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Developmental Toxicity of Alkylating Agents in  
Differentiating Cell Cultures

by Marguerite R. Seeley

A dissertation submitted in partial fulfillment  
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1996

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Program Authorized  
to Offer Degree Environmental Health

Date August 16, 1996

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Date August 16, 1996

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Abstract

Developmental Toxicity of Alkylating Agents in  
Differentiating Cell Cultures

by Marguerite R. Seeley

Chairperson of the Supervisory Committee: Professor Elaine M. Faustman  
Department of Environmental Health

Alkylating agents are well known carcinogens and teratogens. Highest exposures occur in occupational settings, although other lifestyle factors also contribute to exposure. The four alkylating agents used in this study were methylmethanesulfonate (MMS), methylnitrosourea (MNU), ethylmethanesulfonate (EMS), and ethylnitrosourea (ENU). In the micromass rat whole embryo cell culture system, significant concentration-dependent decreases in viability and differentiation were observed for both midbrain (CNS) and limb bud (LB) cells. CNS cells were more sensitive than LB cells, and effects on differentiation occurred at lower concentrations than effects on viability. The order of potency was MMS>MNU>ENU>EMS. Next we adapted the P19 embryonal carcinoma cell line as a system for studying mechanisms of developmental toxicity. Using all-trans retinoic acid to induce neuronal differentiation, we confirmed that uptake of [<sup>3</sup>H]-GABA is a good indicator of neuronal differentiation in P19 cells. As in the micromass cells, significant concentration-dependent decreases in both viability and differentiation were observed in alkylating agent-treated P19 cells. Furthermore, effects in the P19 cells occurred at concentrations which were very comparable to concentrations causing similar effects in the CNS cells, for all agents except ENU, which was much less toxic in P19 cells as compared with CNS cells. We also used O<sup>6</sup>-benzylguanine (O<sup>6</sup>-Bg) to inhibit the activity of the O<sup>6</sup>-alkylguanine-DNA-alkyltransferase protein which repairs adducts at the O<sup>6</sup> position of guanine. O<sup>6</sup>-Bg caused substantial, significant decreases in both viability and differentiation of cells treated with the methylating agents, MMS and MNU. Although O<sup>6</sup>-Bg did reduce viability and differentiation in cells treated with EMS, the effects were not as great as with the methylating agents, and they were not significant. ENU O<sup>6</sup>-Bg only slightly decreased viability and differentiation. The last aspect of our study was to investigate the mode of cell death in neuronally differentiating P19 cells treated with alkylating agents, using both an ELISA to quantify cytosolic oligonucleosomes, and transmission electron microscopy to observe ultrastructural morphology. We observed significant

concentration-dependent increases in cytosolic oligonucleosomes only for MNU and ENU. Additionally, with all four alkylating agents, increases in lethality were greater than increases in cytosolic oligonucleosomes. The ultrastructural morphology of treated cells showed morphologies characteristic of both apoptosis and necrosis, including condensation of chromatin and cytoplasm, apoptotic bodies, phagocytosis, increased numbers of lysosomes, and myelin bodies.

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## DEDICATION

This thesis is dedicated to my parents, Bob and Char Seeley; my brothers and sisters, Joe and Jan, Lauren and Blaise, and Karl; and my niece and nephews, Jake, Isabelle, and Paul.

## **CHAPTER 1:OVERVIEW AND PERSPECTIVES**

Concerns about alkylating agents stems from their potential for widespread human exposure, as well as their *in vivo* and *in vitro* carcinogenic, mutagenic and teratogenic effects. Exposure to alkylating agents has been shown to cause carcinogenic and teratogenic effects in rodents, and data from human cancer chemotherapeutic exposures suggest that some alkylating agents are also carcinogenic and teratogenic in humans.

### **1.1 Introduction: Alkylating Agents Examples and Exposures**

An early example of biologically active alkylating agents are the sulfur and nitrogen mustard gases, used in World War I and World War II, respectively. Figure 1 shows the structure of the mustard gases, as well as other alkylating agents. Preliminary research on sulfur mustard indicated that it could inhibit cell division and induce tumor regression. However, the clinically effective dose was very close to the toxic dose, thereby limiting its use. The nitrogen mustards were also used for cancer chemotherapy. Following World War II, methanesulphonates were tested for their efficacy in treating cancer, and some of these are still in use today. (Reviewed in Brooks, 1990). Other types of alkylating agents include the nitrosoureas. Examples of nitrosoureas include methylnitrosourea (MNU), which has been used both for cancer chemotherapy and as a plant mutagen; and bis-chloroethylnitrosourea (BCNU), which is still used for treatment of cancer. (van derWall et al., 1995). MNU and BCNU also belong to a broader class of alkylating agents known as N-nitroso compounds (NNOs), which are currently of concern from the standpoint of human exposure. Tricker et. al (1989) have provided an extensive survey of human exposure to NNOs, which is summarized in the following paragraph.

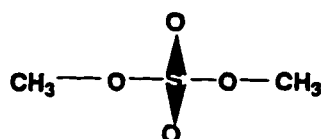
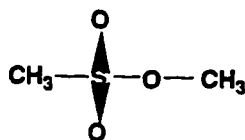
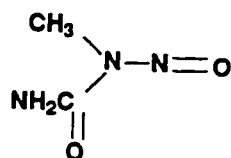
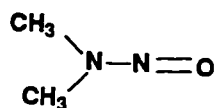
**Mustards****Alkylsulfates****Alkylsulfonates****Alkylnitrosourea****Alkylnitrosamides**

Figure 1: Examples of Alkylating Agents

Occupational settings represent the largest exposure to NNOs. Industries where significant exposures occur include rubber and tire manufacturing, metal cutting and grinding shops which use cutting oils contaminated with NNOs, leather tanneries, and manufacturers of rocket fuel. N-nitrosodimethylamine (NDMA) is an NNO commonly found in occupational settings. Outside of the workplace, a major source of exposure to NNOs occurs through inhalation of tobacco smoke, which also contains NDMA. Use of cosmetics and toiletries which contain laurylsulphates (di- and tri- ethanolamine and their salts) or diethanolamides of fatty acid (a detergent) also represents an exposure to NNOs, through contamination of these ingredients with N-nitrosodiethanolamine. Other products which can be contaminated with NNOs include PABA-containing sunscreens, pharmaceutical products, pesticides, and deionized water prepared from nitrosamine-contaminated anion exchange resins. Well water which contains nitrate can also be contaminated with NNOs. Another source of exposure to NNOs is through consumption of certain food and beverages. A recent survey of daily intake of NDMA in France revealed that alcoholic beverages represented the largest source of exposure in this food and beverage category. Other significant exposures occurred through consumption of vegetables, especially tomatoes; meats, especially cured, salted or canned meats; cheese and fish (Biaudet et al., 1994). Finally, exposure to NNOs can also occur from low levels present in the air and water due to contamination from the above-mentioned sources.

## **1.2 Teratogenic effects of Alkylating Agents**

The effects of gestational exposure to NNOs are of particular interest to our research group. Numerous animal studies have documented that alkylating agents are both carcinogenic and teratogenic. Transplacental exposure of both Fischer 344 and BDIX rat embryos to a single dose of ethylnitrosourea has been shown to result in a

variety of tumors, including glial brain tumors, cranial and spinal nerve schwannomas, and neuroblastomas (Perantoni et al., 1987, Shih et al., 1981). Transplacental exposure of Buf/N rats to methylnitrosourea also results in a variety of tumors including peripheral nervous system schwannomas, central nervous system neurogliomas, epithelial tumors (breast, nasal cavity, Zymbal's gland and transitional cell carcinoma) mesenchymal tumors (kidney, fibrosarcoma, neurofibrosarcoma and spleen undifferentiated sarcoma) and Sertoli cell tumors (Sukumar and Barbacid, 1990). Teratogenic effects of alkylating agents following *in vivo* gestational exposure include mortality; growth retardation; cephalic, CNS, palatal and extremital malformations; as well as anophthalmia (NIOSH, 1987). Exposure of NMRI mice to 20 mg/kg methylnitrosourea on day 2 of gestation produced malformations of vertebrae, ribs, long bones and kidneys (Speilmann et al., 1989). Neurobehavioral deficits have also been observed in rats with microcephaly induced by gestational exposure to N-methyl-N-nitrosourea (Akaike et al., 1994; Hashimoto and Mizutani, 1991). Effects following *in vitro* exposure to alkylating agents are similar to those seen following *in vivo* exposure in that abnormal neurulation, abnormal flexure and optic malformations are observed (Faustman et al., 1989).

In addition to extensive evidence from laboratory animals, some studies also indicate that alkylating agents have both carcinogenic and teratogenic effects in humans. An epidemiology study by Olshan and Faustman (1989) found an increased risk of tumor development in children born to mothers who were exposed to nitrosatable drugs during pregnancy. Exposure to some alkylating agents has also been associated with various birth defects in humans. Various malformations have been observed in infants born to mothers who were treated at some point during their pregnancy with alkylating cancer chemotherapeutic agents, including busulfan (Myeleran), chlorambucil, cyclophosphamide and mechlorethamine. Common malformations observed include growth retardation, cleft palate, abnormalities in the eye and in both the number and

placement of digits, as well as various kidney defects. Risk of malformations due to treatment with these cancer chemotherapeutic agents ranged from 1:2 for chlorambucil, to 1:9 for busulfan (reviewed in Schardein, 1991).

### 1.3 Mechanisms of Action

The toxicity of alkylating agents is due primarily to their electrophilic properties, which enables them to form covalent adducts with nucleophilic sites in the cell, primarily proteins and DNA. Coles (1985) discusses two ways of classifying alkylating agents. One classification is according to whether the reaction of the alkylating agent with nucleophiles occurs by either an  $S_N1$  or an  $S_N2$  mechanism. For alkylating agents which have  $S_N1$  character, the rate determining step for alkylation is generation of a charged electrophile which subsequently reacts with a nucleophile. For alkylating agents with  $S_N2$  character, the rate determining step is formation of a bimolecular transition complex between the alkylating agent and the nucleophile, with subsequent dissociation of a leaving group from the alkylating agent. An alternative classification of alkylating agents is either as hard or soft electrophiles. Hard electrophiles are very polarized with a large, central positive charge density, and react most readily with hard nucleophiles. Conversely, soft electrophiles are readily polarizable with a low positive charge density, and react most readily with soft nucleophiles.

The  $S_N1/S_N2$  character, as well as the hardness/softness of an electrophile, often determines where alkylation will occur. Softer electrophiles, or those with more  $S_N2$  character, react more readily with proteins. Sites on proteins which can be alkylated include primary amino groups of arginine and lysine, secondary amino groups of histidine, sulfur atoms of methionyl residues, and thiol groups of cysteinyl residues (Coles, 1985). Iodoacetamide is an example of an alkylating agent which alkylates proteins, causing a disruption in thiol-disulfide redox status with consequent formation of

disulfide-linked protein aggregates and transcriptional activation of heat shock protein 70 (Liu et al., 1996). Harder electrophiles, or those with more  $S_N1$  character, preferentially alkylate DNA. Sites in DNA which are commonly alkylated include the  $N^3$  and  $N^7$  positions on purines (especially  $N^7$  guanine and  $N^3$  adenine),  $O^6$  position on guanines, and  $O^2$  and  $O^4$  positions of thymines (Figure 2). These sites differ in their nucleophilicity, with the N positions being more nucleophilic than the O positions. The tendency for alkylating agents to form adducts at different sites on DNA can be predicted from the Swain-Scott substrate constant, or  $s$  value. The  $s$  value reflects the selectivity of alkylating agents for sites with high nucleophilic strength, such that agents with a high  $s$  value would preferentially react with N atoms. Studies show that proportion of  $N^7$ -alkylguanine adducts relative to  $O^6$ -alkylguanine adducts increases with the  $s$  value. The relative amount of  $S_N2$  character of an alkylating agent also increases with the  $s$  value. For instance nitrosoureas predominantly donate alkyl groups using an  $S_N1$  nucleophilic substitution reaction, whereas the methanesulfonates predominantly use an  $S_N2$  reaction.

### 1.3.1 DNA Adducts

Although formation of covalent alkyl adducts on proteins can potentially alter their function, resulting in cellular toxicity, the toxic effects of alkylating agents are attributed mainly to formation of DNA adducts (Lawley, 1974). Using a variety of DNA repair inhibitors, Kamendulis and Corcoran (1994) found parallel increases in NDMA-induced DNA damage and toxic cell death in murine hepatocytes, implicating DNA damage as a primary causative factor for alkylating agent-induced cytotoxicity.

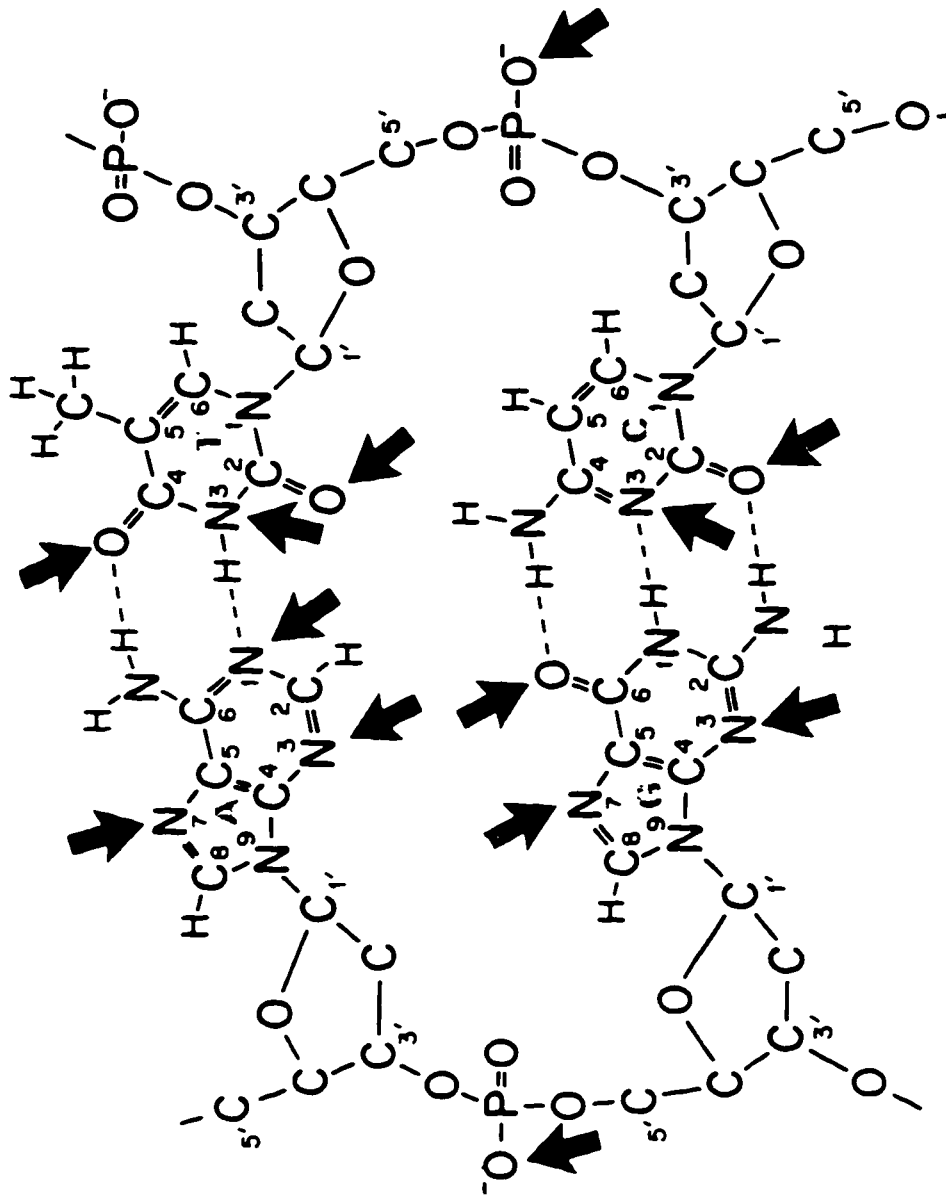


Figure 2: Sites On DNA Which Are Commonly Alkylated  
 Reference: This figure is from an article by Samson, 1992

DNA adducts can be cytotoxic by causing strand breaks and crosslinks, by inhibiting DNA repair, or by initiating an apoptotic response. DNA adducts can also be mutagenic, by causing base-mispairing. Bifunctional alkylating agents, such as cisplatin and BCNU, can form both DNA-DNA and DNA-protein crosslinks, both of which can be toxic. However, the cytotoxicity of these agents is generally attributed to DNA-DNA crosslinking (Bedford et al., 1984; Drewinko et al., 1985; Eder et al., 1987; Zwelling et al., 1979). Adducts at the N<sup>3</sup> and N<sup>7</sup> positions of purines are not very stable, and can lead to depurinated bases at neutral pH. These adducts are more correlated with lethality than mutagenicity, since extensive depurination is lethal to cells, possibly due to formation of strand breaks following depurination (Natarajan et al., 1984). On the other hand adducts at the O<sup>6</sup> position of guanine, and the O<sup>2</sup> and O<sup>4</sup> positions of thymine are more stable, and can interfere with normal hydrogen bonding and base-pairing, which can result in base mispairing and subsequent point mutations if not properly repaired (Figure 3). Alkyl adducts at the O<sup>6</sup> position of guanine are believed to result in G:C to A:T transitions, since O<sup>6</sup>-alkylguanine adducts can form hydrogen bonds with thymine instead of cytosine. Mispairing of thymine with guanine can also occur with adducts at the O<sup>4</sup> position of thymine (Lukash et al., 1991) resulting in A:T to G:C transitions. In addition to their potential for mutagenicity, the O<sup>6</sup>-alkylguanine adduct also has cytotoxic effects (Bignami et al., 1989; Dolan et al., 1989; von Hofe et al., 1992) .

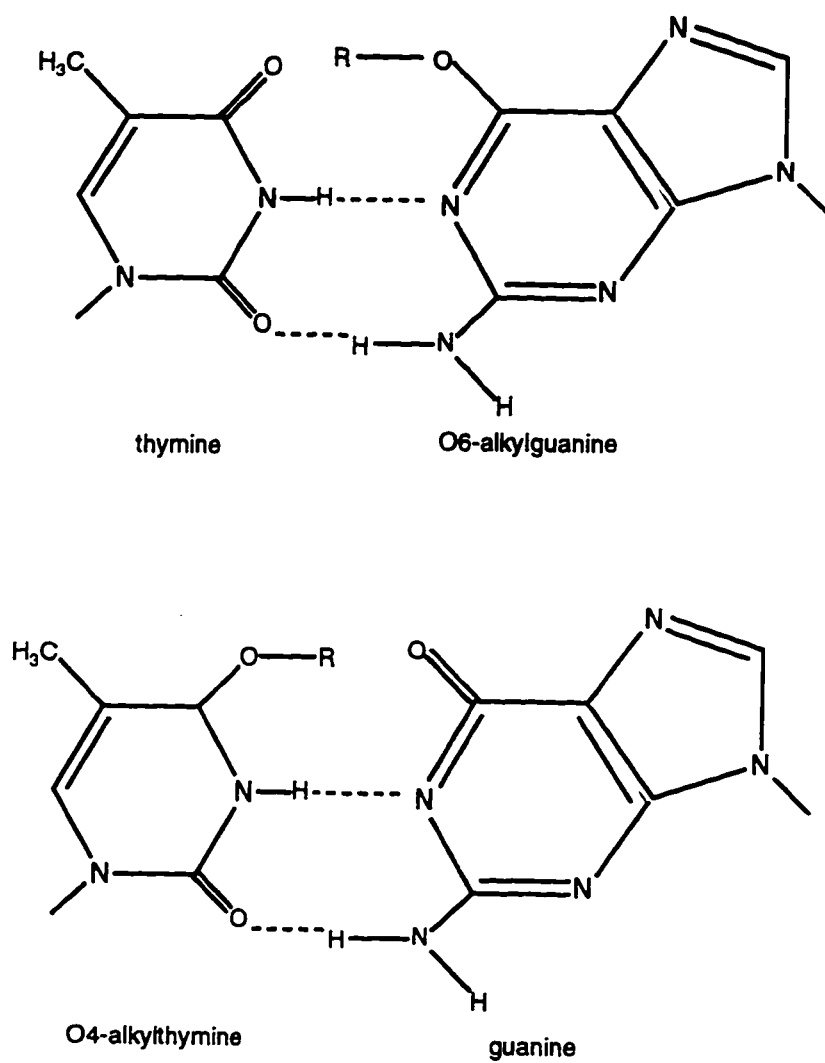


Figure 3: Mismatched Bases

#### 1.4 O<sup>6</sup>-Alkylguanine-DNA-Alkyltransferase

Many studies which support the idea that the O<sup>6</sup>-alkylguanine adduct is both mutagenic and cytotoxic have utilized cells which differ in their expression of O<sup>6</sup>-alkylguanine-DNA alkyltransferase (AT) (Domoradzki et al. 1984; Dunn et al., 1991; Lukash et al., 1991; Maher et al., 1990; Bignami et al., 1989, Kalamegham et al., 1988, Mirzayans et al., 1992). AT is a key DNA repair protein which transfers adducts from the O<sup>6</sup> position of guanine to a cysteine residue in the active site of the protein, resulting in its permanent inactivation such that new AT must be synthesized in order to maintain DNA repair capability. The protein can repair both alkyl and chloroalkyl DNA adducts. However, repair is most efficient for methyl adducts, owing to their small size, and consequently their repair generally occurs faster than repair of bulkier adducts (reviewed in Pegg, 1990).

AT is expressed in a wide range of organisms, from bacteria to mammals. The domain which encompasses the active site of the transferase (about 70 amino acids) is highly conserved among different species (Douglas et al., 1992) and a comparison of rat and human AT proteins shows 68% homology (Rahden-Staron and Laval, 1991). Although the structure of the AT protein from different organisms is overall quite similar, there are interspecies differences in activity and expression levels. For instance, whereas human AT appears to be more efficient than either bacterial or rodent AT at repairing O<sup>6</sup>-methylguanine adducts, it is much less efficient at repairing O<sup>6</sup>-ethylguanine adducts as compared with either rat or *E. coli* AT (Lien et al., 1991; Medcalf and Wade, 1983).

There are also age, tissue and cell specific expression patterns of AT. AT activity in rat liver was higher at 14 or 22 months than in either young rats (1 and 4 months) or old rats (36 months). However, age-related differences in AT activity were not observed in either kidney or white blood cells, nor were activities as high as those in the liver.

(Likhachev et al., 1991). Similarly, AT activity in mice increased between newborns and middle-aged mice, subsequently decreasing in old age (Nakatsuru et al., 1989; Washington et al., 1989). Tissue-specific differences in AT activity have also been observed in human fetal tissue, where activity was highest in liver, followed by kidney, lung, small intestine, large intestine, skin and brain (D'Ambrosio et al., 1987). Intra-organ differences in AT activity among different cell types have also been observed in human kidney, where expression was relatively high in distal tubular and glomerular epithelial cells as compared with cells of Bowman's capsule and the collecting and proximal tubules (Wani et al., 1992). Cell-specific differences in AT expression have also been observed in rat lungs, where constitutive levels of AT in macrophages and type II cells is 2-fold higher than in alveolar small cells and Clara cells (Belinsky et al., 1988).

In addition to differences in constitutive AT expression between species and in different tissues, certain exposures can induce AT expression. In the rat liver, AT can be induced by alkylating agents. However, this may not represent a specific response to alkylating agents, as a similar induction was observed with other non-alkylating agents, as well as by partial hepatectomy (reviewed in Pegg, 1990). In addition to induction in rat liver, Chan et al. (1990) observed induction in kidney, spleen, and brain following treatment with ionizing radiation, although induction levels in these tissues were not as great as in the liver. AT activity also increased 4 to 5-fold following treatment of rat hepatoma cells with ultraviolet or  $\gamma$ -irradiation, heat, cisplatin, or bleomycin (Lefebvre and Laval, 1986). Fritz and Kaina (1992) also saw induction of AT mRNA following treatment of rat hepatoma cells with a variety of agents capable of inducing strand breaks, including alkylating agents, hydrogen peroxide, ultraviolet radiation, x-rays, and restriction enzymes. Their study also suggested that AT mRNA expression can be regulated by protein phosphorylation, as protein kinase inhibitors prevented induction of AT mRNA, while a phosphatase inhibitor prevented induction of AT mRNA by methyl-

N'-nitro-N-nitrosoguanidine. Additionally, both ethanol and acetaldehyde can inhibit AT activity (Espina et al., 1988). Methylation status is another factor which seems to regulate AT activity. Specifically, methylation of a restriction enzyme site in the promoter region of the AT gene can reduce AT expression, while methylation of the coding region of the AT gene can increase its expression (von Wronski and Brent, 1994).

## 1.5 O<sup>6</sup>-Alkylguanine Adducts

### 1.5.1 O<sup>6</sup>-Alkylguanine Adducts and Carcinogenesis

Several lines of evidence relating to the repair of O<sup>6</sup>-alkylguanine adducts suggest that the carcinogenic property of alkylating agents is due to alkylation at the O<sup>6</sup> position of guanine. One line of evidence is from studies showing that tumor development correlates with slow repair of O<sup>6</sup> alkylguanine adducts. For instance, Goth and Rajewsky (1974) observed similar degrees of initial DNA ethylation in both brain and liver, following treatment of rats with ethylnitrosourea. However, removal was much slower in brain tissues which developed tumors, than in liver tissue which was resistant to tumor development. Similarly, the kidney, where the repair rate of O<sup>6</sup>-alkylguanine adducts is intermediate between brain and liver, shows tumor development which is not as frequent as that in the brain (reviewed in Manson, 1981). Another line of evidence for the carcinogenicity of O<sup>6</sup>-alkylguanine adducts is from studies in cells with differing levels of AT activity. For instance, transfection of hypoxanthine phosphoribosyl transferase (HPRT) deficient V79 hamster lung cells with the *E. coli* AT gene reduced the number of revertants at the HPRT locus in cells treated with either MNU or ENU (Fox and Margison, 1988). Similarly, depletion of AT activity in human fibroblasts increased the mutagenicity of MNNG at the HPRT locus (Lukash et al., 1991). Finally, foci forming ability following ENU treatment was lower in cells capable of repairing O<sup>6</sup>-alkylguanine adducts, as compared with cells deficient in AT activity (Thomale et al.,

1990). A third line of evidence demonstrating that O<sup>6</sup>-alkylguanine adducts are carcinogenic comes from studies using transgenic mice. Expression of an AT transgene protects mice from development of both MNU-induced thymic lymphomas (Dumenco et al., 1993) and hepatocarcinomas induced by either dimethylnitrosamine or diethylnitrosamine (Nakatsuru et al., 1993).

