Title
Cell cycle phase specificity of chloroethylnitrosoureas

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Publication Date
1986

Peer reviewed|Thesis/dissertation

CELL CYCLE PHASE SPECIFICITY OF CHLOROETHYLNITROSOUREAS

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Experimental Pathology

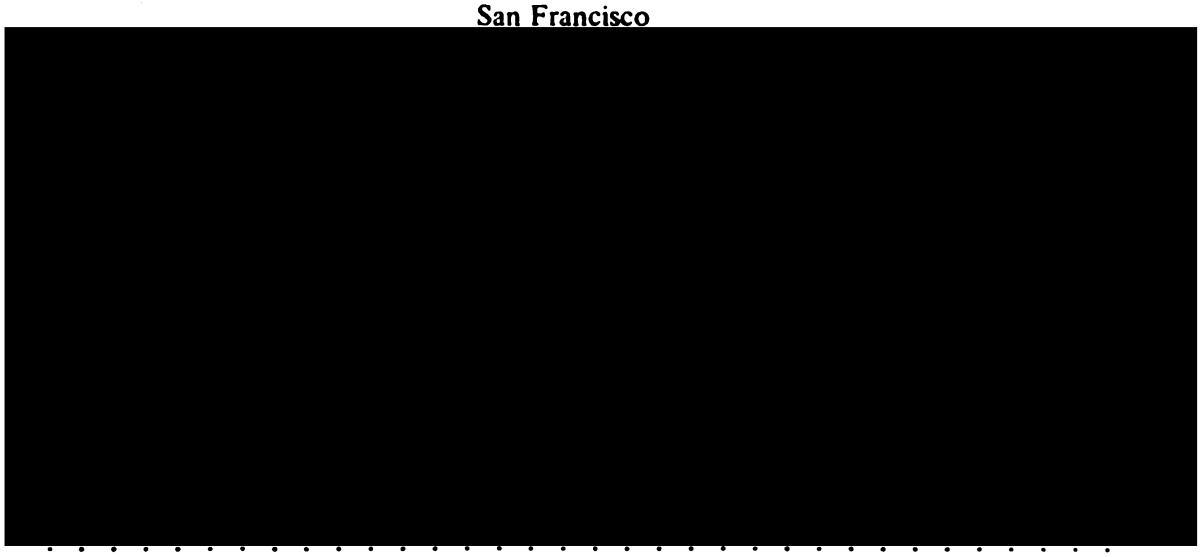
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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DEDICATION

This work is dedicated to the people who helped make my dream a reality: my father, who taught me about compassion and commitment; my mother, who taught me patience; Larry, who encouraged me; Louise, who believed in me. Special thanks to Dennis, who taught me science, and whose friendship, unique wit, and philosophy provided the most important lessons.

CELL CYCLE PHASE SPECIFICITY OF CHLOROETHYLNITROSOUREAS

Peter Allen Linfoot, University of California, San Francisco

Athough the cancer chemotherapeutic agent 1,3-bis(2-cholorethyl)-1-nitrosourea (BCNU) is considered a non-cell cycle phase specific drug, it has been shown to produce differential cell killing in G_1, S, and G_2/M phase cells, with S phase cells appearing relatively resistant. The lack of knowledge regarding the biochemical mechanisms underlying the phase specificity of BCNU provided the impetus for this dissertation. Because BCNU is thought to kill cells via a DNA cross-linking mechanism, experiments were designed to test whether phase-specific survival, measured by colony forming ability, in BCNU-treated 9L cells reflects phase-specific differences in DNA cross-linking, and whether intrinsic cellular drug sensitivity, guanine O^6-alkyltransferase activitity, cellular thiol content, or intracellular drug dose, which are all known to affect BCNU-mediated cell kill, could be correlated with cell cycle phase specificity.

Studies of cell cycle phase specific cell killing, produced by nitrosoureas with different chemical reactivities, clearly indicated that the ability of compounds to cross-link DNA was important in determining their phase specificity. Cells that lacked guanine O^6-alkyltransferase activity showed similar patterns of BCNU phase specificity regardless of their intrinsic sensitivity to BCNU. It was also clear that guanine O^6-alkyltransferase activity did not explain the pattern of phase specificity in 9L or Chinese hamster ovary cells, nor did the steady state level of non-protein sulfhydryls correlate with cell survival in 9L or BTRC-19 cells. DNA inter-strand cross-linking, as measured by alkaline elution, was similar in cells exposed to BCNU in G_1 or S phase. ^{3}H [1-chloroethyl-1nitrosourea]

binding to DNA was the same in G_1, S and G_2/M phase cells indicating that phase-specific differences in drug uptake and intracellular drug dose were not responsible for phase-specific cell kill. These studies suggest that cross-link lesions, other than DNA inter-strand cross-links, and/or effects on DNA repair, other than guanine O^6-akyltransferase, are additional important determinants of BCNU phase specific cell killing.

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I. SPECIFIC AIMS

Many cancer chemotherapeutic agents have been shown to produce differential toxicity towards cells in specific phases of the cellular growth cycle. This information has been used in conjunction with cell kinetic data, such as the tumor mitotic index, growth fraction, and cell cycle time, to design kinetics-based protocols for the combination chemotherapy of solid tumors. To date, this approach has had limited clinical success. Major problems have been caused by a lack of knowledge regarding the factors that determine the phase specificity of many agents and an inability to accurately predict how agents will interact when they are given in combination.

The compound, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a chloroethylnitrosourea (CENU) with demonstrated activity against solid tumors, is a bifunctional alkylating agent that is thought to kill cells via a DNA cross-linking mechanism. BCNU is classified as a cell cycle phase non-specific agent, but it is less active toward cells that are undergoing DNA synthesis at the time of treatment when compared to cells that reside in other phases of the cell cycle. The reason for this phase specificity is uncertain: the goal of this dissertation project was to gain a better understanding of the biological factors that determine CENU phase specificity so that these compounds might be used more rationally in experimental and clinical protocols. The scientific approach was designed to test several hypotheses:

1) whether phase-specific survival in BCNU-treated cells reflects phase-specific differences in DNA cross-linking 2) whether intrinsic cellular BCNU sensitivity affects the pattern of phase-specific survival after BCNU treatment 3) whether guanine O^6-alkyltransferase activity varies during the

cell cycle in parallel with BCNU sensitivity 4) whether cellular thiol content can be correlated with phase-specific survival 5) whether phase-specific survival reflects phase-specific differences in intracellular drug dose.

The specific aims were to:

1. Determine the phase-specific survival pattern for 9L cells treated with BCNU and other nitrosoureas[a] that have different chemical reactivities.

2. Measure DNA inter-strand cross-linking caused by BCNU throughout the cell cycle using alkaline elution in order to evaluate the expression of drug damage in each cell cycle phase.

3. Determine the phase-specific survival pattern in CENU-resistant cells and compare it to that seen in 9L cells to see whether cellular repair of CENU damage plays a role in phase specificity.

4. Measure cellular glutathione and guanine O^6-alkyltransferase levels throughout the cell cycle and correlate levels with phase specific survival.

5. Measure [^{3}H] CNU binding during the 9L cell cycle to determine whether the effective intracellular drug exposure dose varies with cell cycle position.

a-The nitrosoureas studied were: 1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU; N,N'-bis(trans-4-hydroxycyclohexyl)-N-nitrosourea, BHCNU; 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, CCNU; 1-chloroethyl-1-nitrosourea, CNU; 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose, CHLZ; 1-ethyl-1-nitrosourea, ENU; 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea, MeCCNU; 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea, PCNU.

II. BACKGROUND

II.1. Mammalian cell division cycle

Mammalian cells growing in tissue culture systems and in vivo show a characteristic pattern of growth. The growth rate may vary between different cell lines and different tumors, but all cells must duplicate their genomic DNA prior to cell division. DNA synthesis occurs during a distinct period in the cell growth cycle, termed S phase, which is preceded by a gap phase, termed G_1, and followed by a second gap phase, termed G_2. Mitosis follows G_2 phase, lasts approximately 1 hour, and produces two G_1 phase cells. Cells that are viable, but apparently non-cycling can be found with DNA content equivalent to G_1, S, and G_2 phase cells; these are operationally defined as quiescent cells (FIGURE 1)(1). Although the biochemistry of DNA synthesis has been studied in depth (2) the terms G_1 and G_2 reflect the current lack of understanding of the specific reactions that characterize these phases of the cell cycle. The duration of S phase in most mammalian cells is approximately 8 hours, whereas the duration of the G_1 phase and G_2 phase is variable (3). Large variations in the length of G_1 phase can be observed between the cells of different species with variations in the duration of G_2 being less and G_2 usually being the shortest phase of the cell cycle. The specific factors that determine the length of G_1 phase are not known, but subcompartments within G_1, G_{1a} and G_{1b}, can be identified based on cellular RNA content (1). Progression through the cell cycle is thought to involve control points in G_1 and G_2 phases and is closely tied to RNA metabolism. G_{1b} cells are believed to be commited to initiate DNA synthesis, while G_{1a} cells are believed to be capable of becoming quiescent and later reentering the cell cycle under an appropriate stimulus. It is currently not known whether

quiescent S and G_2 phase cells can reenter the cell cycle or whether S phase cells that appear to be quiescent are actually cycling very slowly.

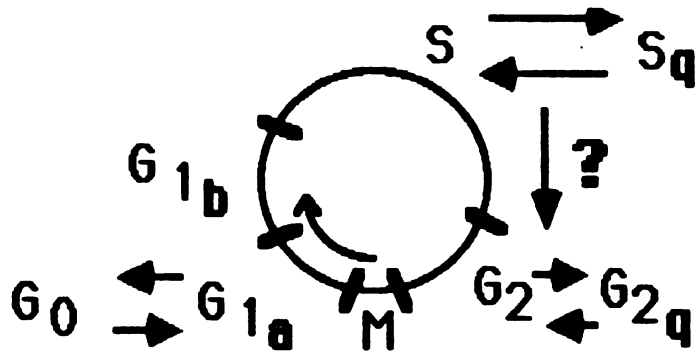


FIGURE 1. Subcompartments of the mammalian cell cycle. G_{1a}, G_{1b}, S, G_2, and M represent cycling cell compartments. G_0, S_q, and G_{2q} represent quiescent cell compartments. The arrows represent cells in transition from one cell cycle compartment to another. Modified from Z. Darzynkiewicz, Pharmac. Ther. 21, 143-188 (1983).

II.1.a. Monolayer culture

The simplest system for studying cellular growth is a monolayer culture. Growth of cell lines in unfed monolayer cultures on plastic can be described by a sigmoid growth curve when cell number is plotted versus time on a semi-logarithmic chart. An initial lag phase, characterized by slow growth, is typically followed by an exponential or log phase, and then a plateau phase, characterized by maintainance of a constant number of viable cells. Most cells will remain in plateau phase until nutrients in the culture medium become depleted causing cells to die, but this pattern can be altered by replenishing the growth medium (feeding) (4,5). The growth of cells in monolayer culture is influenced by the environmental conditions of pH, temperature, and medium composition, particularly serum concentration

(6,7,8,9,10). Monolayer cultures can be established on biological (11) and synthetic matrix (12), in order to model cell-substrate interactions, and in defined serum-free medium (13); different culture environments can profoundly influence gene expression (14). During the exponential phase nearly 100% of the cells will be growing and the cell number will double over each period of the cell cycle. Exponentially growing cells have been widely used in studies of cancer chemotherapeutic agents because they are thought to model cycling cells in tumors. It is known that not all cells in a tumor are actively dividing at any given point in time (15), consequently a monolayer is not an appropriate model for a human tumor; however, because the environmental conditions can be rigorously controlled in monolayer cultures they are valuable for the study of drug mechanisms.

II.1.b. Spheroid culture

Under appropriate conditions cells growing in vitro can be induced to form cellular aggregates that continue to grow in culture (16). These aggregates, termed multicellular tumor spheroids (MTS), can show tumor-like growth (17) and provide an experimental tumor model that is of intermediate complexity between monolayers and in vivo tumors. MTS, in contrast to monlayers, have cells growing in three dimensional contact, they posess gradients of pH, oxygen tension, and nutrients, and are comprised of a large number of noncycling cells (18). Most of these cells have a DNA content similar to that found in G_1 phase cells (19), thus they are arrested prior to DNA synthesis. The MTS model provides an interesting alternative to monolayer cultures, but will not be used for experiments in this dissertation.

II.1.c. Tumors

Tumors growing in vivo are complicated by host related factors such as the host immune response to tumor antigens (20), variations in tumor blood supply and microenvironment (21,22), and tumor cell heterogeneity (23). These factors are highly variable amongst individuals and tumors, and can change in response to therapy (24,25,26) or during tumor progression (27,28). Tumors, like spheroids, contain a large number of cells that are non-cycling; however, its is thought that these cells can be recruited into the cell division cycle following therapy (29,30,31). Although, direct extrapolation of in vitro phase specificity studies to in vivo tumors may not be possible (32) this dissertation will focus on understanding fundamental mechanisms underlying the phase specificity of CENUs.

II.1.d. Biochemistry of the mammalian cell cycle

The biochemical events that occur as mammalian cells progress through the cell cycle are of two types: 1) maintainance functions, that are persistent throughout the cell cycle and required for cell survival, but not necessarily cell growth, for example the production of ATP and 2) phase specific or sequential functions that are under tight genetic control, such as the synthesis of DNA (33). Because of the widespread belief that malignant cells have escaped from normal growth regulation, research has focused on phase specific functions in order that the factors that control cell proliferation might be elucidated. Several extensive reviews are availiable describing the biochemical changes that occur in each cell cycle phase (1, 3,34). Mitchison (34) suggested that the cell division cycle can be thought of as two overlaping cycles 1) the growth cycle (G_1, S, G_2, and M phases) and 2) the chromosome cycle (S, G_2, and M phases); recent findings indicate that

each cycle responds to different regulatory controls as evidenced by the insensitivity of the chromosome cycle to protein synthesis inhibitors (35), and the dissociation between growth in cell size and DNA replication seen under certain conditions (36).

Some of the enzymes involved in DNA replication vary in a phase specific manner as would be expected; however, not all DNA synthetic enzymes appear to be coupled with S phase. Further, the variation in enzyme activity depends on the cell type, and method of synchronization that is used. In 3924A hepatoma cells that are stimulated to proliferate, the specific activity (units of activity/unit protein) of the enzymes thymidine kinase, ribonucleotide reductase, and orotate phosporibosyltransferase all increase with cell growth rate, but show different kinetics, whereas the specific activity of dihydrothymine dehydrogense decreases (37). In contrast, when elutriation methods are used thymidine kinase increases prior to S phase and peaks during S phase, but the specific activities of orotate phosphoribosyltransferase and dihydrothymine dehydrogenase show little change during the cell cycle (38). Engstrom et al. (39), using cells synchronized by either isoleucine deprivation or centrifugal elutriation, have shown that ribonucleotide reductase activity is S phase specific, and also that the individual subunits of the protein are regulated differently during the cell cycle. As might be expected the levels of the nucleotide precursors dATP, dCTP, dGTP, and dTTP are extremely low in G_1 phase cells, they increase just prior to DNA synthesis and peak during S phase, but they do not occur in equimolar concentrations (40,41), and there is evidence that cellular nucleotide pools may be compartmentalized (42,43). Total DNA polymerase activity is increased during S phase, but the activity of specific enzymes varies; beta-polymerase activity appears in early S phase, whereas

alpha-polymerase activity appears in late S (44). Specific activity of DNA topoisomerase I increases during S phase and is insensitive to hydroxyurea, whereas specific activity of topoisomerase II is invariant during the cell cycle (45). These few examples indicate that the regulation of the enzymes involved in DNA synthesis is complicated and that it is important to distinguish between phase-specific and proliferation-specific changes in enzyme activity .

The polyamines putrescine, spermidine, and spermine, which are thought to stabilize DNA, are involved in growth regulation in normal and neoplastic cells (46) and are essential for chromosome condensation prior to mitosis (47). Polyamine levels fluctuate during the cell cycle (48,49,50,51,) as does activity of the polyamine associated enzymes ornithine decarboxylase (48,49,50), s-adenosylmethionine decarboxylase (48,50), and methylthioadenosine phosphorylase (52). In some cells the levels of polyamines increase continuously between early G_1 phase and G_2 phase (48,51), while other cells show a biphasic pattern increasing between G_1 and S phase and then again just prior to mitosis (49,50). The synthesis of histones is tightly coupled to DNA synthesis (53,54), but may occur during G_1 phase (55,56). Methylation of cytosine residues, which is thought to control the expression of some genes (57), has been implicated in cellular differentiation (58), and the activity of the enzyme that regulates DNA methylation, DNA methylase, is coordinated with DNA synthesis (59). Because the activities of these enzymes fluctuate during the cell cycle, therapeutic approaches targeting these enzymes may show phase specificity.

Although information on phase-specific repair of DNA damage is limited, the activities of some DNA repair enzymes have been studied during the cell cycle. The activity of uracil-DNA glycosylase, which converts uracil

bases in DNA to apurinic sites, is coordinated with DNA synthesis in WI-38 fibroblasts and V79-AO3 cells (60,61). Uracil-DNA glycosylase activity in V79-AO3 cells is not affected by hydroxyurea or excess thymidine, whereas UV-sensitive, V79-UC, cells show less variation of activity during the cell cycle and the pattern of activity is altered by hydroxyurea and excess thymidine. These observations indicate that the peak of uracil-DNA glycosylase activity occurs during DNA synthesis, but that the two events can be dissociated from one another. Further, altered cell cycle regulation of glycosylase activity is associated with the UV-sensitive phenotype indicating that a genetic defect in UV repair can alter the expression of an enzyme that is not thought to be involved in the repair of UV damage. The activity of guanine O^6-alkyltransferase, which removes alkylation products from O^6-guanine thereby restoring the guanine base, appears to increase in late G_1 phase, peak in early S phase and then rapidly decline in late S and G_2 phase in regenerating rat liver (62), while removal of O^6-guanine adducts in 10T1/2 cells released from confluence occurs prior to S phase but not during DNA synthesis (63,64). Because guanine O^6-alkyltransferase activity has been shown to be an important factor influencing cell survival after CENU treatment (discussed in a later section) and because both of the above studies may reflect proliferation-specific changes in enzyme activity the variation of guanine O^6-alkyltransferase activity during the 9L cell cycle has been studied as part of this dissertation.

Cellular enzymes that are targets of anti-cancer agents can also vary during growth. The activities of the enzymes that are inhibited by anti-folate chemotherapy including dihydrofolate reductase, thymidylate synthetase, and thymidine kinase peak during log phase in RPMI 4265 lymphoblasts (65).

In conclusion, the activity of important enzymes that affect cellular growth and response to chemotherapeutic agents can vary during the cell cycle; these variations may contribute to the phase specificity of anti-cancer drugs and influence the effectiveness of therapy.

Recent studies on biological growth factors and "oncogenes" suggest that the transition from quiescence to growth is regulated by a small number of proteins. There are similarities between some cell cycle phase dependent genes and cellular and viral oncogenes in that they code for growth factors, growth factor receptors, or regulatory proteins. The epidermal growth factor (EGF) receptor is the product of the c-erbB proto-oncogene, the c-sis proto-oncogene codes for the B-chain of platelet derived growth factor (PDGF), and the colony stimulating factor (CSF-1) receptor is the product of the c-fms proto-oncogene (66). The growth factors PDGF, EGF, and insulin-like growth factor appear to regulate discrete portions of the G_1 phase of the cell cycle and the transition from quiescence to proliferation (67,68). Further, initiation of DNA synthesis or the start of the chromosome cycle appears to commit fibroblasts to undergo cell division (69). Expression of c-myc oncogene, as measured by mRNA level, increases following induction of proliferation in quiescent T lymphocytes, but is invariant during the cell cycle in exponentially growing cells, and density arrested cells maintained with serum growth factors continue to express c-myc RNA at levels comparable to exponentially growing cells (70). The c-myc gene is apparently transcribed in G_0-arrested fibroblasts and is post transcriptionally regulated by growth factors (71). In contrast, c-myb expression appears to fluctuate during the cell cycle in parallel with S phase and show post-transcriptional regulation in mature T cells, but not in immature thymocytes, which appear to regulate expression at the level of

transcription (72). Thus, there are oncogene products that appear to be associated with proliferation specific functions and others that may have cell cycle phase specific functions.

Clearly the biochemistry of the mammalian cell cycle is extremely complex; the transition from quiescence to growth and between each cell cycle phase involves many biochemical changes. Because this dissertation is aimed at understanding the mechanism underlying the phase specificity of CENU-mediated cell killing, experiments will focus on the factors that are most likely to affect cell survival following CENU exposure.

II.2. Cell kinetic terminology.

Technological advances in quantitative cytokinetics, particularly the development of monoclonal antibodies to BrdU-substituted DNA (73,74), have improved the accuracy of cell kinetic measurements on tumor and normal cell populations in vitro and in vivo, and have renewed interest in the relevance of cell kinetics to cancer therapy. Several cell kinetic terms that are used in this dissertation are defined below.

The cell cycle time is the average time it takes a cell to progress through one cell cycle. It can be estimated by pulse labeling a cohort of S phase cells and determining, by sampling at various times post exposure, how long it takes the cohort to return to S phase.

Doubling time of a population refers to the time it takes to increase the population size by a factor of two. Doubling time is estimated from a cell growth curve and is equal to the cell cycle time only if all the cells in the population are cycling and there is no cell loss associated with progression through the cell cycle or over time.

The labeling index (LI) describes the fraction of cells in a population that is undergoing DNA synthesis at a given time point. LI is determined by detecting which cells in a population have incorporated DNA precursors during a pulse exposure to exogenous nucleotides. Incorporation can be measured using radiolabeled nucleotides or with fluorescent antibodies. An increase in the LI indicates a greater proliferative potential.

The growth fraction (GF) is the fraction of cells in a population that is cycling. GF can be estimated by measuring the LI in a cell population that has been pulsed for a period exceeding the cell cycle time. This assumes that all cycling cells have similar cell cycle times and would therefore become labeled over the course of one cell cycle. The GF in exponentially growing monolayer cultures approaches 1 (5), whereas in spheroids and tumors it is considerably less and varies with size (75,76).

The problems associated with measuring each of these population parameters have been eloquently critiqued by Gray (77).

II.3. Applications of cell kinetic data

II.3.a Prediction of tumor responsiveness

The value of cell kinetic information as a predictor of tumor responsiveness or patient survival is equivocal. High proliferative activity of tissues as assayed by LI may indicate a poor prognosis in patients with breast cancer (78) or brain tumors (79), but may be of little value in colorectal cancer (80). Interpretation of these results is complicated by many factors including the fact that LI of tumors varies with tumor size (76), LI may vary in different regions within a tumor (81), and current labeling methods may not adequately label all S phase cells (79,82). Because correlative studies cannot define cause and effect relationships it is possible

that other factors that are similarily correlated may be of greater importance, for example, a larger tumor is itself predictive of a poorer response (), and estrogen receptor status, which affects response to hormone therapy in breast tumors, appears to be inversely related to LI (83). Despite these problems, new indicators of prognosis that combine cell kinetic information with traditional pathologic criteria may prove to be of more value than previous methods (84).

II.3.b. Kinetically-directed tumor therapy

The relevance of cell kinetic concepts to the design of cancer treatment protocols has been hotly debated (85,86,87). One reason for the failure of kinetically designed protocols has been the limited degree of cell cycle synchronization that can be achieved in vivo and its short duration (88,89). A second reason is that the biochemical mechanisms that are responsible for phase specific killing remain unknown for many agents, and combinations of agents often produce unexpected results. Bhuyan (90) has shown that the combination of several S phase specific agents, which might not be expected to interact, produce not only additive cell killing, but also antagonistic and synergistic interactions. Although the clinical results of kinetically directed therapy have not been encouraging, alternative methods for achieving synchony are being investigated (91,92,93,94), and both synchrony and an increased therapeutic effect can be achieved in vivo when appropriate drugs are chosen and drug sequencing is rigorously controlled (95,96,97,98); these developments combined with improved understanding of drug mechanisms and more accurate measurement of cell kinetics in tumors and normal tissues should enable rational implementation of cell kinetic concepts in cancer therapy.

II.4. Chloroethylnitrosoureas (CENUs)

CENUs are compounds that are commonly used for the treatment of malignant brain tumors, lymphomas, and gastrointestinal tumors (99). They are classified as non-phase-specific alkylating agents because they kill cells in all cell cycle phases; however, BCNU, CCNU, and MeCCNU have been shown to preferentially kill cells that are in G_1 and G_2/M phases at the time of treatment (100).

II.4.a. CENU chemistry

All nitrosoureas undergo aqueous hydrolysis to form two types of products 1) alkylation products thought to arise via reactions of chloroethyldiazohydroxide and 2) carbamoylation products thought to result from reaction of substituted isocyanates (FIGURE 2) (101): the specific reactive species formed is dependent on the structure of the parent compound (FIGURE 3).

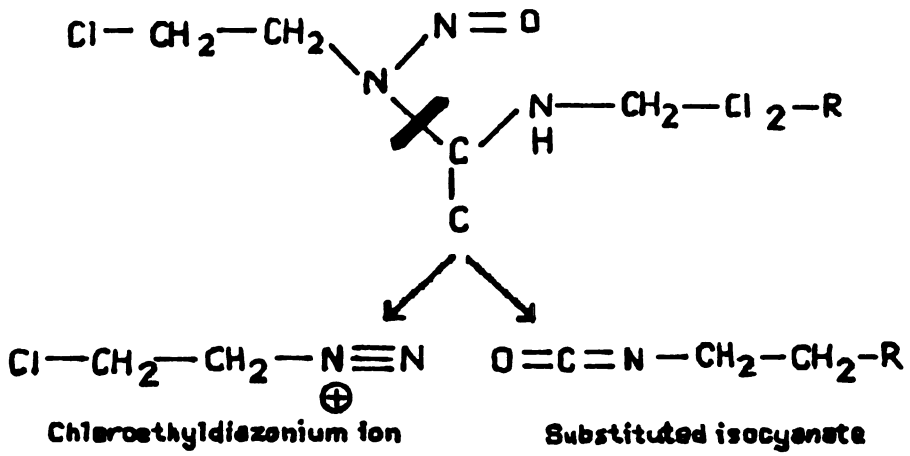
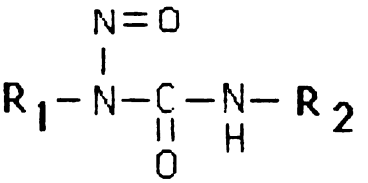


FIGURE 2. Proposed reactive species produced upon aqueous hydrolysis of CENUs.



