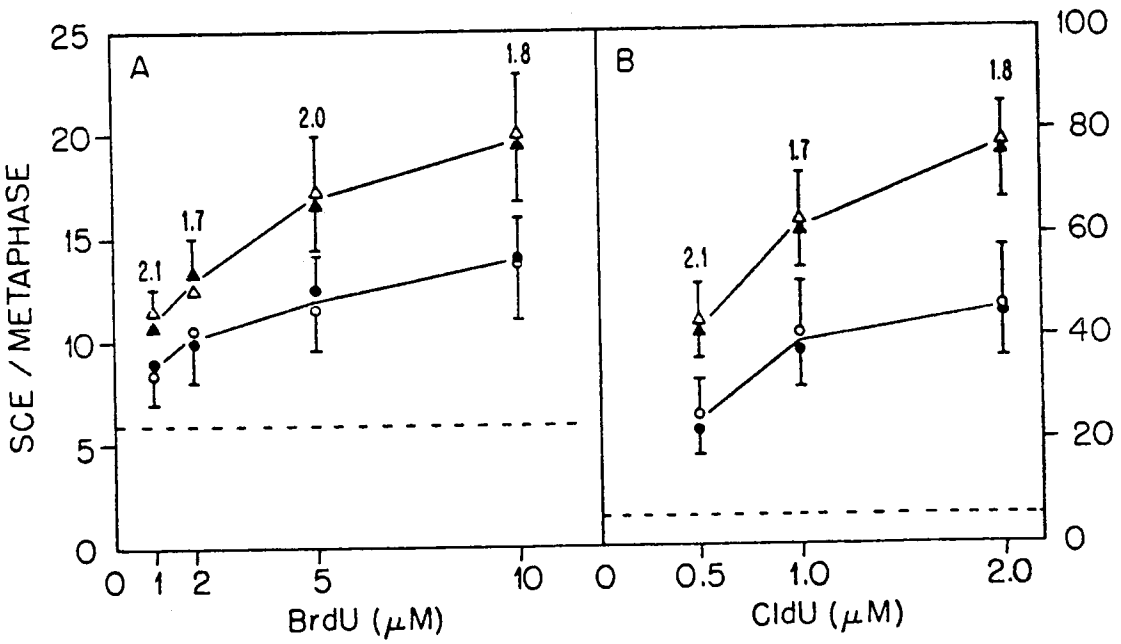

A
B
SCE / METAPHASE
BrdU (μM)
CldU (μM)
2.1
1.7
2.0
1.8
0
1
2
5
10
0.5
1.0
2.0
25
20
15
10
5
0
100
80
60
40
20
0

Figure 10.

24 hours incubation, the cultures were washed and fresh medium containing only dT added. After 25-27 hours incubation, the mitotic cells were collected. This procedure results in M2 chromosomes containing one unsubstituted and one unifilarly substituted chromatid.

Determination of Mutation Induction

Cultures were incubated with different concentrations of BrdU or CldU for 25 hours, and phenotypic expression and selection for 6-thioguanine resistant ($TG^r$) mutants were performed as described previously (Heartlein et al., 1982a). In addition, cultures were grown in the presence of BrdU or CldU for longer time intervals to assess the mutagenicity under conditions of continued replication of analogue substituted DNA. Two experiments (Table 4, 1 and 2) were performed in a manner similar to that used for the SCE determination. Cultures were plated at a density of $2.5 \times 10^5$ cells per 100 mm dish in 10 ml of medium containing BrdU or CldU. After three days growth, $1 \times 10^6$ cells were subcultured in analogue containing medium, and subcultured again three days later. After a 24 hour incubation in analogue containing medium, the cultures were washed and medium containing 0.1% FBS added. Phenotypic expression was accomplished by the arrest method described previously (O'Neill and Hsie, 1979; O'Neill et al., 1981). After 11 days expression, cells were plated for selection of $TG^r$ mutants at $4 \times 10^5$ cells per 100 mm dish in quintuplicate and for cloning efficiency at 200 cells per 60 mm dish in triplicate. Mutant frequency is given as mutants per $2 \times 10^6$

TABLE 4

MUTATION INDUCTION AFTER EXTENDED GROWTH WITH BrdU OR CldU

| | | Concentration (μM)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| A. BrdU | | 0 | 0.25 | 0.5 | 1 | 2 | 5 | 10 |
| 1 | Mutants/2 x $10^6$ cells | 1.6 | -- | 4.4 | 8.8 | 9.0 | 41.0 | 124 0 |
| | Replications x $10^6$ | 13.2 | -- | 13.1 | 13.1 | 12.3 | 11.8 | 9.2 |
| 2 | Mutants/2 x $10^6$ cells | 1.3 | -- | -- | 5.0 | 27.8 | 33.8 | 59.4 |
| | Replications x $10^6$ | 8.7 | -- | -- | 8.2 | 7.6 | 6.4 | 5.0 |
| 3 | Mutants/4 x $10^6$ cells | 1.4 | -- | -- | 17.2 | 16.7 | 21.4 | 58.0 |
| | Replications x $10^6$ | 25.2 | -- | -- | 27.2 | 24.8 | 22.1 | 14.7 |
| B. CldU | | 0 | 0.25 | 0.5 | 1 | 2 | 5 | 10 |
| 1 | Mutants/2 x $10^6$ cells | 1.6 | 8.6 | 36.2 | 94.4 | -- | -- | -- |
| | Replications x $10^6$ | 12.7 | 9.8 | 8.6 | 7.9 | -- | -- | -- |
| 2 | Mutants/2 x $10^6$ cells | 3.6 | 7.4 | 51.1 | 57.3 | -- | -- | -- |
| | Replications x $10^6$ | 13.6 | 13.4 | 11.8 | 10.9 | -- | -- | -- |
| 3 | Mutants/4 x $10^6$ cells | 1.4 | 9.7 | 28.8 | 95.5 | 249.4 | -- | -- |
| | Replications x $10^6$ | 24.1 | 27.1 | 23.1 | 17.6 | 8.1 | -- | -- |

[a]Cultures were grown in medium containing BrdU or CldU for 5-9 days, held arrested for 11 days to allow phenotypic expression and plated for selection of $TG^r$ mutants.

cells, correcting for the cloning efficiency of the cultures at the time of selection. A third experiment (Table 4) employed 150 mm dishes; cultures were plated at 2.5 x $10^5$ cells per dish in 20 ml of analogue containing medium. Fresh analogue containing medium was added after two and three days incubation. On day four, 4 x $10^6$ cells were subcultured in analogue containing medium and transferred 24 hours later to medium containing 0.1% FBS. After 11 days expression, 4 x $10^6$ cells were plated in selection medium at a density of 4 x $10^5$ cells per 100 mm dish. The mutant frequency is given as mutants per 4 x $10^6$ cells. In both types of experiments, the cell number at each subculture was determined to estimate the total number of cell replications during the growth period. The mutuant frequency in the selected cells, the total cell number and the number of replications was used to estimate the mutation rate by dividing the calculated total number of induced mutants by the number of replications. The rate is expressed as mutations per cell per generation x $10^{-7}$. In all these experiments, at the time of the initial plating of the 2.5 x $10^5$ cells in the analogue-containing medium, an aliquot of 1 x $10^6$ cells was plated in analogue-free medium, shifted to medium containing 0.1% FBS after 24 hours incubation, held for 11 days for phenotypic expression and then plated for selection of $TG^r$ mutants. No pre-existing mutants were found, showing that the final mutant frequencies given in Table 4 are a result of mutation induction during the growth period and, therefore, represent mutation rates.

## Results and Discussion

Growth of CHO cells for two rounds of replication in the presence of CldU induces more SCE than are found with equimolar BrdU (Table 3, lines 1 and 2, page 44; Heartlein et al., 1982a). This is not due to differences in incorporation into DNA, as both analogues show similar incorporation at equimolar extracellular concentrations (Heartlein et al., 1982b). Thus the differences in SCE frequency appear to be due to the nature of the halogenated analogue that is incorporated into the DNA in place of dT. Incubation for two rounds of DNA replication results in incorporation of the analogue into DNA during the first round, followed by both replication of the substituted DNA as well as incorporation of the analogue during the second round. In an effort to separate these two aspects of replication in the presence of the analogues, the two step incubations listed in Table 3 were employed. Cells were grown for one round of replication (12.5 hours) in the presence of either BrdU or CldU, washed and then incubated for the second round in the presence of BrdU, CldU or dT alone. An analogue concentration of 5 μM was used because this results in 50-55% substitution for dT with both BrdU and CldU and yields a reasonably analyzable number of SCE with CldU. In addition, the difference in SCE frequency after two rounds with BrdU or CldU is four to five-fold (Table 3, lines 1 and 2) which is a large enough difference to make it possible to detect differential effects. The data of Table 3 show that the SCE frequencies in cells

grown in BrdU and then dT are very similar to those found when both replications are in BrdU, and likewise for CldU and dT compared to two replications in CldU. This suggests that the type of nucleoside incorporated into DNA during the second replication has no effect on the SCE frequency. This is further substantiated by the analogue shift results (Table 3, lines 5 and 6, page 44). In general, the SCE frequency is dependent on the nature of the analogue incorporated into the DNA during the first replication and then replicated in the substituted DNA during the second replication. Since the incorporation of the analogues into DNA during the second replication has little or no effect on the observed SCE frequency (Table 3, lines 5 and 6), the replication of the analogue substituted DNA appears to be the major factor involved in the SCE induction. Replicating 50-55% CldU substituted DNA results in 50-55 SCE/cell. The SCE frequencies at other substitution levels are linearly proportional to the amount of substitution for dT (Heartlein et al., 1982b). Several other studies have addressed the induction of SCE by BrdU and have reached conflicting conclusions. Two reports have suggested that more SCE are induced during two rounds of replication in the presence of BrdU than during one round with BrdU followed by the second round without BrdU (Schvartzman et al., 1979; Gutierrez and Calvo, 1981). However, these studies employed high concentrations of BrdU and the SCE induction may have been complicated by a deoxyribonucleotide pool imbalance (Ashman and Davidson, 1981). Two other studies have reported similar SCE frequencies with both BrdU incubation methods

(Miller et al., 1981; Natarajan et al., 1981), consistent with the data of Table 3, page 44. The results obtained with CldU, however, seem more convincing because of the greater SCE induction obtained with this analogue. The induction of SCE by inhibitors of poly(ADP-ribose) polymerase also appears to be dependent on the replication of analogue substituted DNA (Natarajan et al., 1981).

To test further the proposal that SCE are induced by the replication of BrdU or CldU substituted DNA, CHO cells were grown for seven days, with subculture, in the presence of different concentrations of the two analogues to yield cells that contain predominantly bifilarly substituted DNA. After a change to medium containing no analogue, and a 25 hour incubation to allow two rounds of DNA replication, M2 chromosomes containing one unifilarly substituted and one unsubstituted chromatid were obtained, and differentially stained. These chromsomes resulted from the replication of bifilarly substituted DNA during the first replication and the second replication was of unifilarly substituted DNA, since only dT was incorporated into the newly synthesized DNA during these final two rounds of replication. This contrasts with the more usual two rounds of replication in the presence of the analogues that results in the replication of unsubstituted DNA during the first round and unifilarly substituted DNA during the second round. The former method therefore results in the replication of twice as much analogue substituted DNA as does the latter, more usual method. A two-fold difference in SCE frequency would be anticipated with each

concentration based on the proposal that the SCE are induced by the replication of substituted DNA and are quantitatively related to the amount of substitution. The results of two determinations with BrdU and CldU are shown in Figure 10, page 45. With four concentrations of BrdU (A) and three of CldU (B), incubation for two rounds of replication resulted in SCE frequencies similar to those reported previously (Heartlein et al., 1982a, 1982b). These are represented by the circles in Figure 10A and 10B. Extended growth of cultures in the presence of the analogues, followed by two rounds of replication with dT alone, resulted in higher SCE frequencies with each concentration of both BrdU and CldU (Figure 10A and 10B, triangles) than observed with two replications in the presence of analogue. To test the prediction that a two-fold increase in SCE frequency would result from the replication of twice as much analogue substituted DNA, the spontaneous SCE frequency, i.e., the number of SCE that occur in the absence of analogue substitution, must be subtracted to allow comparison of the induced frequencies at each analogue concentration. This spontaneous frequency is approximately six SCE/cell as calculated by extrapolation of the linear relationship between SCE frequency and per cent substitution to zero analogue substitution, for both BrdU and CldU (Heartlein et al., 1982a). The ratios of induced SCE frequency are listed in Figure 10, page 45, for each concentration of BrdU (A) and CldU (B), and are close to the predicted value of two. These results suggest that the replication of analogue substituted DNA induces an increase in the frequency of SCE, and

that this increase is proportional to the degree of substitution. At equal substitution, the presence of CldU results in seven to eight-fold higher SCE induction than is found with BrdU (Figure 10, page 45, and Heartlein et al., 1982b). These results suggest that the ratio of single to twin SCE in M2 cells as well as the frequency of reciprocal SCE in M3 cells would be dependent on the concentration of analogue employed (Tice et al., 1975; Wolff and Perry, 1975). These concentration effects would be greater for CldU than for BrdU.