#### 1.5.2 O<sup>6</sup>-Alkylguanine Adducts and Teratogenesis

Whereas a strong correlation has been established between alkylation at the O<sup>6</sup> position of guanine and carcinogenicity (Domoradzki et al. 1984; Dumenco et al., 1993; Dunn et al., 1991; Lukash et al., 1991; Maher et al., 1990), studies by Bochert et al. (1991) are the only ones which show a relationship between adduct levels at the O<sup>6</sup> position of guanine and teratogenic potency. In these studies, the teratogenic potency of methylnitrosourea, acetoxymethyl-methylnitrosamine, and ethylmethanesulfonate for eliciting skeletal abnormalities in day 11 mouse embryos was correlated with initial adduct levels at N7 guanine and O<sup>6</sup>-guanine. At both the ED50 and ED90, adduct levels at the O<sup>6</sup> position of guanine varied by less than 12%, while adduct levels at the N7 position of guanine varied by greater than 66%. At the ED10, O<sup>6</sup> and N7 guanine adducts varied by 27% and 61%, respectively. Studies recently completed in our lab (Kidney and Faustman, 1995) also support the idea that adducts at the O<sup>6</sup> position of guanine may have particular relevance for developmental toxicity of some alkylating agents. That study used O<sup>6</sup>-benzylguanine (O<sup>6</sup>-Bg), which is a potent and specific inhibitor of AT. They found that O<sup>6</sup>-Bg greatly potentiated the ability of MNU to inhibit differentiation of micromass cells. However, O<sup>6</sup>-Bg had no effect on cytotoxicity of MNU. Additionally, O<sup>6</sup>-Bg had no effect on either inhibition of differentiation or cytotoxicity of ENU in these studies.

## 1.6 Purpose and Specific Aims

A current goal in toxicology research is to understand mechanisms of toxicity. The idea is that if we can understand how toxicants elicit their effects, then we can better predict the outcomes of specific exposures. The purpose of the research undertaken for this study was to investigate mechanisms involved in the developmental toxicity of alkylating agents. The hypothesis that we wanted to test for this research was that, as with carcinogenesis, O<sup>6</sup>-alkylguanine adducts are a primary causative factor in the developmental toxicity of alkylating agents. The four alkylating agents chosen for evaluation were: two methylating agents, methylnitrosourea (MNU) and methylmethanesulfonate (MMS); and two ethylating agents, ethylnitrosourea (ENU) and ethylmethanesulfonate (EMS) (Figure 4). These agents differ in their s value, and in the ratio of O<sup>6</sup>/N<sup>7</sup> guanine adducts (Table 1). Another difference is that the nitrosoureas are capable of carbamylating proteins in addition to alkylating proteins and DNA.

**Table 1: Alkylating Agents: Reactivity and Affinity for DNA Oxygen Atoms**

Agent	S	Nucleophilic mechanism	O <sup>6</sup> :N <sup>7</sup> guanine ratio
MNU	0.42	SN1	0.1
ENU	0.26	SN1	0.6
MMS	0.83	SN2	0.003-0.004
EMS	0.64	SN2/SN1	0.03

Adapted from Beranek., 1990

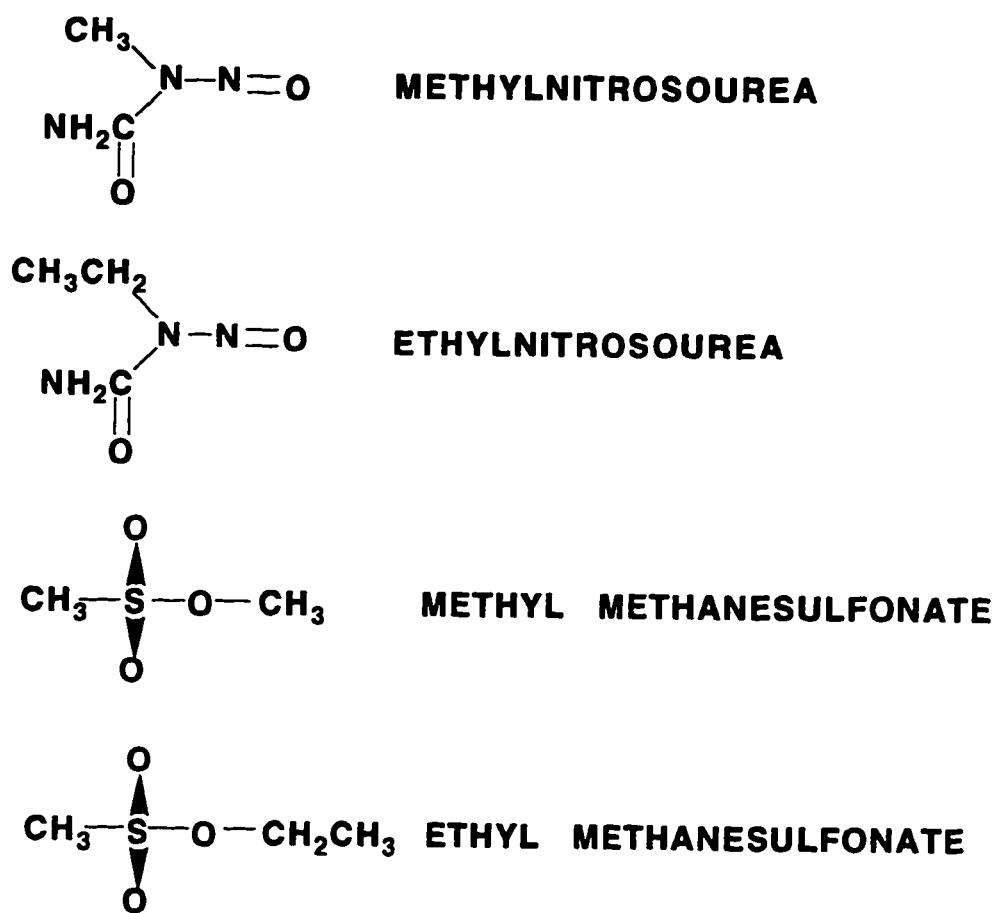


Figure 4: Structures of Alkylating Agents Used in This Study

Cell culture systems are valuable for studying mechanisms of toxicity. One cell culture system which is currently used for research in developmental toxicology is the micromass cell culture system developed by Oliver Flint (1986). The first specific aim of this research was to establish concentration/response curves for the four alkylating agents in the micromass rat embryo primary cell culture system. Results from the micromass system were compared with results from the rat whole embryo culture system. The results from this part of the study are reviewed in Chapter 2.

We also wanted to establish the use of a continuous differentiating cell line for assessing effects of developmental toxicants on viability and differentiating, and also for studying mechanisms of developmental toxicity. One advantage of using cell lines is that gene expression can be regulated using transfections, to determine how alterations in specific genes can affect development. Additionally, use of cell lines for studying mechanisms of developmental toxicity reduces the use of laboratory animals and also reduces overall costs. The cell line which we decided to use is the P19 embryonal carcinoma cell line, which can be induced to differentiate into neuronal or mesodermal-type cells. As such the second specific aim of this study was to establish neuronally differentiating P19 embryonal carcinoma cells as a system for studying mechanisms of developmental toxicity. The first sub-aim for specific aim number 2 was to find a good marker of neuronal differentiation. The second sub-aim was to compare effects of the four alkylating agents in the P19 cells with those observed previously in the micromass cell culture system. Chapter three reviews the results for the second specific aim.

Once we had established the P19 cells for studying mechanisms of developmental toxicity, we wanted to use these cells to study the relevance of O<sup>6</sup>-guanine adducts in the developmental toxicity of alkylating agents. Chapter four presents results from specific aim three, which was to modulate toxicity of the alkylating agents using O<sup>6</sup>-Bg.

The fourth specific aim was to quantify N7 and O<sup>6</sup> guanine adducts at specific effects levels for each of the alkylating agents. Unfortunately, we were not able to detect any adducts, possibly due to limitations in the sensitivity of our assay.

In addition to studying the relevance of O<sup>6</sup>-alkylguanine adducts for developmental toxicity, we also wanted to investigate mechanisms of cell death occurring in neuronally differentiating P19 cells treated with alkylating agents, as specific aim five. In particular, we wanted to determine if treatment with alkylating agents induces an apoptotic or necrotic cell death.

## **CHAPTER 2: TOXICITY OF ALKYLATING AGENTS IN RAT EMBRYO PRIMARY CELL CULTURES**

### **2.1 Introduction**

For the first part of this study the potency of four alkylating agents was tested in the micromass primary cell culture system, and compared with effects seen previously in the post-implantation whole embryo culture system. Our aim was to ascertain if primary cell cultures, such as the micromass embryo cell system, would be useful for investigating the developmental toxicity of a series of alkylating agents. In particular, we were interested in comparing the effects of these agents in the micromass system, with earlier observations in the more complex whole embryo culture system. As mentioned in chapter one, the four alkylating agents chosen for evaluation were: two methylating agents, methylnitrosourea (MNU) and methylmethanesulfonate (MMS); and two ethylating agents, ethylnitrosourea (ENU) and ethylmethanesulfonate (EMS). The nitrosoureas have more  $S_N1$  character than the nucleophilic methanesulfonates, and also have the ability to carbamylate proteins in addition to alkylating proteins and DNA. In the whole embryo culture system, potency of these agents was  $MNU > ENU > MMS > EMS$ . Thus in the whole embryo culture system alkylating agents which use an  $S_N1$  substitution mechanism and carbamylate proteins are more potent than those using an  $S_N2$  mechanism. Additionally, within a given class of alkylating agent (nitrosourea vs. methanesulfonate) methylating agents are more potent than ethylating agents. The dysmorphogenic effects observed in the whole embryo culture system were similar for all agents except that abnormal flexure was not seen in embryos exposed to MNU (Faustman et al., 1989). Notable in our alkylating agent exposed embryos was the preponderance of embryos with malformations affecting neuronal tissues.

Our aim in comparing effects between the whole embryo culture system and the micromass primary cell culture system was to provide investigators more options in choosing valid embryo culture systems for mechanistic studies. Numerous studies in the literature have shown that the whole embryo culture system is a valuable *in vitro* tool, as the developmental processes occurring *in vitro* closely mimic *in vivo* events (New et al., 1976). However, it can be difficult to discern mechanisms involved in developmental toxicity at the cellular level, due to the simultaneous development of multiple organ systems. Thus, our interest in testing these compounds in the micromass cell culture system stems from our belief that it would be advantageous to have a system in which normal differentiation processes occur, which is not as complex as the whole embryo culture system. If we observe in the micromass cell culture system that methylating agents are more potent than ethylating agents, that the nitrosoureas are more potent than the methanesulfonates, and that effects on differentiation are observed at concentrations lower than those affecting cell viability, this would indicate that the relevant mechanisms of toxicity in the micromass system may be similar to those in the whole embryo culture system. Consequently, the micromass system may be able to provide useful information regarding cellular mechanisms of developmental toxicity of alkylating agents which may be relevant to the whole animal.

## **2.2 Materials and Methods**

### **2.2.1 Cell Culture Preparation**

Micromass cell cultures of limb bud (LB) and central nervous system (CNS) tissue were prepared according to the modified procedure of Flint (1983) outlined by Whittaker and Faustman (1992b) (Figure 5). According to the procedure, midbrains and forelimbs are removed from day 12 embryos and then trypsinized to prepare single cell suspensions in culture media (Ham's F12, 10% fetal bovine serum, 50 IU/ml penicillin,

## Micromass Cell Culture System

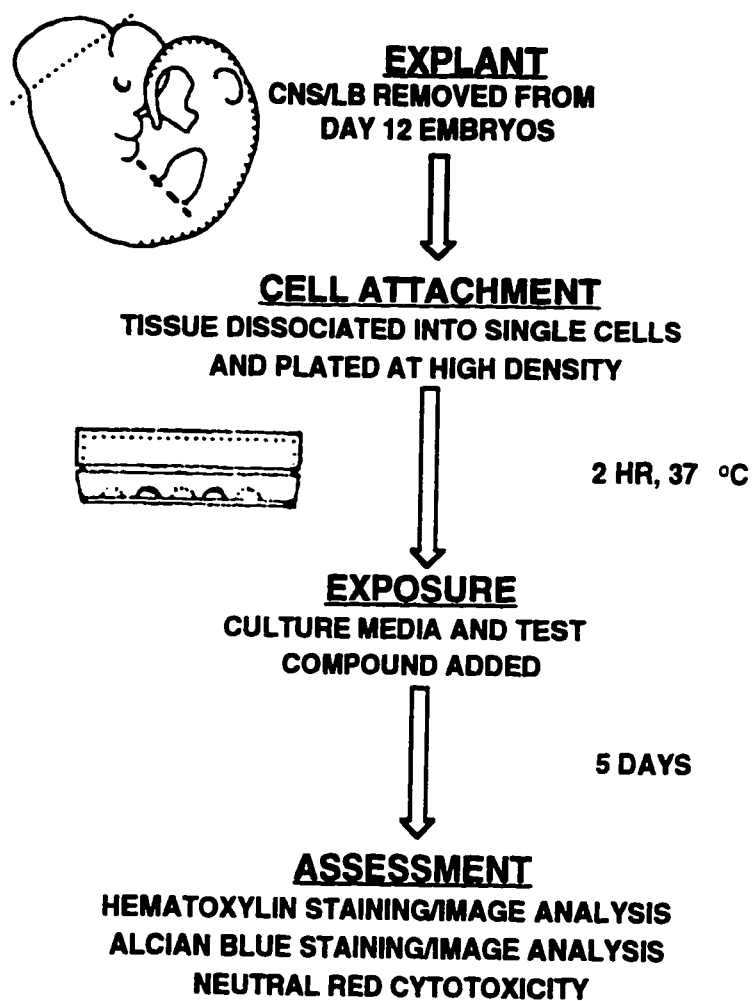


Figure 5: Micromass Cell Culture System

5  $\mu\text{g/ml}$  streptomycin, and 5.8 mg/ml L-glutamine). For assessment of differentiation, 5 or 4 ten  $\mu\text{l}$  droplets of CNS or LB cell suspensions, respectively, were plated on pre-warmed ( $37^\circ\text{C}$ ) 35-mm primaria coated plastic culture dishes. For cytotoxicity assessment, 10  $\mu\text{l}$  droplets of each cell type were plated into wells of pre-warmed ( $37^\circ\text{C}$ ) 96-well tissue culture dishes which had been collagen-coated as described by Whittaker and Faustman (1992b).

### 2.2.2 Chemical Exposure Conditions

The test chemicals were all purchased from Sigma Chemical Co. Stock solutions of test chemicals were prepared immediately prior to use, using the following buffers: MNU was dissolved in 11 mM sodium citrate, pH 6.0; ENU, in 0.1 M sodium acetate, pH 6.5; MMS in Dulbecco's phosphate buffered saline (PBS) (GIBCO); EMS in Hank's Balanced Salt Solution (GIBCO) pH 7.4.

### 2.2.3 Assessment of Differentiation

Alcian blue staining of sulfated proteoglycans was used as an indicator of differentiation in LB cultures. According to this procedure, media was removed following five days in culture and cell cultures were fixed with a 10% (v/v) formaldehyde / 0.5% (w/v) cetylpyridinium chloride solution for 20 minutes. Following a tap water rinse, cell cultures were treated with 3% acetic acid in 1N HCl for one hour, and then stained overnight with 1% (w/v) Alcian Blue 8GX (Sigma). Three percent acetic acid in 1N HCl was used to destain the cultures. Intensity of Alcian Blue staining was quantified as grains/area using an automated image analyzer (American Innovision-Videometric 150). Alcian blue specifically stains sulfate groups, which are present in the sulfated proteoglycans that are part of the mucopolysaccharide matrix produced by chondrogenic cells (Ede et al., 1977). In the micromass system, LB cultures which stain with alcian

blue consist mainly of chondrocytes (Flint and Orton, 1984). Furthermore, staining by alcian blue in LB cultures corresponds highly with uptake of  $^{35}\text{S}$  (Whittaker and Faustman, 1991).  $^{35}\text{S}$  is incorporated into sulfated proteoglycans of chondrocytes, such that its uptake can be used as an alternative measure of LB differentiation.

Haematoxylin staining was used as an indicator of neuronal differentiation of CNS cultures. After five days in culture, media was removed and cell cultures were fixed with a 10% (v/v) formaldehyde solution for 20 minutes. Cells were then rinsed with tap water and stained with Delafield's haematoxylin (Cabisco) for 2-3 minutes. Cultures were destained with tap water and then immersed in tap water for five minutes to one hour for color development. As with LB cultures, intensity of hematoxylin staining was quantified on the image analyzer. Haematoxylin, which is an acidic stain (Stevens and Williams, 1990), darkly stains neuronal nuclei. Intensity of haematoxylin staining in CNS cultures is greatest in areas of the cultures where there are large numbers of neuronal cells (Flint, 1983). Additionally,  $^3\text{H}$ -gamma amino butyric acid (GABA) uptake, which is also used as an indicator of neuronal differentiation, is correlated very closely with intensity of haematoxylin staining in cultures of CNS cells (Whittaker and Faustman, 1991).

#### 2.2.4 Viability Assessment

Viability was assessed according to the neutral red vital dye assay described by Whittaker and Faustman (1992b). Neutral red accumulates in lysosomes of viable cells (reviewed in Whittaker and Faustman, 1992a). After five days in culture, media was removed from cells cultured in 96-well collagen-coated plates and cultures were fixed in 4.5% (v/v) glutaraldehyde for 20 minutes. Fixative was removed, cells were washed once with Dulbecco's PBS, and 200  $\mu\text{l}$  of freshly prepared neutral red/PBS (0.05% w/v) (Fluka Chemical) was added to each culture well. Following a one hour neutral red

exposure, cells were rinsed with PBS twice, and neutral red was eluted for one hour with 200  $\mu$ l of a 0.5% (v/v) acetic acid in ethanol solution. Absorption was measured at 540 nm on a kinetic microplate reader (Molecular Devices Corp.). The neutral red assay compares favorably with the MTT assay, which measures mitochondrial reduction of tetrazolium-MTT salt to MTT-formazan. Effects on viability of LB and CNS cells were identical, as determined by either the neutral red assay or the MTT assay (Whittaker and Faustman, 1992a).

#### 2.2.5 Statistical Procedures

The method of Litchfield and Wilcoxon (1949) was used to calculate EC50 values, 95% confidence intervals, and statistical significance between individual EC50 values ( $p \leq 0.05$ ). Statistical differences between individual concentration-response curves were determined by analysis of variance (ANOVA), using the SYSTAT software package (SYSTAT, Inc.).

### 2.3 Results

Figures 6a-d show the micromass CNS and LB concentration-response curves for cytotoxicity and inhibition of differentiation for MMS, MNU, ENU and EMS. Table 2 lists the effective concentrations causing a 50% decrease in either viability or differentiation (EC50's) for these cultures. Viability and differentiation exhibited significant concentration-dependent decreases in both CNS and LB cell cultures following exposure to all four alkylating agents. LB viability was the least sensitive endpoint and the concentration-response curves for viability were significantly less steep than curves for the other cell culture endpoints for all of the four test chemicals.

Several other consistent patterns of responses were observed. For example, the EC50's for viability were always greater than those for differentiation, with the exception

of CNS response to MMS. The EC50 values for viability ranged from about 1.5 fold greater than EC50 values for differentiation in MMS exposed CNS cells, to about 2.5 fold greater in MNU exposed LB cells. LB cell cultures were also consistently less sensitive to chemical treatment than CNS cell cultures, for both viability and differentiation, as evidenced by EC50 values which were approximately 1.5 - 3 fold greater in LB as compared with CNS cells.

EC50 values for MMS viability were 140  $\mu\text{M}$  (107-183  $\mu\text{M}$ ) in LB and 64  $\mu\text{M}$  (29-139  $\mu\text{M}$ ) in CNS. These values were not significantly greater than the 50% response levels for differentiation, which were 75  $\mu\text{M}$  (51-110  $\mu\text{M}$ ) in LB and 60  $\mu\text{M}$  (38-95  $\mu\text{M}$ ) in CNS. However, CNS cells exposed to this agent were significantly more sensitive than LB cells with respect to both viability and differentiation. There were also differences in the concentration required to cause total inhibition of differentiation. Virtually complete inhibition of CNS differentiation was observed at an MMS concentration of 200  $\mu\text{M}$ . CNS lethality and inhibition of LB differentiation approached 95% at 250  $\mu\text{M}$ . However, concentrations greater than 500  $\mu\text{M}$  were required to cause similar lethality of LB cells.

In contrast with MMS, EC50's for MNU viability were significantly greater than those for differentiation. EC50 values for viability were 700  $\mu\text{M}$  (399-1228  $\mu\text{M}$ ) in LB and 300  $\mu\text{M}$  (155-582  $\mu\text{M}$ ) in CNS, whereas EC50's for inhibition of differentiation were 260  $\mu\text{M}$  (207-327  $\mu\text{M}$ ) in LB and 93  $\mu\text{M}$  (47-183  $\mu\text{M}$ ) in CNS. The EC50 for LB differentiation was also significantly greater than that for the CNS cultures. Although the EC50 for LB viability was greater than that observed in the CNS, the difference was not significant. For both CNS and LB cells, differentiation was almost completely inhibited by 500  $\mu\text{M}$ , whereas percentage of viable cells at this dose was approximately 40 and 70%, respectively, for CNS and LB. Comparable effects on viability were not observed

until MNU concentrations were 1764  $\mu\text{M}$ , where 97% lethality was observed for LB and 90% lethality was observed for CNS.

EC50's for ENU viability were 1000  $\mu\text{M}$  (605-1652  $\mu\text{M}$ ) in LB and 455  $\mu\text{M}$  (288-719  $\mu\text{M}$ ) in CNS, which were greater than those for differentiation, in which case EC50's were 580  $\mu\text{M}$  (465-723  $\mu\text{M}$ ) in LB and 315  $\mu\text{M}$  (249-398  $\mu\text{M}$ ) in CNS. However, the difference was not significant for either cell type. A significant difference in sensitivity between the cell types was observed with respect to differentiation, where CNS cells were more sensitive than LB cells. Nevertheless, due to differences in the slopes of the dose response curves, complete inhibition of differentiation for both CNS and LB cells was observed at a comparable concentration of about 800  $\mu\text{M}$ . Concentrations greater than 880  $\mu\text{M}$  and 1750  $\mu\text{M}$  were required to completely inhibit CNS and LB cell growth, respectively.

For EMS, significant differences between EC50's were only observed for LB viability versus differentiation, and for LB versus CNS viability. EC50's for LB viability and differentiation were 3050  $\mu\text{M}$  (1803-5161  $\mu\text{M}$ ) and 1370  $\mu\text{M}$  (1089-1723  $\mu\text{M}$ ), respectively; EC50's for CNS viability and differentiation were 1100  $\mu\text{M}$  (701-1762  $\mu\text{M}$ ) and 680  $\mu\text{M}$  (369-1252  $\mu\text{M}$ ), respectively. CNS differentiation was almost completely inhibited at 1750  $\mu\text{M}$ , whereas inhibition of LB differentiation was only 93% of control at 10,000  $\mu\text{M}$ , which was the highest concentration tested. CNS cell growth was also completely inhibited at 10,000  $\mu\text{M}$ . Concentrations greater than 10,000  $\mu\text{M}$  would be required to completely inhibit LB cell growth.

Figure 7 is a graphic comparison of the effective concentrations causing a 50% decrease in viability or differentiation in micromass cells versus whole embryo culture. The specific EC50's for the analogous endpoints in the whole embryo culture system: lethality and malformations, are presented in Table 2.