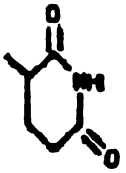
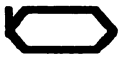
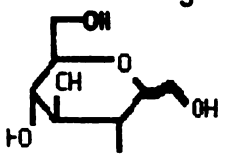
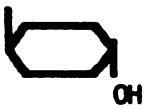
Compound	R_1	R_2	Activity
BCNU	CH_2CH_2Cl	CH_2CH_2Cl	A, C, C-L
PCNU	CH_2CH_2Cl		A, C, C-L
CCNU	CH_2CH_2Cl		A, C, C-L
MeCCNU	CH_2CH_2Cl		A, C, C-L
CHLZ	CH_2CH_2Cl		A, C-L
CNU	CH_2CH_2Cl	H	A, C-L
ENU	CH_2CH_3	H	A, C
BHCNU			C

FIGURE 3. Structures and chemical reactivities of nitrosoureas. A = alkylating activity; C = carbamoylating activity; C-L = cross-linking activity.

Substitution at the R_1 position determines which alkylating products will form, and substitution at the R_2 position determines the reactivity of the isocyanate product. All CENUs have the chloroethyl group at R_1 and different substitutions at R_2. Radiolabel in the R_1 portion of the CENU molecule reacts primarliy with DNA, whereas label in the R_2 portion reacts primarily with cellular protein (102,103). When the reactivity of CENUs is quantitated in vitro they can be classified on the basis of their relative alkylating and carbamoylating activity (TABLE 1) (104). This classification has been widely used to determine the biological role of CENU-mediated alkylation and carbamoylation reactions in cells due to the fact that stuctural analogs are available that have limited chemical reactivities.

TABLE 1. Relative alkylating and carbamoylating activities of CENUs.

Compound	$t_{1/2}$ (min)	Relative A[a]	Relative C[b]
1. CCNU	53[c]	0.22	1.0
2. MeCCNU	58[c]	0.22	0.91
3. BHCNU	10[d]	0	0.87
4. BCNU	50[c]	0.59	0.68
5. PCNU	26[c]	0.79	0.24
6. CHLZ	22[d]	1.0	0.03

a-Alkylating activity relative to CHLZ.
b-Carbamoylating activity relative to CCNU.
c-From Weinkam and Deen, Cancer Res. 42, 1008-1014 (1982).
d-Deen and Williams, unpublished results.

II.4.b. CENU-mediated cell killing

CENUs are currently thought to kill cells via a DNA cross-linking mechanism that only requires activity in the alkylating portion of the molecule. This belief stems from the fact that CENU analogs that lack carbamoylating activity retain anti-tumor activity (105,106), and pure carbamoylating nitrosoureas are ineffective as anti-tumor agents (107). Nitrosoureas that are pure alkylators, for example ENU, produce cell kill at high doses, but are much less effective, on a molar basis, when compared to cross-linking nitrosoureas (108). Further an increase in the relative in vitro carbamoylating activity of CENUs has been correlated with increased toxicity towards bone marrow cells (109,110), and the severity of bone marrow depression in patients undergoing chemotherapy (111).

II.4.b.i. CENU dose concept

The biological activity of CENUs in vitro has been described quantitatively with a dose concept that predicts that cell killing will be proportional to the concentration of active species formed by hydrolysis during the exposure interval (112). Because the stoichiometry of CENU hydrolysis produces reactive species in a 1:1 ratio with parent compound, the concentration of active species is equal to ΔC or the change in concentration of the parent compound. The ΔC concentration can be determined when the drug half life, $t_{1/2}$, exposure dose, C_0, and the exposure interval, t, are known by using the following equation :

$$\Delta C = C_0 (1 - e^{-kt}) \qquad k = (\ln 2) / t_{1/2}$$

The ΔC concept was developed, using 9L rat brain tumor cells, by showing that the multiple survival curves seen when cells were treated with different CENUs converged to a single curve when the drug dose was corrected for the exposure interval and the individual drug half lives. The ΔC concept has since been validated for other measures of cytotoxicity in 9L cells including SCE induction, DNA inter-strand cross-linking, and phase specific survival, and in other cell lines (113).

II.4.b.ii. Monoadduct formation

CENUs, like the monofunctional nitrosoureas, methylnitrosourea (MNU) and ethylnitrosourea (ENU), produce a spectrum of monoadducts in cellular DNA. It is currently thought that a small proportion of the large number of adducts that form can react further to produce cross-links, which are thought to be lethal (114,115,116). The major alkylation products that have been isolated in ENU-treated DNA are ethylphosphodiesters (51%), N^7-ethylguanine (14%), O^6-ethylguanine (9%), N^3-ethyladenine (5%), O^2-ethylthymidine (7%), O^4-ethylthymidine (2%), and O^2-ethylcytosine (5%) (117). BCNU and CCNU are known to produce some of the same adducts (TABLE 2) (118,119); however, the full spectrum of adducts produced by CENUs and their relative proportions are unknown. Most of the monoadducts that have been identified are thought to form as a result of reaction between the chloroethyldiazonium ion and various nucleophillic sites on DNA bases. Although the alkylating portion of the molecule is identical for all CENUs there appear to be subtle differences between structural analogs, including differences in lipophilicity, decomposition rate, and anticancer activity (105,106). Similarly, differences in monoadduct formation have been described in a limited number of studies. BCNU may be unique amongst the

CENUs in that the carbamoylating species, may react to produce unique adducts in DNA, including aminoethyl adducts at N^7-guanine (120).

TABLE 2. Monoadducts formed in CENU-treated DNA.

O^6-hydroxyethylguanine
O^6-chloroethylguanine[a]
7-hydroxyethylguanine
7-aminoethylguanine
3-chloroethylcytosine[a]
3-hydroxyethylcytosine
3, N^4-ethanocytosine
1-chloroethyladenine[a]
1-hydroxyethyladenine
1, N^6-ethanoadenine

a- Fluoroethyladduct has been isolated.

The biological significance of specific adducts remains to be determined, but it is well established that O^6-alkylguanine and O^4-alkylthymidine in DNA are highly mutagenic (121). CENUs produce hydroxyethyl and chloroethyl adducts in DNA with the former predominating (106) and show a sequence specificity for alkylation at sites with adjacent guanine bases (122). It has been proposed that O^6-chloroethylguanine is a precursor lesion that can react further to produce lethal DNA interstrand cross-links (123).

II.4.b.iii. DNA cross-linking

The evidence supporting the hypothesis that a cross-linking mechanism is responsible for CENU-mediated cell killing is convincing. Cross-linking assayed in cellular DNA using alkaline elution is highly correlated to cell survival (114,115,116). Both DNA-DNA cross-links and

DNA-protein cross-links can be detected, but it is the former that has received the most attention in the literature. Human cells that are resistant to CENU-mediated cell killing, designated Mex $^+$ or Mer $^+$, have fewer inter-strand cross-links in their DNA relative to CENU-sensitive cells, designated Mex $^-$ or Mer $^-$ (124). Several agents that have been used experimentally to potentiate CENU-mediated cell killing, including X rays (125), hyperthermia (126), 6-thioguanine (127), and the polyamine biosynthesis inhibitor alpha-difluoromethylornithine (128) have also been shown to increase DNA inter-strand cross-linking (127,129,130,131).

Pretreatment or simultaneous exposure to monofunctional agents, such as 1-methyl-1-nitrosourea (MNU) (132,133), ENU (134), and N'-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (135) can increase cell kill (132,134,135), SCE induction (133), and inter-strand cross-linking (132,135) produced by BCNU. In some cells these enhanced effects may be due to inhibition of guanine O^6-alkyltansferase (discussed in a later section) (133), but in other experimental systems the increased cell kill seems to be unrelated to inter-stand cross-linking (132). It is not known whether the presence of specific monoadducts in DNA can directly enhance the cross-linking reactions of CENUs.

It is clear that DNA cross-linking is important for CENU activitity, but the identity of the cross-link lesion(s) remains elusive. Ludlum and Tong have isolated 1,2-(diguan-7-yl)ethane (136) and 1-[N^3-deoxycytidal],2-[N^1-deoxyguanosinyl]-ethane (137) from BCNU-treated DNA as putative cross-link lesions. Diguanylethane is thought to arise via 2-chloroethyl-mediated alkylation at N^7-guanine, whereas the cytosine-guanine cross-link may form as a consequence of a two step reaction following alkylation at O^6-guanine. The chloroethyl adduct at O^6-guanine is thought to react with N^1-guanine to

produce a cyclized intermediate which subsequently reacts with O^3-cytosine on the opposite helix to form an interstrand cross-link (FIGURE 4) (123).

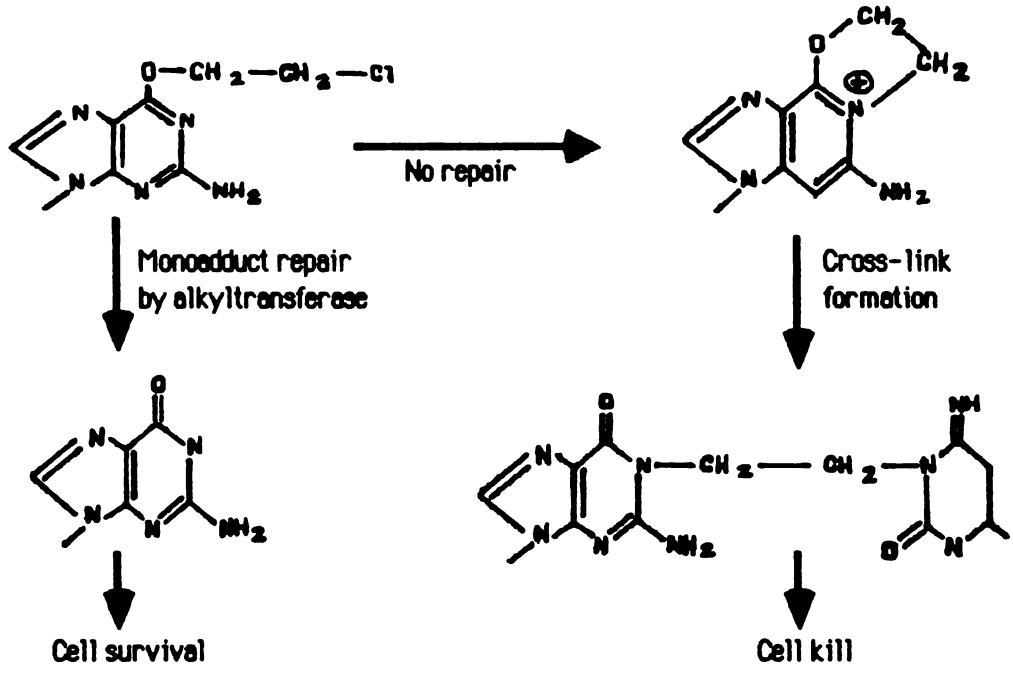


FIGURE 4. Proposed mechanism for interstrand cross-link formation following alkylation at O^6-guanine. Monoadducts are repaired in Mer $^+$ and Mex $^+$ cells prior to cross-link formation.

Monoadduct formation appears to be rapid in contrast to cross-linking which does not usually reach a maximum until 6 hrs after drug exposure (123). It has been suggested that the kinetics of cross-linking reflect the two step nature of the cross-linking process, but while the proposed precursor, O^6-chloroethylguanine and the final cross-linked product have been identified, and a rearrangment of O^6-(2-fluorethyl)guanosine to N_1-(2-hydroxyethyl) guanosine occurs upon hydrolysis (138), the cyclic form has not been found, consequently this proposed mechanism for cross-link formation has not been verified. Choroethyl adducts at O^3-cytosine have been isolated (119), so it is possible that the cytosine-guanine cross-link forms directly from the

cytosine monoadduct. Neil Buckley (139) has developed a reaction scheme for CENUs that explains their specificity of alkylation and suggests that they react with DNA without generating free diazohydroxides or isocyanates. This model also proposes that chloroethylcarbonate adducts form in DNA, from the carbamoylating portion of the molecule, and that they have the potential to form DNA cross-links. Thus, it seems likely that multiple cross-linking lesions form in BCNU-treated DNA.

II.4.c. Factors that affect CENU-mediated cell killing in vitro

Many environmental and intrinsic cellular factors are known to influence cell survival after exposure to CENUs in vitro. Each of these factors will be discussed in the following paragraphs with emphasis on its particular relevance to CENU phase-specific survival: experimental manipulation or control of each factor is also described.

II.4.c.i. Temperature

Temperature changes have been shown to affect the survival after CENU treatment in two ways. First, elevated environmental temperatures during drug exposure increase the rate of CENU hydrolysis, thereby increasing the drug exposure dose in any given time interval (140). Second, an elevation of environmental temperature in the period following drug exposure causes potentiation of DNA inter-strand cross-linking (130). Similar temperature effects have been observed in Sprague-Dawley rats treated with BCNU at elevated total body temperatures (141). In order to minimize temperature effects on phase-specific survival, the environmental temperature was controlled in all phases of experimentation and cells in various phases of the cell cycle were handled identically.

II.4.c.ii. pH

The hydolysis of CENUs is known to be affected by environmental pH. Hydrolysis is rapid in both highly acidic (pH < 2) and alkaline (pH > 8) buffers (101): CENUs are inactive at these pH extremes; however, drug exposure at a slightly elevated pH within the biological range will have an effect similar to treatment at elevated temperature in that the drug dose will be higher. Further, cell killing produced by BCNU and hyperthermia is strongly pH dependent between pH 6.5 and 8.5 (142). Intracellular or intranuclear pH is likely to be more relevant than extracellular pH due to the fact that the products of CENU hydrolysis, particularly the isocycanates, are very reactive and likely to bind rapidly to molecules in the vicinity of the hydrolysis site (143,144). The relationship between extracellular pH and intracellular pH distribution have been studied in yeast (145) and maintainance of intracellular pH has been studied in hamster fibroblasts (146). Intracellular pH increases from pH 7.2 to 7.4, in parallel with DNA synthesis rate, in mitogen stimulated lymphocytes (147), and chinese hamster fibroblasts respond to growth factors only after an elevation of intracellular pH (148). Based on these observations it appears that intracellular pH may increase slightly between G_1 and S phase. If a similar increase occured in 9L cells, the rate of CENU hydrolysis would increase in S phase cells thereby increasing the intracellular drug dose and presumably decreasing survival of cells treated in S phase. Because the exact opposite effect is observed for cells treated with BCNU in vitro, phase specific differences in intracellular pH do not appear to be a determinant of phase specific survival.

A decrease in the extracellular pH from 7.3 to 6.5 has a negligible effect on the intracellular pH in chinese hamster fibroblasts, yet it enhances

the accumulation and cytotoxicity of clorambucil (149). Chlorambucil acts as a weak base and more readily enters cells at pH 6.5 because more drug exists in the unionized form at low pH. Uptake of CENUs does not appear to be pH dependent (discussed in a later section); however, in order to minimize pH effects, extracellular pH was maintained between 7.2 and 7.4 during all drug treatments and cells in each cell cycle phase were handled identically.

II.4.c.iii. Serum

The in vitro hydrolysis of CENUs can also be modified by the presence of serum proteins. Rates of hydrolysis of BCNU, CCNU, MeCCNU, but not PCNU, are accelerated by albumin (150), and CENU degradation is stabilized by lipoproteins (151). Lipoproteins are thought to stabilize CENUs by partitioning drug within hydrophobic regions that prevent protein binding and aqeuous hydolysis (149), and as described by Weinkam et. al. (148) a reaction involving non-specific formation of a protein-CENU complex catalyzes the breakdown of inactive BCNU into two products 1) chloroethyldiazohydroxide, which forms the chloroethldiazonium ion, and 2) chloroethylisocyanate. These factors are likely to be important determinants of in vivo CENU activity, but of minimal importance for in vitro comparisons as long as the serum source and concentration are controlled.

II.4.c.iv. Hypoxia

Environmental hypoxia is a major determinant of survival following radiation exposure (152), but its importance in CENU treated cells is less well understood. It has been suggested that BCNU preferentially spares hypoxic cells (153); however, Teicher et al. (154) have shown that hypoxia causes a

slight enhancement of BCNU cell kill in tissue culture. Exposure to hypoxic conditions alters the distribution of cells throughout the cell cycle by profoundly inhibiting progression of S phase cells and causes cells to accumulate in G_1 phase (155,156). Furthermore, the combination of hypoxia and low pH is is very toxic to cells (157). The effects of hypoxia on cell cycle progression can be reversed by the addition of deoxynucleotides to the cell culture medium; however, the normal coordination between DNA, RNA, and protein synthesis that occurs during the cell cycle is lost under these conditions (158). To avoid hypoxic effects on cell kill and cell progression all experiments used fully oxygenated log phase cells.

II.4.c.v. Growth state

There have been conflicting reports regarding whether non-cycling cells differ from cycling cells in their sensitivity to CENUs. This question has been addressed using density inhibited or serum deprived plateau phase cultures, and MTS. Studies with plateau phase cultures have shown increased sensitivity in cycling cells (159), no effect (160,161), and a large increase in cell kill in non-cycling cells (162,163); some of these observations may be due to serum effects (164). Cell survival is less for 9L cells when they are exposed to BCNU as spheroids, rather than monolayers, and this difference has been attributed to the greater sensitivity of non-cycling cells, which are present in large numbers in MTS (165). Recent development of new methods for detecting CENU damage in cycling versus non-cycling cells may help resolve this issue (166).

Cell survival following CENU treatment can also be affected by the cell density at the time of treatment; BCNU-mediated cell kill increases with increasing cell density in exponentially growing 9L cells (167). As some

cells growing in culture show an ability to communicate with one another and can establish gap junctions that permit the exchange of small molecules between cells (168) this density effect may be due to intercellular communication. Tofilon et al. (169) have shown that cell-cell interactions are evident in heterogeneous MTS, composed of mixtures of sensitive and resistant 9L cells. BCNU induces many more SCEs/cell, on a molar basis, in 9L MTS (BCNU-sensitive) when compared to R_3 MTS (BCNU-resistant). Cells from heterogeneous MTS show a bimodal distribution of SCEs/metaphase that reflects the relative proportion of each cell type present; however, fewer SCEs are induced in the sensitive cells of heterogeneous MTS indicating that the resistant cells in the MTS have confered partial resistance on the sensitive cells. Further, MTS composed of 100% sensitive cells grow more rapidly than MTS composed of only resistant cells, but MTS containing mixtures of both cell types show a growth rate similar to sensitive MTS. The effects of growth state and cell density at the time of treatment were minimized in the experiments to be described by using exponentially growing cells at similar cell densities for all experiments.

II.4.c.vi. Cell progression/Potentially lethal damage repair.

Treatment of cells with CENUs is known to alter cellular progression; specifically, cells treated in S phase and G_2 phase progress relatively undisturbed through one cell division, but exhibit prolonged growth delay in the next cell cycle. In contrast, cells treated in G_1 phase progress through the cell cycle to G_2 phase where they accumulate prior to their first mitosis; after division their growth through the next cell cycle is normal (170,171). When treated with X rays, cells also tend to accumulate in either G_1 or G_2 phase and the transitions from G_1 phase to S and from G_2 to mitosis are

believed to be important in the fixation of UV- and X-irradiation-induced DNA damage (172,173). It is thought that this observed delay reflects DNA repair that must be completed before resuming cell progression. The repair thought to occur under these circumstances has been studied by observing the phenomenon of potentially lethal damage (PLD) repair. PLD repair refers to the decrease in cell kill seen when cells are allowed a post treatment incubation period usually in either balanced salt solution or culture medium prior to plating for cell survival (174). It has been suggested that the variations in cell survival observed during the cell cycle in X-irradiated Ehrlich ascites tumor (EAT) cells reflects the final amount of PLD expressed as cells progress through the cell cycle. In EAT cells PLD repair occurs in cells arrested in all cell cycle phases and cells progressing through the cell cycle as long as they have not progressed past the G_1/S phase transition point or through mitosis (175). Thus, G_1 cells irradiated just prior to the initiation of S phase and G_2 cells irradiated just prior to mitosis are the most sensitive cells in the cell cycle because they have very little time to repair damage prior to its fixation. The mechanism responsible for PLD repair has not been defined and may not be the same for different agents, but it does not occur in 9L (134) or other monolayer cultures treated with CENUs (176), consequently it is not likely to be a determinant of CENU phase specificity in the studies to be described. PLD repair does occur in 9L spheroids treated with BCNU (177), indicating that this phenomenon can be strongly influenced by the experimental conditions.

II.4.c.vii. Guanine O^6-alkyltransferase

Guanine O^6-alkyltransferase (AT) is a repair protein that removes monoadducts from O^6-guanine in mammalian cells. It has a high degree of

substrate specificity in that it readily removes O^6 alkyl lesions, but it is much less efficient at removing O^4 alkyl lesions (178), and is not catalytic like an enzyme, rather each protein molecule is inactivated after removal of a single monoadduct (179). The presence of AT activity determines the Mex $^+$ phenotype that is characteristic of CENU-resistant human cells (180). A similar cell phenotype, termed Mer $^+$, has been described that is characterized by an increased ability to reactivate MNNG treated adenovirus DNA (181). Mer $^-$ cells typically show increased sensitivity to CENUs, reduced levels of DNA cross-linking following CENU exposure, and low levels of AT (182). Cellular resistance in Mex $^+$ and Mer $^+$ cells is thought to occur because AT activity can effectively remove O^6 monoadducts before they react to form DNA cross-links. Support for this mechanism comes from the strong correlation between AT activity and CENU sensitivity, from the fact that CENU resistance can be overcome by pretreatment of cells with MNNG, which forms O^6-guanine adducts, or by pretreatment with O^6-alkylguanine (134,183), and from the fact that AT activity cannot repair cross-links (184). CENUs can inactivate AT by the formation of O^6-guanine in DNA or by direct alkylation of the enzyme (185), thus they can modify their own repair. The terms Mex $^+$ and Mer $^+$ are often used interchangeably to describe CENU-resistant cells, but there is evidence that Rem $^+$ and Rem $^-$ subtypes of the Mer $^+$ phenotype exist (186,187). Both Rem $^+$ and Rem $^-$ cells have AT activity (Mer $^+$); however, they differ in their sensitivity to MNNG. Further, there is evidence that different gene products are involved in the repair of CENU and MNNG treated DNA (188). Cellular resistance mediated by AT is highly specific for agents that produce O^6-alkylguanine lesions as evidenced by the fact that MNNG pretreatment of CENU-resistant cells does not affect *cell* kill produced by nitrogen mustards, or cis-platinum (189). Further,

resistance to CENUs does not imply cross-resistance to other alkyating agents (190,191).

AT activity has been reported to increase in late G_1 phase, reach a maximum in early S phase, and subsequently decline in late S (62). This result is consistent with earlier reports on AT activity in regenerating rat liver (192), but contrasts with the finding that AT activity is higher in CH3 10T1/2 mouse cells during pre S phase and constant throughout the S phase (64). A recent report shows that AT activity increases before S phase in normal human cells, but not in Bloom's syndrome cells (193). Cells from patients with Bloom's syndrome show an increased mutation rate (194) and an altered pattern of excision repair during the cell cycle (195). These findings indicate that cell cycle variation in AT activity is dependent on the cell type studied and the experimental conditions. Because of the many differences between these model systems it is difficult to ascertain the relevance of these results to one another, and consequently AT activity throughout the cell cycle was assayed as part of this dissertation.

II.4.c.viii. Glutathione

Glutathione (GSH), the major non-protein sulphydryl compound in cells, can affect cellular response to drugs and radiation (196). GSH is thought to protect cells from CENU-induced damage via detoxification of active species. The detoxification reactions mediated by GSH include oxidation-reduction reactions and thioether formation (FIGURE 5). Glutathione reductase (GR), the enzyme that regenerates reduced GSH from the oxidized form, GSSG, is irreversibly inhibited by the isocyanates generated from BCNU (197), and inhibition may be specific for BCNU as other CENUs vary in their ability to alter cellular thiol levels (198).

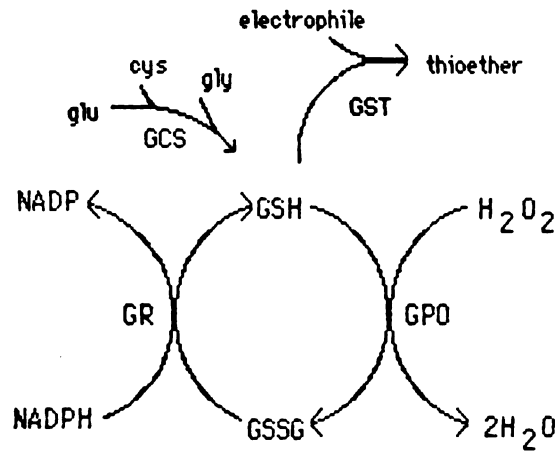


FIGURE 5. Glutathione metabolism in mammalian cells. Oxidation-reduction cycle and thioether formation. The enzymes shown are: ∂-glutamyl-cysteine synthetase, (GCS); glutathione S-transferase, (GST); glutathione reductase, (GR); glutathione peroxidase, (GPO). From B. A. Arrick and C. F. Nathan, Cancer Res. 44, 4224-4232 (1984).

Inhibition of GR is thought to cause membrane changes and karyotypic abnormalities (199), and surface bleb formation, suggesting membrane damage, occurs in sensitive, but not resistant human glioma cells treated with BCNU (200); BCNU also decreases GSH in resistant, but not in sensitive 9L rat brain tumor cells (201). Thus, it is clear that cellular thiols can influence CENU-induced damage and that the active species produced by CENUs can deplete thiol levels, thereby enhancing their own cytotoxicity or the cytotoxicity of other agents administered simultaneously or subsequently.