When cells were subcultured for the extended period of seven days in the presence of BrdU or CldU to obtain cells with bifilarly substituted DNA, we observed an apparent inhibition of growth relative to the control culture with no analogue. At these concentrations of BrdU and CldU, no growth inhibition or cytotoxicity was observed after incubation for only two cell divisions (San Sebastian et al., 1980; Heartlein et al., 1982a). The effects of this seven day incubation are shown in Figure 11. Both BrdU and CldU show concentration dependent growth inhibition (Figure 11A) and cytotoxicity (Figure 11B), with CldU about five times more effective than BrdU. These effects limited the range of concentrations that could be used in the SCE studies reported in Figure 10, since no mitotic cells were obtained after the shift to analogue free medium with cultures grown for seven days in greater than 10 μM BrdU or greater than 2 μM CldU. Because no growth inhibition and little cytotoxicity was observed during the first three cell divisions in the presence of the analogues,

Figure 11. Growth inhibition and cytotoxicity induced by extended growth in BrdU or CldU.

Cells were grown for seven days with subculture in medium containing BrdU (triangles) or CldU (circles). Based on cell number determinations during the growth period, the number of population doublings was determined and is expressed relative to that of the control culture in panel A for BrdU (triangles; 100% = 10.9 and 11.4 for △ and ▲, respectively). The cloning efficiencies of the cultures at the end of the growth period are expressed relative to the control culture in panel B for BrdU (triangles; 100% = 0.72 and 0.81 for ○ and ●, respectively) and CldU (circles; 100% = 0.72 and 0.88 for △ and ▲, respectively).

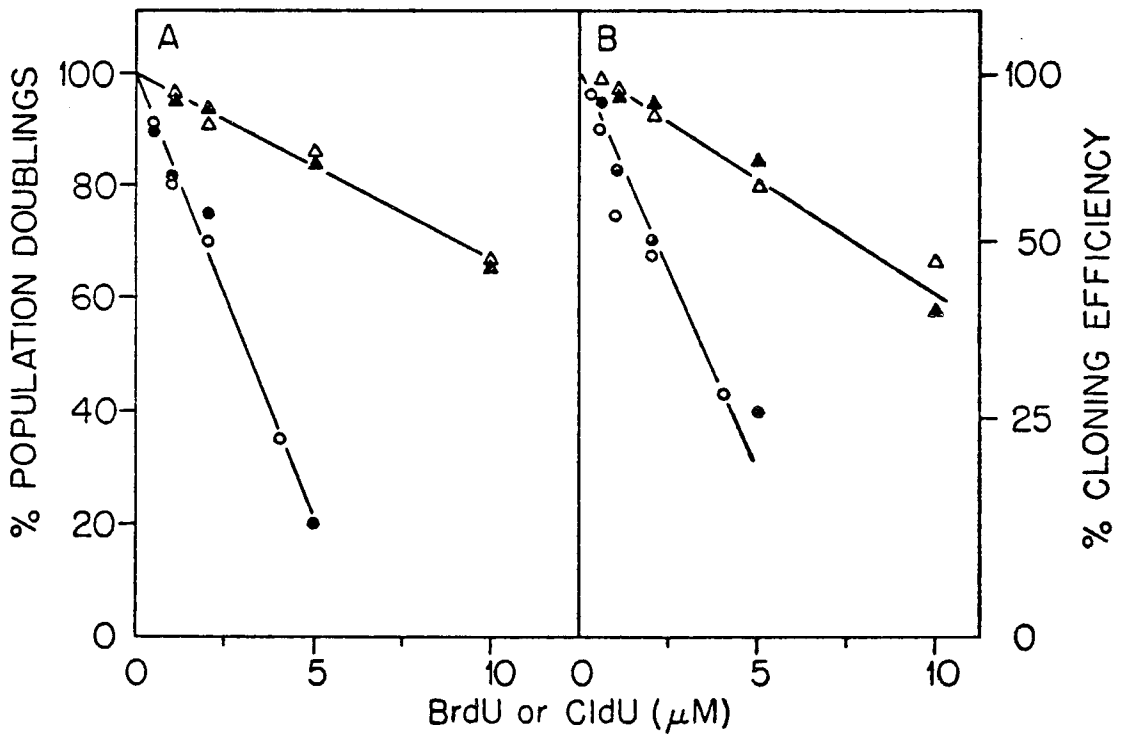

A
B
% POPULATION DOUBLINGS
% CLONING EFFICIENCY
100
80
60
40
20
0
50
25
0
5
10
BrdU or CldU (μM)

Figure 11.

This suggests that growth inhibition and cytotoxicity are due to the replication of analogue substituted DNA. Replication of analogue substituted DNA in this way also induces SCE (Figure 10, page 45) as well as chromosome and chromatid aberrations (data not shown). These observations prompted a study of mutation induction, using resistance to TG as a measure of mutation at the hypoxanthine-guanine phosphoribosyl transferase (hgprt) locus in CHO cells.

Earlier studies have shown that incubation of cells with BrdU or CldU for 25 hours, i.e., two rounds of replication, does not result in mutation induction with concentrations of these analogues less than 50 μM (San Sebastian et al., 1980; Heartlein et al., 1982a). Results of two such experiments are given in Table 5. Mutation induction by BrdU and CldU only at concentrations greater than 50 μM has led to the conclusion that replication of BrdU substituted DNA does not induce mutations through replication errors in mammalian cells (Abersold, 1976; Davidson and Kaufman, 1978) in contrast to results reported for prokaryotic systems (Freese, 1959; reviewed in Kimball, 1978). Mutation induced by higher concentrations of BrdU has been proposed to be due to errors of incorporation of BrdU into DNA in place of deoxycytidine (Ashman and Davidson, 1981). It is clear that BrdU is incorporated into the hgrpt gene during incubation with medium concentrations of 1-10 μM, since subsequent exposure of cultures to black light result in increases in the frequencies of $TG^r$ mutants (Lynn and O'Neill, 1982). However, an alternative explanation for the results of Table 5 might be that the frequency

TABLE 5

MUTATION INDUCTION AFTER 25 H INCUBATION WITH BrdU OR CldU

| Concentration (μM) | Mutant Frequency (Mutants/$10^6$ Cells)[a] | | | |
|---|---|---|---|---|
| | BrdU | | CldU | |
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| 0 | 5.9 | 5.6 | 3.8 | 5.6 |
| 1 | 4.6 | 3.7 | --- | 4.2 |
| 2 | 4.8 | 5.2 | --- | 8.1 |
| 5 | 5.9 | 4.6 | --- | 5.3 |
| 10 | 7.1 | 6.1 | 5.8 | 7.2 |
| 20 | 3.7 | 9.1 | 10.8 | 9.3 |
| 50 | 31.7 | 10.7 | 18.2 | 28.6 |
| 100 | 550.6 | 621.2 | 207.3 | 263.4 |

[a]Cultures were incubated in medium with BrdU or CldU for 25 h, subcultured for phenotypic expression and plated on day 9 for selection of $TG^r$ mutants.

of mutagenic replication errors is small as a consequence of the limited number of replications that occur. Incubation for 25 hours with these analogues result in the replication of dT containing DNA in the first round, that of unifilarly substituted DNA in the second round, and then half unifilarly and half bifilarly substituted DNA in the third round. The analogue substituted DNA is diluted in half during the third and each subsequent replication due to the absence of analogue incorporation after the second round. Mutagenic replication errors might be more sensitively investigated by determination of the mutant frequency after more extended replication of cells containing analogue substituted DNA, in a manner similar to the approach used in the SCE studies of Figure 10, page 45. The results of three such experiments with BrdU and CldU are given in Table 4, page 48. Cultures were grown as described in the Materials and Methods section in the presence of different concentrations of the analogues, held for phenotypic expression in medium containing 0.1% FBS and plated for selection of $TG^r$ mutants (O'Neill et al., 1981). The total number of cell replications was calculated from the increase in cell number during the growth period. The resulting mutant frequencies are given as mutants per number of selected cells, either $2 \times 10^6$ or $4 \times 10^6$, correcting for the cloning efficiencies of the cultures at the time of selection (see Figure 11B). After extended growth in both BrdU (Table 4A, page 48) and CldU (Table 4B), a concentration dependent increase in the frequency of $TG^r$ mutants is observed in three independent determinations, and CldU

appears to be more mutagenic than BrdU. Several observations suggest that these increases in mutant frequency result from the replication of BrdU or CldU-substituted DNA. Cultures plated for selection at the end of the growth period with no time allowed for phenotypic expression showed no increase in mutant frequency. This would be expected because of the length of time necessary for expression of the induced mutant genotype (O'Neill and Hsie, 1979; O'Neill et al., 1981). In addition, there was no increase in mutant frequency in cultures grown for two days in analogue containing medium and then allowed time for phenotypic expression. During this time the cell number increased from $2.5 \times 10^5$ to $2 \times 10^6$ cells, but only the last cell cycle involved the replication of analogue substituted DNA. Further incubation in the presence of the analogue will then result in repeated replication of the substituted DNA. From the data of Table 4, page 48, the mutation rate was estimated, and the average values are presented in Figure 12. The mean mutation rate in control cultures was $7.9 \times 10^{-7}$ mutations per cell per generation found in an earlier fluctuation analysis study (O'Neill et al., 1981). With both BrdU and CldU, concentration dependent increases in the mutation rate are found, with greater increases induced by CldU. A comparison of the slopes of the two lines shown in Figure 12 suggests that, at equimolar concentrations (i.e., approximately equal substitution for dT), the replication of CldU substituted DNA induces eight-fold more mutations to TG resistance than does the replication of BrdU substituted DNA. This is similar to the seven to eight-fold

Figure 12. Increase in mutation rate induced by BrdU or CldU.

The mutation rates were calculated from the data of Table 4, page 48, for cultures grown in the presence of BrdU (△) or CldU (○) and are expressed as mutants/cell/generation x $10^{-7}$. The symbols represent the average of the three determinations. The average spontaneous rates were 6.0 and 9.8 for △ and ○, respectively. In all experiments, at the time of initial plating in the analogues, 1 x $10^6$ cells were held for expression to determine the time zero mutant frequency and no mutants were detected.