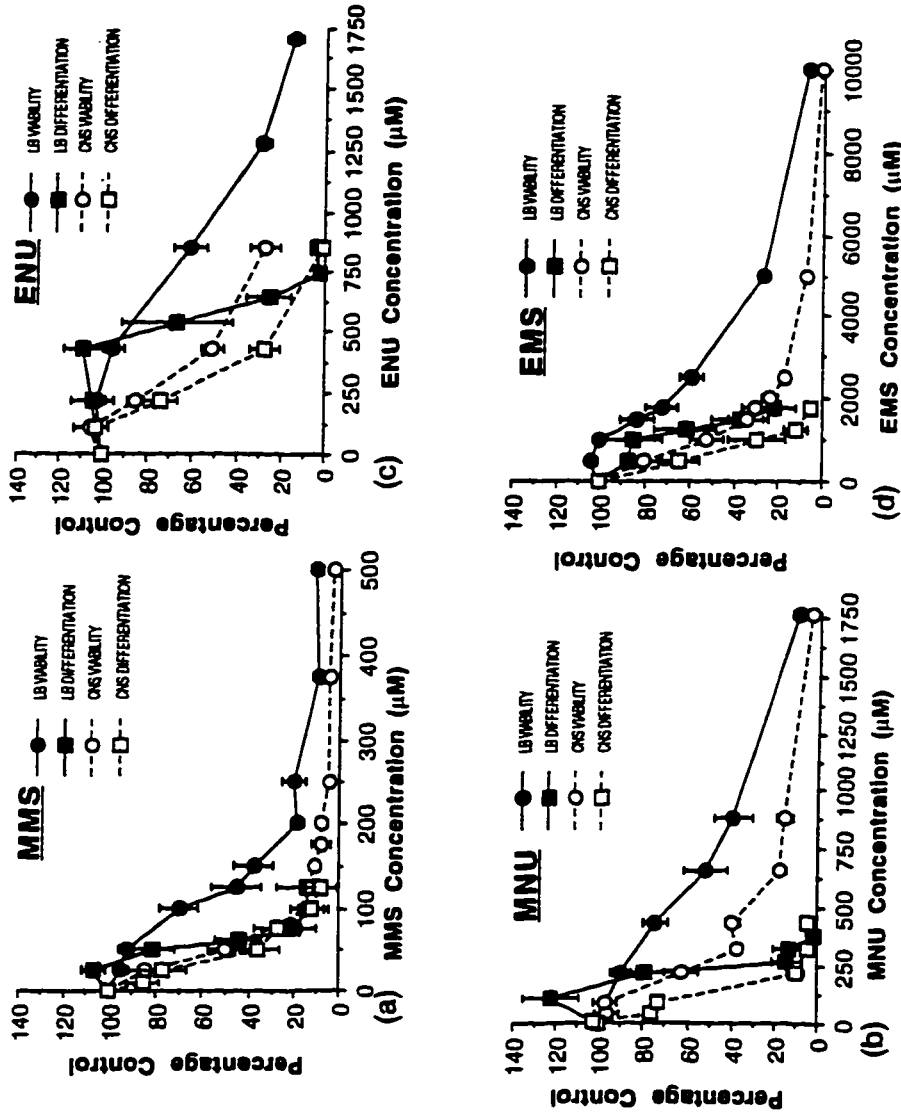


Figure 6: Concentration-Response Curves in CNS and LB Primary Cell Cultures  
 Each point represents the mean of at least three experiments which used cells from a minimum of 5 rats. Error bars are for standard error of the mean. If error bars are not visible then their range is smaller than the size of the data point. Significant differences ( $p < 0.05$ ) were observed between concentration-response curves.

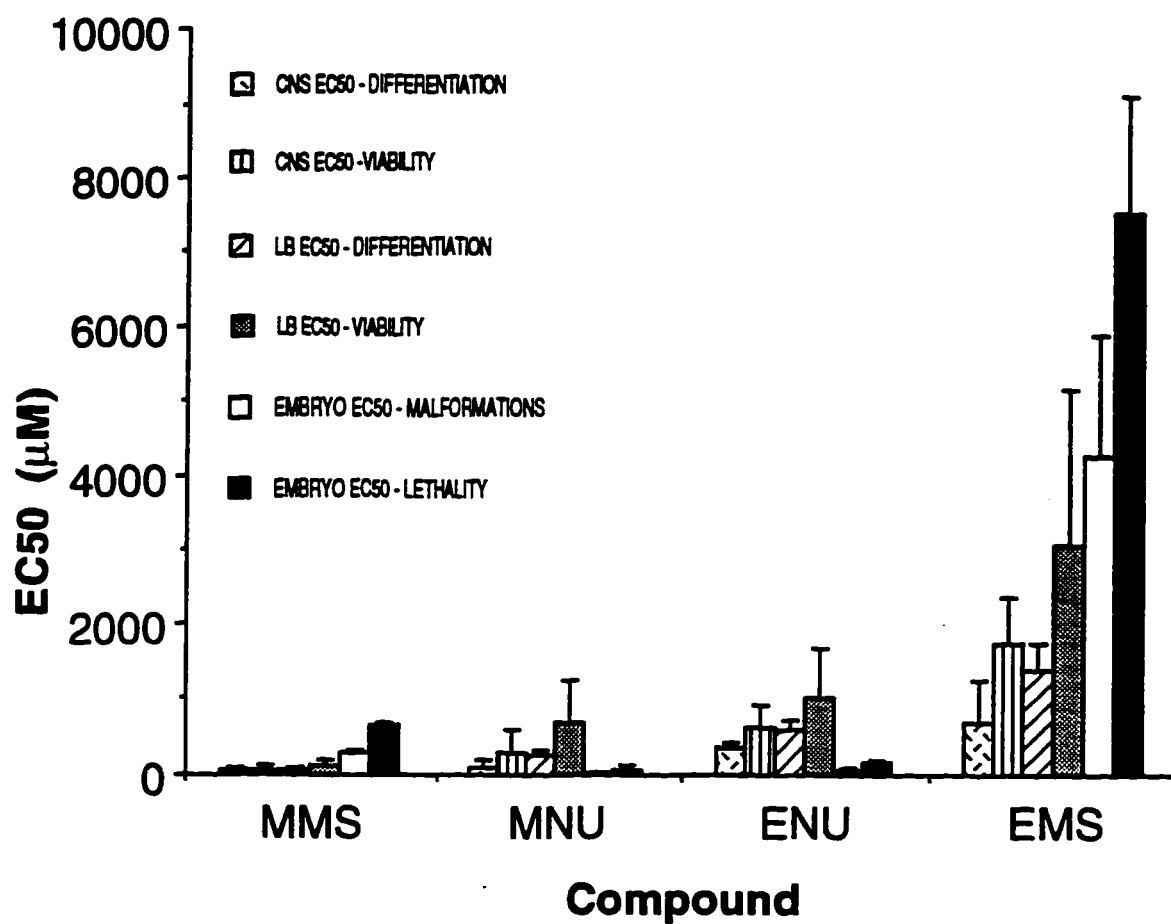


Figure 7: EC50's for CNS and LB Viability and Differentiation: Comparisons with Whole Embryo Culture  
 Error bars represent 95% confidence intervals for at least three experiments, which used cells from a minimum of 5 rats.

**Table 2**  
**EC50's for CNS and LB Viability and Differentiation:**  
**Comparisons with Whole Embryo Culture**

Chemical	LB EC50 <sup>a</sup>		CNS EC50 <sup>a</sup>		Whole Embryo <sup>b</sup>	
	Viability	Differentiation	Viability	Differentiation	Lethality	Malformations
MMS	140 <sup>c</sup> (107-183)	75 <sup>d</sup> (51-110)	64 <sup>c</sup> (29-139)	60 <sup>d</sup> (38-95)	637 (585-692)	300 (259-333)
MNU	700 <sup>e</sup> (399-1228)	260 <sup>e,g</sup> (207-327)	300 <sup>f</sup> (155-582)	93 <sup>f,g</sup> (47-183)	80 (62-116)	33 (24-40)
ENU	1000 <sup>h</sup> (605-1652)	580 <sup>i</sup> (465-723)	455 <sup>h</sup> (288-719)	315 <sup>i</sup> (249-398)	159 (143-184)	70 (56-82)
EMS	3050 <sup>j,k</sup> (1803-5161)	1370 <sup>j,l</sup> (1089-1723)	1100 <sup>k,m</sup> (701-1762)	680 <sup>l,m</sup> (369-1252)	7517 (6450-9135)	4266 (3204-5863)

Note. All concentrations are in  $\mu\text{M}$ ; values in parentheses are 95% confidence intervals.

<sup>a</sup> Data generated in present study, except ENU CNS data (Faustman and Sweeney, 1993).

<sup>b</sup> Numbers are EC50 values and are reprinted here for comparison with the micromass values with permission of authors (Faustman et al., 1989).

<sup>c-m</sup> EC50 values for the micromass cell culture system sharing the same superscript are significantly different from each other.

## 2.4 Discussion

The four alkylating agents tested elicited significant concentration-dependent responses in the micromass cell culture system over dose ranges which varied from 200  $\mu\text{M}$  for MMS to 4000  $\mu\text{M}$  for EMS. Although the four alkylating agents tested exhibited the same order of potency in both cell types, EC50's for LB cells were consistently higher than EC50's for CNS, for both cytotoxicity and inhibition of differentiation, suggesting that CNS cells are more sensitive to the effects of these alkylating agents than LB cells. The sensitivity of CNS cells in the micromass system is similar to the situation in the whole embryo culture system, where a high proportion of malformations occur in neuronal tissue. In contrast with the CNS cells, there seemed to be a population of LB cells which were resistant to the cytotoxic effects of the agents, as approximately 20% of LB cells continued to be viable at concentrations which were greater than two fold above those that caused 100% inhibition of differentiation. This resistant population of LB cells also has been observed following exposure to colchicine, nocodazole, mebendazole, and thiabendazole (Whittaker and Faustman, 1992). As in the embryo culture system, where concentrations causing a 50% increase in lethality were greater than those causing a 50% increase in malformations, EC50's for viability in the micromass culture system were significantly greater by 1.5 to 3 fold than those for differentiation, with the exception of MMS.

One difference between the two culture systems is the order of potency. In the micromass system the order of potency was MMS>MNU>ENU>EMS. The order of potency was MNU>ENU>MMS>EMS in the embryo culture system. Therefore, the observation from previous studies in the embryo culture system that the nitrosoureas (which alkylate via an SN1 substitution mechanism and have carbamylating potential) were more potent than the methane sulfonates (which have more SN2 character in their substitution mechanism and don't have carbamylating potential) was not borne out in the

micromass culture system. In the cell culture system, MMS, a methane sulfonate, was the most potent chemical; EMS, the comparable ethylating agent, was the least potent chemical. Although the relative order of potency differed between the micromass and the embryo culture systems, in both culture systems the results obtained for MNU, ENU and EMS are reasonably consistent with potencies observed in both yeast (Lee et al., 1992) and mammalian cells (Kaina et al., 1991). The variation between the whole embryo and cell culture systems in the relative potency of MMS was also observed in two different types of hamster cells, where the potency of MMS was roughly equivalent to that of MNU in Chinese Hamster Ovary cells, but closer to that of ENU in V79 hamster lung cells (Natarajan et al., 1984). The wide variation in potency observed with MMS could be related to differences in ability to alkylate proteins, since MMS has a greater tendency to alkylate proteins than the other agents tested in this study (Coles, 1985). Differences in sensitivity to MMS could also be related to the reactivity of MMS. Reactivities of alkylating agents can be compared by measuring their general alkylating activity towards a common substrate, such as 4(4-nitrobenzyl)pyridine (NBP). Using NBP as a substrate, the general alkylating activity of MMS was quantitatively about one to two orders of magnitude greater than that of the other alkylating agents tested (Bartsch et al., 1982). The potency variation observed with MMS could also be related to differences in the generation and repair of DNA adducts observed with MMS.

Another difference between the two culture systems is that whereas the nitrosoureas were more potent in the embryo culture system as compared with the cell culture system, the methane sulfonates were more potent in the cell culture system as compared with the embryo culture system. The potency reversal may be partially explained by differences in exposure conditions between the two systems. Whereas cell cultures are exposed for 5 days in media with 10% fetal calf serum (FCS), exposure of embryos is for two hours in Hank's Balanced Salt Solution (HBSS) without serum.

Differences in exposure times may be significant, since the half lives of the methane sulfonates are more than one order of magnitude greater than that of the nitrosoureas (Bartsch et al., 1982). After a two hour exposure, less than 15% of the nitrosoureas would remain as the parent compound at pH 7.4, while greater than 75% of the methane sulfonates would remain as the parent compound. If distribution of the sulfonates to embryonic tissues is not complete within a two hour exposure time, then the effective dose in the embryo culture system may be lower than the effective dose in the micromass culture system. Additionally, the presence of 10% FCS in the micromass cell culture system may quench some of the carbamylating activity of the nitrosoureas, resulting in higher effective doses (Yano et al., 1988).

One observation which was consistent in both culture systems is that methylating agents are more potent than ethylating agents for a given class of compound (i.e. nitrosoureas vs. methane sulfonates). This is also consistent with *in vivo* studies in which methylating agents are more potent than ethylating agents (NIOSH, 1987). Differences in potency between methylating and ethylating agents may be related to ability to form alkyl-DNA adducts, ability to repair the adduct, and the ultimate effect of the unrepaired adduct (Beranek, 1990). Alkylating agents can cause toxicity by overwhelming DNA repair machinery and blocking DNA replication and cell division (Manson, 1981). With respect to adduct formation, methylating agents are more reactive towards nucleic acids than ethylating agents (Singer and Kusmierik, 1982). This may account for the greater potency of methylating agents, since the shorter *in vivo* half lives of methyl adducts (reviewed in Beranek, 1990) suggests that they are repaired more rapidly than ethyl adducts. Additionally, differences in effect of unrepaired methyl versus ethyl adducts could also contribute to the differential potency of alkylating agents. For instance, the methylating agents produce a greater proportion of cytotoxic N3-adenine and N7-guanine adducts than their ethylating counterparts (reviewed in Beranek, 1990).

## 2.5 Summary

In summary, developmental toxicity of the four alkylating agents tested in the rat embryo cell culture system was similar to that observed previously in the whole embryo culture system in that methylating agents were more potent than the ethylating analogues. Additionally, EC50's for viability were consistently higher than those for differentiation. Thus it appears that the micromass system can detect effects on differentiation which appear to be distinct from those on viability. This is comparable to the whole embryo culture system, which can detect dysmorphogenesis at doses lower than those causing lethality. In addition, whereas CNS cells were more sensitive than LB cells in the micromass system, likewise neuronal tissues were highly sensitive in the whole embryo culture system. One striking difference between the two culture systems was that while MMS was the most potent agent tested in the cell culture system, it was relatively much less potent in the whole embryo culture system. Differences in relative potency of MMS as compared with other alkylating agents have also been observed in two different hamster cell lines (Natarajan et al., 1984) and may be related to the ability of MMS to alkylate proteins to a greater extent than the other agents tested, as well as its overall reactivity.

Since a prevalent aim of research in toxicology is to enable predictions of *in vivo* responses, we would ultimately like to relate effects and mechanisms observed in our culture systems to *in vivo* events. Unfortunately it is difficult to quantitatively compare results from our culture systems with *in vivo* data due to a lack of potency data from relevant species and minimal pharmacokinetic information (NIOSH, 1987). However, we have reason to believe that the qualitative responses of the whole embryos to alkylating agent exposures are reflective of *in vivo* responses. First, the development which occurs *in vitro* in the whole embryo culture system very closely parallels that

which occurs *in vivo* during the same time period, with respect to growth and morphology (New et al., 1976). Second, the dysmorphogenic effects of the alkylating agents tested in the whole embryo culture system are qualitatively similar to those observed *in vivo*, especially with respect to the sensitivity of the cephalic and neuronal tissue in rodent models. Similarities between the micromass and the whole embryo culture systems suggest the usefulness of this cell system for studying mechanisms of developmental toxicity of alkylating agents which are relevant to other *in vitro* systems and to *in vivo* development.

Although we believe the micromass cell culture system is useful for studying mechanisms of developmental toxicity, we were also interested in using a continuous cell line for studies of developmental toxicity. In chapter three we discuss the adaptation of the P19 cells as a system for evaluating mechanisms of developmental toxicity.

## **CHAPTER 3: EVALUATION OF P19 CELLS FOR STUDYING MECHANISMS OF DEVELOPMENTAL TOXICITY**

### **3.1 Introduction**

Research in toxicology includes a progression of observations, from effects of toxicants on whole animals, to studying mechanisms of toxicants at the cellular and molecular level. Understanding mechanisms by which toxicants induce their effects will improve our ability to predict effects of toxicants on human health and the environment. In the field of developmental toxicology, use of *in vitro* whole embryo culture can provide relevant toxicological information, owing in part to similarities between *in vitro* and *in vivo* development (New et al., 1976). Although animal models are valuable for studying effects of developmental toxicants, use of cell culture can facilitate studying mechanisms by which developmental toxicants elicit their effects at the cellular and sub-cellular level. For the purposes of studying developmental toxicity, optimal cell culture systems are those which undergo differentiation with patterns similar to those occurring *in vivo*. Thus, cellular morphology and expression of tissue-specific markers in cell culture systems should reflect *in vivo* morphology and gene expression. One such system that reflects *in vivo* patterns of differentiation is the micromass primary cell culture, developed by Oliver Flint (1983). In this system, cells isolated from mid-brains (CNS) and/or limb buds of rat embryos on gestation day 12 are plated at high cell density, and assessed after five days in culture, during which time differentiation occurs. The micromass system is a well-established method for investigating developmental toxicity, in which greater than 90% of chemicals tested have been properly identified with respect to teratogenicity (Flint, 1986). Another system that has been proposed as an *in vitro* screening assay for teratogens compares cytotoxicity in undifferentiated blastocyst-derived murine embryonic stem cells, with cytotoxicity in differentiated "adult"

fibroblasts from day 14 mouse embryos (Laschinski et al., 1991). Additional cell systems that could potentially be useful are pluripotent embryonic carcinoma (EC) cell lines, which can undergo differentiation into a variety of cell types, depending on culture conditions. An obvious advantage of using EC cell lines over primary cell cultures is that they reduce the number of animals required for toxicity testing. Another advantage of using cell lines is the relative ease with which they can be transfected, for studying the effects of gene expression or repression on differentiation. However, use of EC cells for studying mechanisms of developmental toxicity currently is not very widespread. This is possibly due to limited data regarding whether EC cells respond to developmental toxicants in a manner similar to that observed in more established systems such as the micromass cell culture system.

The second aim of this study was to evaluate the use of P19 embryonal carcinoma cells as a system for studying developmental toxicity, by comparing effects of the four alkylating agents on viability and differentiation, with results previously obtained in the CNS micromass system. P19 cells, originally isolated by McBurney and Rogers (1982) are a pluripotent murine cell line isolated from a teratocarcinoma induced in a C3H/He mouse. P19 cells can alternately be induced to differentiate into cells of all three germ layers, by altering culture conditions, although most studies use retinoic acid to induce neuronal differentiation. P19 cells have been used extensively in studies of proto-oncogene expression (Boulter and Wagner, 1988; Dony and Gruss, 1988; deGroot et al., 1990; Ingraham et al., 1989; Nakamura and Hart; 1989), and retinoic acid receptors (Jonk et al., 1992; Leroy et al., 1991; Pratt et al., 1990; Song and Siu, 1989). Additionally, P19 cells induced to differentiate along a neuronal pathway have been well characterized with respect to protein expression and morphology. P19 neurons express several neuronal-specific proteins commonly found in the central nervous system, and display a distinct neuronal morphology (Dinsmore and Solomon, 1991; Falconer et al.,

1989; Husman et al., 1989; Johnson et al., 1992; Maruyama et al., 1991; Staines et al., 1994). The similarities between the CNS and neuronally differentiated P19 cells suggest that this EC cell line could be useful for studying mechanisms of developmental toxicity.

## **3.2 Materials and Methods**

### **3.2.1 Cell Culture**

P19 cells were cultured as outlined in Rudnicki and McBurney (1987). This is shown in Figure 8. On culture day 0, exponentially growing P19 cells were allowed to aggregate by plating in bacteriological grade petri dishes at high cell density and concurrently treating with 0.3  $\mu$ M all trans retinoic acid (RA) (SIGMA) in Dulbecco's Minimal Essential Medium (DMEM) (GIBCO) with 2.5% fetal calf serum and 7.5% bovine calf serum. Cells were replated in fresh medium containing RA after 48 hours, on culture day 2. Following a 96 hour exposure to RA (culture day 4), the aggregates were replated on poly-d-lysine coated tissue culture dishes. Aggregates were allowed to attach for 24 hours, and then exposed to 5  $\mu$ g/ml cytosine B- $\delta$  arabinofuranoside (ara-C) on culture day 5, to inhibit cycling cells and thus enrich the cultures for neuronal cells. Assessments of viability were also made at this time. This medium was replaced after 36 hours (culture day 7), to remove dead, lytic cells. Differentiation of cultures was assessed two days later, on culture day 9, as outlined below. Treatment of cultures with dimethylsulfoxide (DMSO) was used as a negative control for neuronal differentiation, and was analogous to that of RA-treated cultures except that cultures were exposed to 0.1% DMSO, which induces mesodermal differentiation (Edwards and McBurney, 1983; Edwards et al., 1983).

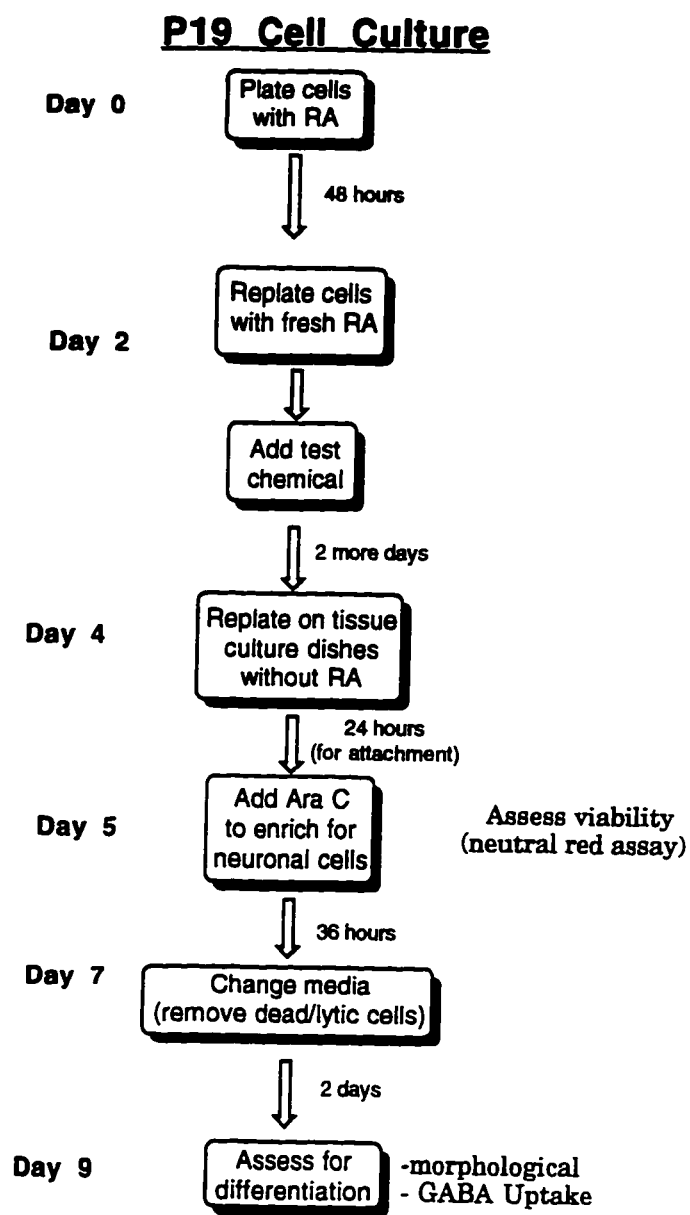


Figure 8: P19 Cell Culture System

### 3.2.2 Chemical exposure conditions

Stock solutions of test chemicals (purchased from SIGMA) were prepared immediately prior to use, using the following buffers: 11 mM sodium citrate (pH 6.0) for MNU; 0.1 M sodium acetate (pH 6.5) for ENU; Dulbecco's Phosphate Buffered Saline (DPBS) (pH 7.4) for MMS; and Hanks Balanced Salt Solution (pH 7.4) for EMS. Test chemicals were added directly to culture dishes, following replating on culture day 2.

### 3.2.3 Assessment of Viability

Viability was assessed on culture day 5 using a neutral red uptake assay. Cell cultures were incubated at 37°C for three hours with medium containing 50 µg/ml neutral red (Fluka Chemical), and subsequently fixed for two minutes with 4.5% (v/v) glutaraldehyde. Neutral red was eluted from the cells with a solution of 0.5% acetic acid (v/v) in ethanol. Absorbance was measured on a kinetic microplate reader at 540 nm (Molecular Devices Corp.).

### 3.2.4 Assessment of Differentiation

Differentiation was assessed on culture day 9 by observing morphology and by using uptake of [<sup>3</sup>H]-γ-aminobutyric acid (GABA). Cell cultures were rinsed twice with DMEM, and allowed to pre-incubate with shaking at 37°C for 15 minutes in DMEM. Cells were exposed to 0.01 µM [<sup>3</sup>H]-GABA (2 µCi) (Amersham) for 20 minutes on a shaking water bath at 37°C. After two rinses in cold DMEM, cells were harvested in calcium/magnesium-free DPBS and lysed for at least one day in an equal volume of hyamine hydroxide (ICN Biomedicals, Inc.). Counts were assessed on a liquid scintillation counter (Beckman). For inhibition of neuronal-specific uptake, 1 mM of 2,4-diaminobutyric acid (DABA) was added to the cultures during the pre-incubation period.

### 3.2.5 Statistical Procedures

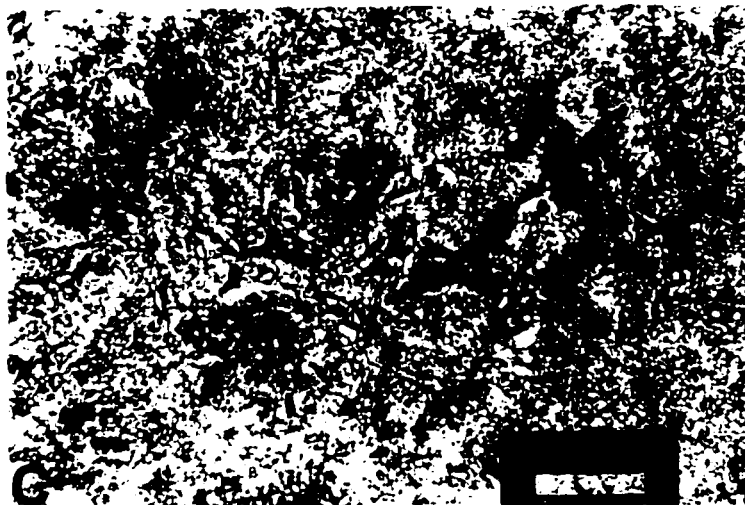
EC50 values were determined according to the method of Litchfield and Wilcoxon (1949). ANOVA's were performed using SYSTAT, to determine statistical differences between individual dose-response curves.  $P \leq 0.05$  was set as the level of statistical significance.