The variation in non-protein sulfhydryl levels during the HeLa cell cycle corresponds closely to the phase specific survival pattern seen following exposure to radiation (202). Higher thiol levels are associated with increased survival for all phases of the cell cycle, except for during mitosis

when thiol levels are elevated and sensitivity of cells is greatest. This inconsistency may reflect the extreme sensitivity of the mitotic spindle to radiation damage independent of thiol status. It has also been shown that addition of cysteamine prior to irradiation reduces the magnitude of survival differences between cell cycle phases (203). Based on these observations thiol levels were measured in 9L cells during the cell cycle and correlated with CENU phase specific survival.

I.4.c.ix. Drug uptake

One of the common causes of cellular resistance to chemotherapeutic agents is reduced intracellular drug dose (204). The effective concentration of drug within cells is influenced by the competing processes of drug uptake, detoxification, and eflux; resistance can be due to an alteration in any of these processes. Treatment with some alkylating agents can also alter the uptake of other alkylating agents (205). Uptake of BCNU and CCNU, which are lipophillic, occurs rapidly by passive diffusion in L5178Y lymphoblasts; equilibrium is established between intracellular and extracellular compartments within one minute (206) and is not thought to contribute to CENU resistance. Likewise, the water soluble CENU, CHLZ, seems to enter cells by passive diffusion, in spite of the presence of the glucose moeity (207), though its uptake may not be as rapid as the lipophilic CENUs (208).

Although, the surface glycopetides and the structure of HeLa cell membranes changes during the cell cycle (209) variation in CENU uptake during the cell cycle has not been studied. Levin et al. have shown that the uptake of urea, manitol, and methotrexate is not affected by cell cycle position in S49 cells (210). This observation in conjunction with the chemical properties of CENUs suggests that uptake through the plasma membrane is

not likely to vary between cell cycle phases; however, the phase specificity of CENUs could be due to reduced levels of alkylation- or carbamoylation-mediated damage in S phase cells relative to G_1 and G_2/M phase. Studies of [^{3}H] CNU binding have attempted to address the issue of phase specific monoadduct formation.

II.4.c.x. Chromatin structure/DNA conformation

As cells progress through the cell cycle their DNA undergoes cyclic changes in structural conformation in order to replicate the genome and to condense chromatin into chromosomes during mitosis (211). Variations in conformation can be dectected as alterations in the pattern of chromatin digestion by DNAse I (212), sensitivity of DNA to acid or thermal denaturation in situ (213,214), and binding of intercalating dyes (215). Cells in G_1 phase have more easily digestable DNA than do S phase cells and digestability is regulated by a cytoplasmic factor in G_1 phase and a nuclear factor in S phase (216,217). Quiescent lymphocytes have DNA that is less resistant to acid denaturation in comparison to cycling cells further, indicating that chromatin structure may be cell cycle phase specific. Changes in DNA conformation can influence the reactions of alkylating agents with DNA as evidenced by the fact that ENU-mediated formation of O^6-alkylguanine in DNA is decreased in chromatin fibers with higher orders of folding (218) and binding of polyamines to DNA, which may alter DNA structure (219), inhibits MNU-mediated alkylation at O^6-guanine, N^3-adenine, and N^7-guanine (220). The repair of DNA damage is also dependent on DNA conformation; a mammalian DNA-repair endonuclease has been shown to act only on supercoiled DNA (221), and O^6-methylguanine and ring opened N^7-methylguanine are not repaired by E. coli enzymes when the

lesions are present in polynucleotides that exist in the Z-DNA conformation (222,223). These results suggest that the conformation of DNA in cells treated with alkylating agents can influence the survival level following treatment.

The effect of DNA conformation on CENU-mediated cell killing has been studied indirectly. Depletion of cellular polyamines by the ornithine decarboxlyase inhibitor, alpha-difluoromethylornithine (DFMO), enhances BCNU cell kill and DNA interstrand cross-linking (131) and low doses of X rays, which also alter DNA conformation (224), have a similar effect (129). DFMO and X rays are thought to increase cell kill by altering the conformation of DNA such that DNA cross-linking is facilitated. In support of this hypothesis cells treated with MeCCNU and DFMO show increased cell killing over MeCCNU alone, but DNA alkylation is the same in untreated and DFMO pretreated cells indicating that DFMO affects cross-linking and not initial monoadduct formation (225). It has also been shown that the chemical structure of CENUs can influence the specificity of binding to chromatin. Pretreatment of cells with sodium butyrate, which induces chromatin condensation, prior to CCNU or CHLZ exposure increased the uptake of both drugs and the extent of DNA alkylation and carbamoylation (226). CCNU preferentially carbamoylates non-histone proteins, and CCNU alkylates DNA in the core region of nucleosome particles, whereas CHLZ preferentially alkylates chromatin in the histone H1 linker region (227). The relevance of these observations to the phase specificity of CENUs is uncertain, but the question of phase specific alkylation was addressed by measuring binding of [^{3}H] CNU to DNA throughout the cell cycle.

II.4.c.xi. Excision repair

Alkylation damage in DNA is repaired by DNA-repair enzymes in a multiple step pathway that involves: 1) recognition of either a damaged base, an apurinic site, or a strand break: 2) excision of the damage by a damage specific endo- or exonuclease: 3) DNA polymerization to fill the gap that is created by excision: and, 4) ligation of free DNA strands to restore continuity to the DNA helix (228). Subsequent to the excision repair process, the repair patch must undergo a structural rearrangement in order to acquire the same stapphylococcal nuclease sensitivity as bulk chromatin (229). Because CENUs are thought to exert their lethal effects via DNA cross-linking after alkylation at O^6-guanine, they have not been widely used in excision repair studies; however, Thompson et. al have isolated mutants of Chinese hamster ovary (CHO) cells that are hypersensitive to ultraviolet radiation (UV) exposure (230). These UV excision repair mutants fall into five complementation groups, two of which are also 30 to 90 fold more sensitive to DNA cross-linking agents than is the parent cell line (231,232). Two of the cross-link sensitive mutants, UV-20 (233) and UV-4 (234), have defects in cross-link removal which suggests that cross-link repair and UV excision repair share some common features and/or enzymes. Recent characterization of another cross-link sensitive CHO mutant, UV-1, shows that sensitivity to UV and mitomycin C is due to more than one genetic alteration (235). This result is consistent with the findings that Mer $^+$ cells can have different sensitivities to MNNG-mediated cell killing (Rem $^+$ or Rem $^-$) (186) and that the Mex $^+$ and Mer $^+$ phenotypes are not identical (188). Phase specific survival in the CHO parent line, AA8, and the mutant, UV-4,was studied to determine the role of excision repair as a determinant of the phase specificity of BCNU.

II.4.c.xii. Nucleotide pools

In vitro and in vivo studies in bacteria and mammalian cells have shown that the fidelity of DNA replication depends on the proper balance of DNA precursors (236). It has also been shown that survival of CHO cells after exposure to alkylating agents can be influenced by the balance of deoxyribonucleotides with survival increasing two to ten fold when cells are treated under conditions where the ratio of dCTP to dTTP is high (237). These results suggest that enhanced cell killing can be achieved by combining CENUs and drugs that alter nucloetide pool balance, but this has not been studied mechanistically. It is not clear how nucleotide pool imbalances would determine the phase specificity of CENUs, but if alkylation of the precursor pool were a significant target for intracellular reactive species, as has been suggested for MNU (238), one might expect S phase, where cells are rapidly incoporating nucleotides, to be the most sensitive cell cycle phase. This is not the case for BCNU-treated cells and consequently nucleotide pool effects were not addressed in this dissertation.

III. MATERIALS AND METHODS

III.1. Cells and culture conditions

Monolayers of 9L rat brain tumor cells and CHO cells were maintained at 37° C in a 5% CO_2-95% air environment. 9L cells were cultured in complete medium (CMEM) consisting of Eagle's minimal essential medium supplemented with non-essential amino acids, and gentamicin (50 ug/ml), and one of three types of serum, where indicated: 1) 10% newborn calf serum (NCS) 2) 10% calf serum containing insulin, transferin, and selenium (SCS) 3) 10% Nu-serum (NS). Cell survival following BCNU treatment was similar regardless of which serum was used. The cell line designated BTRC-19 was isolated as a BCNU-resistant clone following treatment of 9L spheroids with 45 uM BCNU (239). CHO cells were obtained from Larry Thompson at Lawrence Livermore National Laboratory and cultured in alpha-minimal essential medium, without nucleosides, supplemented with 10% fetal calf serum and gentamicin (50 ug/ml). The cell line designated UV-4 was isolated as a UV sensitive clone following exposure of AA8 cells to the mutagen ENU and found to also be sensitive to DNA cross-linking agents (231).

To remove cells from 75 cm^2 plastic flasks, cultures were rinsed with 3 ml of STV (Saline A containing 0.05% trypsin and 0.02% versene). After decanting the rinse, another 3 ml of STV was added and the flasks were incubated at 37° C for 5 min. Enzymatic action was stopped by adding 10 ml of CMEM, then the flask was struck sharply and the cell suspension was pipeteted several times to obtain a single cell suspension. 9L cells growing in 850 cm^2 roller bottles were removed using enzyme cocktail following a rinse with 50 ml of Hanks' balanced salt solution (HBSS). The enzyme cocktail (0.05 % pronase, 45 PKU/ml; 0.02% DNAse, 7 x 10^4 dornase units/mg;

and, 0.02% collagenase II, 139 units/mg) was added for 30 minutes at 37° C after which the suspension was diluted with ice cold CMEM. The pellet was resuspended in fresh CMEM and passed through a 25 um nylon mesh screen to obtain a single cell suspension. CHO cells growing in 75 cm^2 plastic flasks and 850 cm^2 roller bottles were both handled using the STV procedure.

III.2. Drug treatment

All CENUs were stored at -70° C. Immediately before use BCNU, CCNU, MeCCNU, PCNU, BHCNU and CNU were dissolved in a small amount of 100% ethanol; CHLZ and ENU were dissolved initially in DMSO, then ethanol was added. All drugs were subsequently diluted in CMEM in treatment flasks. The concentration of solvent was always less than 1% and did not affect plating efficiency (PE). Extracellular pH was maintained between 7.2 and 7.4 at all times and all drug incubations were carried out at 37° C. After exposure to drug cultures were rinsed with fresh CMEM and then processed for cell survival or alkaline elution.

III.3. Cell survival assay

A colony forming efficiency (CFE) assay, rather than dye exclusion or growth inhibition, was used to determine cell survival because it directly measures the clonogenicity of cells after treatment. The CFE assay has been described (125). Briefly, cells were counted, diluted and plated (in quadruplicate) into Petri dishes containing 5 x 10^4 irradiated (40 Gray) 9L feeder cells. After incubation for 12 to 14 days, colonies were fixed with ethanol, stained with crystal violet and counted. PE was calculated as the ratio of the number of colonies counted to the number of cells plated times 100.

III.4. Centrifugal elutriation

Cell synchrony was achieved using the technique of centrifugal elutriation. This technique, which separates cells on the basis of their sedimentation properties in a continuous flow system, had a minimal effect upon cell viability, and does not require the use of protein or DNA synthesis inhibitors, or mitotic selection. After drug exposure, cultures were disaggregated to single cells and placed in the elutriator flow system and allowed to equilibrate in the separation chamber, then fractions enriched in G_1, S, and G_2/M phase cells were collected by reducing the centrifuge rotor speed.

III.4.a. Methodology

Cells were elutriated using a Beckman J2-21 centrifuge equipped with a JE-6 elutriator rotor. Before each run the elutriator system was sterilized with 70% ethanol and rinsed with an equal volume of sterile saline. Before injection of the cell suspension the flow system was filled with ice cold MEM without additives. The fluid reservoir was maintained on ice and the centrifuge was kept at 4° C during the elutriation procedure. The rotor speed and fluid flow rate were determined empirically for each cell line and cell cycle phase enriched populations were obtained by reducing the rotor speed in 100 rpm decrements and collecting 270 ml fractions at each speed setting. After elutriation, cells were centrifuged at 1000 rpm for 15 min then resuspended in ice cold CMEM and held on ice until they could be assayed for cell survival. The cell number and median cell volume of each elutriator fraction were monitored using a Coulter counter equiped with a channelyzer.

The enrichment of each fraction was determined by analyzing single parameter DNA histograms obtained using flow cytometry (FIGURE 6).

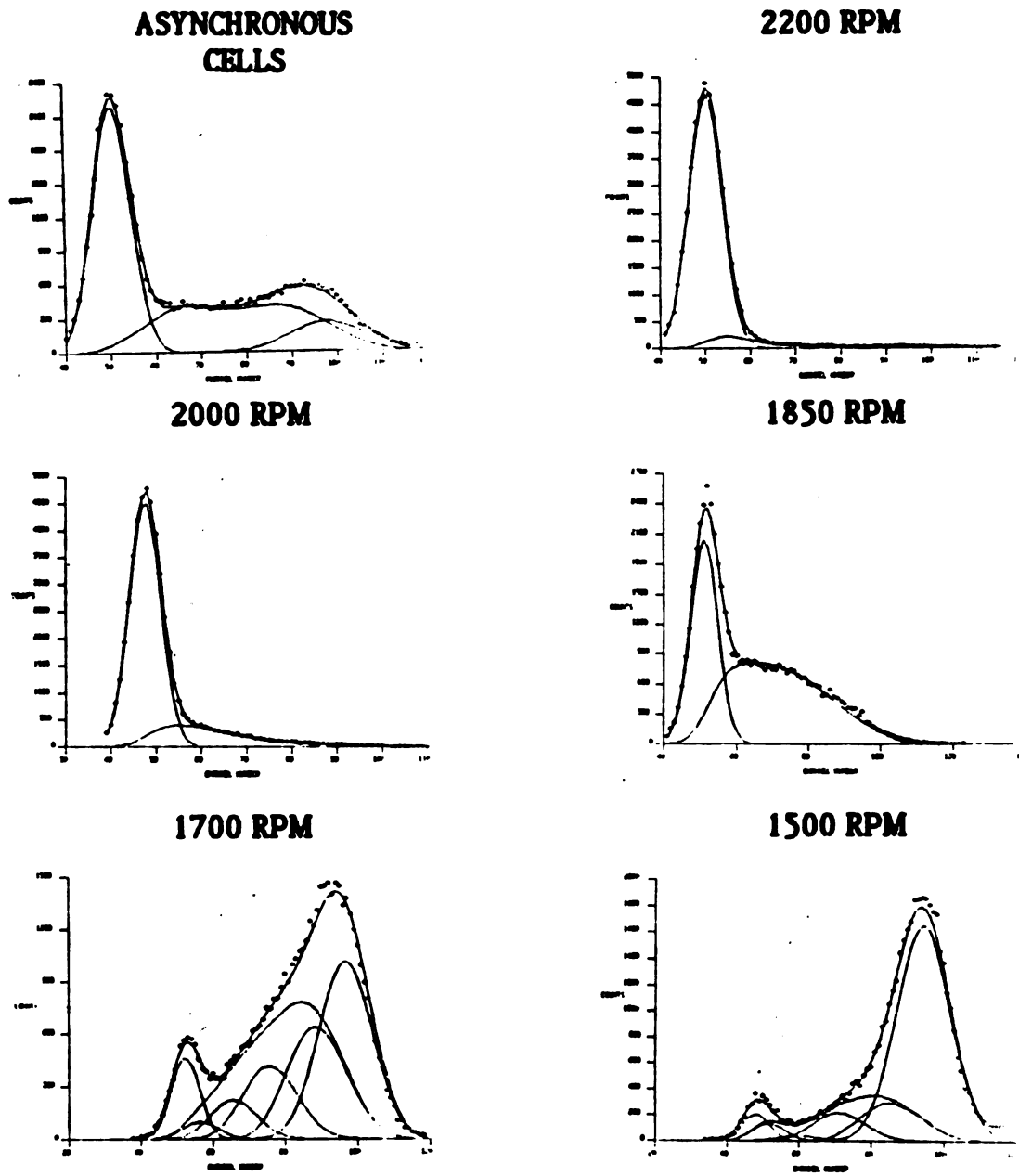


FIGURE 6. Computer analysis of single parameter DNA histograms. Figure shows selected elutriator fractions and the curves used to estimate the phase enrichment of G_1, S, and G_2/M phase cells.

5×10^5 to 1×10^6 cells were fixed in 70% ethanol and stained with 1 ml of chromomycin A_3 solution (10 mg chromomycin A_3, 1.5 g $MgCl_2 \cdot 6H_2O$, in 500 ml distilled water). Samples were analyzed using a FACS III flow cytometer with a 5 watt argon laser set at 457 nm and adjusted to emit 160-200 milliwatts. The resulting fluorescence was passed through a Schott KV-250 nm long wave pass filter. The DNA distributions were analyzed using the computer program PEAKS3 at Lawrence Livermore National Laboratory. The mathematical model used in the analysis represented G_1 and G_2/M phases with Gaussian functions and S phase with a series of Gaussian-broadened rectangles. A non-linear, least squares curve fitting technique was used to fit the combined function to the data. G_1 and S phase fractions were obtained with good precision; the average error associated with estimates of G_1 and S phase enrichment in each elutriated fraction was ± 1.4%. Estimates of G_2/M phase enrichment were somewhat variable, especially if the fraction was small; the average error associated with estimation of the percentage of G_2/M phase cells was ± 5.1%.

III.4.b. Estimation of subpopulation plating efficiency

Pure populations of G_1, S, and G_2/M phase cells were not obtained with elutriation so that true phase specific PEs could not be determined experimentally. Instead these data were used to compute estimates of the PEs for pure populations of G_1, S, and G_2/M phase cells (240). In this procedure, the purity of each elutriated fraction is determined from single parameter DNA histograms. The total PE of any fraction is thought to reflect the sum of PEs of the subpopulations weighted by their individual phase enrichment. We assume that PE within each phase is constant and that the total PE for any elutriator fraction i is:

$$PE^i = (PE_{G1} \times F_{G1}) + (PE_S \times F_S) + (PE_{G2/M} \times F_{G2/M})$$

where F_{G1}, F_S, and $F_{G2/M}$ are the fractions of cell in the G_1, S, and G_2/M phases determined by analysis of the DNA distribution for fraction i, and PE_{G1}, PE_S, and $PE_{G2/M}$ are the plating efficiencies that would be measured for pure populations of G_1, S, and G_2/M phase cells, respectively. The "true" plating efficiencies PE_{G1}, PE_S, and $PE_{G2/M}$ were estimated using a least squares best fit procedure in which these values were adjusted to minimize the quantity:

$$[PE^i - (PE_{G1} \times F_{G1}) - (PE_S \times F_S) - (PE_{G2/M} \times F_{G2/M})]^2$$

wher PE^i is the experimentally measured PE for the elutriator fraction i; the other quantities are defined above.

III.5. Alkaline elution assay of DNA cross-linking

The alkaline elution assay described by Kohn et al. (241) was used to assay damage in the DNA of treated cells because of its high sensitivity for detection of single strand breaks and ability to detect DNA-DNA cross-links and DNA-protein cross-links. The specific procedure measured the amount of DNA in each fraction by quantitating the fluorescence of bound Hoechst 33258 dye.

9L cells growing in two 850 cm^2 roller bottles were dissociated to single cells, elutriated as described, and then placed in water jacketed spinner flasks containing 37° C CMEM. Cells were treated in suspension with 120 uM BCNU for 30 min after which they were centrifuged at 1000 rpm for 5 min, resuspended in 37° C CMEM and maintained in suspension culture for

6 hrs. 4×10^6 to 7×10^6 cells, depending on the elutriator fraction, were deposited on polycarbonate filters (2 um pore size) and lysed with a solution containing 2 M NaCl, 0.04 M Na_2EDTA, 0.2% Sarkosyl (pH 10.0), and 0.5 mg/ml proteinase K. The filter was washed with 0.02 M Na_2EDTA (pH 10.3), and the DNA was eluted in the dark with tetrapropylammonium hydroxide-0.02 M H_4EDTA (pH 12.2) at a flow rate of 0.036 to 0.038 ml/min. Fractions were collected every 90 min for 18 hr. After the elution procedure, DNA was removed from the filters by heating at 65° C for 20 min in 5 ml of the eluting buffer. The amount of DNA in each fraction , including the filter and wash solution, was determined as described by Murray and Meyn (242). The cross-linking factor (CLF) was calculated as described by Ewig and Kohn (243) according to the following equation:

$$1000 \times [((1 - R_0) / (1 - R_1))^{1/2} - 1]$$

where R_0 is the fraction of the DNA remaining on the filter after 30 ml of elution for control irradiated cells (4 Gray on ice) and R_1 is the fraction of DNA remaining on the filter after 30 ml of elution for BCNU treated and irradiated cells. The retardation in the rate of elution seen in BCNU-treated cells that are irradiated just prior to placement on the filters is proportional to DNA cross-linking, and an increase in the CLF corresponds to an increase in cross-linking. Because proteinase K is included in the lysis solution the CLF reflects DNA-DNA croslinks, as the contribution of DNA-protein cross-links is eliminated by enzyme treatment.

III.6. Guanine O^6-alkyltransferase assay

The transfer of ^3H-labeled methyl groups from O^6-guanine in DNA to the AT acceptor protein was determined from the appearance of tritium in an insoluble protein fraction essentially as described by Myrnes et al. (244). About 1 ug of [^3H] MNU-treated DNA was incubated with the transferase at 37° C for 30 min in 200 ul of buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.02% sodium azide and 10% glycerol. The reaction was stopped by adding 2.5 volumes of 5% tricholoacetic acid (TCA) to precipitate both DNA and protein. The DNA was selectively hydrolyzed by heating for 30 min at 80° C, which depurinated all of the O^6-methylguanine, then cooled on ice for at least 5 min, and when less than 100 ug of protein was present (protein assay descibed below) 100 ug of BSA was added. The protein precipitate was collected by filtration on 2.5 cm Whatman GF-F glass fiber filters. The filters were washed twice with 15 ml of 5% TCA and once with 95% ethanol. Each filter was placed in 200 ul of solubilizer (NCS, Amersham) in a scintillation vial for about 1 min before addition of toluene-based nonaqueous scintillation cocktail for scintillation counting. A unit of transferase activity was equivalent to 1 pmol of [^3H] methyl bound to protein in this assay. Samples were run in duplicate, when posible, and AT activity was calculated from the regression line comparing picomoles of AT activity per ul assay volume at 3-5 different assay volumes. Each sample was also normalized for the number of cells/ml and the protein content/ml.

III.7. Glutathione and protein determination

Total non-protein sulfhydryl (GSH + GSSG) contents of 9L cells and BTRC-19 cells were determined throughout the cell cycle. Cells (1×10^6) from each elutriator fraction were washed with 5 ml of phosphate buffered

saline (PBS), centrifuged at 2000 rpm, then the supernatant was removed and the pellet suspended in 250 ul of 8% Sulpho-salycilic acid. The cell suspension was sonicated for 30 sec, then centrifuged at 2000 rpm for 5 min and the supernatant assayed for thiol content using a modified Tietze assay (245). 720 ul of NADPH (1 mg/ml in pH 7.5 buffer), 100 ul of triethanolamine (1 mM adjusted to pH 8), 50 ul of GSH standard or sample, 10 ul of glutathione reductase (50 U/ml in pH 7.5 buffer), and 100 ul of 5,5'-dithiobis-(2-nitrobenzoic acid) (6 mM in pH 7.5 buffer) were reacted directly in a 1 ml cuvette and the ΔOD at 412 nm was determined.

For protein determination 5 x 10^5 cells from each elutriator fraction were washed with 5 ml of PBS, centrifuged, then the pellet was resuspended in 500 ul of PBS. The cell suspension was sonicated for 30 sec and total cellular protein content was determined using the method of Lowry et. al. (246) with bovine serum albumin as the standard.

III.8. Measurement of ^{3}H-CNU binding

Exponentially growing 9L cells were removed from roller bottles using enzyme cocktail and placed in suspension culture at 37° C. 15 uM CNU containing 0.37 uM [^{3}H] CNU (New England Nuclear 62 Ci/mmole) was added to the cell suspension for one hour after which the cells were washed several times with HBSS to remove unbound radioactivity. 3 x 10^8 cells were elutriated as described. A sample of the asynchronous culture (3.5 x 10^7 cells) and samples enriched in G_1, S, and G_2/M phase (4.0 x 10^7, 2.5 x 10^7, and 8 x 10^7 cells) were analyzed for phase enrichment, and the DNA in each sample was isolated according to Bodell et al. (247). The amount of purified DNA recovered was determined by measuring absorbance at 260 nm and bound radioactivity was determined by scintillation counting.

IV. RESULTS

IV.1. CENU analogs

Phase specificities of CENU analogs were determined in 9L cells using centrifugal elutriation. Typically, 70-90% of the cells injected into the elutriator were recovered. The cell volume increased linearly with fraction number and was reproducible in repeated experiments (FIGURE 7).

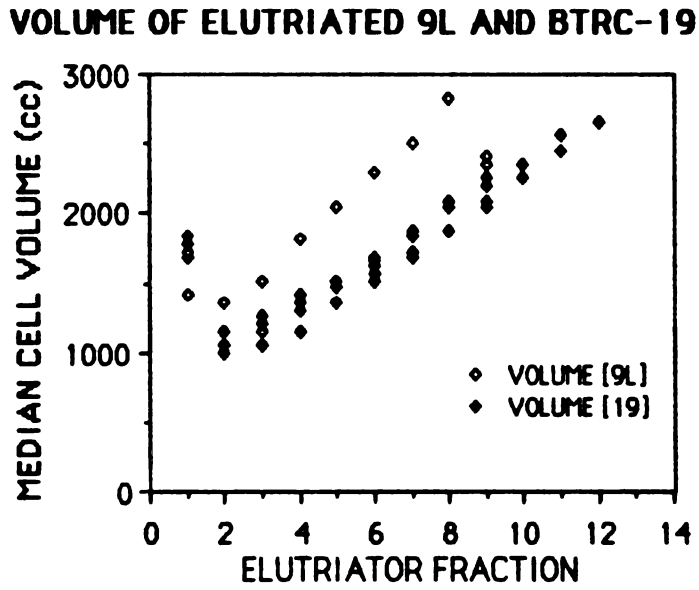


FIGURE 7. Cell volume in elutriated 9L and BTRC-19 cells. Fraction 1 corresponds to asynchronous cells. Figure shows data from 6 experiments, 3 with each cell line.