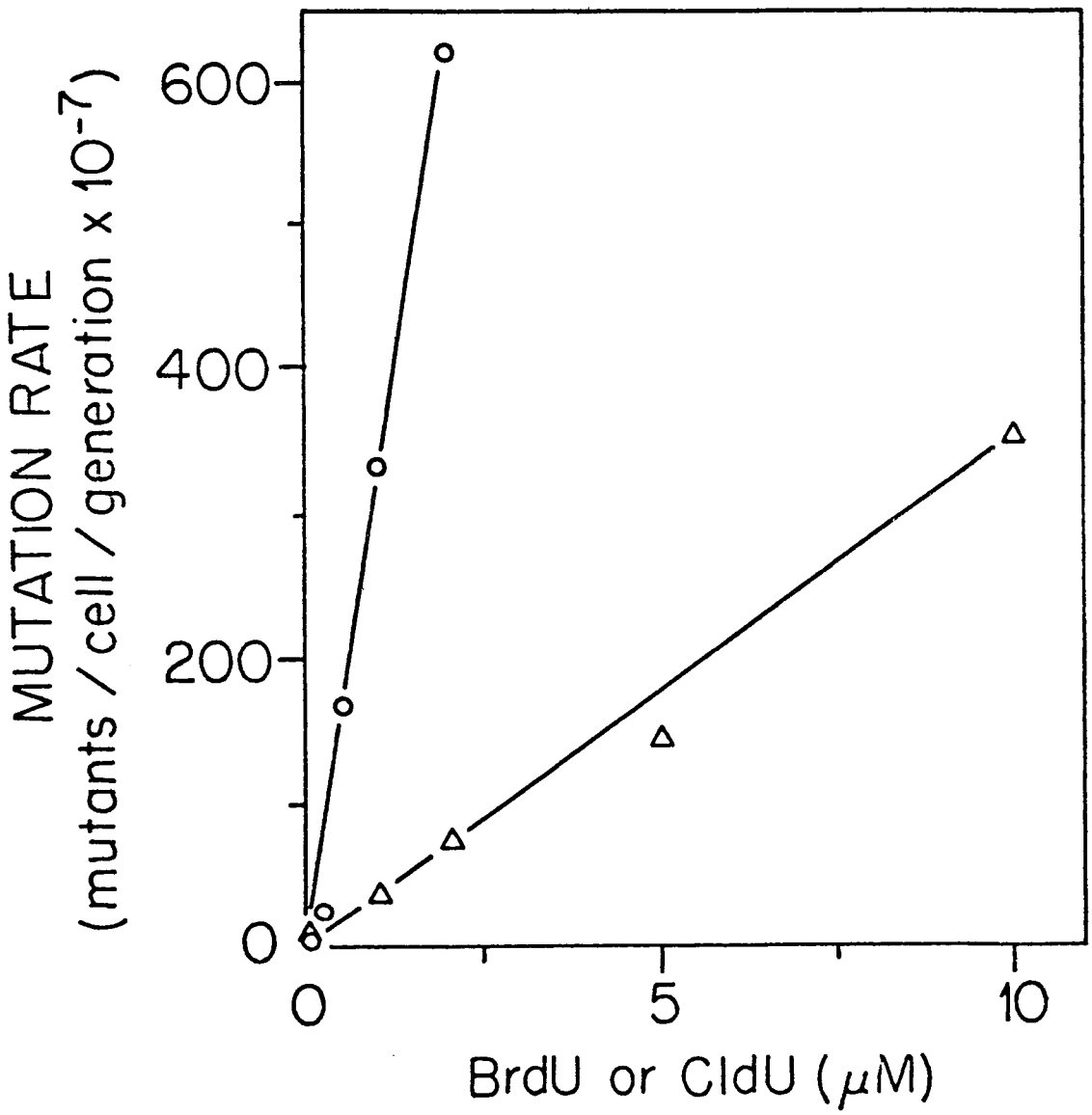

MUTATION RATE
(mutants / cell / generation x $10^{-7}$)
600
400
200
0
0
5
10
BrdU or CldU ($\mu$M)

Figure 12.

greater SCE induction induced with CldU replication as compared to BrdU replication (Figure 10, page 45, and Heartlein et al., 1982b). Therefore, the observation that the ratio of effectiveness of CldU to BrdU is equivalent for both mutation and SCE induction may reflect the involvement of similar mechanisms operating during the replication of analogue substituted DNA. Since both mutations and SCE do occur spontaneously in the absence of analogue substitution, the presence of the analogues in place of dT must cause an increase in the frequency of these events. It remains to be shown whether these are due to the same mechanism, or to different mechanisms which are equally perturbed by the replication of BrdU or CldU substituted DNA.

The results of this study together with previous reports indicate that gene mutations and SCE are induced by these halogenated analogues of dT through errors of both replication and incorporation (Hopkins and Goodman, 1980). The data presented here are consistent with induction through replication errors. Gene mutations are likely to be due to the infrequent mispairing of BrdU or CldU with guanine during DNA synthesis; resulting in base transitions (Freese, 1959). The eight-fold greater mutagenicity of CldU might then be due to an increased probability of such mispairing. The parallel seven to eight-fold higher induction of SCE with CldU replication might be due to a similar mechanism, or to a possible decrease in the rate of DNA replications because of the analogue substitution (Kato, 1980). The greater growth inhibition found with CldU may support this proposal (Figure 11). It was previously reported that the mutagenicity of

BrdU requires higher concentrations of BrdU in the medium, is not correlated with DNA incorporation, and is increased by dT and suppressed by deoxycytidine (Abersold, 1976; Davidson and Kaufman, 1978; San Sebastian et al., 1980). This reported mutagenicity appears to be due to deoxyribonucleotide pool imbalance and the resulting incorporation errors due to misincorporation of BrdU into DNA in place of deoxycytidine (Ashman and Davidson, 1981). The mutagenicity at high concentrations of CldU follows this pattern (Heartlein et al., 1982a). A parallel increase in SCE frequency has been shown with BrdU and this induction is suppressed by deoxycytidine (Davidson et al., 1980). However, the observation that dT does not stimulate SCE induction by BrdU, as well as the small observed increases in SCE frequency at mutagenic concentrations of CldU, suggests the possibility of different mechanisms (San Sebastian et al., 1980; Heartlein et al., 1982a). It is clear, however, that the frequencies of SCE at high concentrations of BrdU and CldU, i.e., greater than 50 μM, are very different, while frequencies of mutants are similar (Heartlein et al., 1982a).

In summary, these results suggest that both SCE and gene mutations are induced by the replication of BrdU and CldU substituted DNA in CHO cells. These results on mutation induction together with earlier studies are consistent with two mechanisms of mutagenicity by these analogues--replication errors as well as errors of incorporation.

# PART II

# THE USE OF INHIBITORS OF DNA REPAIR TO DETERMINE MECHANISMS OF RADIATION-INDUCED CHROMOSOME ABERRATIONS

# CHAPTER V

# AN EXPLANATION OF INTERSPECIFIC DIFFERENCES IN SENSITIVITY TO X RAY-INDUCED CHROMOSOME ABERRATIONS

## Summary

Using 1-β-D-arabinofuranosylcytosine (araC) which is an inhibitor of DNA repair resynthesis, previous studies have shown that the frequency of chromosome-type aberrations is influenced by the rate of repair of araC inhibitable DNA damage. The experiments described here are a further test of this hypothesis and also an attempt to determine if the different sensitivities of lymphocytes of different species to X ray-induced aberrations is related to the rate of repair of DNA damage. Unstimulated lymphocytes from four species were exposed to an X ray dose of 200 rad, and then incubated with araC for 0, 1, 2, 3, or 4 hours. The aberration frequencies increased in all species up to 3-4 hours. It was also clear that the rate of increase was different between species and was approximately proportional to the ratios of X ray-induced aberrations observed in the absence of araC. For example, human lymphocytes are approximately twice as sensitive as rabbit lymphocytes to the induction of aberrations by X rays and the rate of increase of aberrations in the presence of araC was about twice as great in human as rabbit lymphocytes. In addition, using 50, 100, 200, or 300 rad of X rays

and treating human lymphocytes for 0, 1, 2, or 3 hours in araC post-irradiation, we have shown that the rate of increase in aberrations is proportional to the amount of araC inhibitable DNA damage; with a limiting dose at about 50 rad. These results appear to provide a basis for interpreting differences in sensitivities to aberration induction among mammalian species.

## Introduction

Various species of mammals are known to exhibit different sensitivites to X or gamma ray-induced chromosome aberrations (Brewen et al., 1973; Leonard et al., 1982). Extrapolation of results from experimental animals to humans has made the search for an explanation of this differential radiosensitivity of considerable importance. The arm number hypothesis originally proposed by Brewen and colleagues (1973) to predict the sensitivity of mouse lymphocytes (reviewed in Sankaranarayanan, 1976) has received the most attention. Although this hypothesis fit the data for the six species reported by Brewen et al. (1973), it does not appear to be valid for other species. Subsequently, it has been concluded that differences in cellular characteristics such as nuclear volume, DNA content, and effective chromosome arm number cannot explain interspecific radiosensitivity (Leonard et al., 1982). An alternate explanation was therefore required to adequately assess the data.

It has been shown (Sasaki and Tonomura, 1969; Evans and Adams, 1970) that $G_0$ lymphocytes from patients with Down syndrome are more

sensitive to X ray-induced dicentrics than normal cells. An explanation for this increased sensitivity was provided by Preston (1981). The frequency of X ray-induced chromosome aberrations increased more rapidly as a function of the post-irradiation incubation time in araC with Down lymphocytes than normal cells. Since the increase in aberrations in the presence of araC is an indicator of the rate of repair of the X ray-induced DNA damage that can be converted into aberrations, it was concluded that Down cells repaired this damage more rapidly than normal cells. It was further concluded from this and other studies that it was largely the repair of DNA base damages that was involved in the induction of X ray-induced chromosome aberrations. The faster rate of repair of DNA base damages in Down cells compared to normal cells would result in an increased probability of interactions of repairing regions (misrepair) leading to aberrations, and thus give a higher frequency of aberrations in Down cells than normal cells.

These results prompted us to determine if this hypothesis could be extended to explain the different sensitivities to X ray-induced chromosome aberrations of different species of mammals. The experiments to be described were designed to determine if the frequency of chromosome-type aberrations in different species is influenced by the rate of repair of DNA damage that can be converted into aberrations and, in addition, if differences in sensitivity to radiation-induced aberrations among mammalian species is a result of repair rate differences.

The results presented here support these ideas. Using araC as a specific inhibitor of excision repair, we have found that inter-specific radiosensitivity appears to be dependent on repair rate. In addition, we have shown that the rate of repair of araC inhibitable DNA damage that can be converted into aberrations is dependent upon the amount of induced DNA damage, i.e., the radiation dose. Thus, the frequency of X ray-induced aberrations at any particular dose is clearly influenced by the amount of DNA damage that is converted into aberrations, but also by the rate at which it is repaired. This latter fact will be reflected in the non-linear dose response curve obtained for X ray-induced chromosome aberrations. These results also support a mechanism involving base damage in a primary role in the production of chromosome aberrations, at least with X ray doses greater than 50 rad.

## Materials and Methods

Human lymphocyte cultures were established from the buffy coats of freshly drawn heparinised blood samples. Each buffy coat was obtained from a 3 ml blood sample, and was added to 10 ml of Ham's F10 medium (K. C. Biological) containing 10% heat-inactivated fetal bovine serum (Sterile Systems). The cultures were maintained at 37°C in an atmosphere of 5% $CO_2$/95% air. Whole blood cultures from New Zealand White rabbits (Oryctolagus cuniculus), Cotton Top marmosets (Saguinus fuscicollis), and domestic pigs (Sus scrofa)

were also established from freshly drawn heparinised blood samples. Each culture was made by adding 0.5 ml of whole blood to 9.5 ml of Ham's F10 medium (K. C. Biological) containing 10% heat-inactivated fetal bovine serum (Sterile Systems). The cultures were kept at 38°C in an atmosphere of 5% $CO_2$/95% air. All treatments were performed prior to mitogenic stimulation with phytohemagglutinin (PHA).

X-irradiations were with a 320 kVp Gemini Industrial X ray Unit operated at 250 kV and 12 mA with 2.3 mm Aluminum filtration. The dose rate was 100 rad/minute.

In the experiments using different species the appropriate cultures of rabbit, human, marmoset, and pig were treated with 50 μM araC 30 minutes prior to irradiation with 200 rad of X rays in order to equilibrate the nucleotide pools. Post-irradiation incubation with araC was for 1, 2, 3, or 4 hours. In the experiments using different X ray doses, the appropriate cultures of human lymphocytes were treated with 50 μM araC (Sigma) for 30 minutes prior to irradiation with 50, 100, 200, or 300 rad of X rays. Post-irradiation incubation with araC was for 1, 2, or 3 hours. The cultures were then washed twice with Saline G and returned to fresh culture medium. At this time PHA (3%; Wellcome Labs) was added to begin the process of mitogenic stimulation. In addition, there were several different controls: one culture was untreated; one was irradiated with the appropriate dose of X rays, but was not incubated with araC; one was treated with 50 μM araC only. Following two washes with Saline G, PHA was added to each culture. In addition, at this time 100 μM

deoxycytidine (dC, Sigma) was added to the cultures to reverse the inhibitory action of araC. Thus, PHA and dC were added to each culture immediately after the completion of the treastment. The cultures were fixed at 48 hours after the addition of PHA and dC for human and marmoset, at 40 hours for rabbit, and 34 hours for pig. Two hours prior to fixation, colchicine ($1 \times 10^{-7}$ M) was added to each culture. The fixation, slide preparation and staining procedures followed standard methods.

A minimum of 150 cells were analyzed at each treatment, and all classes of chromosome aberrations were recorded.