## 3.3 Results

### 3.3.1 Differentiation of P19 cells

P19 cells were induced to differentiate into neurons by adding 0.3  $\mu\text{M}$  RA to undifferentiated cells which were plated at a density of  $1 \times 10^5$  cells/ml on bacteriological grade petri dishes. Neuronal differentiation was assessed using both morphological and biochemical criteria. Undifferentiated cells were grown as a monolayer and appear fibroblast-like. After four days of exposure to RA in bacteriological-grade petri dishes, the cells form aggregates (Figure 9a). Although the neuronal character of these cells has already been determined at this point, morphologically they do not appear neuronal-like. The P19 cells that eventually develop following RA exposure have a distinct neuronal morphology (Figure 9b), evident as long interwoven neurite extensions which emanate from the cell aggregates. These neurite extensions are visible by day 7. This neuronal morphology differs dramatically from that of DMSO-exposed P19 cells (Figure 9c).

**Figure 9: Morphological Effects of Retinoic Acid and DMSO on P19 Cell Cultures**  
(a) RA-treated, culture day 5 (10X); (b) RA-treated, culture day 9 (20X); (c) DMSO-treated, culture day 9 (20X). Cultures were formaldehyde-fixed and stained with haematoxylin.



McBurney et al. (1988) reported that [<sup>3</sup>H]-GABA uptake was observed in RA-treated cultures, specifically in cells containing neuritic processes. Therefore, we decided to use [<sup>3</sup>H]-GABA as a biochemical marker for differentiation of P19 cells. Uptake of [<sup>3</sup>H]-GABA in RA-treated P19 cells increased approximately 7 fold between the fifth and ninth day in culture. Addition on day 9 of DABA (a neuron-specific inhibitor of GABA uptake) reduced [<sup>3</sup>H]-GABA uptake by 75%. [<sup>3</sup>H]-GABA uptake in DMSO-treated cultures, which differentiate into mesodermal type cells, was only about 25% of uptake in RA-treated cultures on day 9 (Table 3). All differences were significant ( $P < 0.05$ ) except for the difference between [<sup>3</sup>H]-GABA on day 7 versus day 9.

**Table 3: Uptake of [<sup>3</sup>H]-GABA in P19 Cultures**

Condition		Uptake (cpm/mg protein) <sup>a</sup>
RA treated	- Day 5	28 (8)
	- Day 7	135 (15)
	- Day 9	204 (16)
+ DABA inhibition	- Day 9	48 (6)
DMSO treated	- Day 9	52 (4)

<sup>a</sup>Values in parentheses are standard errors of the mean of at least three replicates.

### 3.3.2 Effects of alkylating agents on growth and differentiation of P19 cells

Significant concentration-dependent responses for both growth and differentiation were observed for the four alkylating agents tested in this study ( $p \leq 0.05$ ) (Figure 10). For all agents tested, the effective concentrations for a 50% decrease in viability (EC50) were approximately two times greater than the EC50's for differentiation (Table 4), although these differences were not statistically significant.

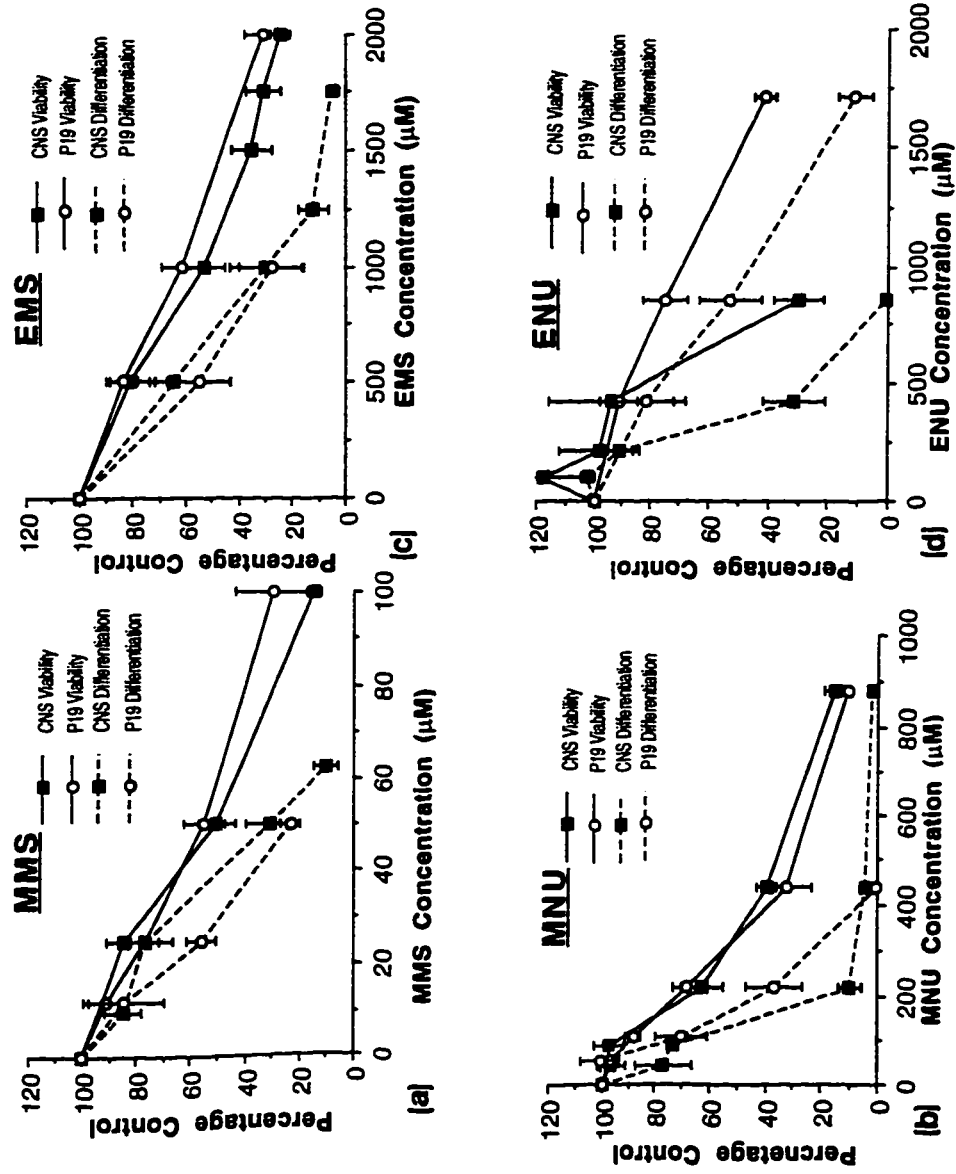


Figure 10: Concentration-Response Curves in P19 Cell Cultures: Comparison with Micromass CNS cells. Each point represents the mean of at least three experiments, with at least two dishes/assay at each dose. Error bars are for standard error of the mean.

With respect to order of potency, methylating agents were substantially more potent than ethylating agents. MMS was the most potent agent tested, with an EC50 for viability of 54  $\mu\text{M}$  (24-123  $\mu\text{M}$ ) and an EC50 for differentiation of 28  $\mu\text{M}$  (14-54  $\mu\text{M}$ ). EC50's for MNU viability and differentiation were 300  $\mu\text{M}$  (135-666  $\mu\text{M}$ ) and 160  $\mu\text{M}$  (81-315  $\mu\text{M}$ ) respectively. EMS and ENU were about 4 times less potent than MNU, with comparable potencies. EC50's for EMS viability and differentiation were 1250  $\mu\text{M}$  (494-3165  $\mu\text{M}$ ) and 560  $\mu\text{M}$  (245-1281  $\mu\text{M}$ ) respectively. EC50's for ENU viability and differentiation were 1450  $\mu\text{M}$  (704-2985  $\mu\text{M}$ ) and 850  $\mu\text{M}$  (403-1799  $\mu\text{M}$ ) respectively. For the sake of comparison, we have also included the concentration-response curves for the CNS cells on Figure 10. EC50's for micromass CNS (Seeley and Faustman, 1995) are also given in Table 4, to enable comparisons with the P19 cells. The EC50 comparisons are presented graphically in Figure 11.

**Table 4: Effects of Alkylating Agents on P19 Cells: Comparisons with Micromass CNS cells**

Chemical	P19 EC50		CNS EC50	
	Viability	Differentiation	Viability	Differentiation
MMS	54 (24-123)	28 (14-54)	64 (29-139)	60 (38-95)
MNU	300 (135-666)	160 (81-315)	300 <sup>a</sup> (155-582)	93 <sup>a</sup> (47-183)
EMS	1250 (494-3165)	560 (245-1281)	1100 <sup>b</sup> (701-1762)	680 <sup>b</sup> (369-1252)
ENU	1450 (704-2985)	850 (402-1799)	455 (288-719)	315 (249-398)

Note. All concentrations are in  $\mu\text{M}$ ; values in parentheses are 95% confidence intervals. <sup>a,b</sup> EC50 values for the micromass cell culture system sharing the same letter are significantly different from each other. There were no significant differences between viability and differentiation EC50's in the P19 cells

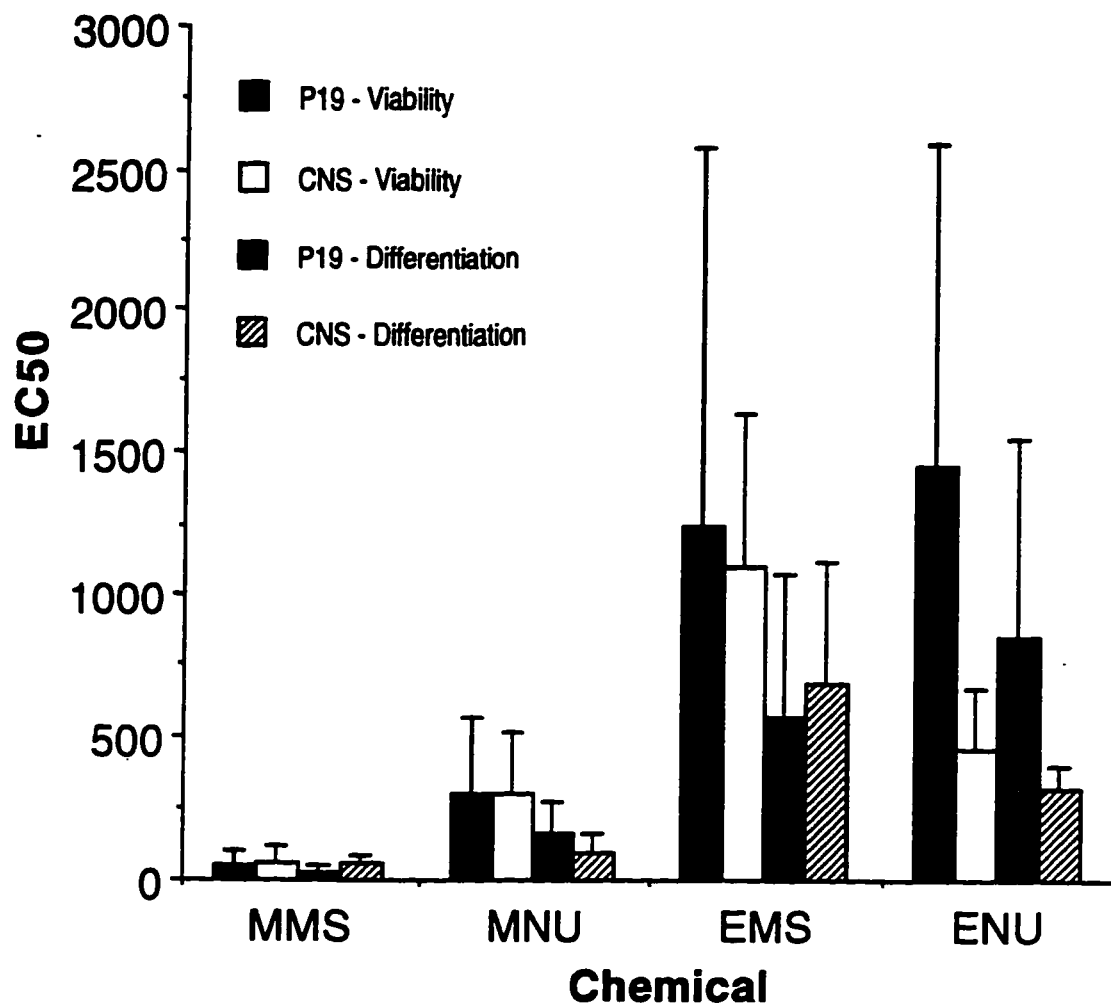


Figure 11: EC50's for P19 Cell Viability and Differentiation: Comparison with Micromass CNS Cells  
Error bars are for the 95% confidence interval of the mean.

### 3.4 Discussion

P19 cells are pluripotent embryonal carcinoma cells that can be induced to differentiate into derivatives of all three germ layers by altering physical and chemical culture conditions (Rudnicki and McBurney, 1987). In these studies we used 0.3  $\mu\text{M}$  RA to induce differentiation into a neuronal phenotype. The neuronal cells that develop from RA-exposed P19 cells share many physical and biochemical characteristics with cells of the central nervous system, including a distinct neuronal morphology (cell bodies with long neurite extensions, shown in Figure 9) expression of numerous neuronal specific markers such as neuron specific enolase, MAP2, and NF-160, and enzymes associated with both GABAergic and cholinergic neurons (Dinsmore and Solomon, 1991; Jones-Villeneuve et al., 1983; McBurney et al., 1988). P19 cells also express MASH1 and MASH2 neural determination genes, which are homologues of the *Drosophila achaete-scute* genes (Johnson et al., 1992).

Many studies have used the P19 cells to characterize events associated with neuronal differentiation, such as expression of retinoic acid receptors and proto-oncogenes. However, use of these cells is not widespread in the field of toxicology. One of the goals of our study was to adapt the P19 cells to use as a system for studying mechanisms of developmental toxicity. Previous studies in our lab used the micromass rat embryo primary cell culture system, developed by Flint (1986) in which cells undergo *in vitro* differentiation with parallels to *in vivo* differentiation. One attribute of the micromass system is its ability to distinguish between effects on viability and effects on differentiation. In the micromass CNS cells, viability is assessed with a neutral red uptake assay and differentiation is assessed either with [ $^3\text{H}$ ]-GABA uptake or with haematoxylin staining.

In adapting the P19 cells we wanted to be able to quantify both viability and differentiation. We chose to make our viability assessments on day five, using the neutral red uptake assay. This timing ensures that we are quantifying viability on both differentiated, non-dividing cells and undifferentiated or partially differentiated cells which are still dividing, since it precedes the addition of ara-c which is toxic to dividing cells. In order to assess differentiation, we tried to find a marker which would be both easy to quantify, and indicative of proper neuronal differentiation and function. A previous study by McBurney et al. (1988) indicated that [<sup>3</sup>H]-GABA uptake was observed in RA-treated cultures, specifically in cells containing neuritic processes. A more recent comprehensive study of morphology and neurotransmitter profile of RA-exposed P19 cell aggregates found that the majority of P19 neurons are GABAergic, with approximately 60% of these cells displaying high affinity uptake for GABA. These P19 neurons also expressed glutamic acid decarboxylase and GABA-transaminase, which are involved in the synthesis and breakdown of GABA, respectively (Staines et al., 1994). GABA is present at a relatively high concentration in the brain ( $10^{-3}$ M) and GABAergic neurons are ubiquitous in all parts of the CNS, comprising anywhere from 20 to 50% of the total neuronal population in most structures (reviewed in Thomas, 1986; Krnjevic, 1974).

Based on the prevalence of GABA uptake in the P19 cells, and the use of GABA uptake as a marker of neuronal differentiation in the micromass CNS cells, we decided to test the specificity of neuronal [<sup>3</sup>H]-GABA uptake in RA-exposed P19 cell aggregates. As indicated in Table 3, significant time-dependent increases in [<sup>3</sup>H]-GABA uptake were observed in RA-exposed P19 aggregates, and these increases correlated well with changes in cell morphology (Figure 9). [<sup>3</sup>H]-GABA uptake has also been shown to increase over time with differentiation in cultures of neonatal rat cortical neurons (Balcar et al., 1989). Furthermore, 75% of this uptake was inhibitable by DABA, which

specifically blocks high affinity neuronal uptake of GABA (Balcar et al., 1979; Iversen and Kelly, 1975). The remaining [<sup>3</sup>H]-GABA uptake which we observed could represent low affinity GABA uptake, which also occurs in neuronal cells (Lasher, 1975), as well as uptake by glial cells, which are present in P19 cultures and which are not inhibitable by DABA. As a negative control, we measured [<sup>3</sup>H]-GABA uptake in P19 cells exposed to 0.1% DMSO instead of RA, which induces mesodermal differentiation. In DMSO-exposed cells, [<sup>3</sup>H]-GABA uptake was approximately 25% of uptake in RA-exposed P19 cells. Based on the time-dependent increase in [<sup>3</sup>H]-GABA uptake in RA-exposed cells, the inhibition of uptake by the neuronal-specific DABA, and the reduced uptake in DMSO-exposed cells, we believe [<sup>3</sup>H]-GABA uptake is a good marker of neuronal differentiation on the P19 cells. For our subsequent studies, uptake was measured on day 9, when maximal [<sup>3</sup>H]-GABA uptake of  $204 \pm 16$  cpm/ $\mu$ g protein was observed. In comparison, average [<sup>3</sup>H]-GABA in CNS cells on day 5 was  $78 \pm 31$  cpm/ $\mu$ g protein.

Once we had a system analogous to the micromass cell culture system in which effects on both viability and differentiation could be determined, we wanted to test the responsiveness of the P19 cells to developmental toxicants by comparing effects in the P19 cells with effects observed previously in the micromass CNS cells. For this comparison we used a series of four alkylating agents, MNU, ENU, MMS, and EMS. Since the responsiveness of cells to toxic insults could depend on the state of differentiation, we wanted the treatment time in the P19 cells to parallel that of the CNS cells with respect to differentiation. Therefore, we decided to treat the P19 cells on day three, at an early stage of differentiation, when neural determination may have already been partially determined, but before the cells had acquired visible characteristics of neuronal cells.

As in the micromass system, significant concentration-dependent effects on growth and differentiation of P19 cells were observed, with methylating agents substantially more potent than ethylating agents, for a given class of agent (i.e. nitrosourea vs. methanesulfonate). In the P19 cells the order of potency was MMS>MNU>EMS>ENU, while in the micromass CNS cells the order of potency was MMS>MNU>ENU>EMS. Except for ENU, effects on growth and differentiation occurred at concentrations that were very comparable to those causing similar effects in micromass CNS cell cultures (Seeley and Faustman, 1995) (Table 4 and Figures 10 and 11). The effects of MMS, MNU, and EMS were similar in both culture systems with respect to both the EC50s and the shape of the concentration-response curves. As with the micromass culture system, EC50's for viability in the P19 cultures were consistently higher than EC50's for differentiation. With the exception of ENU, the order of potency observed in the P19 cells was the same as that observed in the CNS cell cultures. It is not clear why ENU was so much less potent in the P19 cells than in the CNS micromass cells. There may be differences in repair of specific DNA adducts. For instance, ENU forms a greater percentage of adducts at the phosphotriester bond of the DNA backbone than the other chemicals tested (reviewed in Beranek, 1990), and repair of this adduct may be more efficient in P19 cells as compared to CNS cells. Alternatively, the stability of this agent in the two culture systems may have differed. Micromass CNS cells were cultured in F12 media with 10% fetal calf serum, whereas P19 cells were cultured in DMEM with 2.5% fetal calf serum and 7.5% bovine calf serum. In spite of the difference in sensitivity to ENU between the two systems, we felt the overwhelming similarities provide strong justification for using P19 cells in future mechanistic studies.

### 3.5 Summary

In this chapter we have demonstrated that we now have a system in an established cell line that parallels the micromass primary cell culture system in that it can be used to assess effects of developmental toxicants on both viability and differentiation. As in the micromass cell culture system, neuronal differentiation occurs in the P19 cells, as evidenced by development of neuritic morphologies, and increases in [<sup>3</sup>H]-GABA uptake. With the exception of ENU, responses of P19 cells to the four alkylating agents tested in this study were very comparable to those observed in micromass CNS cells. Not only the EC<sub>50</sub>'s, but also the shape of the concentration-response curves, were similar for both viability and differentiation, for MMS, MNU, and EMS. This suggests that effects on growth and differentiation in the two culture systems may be due to similar mechanisms.

It is not clear why ENU was so much less potent in the P19 cells as than in the micromass CNS cells. There may be differences in activities of specific DNA repair proteins. Chapter Three discusses differences between the micromass CNS cells and the P19 cells regarding their response to inhibition of the AT protein. Whereas AT inhibition had no discernible effect on ENU toxicity in the CNS cells (Kidney and Faustman, 1995) AT inhibition did have a very slight, although non-significant effect on ENU toxicity in neuronally differentiated P19 cells. This suggests that the AT protein in P19 cells may be able to repair a small percentage of O<sup>6</sup>-ethylguanine adducts, thereby reducing ENU toxicity relative to cells not capable to repairing O<sup>6</sup>-ethylguanine adducts with the AT protein. Differences in the ability of O<sup>6</sup>-benzylguanine (O<sup>6</sup>-Bg) to inhibit the activity of recombinant AT protein, with mouse AT requiring 4-5 fold higher O<sup>6</sup>-Bg concentrations than rat (Elder et al., 1994) suggest that there could also be differences in their ability to repair O<sup>6</sup>-ethylguanine adducts. Similar differences may exist in activities involved in repair of other ethyl-DNA adducts. For instance, ENU forms a much greater percentage

of adducts at the phosphotriester bond of the DNA backbone than the other alkylating agents tested (reviewed in Beranek, 1990), and repair of this adduct may be more efficient in the P19 cells as compared to micromass CNS cells.

In the next chapter we discuss the use of the P19 cells to investigate the role of O<sup>6</sup>-alkylguanine adducts in the developmental toxicity of alkylating agents.

## **CHAPTER 4: MODULATION OF ALKYLATING AGENT TOXICITY IN P19 CELLS USING O<sup>6</sup>-BENZYLGUANINE**

### **4.1 Introduction**

Although the developmental toxicity of alkylating agents is well documented, the mechanism by which these agents cause developmental toxicity is still unclear (Kimmel et al., 1993). It is generally recognized that these agents are highly electrophilic and thus able to bind to nucleophilic sites in the cell, such as nucleic acids and proteins (Coles, 1985). Redox status may affect toxicity of alkylating agents, since toxicity of melphalan correlated well with intracellular glutathione content (Mulcahy et al., 1995). Developmental and cell-type specific changes in glutathione have been observed in the mouse CNS (Beiswanger et al., 1995) which could be a factor influencing developmental toxicity of alkylating agents. However, a specific target for developmental toxicity has not yet been identified. Whereas a strong correlation has been established between alkylation at the O<sup>6</sup> position of guanine and carcinogenicity (Domoradzki et al. 1984; Dumenco et al., 1993; Dunn et al., 1991; Lukash et al., 1991; Maher et al., 1990), studies by Bochert et al. (1991) are the only ones which show a correlation between adduct levels at the O<sup>6</sup> position of guanine and teratogenic potency. Studies recently completed in our lab (Kidney and Faustman, 1995) support the idea that adducts at the O<sup>6</sup> position of guanine may have particular relevance for developmental toxicity of some alkylating agents. That study used O<sup>6</sup>-benzylguanine (O<sup>6</sup>-Bg), a potent and specific inhibitor of the O<sup>6</sup>-alkylguanine alkyltransferase (AT) protein which repairs adducts at the O<sup>6</sup> position of guanine. They found that O<sup>6</sup>-Bg greatly potentiated the ability of MNU to inhibit differentiation of micromass cells. However, O<sup>6</sup>-Bg had no effect on cytotoxicity of MNU. Additionally, in their study O<sup>6</sup>-Bg had only a very slight effect on differentiation, and no effect on viability of ENU. The third aim of this thesis research

was to further investigate the role of O<sup>6</sup>-alkylguanine adducts in the developmental toxicity of four alkylating agents in the P19 cells.

## **4.2 Materials and Methods**

### **4.2.1 Cell Culture**

P19 cells were cultured as indicated in chapter three, according to the method of Rudnicki and McBurney (1987).

### **4.2.2 Chemical exposure conditions**

Stock solutions of test chemicals were prepared as indicated in chapter three. For the experiments with O<sup>6</sup>-Bg, cell cultures were pretreated with O<sup>6</sup>-Bg dissolved in DMSO for 24 hours prior to treatment with the alkylating agents. Fresh O<sup>6</sup>-Bg was added to cultures at the time of treatment (Figure 12). An equal volume of DMSO was added to parallel cultures at each dose of alkylating agent tested. The final concentration of DMSO in the cultures was 0.2%, and this concentration did not affect either morphology or GABA uptake (unpublished observation and data). Edwards et al. (1983) have also shown that treatment of P19 cells with 0.5% DMSO does not affect RA-induced neuronal differentiation. The O<sup>6</sup>-Bg used in this study was the generous gift of Dr. Moschel at the National Cancer Institute.

### **4.2.3 Assessment of Viability and Differentiation**

Viability was assessed on culture day 5 using a neutral red uptake assay, as outlined in chapter three. Differentiation was assessed on culture day 9 by observing morphology and by using uptake of [<sup>3</sup>H]-GABA. This is described in chapter three.