In selected elutriated fractions, the purity of G_1 phase cells was greater than 90%, while the highest purity of S and G_2/M phase enriched fractions was 65 and 55%, respectively; the PEs of untreated cells from all elutriated fractions were 43-70% (TABLE 3). PEs of untreated and unelutriated 9L cells typically ranged from 40-70% depending on the serum lot.

TABLE 3. Phase enrichment and PE of elutriator fractions determined by computer. Results from a representative separation of untreated 9L cells.

Fraction	Rotor Speed (rpm)	PE[a]	Phase Enrichment (%)		
			G_1	S	G_2/M
1	Asynchronous cells	63.9 ± 10.6	50	39	12
2	2200	65.3 ± 12.5	91	8	1
3	2100	61.2 ± 10.6	89	10	1
4	2000	69.7 ± 7.3	80	19	0
5	1900	59.1 ± 7.9	55	45	0
6	1800	60.9 ± 8.9	39	61	0
7	1700	60.3 ± 3.3	20	65	15
8	1600	45.6 ± 6.7	9	52	38
9	1500	42.8 ± 13.9	5	41	55

a-Mean ± standard deviation of 4-8 Petri dishes.

All drugs were cytotoxic to cells in all elutriated fractions. However, drugs with different chemical reactivities produced different patterns of phase specific cell killing (FIGURES 8-10). All CENUs were more toxic to fractions enriched in G_1 or G_2/M phase cells. A plot of PE vs. elutriation fraction for PCNU, which was typical for the CENUs, is shown in FIGURE 8. Elutriator fractions enriched in S phase cells (Fractions 4-6) showed higher PEs compared to G_1 and G_2/M phase enriched fractions, and the magnitude of the differences increased with increasing dose. ENU was most toxic to elutriated fractions enriched in S and G_2/M phase cells, and the magnitude of the differences in PE between cell cycle phases increased slightly at the higher drug dose (FIGURE 9). Cell kill caused by BHCNU appeared to be similar for cells in all phases of the cell cycle (FIGURE 10). Only one drug dose is shown for cells treated with BHCNU due to the fact that doses

exceeding 300 uM caused extensive cell clumping which prevented obtaining a single cell suspension.

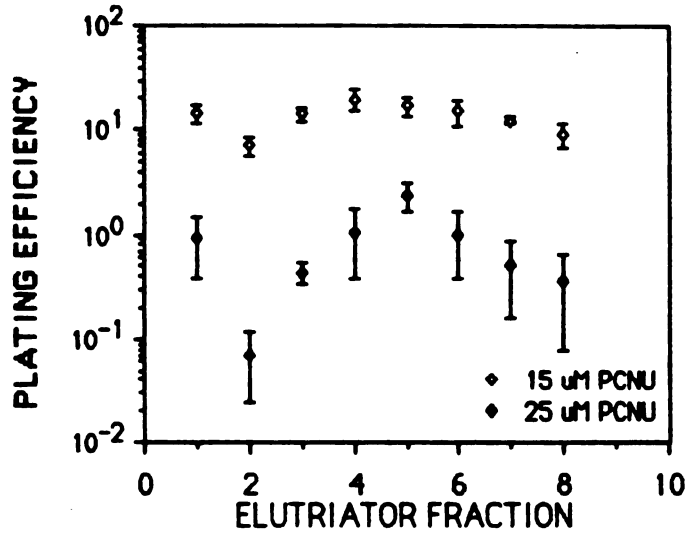


FIGURE 8. PE of elutriated 9L cells treated with PCNU (0.5 hr exposure). Fraction 1 corresponds to asynchronous cells. Each point represents mean ± standard deviation of 4-8 Petri dishes.

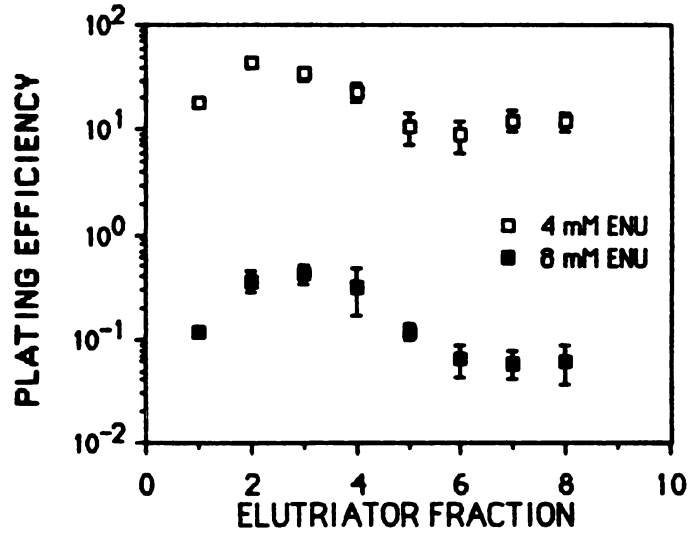


FIGURE 9. PE of 9L cells treated with ENU (1 hr exposure). Fraction 1 corresponds to asynchronous cells. Each point represents mean ± standard deviation of 4-8 Petri dishes.

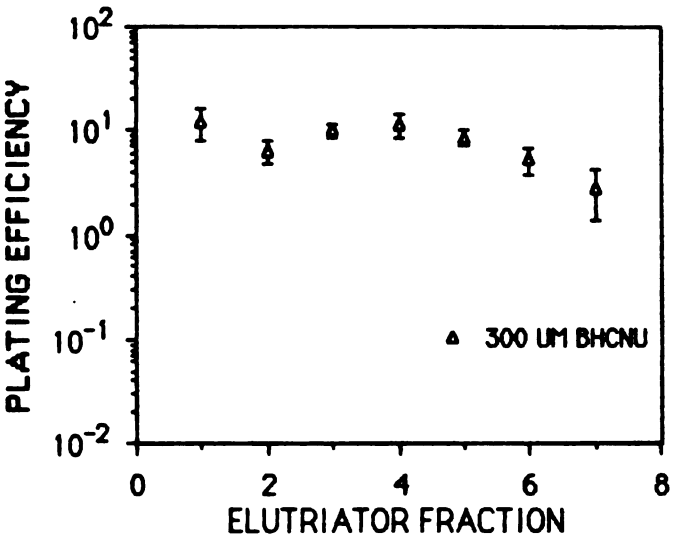


FIGURE 10. PE of 9L cells treated with BHCNU (1 hr exposure). Fraction 1 corresponds to asynchronous cells. Each point represents mean ± standard deviation of 4-8 Petri dishes.

The general pattern of sensitivity for G_1 and S phase cells can be seen on these plots; however, because of the inter-experimental variation in the purity of elutriated fractions, individual phase sensitivities could not be determined accurately. A better estimate of the true PEs of the G_1, S, and G_2/M subpopulations was calculated using the mathematical model described in the Material and Methods section. The magnitude of differences between cell cycle phases became evident when data were analyzed using this method (TABLE 4). G_2/M phase cells treated with PCNU had about the same sensitivity as G_1 phase cells; G_2/M phase cells treated with ENU were less sensitive than S phase cells; and G_2/M phase cells treated with BHCNU were much more sensitive than G_1 or S phase cells. Thus, when phase fraction purity was accounted for, G_2/M phase cells had a phase sensitivity pattern that was not apparent from the plots of the uncorrected data.

TABLE 4. PEs of cell cycle phase subpopulations.

Treatment	Dose (uM)	No. of experiments	Plating efficiency			
			Asynchronous cells	Calculated values[a]		
				G_1	S	G_2/M
Untreated	--	3	67.3 ± 7.3[b]	64.2 ± 4.9	63.9 ± 8.3	53.5 ± 9.3
BCNU[c]	20	2	9.4, 17.4 (13.4)	5.8, 13.3 (9.6)	3.6, 25.0 (19.3)	0, 7.6 (3.8)
	40	1	2.6	0.24	7.1	0[d]
PCNU[c]	15	1	14.5	7.2	20.9	6.4
	25	2	6.4, 0.92 (3.7)	0.12, 0.34 (0.23)	3.5, 2.6 (3.0)	0, 0[d]
CCNU[c]	30	2	3.0, 2.5 (2.8)	2.7, 1.8 (2.2)	5.8, 4.6 (5.2)	0.0002, 0.0001 (0.0001)
	60	2	0.047, 0.031 (0.039)	0.012, 0.0014 (0.0067)	0.062, 0.030 (0.046)	0, 0[d]
MeCCNU[c]	15	1	17.6	11.6	24.5	11.0
	30	1	0.33	0.033	1.4	0.13
CHLZ[c]	25	1	2.6	2.5	5.8	0.96
	60	2	0.11, 0.0067 (0.089)	0.056, 0.041 (0.049)	0.162, 0.069 (0.12)	0, 0.004 (0.002)
CNU[e]	10	1	17.6	12.2	28.6	0
	15[f]	1	0.709	0.393	0.958	0
ENU[e]	4000	2	10.1, 18.6 (14.4)	15.8, 40.9 (28.4)	0.045, 2.45 (1.25)	3.9, 13.3 (8.61)
	8000	2	0.12, 0.80 (0.46)	0.421, 0.481 (0.45)	0, 0	0.025, 1.43 (0.73)
BHCNU[e]	300	2	7.3, 12.4 (9.9)	16.3, 13.4 (14.8)	8.2, 6.8 (7.5)	1.1, 0 (0.54)

[a] Least square estimate of subpopulation plating efficiency as described; average values in parentheses below line when 2 experiments were performed.
[b] Mean ± standard deviation in three experiments.
[c] 0.5 hr drug treatment.
[d] Data predicts PE < 10^{-5}.
[e] 1 hr drug treatment.
[f] Cells treated in suspension.

S phase cells treated with CENUs at various doses that produced 80-90% cell kill (in asynchronous cells) were 2-3 fold less sensitive than G_1 phase cells. For PCNU and MeCCNU, cells in the G_2/M phase had about the same sensitivity as cells in G_1 phase, but for the other CENUs, G_2/M phase cells were more sensitive than G_1 phase cells.

Estimates of phase specific PEs obtained for several drug treatments predicted essentially zero PE for G_2/M phase cells, which would not have been expected from the phase specificity plots. These predicted PEs may reflect either the limit of sensitivity of the CFE assay or an error in the method used to estimate G_2/M phase enrichment. An attempt was made to measure the PE of G_2/M phase enriched elutriator fractions free of contaminating late S phase cells, by simultaneously exposing cells to PCNU and a lethal dose of [^{3}H] thymidine. In these experiments, only S phase cells should have incorporated the radiolabel and been killed; G_1 and G_2/M phase cells should have been unaffected by the [^{3}H] thymidine. Unfortunately, the PE of asynchronous cells treated with PCNU and [^{3}H] thymidine was much lower than for cells treated with PCNU alone, and this difference could not be attributed to the loss of all the S phase cells from the population. Thus, some G_1 and G_2/M phase cells were killed by the combination treatment that would probably have survived treatment with PCNU alone. In order to test the accuracy of computer-derived estimates of phase enrichment, three separate methods were used to estimate phase enrichment in elutriator

fractions of 9L cells obtained from a single elutriation experiment. The three methods were: 1) computer analysis of single parameter DNA histograms: 2) autoradiography: 3) simultaneous measurement of BrdU and DNA content using flow cytometry. Although technical problems were encountered with these experiments, the preliminary findings suggested that the PEAKS3 program tended to underestimate the percentage of S phase cells in G_2/M phase enriched fractions. With this result in mind, the method used to estimate the PEs for pure populations of G_1, S, and G_2/M phase cells may not be accurate. The relative phase specificity patterns for all drugs tested are summarized in TABLE 5.

TABLE 5. Phase specificity patterns in 9L cells.

Compound	Relative phase sensitivity	Chemical reactivity[a]
CENUs		
BCNU	$G_2/M > G_1 > S$	A, C, C-L
PCNU	$G_2/M \geq G_1 > S$	A, C, C-L
CCNU	$G_2/M > G_1 > S$	A, C, C-L
MeCCNU	$G_1 \geq G_2/M > S$	A, C, C-L
CHLZ	$G_2/M > G_1 > S$	A, C-L
CNU	$G_2/M > G_1 > S$	A, C-L
ENU	$S > G_2/M > G_1$	A, C
BHCNU	$G_2/M > S > G_1$	C

a-A = alkylating activity; C = carbamoylating activity; C-L = cross-linking activity.

The differential phase sensitivity became more marked with increasing drug dose for all drugs, except CHLZ and CNU. G_1 and G_2/M phase cells treated

with 4 mM ENU for 1 hr were 22- and 7-fold less sensitive than S phase cells. For cells treated with 300 uM BHCNU for 1 hr, PE decreased as cells progressed from G_1 to G_2/M phase.

IV.2. Alkaline elution

Treatment of elutriated 9L cells with 120 uM BCNU for 30 minutes followed 6 hours later by alkaline elution assay showed that cells treated in G1 phase have increased DNA interstrand cross-linking consistent with the phase specific survival pattern (FIGURE 11).

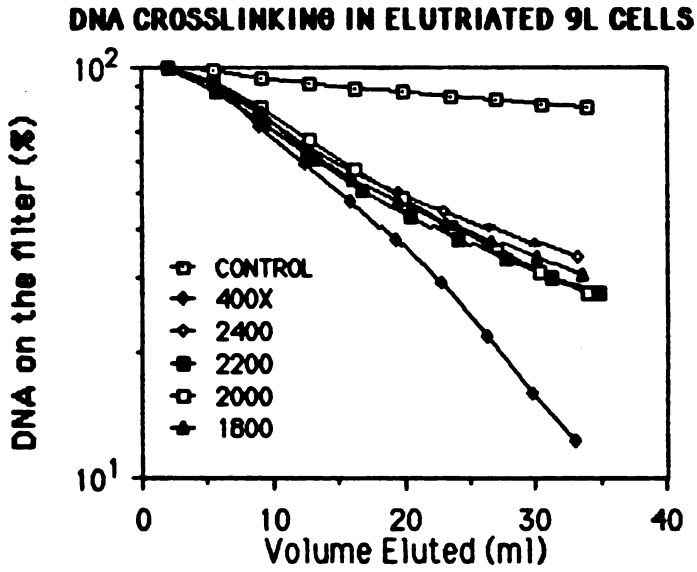


FIGURE 11. Alkaline elution curves for elutriated 9L cells treated with 120 uM BCNU (0.5 hr exposure). The fraction collected at 2400 rpm was enriched in G_1 phase cells; the fractions collected at 2200, 2000, and 1800 rpm were enriched in S phase cells.

Although, small differences in the elution profiles were detected with this assay, the results obtained in repeated experiments were highly variable (TABLE 6).

TABLE 6. Cross-linking factors in elutriated 9L cells treated with 120 uM BCNU for 0.5 hr.

Experiment	Rotor Speed (rpm)	CLF x 10^3	Phase enrichment (%) G_1	S	G_2/M
1. NCS[a]	Asynchronous cells	107	40	54	5
	2200	79	87	13	0
	1900	81	11	88	0
	1600	90	1	60	39
2. NCS[a]	Asynchronous cells	65	52	40	8
	2200	120	95	5	0
	1900	78	27	74	0
	1700	76	3	94	0
3. NCS[a]	Asynchronous cells	79	49	44	7
	2200	78	94	6	0
	1900	71	21	79	0
	1700	79	3	97	0
4. NS[b]	Asynchronous cells	40	34	51	15
	2400	84	85	14	1
	2200	73	49	50	1
	2000	75	13	87	0
	1800	60	2	72	26
5. NS[b]	Asynchronous cells	149	66	24	9
	2400	155	97	3	0
	2200	107	86	13	1
	2000	110	48	51	1
	1800	130	4	57	39

a- Cells maintained and treated in CMEM containing 10% NCS.
b- Cells maintained and treated in CMEM containing 10% NS.

In two of these experiments (experiments 2 and 5) the fraction enriched in G_1 phase cells had a higher CLF than any S phase enriched fraction, and in experiment 5 G_2/M phase cells appear to have a higher CLF than do S phase cells. These results suggest BCNU-mediated DNA inter-strand cross-linking in each cell cycle phase may vary in a manner that is consistent with the pattern of phase secific cell killing, but the differences are not large when assayed by alkaline elution.

IV.3. BTRC variants and CHO mutants

The BCNU dose response curves showing the relative CENU sensitivity for 9L, BTRC-19, AA8, and UV-4 cells are shown in FIGURES 12 and 13. The ratio of the doses that were required to reduce PE to10% (resistant /sensitive) was 5.5 (165/30) for 9L cells and 20 (40/20) for CHO cells. AA8 cells had about the same BCNU sensitivity as 9L cells.

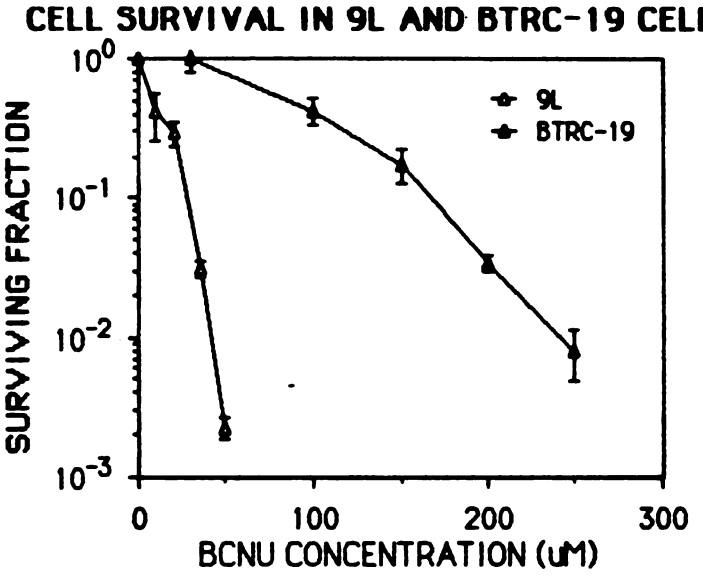


FIGURE 12. Survival curves for 9L and BTRC-19 cells treated with BCNU (1 hr exposure). Each point represents mean ± standard deviation of 4-8 Petri dishes. PE for untreated 9L cells was 69.5 ± 12.4 and the PE for untreated BTRC-19 cells was 37.3 ± 6.7.

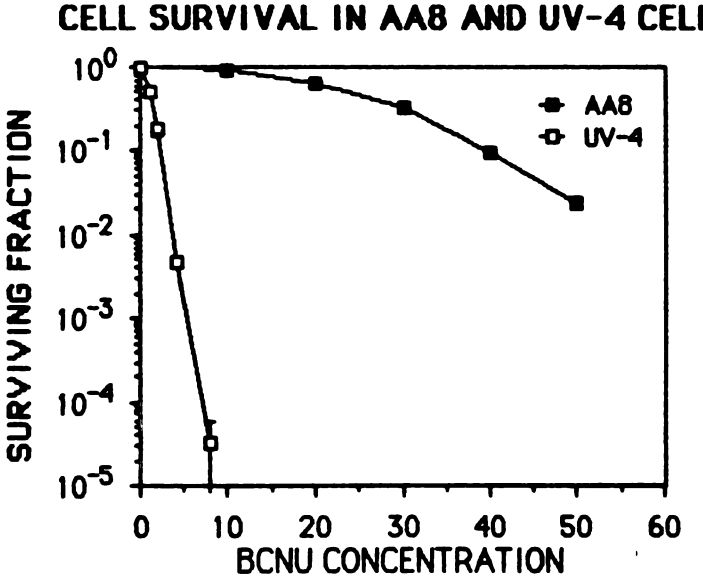


FIGURE 13. Survival curves of AA8 and UV-4 cells treated with BCNU (1 hr exposure). Each point represents the mean ± standard deviation of 4-8 Petri dishes. PE for untreated AA8 cells was 76.7 ± 6.5 and the PE for untreated UV-4 cells was 73.2 ± 11.7.

The phase specific survival pattern for the parent 9L cell line and the BCNU-resistant 9L variant, BTRC-19, are shown in FIGURES 14 and 15.

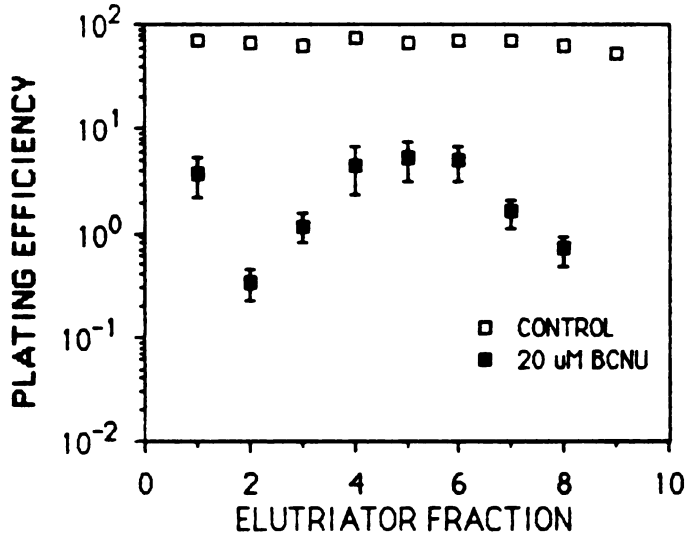


FIGURE 14. PE of 9L cells treated with BCNU (1 hr exposure). Fraction 1 corresponds to asynchronous cells. Control represents untreated elutriated cells. Each point represents the mean ± standard deviation of 4-8 Petri dishes.

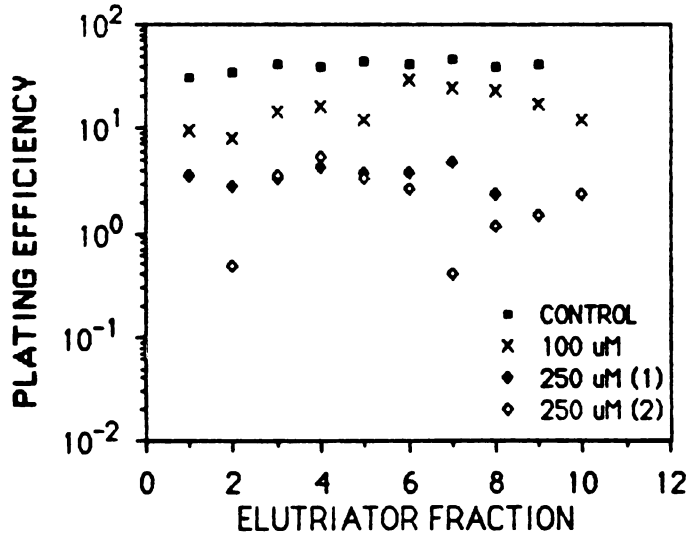


FIGURE 15. PE of BTRC-19 cells treated with BCNU (1 hr exposure). Control represents untreated elutriated cells. Fraction 1 corresponds to asynchronous cells. Each point represents the mean ± standard deviation of 4-8 Petri dishes.

In contrast to the parent line, BTRC-19 cells showed little variation in phase specific survival even at a dose of 250 uM, which reduced the PE of asynchronous cells to 3%. Thus, other CENUs may not show marked phase specificity in these cells.

The phase specific survival pattern for AA8 cells and UV-4 cells are shown in FIGURES 16 and 17.

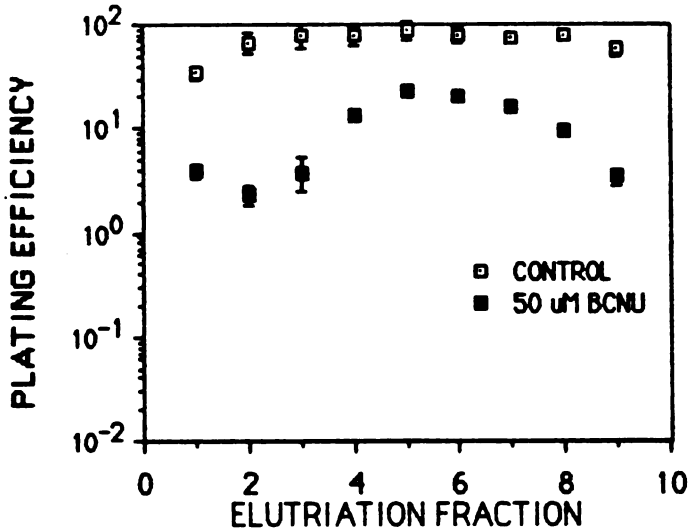


FIGURE 16. PE of AA8 cells treated with BCNU (1 hr exposure). Fraction 1 corresponds to asynchronous cells. Control represents untreated elutriated cells. Each point represents the mean ± standard deviation of 4-8 Petri dishes.

AA8 cells had about the same BCNU sensitivity as 9L cells and the patterns of phase specific cell killing were similar in that S phase cells were less sensitive than were G_1 or G_2/M phase cells.

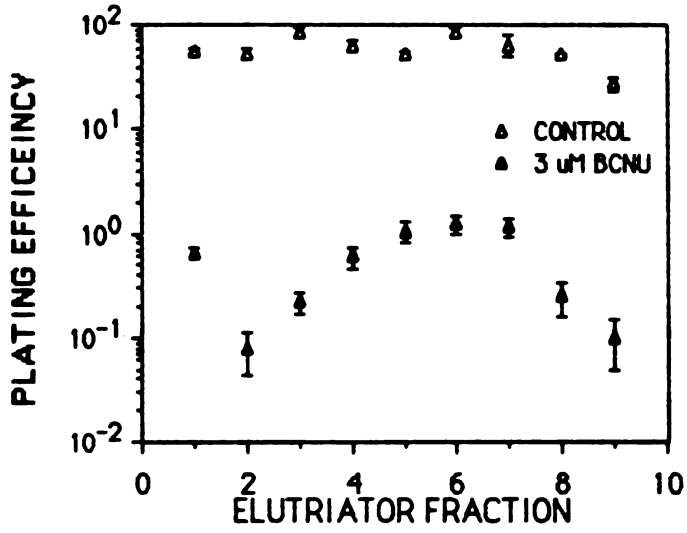


FIGURE 17. PE of UV-4 cells treated with BCNU (1 hr exposure). Fraction 1 corresponds to asynchronous cells. Control represents untreated elutriated cells. Each point represents the mean ± standard deviation of 4-8 Petri dishes.