## Results

In order to test the idea that differences in repair rates reflect differences in radiosensitivity, unstimulated lymphocyte cultures of human, marmoset, pig, and rabbit were held in the presence or absence of araC for 1, 2, 3, or 4 hours following X irradiation. After each treatment dC was added to reverse the inhibitory action of araC. For all four species, a time dependent increase in dicentric frequency was observed (Figure 13). There was little or no effect of araC treatment from 1 to 4 hours in the absence of X rays (data not shown). Furthermore, there was an increase in all classes of chromosome-type aberrations, but most clearly for dicentrics, as previously observed with human lymphocytes (Preston, 1980, 1981). The frequency of dicentrics increased in all species up to about 3-4 hours of post-irradiation incubation in araC. The time-response

Figure 13. The effect of post-irradiation incubation with 50 μm araC for 0, 1, 2, 3, or 4 hours on the frequency of X ray-induced dicentric aberrations in unstimulated lymphocytes of human, marmoset, rabbit, and pig.

The points represent dicentric frequency ± standard error.

□ X rays (200 rad) alone

■ X rays (200 rad) + araC for 1, 2, 3, or 4 hours.

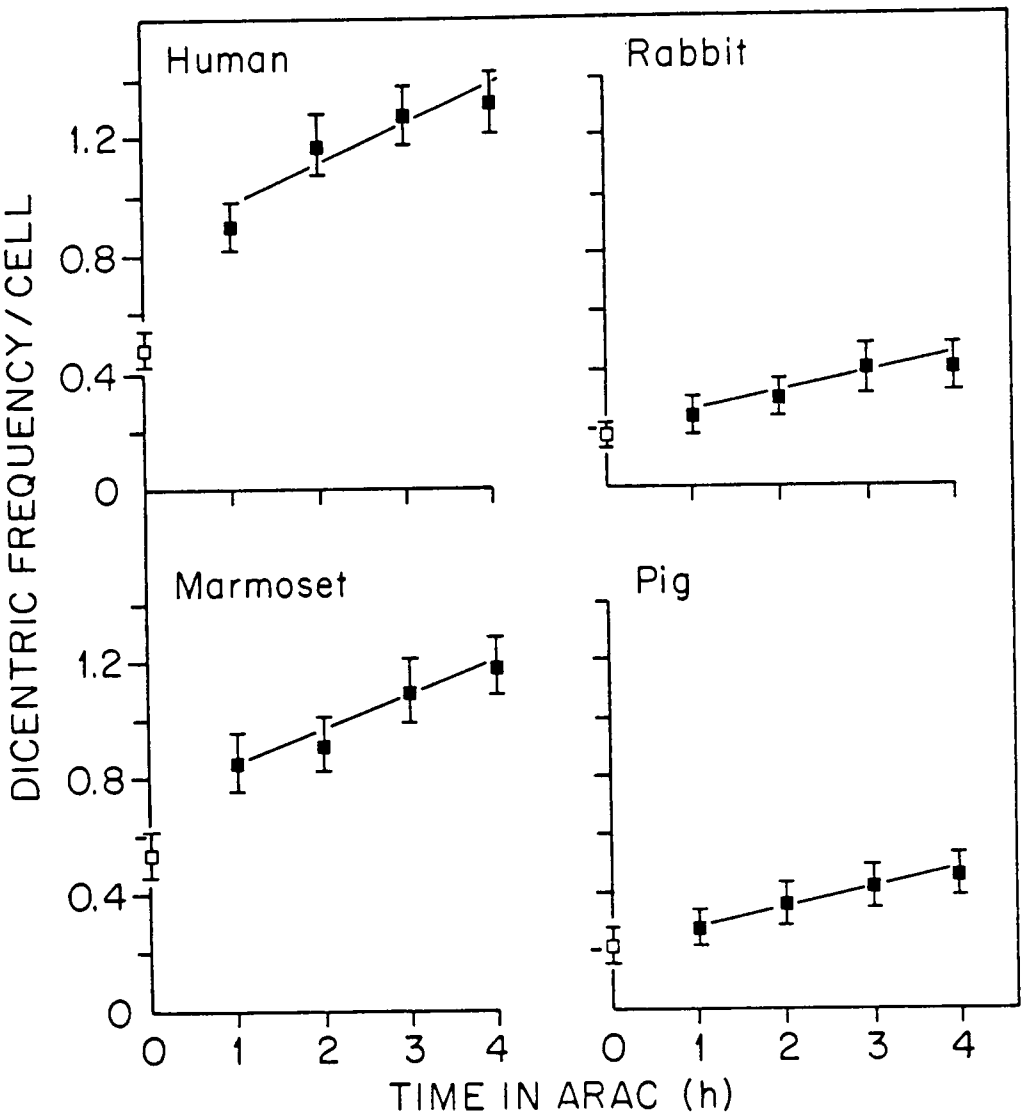

Human
Rabbit
Marmoset
Pig
DICENTRIC FREQUENCY / CELL
0
0.4
0.8
1.2
0
1
2
3
4
TIME IN ARAC (h)

Figure 13.

curves, however, are different among the species. The magnitude of increase in dicentrics with holding cells in araC after irradiation appears to be much larger for human than rabbit, and similarly larger for marmoset than pig (Figure 1, page 2). These differences in araC time-response curves, in addition, appear to be associated with the difference in dicentrics obtained with 200 rad in the absence of araC, i.e., differences in radiosensitivity are correlated with differences in the magnitude of increase in dicentrics obtained by holding cells in araC post-irradiation.

Since these experiments indicated that different rates of repair of DNA damage could account for differences in sensitivity to aberration induction between species, it was also pertinent to determine if different repair rates were also apparent within a species at different amounts of induced DNA damage, i.e., at different doses. Unstimulated human lymphocytes were irradiated with 50, 100, 200, or 300 rad of X rays, and incubated with 50 μM araC for 1, 2, or 3 hours. Deoxycytidine was added to reverse the inhibitory effects of araC. The results for dicentrics are shown in Figure 14. It can be seen that the rate of increase of dicentrics with increasing araC incubation time is highest at 300 rad, and decreases with decreasing dose. At a dose of 50 rad of X rays there is no increase in dicentric frequency when cells were incubated with araC compared to the frequency in cells irradiated alone. The frequency of X ray-

Figure 14. The effect of post-irradiation incubation with 50 μM araC for 0, 1, 2, or 3 hours on the frequency of X ray-induced dicentric aberrations in unstimulated human lymphocytes.

The unconnected point represents the frequency of dicentrics obtained with X rays in the absence of araC. All the points represent the frequency of dicentrics ± standard error.

○ 50 rad

● 100 rad

△ 200 rad

▲ 300 rad.

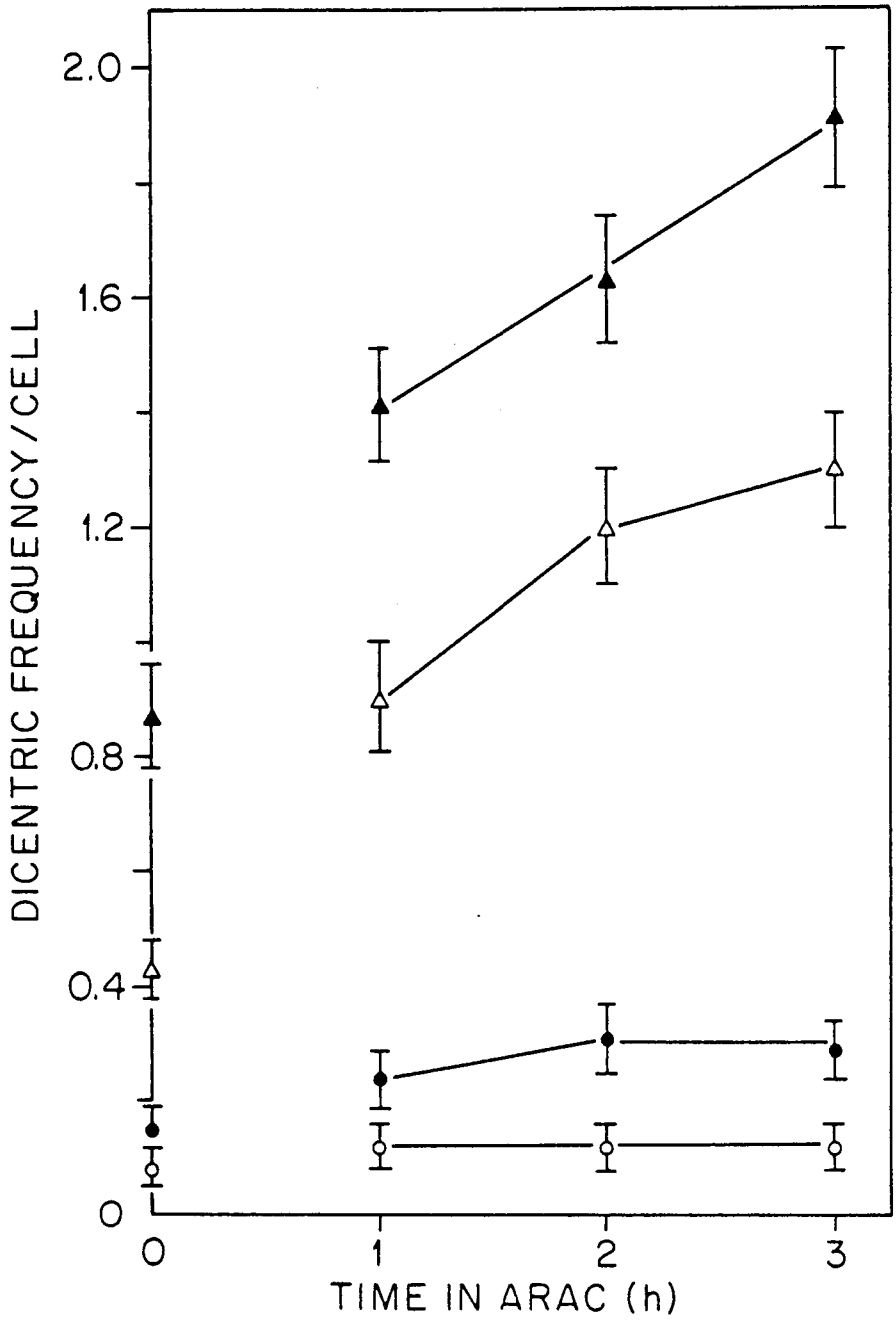



Figure 14.

induced chromosome aberrations is obviously a function of the amount of induced DNA damage, but is also influenced by the rate of repair of damage. These results will be discussed later in terms of the types of DNA damage involved in aberration production at different doses, and the role of different repair rates at different doses on the shapes of dose-response curves.

## Discussion

Various hypotheses have been proposed to explain differences in sensitivity to the induction of chromosome aberrations by chemical and physical agents among mammalian species. The most attention has been given to the arm number hypothesis proposed by Brewen and colleagues in 1973 (for review, see Sankaranarayanan, 1976). This proposal correlated the radiosensitivity of six species to the different effective number of chromosome arms, at several doses of X rays. The hypothesis suggested that the number of chromosome arms available to interchange following DNA damage and repair determined the sensitivity of the cell to radiation-induced chromosome aberrations. Subsequent experiments, however, have shown that this hypothesis does not hold for all species studied, but no additional hypothesis has been proposed. Therefore, in an attempt to provide an alternate explanation for interspecific differences in radiosensitivity to aberration induction, we have examined the effect of inhibition of excision repair by cytosine arabinoside in four mammalian species: human, rabbit, marmoset, and pig. All of these

have somewhat similar DNA contents (Bachman, 1972) and, therefore, presumably contain similar amounts of DNA damage following a particular dose of radiation. Any differences in araC responses should be primarily accountable for by differences in the repair rates of araC inhibitable damage.