### P19 Cell Culture - with O<sup>6</sup>-Bg treatment

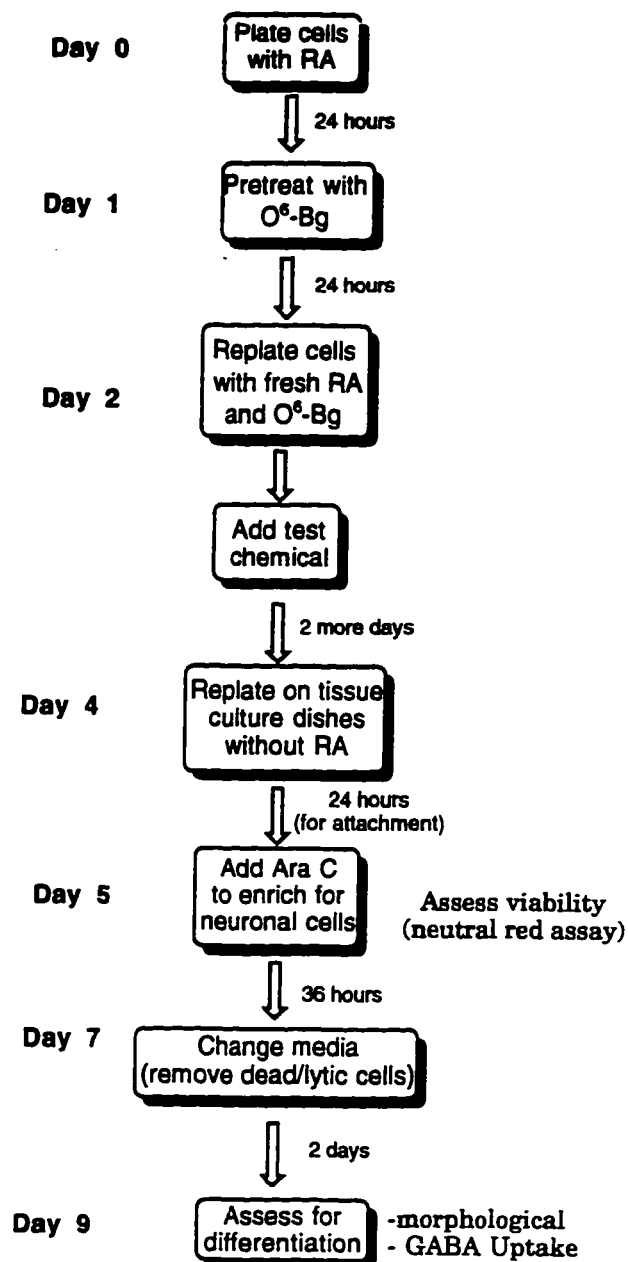


Figure 12: P19 Cell Culture with O<sup>6</sup>-Benzylguanine

#### 4.2.4 AT Assay

AT activity was determined according to the method described of Mymes et al. (1984). Untreated cells were harvested in DPBS on culture day 2 following both the 24 hour pre-treatment with O<sup>6</sup>-Bg and addition of fresh O<sup>6</sup>-Bg, and snap frozen in liquid nitrogen. Cell extracts were prepared by alternately sonicating and centrifuging cells at 10,000 x g, and finally ultra-centrifuging cells at 100,000 x g. Activity of AT was determined by allowing extracts to react with a calf thymus DNA template treated with [<sup>3</sup>H]-MNU. Counts were made on the acid-insoluble fraction of the reaction mixture, which contains the alkyltransferase from the cell extracts and any [<sup>3</sup>H]-methyl groups transferred from the DNA template to the alkyltransferase. Either the molarity or number of molecules is determined from the specific activity of the [<sup>3</sup>H]-MNU, and normalized to either protein content or cell number, respectively.

#### 4.2.5 Statistical Procedures

Student's T-test was used to determine statistically significant effects of O<sup>6</sup>-Bg at each dose tested. The level of significance was indicated as either  $P \leq 0.05$  or  $P \leq 0.001$ .

### 4.3 Results

#### 4.3.1 Effects of O<sup>6</sup>-benzylguanine on growth, differentiation and AT activity of P19 cells

O<sup>6</sup>-benzylguanine was used in this study to inhibit the O<sup>6</sup>-alkylguanine DNA-alkyltransferase (AT) repair protein. O<sup>6</sup>-Bg is a very potent and specific inhibitor of AT, at concentrations which don't cause any overt toxicity. As illustrated in Figure 13, concentrations of 5 and 10  $\mu$ M of O<sup>6</sup>-Bg did not have any readily apparent effects on

either growth or differentiation of P19 cells. However, slight effects on growth and differentiation were observed at O<sup>6</sup>-Bg concentrations of 20  $\mu$ M and higher. Therefore we chose an O<sup>6</sup>-Bg concentration of 10  $\mu$ M for further studies with the alkylating agents. Figure 14 shows cultures treated with either DMSO (as a control) or 10  $\mu$ M O<sup>6</sup>-Bg, and demonstrates that this concentration of O<sup>6</sup>-Bg did not have a noticeable effect on morphology of P19 cultures. However, this concentration of O<sup>6</sup>-Bg did effectively reduce AT activity (Table 5). AT activity in control P19 cultures was  $3949 \pm 634$  molecules AT/cell, vs.  $331 \pm 154$  molecules AT/cell in P19 cultures treated with 10  $\mu$ M O<sup>6</sup>-Bg. In comparison, AT activity was not detectable in the micromass CNS cells (Kidney and Faustman, 1995).

**Table 5: Effect of O<sup>6</sup>-Benzylguanine on Alkyltransferase Activity in P19 Cells**

Treatment	Alkyltransferase Activity	
	AT molecules/cell	fmol AT/mg protein
Control	3949 (634)	115 (23)
O <sup>6</sup> -Bg treated (10 $\mu$ M)	331 (154)	10 (5)

Numbers in parentheses are the standard error of the mean of three experiments.

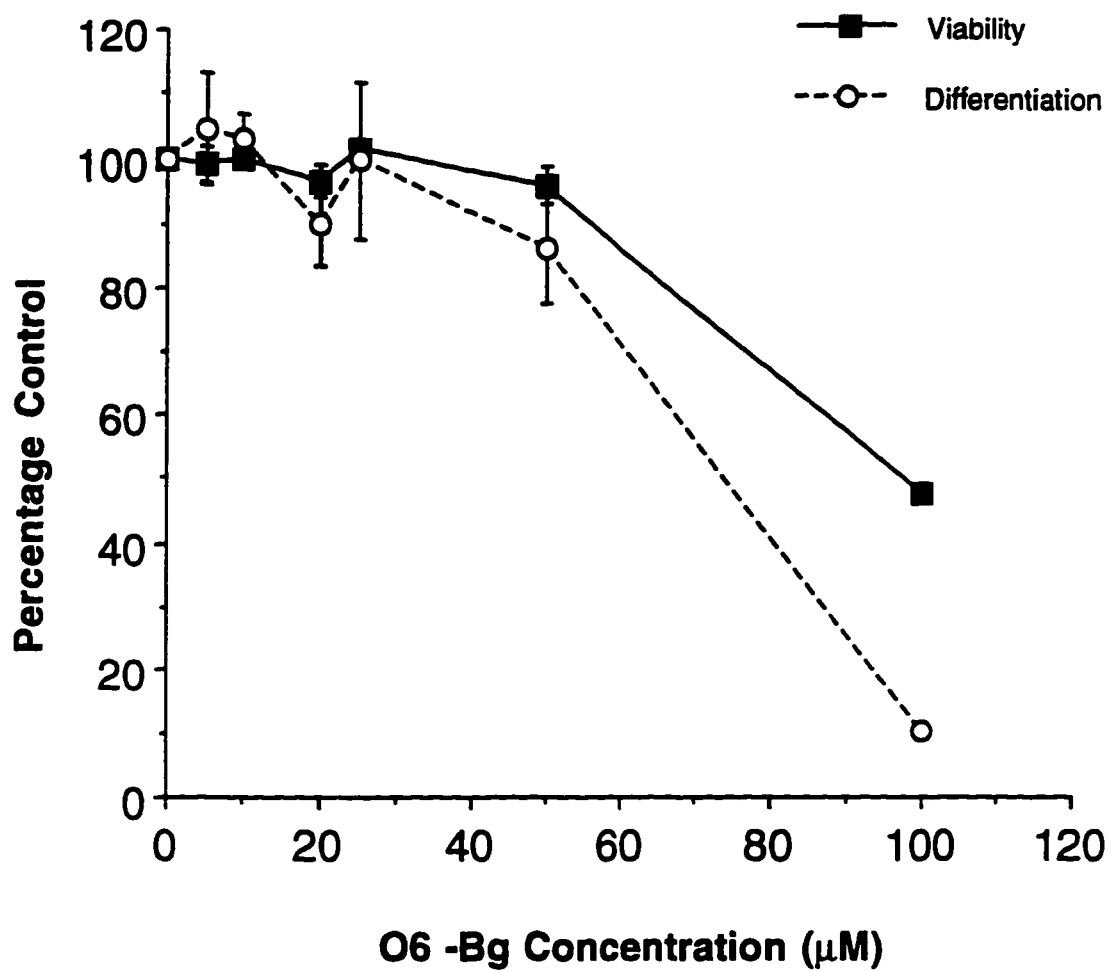
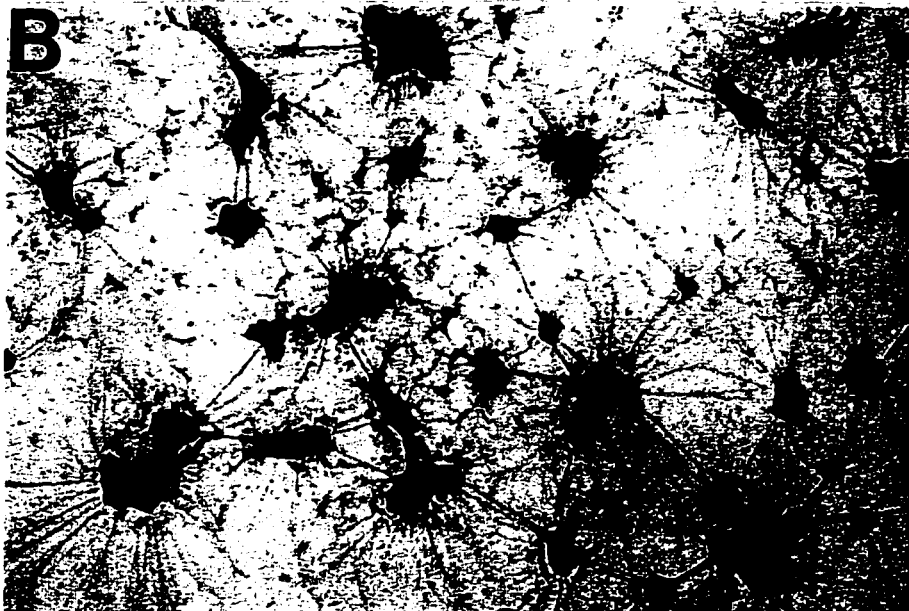
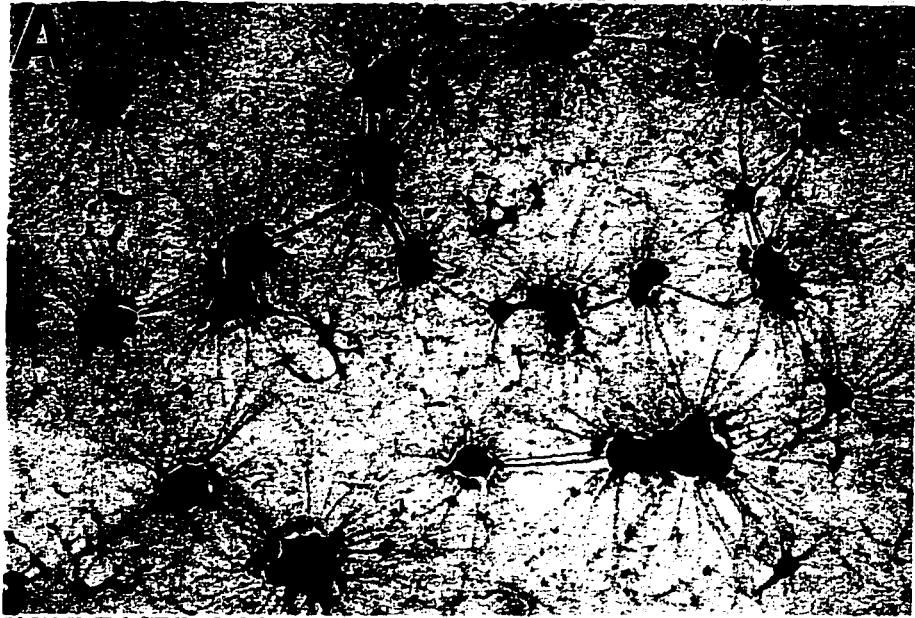


Figure 13: Concentration-Response of O<sup>6</sup>-Benzylguanine in P19 Cells  
Each point represents the mean of at least three experiments. Error bars are for standard error of the mean.

**Figure 14: Effects of 10  $\mu$ M O<sup>6</sup>-Benzylguanine on Morphology of P19 Cells**  
Cultures were formaldehyde-fixed and stained with haematoxylin, on culture day 9.

(a) DMSO treated (b) DMSO + 10mM O<sup>6</sup>-Bg.



#### 4.3.2 Effects of O<sup>6</sup>-Bg on developmental toxicity of alkylating agents

Table 6 shows how viability and inhibition of differentiation induced by MNU, MMS, ENU and EMS is modulated by O<sup>6</sup>-Bg. This is also represented graphically in Figure 15. Treatment of P19 cells with O<sup>6</sup>-Bg had the greatest effect on viability and differentiation of the two methylating agents, MNU and MMS. For both of these compounds, effects of O<sup>6</sup>-Bg were statistically significant at more than half of the doses tested. Effects of O<sup>6</sup>-Bg were much less dramatic with EMS and were not statistically significant at any of the doses tested. Effects of O<sup>6</sup>-Bg with ENU were minimal and statistically insignificant. For all four chemicals, O<sup>6</sup>-Bg had a greater effect on differentiation than on viability. Differentiation was almost completely inhibited by O<sup>6</sup>-Bg in MMS and MNU-treated cultures, at concentrations where differentiation was inhibited by 70-80% in cultures not exposed to O<sup>6</sup>-Bg (Table 6). The effect of O<sup>6</sup>-Bg on EMS-treated cultures was less dramatic, inhibiting differentiation by 90%, as opposed to 70% in cultures not exposed to O<sup>6</sup>-Bg. The effect of O<sup>6</sup>-Bg on ENU-exposed cultures was minimal. The morphology of cultures treated with and without O<sup>6</sup>-Bg is illustrated in Figure 16, and shows that the effects of O<sup>6</sup>-Bg are greater for the methylating agents than for the ethylating agents.

**Table 6: O<sup>6</sup>-Bg Modulation of Alkylating Agent Effects on Viability and Differentiation in P19 Cells**

MMS ( $\mu\text{M}$ )	Viability <sup>a</sup>			Differentiation <sup>a</sup>		
	- O <sup>6</sup> -Bg	+ O <sup>6</sup> -Bg	Relative decrease by O <sup>6</sup> -Bg	- O <sup>6</sup> -Bg	+ O <sup>6</sup> -Bg	Relative decrease by O <sup>6</sup> -Bg
12.5	91	74	19	85	33*	62
25	77	39*	50	56	9**	85
50	55	23**	59	23	1*	95
100	30	14	52	—	—	—
<b>MNU (<math>\mu\text{M}</math>)</b>						
55	97	74	23	101	50*	50
110	88	45**	49	70	6**	92
220	68	22*	67	36	1	97 <sup>b</sup>
441	32	20	38	1	—	—
<b>EMS (<math>\mu\text{M}</math>)</b>						
500	84	67	20	55	34*	38
1000	62	40*	35	28	9	66
2000	30	20*	35	0.7 <sup>b</sup>	0.5 <sup>b</sup>	29
<b>ENU (<math>\mu\text{M}</math>)</b>						
427	91	88	3	81	71	12
854	75	73	3	53	46	12
1708	41	32*	24	11	9	18

<sup>a</sup>Expressed as percentage of viability/differentiation in untreated control cells

<sup>b</sup>only one data point for these treatments due to high cell lethality.

\*Significantly different,  $P \leq 0.05$

\*\*Significantly different,  $P \leq 0.001$

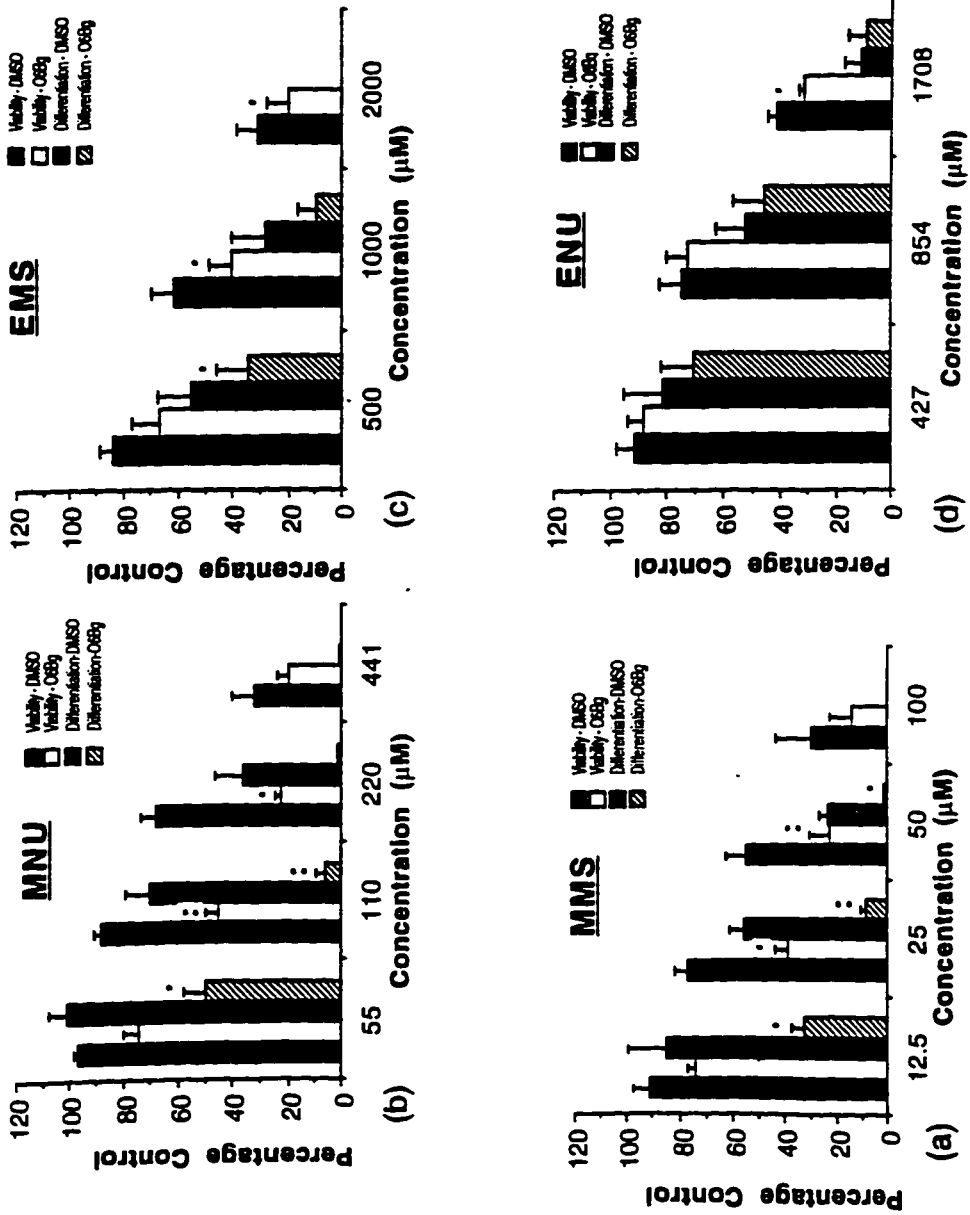
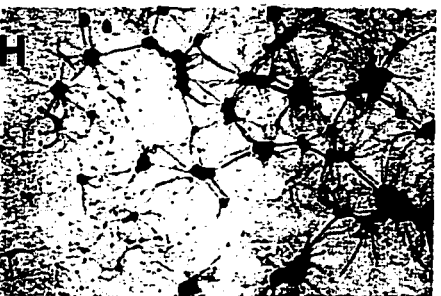
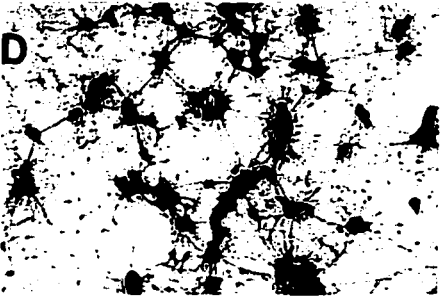
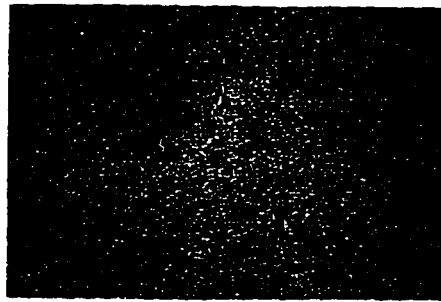
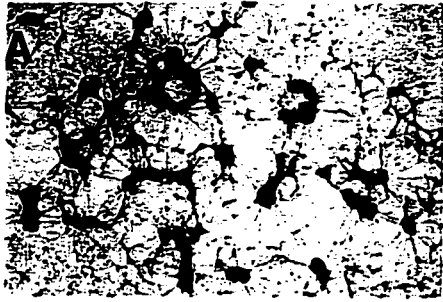


Figure 15: Potentiation of Alkylating Agent Toxicity in P19 Cells by O<sup>6</sup>-Benzylguanine  
 \* -- significantly different from control, p < 0.05; \*\* -- significantly different from DMSO-control, p < 0.001. 60

**Figure 16: Effects of O<sup>6</sup>-Benzylguanine on Morphology of P19 Cells Treated with Alkylating Agents**

(a) MNU, (b) MMS, (c) ENU, (d) EMS, (e) MNU + O<sup>6</sup>-Bg, (f) MMS + O<sup>6</sup>-Bg, (g) ENU + O<sup>6</sup>-Bg, and (h) EMS + O<sup>6</sup>-Bg. Equivalent concentrations of alkylating agents were used for control and O<sup>6</sup>-Bg treated cultures, and were: 110  $\mu$ M for MNU, 25  $\mu$ M for MMS, 854  $\mu$ M for ENU, and 500  $\mu$ M for EMS.



#### 4.4 Discussion

For the mechanistic part of this study, we looked at potentiation of alkylating agent toxicity by O<sup>6</sup>-Bg, a potent and specific inhibitor of the AT protein which repairs adducts at the O<sup>6</sup>-position of guanine. Although there is a high degree of homology of the AT protein between species, especially when comparing mammalian species (Rahden-Staron and Laval, 1991) there are tissue-, cell- and age-specific differences in AT activity (Likachev et al., 1991; Wani et al., 1992; Washington et al., 1989). The AT protein can repair DNA with a variety of alkyl, and chloro-alkyl adducts. However, repair efficiency by the AT protein generally decreases as size of the adduct increases, such that repair of ethyl adducts is slower than repair of methyl adducts (Pegg, 1990). Although O<sup>6</sup>-Bg is a relatively bulky adduct, the bulk of the adduct may be offset by the benzene ring in which charge is delocalized in the transition state, thereby facilitating transfer of the adduct to the AT repair protein resulting in highly effective inhibition of AT (discussed in Dolan et al., 1990).

In our studies with P19 cells, we found that a concentration of 10  $\mu$ M O<sup>6</sup>-Bg effectively reduced AT activity to approximately 10% of activity in untreated cultures, from 115 to 10 fmol/mg protein. This concentration was 4 times greater than the concentration (2.5  $\mu$ M) used to inhibit AT in micromass CNS cells, and may reflect higher AT activity in P19 cells as compared with CNS micromass cells (Kidney and Faustman, 1995). AT activity in P19 cells is about 2-fold higher than activity in human brain (D'Ambrosio et al., 1987) and comparable to activity in CHO cells transfected with the human AT gene (Dunn et al., 1991; Wu et al., 1991), as well as activity in human gliomas (Mineura et al., 1994).

In P19 cells, potentiation of toxicity by O<sup>6</sup>-Bg was most pronounced for MMS and MNU, reducing viability by greater than 50% and differentiation by greater than 90%, from viability and differentiation observed in cultures treated with equivalent

alkylating agent concentrations without O<sup>6</sup>-Bg. The potentiation of toxicity by O<sup>6</sup>-Bg was not as great for EMS, where the largest reductions in viability and differentiation were 35% and 66% respectively. O<sup>6</sup>-Bg was least effective for potentiation of ENU-induced toxicity, causing only a 24% reduction in viability and an 18% reduction in differentiation at the highest concentration of ENU that we tested. At the two lower ENU concentrations, we only saw a 3% reduction in viability and an 11% reduction in differentiation. In comparison with the three other alkylating agents, O<sup>6</sup>-Bg reduced viability by greater than 20%, and differentiation by greater than 35%, at equitoxic doses. In a previous study from our lab, O<sup>6</sup>-Bg significantly potentiated effects of MNU, but not ENU, to inhibit differentiation of the micromass CNS cells (Kidney and Faustman, 1995).

Results from other studies also suggest that viability of cells exposed to ENU may not be dependent on AT activity. In one study survival of CHO cells proficient in AT was similar to that in CHO cells deficient in AT, following exposure to ENU, whereas survival of AT proficient cells was much greater than that of AT-deficient cells following exposure to MNU (Bignami et al., 1989). Similarly, CHO cells transfected with human AT were more resistant than parent cell lines to MNU but not to ENU (Wu et al., 1991). Wu et al. suggest that O<sup>6</sup>-ethyl guanine adducts may not be a major contributor to ENU induced cytotoxicity. Studies by Bronstein et al., (1991, 1992) suggest that the nucleotide excision repair (NER) pathway may cooperate with the AT protein in repair of O<sup>6</sup>-ethyl guanine adducts in some cells. In these studies survival of ENU-treated human lymphoblastoid cells that lack either AT activity or the NER pathway is comparable, and is substantially lower than that of cells that have both repair activities. Furthermore, inhibition of AT activity in NER-deficient cells did not alter survival of ENU-treated cells. These NER-deficient cells may use an alternative repair pathway,

which is independent of AT activity. However, in other cell systems, the NER pathway may be sufficient for repairing O<sup>6</sup>-ethylguanine adducts. For instance in bacteria repair by the NER pathway increases as the size of the alkyl group increases (Warren and Lawley, 1980; Todd and Schendel, 1983). The negligible effect that O<sup>6</sup>-Bg had on the toxicity of ENU in both P19 and micromass CNS cells suggests that lesions other than O<sup>6</sup>-ethylguanine play a much greater role in ENU-induced toxicity in these cultures, or that O<sup>6</sup>-ethylguanine adducts are repaired by pathways which don't involve the AT protein.