UV-4 cells treated in S phase were also relatively resistant to BCNU indicating that the biochemical defect which is responsible for their increased sensitivity to cross-linking agents does not affect CENU phase specificity.

The estimated PEs for pure populations of G_1, S, and G_2/M phase cells are shown in TABLE 7.

TABLE 7. Calculated PEs of elutriated 9L, BTRC-19, AA8, UV-4 cells treated with BCNU.

		Plating efficiency			
			Calculated values[a]		
Cell line	Dose[b] (uM)	Asynchronous cells	G_1	S	G_2/M
9L	---	63.9 ± 10.6	65.0	61.7	44.2
	20	3.76 ± 1.58	0.35	8.09	1.88
BTRC-19	---	31.0 ± 5.0	36.1	47.8	30.7
	250	3.50 ± 0.5	2.7	4.8	1.1
AA8	---	34.1 ± 5.4	76.3	85.5	25.5
	50	3.93 ± 0.60	1.55	27.1	0
UV-4	---	56.3 ± 4.1	61.4	99.5	0
	3	0.66 ± 0.08	0.25	1.38	0

a-Estimated PEs for pure populations of G_1, S, and G_2/M phase cells. Calculated as described in Materials and Methods.
b-1 hr treatment.

The estimated PEs of G_1, S, and G_2/M phase cells that were not treated with BCNU were different for 9L, BTRC-19, AA8, and UV-4 cells. Asynchronous 9L and BTRC-19 cells that were elutriated, but did not receive any drug, had PEs of 63.9 and 31.0 % respectively, whereas asynchronous

AA8 and UV-4 cells had PEs of 34.1 and 56.1 %. These PEs were similar to those for 9L and BTRC-19 cells in FIGURE 13, but values for AA8 and UV-4 cells were considerably lower than in FIGURE 14.

The estimated PE for elutriated 9L cells in G_1 phase was the same as that predicted for S phase cells, but G_2/M phase cells had a slightly lower PE after elutriation. Untreated BTRC-19 cells in S phase had a slightly higher expected PE than in G_1 or G_2/M phase when elutriated. The estimated PEs for untreated and elutriated AA8 and UV-4 cells in G_2/M phase was considerably less than that predicted for G_1 and S phase cells, suggesting that the elutriation process produced some toxicity in CHO cells.

The patterns of cell cycle phase sensitivity were similar in 9L, AA8, and UV-4 cells. 9L cells in S phase were predicted to be 23 fold less sensitive than G_1 phase cells; AA8 cells in S phase were 17 fold less sensitive than G_1 phase cells; UV-4 cells in S phase were 5 fold less sensitive than G_1 phase cells. There was only a 2 fold difference between the PEs of G_1 and S phase BTRC-19 cells. AA8 and UV-4 cells in G_2/M phase at the time of treatment would not be expected to form colonies after treatment with 50 and 3 uM BCNU, respectively.

IV.4. Guanine O^6-alkyltransferase

Guanine O^6-alkyltransferase (AT) activity was assayed in 9L, BTRC-19, AA8, and UV-4 cells. No activity was detected in 9L, AA8, or UV-4 cells indicating that they have a phenotype similar to Mex $^-$ cells, but high levels of AT activity were found in BTRC-19 (2.24 picomoles activity/mg protein). This level of activity is comparable to the lymphoblast cells used by Brent (185) as a positive control.

AT activity in elutriated BTRC-19 cells increased about twofold with increasing fraction number (FIGURE 18).

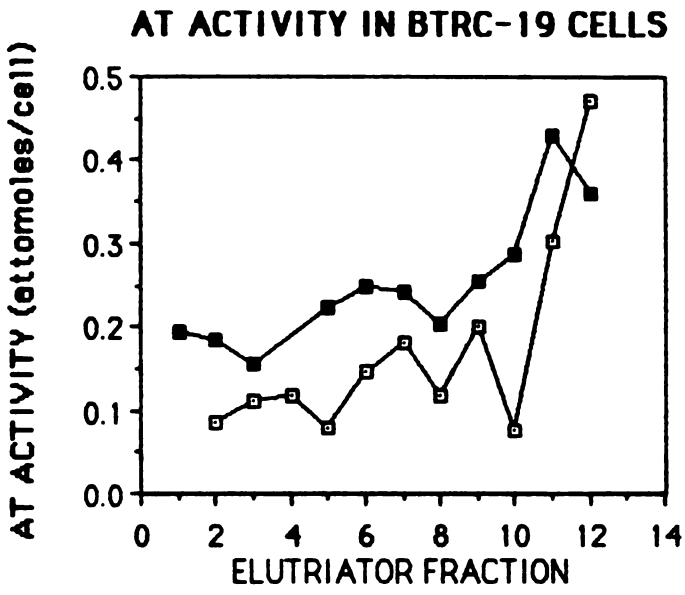


FIGURE 18. Guanine O^6-alkyltransferase activity in elutriated BTRC-19 cells. Fraction 1 corresponds to asynchronous cells. Figure shows the results of two separate experiments.

When normalized for cellular protein the activity was fairly constant throughout G_1 and S phase, but increased slightly in the fractions enriched in G_2/M phase cells (FIGURE 19).

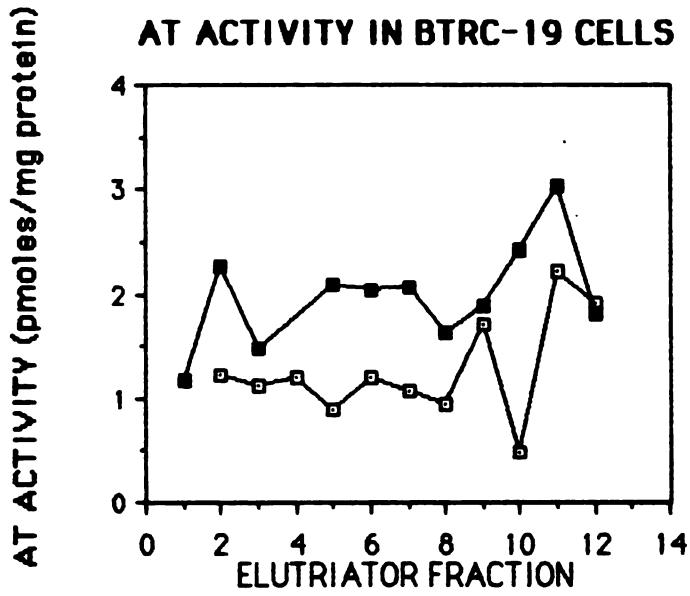


FIGURE 19. Guanine O^6-alkyltransferase activity in elutriated BTRC-19 cells. Fraction 1 corresponds to asynchronous cells. Figure shows the results of two separate experiments.

Since fraction 10 contained 67.8% and 64.1% S phase cells and 30.7 and 32.5 % G_2/M phase cells in experiment 1 and 2. respectively. phase enrichment in fractions 11 and 12 could not be analyzed accurately in these experiments. These results suggest that BTRC-19 cells in late S and G_2/M phases should be the most resistant to BCNU.

IV.5. Glutathione

Levels of total non-protein sulfhydryls (GSH and GSSG) were measured in 9L (FIGURE 20) and BTRC-19 cells (FIGURE 21).

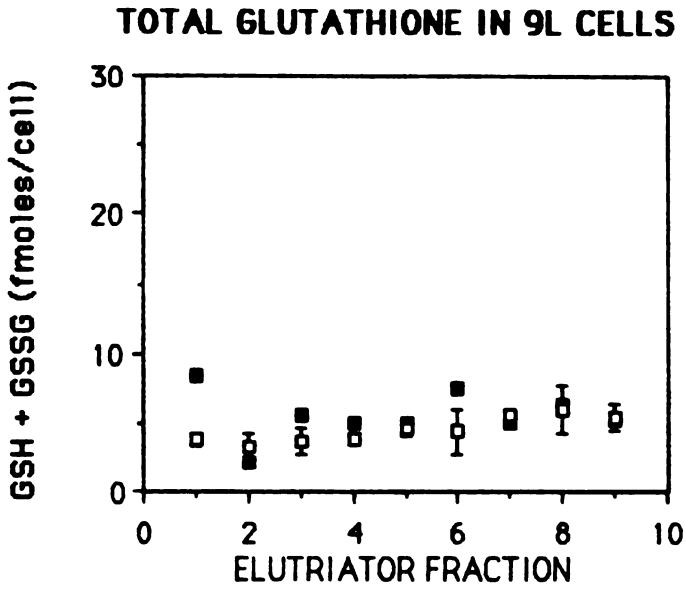


FIGURE 20. Total non-protein sufhydryl levels in elutriated 9L cells. Figure shows results of 2 separate experiments. Error bars indicate standard deviation of 2-3 detetminations at each point.

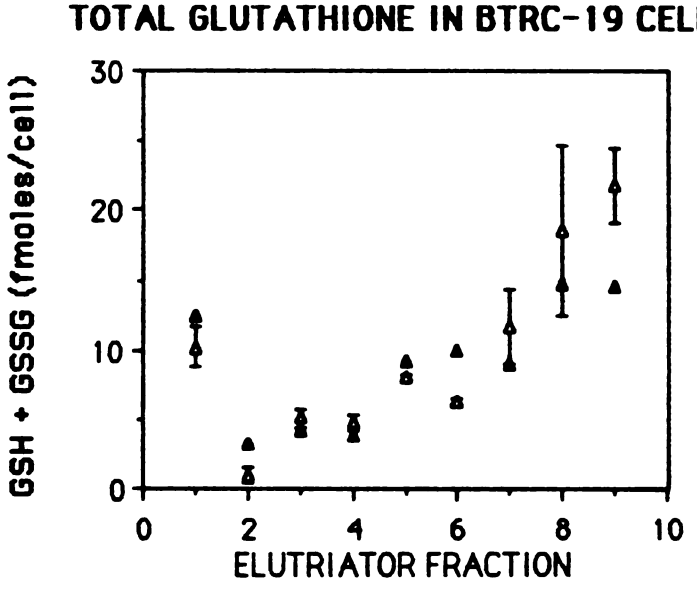


FIGURE 21. Total non-protein sufhydryl levels in elutriated BTRC-19 cells. Figure shows results of 2 separate experiments. Error bars indicate standard deviation of 2-3 determinations at each point.

Thiol levels increased slightly with elutriator fraction number in 9L cells, but increased almost 10 fold in BTRC-19 cells. The total amount of protein was

similar in each cell line (FIGURES 22 and 23); although, protein determinations on elutriated 9L cells were much more variable than elutriated BTRC-19 cells. The reason for the greater variability of protein content in 9L cells is unknown.

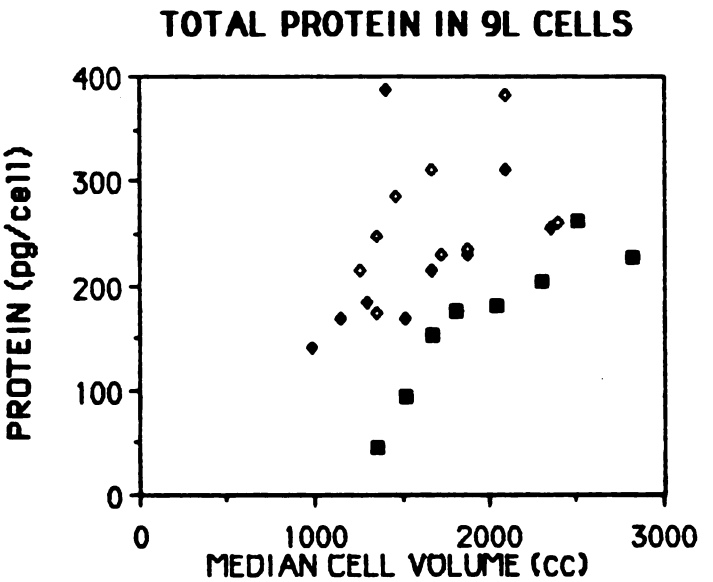


FIGURE 22. Protein content of elutriated 9L cells. Figure shows results from 3 separate experiments.

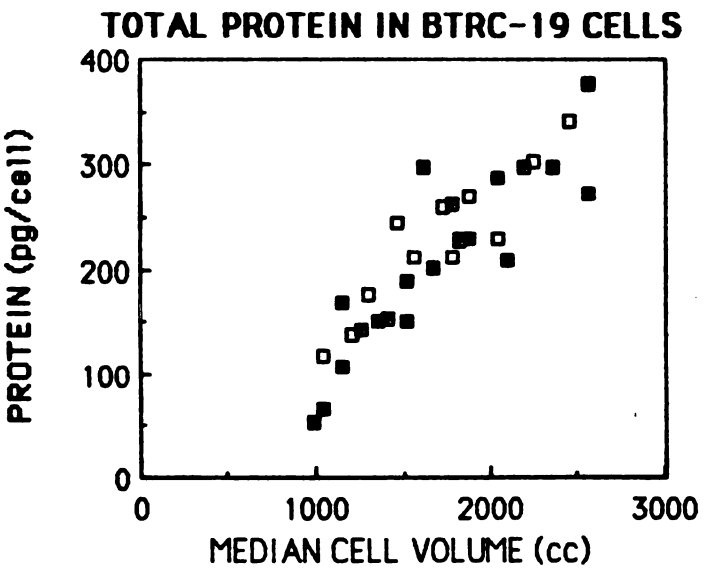


FIGURE 23. Protein content of elutriated BTRC-19 cells. Figure shows results of 3 separate experiments.

When normalized for protein content per cell total glutathione level was variable throughout the cell cycle in both cell lines (FIGURE 24).

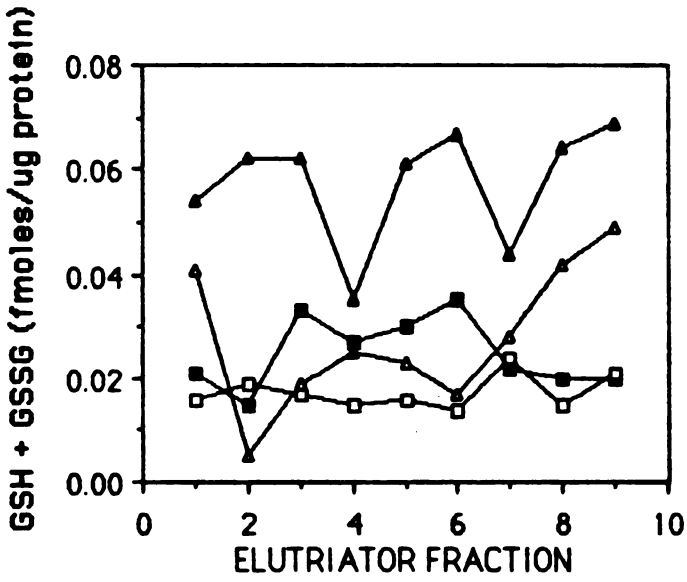


FIGURE 24. Total non-protein sulfhydryl levels in elutriated 9L and BTRC-19 cells normalized for protein content per cell. Symbols correspond to the same experiments plotted in FIGURES 24 and 25. Triangles, BTRC-19 cells; Squares, 9L cells.

Thiol levels were not consistenly higher in 9L cells that were in S phase, and consequently they did not correlate with BCNU phase specific cell kill. Likewise, the fluctuation of thiols in BTRC-19 cells during the cell cycle did not appear to be related to the pattern of BCNU-mediated cell killing in these cells. BTRC-19 cells had two to three times as much glutathione per ug protein. When normalized for cell volume, the thiol levels in 9L cells were constant throughout the cell cycle in 9L cells (FIGURE 25) and increased with volume in BTRC-19 cells (FIGURE 26), thus, the variabilty seen between the experiments in FIGURE 24 was likely due to poor reproducibility of protein measurements.

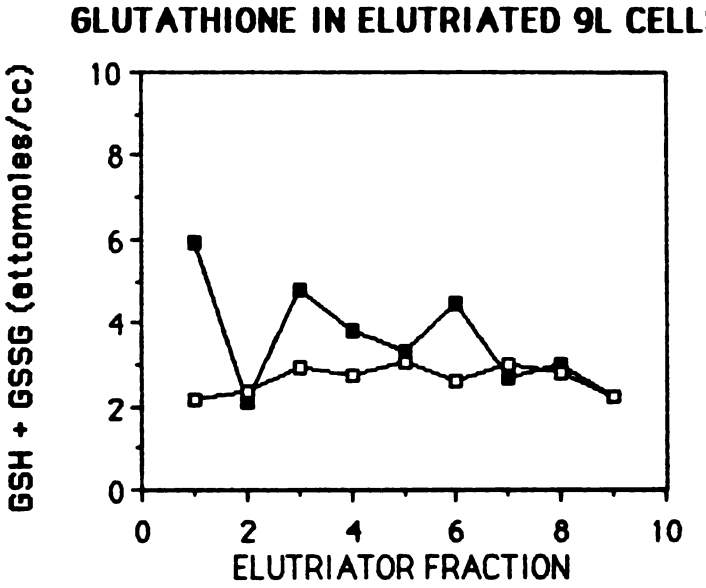


FIGURE 25. Total non-protein sufhydryl levels in elutriated 9L cells. Fraction 1 corresponds to asynchronous cells. Figure shows results of 2 separate experiments.

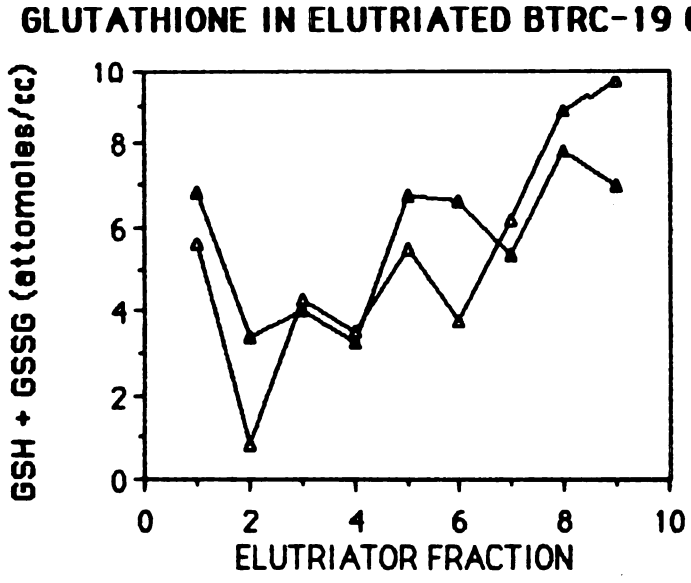


FIGURE 26. Total non-protein sufhydryl levels in elutriated BTRC-19 cells. Fraction 1 corresponds to asynchronous cells. Figure shows results of 2 separate experiments.

IV.6. ^{3}H-CNU binding

CENU monoadduct formation during the cell cycle was estimated by measuring binding of ^{3}H-CNU to cellular DNA in elutriated 9L cells. CNU-treated 9L cells showed a phase sensitivity pattern similar to other CENUs (TABLES 4 and 5). The protocol that was used for labeling cellular DNA, which necessitated treating cells as a single cell suspension, increased the cell kill caused by a one hour exposure to 15 uM CNU; the PE of asynchronous cells treated as monolayers prior to trypsinization was 3.15 ± 1.1 and the PE of cells dissociated with enzyme cocktail prior to drug exposure and treated in suspension culture was 0.071 ± 0.06; however, the pattern of cell cycle phase sensitivity was similar to that seen in BCNU-treated cells in that the S phase enriched fractions had the highest PEs (FIGURE 27).

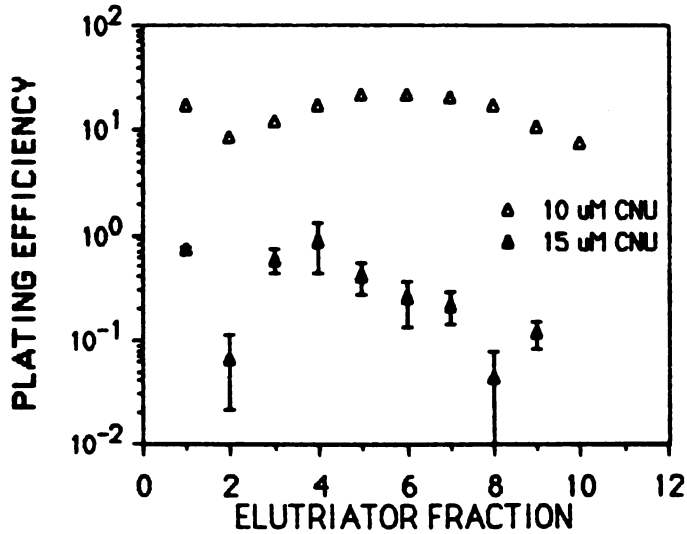


FIGURE 27. PE of elutriated 9L cells treated with CNU. Fraction 1 corresponds to asynchronous cells. Each point represents mean ± standard deviation of 4- 8 petri dishes. Open triangles, 1 hr exposure in monolayer; Closed triangles, 1 hr exposure in suspension.

The amount of ^{3}H-CNU bound to DNA was similar for asynchronous cells and fractions enriched in G_1, S. and G_2/M phase cells indicating that the intracellular drug dose was the same in each cell cycle phase (TABLE 8).

TABLE 8. [^{3}H] CNU binding to DNA in elutriated 9L cells.

Fraction	OD	CPM	CPM/ug DNA	Phase enrichment (%) G_1	S	G_2/M
1. Asynchronous cells	0.92	456	9.9	53	32	15
	0.93	478	9.6			
	0.85	411	9.7			
2. G_1 phase cells	0.91	457	10.0	66	31	3
	0.92	469	10.2			
	0.87	432	9.9			
3. S phase cells	0.76	382	10.1	17	54	29
	0.76	393	10.3			
	0.76	405	10.6			
4. G_2/M phase cells	0.32	165	10.3	3	16	81
	0.40	220	10.9			
	0.40	209	10.5			

V. DISCUSSION

The cell cycle phase sensitivity of 9L cells to the nitrosoureas studied was similar to results reported for BCNU, CCNU, and MeCCNU using 9L, CHO, and DonC cells (reviewed in reference100). While cells in all cell cycle phases were killed, G_1 and G_2/M phase cells had lower PEs after treatment. Higher concentrations of BCNU, CCNU, MeCCNU, and PCNU produced a greater differential in PE between cell cycle phases, whereas CNU and CHLZ did not show a dose modified effect. ENU was most toxic to S phase cells, and the toxicity of BHCNU was greatest for cells treated in G_2/M phase and least for cells treated in G_1 phase (TABLE 4).

Estimates of phase specific PEs obtained for several drug treatments predicted essentially zero PE for G_2/M phase cells. These predicted PEs may reflect either the limit of sensitivity of the CFE assay or an error in the method used to estimate the percentage of G_2/M phase enrichment. Comparison of several methods for estimating the cell cycle phase enrichment of elutriator fractions suggested that the PEAKS3 program tended to underestimate the percentage of S phase cells in G_2/M phase enriched fractions. Since similar results have been reported in 9L cells by Nagashima and Hoshino (248) the mathematical method used to estimate the PEs of pure populations of G_1, S, and G_2/M phase cells may not be valid.

The phase specificity pattern seen with all CENUs was characteristic in that S phase cells were relatively resistant to cell killing when compared to other cell cycle phases. Since CENUs differ from the other nitrosoureas studied principally in their ability to cross-link DNA (TABLE 3), these results suggest that CENU-mediated cell killing is caused by DNA cross-linking and that the effectiveness of cross-linking depends on a cells position in the cell cycle at the time of treatment. Further, if the cytotoxicity of CENUs is the

result of DNA inter-strand cross-linking, then inter-strand cross-linking would appear to be greatest in G_1 and G_2/M phases.

The results for ENU and BHCNU indicate that entirely different mechanisms are responsible for the observed phase specific cell killing. As reported by Goth-Goldstein and Burki (249), CHO cells synchronized by mitotic selection are most sensitive to ENU in early S phase implying that DNA synthesis is required for effective cell kill. If alkylation of the DNA precusor pool is an important target for monofunctional alkylating agents, as suggested by Topal and Baker (250) for MNU, then incorporation of modified nucleosides into DNA during S phase could result in base mispairing and lethal mutation (251). This could explain the S phase specificity of ENU-mediated cell killing. Other explanations are certainly possible as a high percentage (50%) of ENU reacts with phosphotriesters in DNA (117) and these lesions are repaired very slowly in cells (252). Further, these phosphotriesters and other monoadducts are labile in alkali and thus can give rise to single strand breaks in the DNA backbone (253).

BHCNU, which is a pure carbamoylating agent and relative to CENUs is ineffective as a cytotoxic agent, probably has little or no effect on DNA. It does; however, cause an alteration in cell adherance and cell size (254), and thus may cause cell death via an effect on cell membranes. Tew et al. (255) have shown that treatment of Walker carcinoma cells with BHCNU causes a dose dependent inhibition of glutahione reductase and changes in plasma and nuclear membranes that are consistent with depletion of cellular glutathione. They also found that BHCNU interferes with mitotic spindle formation which is in good agreement with the observation that G_2/M phase is the most sensitive phase in 9L cells treated with BHCNU.