It is generally assumed that araC inhibits the resynthesis step of DNA excision repair. Since a large fraction of the total DNA damage induced by X rays is comprised of base damages (the repair of which is araC inhibitable) it is reasonable to assume that the increases of aberration frequency in cells X irradiated and incubated with araC provide an estimate of the rate of repair of DNA base damage that is converted into aberrations. If araC inhibits repair resynthesis by being incorporated into DNA thereby providing a poor primer terminus for further DNA resynthesis (Major, 1982), it will cause the accumulation of gaps in the DNA after X-irradiation. These gaps accumulate primarily as a result of the inhibition of repair of base damage since araC presumably does not inhibit the repair of most directly-induced strand breaks (Hiss and Preston, 1977). The larger the number of these gaps, the greater the probability that two of them misrepair to form an aberration when the inhibitory effect is reversed by dC. Consequently, the greater the length of time cells are held in araC, the higher the frequency of aberrations. This is directly related to the repair rate because the production of gaps by araC is regulated by the rate of incision of base damage. A more rapid repair rate, therefore, will increase the frequency of

gaps per unit time produced from repair inhibition, and thus the probability that two can interact to produce aberrations following reversal of the repair inhibition. In the case of X-irradiation alone, with no araC incubation, the repair rate will affect the probability that two DNA lesions will be repairing coincidentally and thus able to interact to form an aberration. Assuming no change in induced damages, the faster the repair rate the greater the probability of this coincident repair, and the higher the aberration frequency. Evidence in support of the role of the repair of DNA base damage in the production of chromosome aberrations comes in part from experiments in which monoclonal antibody to thymine glycol (a major radiolysis product) was used to show the temporal loss of antibody reactive sites in DNA which contained this base damage (Leadon and Hanawalt, 1983). The majority of radiation-induced thymine glycols are removed in about 2 hours, and this associates well with our findings that the maximum increase in dicentric yield with post-irradiation incubation in araC occurs at 2-3 hours. Therefore, inhibition of the repair of base damage seems to be associated with the increase in dicentrics as a result of incubation in araC following X-irradiation.

Previous studies (Preston, 1981) have used an experimental approach similar to the design described in this report to study the differences in radiosensitivity between Down syndrome cells and normal human cells. For example, the frequency of dicentric chromosomes with 200 rad of X ray in the absence of araC in Down cells is about

0.60 whereas in normal cells the frequency is 0.45. Down cells are therefore about 1.5x more sensitive to X ray-induced dicentrics than normal human cells. If cells are held in araC following X-irradiation, the repair rates of DNA damage converted into aberrations can be estimated. It was found that Down cells reach the same frequency of dicentrics as in normal cells about 1.5 times faster. For example, it takes Down cells one hour to reach a frequency of dicentrics of 1.3, whereas it takes normal cells about 1.5 hours to reach the same frequency of 1.3. Therefore, the rate of repair of DNA damage converted into aberrations is greater in Down as compared to normal cells and can account for the observed difference in sensitivity to X rays.

The results of these experiments prompted us to examine the difference in X ray-induced aberrations among various mammalian species using a similar experimental approach to that with the Down vs. normal lymphocytes. Therefore, using 200 rad of X rays and incubating in araC from 1-4 hours after irradiation, we have reported here that the frequency of dicentrics increase in all species up to about 3-4 hours in araC (Figure 13). The magnitude of increase, however, is different among the species and seems to be reflected in the degree of increase in dicentrics that were generated by holding the cells in araC after X-irradiation. These differences in araC responses may reflect differences in repair rates of DNA damage that can be converted into dicentrics. At the dose of 200 rad, for example, human lymphocytes are approximately twice as

sensitive to X ray-induced dicentrics as rabbit lymphocytes (Figure 13, page 72). A rough correlation can be made between this increase in sensitivity to dicentric induction in the absence of araC to a much more pronounced araC response with human compared to rabbit.

If it is assumed that araC inhibits the resynthesis of excised damaged DNA (Major et al., 1982), then the increased araC response during the time following X ray treatment may be indicative of an increased repair rate in human lymphocytes relative to rabbit lymphocytes. Human cells are, therefore, repairing damage that can be converted into dicentrics more efficiently than rabbit lymphocytes. This also accounts for the difference in radiosensitivity. A similar pattern is seen with both the marmoset and pig, where a difference in radiosensitivity is reflected in a difference in repair rate (the higher radiosensitivity is associated with the higher repair rate).

In addition to the demonstration that interspecific differences in sensitivity to X rays may result from repair rate differences, the above experiments reinforce the notion that with X rays, base damage is the major component of DNA damage involved in aberration formation. This is further made apparent by the results shown in Figure 14. Because araC does not inhibit the rejoining of directly-induced strand breaks, the dose dependent increase in the rate of dicentric induction would seem to be due to the inhibition of base damage repair, or inhibition of some other excisable radiogenic lesion (Figure 14). In addition, an association can be made between

the amount of DNA base damage (proportional to X ray dose), its repair inhibition by araC, and the rate of induction of dicentric chromosome aberrations. Since the slope of the curve of dicentric frequency as a function of araC incubation time increases with increasing X ray dose (Figure 14), it appears that the rate of repair of DNA damage that is converted into aberrations increases with increasing X ray dose. At 50 rad the amount of randomly dispersed base damage is low and, therefore, the probability is low that there will be DNA damages that are being coincidentally repaired in time and space (within the rejoining distance) that can interact to produce aberrations. Thus, it would be expected that there would be little or no increase in dicentric frequency with post-irradiation incubation in araC following 50 rad. This is what was observed as shown in Figure 14.

These results can be utilized in the interpretation of the experiments conducted with the different species (Figure 13, page 72). In these experiments, the amount of DNA damage in the cells from the different species will be quite similar since a single X ray dose was used. As suggested here, the interactive effect of X rays and araC on dicentric induction is dependent both on the amount of DNA damage and the repair rate. Therefore, the simplest explanation for the different araC responses among the species, where DNA damage is constant, is that there are different repair rates of araC inhibitable X ray damage. These different repair rates,

therefore, appear to explain differential interspecific sensitivity to the induction of chromosome aberrations by X rays.

# CHAPTER VI

## THE EFFECT OF 3-AMINOBENZAMIDE ON THE FREQUENCY OF X RAY OR NEUTRON-INDUCED CHROMOSOME ABERRATIONS IN CYCLING OR NON-CYCLING HUMAN LYMPHOCYTES


### Summary

3-Aminobenzamide (3-AB) is a potent inhibitor of poly(ADP-ribose) synthesis in mammalian cells. It was found to cause a two-fold increase in dicentric frequency following X-irradiation of 9-18 hour PHA stimulated human lymphocytes, while 3-AB by itself had no effect on aberration frequency. In contrast to previously reported data, however, our results indicate that 3-AB does not increase the frequency of aberrations following either neutron or X ray exposure of unstimulated ($G_0$) human lymphocytes. Although 3-AB incubation after X ray exposure in $G_1$ caused a large increase in dicentrics, there was no effect of 3-AB incubation following neutron exposure in $G_1$. Although the implications of these experiments are presently uncertain, they do, however, suggest the importance of cycling cells for 3-AB to exert its enhancement effect, presumably on some step of DNA repair. Furthermore, these data support the hypothesis that there are different mechanisms of chromosome aberration induction with fission nuetrons and X rays (at X ray doses above 50 rad).

## Introduction

3-Aminobenzamide, an analogue of nicotinamide adenine dinucleotide (NAD), is a potent inhibitor of poly(ADP-ribose) synthesis in mammalian cells. The mechanism of inhibition is that 3-AB competes with NAD, the normal substrate for poly(ADP-ribose) polymerase. Although the specificity of inhibition has come into question because 3-AB may affect other metabolic processes (Milam and Cleaver, 1984), it remains a useful tool in the examination of the role of poly(ADP-ribose) in cellular metabolism, since it is an effective inhibitor and the most specific of the poly(ADP-ribose) inhibitors (Mandel, 1982).

In recent years, a number of reports have suggested that poly(ADP-ribose) is involved in several diverse, and seemingly conflicting, cellular metabolic processes (for reviews see Mandel, 1982; Lindahl, 1982; Sugimura, 1983). Briefly, the polymer has been shown to play a regulatory role in gene expression and differentiation (Nishio et al., 1983; Tanuma et al., 1983), modulation of enzyme activities (Creissen and Shall, 1982; Ferro and Olivera, 1984), chromatin structure (Wong et al., 1983) and DNA repair (Lindahl, 1982). The frequencies of sister chromatid exchanges (Morgan and Cleaver, 1982) and chromosome aberrations (Natarajan et al., 1982) have also been reported to be affected by inhibition of poly(ADP-ribosyl)ation.

The results of Natarajan et al. (1982) prompted us to examine further the mechanism of aberration formation with 3-AB following

X-irradiation. Results of recent experiments (Preston and Heartlein, unpublished) with the excision repair resynthesis inhibitor cytosine arabinoside (araC) have suggested that there are different mechanisms of aberration induction by fission neutrons and X rays (at X ray doses above 50 rad). The difference in mechanisms stems from the difference in the spectrum of DNA damage caused by these two types of radiation.

The use of an inhibitor such as 3-AB, which seems to affect a later step than resynthesis in excision repair (Lindahl, 1982) could provide additional evidence for these different mechanisms of aberration formation. Furthermore, the fact that poly(ADP-ribose) may play a regulatory role in the temporal expression of enzymes that appear to be involved in DNA repair and chromatin structure [DNA ligase (Ohashi et al., 1983; Creissen and Shall, 1982), topoisomerase (Ferro and Olivera, 1984), for example], allowed a consideration to be made of the effect of inhibition of poly(ADP-ribose) synthesis in different cell cycle stages on the induction of chromosome aberrations.

The initial results of the present experiments were somewhat unexpected in that we could not demonstrate an effect of 3-AB on aberration frequency after X-irradiation of unstimulated human lymphocytes. These results are contrary to previously reported data (Natarajan et al., 1982). It was found, however, that treatment with 3-AB following X-irradiation could cause a large increase in dicentrics compared to that with X-irradiation alone provided the

cells were treated in the $G_1$ stage of the cell cycle. No effect on aberration frequency was observed when 3-AB treatment following irradiation of $G_0$ or $G_1$ cells with fission neutrons. This evidence supports the hypothesis that the mechanism of aberration formation is different for fission neutrons and X rays.

## Materials and Methods

### Unstimulated Lymphocytes ($G_0$ Irradiated)

Lymphocyte cultures were established from the buffy coats of freshly drawn heparinized human blood samples. Each buffy coat was obtained from a 3 ml blood sample, and was added to 10 ml of Ham's F10 medium (K. C. Biological) containing 10% heat-inactivated fetal bovine serum (Sterile Systems). All treatments were performed prior to the addition of phytohemagglutinin (PHA).

X-irradiations were performed with a 320 kVp Gemini Industrial X Ray Unit operated at 250 kV and 12 mA with 2.3 mm Aluminum filtration. The dose rate was 100 rad/minute. Fission neutrons (1.2-1.3 MeV) were generated by the Health Physics Research Reactor, Oak Ridge National Laboratory. The dose rate was about 4 rad/minute.

The appropriate cultures were treated with 5 mM 3-Aminobenzamide (Sigma) for 30 minutes prior to irradiation with 200 rad of X rays. Post-irradiation incubation with 3-AB was for 1, 2, or 6 hours. The cultures were then washed twice with Saline G and returned to fresh culture medium. At this time PHA (3%; Wellcome Labs) was added to begin the process of mitogenic stimulation. In

addition, there were several different controls: one culture was untreated; one was irradiated with 200 rad of X rays, but was not incubated with 3-AB; one was treated with 5 mM 3-AB for different periods of time. Following two washes with Saline G, PHA was added to each culture. Thus, PHA was added to each culture immediately after the completion of the treatment, and each was fixed 48 hours after the addition of PHA. The cultures were incubated at 37°C in an atmosphere of 5% $CO_2$/95% air. Two hours prior to fixation, colchicine ($1 \times 10^{-7}$ M) was added to each culture. The fixation, slide preparation and staining procedures followed standard methods.

The cells irradiated with fission neutrons were handled in the same way as those that were X-irradiated, except that they were maintained at 37°C in a portable incubator during transportation to the reactor. The neutron doses were 24 and 28.3 rad for the experiments reported.

A minimum of 100 cells was analyzed at each treatment, and all classes of chromosome aberrations were recorded.

Stimulated Lymphocytes ($G_1$ Irradiated)

The cultures to be treated in $G_1$, following the initiation of mitogenic stimulation with PHA; were established in the same way as described for unstimulated lymphocytes above. Cells were irradiated 3 to 24 hours after the addition of PHA. In those cultures incubated with 3-AB after irradiation, the 3-AB (5 mM) was added one-half hour prior to irradiation. The post-irradiation incubation in the presence

3-AB was two hours. All cells were washed twice with Saline G before being returned to fresh culture medium. The cultures were fixed 48 hours after the addition of PHA irrespective of the time of treatment, following a two hour colchicine treatment. The cell fixation and slide preparation procedures followed standard methods.