In our alkylating agent studies with P19 cells we observed that O<sup>6</sup>-Bg had a greater effect on differentiation than on viability. At most concentrations tested, we saw reductions in differentiation that were approximately two times greater than reductions in viability. However, the effects which we observed on differentiation may be a consequence of effects on viability, as the relationship between differentiation and viability for a given effect on viability was similar to what was seen in the absence of O<sup>6</sup>-Bg.

In a previous study by Kidney and Faustman (1995) the magnitude of effect that O<sup>6</sup>-Bg had on differentiation of MNU-treated CNS cells was comparable to what we observed in MNU-treated P19 cells. In the P19 cells O<sup>6</sup>-Bg reduced differentiation by approximately 90% relative to cells not pre-treated with O<sup>6</sup>-Bg, while in the CNS cells O<sup>6</sup>-Bg reduced the EC<sub>50</sub> for differentiation by approximately 83%, relative to cells not pre-treated with O<sup>6</sup>-Bg. However, unlike the P19 cells, inhibition of AT did not affect viability of the CNS cells. Thus it appears that the CNS cells were somehow resistant to the cytotoxic effects of AT inhibition. One factor which could contribute to this resistance is that viability in the micromass CNS cells is determined on cells grown on collagen coated 96-well tissue culture plates, whereas differentiation is determined on cells grown on Primaria coated dishes. Accordingly, the growth characteristics, cell cycling, or

differentiation pattern may differ between the 96 well plates and the Primaria dishes, such that the CNS cells grown on the 96-well plates are rendered resistant to the effects of AT inhibition. For instance, if cell cycling times are slower on the 96-well plates, there may be sufficient time prior to cell replication to replenish cellular AT and repair O<sup>6</sup>-alkylguanine adducts. Alternatively, our apparent inability to detect a specific effect on differentiation may be related to the treatment time which we chose for these studies. At the time that we treated our cells, cell division may have been rapid such that the presence of O<sup>6</sup> adducts would be toxic by virtue of impairing DNA replication. At later treatment times, cell division may be slower and proper differentiation may depend on carefully coordinated gene expression. At this time, the persistence of the relatively stable O<sup>6</sup> adducts may not be cytotoxic, but may impact gene expression by interfering with the binding of transcription factors to DNA.

Although not significant, O<sup>6</sup>-Bg did potentiate toxicity of EMS in P19 cells. This suggests that unlike ENU, the AT protein may play a role in protecting the cell from alkylation by EMS. This result was somewhat surprising since we expected that the AT protein should equally be able to repair O<sup>6</sup>-ethylguanine adducts, regardless of whether cells were treated with ENU or EMS. Kaina et al., (1991) also found that the AT protein may be involved in repairing EMS adducts. In that study, resistance to the cytotoxic effects of EMS was increased by transfection of CHO cells with human AT.

Another surprising result from the current study was that O<sup>6</sup>-Bg potentiated toxicity of MMS to approximately the same extent as that of MNU. Since the O<sup>6</sup>/N<sup>7</sup> guanine ratio of MMS is so much lower than that of MNU, we expected potentiation of MMS toxicity to be substantially lower than that observed with MNU. Although transfection of CHO cells with either human or E. coli AT can both increase survival (Dunn et al., 1991) and reduce incidence of sister chromatid exchange (White et al., 1986) in cells exposed to MMS, the magnitude of the effect in those studies was only

about 50% of the effect observed in MNU-exposed cells. One explanation for the discrepancy between our study and previous studies is that in our study treatment with MMS and O<sup>6</sup>-Bg occurred while the P19 cells were undergoing differentiation, whereas other studies did not use differentiating cell cultures. During development, terminal differentiation is preceded by rapid cell growth. In our system, treatment occurred at a time when many cells may have been in this period of rapid cell growth. Differences between our results and other studies may be due to shorter cell cycle times in the P19 cells, such that there is insufficient time for DNA repair.

#### 4.5 Summary

Our results using O<sup>6</sup>-Bg suggest that for MMS, MNU and EMS, adducts at the O<sup>6</sup>-position of guanine contribute to reductions in viability and differentiation. Although O<sup>6</sup>-Bg appeared to have a greater effect on differentiation than on viability, the effects on differentiation may be due to reductions in viability caused by O<sup>6</sup>-Bg as the relationship between viability and differentiation was the same both with and without O<sup>6</sup>-Bg. Thus from this study we can't conclusively say that differentiation is more sensitive to the presence of O<sup>6</sup>-alkylguanine adducts, or that the effects of the alkylating agents on differentiation correlate with O<sup>6</sup>-alkylguanine adducts. Future studies could investigate the effect of treatment time on the specific sensitivity of differentiation to O<sup>6</sup>-alkylguanine adducts. Results from this study are somewhat in contrast with results from the micromass CNS cell cultures, where O<sup>6</sup>-Bg had the same magnitude of effect on differentiation as in the P19 cells, but did not alter the viability response. Future studies could also address why the CNS cells are resistant to the effects of inhibiting the AT protein, as cell survival does seem to depend on expression levels of the AT protein in other systems.

## **CHAPTER 5: MODE OF CELL DEATH IN NEURONALLY DIFFERENTIATING P19 CELLS**

### **5.1 Introduction**

In recent years, interest in apoptosis, has burgeoned. Although the phenomena of apoptosis has been recognized for many years, the importance of this process in such diverse aspects of biology as embryogenesis, clonal T cell selection, cancer development and prevention, and chemically induced toxicity, has added momentum to research in this field. Although the apoptotic process varies somewhat depending on which system is being studied, there are certain features of apoptotic cells which are consistently observed in most apoptotic cells. Classical morphological changes which occur include chromatin condensation at the periphery of the nucleus, condensation of the cytoplasm, and membrane blebbing. All these changes can occur without significant effects on intracellular organelles, and without disruption of the plasma membrane. At later stages of apoptosis, the nucleus can become very electron dense, and sometimes breaks up into 'apoptotic bodies'. Eventually the apoptotic cell may be phagocytosed by neighboring cells. Another common feature of apoptosis is activation of an endogenous endonuclease which cleaves DNA in the internucleosomal region, resulting in cytosolic oligonucleosomal-length DNA fragments which can be detected on an agarose gel as a DNA ladder. A distinguishing characteristic of apoptosis is that it is a tightly controlled energy and protein dependent process, and can be prevented in many cases by metabolic and protein synthesis inhibitors. Finally, apoptosis usually occurs in scattered cells without accompanying inflammation.

The apoptotic process is in stark contrast with the necrotic process, which is usually characterized by swelling of the cell and organelles, and breakdown of the cell membrane. Necrosis can occur when cellular homeostasis is not maintained, such as

when there is a disruption in ATP supply resulting in an inability to regulate osmotic and ionic gradients. Additionally, necrosis is usually accompanied by inflammation and damage to surrounding tissues.

In the field of embryogenesis, researchers have recognized for many years that many cells die during the course of normal development, in order to provide the developing embryo with the proper form and function by maintaining appropriate numbers and types of cells, and eliminating cells which are no longer necessary (Glucksmann, 1951). This cell death occurs in a very controlled manner, and has been called physiological or programmed cell death (PCD). PCD is observed in almost all tissues during development, and often occurs concurrently with cell division and differentiation. Developmental processes which involve PCD include formation of the palate (Taniguchi et al, 1995), and cleft palate can occur due to a decrease in physiological PCD (Abbott et al., 1989). Other tissues where PCD is significant include the kidneys (Coles et al., 1993), the interdigital tissue of developing limbs (Garcia-Martinez, 1993), and in the nervous system (Oppenheim, 1991). In the nervous system, as many as 50% of postmitotic neurons are lost to PCD during embryogenesis (Lo et al., 1995).

As people begin to study neuronal PCD, it is becoming evident that in many cases this cell death occurs via a classical apoptotic process. For instance, the PCD which occurs in the globus pallidus of the rat, mouse and marmoset, as well as the mouse cerebellum, appears apoptotic (Waters et al., 1994; Wood et al., 1993). Likewise, O2-A progenitor cells, which develop in vitro into either oligodendrocytes or type-2 astrocytes, underwent cell death bearing many morphological characteristics of apoptosis when deprived of the necessary growth factors (Barres et al., 1992). The embryonic expression in neuroectoderm derived tissues, of genes such as Nedd2 which appear to induce an apoptotic cell death also suggests that apoptosis occurs during neuronal PCD (Kumar et al., 1994).

Apoptosis is also an important phenomena in the field of cancer research. It is now becoming clear that many cancers are due to dysfunction in genes which either cause apoptosis, such as the P53 tumor suppressor gene, or which prevent apoptosis, such as the bcl-2 oncogene. P53 may induce cells to die when there is severe DNA damage (reviewed in Hooper, 1994), by activating the endogenous endonuclease which cleaves DNA in the internucleosomal region. Another finding in the field of cancer research is that the efficacy of many DNA-reactive cancer chemotherapeutic agents, including topoisomerase inhibitors, nucleotide analogues, and alkylating agents, is related to their ability to induce apoptosis in tumor cells (Miyashita and Reed, 1993). Other cytotoxic agents which damage DNA can also induce apoptosis. For instance, apoptosis has been induced in a murine IL-3 dependent cell line following exposure to either nitrogen mustard or cisplatin, and in rat hepatocytes following exposure to dimethylnitrosamine (Walton et al., 1993; Pritchard and Butler, 1989, Piacentini et al., 1991).

For specific aim number four, we wanted to determine if apoptosis occurs in neuronally differentiating P19 cells, and if the alkylating agents caused any increases in apoptosis. Since apoptosis is known to accompany neuronal differentiation in many instances, we expected that apoptosis might occur in our cultures, even in the absence of alkylating agents. Additionally, since evidence suggests that agents which damage DNA, such as the direct-acting alkylating agents, can cause apoptosis, we expected that exposure to alkylating agents might increase the extent of apoptosis in our cultures.

We studied apoptosis both morphologically and biochemically, using transmission electron microscopy to look at ultrastructural features of cells, and an ELISA to detect cytosolic oligonucleosomal DNA fragments. For this study we used the same alkylating agents used in previous studies with the rat micromass primary cell and P19 EC cell culture systems.

## **5.2 Materials and Methods**

### **5.2.1 Cell Culture**

Undifferentiated P19 cells were induced to differentiate into neuronal-like cells by allowing cells to aggregate on bacteriological-grade dishes and treating with 0.3  $\mu$ M retinoic acid (RA). After 48 hours of exposure to RA, (culture day 2) the aggregates were replated with fresh RA and treated with either MNU, MMS, ENU or EMS, at concentrations previously determined to cause either a 25% or 50% decrease in viability, at the EC25 or EC50, respectively. Aggregates were harvested for determinations of apoptosis following a 24 hour exposure to the alkylating agents, on culture day 3. In one experiment, cells were fixed and prepared for transmission electron microscopy. In another set of experiments, the cytosolic fraction of the cells was isolated, for determinations of oligonucleosomal DNA fragments. For viability determinations, parallel cell cultures were transferred to lysine coated dishes on culture day 4, and viability was measured on culture day 5. This scheme is indicated in Figure 17.

### **5.2.2 Chemical Exposure Conditions**

We prepared stock solutions of test chemicals (purchased from SIGMA) immediately prior to treating the cells. MMS was dissolved in Dulbecco's Phosphate Buffered Saline (DPBS), pH 7.4; MNU was dissolved in 11 mM sodium citrate, pH 6.0; EMS was dissolved in Hanks Balanced Salt Solution, pH 7.4; and ENU was dissolved in 100mM sodium acetate, pH 6.5. Test chemicals were added directly to the culture dishes, following replating on culture day 2. Control cells were treated with an equal volume of the appropriate buffer.

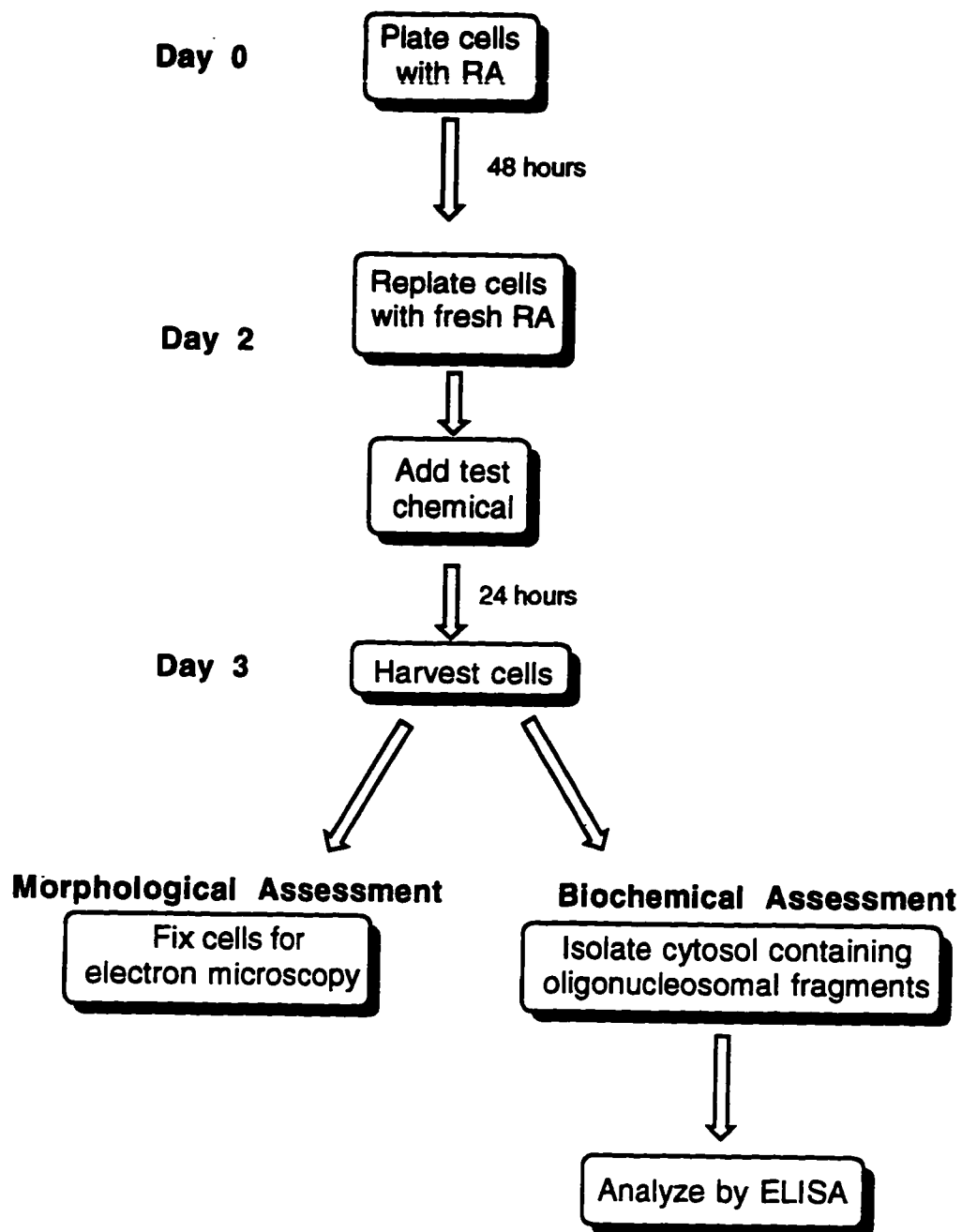


Figure 17: Experimental Design For Detection of Cell Death

### 5.2.3 Viability Assessment

Viability was assessed using the neutral red uptake assay, which is an indicator of lysosomal activity in viable cells. For this assay, media was removed and replaced with media containing 0.05 mg/ml neutral red (Fluka). After incubating the cells for 3 hours at 37°C, the media with neutral red was removed, and cells were fixed for two minutes with glutaraldehyde (4.5ml/100ml). Neutral red taken up by cells was eluted for 2 hours using 0.5% acetic acid (v/v) in ethanol, and absorbance was measured on a kinetic microplate reader at 540 nm (Molecular Devices Corporation).

### 5.2.4 Transmission Electron Microscopy

Cell aggregates were fixed with 0.4% glutaraldehyde/0.16M sodium cacodylate in PBS, and post-fixed with 1% osmium tetroxide/0.16M sodium cacodylate in PBS. Aggregates were subsequently dehydrated in a graded series of acetone, from 30 to 100 percent, and embedded in medcast. Thin sections of the aggregates were prepared and stained with uranyl lead acetate, and observed by transmission electron microscopy on a Zeiss electron microscope.

### 5.2.5 ELISA for Oligonucleosomal DNA fragments

Cells were harvested, centrifuged, and resuspended in fresh media.  $2.5 \times 10^5$  cells were then centrifuged, and resuspended in incubation buffer (Boehringer Mannheim trademark) for 30 minutes at 4°C. After centrifuging at 14,000 rpm for 10 minutes at 4°C, the supernatant containing the cytosolic oligonucleosomes was collected and stored at -20°C for future analysis. For detecting the relative quantity of oligonucleosomes in each sample, specially coated microtiter plates (Boehringer Mannheim, TM) were incubated overnight at 4°C with an anti-histone antibody. This antibody recognizes

histones H2a, H2b, H3 and H4 (the core histones) from rat, mouse, human, hamster, cow, opossum, and frog. After rinsing the wells three times, they were incubated for 90 minutes at room temperature (RT) with incubation buffer containing a 1 to 40 dilution of sample. Wells were rinsed again (x3) and then incubated for 90 minutes at RT with a peroxidase-conjugated anti DNA antibody. This antibody recognizes both single and double-stranded DNA. Following a final rinse (x3) wells were incubated with substrate (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate) at RT for sufficient color development (10-20 minutes). Relative amounts of oligonucleosomes were quantified by measuring the absorbance at 405 nm. Enrichment factors were determined as the absorbance at 405 nm of cytosol from alkylating agent-treated cells, divided by the absorbance of cytosol from untreated cells (Figure 18).

### ELISA for Detection of Oligonucleosomal Fragments

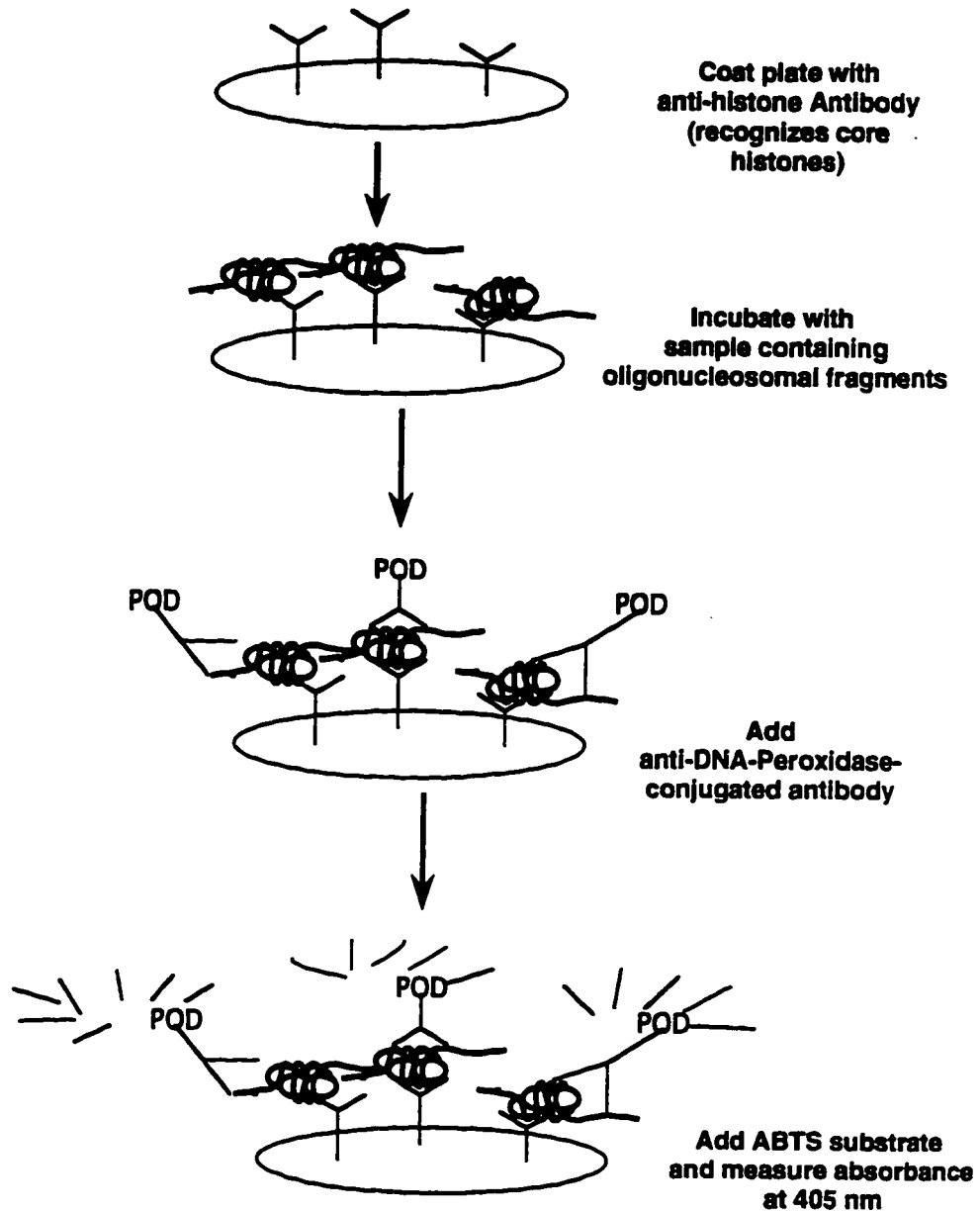


Figure 18: ELISA for Detecting Cytosolic Oligonucleosomal DNA Fragments

## 5.3 Results

### 5.3.1 Biochemical Assessment

We used an ELISA to quantify cytosolic oligonucleosomal DNA fragments in RA-exposed P19 cell aggregates treated with alkylating agents. Aggregation of P19 cells on bacteriological grade dishes and exposure to 0.3  $\mu\text{M}$  RA increased oligonucleosomal fragments DNA by about 2 fold relative to undifferentiated cells ( $2.4 \pm 0.4$ ). Treatment of RA-exposed cell aggregates with alkylating agents resulted in further increases in oligonucleosomal DNA fragments. The enrichment factors for RA exposed cells treated with alkylating agents are given in Table 7, and are increased over background. Enrichment factors at the EC25 ranged from 1.9 to 2.7. At the EC50, enrichment factors ranged from 1.8 to 3.8. Significant concentration-dependent increases in oligonucleosomes were observed for the nitrosoureas, MNU and ENU. AT 331  $\mu\text{M}$  MNU and 1281  $\mu\text{M}$  ENU the increases in oligonucleosomes were significantly different from control. Figure 19 is a graphical representation of the enrichment factors with comparison to cell lethality. This figure shows that whereas lethality increased by approximately two fold between the EC25 and EC50, the increase in the enrichment factor between the EC25 and the EC50 was only 1.4 fold for both MNU and ENU. For MMS and EMS the enrichment factor did not increase between the EC25 and the EC50.

**Table 7: Enrichment of Cytosolic Oligonucleosomes in P19 cell aggregates**

Chemical	Enrichment Factor <sup>a</sup>	
	EC25 <sup>b</sup>	EC50 <sup>b</sup>
MMS	1.9(0.5) <sup>c</sup>	1.8(0.3)
MNU	2.7(0.2)	3.8(0.4) <sup>d</sup>
EMS	2.2(0.3)	2.2(0.4)
ENU	1.9(0.3)	2.6(0.3) <sup>d</sup>

<sup>a</sup> Enrichment factor is the absorbance of cytosolic oligonucleosomes from cell aggregates treated with alkylating agent, divided by the absorbance of cytosolic oligonucleosomes from vehicle-control aggregates, for equivalent numbers of cells. EC25's and EC50's for the four agents were as follows: MMS - 25 and 50  $\mu$ M; MNU - 165 and 331  $\mu$ M; EMS - 500 and 1000  $\mu$ M; ENU - 854 and 1281  $\mu$ M. Enrichment factor for RA control cells, relative to undifferentiated cells was 2.4 (0.4)

<sup>b</sup> EC25 and EC50 are the target concentrations causing a 25% and 50% reduction in viability, respectively.

<sup>c</sup> Numbers in parentheses represent the standard error of the mean.