Because cell survival, as measured by a CFE assay, reflects both the initial level of damage and the cells response to that damage, the apparent cell cycle phase specific differences in CENU-mediated cross-linking could be due to the induction of more monoadduct damage and/or cross-links in G_1 and G_2/M phases or less repair of the damage, when compared to S phase. Although cross-linking activity seems to be the most important determinant of CENU phase specificity, there is evidence that carbamoylating activity can affect the magnitude of differences in PE seen between cell cycle phases. Of the CENUs studied, both CHLZ and CNU failed to show a dose dependent increase in differential cell killing. For both drugs and at both doses studied, G_1 cells were 2-4 fold more sensitive than S phase cells and G_2/M phase cells were much more sensitive than S phase cells. Since both CHLZ and CNU lack significant carbamoylating activity it may be that the dose dependence seen with other CENUs reflects repair inhibition mediated by CENU-derived isocyanates. As mentioned earlier, BCNU-derived chloroethylisocyanate inhibits glutathione reductase (197), but it can also inhibit DNA polymerase (256) and DNA ligase (257). The inhibition of DNA ligase has been shown to potentiate DNA damage caused by ionizing radiation (258) and nitrosoureas that have carbamoylating activity can inhibit the repair of both drug and X-ray-induced damage in Mer $^+$ human embryo fibroblasts (259). Further, the difference in cell killing observed between Mer $^+$ and Mer $^-$ cells is less pronounced for CENUs that cause increased levels of carbamoylation in vitro (187). Although 9L cells have undetectable levels of AT activity, carbamoylation of other repair enzymes might contribute to cell killing at high drug doses. If so, CHLZ and CNU may not show dose dependent potentiation of relative phase sensitivity because their lack of carbamoylating activity makes them unable to inhibit cellular repair activity.

The phase specificity studies with the various nitrosoureas strongly imply that cross-linking is responsible for the phase specificity of CENUs, but alkaline elution assay of DNA inter-strand cross-linking does not confirm this hypothesis. The experiments do not demonstrate a consistent difference in CLF between G_1 and S phase cells, and when there is a difference it is small. It may be that the levels of inter-strand cross-linking are similar in each cell cycle phase; however, there are several explanations for why the CLF might not parallel the pattern of cell survival. First, it is possible that the lethal lesion(s) that is responsible for the phase specificity of CENUs is not an inter-strand cross-link. The important lesion could be a specific monoaddduct, a DNA-protein cross-link, or a DNA intra-strand crosslink. All of these lesions form in CENU-treated cells (260) and there is growing evidence that inter-strand cross-linking may not be the only or even the most important lethal lesion in CENU-treated cells (260.261.262..263). A second possibility is that the technical considerations, which necessitated treating the cells in suspension culture following elutriation, adversely affected the cells used for alkaline elution, as it is well known that trypsinization prior to drug exposure can alter the measurement of cell survival (264). If the phase specificity of CENUs were a reflection of the time elapsed between drug exposure and the lethal fixation of damage, as shown for X rays (175), then cell progression could profoundly affect survival. Even though there is about a 4 hr growth delay when 9L cells are dissociated with enzyme cocktail and elutriated (265), the protocol used for determining the CLF, which was similar to that used for measurement of ^{3}H CNU binding, did not appear to alter the phase specific survival pattern (FIGURE 25). Thus, the growth delay caused by enzymatic dissociation and elutriation prior to drug treatment did not affect the phase specificity of CNU.

The experiments with the CENU-resistant BTRC-19 cells and the CHO cells, AA8 and UV-4, showed that the phase specific survival pattern seen with 9L cells was typical of cell lines that lack AT activity. AT activity was undetectable in 9L, AA8, and UV-4 cells, and S phase cells from cultures treated with BCNU at doses that produced a 1-2 log cell kill in asynchronous cells were more resistant than cells in other cell cycle phases (FIGURES 14, 16, and 17). In contrast, BTRC-19 cells showed very little variation in cell kill throughout the cell cycle at a similar asynchronous survival level (FIGURE 15). Although the pattern of cell cycle phase sensitivity is similar in 9L, AA8, and UV-4 cells, the estimated PEs for pure populations of G_1, S, and G_2/M phase cells (TABLE 7) indicate that there are differences between these cell lines. The estimated PEs for cells that were elutriated , but not exposed to drug, were different in each cell line. Untreated 9L and BTRC-19 cells that were elutriated had different estimated PEs, but they were similar in that G_1 phase cells had about the same PE as asynchronous cells. The estimated PE of S phase BTRC-19 cells was higher than G_1 or G_2/M phases and untreated 9L cells in G_2/M phase appeared to be slightly more sensitive than G_1 or S phase cells. Analysis of data from untreated AA8 and UV-4 cells, that had been elutriated, predicted that S phase cells had the highest PE followed by G_1 phase cells and then G_2/M phase cells. This suggests that the elutriation process itself may show some phase specific cell killing in CHO cells, particularly in G_2/M phase. The low predicted PEs for untreated G_2/M phase cells could be a result of an inablility to distiguish between G_2 and mitotic cells with the flow cytometric methods used, or, as discussed earlier, an error in estimating G_2/M phase enrichment in the CHO cell lines. Due to the shorter cell cycle phase durations of CHO cells it may be more difficult to estimate the percentage of G_2/M phase cells in elutriation experiments .

The cell cycle time of exponentially growing 9L cells is approximately 20 hr, with the duration of G_1 and S phases each being about 8 hr, the duration of G_2 phase being about 3.5 hr, and mitosis being about 0.5 hr (266). The doubling time of BTRC-19 cells is similar to 9L cells, but they have a longer lag phase (239). This may be due to the fact that a lower untreated PE, ie. 69.5 for 9L cells and 37.3 for BTRC-19 cells (FIGURE 13), would tend to slow the initial rate of growth when cells were replated at low densities. After BTRC-19 cells attach to the culture flask they apparently grow at a rate that is indistinguishable from 9L cells. The cell cycle phase durations have not been determined in BTRC- 19 cells, but since the percentages of G_1, S, and G_2/M phase cells, during exponential growth are similar to 9L cells and the doubling times are similar, the phase durations are assumed to be similar.

Both AA8 and UV-4 cells have doubling times of 13-14 hr (231), considerably shorter than 9L and BTRC-19 cells, and they have similar untreated PEs (76.7 for AA8 cells and 73.2 for UV-4 cells from FIGURE 14 legend). The cell cycle phase durations have not been determined in these cell lines; however, reports in the literature for CHO cells (267,268) would suggest that the duration of S phase is between 5.5 and 6.8 hr, the duration of G_1 is probably between 2.2 and 4 hr long and G_2 phase and mitosis would occupy the remaining 2-3 hr. Because mitosis takes about 1 hr to complete, the G_2 phase would be shorter in both AA8 and UV-4 cells, compared to 9L cells, therefore it would be difficult to separate late S phase cells and G_2 phase cells by volume. Since S phase enrichment is the most difficult to quantitate using computer modeling (269), an inability to separate G_2 phase cells would make estimating G_2/M phase enrichment even more difficult. In

light of this difficulty, the results presented for CHO cells in TABLE 7 must be interpreted with caution.

The PEs of asynchronous AA8 and UV-4 cells in TABLE 7 are also considerably lower than the predicted PEs of untreated S phase cells and the observed PEs for untreated asynchronous cells in FIGURES 13 and 14. Because the PE of untreated AA8 cells declines progressively over 6 hours when held in suspension (270), the decreased PEs of of the asynchronous cells from elutriation experiments is likely due to prolonged holding at ice temperatures (4-6 hrs) during the elutriation procedure.

Of the four cell lines studied only BTRC-19 cells had detectable levels of AT activity and a markedly dampened phase sensitivity pattern. The lack of AT activity in 9L, AA8, and UV-4 cells indicates that AT-mediated repair cannot be involved in determining the phase sensitivity pattern in these cells. AT activity in BTRC-19 cells was comparable to values reported for Mex $^+$ cells (271) which suggests that CENUs may not show much phase specific killing in other Mex $^+$ cell lines. This finding may have important clinical implications because the majority of non-transformed human cell lines examined to date have the Mex $^+$ or Mer $^+$ phenotype (272). AT activity (attomoles/cell) increased 2-3 fold with increasing elutriator fraction number in BTRC-19 cells indicating that S phase and G_2/M phase cells have more repair capability than G_1 phase cells (FIGURE 18). This is consistent with the results of Dunn et al. (273) who found that AT activity (molecules/cell) decreased initially in C3H/10T1/2 cells released from confluence, but increased progressively from early S to G_2/M phase. When normalized for cellular protein content, AT activity throughout the cell cycle was relatively constant, possibly increasing slightly in the later G_2/M phase enriched fractions. Since by definition DNA content doubles between G_1 and

G_2 phase, the DNA content of fraction 12 should be twice that of fraction 2. This would suggest that AT activity increases with DNA content maintaining a fairly constant AT/DNA ratio. There is some evidence that cellular sensitivty to CENUs and AT activity is proportional to DNA content. Reeve et al. (274) have shown that cellular CENU sensitivity in RIF-1 clones is related to the ploidy level with tetraploid cells being resistant and diploid cells being sensitive. This result is consistent with an AT gene dosage effect in tetraploid cells although it remains to be proven. A second line of evidence suggesting that AT activity per unit DNA is the most relevant predictor of cellular CENU sensitivity is provided by the work of Gerson et al. (275), who have shown that when AT activity in various tissues is normalized for DNA content, rather than protein content, the rank order for tissue susceptibility to O^6-alkylguanine lesions is different. AT activity is low in brain tissue regardless of whether data is normalized to DNA or protein content, which is consistent with the observed susceptabilty of developing nervous tissue to mutation and carcinogenesis after treatment with agents that produce O^6-alkylguanine (276). When based on protein content, bone marrow cells have an intermediate to high level of AT activity; however, when based on DNA content they have a low level of activity. Clinically, bone marrow depression produced by CENUs often limits the effectiveness of chemotherapy (277) and therefore a low level of AT activity in bone marrow is more in keeping with the observed clinical sensitivity of these cells. Thus, AT activity per unit of DNA appears to be a better measure of cellular sensitivity than does AT activity per unit protein. As mentioned earlier, AT activity in BTRC-19 cells appears to increase 2 fold in parallel with a 2 fold increase in DNA content. The lack of BCNU phase specificity in BTRC-19 cells is understandable therefore, if O^6-alkylguanine lesions are related to cell killing and one makes

two other assumptions; first, that the AT/DNA ratio is relatively constant, suggested by the above data, and second, that O^6-alkylguanine formation is proportional to DNA content (278).

Measurement of non-protein sulfhydryls in 9L and BTRC-19 cells indicated that glutathione levels in asynchronous cells were at least 2 fold higher in BTRC-19 cells. While elevated thiols may contribute to CENU resistance they did not seem to correlate with the pattern of BCNU phase specific killing in either cell line. The thiol levels measured in elutriated 9L cells were similar to those reported in elutriated CHO cells by Murray and Meyn (279). In studying the mechanism underlying the phase specificity of nitrogen mustards, they found that thiol levels increased slightly with increasing fraction number, but were constant throughout the cell cycle when normalized for cell volume. Their overall conclusions were similar to those reported here in that the phase specificity of nitrogen mustard could not be attibuted to phase differences in DNA damage and repair within the confidence limits of the availiable assays.

[^{3}H] CNU binding to DNA was similar in asynchronous 9L cells and elutriator fractions enriched in G_1, S, and G_2/M phases, providing a clear indication that CENU uptake through the plasma membrane was not responsible for the pattern of phase specific cell killing. Further, it was apparent that the amount of CNU that reacted per ug of DNA was similar in each cell cycle phase. This result is in keeping with the findings of Levin et al. (), who observed that uptake of urea, mannitol, and methotrexate did not vary with cell cycle position in elutriated S49 cells. Further, studies on the mechanism of cellular CENU resistance in TLX5 lyphoma (280,281) and L1210 () cells show that CENU uptake and binding is the same in sensitive and resistant cells. Differences in CENU uptake have been found in elutriated

bone marrow cells (282); however, no information is availiable on cell cycle phase enrichment in these studies, consequently the observed differences in uptake may simply reflect the variation in enrichment of different bone marrow cells in each elutriator fraction.

While the exact mechanism underlying the phase specificity of CENUs remains to be determined, the results presented here suggest further experimentation. With regard to the specific hypotheses tested, the findings were: 1) no significant difference in DNA inter-strand cross-linking could be detected between G_1 and S phase cells indicating that other types of cross-links may be important for BCNU phase specificity: 2) intrinsic cellular BCNU sensitivity did not alter the phase specificity pattern in cell lines that lacked AT activity: 3) O^6-guanine alkyltransferase activity increased during the cell cycle in BTRC-19 cells and may expain the lack of marked phase specificity in these cells: 4) levels of non-protein sulfhydryls did not correlate with phase specificity: 5) [^{3}H] CNU binding was the same in G_1, S, and G_2/M phase cells indicating that intracellular drug dose does not vary with position in the cell cycle at the time of treatment.

VI. CONCLUSIONS

1) All cross-linking nitrosoureas show a charateristic pattern of cell cycle phase specificity in cell lines that lack AT activity, with S phase cells being relatively resistant, compared to other cell cycle phases.

2) Carbamoylating activity of CENUs can alter the magnitude of survival differences seen between cell cycle phases.

3) No significant differences in DNA inter-strand cross-linking can be detected btween 9L cells in different phases of the cell cycle.

4) AT activity cannot explain the pattern of phase specific survival in 9L, AA8, and UV-4 cells, but may explain the lack of marked phase specificity in BTRC-19 cells.

5) AT activity appears to increase in parallel with DNA content in BTRC-19 cells.

6) Thiol levels in 9L cells are fairly constant during the cell cycle, but increase progessively through the cell cycle in BTRC-19 cells.

7) Total glutathione level does not correlate with phase specific survival in either 9L or BTRC-19 cells.

8) CNU uptake is not responsible for phase specific cell kill.

9) The amount of CNU bound per ug of DNA is similar in G_1, S, and G_2/M phase cells.

VII. FURTHER STUDIES

The cause of CENU phase specificity remains an open question that can be approached from several directions depending on one's point of view. Two hypotheses that might direct future studies are as follows: 1) the type of damage is different in the various phases of the cell cycle, ie. more lethal crosslinks form in G_1 and G_2/M phase cells: 2) cross-link precursor and/or cross-link repair varies between cell cycle phases.

The fact that CNU binding is the same in G_1, S, and G_2/M phase cells argues against a phase-specific difference in monoadduct formation; however, the assay of total drug binding provides no information about specific adducts, which might form with different effciencies in each cell cycle phase. In addition, only DNA inter-strand cross-linking was measured in the above experiments, consequently other types of cross-links may be responsible for the phase specificity of BCNU or different cross-links may form with different efficiencies in each cell cycle phase.

Several studies with DNA repair deficient CHO cells indicate that the phase-specific cell killing produced by UV light (283) and 7-bromomethyl-benz[a]anthracene (284) is probably due to phase-specific differences in damage repair. Two observations in CENU-treated cells are relevant to the repair hypothesis. In 9L cells depleted of polyamines by DFMO pretreatment, MeCCNU-mediated cell killing is enhanced, but monodduct formation is the same with or without DFMO pretreatment (225). This finding suggests that decreased cross-link repair, or increased cross-link formation is responsible for the potentiation of MeCCNU cell kill seen with DFMO pretreatment. Polyamine depletion is currently thought to alter the conformation of DNA such that DNA cross-linking is facilitated, ie. more monoadducts successfully form lethal crosslinks in DFMO treated cells. Since

the conformation of DNA is different in each cell cycle phase, it seems possible that a similar effect on cross-linking could occur during the cell cycle.

Barcellos et al. (285) have recently found that the increased BCNU sensitivity of 9L MTS is due to the greater sensitivity of the non-cycling cell population. Further, when non-cycling cells are allowed to remain as spheroids for 24 hr, rather than dissociated immediately following drug exposure and placed in monolayer culture, they are able to repair some of the PLD. Because PLD repair does not occur in exponentially growing monolayer cultures, and since non-cycling cells in MTS rapidly enter the cell cycle when placed in monolayer culture (), it may be that PLD becomes fixed when CENU damaged cells progress through the cell cycle. Thus, the cell cycle phase specificity of CENUs may be a reflection of the time availiable for repair prior to damage fixation in a manner analagous to the situation seen with X rays (). These studies indicate that further experimentation should be aimed at clarifying the role of DNA repair in the phase specificity of CENUs.

VIII. LITERATURE CITED

1. Z. Darzynkiewicz, Molecular interactions and cellular changes during the cell cycle. Pharmac. Ther. **21**, 143-188 (1983).

2. M. L. Pamphilis and P. M. Wasserman, Replication of eukaryotic chromosomes: A close-up of the replication fork. A. Rev. Biochem. **49**, 627-666 (1980).

3. A. B. Pardee, R. Dubrow, J. L. Hamlin, and R. F. Kletzein, Animal cell cycle. A. Rev. Biochem. **47**, 715-750 (1978).

4. G. M. Hahn, J. R. Stewart, S. J. Yang, and V. Parker, Chinese hamster cell monolayer cultures. I. Changes in cell dynamics and modification of the cell cycle with the period of growth. Exp. Cell Res. **49**, 285-292 (1968).

5. J. W. Sheridan and R. J. Simmons, Studies on a human melanoma cell line: Effect of cell crowding and nutrient depletion on the biophysical and kinetic characteristics of the cells. J. Cell. Physiol. **107**, 85-100 (1981).

6. H. Eagle, The effect of environmental pH on the growth of normal and malignant cells. J. Cell. Physiol. **82**, 1-8 (1968).

7. H. Zaitsu and G. Kimura, Advance toward S phase and retreat toward deeper "G0" states in resting 3Y1 cells with environmental changes. J. Cell. Physiol. **120**, 181-187 (1984).

8. D. Paul, Growth control in Hela cells by serum and anchorage. Exp. Cell Res. **114**, 434-438 (1978).

9. L. Schiaffonati and R. Baserga, Different survival of normal and transformed cells exposed to nutritional conditions nonpermissive for growth. Cancer Res. **37**, 541-545 (1977).

10. J. F. Lechner, Interdependent regulation of epithelial cell replication by nutrients, hormones, growth factors, and cell density. Fed. Proc. **43**, 116-120 (1984).

11. I. Vlodavsky, G. M. Lui, and D. Gospodarowicz, Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix versus plastic. Cell **19**, 607-616 (1980).

12. F. L. Baker, G. Spitzer, J. A. Ajani, W. A. Brock, J. Lukeman, S. Pathak, B. Tomasovic, D. Thielvolt, M. Williams, C. Vines, and P. Tofilon, Drug and radiation sensitivity measurements of successful primary monolayer culturing of human tumor cells using cell-adhesive matrix and supplemented medium. Cancer Res. **46**, 1263-1274 (1986).

13. G. D. Shipley and R. G. Ham, Multiplication of Swiss T3 cells in serum free medium. Exp. Cell Res. **146**, 249-260 (1983).

14. M. J. Bissel, The differentiated state of normal and malignant cells or how to define a "normal" cell in culture. Int. Rev. Cytol. **70**, 27-100 (1981).

15. B. T. Hill, Principles of tumor growth. In: Scientific Foundations of Respiratory Medicine, pp 593-602, J. G. Scadding and G. Cumming (eds), W. B. Saunders Co., Philadelphia.

16. R. M. Sutherland, J. A. McCredie, and W. R. Inch, Growth of multi-cell spheroids in tissue culture as a model of nodular carcinomas. J. Natl. Cancer Inst. **46**, 113-120 (1970).

17. R. E. Durand, Cell cycle kinetics of an in vitro tumor model. Cell Tissue Kinet. **9**, 403-412 (1976).

18. R. M. Sutherland and R. E. Durand, Radiation response of multicell spheroids--an in vitro tumor model. Curr. Top. Radiat. Res. Quart. **11**, 87-119 (1976).

19. D. C. Alison, J. M. Yuhas, P. F. Ridolpho, S. A. Andersen, and T. S. Johnson, Cytophotometric measurement of the cellular DNA content of [^{3}H]thymidine-labeled spheroids. Demonstration that some non-labeled cell have S and G_2 DNA content. Cell Tissue Kinet. **16**, 237-246 (1983).

20. K. Tanaka, K. J. Isselbacher, G. Khoury, and G. Jay, Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene. Science **228**, 26-30 (1985).

21. J. Folkman, Angiogenesis. In: Biology of Endothelial cells. E. A. Jaffe (ed), pp. 412-428, Nijhoff, Boston, 1984.

22. Mediators in Cell Growth and Regulation. R. Ford and A. Maizel (eds), Raven Press, New York, 1986.

23. G. Poste and R. Greig, The experimental and clinical implications of cellular heterogeneity in malignant tumors. J. Cancer Res. Clin. Oncol. **106**, 159-170 (1983).

24. H. I. Bicher, F. W. Hetz, T. S. Sandhu, S. Frinak, P. Vaupel, M. D. O'Hara, and T. O'Brien, Effects of hyperthermia on normal and tumour microenvironment. Radiology **137**, 523-530 (1980).

25. M. J. Mantyla, J. T. Toivanen, M. A. Pikanen, and A. H. Rekonen, Radiation induced changes in regional blood flow in human tumours. Int. J. Radiat. Oncol. Biol. Phys. **8**, 1711-1717 (1982).

26. C. Klintenberg, K. Bjelenkrantz, J-C. Mansson, D. Killander, and B. Nordenskjold, ^{3}H-thymidine autoradiography and cytophotometric analysis of needle aspirates from human tumors during radiation therapy, endocrine therapy and chemotherapy. ACTA Radiol. Oncol. **24**, 117-122 (1985).

27. P. C. Nowell, The clonal evolution of tumor cell populations. Acquired genetic lability permits stepwise selections of variant sublines and underlies tumor progression. Science **194**, 23-28 (1976).

28. P. C. Nowell, Mechanisms of tumor progression. Cancer Res. **46**, 2203-2207 (1986).

29. M. Potmesil and A. Goldfeder, Cell kinetics of irradiated experimental tumors: Cell transition from the non-proliferating to the proliferating pool. Cell Tissue Kinet. **13**, 563-570 (1980).

30. B. W. Greider, R. F. Kallman, and A. J. Franko, Recruitment of noncycling tumor cells into proliferation by isoproterenol. Cancer Res. **43**, 1501-1503 (1983).

31. S. Tsubouchi and C. S. Potten, Recruitment of cells in the small intestine into rapid cell cycle by small doses of external gamma- or internal beta-radiation. Int. J. Radiat. Biol. **48**, 361-369 (1985).

32. P. C. Keng, D. W. Siemann, and K. T. Wheeler, Comparison of tumour age response to radiation for cells derived from tissue culture or solid tumours. Br. J. Cancer **50**, 519-526 (1984).

33. B. T. Hill, Biochemistry of the cell cycle. In: Scientific Foundations of Oncology, pp 63-72, T. Symington and R. L. Carter (eds), William Heinemann Medical Books, Ltd., London, 1976.

34. J. M. Mitchison, The biology of the cell cycle. Cambridge University Press, London, 1971.

35. R. S. Wu and W. M. Bonner, Mechanism for differential sensitivity of the chromosome and growth cycles of mamalian cell to the rate of protein synthesis. Mol. Cell. Biol. **5**, 2959-2966 (1985).

36. R. Baserga, Growth in size and cell DNA replication, Exp. Cell Res. **151**, 1-5 (1984).

37. E. Olah, Biochemistry of cycling and resting cancer cells. In: Progress in Clinical and Biological Research, Vol. 132C, E. A. Mirard, W B. Hutchinson, and E. Mihich (eds), pp 179-188, Alan R. Liss Inc. New York, 1983.

38. A. A. Piper, M. H. N. Tattersall, and R. M. Fox, Activities, at different stages of the cell cycle, of enzymes of pyrimidine and purine metabolism. In: Nuclosides and Cancer Treatment, (eds), pp 57-70, Academic Press, Australia, 1981.

39. Y. Engstrom, S. Eriksson, I. Jildevik, S. Skog, L. Thelander, and B. Tribukait, Cell cycle-dependent expression of mammalian ribonucleotide reductase. J. Biol. Chem. **260**, 9144-9116 (1985).

40. K. L. Skoog, B. A. Nordenskjold, and K. G. Bjursell, Deoxyribonucleoside-triphosphate pools and DNA synthesis in synchronized hamster cells. Eur. J. Biochem. **33**, 428-432 (1973).

41. R. A. Walters, R. A. Tobey, and R. L. Ratliff, Cell-cycle-dependent variations of deoxyribonuclueoside triphosphate pools in Chinese hamster cells. Biochim. Biophys. ACTA **319**, 336-347 (1973).

42. G. P. V. Reddy and A. B. Pardee, Coupled ribonucleoside diphosphate reduction, channeling, and incorporation into DNA of mammalian cells. J. Biol. Chem. **257**, 12526-12531 (1982).

43. J. M. Leeds, M. B. Slabaugh, and C. K. Mathews, DNA precursor pools and ribonuclotide reductase activity: Distribution betweeen the nucleus and cytoplasm of mammalian cells. Mol. Cell. Biol. **5**, 3443-3450 (1985).

44. C. Delfini, E. Alfani, V. De Venezia, G. Oberholtzer, C. Tomasello, T. Eremenko, and P. Volpe, Cell-cycle dependence and properties of the HeLa cell DNA polymerase system. Proc. Natl. Acad. Sci. USA **82**, 2220-2224 (1985).

45. J. M. Tricoli, B. M. Sahai, P. J. McCormick, S. J. Jarlinski, J. S. Bertram, and D. Kowalski, DNA topoisomerase I and II activities during cell proliferation and the cell cycle in cultured mouse embryo fibroblast (C3H 10T1/2) cells. Exp. Cell Res. **158**, 1-14 (1985).

46. Polyamines in Biology and Medicine. D. R. Morris and L. J. Marton (eds), Dekker, New York, 1981.

47. P. S. Sunkara, C. C. Chang, and N. J. Prakash, Role of polyamines during chromosome condensation of mammalian cells. Cell Biol. Int. Rep. **7**, 455-465 (1983).

48. D. H. Russell and P. J. Stambrook, Cell cycle specific fluctuations in adenosine 3':5'-cyclic monophosphate and polyamines of Chinese hamster cells. Proc. Natl. Acad. Sci. USA **72**, 1482-1486 (1975).