A minimum of 150 cells were analyzed at each treatment, and all classes of chromosome aberrations were recorded.

## Results

When $G_0$ human lymphocytes were irradiated with 200 rad of X rays and incubated with 5 mM 3-AB for 2 to 6 hours, the aberration frequency was no higher than that for cells that were irradiated only. The results of two experiments are shown in Table 6. A similar result was also found following irradiation of $G_0$ lymphocytes with 24 rads of fission neutrons. The frequency of chromosome aberrations was the same whether or not the cultures were incubated with 3-AB (1 or 2 hours) after irradiation (Table 7).

It has been suggested that poly(ADP-ribose) metabolism changes throughout the cell cycle and especially in $G_1$ (Surowy and Berger, 1982) and it is known that certain enzymatic activities also change as a function of the cell cycle. It seemed pertinent, therefore, to study the effect of 3-AB inhibition of poly(ADP-ribosyl)ation on chromosome aberration production throughout $G_1$.

The experiments were performed by treating lymphocyte cultures with PHA in order to stimulate T-cells to enter the cell cycle

TABLE 6

THE EFFECT OF 3-AB INCUBATION ON THE FREQUENCY OF X RAY-INDUCED ABERRATIONS IN $G_0$ HUMAN LYMPHOCYTES

| | Number of Cells | Dicentric Frequency[a] | Ring Frequency | Deletion Frequency |
|---|---|---|---|---|
| Experiment I | | | | |
| Untreated | 100 | 0.00 | 0.00 | 0.01 ± 0.01 |
| 3-AB | 100 | 0.01 ± 0.01 | 0.00 | 0.02 ± 0.02 |
| X Ray | 200 | 0.45 ± 0.05 | 0.07 ± 0.02 | 0.29 ± 0.04 |
| X Ray + 3-AB (2 Hours) | 200 | 0.54 ± 0.05 | 0.11 ± 0.02 | 0.28 ± 0.04 |
| Experiment II | | | | |
| Untreated | 100 | 0.00 | 0.00 | 0.07 ± 0.02 |
| 3-AB | 100 | 0.00 | 0.00 | 0.01 ± 0.01 |
| X Ray | 100 | 0.64 ± 0.08 | 0.11 ± 0.03 | 0.34 ± 0.06 |
| X Ray + 3-AB (6 Hours) | 144 | 0.60 ± 0.06 | 0.15 ± 0.03 | 0.47 ± 0.06 |

[a]Frequency of aberration type ± standard error.

TABLE 7

THE EFFECT OF 3-AB INCUBATION ON THE FREQUENCY OF NEUTRON-INDUCED CHROMOSOME ABERRATIONS IN $G_o$ HUMAN LYMPHOCYTES

| | Number of Cells | Dicentric Frequency[a] | Ring Frequency | Deletion Frequency |
|---|---|---|---|---|
| Untreated | 150 | 0.00 | 0.00 | 0.01 ± 0.01 |
| 3-AB | 100 | 0.00 | 0.00 | 0.01 ± 0.01 |
| Neutron | 100 | 0.24 ± 0.05 | 0.05 ± 0.02 | 0.17 ± 0.04 |
| Neutron + 3-AB (1 Hour) | 100 | 0.23 ± 0.05 | 0.03 ± 0.02 | 0.15 ± 0.04 |
| Neutron + 3-AB (2 Hours) | 100 | 0.27 ± 0.05 | 0.03 ± 0.02 | 0.18 ± 0.04 |

[a]Frequency of aberration type ± standard error.

(from about 3 to 16 hours after PHA, most of the cells are in $G_1$ of the cell cycle). The cultures were treated with 200 rad of X rays 10 hours after the addition of PHA, a time when the majority of cells are in $G_1$, and then incubated in the presence of absence of 3-AB for 2 hours. The results are shown in Table 8. A striking increase in aberration frequency is seen in all classes of chromosome-type aberrations, but it is most striking for dicentrics. The frequency of dicentrics increase nearly two-fold after a 2 hour treatment with 3-AB following X-irradiation. This result is in contrast to experiments conducted on cells in $G_0$ where no enhancement of X ray-induced aberrations was seen with 3-AB.

A more detailed study of this presumptive $G_1$ effect is shown in Figure 15. In these experiments, all cultures were treated with PHA and then at various times afterwards they were X-irradiated with 200 rad, and incubated with or without 5 mM 3-AB for 2 hours. All the cultures were then fixed at 48 hours after PHA addition. For simplicity, and since the major effect is on dicentric induction, only the frequency of dicentrics is plotted in Figure 15.

From Figure 15 it is evident that there is an increase in dicentric frequency which seems to begin about 9 hours after PHA stimulation and continues to 18 hours, after which the level of dicentrics decrease to the X ray control level. From 9 to 18 hours after PHA, the majority of cells are in $G_1$, since this first cell cycle is rather synchronous. Because the frequency of chromatid-type aberrations was significantly increased at the 18 and 24 hour

TABLE 8

THE EFFECT OF 3-AB INCUBATION ON THE FREQUENCY OF X RAY-INDUCED CHROMOSOME ABERRATIONS IN $G_1$ HUMAN LYMPHOCYTES

| | Number of Cells | Dicentric Frequency[a] | Ring Frequency | Deletion Frequency |
|---|---|---|---|---|
| Untreated | 150 | 0.00 | 0.00 | 0.05 ± 0.02 |
| 3-AB | 150 | 0.01 ± 0.01 | 0.00 | 0.05 ± 0.02 |
| X Ray | 150 | 0.49 ± 0.06 | 0.13 ± 0.03 | 0.33 ± 0.05 |
| X Ray + 3-AB | 150 | 1.03 ± 0.08 | 0.22 ± 0.04 | 0.47 ± 0.06 |

[a]Frequency of aberration type ± standard error.

Figure 15. The effect of post-irradiation incubation with 5 mM 3-AB (2 hours) on the frequency of X ray-induced dicentric aberrations in $G_1$ human lymphocytes.

Cells were irradiated at different times after the addition of PHA.

○ X rays (200 rad) + 3-AB for 2 hours

■ X rays (200 rad) alone.

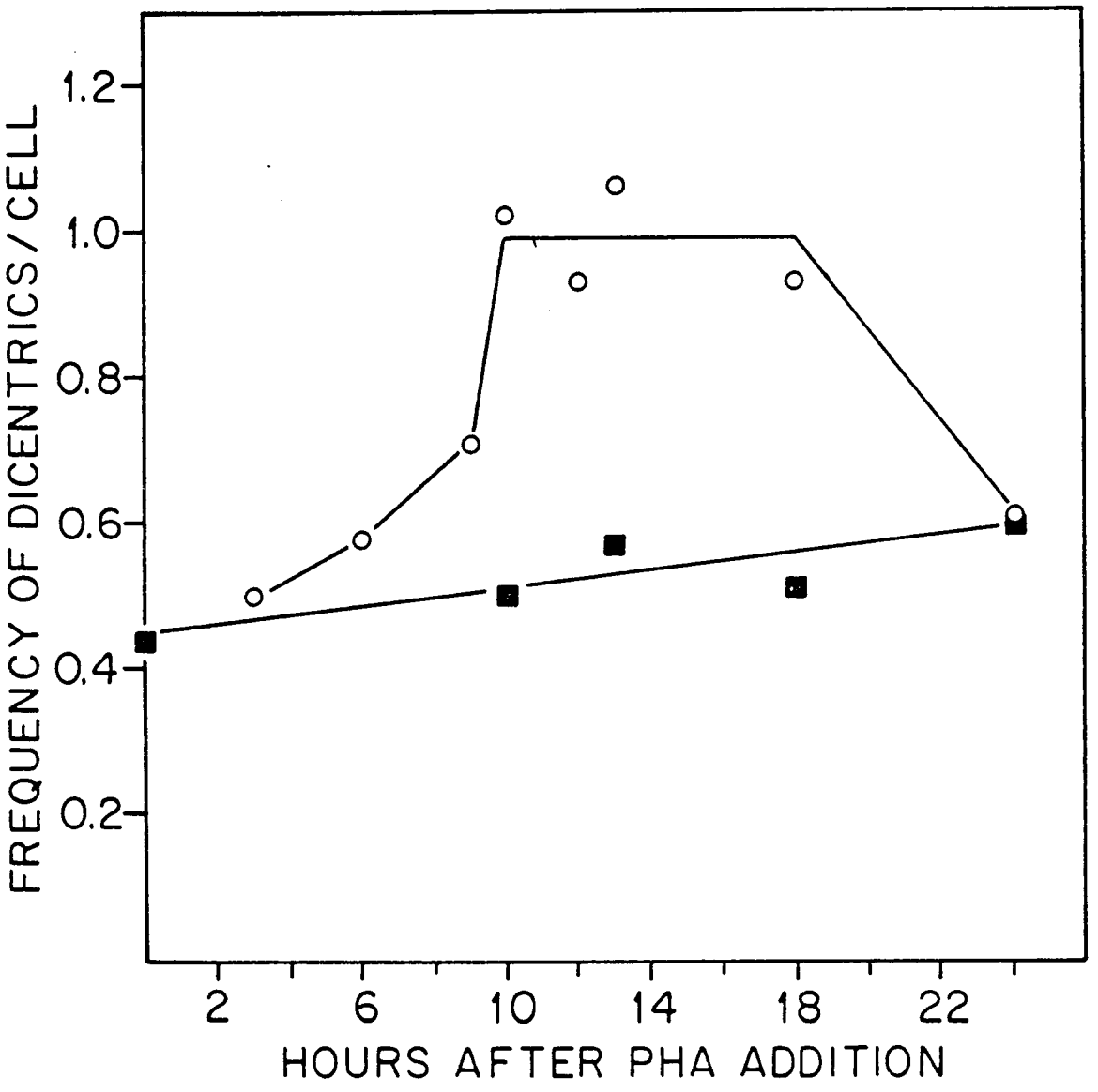

FREQUENCY OF DICENTRICS/CELL
1.2
1.0
0.8
0.6
0.4
0.2
2
6
10
14
18
22
HOURS AFTER PHA ADDITION

Figure 15.

time points in both X ray and X ray + 3-AB treated cultures (data not shown), it would appear that some proportion of the cells were at the $G_1$/S border or in S at the time of irradiation. Therefore, the effect of 3-AB seems to be a $G_1$-specific phenomenon. The frequency of dicentrics with X rays alone also increases slightly throughout $G_1$ (Figure 15). This is a consistent observation and one that has been reported by other authors (Leonard and Merz, 1983). In addition, 3-AB by itself did not have any detectable effect on aberration frequency (Tables 6-9). It was also observed, as others have suggested (Schwartz et al., 1983), that 3-AB did not cause a change in mitotic index (data not shown).

Similar experiments on $G_1$ cells were performed with fission neutrons, and the results are shown in Table 9. Cells were initiated with PHA and 13 hours later were irradiated, and incubated for 2 hours with or without 3-AB. There was no enhancement of the frequency of neutron-induced dicentrics when cells were incubated with 3-AB compared to the frequency in cells irradiated alone. As an additional control, it was shown that $G_1$ cells (13 hours after PHA) irradiated with X rays and incubated with 3-AB showed the two-fold increase in dicentric frequency reported above.

## Discussion

The present set of experiments clearly show that with human peripheral blood lymphocytes there is an enhancing effect of 3-AB on X ray-induced dicentric frequency in the $G_1$ stage of the cell

TABLE 9

THE EFFECT OF 3-AB INCUBATION ON THE FREQUENCY OF NEUTRON-INDUCED CHROMOSOME ABERRATIONS IN $G_1$ HUMAN LYMPHOCYTES

| | Number of Cells | Dicentric Frequency[a] | Ring Frequency | Deletion Frequency |
|---|---|---|---|---|
| Untreated | 150 | 0.00 | 0.00 | 0.01 ± 0.01 |
| 3-AB | 150 | 0.00 | 0.00 | 0.01 ± 0.01 |
| Neutron | 150 | 0.27 ± 0.04 | 0.06 ± 0.02 | 0.24 ± 0.04 |
| Neutron + 3-AB | 150 | 0.34 ± 0.05 | 0.05 ± 0.02 | 0.22 ± 0.04 |
| X Ray | 150 | 0.60 ± 0.06 | 0.14 ± 0.03 | 0.31 ± 0.05 |
| X Ray + 3-AB | 150 | 1.15 ± 0.09 | 0.21 ± 0.04 | 0.29 ± 0.04 |

[a]Frequency of aberration type ± standard error.

cycle, but not in the $G_0$ stage. The possibility exists, however, that this effect may be peculiar to the first $G_1$ after PHA addition. For example, it would be important to examine the effect of 3-AB on radiation induced aberrations in $G_2$ and $G_1$ of either the second or subsequent cell cycle after stimulation with PHA, or with synchronized cells in culture.