<sup>d</sup> Significantly different from control

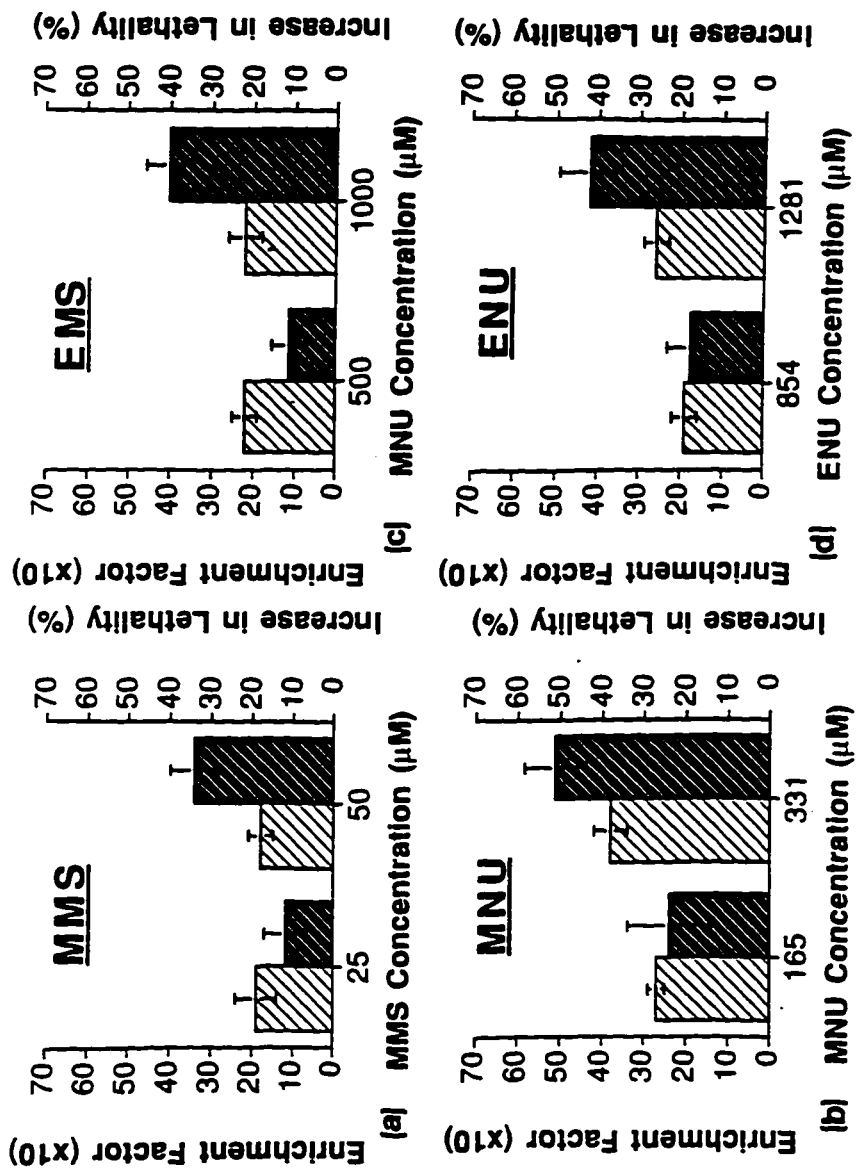


Figure 19: Enrichment Factor and Lethality in Alkylating Agent-Treated P19 Cells  
 Enrichment factor (▨) and increase in lethality (■) for RA-exposed P19 cells treated with either MMS (a), MNU (b), EMS (c) or ENU (d). Error bars represent standard error of the mean for at least three experiments. 77

### 5.3.2 Morphological Assessment

The effects of alkylating agents on the ultrastructural morphology of RA-exposed P19 cell aggregates was studied using transmission electron microscopy. Control P19 aggregates exposed to 0.3  $\mu\text{M}$  RA alone (Figure 20a) are tightly compacted with few intercellular spaces and are characterized by their polygonal shape, a large nucleus (nu) to cytoplasm ratio, and large and sometimes multiple nucleoli (no). The predominant organelle in these cells is the mitochondria (mt), of which there are many. Although ribosomes are mainly loose in the cell, an occasional endoplasmic reticulum (er) can be seen, as well as some golgi apparatus (not shown in this micrograph). Some cells also have myelin figures (my) and lysosomes (ly). Though not shown in this micrograph, some control cells did have condensed cytoplasm and nuclei.

Treated cells show multiple signs of distress, including an increase in both the number and size of lysosomes, although the distressed cells are generally dispersed amongst normal, healthy looking cells. A characteristic of many treated cells is an increase in both the number and size of lysosomes. Figure 20b, depicting cells treated with 25  $\mu\text{M}$  MMS shows a number of cells with chromatin condensation (ch) at the periphery of the nucleus. In one cell it appears as if the nucleus has broken down into apoptotic bodies (ab). Cells treated with 331  $\mu\text{M}$  MNU are shown in figure 20c. A golgi apparatus (go) can be seen in one cell, and mitotic figures (mf) in another. There are also some myelin bodies, a number of intercellular spaces (is) and one cell with a large lysosome which appears to have phagocytosed (ph) another cell that is at an advanced stage of deterioration. A phagocytosed cell is also depicted in Figure 20d, which depicts cells treated with 500  $\mu\text{M}$  EMS. There is also a fairly large intercellular space in this figure. A distinguishing feature of cells depicted in Figure 20e, which were treated with 854  $\mu\text{M}$  ENU, are the misshapen nuclei (mn), extensive vacuolization (vc) and darkly

staining condensed cytoplasm (cy). Note that the organelles (mitochondria and endoplasmic reticulum) still appear normal in cells with oddly shaped nuclei and condensed cytoplasm. One cell in this figure appears to have fragmented, with some of the fragments containing apoptotic bodies.

**Figure 20: Ultrastructural Morphology P19 Cells**

(a) Control cells: Characterized by their large nucleus (NU) and prominent nucleolus (NO). Cytoplasm contains many mitochondria (MT), and loose ribosomes, with sparse endoplasmic reticulum (ER)

CONTROL

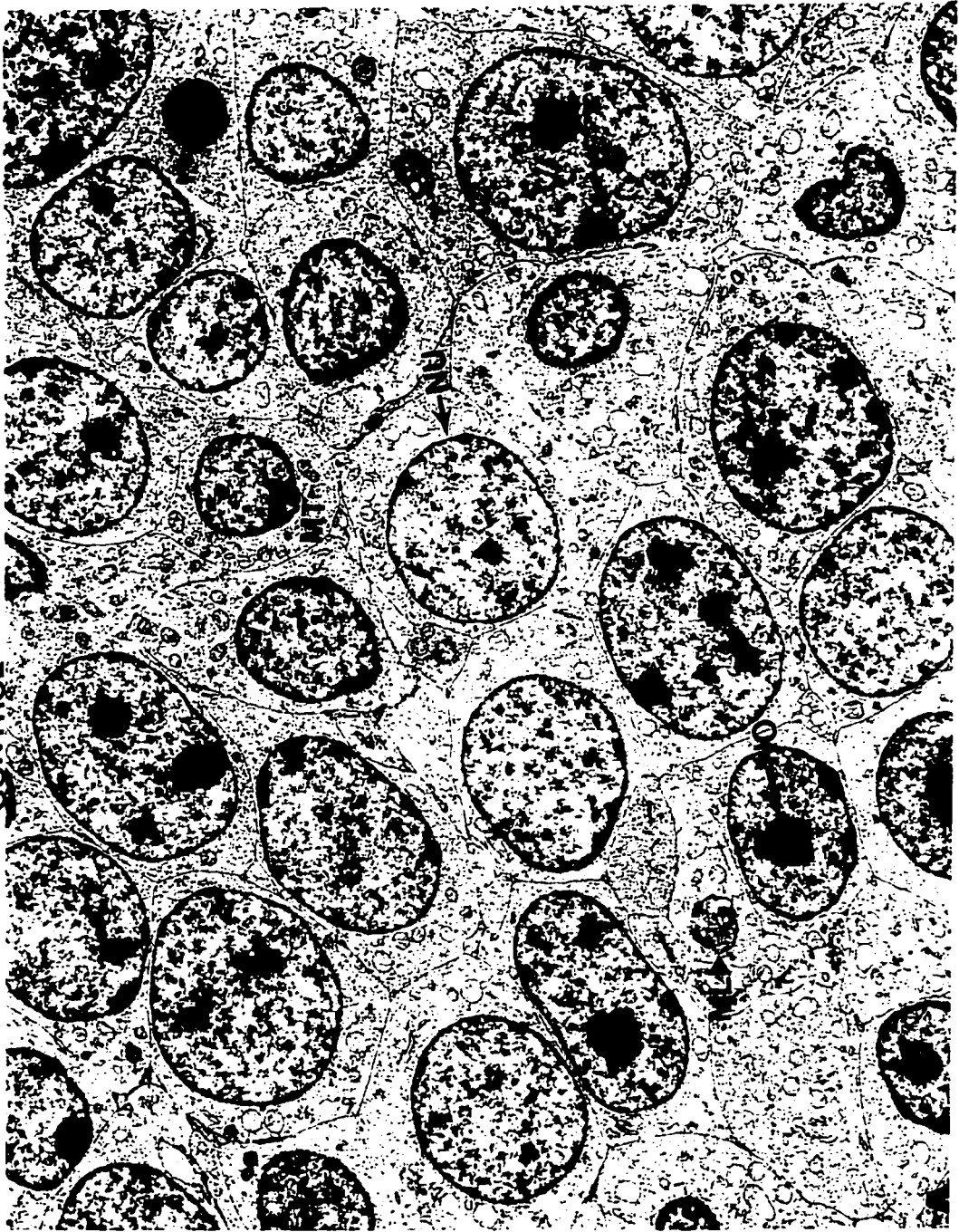
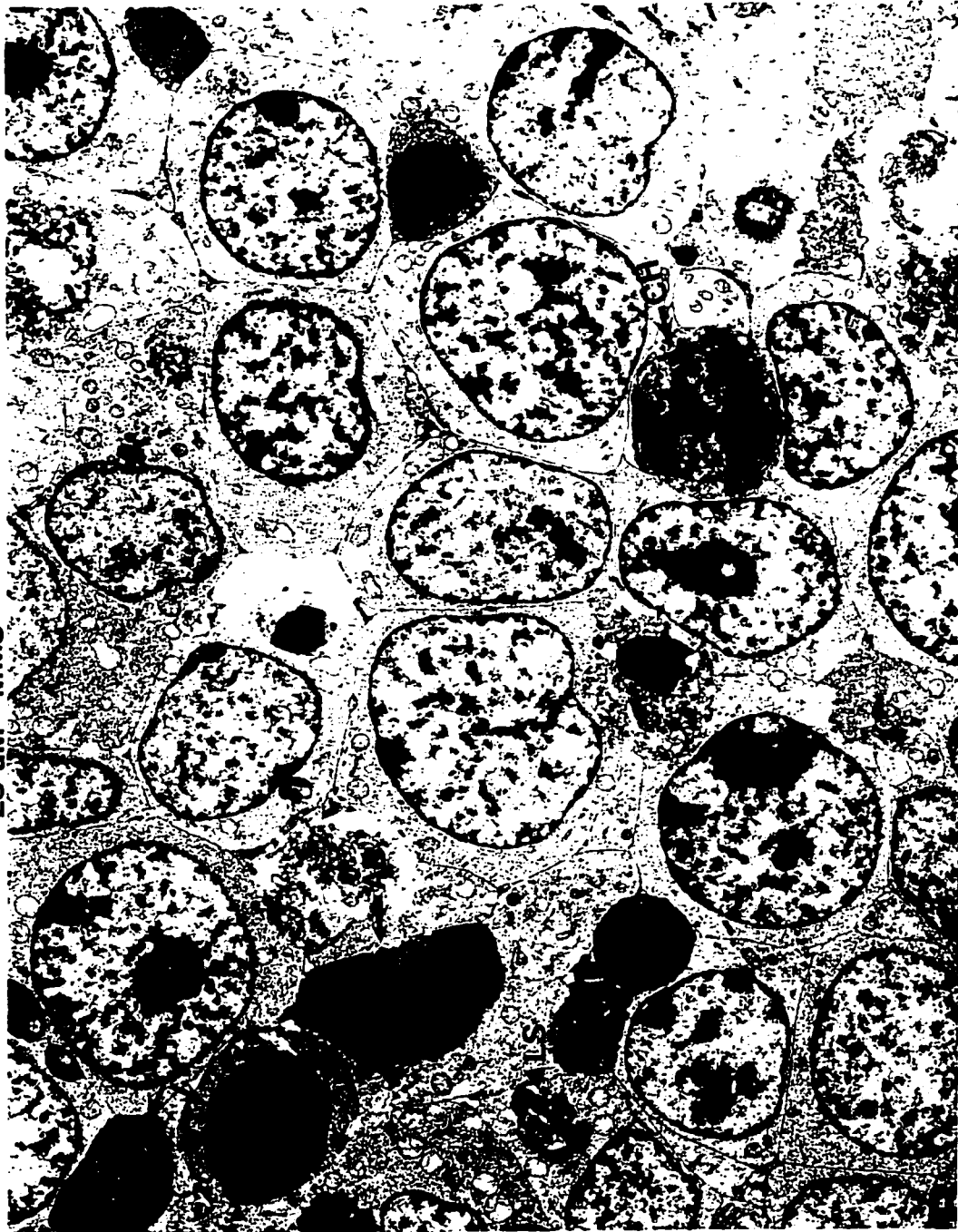


Figure 20 (continued): Ultrastructural Morphology of P19 Cells

(b) 25  $\mu\text{M}$  MMS: Several cells with condensed chromatin (CH) and one with apoptotic bodies (AB) can be observed. There are also numerous lysosomes (LS) and myelin bodies (ML)

25  $\mu$ m MMS



**Figure 20 (continued): Ultrastructural Morphology of P19 Cells**

(c) 331  $\mu\text{M}$  MNU: Cells contain numerous lysosomes (LY) and myelin bodies (ML), and large intercellular spaces (IS). One cell contains a golgi apparatus (GO), one contains mitotic figures (MF), and one contains a large lysosome and has phagocytosed another cell at a highly advanced stage of deterioration

331  $\mu$ M MNU

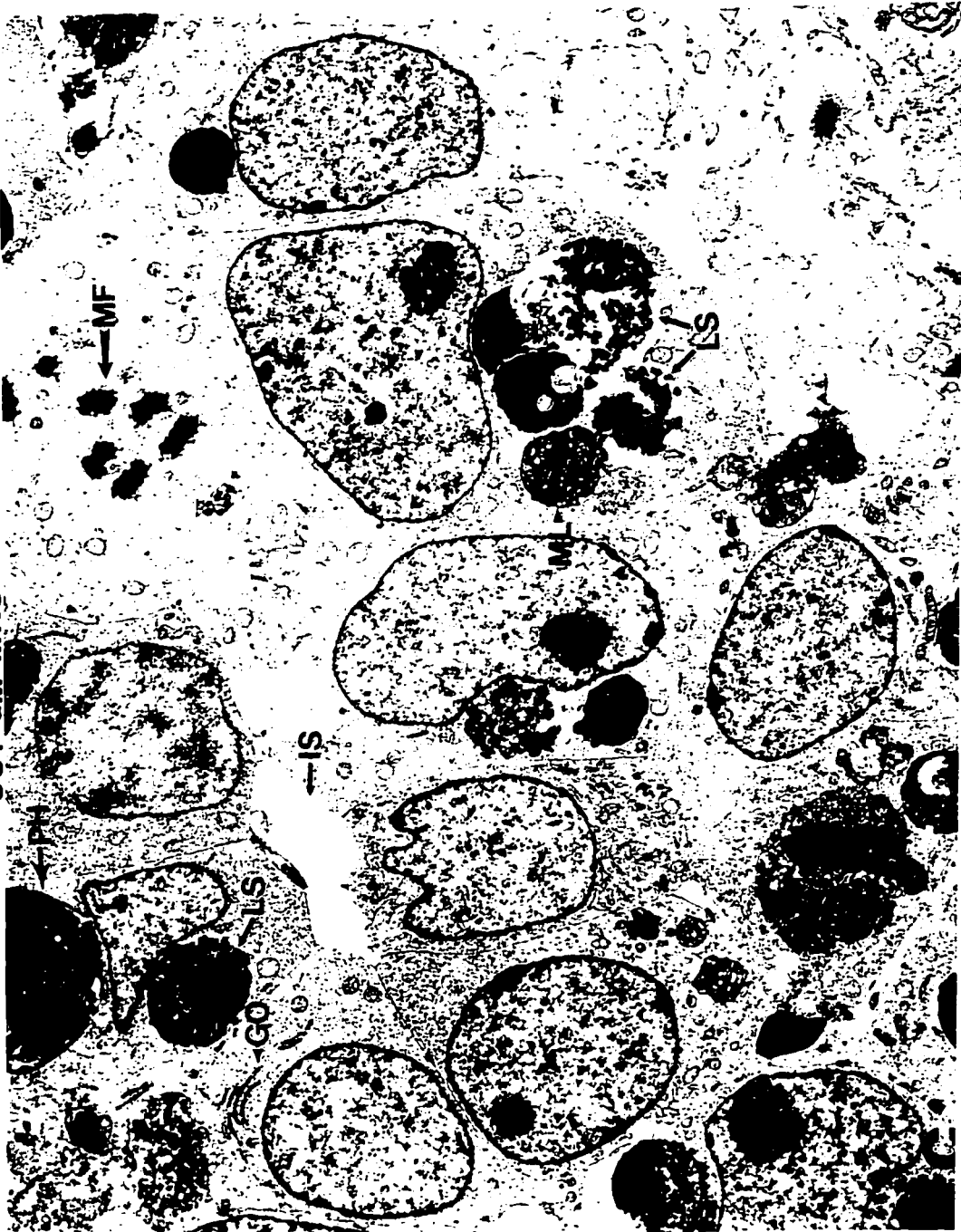
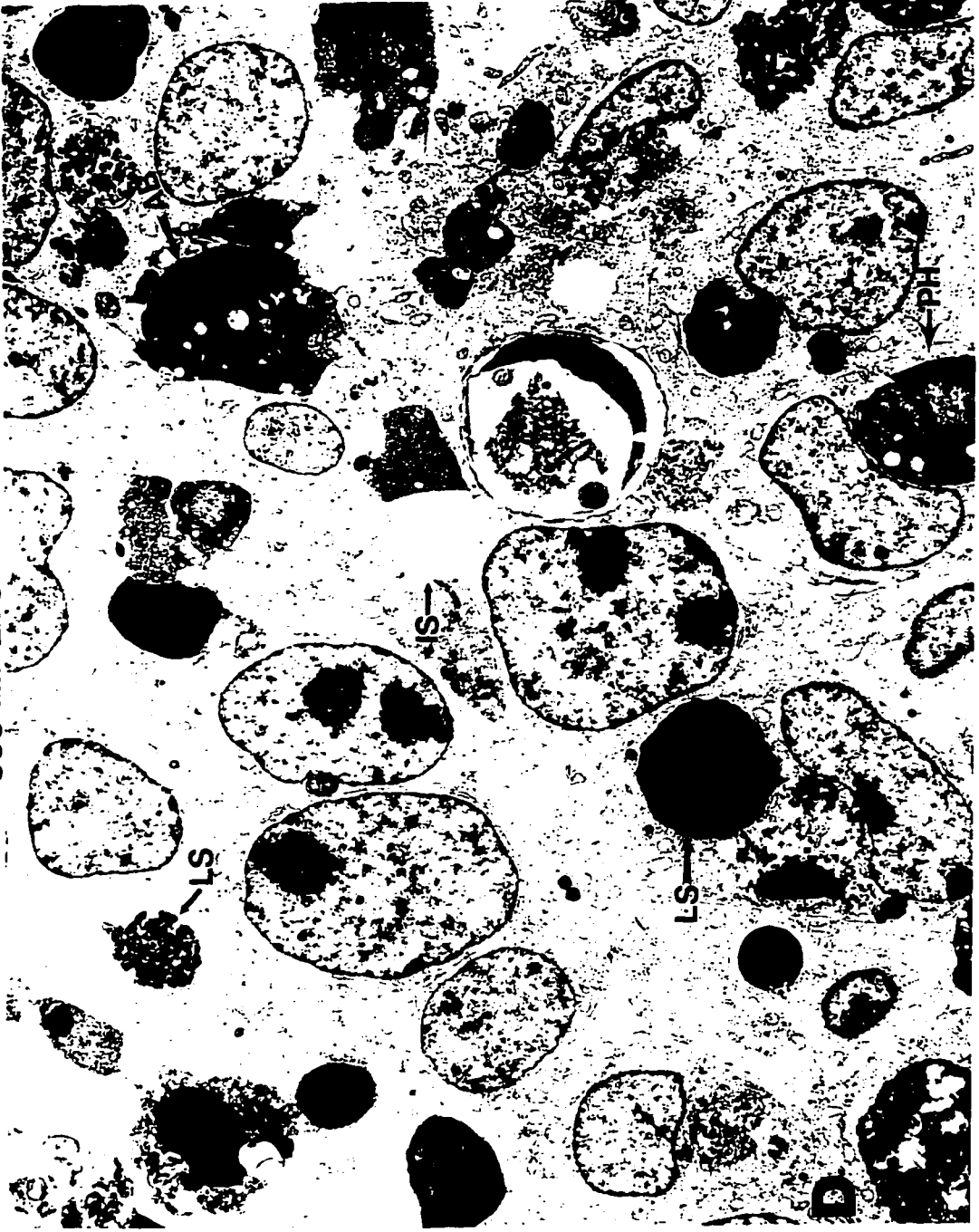


Figure 20 (continued): Ultrastructural Morphology of P19 Cells  
(d) 500  $\mu\text{M}$  EMS: Note presence of lysosomes (LY) and intercellular spaces (IS)

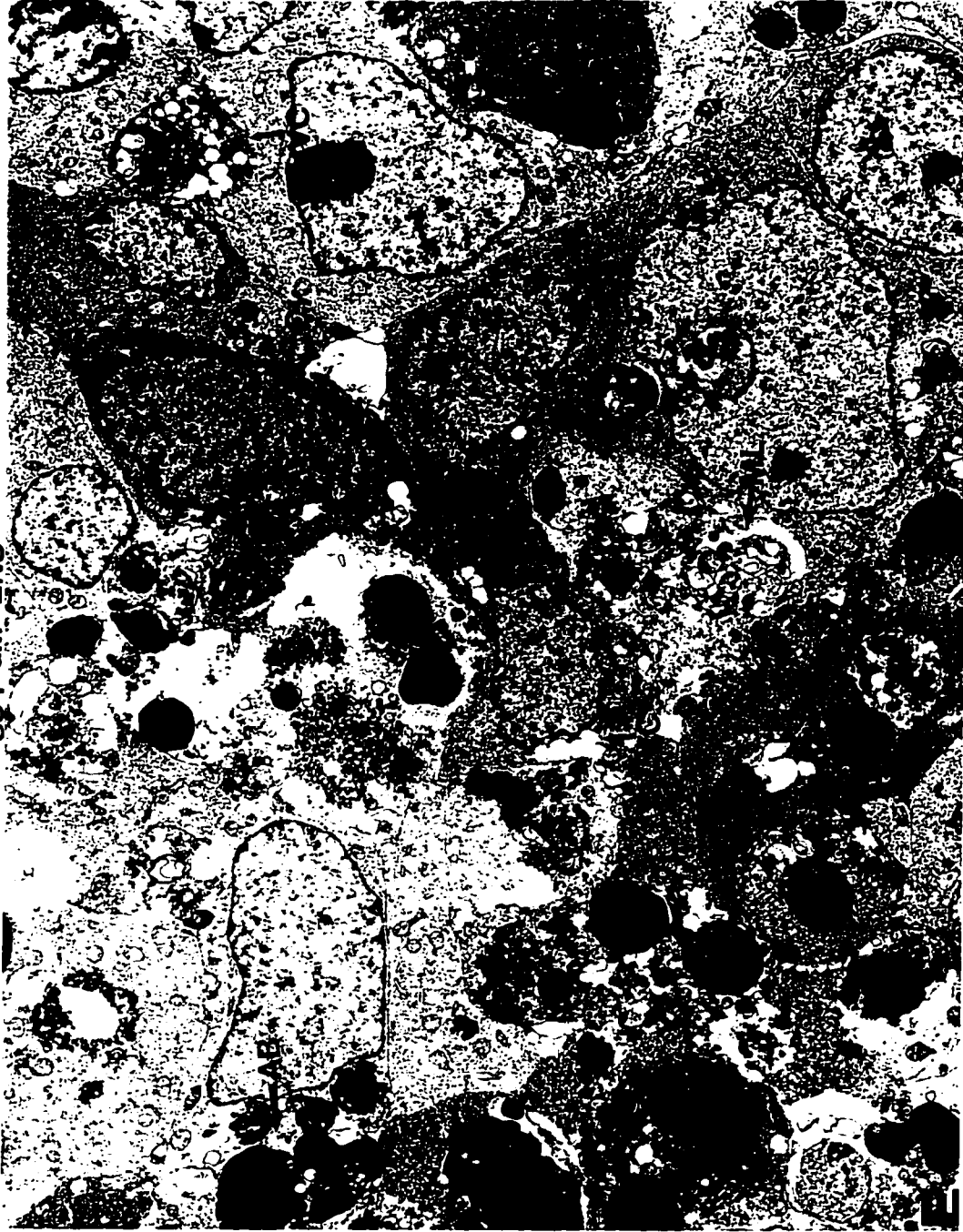
500  $\mu$ m EMS



**Figure 20 (continued): Ultrastructural Morphology of P19 Cells**

**(e) 854  $\mu$ M ENU: These cells contain numerous lysosomes (LY) and myelin bodies (ML), also cells with cytoplasmic vacuolization (VC), oddly shaped nuclei (MN) and condensed cytoplasm (CY)**

854  $\mu$ M ENU



#### 5.4 Discussion

Ultrastructural morphology is considered to be the gold standard for assessing apoptosis. Morphological features characteristic of apoptotic cells include chromatin condensation at the periphery of the nucleus in crescent-shaped masses, shrinkage of the cytoplasm, and membrane blebbing. In some apoptotic cells, the nucleus can be extremely condensed and pyknotic, and can also break down into apoptotic bodies. Biochemically, cytosolic oligonucleosomal DNA fragmentation is considered a classical hallmark of apoptosis. The presence of oligonucleosomal DNA fragments in the cytosol is attributed to the action of an endogenous endonuclease which is activated in cells undergoing apoptotic cell death. This endonuclease cleaves DNA in the internucleosomal region, resulting in DNA fragments composed of one or more nucleosomes. The oligonucleosomal fragments are small enough to pass through the nuclear pores, and consequently can be isolated from the cytosolic fraction of cells. These fragments can be detected as a DNA ladder on agarose gels, or they can be quantified using a sandwich ELISA which recognizes both histones and double-stranded DNA.