49. O. Heby, L. J. Marton, J. W. Gray, P. A. Lindl, and C. B. Wilson, Polyamine metabolism in synchronously growing mammalian cells. In: Proceedings of the Ninth Congress of the Nordic Society for Cell Biology. F. Bierring (ed), Odense University Press, Denmark, 1975.

50. P. S. Sukara, S. Ramakrishna, K. Nishioka, and P. N. Rao, The relationship between level and rates of synthesis of polyamines during the mamalian cell cycle. Life Sci. **28**, 1497-1506 (1981).

51. S. M. Oredsson, J. W. Gray, and L. J. Marton, Progressive increase in polyamine levels in 9L cells in vitro during the cell cycle: Comparison between cells isolated by centrifugal elutriation and cells grown in synchrony. Cell Tissue Kinet. **17**, 437-444 (1984).

52. P. S. Sunkara, C. C. Chang, and P. J. Lachman, Cell proliferation and cell cycle dependent changes in the methylthioadenesine phosporylase activity in mammalian cells. Biochem. Biophys. Res. Commun. **127**, 546-551 (1985).

53. I. Isenberg, Histones. Ann. Rev. Biochem. **48**, 149-151 (1979).

54. A. M. Delagene and A. S. Lee, Coupling of histone and DNA synthesis in the somatic cell cycle. Science **215**, 79-81 (1982).

55. V. E. Groppi and P. Coffino, G_1 and S phase mammalian cells synchronize histones at equivalent rates. Cell **21**, 195-204 (1980).

56. R. S. Wu, and W. M. Bonner, Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. Cell **27**, 321-330 (1981).

57. A. D. Riggs and P. A. Jones, 5-methylcytosine, gene regulation and cancer. Adv. Cancer Res. **40**, 1-30 (1983).

58. DNA methylation: Biochemistry and Biological Significance. A. Razin, H. Cedar, and A. D. Riggs (eds), Springer-Verlag Inc., New York, 1984.

59. M. Szyf, F. Kaplan, V. Mann, H. Giloh, E. Kedar, and A. Razin, Cell cycle-dependent regulation of eukaryotic DNA methylase level. J. Biol. Chem. **260**, 8653-8656 (1985).

60. P. K. Gupta and M. A. Sirover, Stimulation of the nuclear uracil DNA glycosylase in proliferating human fibroblasts. Cancer Res. **41**, 3133-3136 (1981).

61. M. P. Hagan, E. V. Holohan, D. P. Dodgen, and M. H. Schneiderman, Coupling of uracil-DNA glycosylase activity to cell cycle progression. Abstracts of the 33rd Annual Meeting of the Radiation Research Society, May , 1985, Los Angeles, CA.

62. C. Schuster, G. Rode, and H. M. Rabes, O^6-methylguanine repair of methylated DNA in vitro: Cell cycle-dependence of rat liver methyltransferase activity. J. Cancer Res. Clin. Oncol. **110**, 98-102 (1985).

63. G. J. Smith, D. G. Kaufman, and J. W. Grisham, Decreased excision of O^6-methylguanine during the S phase in 10T1/2 cells. Biochem. Biophys. Res. Commun. **92**, 787-794 (1980).

64. G. J. Smith, J. W. Grisham, and D. G. Kaufman, Cycle-dependent removal of certain methylated bases from DNA of 10T1/2 cells treated with N-methyl-N'-nitro-N-nitrosoguanidine. Cancer Res. 41, 1373-1378 (1981).

65. P. L. Chello, C. A. McQueen, L. M. DeAngelis, and J. R. Bertino, Comparative effects of folate antagonists versus enzymatic folate depletion on folate and thymidine enzymes in cultured mammalian cells. Cancer Treat. Rep. **61**, 539- (1977).

66. A. S. Goustin, E. B. Leof, G. D. Shipley, and H. L. Moses, Growth factors in cancer. Cancer Res. **46**,1015-1029 (1986).

67. E. J. O'Keefe and W. J. Pledger, A model of cell cycle control: Sequential events regulated by growth factors. Mol. Cell. Endocrinol. **31**, 167-186 (1983).

68. O. Larsson, A. Zetterberg, and W. Engstrom, Cell-cycle-specific induction of quiescence achieved by limited inhibition of protein synthesis: Counteractive effect of addition of purified growth factors. J. Cell Sci. **73**, 375-387 (1985).

69. W. Wharton, Hormonal regulation of discrete portions of the cell cycle: Commitment to DNA synthesis is commitment to cellular division. J. Cell. Physiol. **117**, 423-429 (1983).

70. C. B. Thompson, P. B. Challoner, P. E. Neiman, and M. Groudine, Levels of c-myc oncogene mRNA are invariant throughout the cell cycle. Nature **314**, 363-366 (1985).

71. J-M. Blanchard, M. Piechaczyk, C. Dani, J-C. Chambard, A. Franchi, J. Pouyssegur, and P. Jeanteur, C-myc gene is transcribed at high rate in G_0-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. Nature **317**, 443-445 (1985).

72. C. B. Thompson, P. B. Challoner, P. E. Neiman, and M. Groudine, Expression of the c-myb proto-oncogene during cellular proliferation. Nature **319**, 374-380 (1986).

73. F. Dolbeare, H. Gratzner, M. G. Pallavicini, and J. W. Gray, Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc. Natl. Acad. Sci. USA **80**, 5573-5577 (1983).

74. P. N. Dean, F. Dolbeare, H. Gratzner, G. C. Rice, and J. W. Gray, Cell cycle analysis using a monoclonal antibody to BrdUrd. Cell Tissue Kinet. **17**, 427-36 (1984).

75. T. S. Johnson, E. Bain, M. R. Raju, and J. C. Martin, Correlation of the growth of Chinese hamster $V_2$79-171b multicellular spheroids with cytokinetic parameters. Cytometry **1**, 65-70 (1985).

76. L. Norton and R. Simon, Tumor size, sensitivity to therapy, and the design of treatment schedules. Cancer Treat. Rep. **61**, 1307-1317 (1977).

77. J. W. Gray, Quantitative cytokinetics: Cellular response to cell cycle specific agents. Phamac. Ther. **22**, 163-197 (1983).

78. M. Tubiana, M. H. Pejovic, N. Chavaudra, G. Contesso, and E. P. Malaise, The long term significance of the thymidine labeling index in breast cancer. Int. J. Cancer **33**, 441-445 (1984).

79. T. Hoshino and C. B. Wilson, Cell kinetic analysis of human malignant brain tumors (gliomas). Cancer **44**, 956-962 (1979).

80. H. Bleiberg, M. Buyse, B. Van Den Heule, and P. Galand, Cell cycle parameters and prognosis of colorectal cancer. Eur. J. Clin. Oncol. **20**, 391-396 (1984).

81. E. Hamilton and J. Dobbin, The validity of the labeling index in tumour studies. Br. J. Cancer **51**, 15-21 (1985).

82. D. C. Allison, P. F. Ridolpho, S. Anderson, and K. Bose, Variations in the [^{3}H]thymidine labeling of S-phase cells in solid mouse tumors. Cancer Res. **45**, 6010-6016 (1985).

83. J. S. Meyer and J. Y. Lee, Relationships of S-phase fraction of breast carcinoma in relapse to duration of remission, estrogen receptor content, therapeutic responsiveness, and duration of survival. Cancer Res. **40**, 1890-1896 (1980).

84. H. Baisch, U. Otto, and G. Kloppel, Malignancy index based on flow cytometry and histology for renal cell carcinomas and its correlation to prognosis. Cytometry **7**, 200-204 (1986).

85. I. Tannock, Cell kinetics and chemotherapy: A critical review. Cancer Treat. Rep. **62**, 1117-1133 (1978).

86. B. T. Hill, Cancer Chemotherapy. The relevance of certain concepts of cell cycle kinetics. Biochim. Biophys. ACTA **516**, 389-417 (1978).

87. L. Norton, Implications of kinetic heterogeneity in clinical oncology. Sem. Oncol. **12**, 231-249 (1985).

88. L. M. van Putten, H. J. Keizer, and J. H. Mulder, Perspectives in cancer reserach. Synchronization in tumour chemotherapy. Eur. J. Cancer **12**, 79-85 (1976).

89. L. A. Dethlefsen, J. D. Ohlsen, and J. L. Roti Roti, Cell synchronization in vivo: fact or fancy? In: Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells. B. Drewinko and R M. Humphrey (eds), pp 491-507, Williams and Wilkins, Baltimore, 1977.

90. B. K. Bhuyan, Cell cycle-related cellular lethality. In: Growth Kinetics and Biochemical Regulation in Normal and Malignant Cells. B. Drewinko and R. M. Humphrey (eds), pp 363-375, Williams and Wilkins, Baltimore, 1977.

91. S. C. Barranco, J. T. May, W. Boerwinkle, S. Nichols, K. M. Hokanson, J. Schumann, W. Gohde, J. Bryant, and L. F. Guseman, Enhanced cell killing through the use of cell kinetics-directed treatment schedules for two-drug combinations in vitro. Cancer Res. **42**, 2894-2898 (1982).

92. L. P. Colly, D. W. van Bekkum, and A. Hagenbeek, Cell kinetic studies after high dose ara-c and adriamycin treatment in a slowly growing rat leukemia model (BNML) for human acute myelocytic leukemia. Leukemia Res. **8**, 945-952 (1984).

93. C. K. Osborne, D. H. Boldt, and P. Estrada, Human breast cancer cell cycle synchronization by estrogens and antiestrogens in culture. Cancer Res. **44**, 1433-1439 (1984).

94. V. Hug, D. Johnston, M. Finders, and G. Hortobagyi, Use of growth-stimulatory hormones to improve the in vitro therapeutic index of doxorubicin for human breast tumors. Cancer Res. **46**, 147-152 (1986).

95. P. G. Braunschweiger, L. L. Schenken, and L. M. Schiffer, Kinetically directed combiantion therapy with adriamycin and X-irradiation in a mammary tumor model. Int. J. Radiat. Oncol. Biol. Phys. **7**, 747-753 (1981).

96. S. C. Barranco, C. M. Townsend, J. J. Costanzi, J. T. May, R. Baltz, A. G. O'Quinn, B. Lepzig, K. M. Hokanson, L. F. Guseman, and W. R. Boerwinkle, Use of 1,2:5,6-dianhydrogalactitol in studies on cell kinetics-directed chemotherapy schedules in human tumors in vivo. Cancer Res. **42**, 2899-2905 (1982).

97. L. P. Colly, D. W. van Bekkum, and A. Hagenbeek, Enhanced tumor load reduction after chemotherapy induced recruitment and synchronization in a slowly growing rat leukemia model (BNML) for human acute myelocytic leukemia. Leukemia Res. **8**, 953-963 (1984).

98. P. F. Conte, G. Fraschini, A. Alama, A. Niclin, E. Corsaro, G. Canavese, R. Rosso, and B. Drewinko, Chemotherapy following estrogen-induced expansion of the growth fraction of human breast cancer. Cancer Res. **45**, 5926-5930 (1985).

99. E. P. Mitchell and P. S. Schein, Contributions of nitrosoureas to cancer treatment. Cancer Treat. Rep. **70**, 31-41 (1986).

100. R. E. Meyn and D. Murray, Cell cycle effects of alkylating agents, Pharmac. Ther. **24**, 147-163 (1984).

101. R. J. Weinkam and H-S. Lin, Chloroethylnitrosourea cancer chemotherapeutic agents. Adv. Pharmacol. Chemother. **19**, 1-33 (1982).

102. C. J. Cheng, S. Fujimura, D. Grunberger, and I. B. Weinstein, Interaction of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037) with nucleic acids and proteins in vivo and in vitro. Cancer Res. **32**, 22-27 (1972).

103. P. V. Woolley, R. L. Dion, K. W. Kohn, and V. H. Bono, Jr., binding of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea to L1210 cell nuclear proteins. Cancer Res. **36**, 1470-1474 (1976).

104. G. P. Wheeler, B. J. Bowden, J. A. Grimsley, and H. H. Lloyd, Interrelationships of some chemical, physiochemical, and biological activities of several 1-(2-haloethyl)-1-nitrosoureas. Cancer Res. **34**, 194-200 (1974).

105. J. A. Montgomery, Chemistry and structure-activity studies of the nitrosoureas. Cancer Treat. Rep. **60**, 651-664 (1976).

106. M. Colvin, R. B. Brundett, W. Cowens, I. Jardine, and D. B. Ludlum, A chemical basis for the antitumor activity of chloroethylnitrosoureas. Biochem. Pharmacol. **25**, 695-699 (1976).

107. T. P. Johnston and J. A. Montgomery, Relationship of structure to anticancer activity and toxicity of the nitrosoureas in animal systems. Cancer Treat. Rep. **70**, 13-30 (1986).

108. P. J. Tofilon, M. E. Williams, and D. F. Deen, Nitrosourea-induced sister chromatid exchanges and correlation to cell survival in 9L rat brain tumor cells. Cancer Res. **43**, 473-475 (1983).

109. P. Schein, J. Bull, D. Doukas, D. Hoth, Sensitivity of human and murine bone marrow precursor cells to 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose and 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res. **38**, 257-260 (1978).

110. F. Ali-Osman, J. Giblin, M. Berger, M. J. Murphy, Jr., and M. Rosenblum, Chemical structure of carbamoylating groups and their relationship to bone marrow toxicity and antiglioma activity of bifunctionally alkylating and carbamoylating nitrosoureas. Cancer Res. **45**, 4185-4191 (1985).

111. R. B. Weiss and J. S. MacDonald, Toxicities associated with nitrosourea treatment. In: Nitrosoureas in Cancer Treatment, INSERM Symposium No. 19, B. Serrou, P. S. Schein, and J-L. Imbach (eds), Elsevier/North Holland Biomedical Press, Amsterdam, 1981.

112. R. J. Weinkam and D. F. Deen, Quantitative dose-response relations for the cytotoxic activity of chloroethylnitrosoureas in cell culture. Cancer Res. **42**, 1008-1014 (1982).

113. N. Buckley, T. P. Brent, L. J. Marton, and D. F. Deen, Manuscript in preparation, 1986.

114. R. A. G. Ewig and K. W. Kohn, DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. Cancer Res. **37**, 2114-2122 (1977).

115. L. C. Erikson, M. O. Bradley, and K. W. Kohn, Measurements of DNA damage in Chinese hamster cells treated with equitoxic and equimutagenic doses of nitrosoureas. Cancer Res. **38**, 3379-3384 (1978).

116. L. C. Erickson, M. O. Bradley, J. M. Ducore, R. A. G. Ewig, and K. W. Kohn, DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. Proc Natl. Acad. Sci. USA **77**, 467-471 (1980).

117. B. Singer, W. J. Bodell, J. E. Cleaver, G. H. Thomas, M. F. Rajewsky, and W. Thon, Oxygens in DNA are main targets for ethylnitrosourea in normal and xeroderma pigmentosum fibroblasts and fetal rat brain cells. Nature **276**, 85-88 (1978).

118. D. B. Ludlum and W. P. Tong, Modification of DNA and RNA bases by the nitrosoureas. In: Nitrosoureas in Cancer Treatment. B. Serrou, P. S. Schein, and J-L Imbach (eds), pp21-31, Elsevier/North Holland Biomedical Press, Amsterdam, 1981.

119. W. P. Tong, K. W. Kohn, and D. B Ludlum, Modifications of DNA by different haloethylnitrosoureas. Cancer Res. **42**, 4460-4464 (1982).

120. C. T. Gombar, W. P. Tong, and D. B. Ludlum, Mechanism of action of the nitrosoureas --IV. Reactions of bis-chloroethyl nitrosourea and chloroethyl cyclohexyl nitrosourea with deoxyribonucleic acid. Biochem. Pharmacol. **29**, 2639-2643 (1980).

121. R. Saffhill and J. A. Hall, The incorporation of O^6-methyldeoxyguanosine monophosphate and O^4-methyldeoxythymidine monophosphate into polynucleotide templates leads to errors during subsequent in vitro DNA synthesis. Chem.-Biol. Interactions **56**, 363-370 (1985).

122. J. A. Hartley, N. W. Gibson, K. W. Kohn, and W. B. Mattes, DNA sequence selectivity of guanine-N7 alkylation by three antitumor chloroethylating agents. Cancer Res. **46**, 1943-1947 (1986).

123. K. W. Kohn, Interstrand cross-linking of DNA by 1,3-bis(2-chloroethyl)-1-nitrosourea and other 1-(2-haloethyl)-1-nitrosoureas. Cancer Res. **37**, 1450-1454 (1977).

124. L. C. Erickson, G. Laurent, N. A. Sharkey, and K. W. Kohn, DNA cross-linking and monoadduct repair in nitrosourea-treated human tumor cells. Nature **288**, 727-729 (1980).

125. K. T. Wheeler, D. F. Deen, C. B. Wilson, M. E.Williams, and S. Sheppard, BCNU modification of the in vitro radiation response of 9L rat brain tumor cells of rats. Int. J. Radiat. Oncol. Biol. Phys. **2**, 79-88 (1977).

126. V. Da Silva, P. J. Tofilon, P. H. Gutin, W. C. Dewey, N. Buckley, and D. F. Deen, Comparative study of the effects of hyperthermia and BCNU on BCNU-sensitive and BCNU-resistant 9L rat brain tumor cells. Radiat. Res. **103**, 363-372 (1985).

127. W. J. Bodell, W. F. Morgan, J. Rasmussen, M. E. Williams, and D. F. Deen, Potentiation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-induced cytotoxicity in 9L cells by pretreatment with 6-thioguanine. Biochem. Pharmacol. **34**, 515-520 (1985).

128. D. T. Hung, D. F. Deen, J. Seidenfeld, and L. J. Marton, Sensitization of 9L rat brain gliosarcoma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea by alpha-difluoromethylornithine, an ornithine decarboxylase inhibitor. Cancer Res. **41**, 2783-2785 (1981).

129. P. J. Tofilon, M. E. Williams, and D. F. Deen, The effects of X rays on BCNU-induced DNA crosslinking. Radiat. Res. **99**, 165-174 (1984).

130. P. J. Tofilon, V. Da Silva, P. H. Gutin, and D. F. Deen, Effects of hyperthermia on DNA interstrand crosslinking after treatment with BCNU in 9L rat brain tumor cells. Radiat. Res. **103**, 373-382 (1985).

131. P. J. Tofilon, D. F. Deen, and L. J. Marton, Alpha-difluoromethyl ornithine-induced polyamine depletion of 9L tumor cells modifies drug induced DNA crosslink formation. Science **222**, 1132-1135 (1983).

132. W. J. Zeller, M. R. Berger, T. Henne, and E. Weber, Pretreatment of human colon tumor cells with DNA methylating agents inhibits their ability to repair chloroethyl monoadducts. Carcinogenesis **5**, 83-87 (1984).

133. J. K. Weinke and W. J. Bodell, N-methyl-N-nitrosourea potentiation of cytogenetic damage induced by 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res. **45**, 4798-4803 (1985).

134. P. A. Linfoot, P. J. Tofilon, L. J. Marton, and D. F. Deen, effect on cell kill, sister chromatid exchange, crosslink formaation, and phase specific survival of simultaneous treatment of 9L cells with BCNU and ENU. Submitted to Carcinogenesis.

135. C. Ziotogorski and L. C. Erickson, Pretreatment of normal human fibroblasts and human colon carcinoma cells with MNNG allows chloroethylnitrosourea to produce DNA interstrand crosslinks not observed in cells treated with chloroethylnitrosourea alone. Carcinogenesis **4**, 759-763 (1983).

136. W. P. Tong and D. B. Ludlum, Formation of the crosslinked base, diguanylethane, in DNA treated with N,N'-bis(2-chloroethyl)-N-nitrosourea. Cancer Res. **41**, 380-382 (1981).

137. W. P. Tong, M. C. Kirk, and D. B. Ludlum, Formation of the cross-link 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosinyl]-ethane in DNA treated with N, N'-bis(2-chloroethyl)-N-nitrosourea. Cancer Res. **42**, 3102-3105 (1982).

138. W. P. Tong, M. C. Kirk, and D. B. Ludlum, Mecahnism of action of the nitrosoureas--V. Formation of O^6-(2-fluoroethyl) guanine and its probable role in the crosslinking of deoxyribonucleic acid. Biochem. Pharmacol. **32**, 2011-2015 (1983).

139. N. Buckley, Personal communication, 1986.

140. R. J. Weinkam and M. E. Dolan, An analysis of the effects of temperature on chloroethylnitrosourea activity in cell culture. Proc. Am. Assoc. Cancer Res. **23**, 163 (1982).

141. R. G. Selker, E. Bova, M. Kristofik, E. Jones, D. Iannuzzi, A. Landray, and F. Taylor, Effect of total body temperature on toxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Neurosurgery **4**, 157-161 (1979).

142. G. M. Hahn and E. C Shiu, Effect of pH and elevated temperatures on the cytotoxicity of some chemotherapeutic agents on Chinese hamster cells in vitro. Cancer Res. **43**, 5789-5791 (91983).

143. A. Streitweiser, Jr. and W. D. Schaeffer, Stereochemistry of the primary carbon. VI. The reaction of optically active 1-aminobutane-1-d with nitrous acid. Mechanism of the amine-nitrous acid reaction. J. Amer. Chem. Soc. **79**, 2888-2893 (1957).

144. J. K. Snyder and L. M. Stock, Reactions of alkylnitrosoureas in aqueous solution. J. Org. Chem. **45**, 1990-1999 (1980).

145. J. Slavik, Intracellular pH topography: determination by a fluorescent probe. FEBS Letters **156**, 227-230 (1983).

146. G. L'Allemain, S. Paris, and J. Pouyssegur, Role of Na^+-dependent Cl^- HCO_3^- excange in regulation of intracellular pH in fibroblasts. J. Biol. Chem. **260**, 4877-4883 (1985).

147. D. F. Gersen and H. Kiefer, Intracellular pH and the cell cycle of mitogen-stimulated murine lymphocytes. J. Cell Physiol. **114**, 132-135 (1983).

148. J. Pouyssegur, A. Franchi, G. L'Allemain, and S. Paris, Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. FEBS Letters **190**, 115-119 (1985).

149. R. B. Mikkelsen, C. Asher, and T. Hicks, Extracellular pH, transmembrane distribution and cytotoxicity of chlorambucil. Biochem. Pharmacol. **34**, 2531-2534 (1985).

150. R. J. Weinkam, T-Y J. Liu, and H-S Lin, Protein mediated chemical reactions of chloroethylnitrosoureas. Chem-Biol. Interactions **31**, 167-177 (1980).

151. R. J. Weinkam, A. Finn, V. A. Levin, and J. P. Kane, Lipophillic drugs and lipoproteins: partitioning effects on chloroethylnitrosourea reaction rates in serum. J. Pharmacol. Exp. Ther. **214**, 318-323 (1980).

152. A. Russo, J. Mitchell, T. Kinsella, G. Morstyn, and E. Glatstein, Determinants of radiosensitivity. Sem. Oncol. **12**, 332-349 (1985).

153. R. P. Hill and J. A. Stanley, The response of hypoxic B16 melanoma cell to in vivo treatment with chemotherapeutic agents. Cancer Res. **35**, 1147-1153 (1975).

154. B. A. Teicher, J. S. Lazo, and A. C. Sartorelli, Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Res. **41**, 73-81 (1981).

155. E. O. Pettersen and T. Lindmo, Inhibition of cell-cycle progression by acute treatment with various dgrees of hypoxia: modifications induced by low concentrations of mizonidazole present during hypoxia. Br. J. Cancer **48**, 809-817 (1983).

156. I. J. Spiro, G. C. Rice, R. E. Durand, R. Stickler, and C. C. Ling, Cell killing, radiosensitization, and cell cycle redistribution induced by chronic hypoxia. Int. J. Radiat. Oncol. Biol. Phys. **10**, 1275-1280 (1984).

157. I. F. Tannock, Experimental chemotherapy and concepts related to the cell cycle. Int. J. Radiat. Biol. **49**, 335-355 (1986).

158. M. Loffler, Characterization of the deoxynucleotide-dependent reversal of hypoxia-induced inhibition of cell cycle progression in Ehrlich asicites tumor cells. Eur. J. Cell Biol. **39**, 198-204 (1985).

159. H. S. Maker, H. H. Syed, and G. M. Leher, Environmental factors in glial tumor response to 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea: effect of cell confluence. J. Natl. Cancer Inst. **59**, 1427-1429 (1977).

160. R. F. Hageman, L. L. Schenken, and S. Lesher, Tumor chemotherapy: effficacy dependent on mode of growth. J. Natl. Cancer Inst. **50**, 467-474 (1973).

161. B. Drewinko, B. Barlogie, and E. J. Freireich, Response of exponentially growing, stationary-phase, and synchronized cultured human colon carcinoma cell to treatment with nitrosourea derivitives. Cancer Res. **39**, 2630-2636 (1979).

162. S. C. Barranco, J. K. Novak, and R. M. Humphrey, Response of mammalian cells following treatment with bleomycin and 1,3-bis(2-chloroethyl)-1-nitrosourea during plateau phase. Cancer Res **33**, 691-694 (1973).

163. P. R. Twentyman and N. M. Bleehen, The sensitivity of cells in exponential and stationary phases of growth to bleomycin and to 1,3-bis(2-chloroethyl)-1-nitrosourea. Br. J. Cancer **28**, 500-507 (1973).

164. G. M. Hahn, L. F. Gordon, and S. S. Kurkjian, Responses of cycling and non-cycling cells to 1,3-bis(2-chloroethyl)-1-nitrosourea and to bleomycin. Cancer Res. **34**, 2373-2377 (1974).

165. T. Hoshino, D. F. Deen, M. E. Williams, and Y. Sano, Differential response of elutriated 9L cells to treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res. **41**, 4404-4407 (1981).

166. R. Jostes and D. F. Deen, Double elution analysis of cross-link formation and repair in cycling and noncycling 9L spheroid cells. Cancer Res. **46**, 2352-2355 (1986).

167. D. F. Deen and M. E. Williams, unpublished results.

168. G. V. Vitkauskas and E. S. Canellakis, Intercellular communication and cancer chemotherapy. Biochim. Biophys. Acta **823**, 19-34 (1985).