There was no detectable increase in aberrations when $G_0$ cells were incubated with 3-AB after X-irradiation, or when cells were in the S-phase of the cell cycle at the time of radiation treatment. These results are in contrast to those of Natarajan et al. (1982) who reported that post-incubation with 3 mM 3-AB for 6 hours caused a two-fold increase in X ray-induced dicentrics, rings, and deletions in $G_0$ human peripheral blood lymphocytes. The reason for this discrepancy is unknown. However, our experiments have repeatedly failed to demonstrate any effect on X ray-induced aberrations with 3-AB in $G_0$.

The results for neutron irradiation of cells in $G_0$ or $G_1$ contrast with the results of 3-AB effects on X-irradiated cells and, in addition, allow an interpretation of the mechanism of the interactive effect of 3-AB with X ray-induced DNA damage that leads to chromosome aberrations. That is, post-irradiation incubation with 3-AB had no effect on the frequency of neutron induced aberrations in $G_0$ or $G_1$. Similar results for $G_0$ cells have also been observed with cytosine arabinoside (araC) which is another excision repair inhibitor (Preston and Heartlein, unpublished results). If cells were irradiated in $G_0$ with fission neutrons and incubated with araC for 1-6 hours there

was no increase in aberration frequency compared to cells irradiated alone, whereas with X rays a 3-4 fold increase in dicentric frequency was observed with post-irradiation araC incubation.

These results have been interpreted in terms of the different spectra of DNA damages induced by X rays and fission neutrons, and the probabilities of these different damages being involved in the production of chromosome aberrations. It appears that araC can inhibit excision repair during the resynthesis step, but does not inhibit the repair of directly-induced strand breaks (Hiss and Preston, 1977). Thus, the increase in aberrations in cells exposed to X rays and incubated with araC would be a consequence of the inhibition of repair of DNA base damage by araC (Preston, 1980, 1982). Most X ray-induced aberrations are, therefore, the result of misrepair of coincidentally repairing base damages. The probability of directly-induced DNA double strand breaks interacting to produce aberrations is low following X-irradiation, largely because double strand break frequency is low at the dose levels used. In contrast, with fission neutrons, the proportion of double-strand breaks is much higher than with X rays, and consequently the probability of their misrepairing to produce aberrations is much higher. Thus, the frequency of aberrations is not significantly increased when neutron-irradiated cells are incubated with araC. If it is assumed that 3-AB inhibition of poly(ADP-ribose) synthesis changes the regulation of a step in excision repair, either through a DNA ligase (Creissen and Shall, 1982; Ohashi et al., 1983) or some chromatin interaction,

such as with histone H1 (Surowy and Berger, 1983; Wong et al., 1983; Jump and Smulson, 1980), then it is quite possible that 3-AB and araC act by similar mechanisms altering DNA repair, and influencing chromosome aberration frequencies. Thus the primary role of poly(ADP-ribose) that is inhibited by 3-AB, resulting in an increase in chromosome aberrations may involve a step in the excision repair of base damage.

The ratio of base damages to directly-induced double strand breaks may be much higher for X rays than for neutrons (and other high LET radiations). If poly(ADP-ribose) is involved in excision repair of base damages, this would explain why inhibition of this repair via 3-AB may effectively increase the lesions available for interaction and, therefore, increase the yield of aberrations after X-irradiation. Since neutrons may produce relatively smaller proportions of base damages at a given dose, there would be little opportunity for 3-AB to interact. This conclusion rests on the assumption that poly(ADP-ribose) participation in base damage excision repair is of primary importance in aberration formation with 3-AB and X rays. This assumption is strengthened by a number of recent reports (Das et al., 1984; Durkacz et al., 1980) which suggest that poly(ADP-ribose) may indeed be involved in excision repair.

The reason for the confinement of the 3-AB interaction with X rays to $G_1$ of the cell cycle is presently unclear. It may be,

however, that the enzymes that are responsible for the step(s) of excision repair that are under poly(ADP-ribose) regulation, are temporally expressed during the cell cycle. Recently, it was shown that poly(ADP-ribose) regulates the catalytic activity of a topoisomerase purified from calf thymus (Ferro and Olivera, 1984). It has also been suggested that there is an involvement of topoisomerase in DNA repair and the modulation of chromatin structure (Cleaver, 1982; Mattern et al., 1982; Hancock and Boulikas, 1982). Preliminary experiments in our laboratory with novobiocin, which has been shown to be a topoisomerase inhibitor, have demonstrated that it can abolish the 3-AB enhancement of X ray-induced dicentrics (Heartlein and Preston, unpublished results). Therefore, cell cycle dependent expression of a topoisomerase that is regulated by poly(ADP-ribosyl)ation may explain the interaction of 3-AB with X ray damage induced in $G_1$.

Various other possibilities also exist to explain the cell cycle dependence of aberration formation caused by poly(ADP-ribose) inhibition. For example, the geometry of chromatin in the nucleus has been shown to change as a function of the stage of the cell cycle (Hancock and Boulikas, 1982). These conformational changes may allow interaction of 3-AB with damage to produce aberrations only at certain cell stages ($G_1$, for example). In addition, it has been shown that there is a cell cycle dependence of both 3-AB action and poly(ADP-ribosyl)ation of certain proteins (Kun et al., 1983; Tanuma and Kanai, 1982). Given the variety of possible mechanisms

of action, however, clarification of the role of 3-AB and other repair inhibitors in aberration induction will be dependent upon molecular approaches to further test these various hypotheses.

In summary, if cells are X-irradiated in $G_1$ and incubated with 3-AB there is a two-fold increase in dicentric frequency compared to cells irradiated alone. There is no similar enhancement in cells treated in $G_0$ or S, although the latter may only occur during the first *in vitro* S phase. There is no enhancement of dicentric frequency in cells irradiated with fission neutrons and incubated with 3-AB.

# CHAPTER VII

## DIFFERENT MECHANISMS OF INDUCTION OF CHROMOSOME ABERRATIONS BY FISSION NEUTRONS AND X RAYS


### Summary

When $G_0$ human and pig lymphocytes were irradiated with fission neutrons and incubated with araC for 1 to 5 hours, no increase in aberrations was observed compared to cells irradiated with neutrons alone. These results contrast with similar experiments conducted with X rays, where post-irradiation incubation in araC caused an approximately linear increase in aberrations with increasing araC incubation times. The RBE for the fission neutrons compared to X rays was about five for human and about eight for pig. Since we have shown previously that araC does not inhibit the repair of directly-induced DNA strand breaks, it appears that the chromosome aberrations induced by neutrons result from the interaction of directly-induced DNA strand breaks, in contrast to their induction with X rays where base damage repair appears to be important. These experiments also suggest that for any particular cell type the RBE for chromosome aberrations will be proportional to the ratio of misrepaired double-strand breaks to misrepaired base damage, and can vary from cell type to cell type or from species to species for a particular radiation quality.

## Introduction

For a number of years, there has been debate over the mechanism of radiation-induced chromosome aberrations. Essentially, there are two hypotheses. The first suggests that all aberrations are the result of the misrepair of directly-induced double strand breaks (Obe et al., 1982). The second hypothesis invokes the repair of coincident base damages and their conversion into aberrations (Preston, 1981; Bender and Preston, 1982). Recent experiments (Preston, 1981; Heartlein and Preston, submitted for publication) using the excision repair inhibitor, cytosine arabinoside (araC) have shown that incubation in araC following X-irradiation results in a time- and dose-dependent increase in aberration frequency. Since araC does not inhibit the repair of directly-induced double or single strand breaks (Hiss and Preston, 1977), these results suggest that base damage repair influences the rate of chromosome aberration formation with X rays. Other experiments (Heartlein and Preston, submitted for publication) have shown that the differences in sensitivity to X ray-induced chromosome aberrations in the lymphocytes of different species (human, marmoset, pig, and rabbit) is the result of differences in the rate of repair of araC inhibitable damage (base damage). Aberration formation with X rays, therefore, depends on both the amount and repair rate of base damage. Because the above studies suggest that directly-induced strand breaks are not the important lesions in aberration formation with X rays, it

seemed pertinent to examine the effects of araC on neutron-induced aberrations, since neutrons produce far less base damage and more directly-induced double strand breaks than X rays due to the different ionization properties of these two radiation qualities. The results reported here show that chromosome aberrations induced by neutrons result from the interaction of directly-induced DNA strand breaks, in contrast to the induction of aberrations by X rays. Therefore, the ratio of the misrepair of base damage to misrepair of directly-induced strand breaks determines both radiation sensitivity and yield of aberrations for a particular radiation quality and for a particular cell type.

## Materials and Methods

Human lymphocyte cultures were established from the buffy coats of freshly drawn heparinized blood samples. Each buffy coat was obtained from a 3 ml blood sample, and was added to 10 ml of Ham's F10 medium (K. C. Biological) containing 10% heat-inactivated fetal bovine serum (Sterile Systems). The cultures were maintained at 37°C in an atmosphere of 5% $CO_2$/95% air. Whole blood cultures from domestic pigs (Sus scrofa) were established from freshly drawn heparinized blood samples. Each culture was made by adding 0.5 ml of whole blood to 9.5 ml of Ham's F10 medium containing 10% heat-inactivated fetal bovine serum. The cultures were kept at 38°C in an atmosphere of 5% $CO_2$/95% air. All treatments were performed prior to mitogenic stimulation with phytohemagglutinin (PHA).

X-irradiations were with a 320 kVp Gemini Industrial X Ray Unit operated at 250 kV and 12 mA with 2.3 mm Aluminum filtration. The dose rate was 100 rad/minute. Fission neutrons (1.2-1.3 MeV) were obtained from the Health Physics Research Reactor, Oak Ridge National Laboratory. The dose rate was about 4 rad/minute.

The appropriate cultures were treated with 50 μM araC (Sigma) for 30 minutes prior to irradiation with 30 rad of fission neutrons in order to equilibrate the nucleotide pools. During transportation to the reactor, the cultures were maintained at 37°C in a portable incubator. Post-irradiation incubation with araC was for 1, 2, 3, 4, or 5 hours. In addition, there were several different controls: one culture was untreated; one was irradiated with 30 rad of fission neutrons, but was not incubated with araC; one was treated with 50 μM araC only. The cultures were then washed twice with Saline G and returned to fresh culture medium. At this time PHA (3%; Wellcome Labs) was added to begin the process of mitogenic stimulation, and 100 μM deoxycytidine (dC, Sigma) was added to reverse the inhibitory action of araC. Thus, PHA and dC were added to each culture immediately after the completion of the treatment. After the addition of PHA and dC, the cultures were fixed at 48 hours for human, and 34 hours for pig. Two hours prior to fixation, colchicine ($1 \times 10^{-7}$ M) was added to each culture.

The cells irradiated with X rays were handled in the same way as those that were neutron-irradiated. The X ray dose was 200 rad for the experiments reported.

The fixation, slide preparation, and staining procedures followed standard methods. A minimum of 200 cells was analyzed at each treatment, and all classes of chromosome aberrations were recorded.

## Results

Unstimulated lymphocytes from human and pig were held in 50 μM araC from 1 to 5 hours following irradiation with 30 rad of fission neutrons. There was no increase in the frequency of dicentrics compared to the dicentric frequency in cells treated with neutrons in the absence of araC (Figure 16). This is in contrast to results with 250 kV X rays (Heartlein and Preston, submitted; Figure 16, dashed lines). When unstimulated lymphocytes from human and pig were held in araC from 1 to 5 hours following irradiation with 200 rad of X rays, there was a time-dependent, linear increase in dicentrics up to about 3 to 4 hours in araC. The relative biological effectiveness (RBE) for the fission neutrons compared to X rays for human was about five, whereas for pig, the RBE was about eight.

## Discussion

It is a commonly accepted idea that all aberrations result from the misrepair of directly-induced strand breaks (Obe et al., 1982). High linear energy transfer (LET) radiations such as fission neutrons, produce an entirely different spectrum of DNA damage as compared to X rays. That is, the ratio of directly-induced double

Figure 16. The effect of post-irradiation incubation with araC on dicentric frequency.

Unstimulated lymphocytes of human (●) or pig (○) were exposed to either 200 rad of X rays (dashed lines) or 30 rad of fission neutrons. The unconnected points represent the dicentric frequency obtained with fission neutrons in the absence of araC. Each point represents the mean frequency of dicentrics obtained from 200 cells ± standard error.

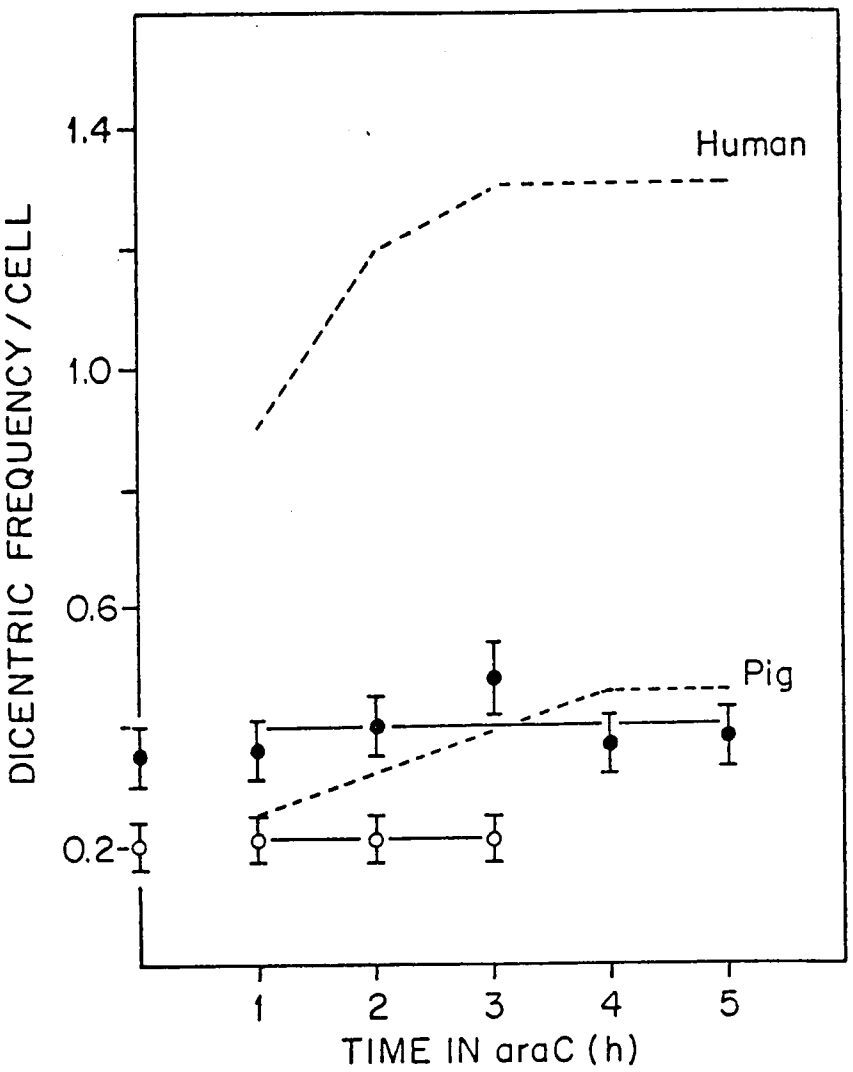

DICENTRIC FREQUENCY / CELL
1.4
1.0
0.6
0.2
Human
Pig
1
2
3
4
5
TIME IN araC (h)

Figure 16.

strand breaks to base damage is far greater for neutrons than X rays. Repair of these directly induced breaks is known to be insensitive to araC (Hiss and Preston, 1977). Therefore, experiments in which unstimulated lymphocytes were irradiated with 30 rad of fission neutrons, then incubated in the presence of araC from 1 to 4 hours (Figure 16) showed that in contrast to the results with X rays, araC had no effect on neutron-induced dicentrics for both human and pig. This is also true for higher doses of neutrons where more base damage is produced (data not shown). Directly-induced double strand breaks, however, are also proportionately induced and, therefore, the likelihood of actually observing an effect of araC on base damage repair with neutrons and their conversion into aberrations, would be minimal.

An experiment similar to this was conducted on human lymphocytes using different doses of X rays (Heartlein and Preston, submitted for publication). It was found that the synergistic effect of araC on dicentric frequency is apparently dependent upon the amount of base damage induced at a particular X ray dose, such that at 50 rad there was little or no effect of araC, whereas at 300 rad, the araC-mediated increase in dicentrics remained linear up to at least 3 hours of incubation in araC following irradiation.

Since there appears to be a limiting dose at about 50 rad on the effect of araC with X rays, this may be analogous to the situation with neutrons. For example, at 50 rad very little base damage is produced and, therefore, the probability is low that there will

be coincidentally repairing regions both in time and in space to produce aberrations. The amount of randomly dispersed base damage produced by neutrons is also presumably low (Van der Schans et al., 1983) which may explain why there is no effect of araC on abberations after neutron exposure. In contrast to X rays, therefore, directly-induced double strand breaks appear to be a major component of neutron-induced damage and this accounts for both the lack of an effect with araC on dicentric induction and the production of aberrations with neutrons.

Experiments with another excision repair inhibitor, 3-aminobenzamide [(3-AB); Heartlein and Preston, submitted for publication)] have produced results similar to those with araC, with neutrons and X rays. Although the effect of 3-AB appears to be specific for $G_1$ of the first cell cycle after PHA stimulation, it was shown that 3-AB incubation after X ray exposure caused a large increase in dicentrics. There was no effect, however, as also seen with araC, of incubation in 3-AB following neutron exposure. The above results taken together with data reported here, strongly support the hypothesis that directly-induced double strand breaks are important in dicentric formation with neutrons, whereas with X rays, repair of coincident base damages determines the yield of aberrations.

The results presented here also bear on the subject of relative biological effectiveness (RBE). Since double strand breaks are important in neutron aberration formation, while base damage appears instrumental in X ray aberration induction, and neutrons are five

to eight times more effective at inducing dicentric aberrations, RBE will depend upon the ratio of directly-induced double strand breaks to base damage for a particular radiation quality. Any radiation, therefore, that produces a greater proportion of directly-induced double strand breaks than base damage will be more effective at producing aberrations than X rays, for example. Radiations that produce mainly base damages will be less effective at aberration formation since for aberrations to be produced there must be either coincidentally repairing regions or single strand enzymatic DNA scissions opposite base damages (Ahnstrom and Bryant, 1982), thereby effectively producing a double strand gap, i.e., after excision of the damaged base. In addition, there must be two double strand gaps in time and space to produce an exchange. Whereas, with directly-induced double strand breaks, no repair is presumably necessary since the already broken DNA strands are free to interact, and can thereby produce an exchange with greater efficiency than with base damage repair.

Two predictions can be made from the above explanations of RBE. Neutron-induced aberrations should not be dependent upon repair rate. This is in contrast to X ray-induced aberrations. The results of experiments conducted with various mammalian species which show different sensitivities to X ray-induced aberrations and with experiments using different doses of X rays, demonstrated that the production of aberrations with X rays at doses above 50 rad is dependent on both the rate of repair and amount of DNA base damage (Heartlein

and Preston, submitted for publication). Secondly, the differences in RBE for neutrons observed among various species should be primarily accountable by differences in DNA contents. This roughly appears to be the case for the difference in RBE between human and pig where a 30% difference in DNA content (Bachman, 1972) is reflected in a 40% difference in RBE.

In summary, although the results of the experiments presented here do not entirely refute the idea that directly-induced strand breaks can account for all aberration formation with ionizing radiation, they do add an additional explanation in terms of the importance of the repair of base damage in aberration formation. It is clear from the results of the effects of araC with fission neutrons, however, that directly-induced double strand breaks may be a minor factor in X ray aberration formation while base damage repair may be a minor component in the formation of neutron-induced aberrations. In addition, the RBE for any cell will be dependent upon the ratio of directly-induced double strand breaks to base damage for a particular radiation quality.

# CHAPTER VIII

## SUMMARY AND IMPLICATIONS

### Part I

Initially, the objective of the experiments described in part I (Chapters II-IV) were to determine why CldU caused a seven-fold elevation in SCE frequency. The experiments led, however, to an explanation of the mechanism of induction of SCE and mutation by both BrdU and CldU and, in addition, this mechanism may hold for other agents which induce SCE.

The following points can be made from the results presented in Chapters II-IV. CldU causes seven times more SCE than BrdU, even though both analogues replace thymidine in DNA throughout the concentration range of 0.5-20 μM, in a linear and equal fashion. Based upon extrapolation of this linear relationship between substitution for thymidine and SCE induction, caused by both BrdU and CldU, a spontaneous SCE frequency of about six/cell was demonstrated. In addition, at these concentrations, the mutagenicity of both analogues were similar--with CldU slightly lower than BrdU. Mutation in this case (i.e. at high analogue concentration), is accomplished by errors of incorporation. In Chapter IV, different types of SCE and mutation experiments were employed in order to demonstrate that replication of CldU-substituted DNA could result in a seven-fold increase in both mutations and cytotoxicity as

compared to replication of BrdU substituted DNA. It was also shown that SCE are induced by these analogues during replication of analogue-substituted DNA. SCE as well as mutation, therefore, are induced by replication errors, with CldU substituted DNA being seven times more prone to misreplication than BrdU containing DNA. Mutations are also induced by these analogues through errors of incorporation, but both BrdU and CldU are similar in this respect, and errors of incorporation appear to be detected only at high concentrations of these nucleoside analogues.

There is at least one fundamental question left unanswered by these experiments. Although it was conclusively demonstrated in Chapters II-IV that it appears that SCE and mutation are induced by replication errors, the question remains, what is the nature of this halogen (Cl) at the five position of uridine which perturbs the DNA replication machinery to cause SCE and mutations seven to eight times more effectively than the same nucleoside with Br at the five position? In addition, it remains to be shown which step(s) in replication are affected when halogen-substituted DNA is used as a template, and whether or not SCE and mutation are associated with this presumed alteration.

Another problem precipitated by these studies is the biological significance of SCE and what the study of their mechanisms of induction reveal about other genetic processes. It is tempting to assume that SCE may be a somatic manifestation of meiotic recombination since there are many similar aspects of both

processes. At present, however, the evidence is only indirect. The demonstration in Chapter III that there appears to be spontaneous SCE may suggest that this exchange process is either a necessary event for maintenance of replication or a manifestation of some other process that has little to do with recombination or replication.

## Part II

Chapters V-VII taken together describe experiments concerning the induction of chromosome aberrations by ionizing radiation. The results have bearing on several subjects including: the relative biological effectiveness (RBE) of different radiation qualities, sensitivities of various species to induction of aberrations, and elucidation of mechanisms of X ray- and neutron-induced chromosome aberrations.

The results can be distilled into a few general points. Differences in sensitivity of cells to X ray-induced chromosome aberrations are due to differences in repair rates, such that cells that repair base damage more efficiently, are more sensitive to the induction of aberrations by X rays. The induction of chromosome aberrations by radiation depends upon the ratio of directly-induced double strand breaks to base damage. With neutrons, directly-induced double strand breaks are primarily responsible for the induction of aberrations, whereas repair of base damage can account for the formation of aberrations with X ray doses greater than 50 rad. This is primarily due to the ionization properties of these two different

radiation qualities. The RBE of a particular radiation quality is also related to the type of damage induced. Neutrons, for example, have a high RBE compared to X rays. Our interpretation is that neutrons are more effective since they produce mainly directly-induced double strand breaks which presumably do not require excision repair in order to produce aberrations. This is in contrast to X rays where the induction of aberrations appears to be dependent on excision repair rate.

The experiments described in the preceding three chapters may be important in terms of interpreting the risk of human populations to X rays and chemicals. Because we have provided an explanation of interspecific radiosensitivity, it may facilitate extrapolation of results obtained from experiments with laboratory animals to humans. They also suggest that there may be quite a large variation with respect to sensitivity to X rays among the human population since it has been suggested that there are differences in repair rates between human individuals. This is important, since we have shown here that production of aberrations by X rays is, in part, dependent upon the repair rate. Whereas, with high LET radiations such as neutrons, for example, repair rate is probably of relatively lesser importance.

Experiments similar to those described in the preceeding chapters, therefore, may help in identifying human populations at risk to X rays and other agents which require excision repair to produce aberrations.

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# VITA

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