169. P. J. Tofilon, N. Buckley, and D. F. Deen, Effect of cell-cell interactions on drug sensitivity and growth of drug-sensitive and -resistant tumor cells in spheroids. Science **226**, 862-864 (1984).

170. K. Nomura, T. Hoshino, K. Knebel, D. F. Deen, and M. Barker, BCNU-induced perterbations in the cell cycle of 9L rat brain tumor cells. Cancer Treat. Rep. **62**, 747-754 (1978).

171. U. K. Ehmannn and K. T. Wheeler, Cinemicrographic determination of cell progression and division abnormalities after treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea. Europ. J. Cancer **15**, 461-473 (1979).

172. S. Wolff, J. Bodycote, and R. B. Painter, Sister chromatid exchanges induced in Chinese hamster cells by UV irradiation of different stages of the cell cycle: the necessity for cells to pass through S. Mutation Res. **25**, 73-81 (1974).

173. G. Iliakis and M. Nusse, The importance of G1/S-border and mitosis in the fixation of potentially lethal damage. Radiat. Environ. Biophys. **22**, 201-207 (1983).

174. M. Bertrand and D. F. Deen, Factors influencing the recovery from potentially lethal damage (PLD) in mammalian cells in vitro and in vivo. Cancer Treat. Rev. **7**, 1-15 (1980).

175. G. Iliakis and M. Nusse, Evidence that repair and expression of potentially lethal damage cause the variations in cell survival after X irradiation observed through the cell cycle in Ehrlich Ascites tumor cells. Radiat. Res. **95**, 87-107 (1983).

176. S. C. Barranco, J. K. Novak, and R. M. Humphrey, Studies on the recovery from chemically induced damage in mammalian cells. Cancer Res. **35**, 1194-1204 (1975).

177. Y. Sano, T. Hoshino, M. Barker, and D. F. Deen, Response of 9L rat brain tumor multicellular spheroids to single and fractionated doses of 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res. **44**, 571-576 (1984).

178. R. A. Becker and R. Montesano, Repair of O^4-methyldeoxythymidine residues in DNA by mammalian liver extracts. Carcinogenesis **6**, 313-317 (1985).

179. A. L. Harris, P. Karran, and T. Lindahl, O^6-methylguanine-DNA methyltransferase of human lymphoid cells: Structural and kinetic properties and absence in repair deficient cells. Cancer Res. **43**, 3247-3252 (1983).

180. R. Sklar and B. Straus, Removal of O^6-methylguanine from DNA of normal and xeroderma pigmentosum-derived lymphoblastoid lines. Nature **289**, 417-420 (1981).

181. R. S. Day III, C. H. J. Ziolkowski, D. Scudiero, S. A. Meyer, A. S. Lubiniecki, A. J. Giardi, S. M. Galloway, and G. D. Bynum, Defective repair of alkylated DNA by human tumor and SV40-transformed human cell strains. Nature **288**, 724-728 (1980).

182. D. B. Yarosh, R. S. Foote, S. Mitra, and R. S. Day III, Repair of O^6-methylguanine in DNA by demethylation is lacking in Mer^- human tumor cell strains. Carcinogenesis **4**, 199-205 (1983).

183. M. E. Dolan, K. Morimoto, and A. E. Pegg, Reduction of O^6-alkylguanine-DNA alkyltransferase activity in HeLa cells treated with O^6-alkylguanines. Cancer Res. **45**, 6413-6417 (1985).

184. P. Robbins, A. L. Harris, I. Goldsmith, and T. Lindahl, Cross-linking of DNA induced by chloroethylnitrosourea is prevented by O^6-methylguanine-DNA methyltransferase. Nuc. Acids Res. **11**, 7743-7758 (1983).

185. T. P. Brent, Inactivation of purified human O^6-alkylguanine-DNA alkyltransferase by alkylating agents or alkylated DNA. Cancer Res **46**, 2320-2323 (1986).

186. D. A. Scudiero, S. A. Meyer, B. E. Clatterbuck, M. R. Mattern, C. H. J. Ziolkowski, and R. S. Day III, Relationship of DNA-repair phenotypes of human fibroblast and tumor strains to killing by N-methyl-N'-nitro-N-nitrosoguanidine. Cancer Res. **44**, 961-969 (1984).

187. D. A. Scudiero, S. A. Meyer, B. E. Clatterbuck, M. R. Mattern, C. H. J. Ziolkowski, and R. S. Day III, Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylating agents including chloroethylnitrosoureas. Cancer Res. **44**, 2467-2474 (1984).

188. R. Ding, K. Ghosh, A. Eastman, and E. Bresnick, DNA-mediated transfer and expression of a human DNA repair gene that demethylates O^6-methylguanine. Mol. Cell. Biol. **5**, 3293-3296 (1985).

189. N. W. Gibson, C. Zlotogorski, and L. C. Erickson, Specific DNA repair mechanisms may protect some human tumor cells from DNA interstrand crosslinking by cloroethylnitrosoureas but not from crosslinking by other anti-tumor alkylating agents. Carcinogenesis **6**, 445-450 (1985).

190. K. D. Tew and A. L. Wang, Selective cytotoxicity of haloethynitrosoureas in a carcinoma cell line resistant to bifunctional nitrogen mustards. Mol. Pharmacol. **21**, 729-738 (1982).

191. W. J. Bodell, M. Gerosa, T. Aida, M. S. Berger and M. L. Rosenblum, Investigation of resistance to DNA cross-linking agents in 9L cell lines with different sensitivities to chloroethylnitrosoureas. Cancer Res. **45**, 3460-3464 (1985).

192. A. E. Pegg and W. Perry, Stimulation of transfer of methyl groups from O^6-methylguanine in DNA to protein by rat liver extracts in response to hepatotoxins. Carcinogenesis 2, 1195-1200 (1981).

193. S. Kim, T. M. Voberg, J.-Y. Ro, M. Kim, and M. A. Sirover, O^6-methylguanine methyltransferase increases before S phase in normal human cells but does not increase in hypermutable Bloom's syndrome cells. Mutation Res. **173**, 141-145 (1986).

194. H. J. Vijayalaxmi, H. J. Evans, J. H. Ray, and J. German, Bloom's syndrome: Evidence for increased mutation frequency in vivo. Science **221**, 851-853 (1983).

195. P. K. Gupta, and M. A. Sirover, Altered temporal expression of DNA repair in hypermutable Bloom's syndrome cells. Proc. Natl. Acad. Sci. USA **81**, 757-761 (1984).

196. B. A. Arrick and C. F. Nathan, Glutathione metabolism as a determianat of therapeutic efficacy: A review. Cancer Res. **44**, 4224-4232 (1984).

197. J. R. Babson and D. J. Reed, Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. Biochem. Biophys. Res. Commun. **83**, 754-762 (1978).

198. W. R. McConnell, P. Kari, and D. L. Hill, Reduction of glutathione levels in livers of mice treated with N.N'-bis (2-chloroethyl)-N-nitrosourea. Cancer Chemother. Pharmacol. **2**, 221-223 (1979).

199. K. D. Tew, G. Kyle, A. Johnson, and A. L. Wang, Carbamoylation of glutathione reductase and changes in cellular and chromosome morphology in a rat cell line resistant to nitrogen mustards but collaterally sensitive to nitrosoureas. Cancer Res. **45**, 2326-2333 (1985).

200. B. H. Smith, M. Vaughan, M. A. Greenwood, P. L. Kornblith, A. Robinson, N. Shitara, and P. E. McKeever, Membrane and cytoplasmic changes in 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU)-sensitive and resistant human malignant glioma-derived cell lines. J. Neuro-Oncol. **1**, 237-248 (1983).

201. R. E. Talcott and M. T. Smith, Personal communication, 1985.

202. H. Ohara and T. Terasima, Variations of cellular sulfhydral content during the cell cycle of HeLa cells and its correlation to cyclic change of X-ray sensitivity. Exp. Cell Res. **58**, 182-185 (1969).

203. W. K. Sinclair, Protection by cysteamine against lethal X-ray damage during the cell cycle of Chinese hamster cells. Radiat. Res. **39**, 135-154 (1969).

204. B. T. Hill, Biochemical and cell kinetic aspects of drug resistance. In: N. Bruchovsky and J. H. Goldie (eds), Drug and Hormone Resistance in Neoplasia, Vol. 1, CRC Press, New York, 1982.

205. A. D. Martin, R. W. G. Beer, A. G. Bosanquet, and E. D. Gilby, The effect of alkylating agents and other drugs on the accumulation of melphalan by murine L1210 leukaemia cells in vitro. Biochem. Phamacol. **31**, 2727-2732 (1982).

206. A. Begleiter, H-Y. P. Lam, and G. J. Goldenberg, Mechanism of uptake of nitrosoureas by L5178Y lymphoblasts in vitro. Cancer Res. **37**, 1022-1027 (1977).

207. H-Y. P. Lam, M. M. Talgoy, and G. J. Goldenberg, Uptake and decomposition of chlorozotocin in L5178Y lymphoblasts in vitro. Cancer Res. **40**, 3950-3955 (1980).

208. R. J. Weinkam and M. E. Dolan, An analysis of 1-(2-chloroethyl)-1-nitrosourea activity at the cellular level. J. Med. Chem. **26**, 1656-1659 (1983).

209. B. Kalvelage and G. Koch, Studies on HeLa cells surface gylcopeptides alterations in membrane structure during the cell cycle. Eur. J. Cell Biol. **28**, 233-237 (1982).

210. V. A. Levin, D. Dolginow, H. D. Landahl, C. Yorke, and J. Csejtey, Relationship of octanol/water partition coefficient and molecular weight to cellular permeability and partitioning in S49 lymphoma cells. Pharm. Res. **6**, 237-242 (1984).

211. R. Hand, Eukaryotic DNA: Organization of the genome for replication. Cell **15**, 317-325 (1978).

212. D. A. Prentice, R. A. Tobey, and L. R. Gurley, Cell cycle variations in chromatin structure detected by DNase I. Exp. Cell Res. **157**, 242-252 (1985).

213. Z. Darzynkiewicz, F. Traganos, M. Andreff, T. Sharpless, and M. R. Melamed, Different sensitivity of chromatin to acid denaturation in quiesent and cycling cells as revealed by flow cytometry. J. Histochem. Cytochem. **27**, 478-485 (1979).

214. Z. Darzynkiewicz, F. Traganos, Z. A. Arlin, T. Sharpless and M. R. Melamed, Cytofluorometric studies on conformation of nucleic acids in situ. II. Denaturation of deoxyribonucleic acid. J. Histochem. Cytochem. **24**, 49-58 (1976).

215. F. Traganos, Z. Darzynkiewicz, T. Sharpless, and M. R. Melamed, Cytofluorometric studies on conformation of nucleic acids in situ. I. Restriction of acridine orange binding by chromatin proteins. J. Histochem. Cytochem. **24**, 40-48 (1976).

216. D. A. Prentice, R. A. Tobey, and L. R. Gurley, DNAse I and cellular factors that affect chromatin structure. Biochim. Biophys. Acta **741**, 288-296 (1983).

217. P. Nehls and M. F. Rajewsky, Ethylation of nucleophilic sites in DNA by N-ethyl-N-nitrosourea depends on chromatin structure and ionic strength. Mutat. Res. **150**, 13-21 (1985).

218. P. Nehls and M. F. Rajewsky, Differential formation of O^6-ethylguanine in the DNA of rat brain chromatin fibers of different folding levels exposed to N-ethyl-N-nitrosourea in vitro. Cancer Res. **45**, 1378-1383 (1985).

219. D. T. Hung, L. J. Marton, D. F. Deen and R. H. Shafer, Depletion of intracellular polyamines may alter DNA conformation in 9L rat brain tumor cells. Science **221**, 368-370 (1983).

220. S. Rajalakshmi, P. M. Rao, and D. S. R. Sarma, Modulation of carcinogen chromatin-DNA interaction by polyamines. Biochem. Biophys. Res. Commun. **81**, 936-940 (1978).

221. D. Helland, I. F. Nes, and K. Kleppe, Mammalian DNA-repair endonuclease acts only on supercoiled DNA. FEBS Letters **142**, 121-124 (1982).

222. S. Boiteux, R. C. de Oliveira, and J. Laval, The escherichia coli O^6-methylguanine-DNA methyltransferase does not repair promutagenic O^6-methylguanine residues when present in Z-DNA. J. Biol Chem. **260**, 8711-8715 (1985).

223. C. Lagravere, B. Malfoy, M. Leng, and J. Laval, Ring-opened alkylated guanine is not repaired in Z-DNA. Nature **310**, 798-800 (1984).

224. K.-L. W. Wun and R. H. Shafer, Structure changes in mammalian DNA by low-dose X-ray damage and subsequent postirradiation incubation in the presence and absence of caffeine. Radiat. Res. **90**, 310-320 (1982).

225. S. M. Oredsson, A. E. Pegg, L. Alhonen-Hongisto, D. F. Deen, and L. J. Marton, Possible factors in the potentiation of 1-(2-chloroethyl)-3-trans-4-methylcyclohexyl-1-nitrosourea cytotoxicity by alpha-difluoromethylornithine in 9L rat brain tumor cells. Eur. J. Cancer Clin. Oncol. **20**, 535-542 (1984).

226. K. D. Tew, S. Sudhakar, P. S. Schein, and M. E. Smulson, Binding of chlorozotocin and1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea to chromatin and nucleosomal fractions of HeLa cells. Cancer Res. **38**, 3371-3378 (1978).

227. D. Green, K. D. Tew, T. Hisamatsu, and P. S. Schein, Correlation of nitrosourea murine bone marrow toxicity with deoxyribonucleic acid alkylation and chromatin binding sites. Biochem. Phamacol. **31**, 1671-1679 (1982).

228. P. C. Hanawalt, P. K. Cooper, A. K. Ganesan, and C. A. Smith, DNA repair in bacteria and mammalian cells. Ann. Rev. Biochem. **48**, 783-836 (1979).

229. D. J. Hunting, S. L. Dresler, and M. W. Lieberman, Multiple conformational states of repair patches in chromatin during DNA excision repair. Biochemistry **24**, 3219-3226 (1985).

230. L. H. Thompson, J. S. Rubin, J. E. Cleaver, G. F. Whitmore, and K. Brookman, A screening method for isolating DNA-repair deficient mutants of chinese hamster ovary cells. Somatic Cell Gen. **6**, 391-405 (1980).

231. L. H. Thompson, D. B. Busch, K. Brookman, C. L. Mooney, and D. L. Glaser, Genetic diversity of UV-sensitive mutants of chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA **78**, 3734-3737 (1981).

232. C. A. Hoy, E. P. Salazar, and L. H. Thompson, Rapid detection of DNA-damaging agents using repair-deficient CHO cells. Mutation Res. **130**, 321-332 (1984).

233. R. E. Meyn, S. F. Jenkins, and L. H. Thompson, Defective removal of DNA cross-links in a repair deficient mutant of chinese hamster cells. Cancer Res. **42**, 3106-3110 (1982).

234. C. A. Hoy, L. H. Thompson, C. L. Mooney, and A. L. Salazar, Defective DNA cross-link removal in chinese hamster cell mutants hypersensitive to bifunctional alkylating agents. Cancer Res. **45**, 1737-1743, (1985).

235. C. A. Hoy, L. H. Thompson, E. P. Salazar, and S. A. Stewart, Different genetic alterations underlie dual hypersensitivity of CHO mutant UV-1 to DNA methylating and cross-linking agents. Somatic Cell Mol. Genet. **11**, 523-532 (1985).

236. B. A. Kunz, Genetic effects of deoxyribonucleotide pool imbalances. Env. Mutagenesis **4**, 695-725 (1982).

237. M. Meuth, Role of deoxynucleoside triphosphate pool in the cytotoxic and mutagenic effects of DNA alkylating agents. Somatic Cell Genet. **7**, 89-102 (1981).

238. M. D. Topal and M. S. Baker, DNA precursor pool: A significant target for N-methyl-N-nitrosourea in C3H/10T1/2 clone 8 cells. Proc. Natl. Acad. Sci. USA **79**, 2211-2215 (1982).

239. M. H. Barcellos, P. A. Linfoot, T. P. Brent, L. J. Marton, and D. F. Deen, Manuscript in preparation.

240. P. A. Linfoot, J. W. Gray, P. N. Dean, L. J. Marton, and D. F. Deen, effect of cell cycle position on the survival of 9L cells treated with nitrosoureas that alkylate, cross-link, and carbamoylate. Cancer Res. **46**, 2402-2406 (1986).

241. K. W. Kohn, L. C. Erickson, R. A. G. Ewig, and C. A. Friedman, Fractionation of DNA from mammalin cells by alkaline elution. Biochemistry **15**, 4629-4637 (1976).

242. D. Murray and R. E. Meyn, Enhancement of the DNA cross-linking activity of melphalan by mizonidazole in vivo. Br. J. Cancer **47**, 195-203 (1983).

243. R. A. G. Ewig and K. W. Kohn, DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. Cancer Res. **38**, 3197-3203 (1978).

244. B. Myrnes, K. Norstrand, K.-E. Giercksky, C. Sjunneskog, and H. Krokan, A simplified assay for O6-methylguanine-DNA methyltransferase activitiy and its application to human neoplastic and non-neoplastic tissues. Carcinogenesis **5**, 1061-1064 (1984).

245. F. Tietze, Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to blood and other tissues. Anal. Biochem. **27**, 502-522 (1969).

246. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, Protein measurement with the folin reagent. J. Biol. Chem. **193**, 265-275 (1951).

247. W. J. Bodell and M. R. Bannerjee, Reduced DNA repair in mouse sattelite DNA after treatment with methyl-methanesulfonate, and N-methyl-N-nitrosourea. Nuc. Acids Res. **3**, 1689-1701 (1976).

248. T. Nagashima and T. Hoshino, Rapid detection of S-phase cells by anti-bromodeoxyuridine monoclonal antibody in 9L brain tumor cells in vitro and in situ. Acta Neuropathol. **66**, 12-17 (1985).

249. R. Goth-Goldstein and H. J. Burki, Ethylnitrosourea-induced mutagenesis in asynchronous and synchronous Chinese hamster ovary cells. Mutat. Res. **69**, 127-137 (1980).

250. M. D. Topal and M. S. Baker, DNA precursor pool: a significant target for N-methyl-N-nitrosourea in C3H10T1/2 cells. Proc. Natl. Acad. Sci. USA **79**, 2211-2215 (1983).

251. J. S. Eadie, M. Conrad, D. Toorchen, and M. D. Topal, Mechanism of mutagenesis by O^6-methylguanine. Nature **308**, 201-213 (1984).

252. W. J. Bodell, G. H. Thomas, and J. E. Cleaver, Evidence for removal at different rates of O-ethyl pyrimidines and ethylphosphotriesters in two human fibroblast cell strains. Nuc. Acids Res. **6**, 2819-2829 (1979).

253. K. V. Shooter, The kinetics of the alkaline hydrolysis of phosphotriesters in DNA. Chem. -Biol. Interactions **13**, 151-163 (1976).

254. P. A. Linfoot and D. F. Deen, Unpublished results.

255. K. D. Tew, G. Kyle, A. Johnson, and A. L. Wang, Carbamoylation of glutathione reductase and changes in cellular cand chromosome morphology in a rat cell line resistant to nitrogen but collaterally sensitive to nitrosoureas. Cancer Res. **45**, 2326-2333 (1985).

256. B. B. Baril, E. F. Baril, J. Lazlo, and G. P. Wheeler, Inhibition of rat liver DNA polymerase by nitrosoureas and isocyanates. Cancer Res. **35**, 1-5 (1975).

257. A. J. Fornace Jr., K. W. Kohn, and H. E. Kann Jr., Inhibition of the ligase step of excision repair by 2-chloroethyl isocyanate, a decomposition product of 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res. **38**, 1064-1069 (1978).

258. H. E. Kann Jr., M. A. Schott, and A. Petkas, Effects of structure and chemical activity on the ability of nitrosoureas to inhibit DNA repair. Cancer Res. **40**, 50-55 (1980).

259. E. Sariban, L. E. Erickson, and K. W. Kohn, Effects of carbamoylation on cell survival and DNA repair in normal human embryo cells (IMR 90) treated with various 1-(2-chloroethyl)-1-nitrosoureas. Cancer Res. **44**, 1352-1357 (1984).

260. S. W. Dean, A. B. Johnson, and K. D. Tew, A comparative analysis of drug-induced DNA effects in a nitrogen mustard resistant cell line expressing sensitivity to nitrosoureas. Biochem. Pharmac. **35**, 1171-1176 (1986).

261. P. Karran and S. A. Williams, The cytotoxic and mutagenic effects of alkylating agents on human lymphoid cells are caused by different DNA lesions. Carcinogenesis **6**, 789-792 (1985).

262. B. M. Kacinski, W. D. Rupp, and D. B. Ludlum, Repair of haloethylnitrosourea-induced DNA damage in mutant and adapted bacteria. Cancer Res. **45**, 6471-6474 (1985).

263. N. W. Gibson, J. Hartley, R. J. La France, and K. Vaughn, Differential cytotoxicity and DNA-damaging effects produced in human cells of the Mer $^{+}$ and Mer $^{-}$ phenotypes by a series of alkyltriazenylimidazoles. Carcinogenesis **7**, 259-265 (1986).

264. S. C. Barranco, W. E. Bolton, and J. K. Novak, Time-dependent changes in drug sensitivity expressed by mammalian cells after exposure to trypsin. J. Natl. Cancer. Inst. **64**, 913-916 (1980).

265. P. A. Linfoot and D. F. Deen, Unpublished results.

266. K. Nomura, T. Hoshino, D. F. Deen, and K. D. Knebel, Perturbed cell kinetics of 9L rat brain tumor cells following dianhydrogalactitol. Cancer Treat. Rep. **62**, 2055-2061 (1978).

267. A. P. Rao and P. N. Rao, The cause of G2-arrest in chinese hamster ovary cells treated with anticancer drugs. J. Natl. Cancer Inst. **57**, 1139-1143 (1976).

268. J. W. Gray, E. Bogart, D. T. Gavel, Y. S. George, and D. H. Moore II, Rapid cell cycle analysis. II. Phase durations and dispersions from computer analysis of RC curves. Cell Tissue Kinet. **16**, 457-471 (1983).

269. H. Baisch, H-P, Beck, I. J. Christensen, N. R. Hartmann, J. Freid, P. N. Dean, J. W. Gray, J. H. Jett, D. A. Johnston, R. A. White, C. Nicolini, S. Zeitz and J. V. Watson, A comparison of mathematical methods for the analysis of DNA histograms obtained by flow cytometry. Cell Tissue Kinet. **15**, 235-249 (1982).

270. P. A. Linfoot and D. F. Deen, Unpublished results.

271. T. P. Brent, Suppression of cross-link formation in chloroethyl-nitrosourea-treated DNA by an activity in extracts of human leukemic lymphoblasts. Cancer Res. **44**, 1887-1892 (1984).

272. D. B. Yarosh, The role of O^6-methylguanine-DNA methyltransferase in cell survival, mutagenesis, and carcinogenesis. Mutat. Res. **145**, 1-16 (1985).

273. W. C. Dunn, R. S. Foote, R. E. Hand, Jr., and S. Mitra, Cell cycle-dependent modulation of O^6-methylguanine methyltransferase in C3H/10T1/2 cells. Carcinogenesis **7**, 807-812 (1986).

274. J. G. Reeve, K. A. Wright, and P. Workman, Patterns of cross-sensitivity in the responses of clonal subpopulations isolated from the RIF-1 mouse sarcoma to selected nitrosoureas and nitrogen mustards. Br. J. Cancer **50**, 153-158 (1984).

275. S. L. Gerson, J. E. Trey, K. Miller, and N. A. Berger, Comparison of O^6-alkylguanine-DNA alkyltransferase activity based on cellular DNA content in human, rat and mouse tissues. Carcinogenesis **7**, 745-749 (1986).

276. R. Muller and M. F. Rajewsky, Elimination of O^6-ethylguanine from the DNA of brain, liver, and other rat tissues exposed to ethylnitrosourea at different stages of prenatal development. Cancer Res. **43**, 2897-2904 (1983).

277. M. Osband, H. Cohen, J. R. Cassidy, and N. Jaffe, Severe and protracted bone marrow dysfunction following long-term therapy with methyl-CCNU. Am. Soc. Clin. Oncol. Proc. **18**, 303 (1977).

278. H. V. Vadi and D. J. Reed, Effect of 2-chloroethylnitrosoureas on plasmid DNA including the formation of strand breaks and interstrand crosslinks. Chem. Biol. Interact. **46**, 67-84 (1983).

279. D. Murray and R. E. Meyn, Cell cycle-dependent cytotoxicity of alkylating agents: Determination of nitrogen mustard-induced DNA cross-links and their repair in chinese hamster ovary cells synchronized by centrifugal elutriation. Cancer Res. **46**, 2342-2329 (1986).

280. T. A. Conners and J. R. Hare, The binding of ^{14}C labeled 1-(2-chloroethy)-3-cyclohexyl-1-nitrosourea (CCNU) to macromolecules of sensitive and resistant tumours. Br. J. Cancer **30**, 477-480 (1974).

281. N. W. Gibson and J. A. Hickman, The role of isocyanates in the toxicity of antitumour haloalkylnitrosoureas. Biochem. Pharm. **31**, 2795-2800 (1982).

282. P. Byrne, K. Tew, J. Jemionek, T. MacVittie, L. Erickson, and P. Schein, Cellular and molecular mechanisms of the bone marrow sparing effects of the glucose chloroethylnitrosourea Chlorozotocin. Blood **63**, 759-767 (1984).

283. R. D. Wood and H. J. Burki, Repair capability and the cellular age response for killing and mutation induction after UV. Mutat. Res. **95**, 505-514 (1982).

284. L. H. Thompson, K. W. Brookman, and C. L. Mooney, Repair of DNA adducts and the role of repair in cell killing and mutation induction in synchronous cells treated with 7-bromomethylbenz[a]anthracene. Somat. Cell Mol. Genet. **10**, 183-194 (1984).

285. M. H. Barcellos, Personal communication, 1986.

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