In order to determine if treatment of P19 cell aggregates with alkylating agents induces apoptosis, we quantified cytosolic oligonucleosomal DNA fragments using an ELISA, and examined ultrastructural morphology using transmission electron microscopy. For each alkylating agent we used two different concentrations, the EC25 and the EC50, and also assessed viability, in order to determine if apoptosis occurred in a concentration-dependent manner.

Exposure of P19 cell aggregates to 0.3  $\mu$ M RA resulted in increases in cytosolic oligonucleosomal DNA fragments, relative to undifferentiated cells. This suggests that apoptosis is occurring in neuronally differentiating P19 cells. RA-induced differentiation of both human neuroblastoma and F9 embryonal carcinoma cells was also accompanied

by apoptosis (Atencia et al., 1994; Melino et al., 1993; Piacentini et al., 1991). Additionally, RA is involved in expression of the *Ghox-8* homeobox gene, which is expressed in areas of the chick embryo limb bud undergoing PCD (Coelho et al., 1991). During limb morphogenesis, PCD is increased by vitamin A, the naturally occurring form of RA (Schweichel et al., 1971).

In the nervous system, PCD serves to eliminate neurons which don't make proper synaptic connections, or don't migrate to the proper site. In such cases, survival of neurons may depend on the presence of target-derived trophic factors. For instance, sympathetic and sensory neurons may require nerve growth factor derived from their target cells (Levi-Montalcini, 1950; Purves, 1988; reviewed in Barde, 1989). Glial precursor O2-A progenitor cells require insulin-like growth factors and platelet derived growth factor for survival (Barres et al., 1992). Catsicas et al. (1987) suggest that neuronal death during embryogenesis helps eliminate imprecise neuronal connections.

The apoptosis observed in RA-exposed, neuronally differentiating P19 cells may be due to processes similar to the PCD which occurs during development of the nervous system *in vivo*, and may involve expression of specific genes, such as tissue transglutaminase (tTG) (Chiocca et al., 1988). Elevation of tTG accompanied apoptosis in RA-treated mouse embryo limb buds (Jiang and Kochhar, 1992), as well as in both cervical adenocarcinoma and neuroblastoma cell lines (Piacentini et al., 1991; Melino et al., 1994). Other genes involved in neuronal PCD may include interleukin-1 $\beta$  converting enzyme (ICE), which mediates apoptosis in chick dorsal root ganglion neurons following withdrawal of nerve growth factor, and Nedd2, an ICE homologue which induces apoptosis in cells derived from the neuroectoderm (Kumar et al., 1994).

Treatment of RA-exposed P19 cell aggregates with alkylating agents increase cytosolic oligonucleosomal DNA fragmentation relative to vehicle control aggregates by 2 to 4 fold. A significant concentration-dependent increase in cytosolic oligonucleosomes

was only observed for the nitrosoureas, and increases were significantly different from controls at 331  $\mu\text{M}$  MNU and 1281  $\mu\text{M}$  ENU. Overall, enrichment factors for MNU were higher than for the other alkylating agents at both the EC25 and the EC50. This data suggests that although some apoptosis may occur as a general response to DNA damage, ability to induce apoptosis may also be somewhat chemical specific. Although there was a concentration-dependent increase in oligonucleosomes for the nitrosoureas, this increase was only 1.4 fold, whereas lethality increased by approximately two fold between the EC25 and the EC50. This suggests that a greater percentage of cells are dying via necrotic processes at the higher concentration of alkylating agent.

In our system it is possible that the apoptosis which we observed is P53-dependent. In other systems agents which damage DNA have been shown to specifically induce accumulation of P53 protein, followed by an apoptotic cell death (Fritsche et al., 1993; Clarke et al., 1994; Lowe et al., 1993). Although we have not yet looked for the presence of P53 protein in our cultures, the expression pattern in the mouse embryo suggests it would likely be present, as it is expressed in cells during the early stages of differentiation in all tissues, and in brain tissues expression is also observed in post-mitotic neurons (Schmid et al., 1991). Other genes may also be induced by agents which damage DNA. For instance cisplatin induced apoptosis was accompanied by an increase in  $\gamma\text{H2AX}$  activity in a neuroblastoma cell line (Piacentini et al., 1993) and by mRNA for ICE, in three glioma cell lines (Kondo et al., 1995).

It should be noted that our results may have been different if we had quantified cytosolic oligonucleosomes at a different time point. For instance in rat whole embryos exposed to 10  $\mu\text{M}$  phosphoramidite mustard, DNA fragmentation was not observed following 6 and 12 hour exposures, but was observed in rat whole embryos exposed for 24 hours. DNA fragmentation was even more pronounced in embryos exposed for 45 hours. (Chen et al., 1994). The time at which the maximum increase in

oligonucleosomes is observed may also be dose-dependent. Thayer and Mirkes (1995) observed maximal DNA fragmentation at five hours in rat whole embryos exposed to 50  $\mu\text{g/ml}$  N-acetoxy-2-acetylaminofluorene (N-Ac-AAF). However, maximal DNA fragmentation didn't occur until 10 hours in embryos exposed to 5  $\mu\text{g/ml}$  N-Ac-AAF.

The morphology of our control RA-exposed P19 aggregates was characteristic of other embryonal carcinoma cells. These cells are typified by their large nuclei, prominent and sometimes multiple nucleoli, loose ribosomes and a predominance of mitochondria with few other organelles except for occasional lysosomes and golgi apparatus (Andrews et al., 1987). A few nuclei with condensed chromatin were observed in control cells, as well as occasional lysosomes and myelin bodies, which can be observed in healthy cells due to normal cell maintenance and turnover. Treatment of the P19 cell aggregates with alkylating agents resulted in various morphologies indicative of cellular distress. Relative to the untreated control cells, we did see a greater percentage of cells with chromatin condensation at the periphery of the nucleus, which is characteristic of apoptotic cells. In addition, lysosomes and myelin bodies in the alkylating agent-treated cells were larger and more numerous. Pritchard and Butler (1989) also observed an excess of lysosomes in dimethylnitrosamine-treated hepatocytes. Other morphologies characteristic of apoptosis which we observed in our alkylating agent-treated P19 cell aggregates include misshapen nuclei, and heterophagocytosis (Watt et al., 1994; Garcia-Martinez et al., 1993; Waters et al., 1994). Some morphologies, such as cytoplasmic vacuolization, myelin bodies, and lysosomes, can occur in both apoptotic and necrotic cells (Sloviter et al., 1993; Uchiyama et al., 1995).

The presence of cells with pathological morphologies interspersed among normal, healthy looking cells suggests that the cell death is occurring in a relatively controlled manner, which is characteristic of apoptosis. Ratan et al. (1994) report that less than 15% of oxidatively stressed embryonic cortical neurons appear apoptotic at any one time,

possibly due to intercell variability in antioxidant defense mechanisms. Furthermore, some of the cells actually show apoptotic morphologies, such as condensed nucleus and cytoplasm, and breakdown to the nucleus into apoptotic bodies. However, the presence of numerous lysosomes, myelin bodies, and cytoplasmic vacuolization suggests that necrosis may also be occurring. The presence of both apoptotic and necrotic cells is in accordance with our DNA fragmentation data, which suggests that cells are dying via both apoptosis and necrosis, especially at the higher concentration of alkylating agent. The simultaneous occurrence of both apoptosis and necrosis has been observed previously in rat livers exposed to 1,1-dichloroethylene (Reynolds et al., 1984) and due to dimethylnitrosamine and thioacetamide exposure (reviewed in Corcoran et al., 1994). Thayer and Mirkes (1995) also observed both apoptotic and necrotic cells in rat whole embryos exposed to N-Ac-AAF.

The heterogeneous nature of the alkylating agent-treated P19 cells may be partly attributable to the variable response of P19 cells to RA, resulting in cells at various stages of differentiation. The exact phenotype and pattern of gene expression may partly determine whether a cell undergoes apoptosis or necrosis due to a specific insult. For instance, the induction of P53, which has been implicated in apoptosis following genotoxic exposures is tissue specific in mouse embryos (Wubah et al., 1996). Studies by Lowe et al., (1993) show that induction of P53-dependent apoptosis by DNA-damaging agents in primary mouse embryo fibroblasts required the co-expression of the E1A viral oncogene. Conversely, induction of P53-dependent apoptosis in a murine IL-3 dependent cell line was inhibited by the presence of IL-3, or the co-expression of either the raf-1 or src oncogenes (Canman et al., 1995). Since the P19 cells used in our studies are at various stages of differentiation and patterns of gene expression can change during the course of differentiation, the response of individual P19 cells to the alkylating agents may depend on the expression pattern of these proto-oncogenes.

## 5.5 Summary

In summary, there was an increase in cytosolic oligonucleosomes in RA-exposed neuronally differentiating P19 cells, relative to undifferentiated cells. This increase may be due to processes similar to PCD which normally occurs during neuronal differentiation. Treatment with alkylating agents resulted in further increases in cytosolic oligonucleosomes. Additionally, pathological morphologies of alkylating agent-treated cells suggest that apoptosis is enhanced by the alkylating agents, although necrosis may also be occurring. Areas of the body which undergo developmental PCD during embryogenesis seem to coincide with areas where there is a high incidence of malformations, suggesting that alterations in normal patterns of PCD could be teratogenic (Menkes et al., 1970). Sulik et al. (1988) has observed that various teratogenic insults can increase the extent of PCD in embryonic tissues which normally undergo apoptosis. Similarly, the RA-exposed P19 cell aggregates may have been particularly susceptible to apoptosis induced by the alkylating agents. Future studies could examine the time course for induction of apoptosis by the alkylating agents. Additional studies could also address the issue of whether the induction of apoptosis differs between the nitrosoureas and the methanesulfonates. For instance, induction patterns of genes involved in apoptosis, such as p53, tTG, Nedd2, and ICE, may be different for the nitrosoureas and the methanesulfonates.

## **CHAPTER 6: CONCLUDING REMARKS**

Concentration/response curves for alkylating agent exposure in micromass CNS and LB cells have been ascertained and were presented in Chapter 2. The results in the primary cell culture system have been compared with previous toxicity assessments in the whole embryo culture system. In the whole embryo culture system, significant concentration-dependent effects on lethality and malformations were observed, and EC50's for lethality were higher than EC50's for malformations. Furthermore, there was a preponderance of malformations in CNS tissue. Similarly, for both CNS and LB micromass cells significant concentration-dependent decreases in both viability and differentiation were observed for all four chemicals, and EC50's for viability were higher than EC50's for differentiation. CNS cells were universally more sensitive than LB cells for effects on both viability and differentiation, such that EC50 values in the LB cells were approximately 1.5 to 3 fold greater than EC50 values in CNS cells, for both viability and differentiation. Additionally, LB cells were also relatively resistant to the cytotoxic effects of the alkylating agents. Whereas concentrations causing greater than 90% lethality in the LB cells were about 2 fold higher than concentrations which inhibited differentiation by greater than 90% in the LB cells, differentiation and viability were affected by similar concentrations in the CNS cells. Thus, the micromass cell culture system parallels the whole embryo culture system in being able to distinguish between effects on differentiation, and effects on viability, and in being able to detect the sensitivity of CNS tissue. However, the two culture systems differ with respect to the order of potency which was MNU>ENU>MMS>EMS in the whole embryo culture system, and MMS>MNU>ENU>EMS in the micromass cell culture system. Discrepancies between the two culture systems may be due to differences between exposure times (two hours in the whole embryo culture system versus five days in the micromass cell culture system) as well as the presence of 10% fetal calf serum in the

micromass cell culture system which was not present in the whole embryo culture system during the exposure period.

In chapter three we discussed the development of the P19 embryonal carcinoma cell line as an alternative cell culture system for evaluating mechanisms of developmental toxicity. One objective for this part of the study was to establish a system that paralleled the primary CNS cell culture system. To accomplish this, it was necessary to find a marker specific for neuronal differentiation. Based on a previous study by McBurney et al. (1988) in which [ $^3\text{H}$ ]-GABA uptake was observed in neuronally differentiated P19 cells, we decided to verify that [ $^3\text{H}$ ]-GABA uptake is a specific marker for neuronal differentiation. Our results confirmed the neuronal specificity of [ $^3\text{H}$ ]-GABA uptake, as we observed increases in [ $^3\text{H}$ ]-GABA uptake over time. The neuronal specificity of [ $^3\text{H}$ ]-GABA uptake was further confirmed by observing a 75% reduction in [ $^3\text{H}$ ]-GABA uptake in cells induced to differentiate mesodermally, as compared with neuronally differentiated cells; and by observing a 75% reduction of [ $^3\text{H}$ ]-GABA uptake in neuronally differentiated cells pretreated with DABA, which is a specific inhibitor of high affinity neuronal [ $^3\text{H}$ ]-GABA uptake. Our second objective for this part of the study was to compare the effects of the four alkylating agents in the P19 cells with responses observed previously in the micromass CNS cells. As with the CNS cells, we observed significant concentration-dependent decreases in viability and differentiation of P19 cells, and that EC50's for viability were consistently about 2 fold higher than EC50's for differentiation. Finally, both the EC50s and the shape of the concentration-response curves for growth and differentiation were very similar in both cell culture systems, with the exception of ENU. Differences in response to ENU may be due to differences in repair of ENU-specific adducts, such as phosphotriester adducts. Based on similarities in response between the P19 and micromass cell culture systems, we feel that the P19 cell culture system can be useful for studying mechanisms of developmental toxicity of

alkylating agents as well as of other teratogens. Additionally, the differentiation which occurs in P19 cells has many parallels with *in vivo* neuronal differentiation, with respect to expression of neuronal-specific genes, and with respect to functional aspects of neurons, such as GABA uptake, sensitivity to tetrodotoxin, and the ability to generate action potentials. Processes which occur during development of P19 cells are probably similar to processes which occur *in vivo* and *in vitro* in the CNS primary cell cultures. Since the processes are similar, the ability of developmental toxicants to disrupt normal differentiation in the P19 cells could be indicative of ability to elicit functional or behavioral abnormalities in the nervous system *in vivo*.

Once we had established that the P19 cells could be used to assess effects of developmental toxicants on growth and differentiation, we wanted to use the P19 cells to investigate the relationship between O<sup>6</sup>-alkylguanine adducts and the developmental toxicity of alkylating agents, as discussed in chapter four. Towards this aim we used O<sup>6</sup>-Bg to inhibit the AT protein which repairs O<sup>6</sup>-alkylguanine adducts. We found that a non-toxic concentration of 10  $\mu$ M O<sup>6</sup>-Bg effectively inhibited AT activity by approximately 90%. When O<sup>6</sup>-Bg was added to cultures treated with alkylating agents, a dramatic reduction in viability and differentiation was observed for the two methylating agents, MMS and MNU. O<sup>6</sup>-Bg moderately reduced viability and differentiation in cultures treated with EMS, and only slightly affected toxicity of ENU. For all agents, O<sup>6</sup>-Bg affected differentiation to a greater extent than viability. We were surprised to see that O<sup>6</sup>-Bg affected toxicity of MMS to the same extent as MNU, since MMS generates a much smaller proportion of O<sup>6</sup>-guanine adducts relative to total adducts than MNU. However, this could be due to differences in rates of cell proliferation between our cells and cells used in other studies. Another interesting result from our study is that O<sup>6</sup>-Bg affected toxicity of EMS to a greater extent than toxicity of ENU, although the proportion of O<sup>6</sup>-ethylguanine adducts relative to total adducts is greater for ENU than EMS. The

differences between EMS and ENU may depend on whether an S<sub>N</sub>1 or S<sub>N</sub>2 reaction mechanism occurs between the alkylating agent and the nucleophile. Although repair of ethyl adducts occurs slower than repair of methyl adducts, the formation of a bimolecular transition state between EMS, which has more S<sub>N</sub>2 character than ENU, and O<sup>6</sup>-guanine, may facilitate removal of EMS by the AT protein if charge delocalization occurs during transition state formation. As discussed in Dolan et al. (1990) the effectiveness of O<sup>6</sup>-Bg to inhibit the AT protein is due to a delocalization of charge on the benzene ring, such that the benzene group can be transferred to the AT protein even though it is a bulky adduct.

We also wanted to measure O<sup>6</sup> and N7 guanine adducts at equitoxic doses (EC50) for differentiation for the four alkylating agents, both with and without O<sup>6</sup>-Bg. This is described in Appendix A. We developed an HPLC assay for fluorescent detection of these adducts. Even at doses that were approximately 20 times greater than the EC50 for MNU (2841 μM) we were only able to detect adducts at the O<sup>6</sup> position of guanine. However, when we ran our samples treated with biologically relevant doses for MMS and MNU we were not able to detect alkylation at the O<sup>6</sup> position of guanine. Purines such as guanine are detected using excitation and emission wavelengths in the UV range. Since there are other species in our samples which also fluoresce in this range, our baseline was high and the assay was not sensitive enough to detect O<sup>6</sup>-alkylguanine adducts at biologically relevant doses. Most studies which use HPLC to detect low levels of adducts use radiolabeled alkylating agents, since radioactivity can be detected at lower levels than fluorescence of the bases. Immunoslot blots using adduct-specific antibodies can also be used to detect low levels of DNA adducts.

In Chapter Five we discussed mechanisms of cell death occurring in neuronally differentiating P19 cells treated with alkylating agents. We were specifically interested in investigating the occurrence of apoptosis in our P19 cultures. Our interest in studying

apoptosis stemmed from the prevalence of apoptotic programmed cell death of neurons which occurs during embryogenesis, as well as the ability of DNA damaging agents to induce apoptosis. Apoptosis was determined both morphologically, using transmission electron microscopy, and biochemically using an ELISA specific for oligonucleosomal DNA fragments which are considered a classical hallmark of apoptosis. Using the ELISA, we found that in our control P19 cultures, the addition of RA to induce neuronal differentiation resulted in increases in oligonucleosomal DNA fragments relative to undifferentiated cells. Additionally alkylating agent treatment of the P19 aggregates caused further increases in oligonucleosomes. However, although we did see concentration-dependent increases in DNA fragmentation, increases in DNA fragmentation could not account for increases in cytotoxicity, suggesting that more cells may be dying by necrosis at the higher alkylating agent concentration. Likewise, the ultrastructural morphology of alkylating agent treated P19 cells showed morphologies indicative of both necrosis and apoptosis. It is interesting to speculate that the prevalence of apoptosis in neuronal tissue during embryogenesis could render this tissue particularly susceptible to undergo apoptosis following treatment with alkylating agents. Research in the field of apoptosis suggests that there is a delicate balance of signals which determine whether apoptosis will occur. During development, small changes in trophic factors can alter the fate of a cell, and it is easy to imagine that a teratological insult could readily perturb this delicate balance. Thus neuronal cells may be primed for apoptosis such that lower concentrations of cytotoxic agents are required to induce apoptosis in these tissues than in other tissues where apoptosis is not so prevalent. From this standpoint, comparisons of apoptosis in LB and CNS cells would be interesting. Another interesting finding from our apoptosis studies was that DNA fragmentation was somewhat higher with the nitrosoureas than with the methanesulfonates. These two classes of alkylating agents differ in their distribution of DNA adducts, with a higher proportion of O6 adducts

generated by nitrosoureas. Thus, it's possible that certain adducts may be more likely to induce apoptosis than others. Studies using O<sup>6</sup>-Bg could address this possibility. Alternatively, the ability of the nitrosoureas to carbamylate proteins may also contribute to induction of apoptosis. For instance, carbamylation of histone proteins could alter the configuration of the nucleosomes, and possibly facilitate internucleosomal cleavage.

The overall aim of this study was to investigate mechanisms involved in the developmental toxicity of alkylating agents. A study by Bochert et al. (1991) showed a strong correlation between teratogenic potency of three alkylating agents with differing structures, and adduct levels at the O<sup>6</sup>-position of guanine, suggesting that at least certain teratogenic effects of alkylating agents may be due to alkylation at the O<sup>6</sup>-position of guanine. A subsequent study by Kidney and Faustman (1995) also suggested a major role for O<sup>6</sup>-alkylguanine adducts in inhibiting differentiation, but not in eliciting cytotoxicity, for micromass cells. Since the carcinogenic potency of alkylating agents seems to be related to alkylation at the O<sup>6</sup>-position of guanine, it seemed interesting that O<sup>6</sup>-alkylguanine adducts could also be related to the teratogenic potency of alkylating agents. Indeed, there are some similarities between processes involved in carcinogenesis and teratogenesis. For instance, many growth-related proto-oncogenes which are normally expressed during development are aberrantly expressed as oncogenes in tumors, including in tumors induced by alkylating agents. Additionally, many carcinogenic compounds are also teratogenic, and moreover, both tumors and developmental abnormalities are simultaneously induced in laboratory animals treated with alkylating agents (Druckrey et al., 1966). Another common feature is that the brain is one of the most sensitive organs with respect to both the tumorigenic and teratogenic effects of alkylating agents. Expression of AT in the brain, kidney and liver is inversely correlated with tumor incidence in these tissues, implicating a major role for O<sup>6</sup>-alkylguanine adducts in the etiology of alkylating agent induced tumors. Likewise, a similar

correlation may also hold true between AT activity and incidence of malformations in various tissues. It would be interesting to see if transgenic mice expressing the AT gene are less susceptible to the teratogenic effects of alkylating agents.

In our study O<sup>6</sup>-Bg affected differentiation to a greater extent than viability in our study. However, the effects on differentiation may be secondary to effects on viability, as discussed in chapter four. Furthermore, we were not able to detect DNA adducts, due to sensitivity limitation of our HPLC assay. Thus although previous studies did observe a relationship between developmental toxicity and O<sup>6</sup>-alkylguanine adducts, results from our study were inconclusive with respect to this relationship

As discussed in the introduction, O<sup>6</sup>-alkylguanine adducts can be both cytotoxic and mutagenic. Whether the cytotoxic or mutagenic effects of O<sup>6</sup>-alkylguanine adducts prevail may depend on cell proliferation rates, with the cytotoxic effects having more relevance for cells which are proliferating rapidly. Conceivably, we may have observed a stronger relationship between O<sup>6</sup>-alkylguanine adducts and inhibition of differentiation if we had treated our cells at a later stage of differentiation, when cell proliferation is slower and proper differentiation requires the carefully orchestrated expression of specific genes. In this situation, the persistence of O<sup>6</sup>-alkylguanine adducts, which are relatively stable, could disrupt proper gene expression, either by interfering with normal base-pairing, interfering with the interaction between transcription factors and gene promoters or enhancers, or by impeding progress of polymerases.

In spite of our inconclusive results, the results of Bochert (1991), and of Kidney and Faustman (1995), as well as the plausibility that the persistence of O<sup>6</sup>-alkylguanine adducts in the genome could interfere with developmental processes, warrants further investigations to elucidate the specific relationship between O<sup>6</sup>-alkylguanine adducts and developmental toxicity.

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## Appendix 1: Determination of DNA Adducts

Neuronally differentiated cells were treated with either MNU or MMS at the EC50 for differentiation, with and without O<sup>6</sup>-Bg. Treatment levels were as follows: 55 and 165  $\mu$ M MNU, with and without O<sup>6</sup>-Bg, respectively; and 10 and 30  $\mu$ M MMS with and without O<sup>6</sup>-Bg. DNA was isolated after a 1 hour exposure, when we expected to observe maximum levels of alkylation. To quantify the N7 and O<sup>6</sup>-alkylguanine adducts, we originally tried an HPLC assay using fluorescence detection (Dumenco et al., 1991). The limits of detection for this assay were 300 pg for N7-methylguanine (N7-m-gua) and 50 pg for O<sup>6</sup>-methylguanine (O<sup>6</sup>-m-gua). Based on a review of the literature, for cells treated at comparable effect levels, we expected to see adduct levels of approximately 1 ng N7-m-gua/mg DNA, and 150 pg O<sup>6</sup>-m-gua/mg DNA. Therefore, we would need approximately 333  $\mu$ g DNA. I decided to round this up to 500  $\mu$ g DNA to add a 'safety factor' in the event that the P19 cells would have lower alkylation levels. Based on a lower bound estimate for DNA yield of 5  $\mu$ g/10<sup>6</sup> cells, we would need approximately 10<sup>8</sup> cells. From previous experiments, the 100mm dishes should have approximately 1.2 x 10<sup>7</sup> cells dish at harvest time. Therefore we would need 8.3 dishes, which was rounded up to 9 dishes per treatment. The DNA isolation method is outlined below:

### DNA isolation:

1. Cells were harvested, centrifuged for 10 minutes at 800 rpm at 4°C (in the Beckman table top centrifuge in the cell culture room), rinsed 2 times in cold PBS, and then resuspended in 1%SDS/1mM EDTA, adding approximately 1ml/10<sup>8</sup> cells.
2. Add 24  $\mu$ l/ml 1M TRIS-HCl, pH 7.4, and vortex.
3. Add 24  $\mu$ l/ml RNase A (@ 10 mg/ml) and 8  $\mu$ l/ml RNase T1 (@ 5u/ $\mu$ l) and vortex.
4. Incubate at 37°C for 1 hour, with shaking and periodic inversion of the test tubes with cells.
5. Add 84  $\mu$ l/ml Pronase (@ 10 mg/ml) and vortex.
6. Incubate at 37°C for 1 hour, with shaking and periodic inversion of the test tubes with cells.
7. Add 1X vol buffered phenol, pH = 7.4.
8. Invert tubes for 5 minutes.
9. Centrifuge at 3750 rpm (Cell Culture Beckman) at room temperature for 20 minutes.
10. Transfer the top phase to a new tube using a 3 ml transfer pipette with the tip cut off. Make sure not to remove any of the thick interphase, which is white!!
11. Add 0.5 vol buffered phenol and 0.5 vol SEVAG, and repeat steps 8 - 10.
12. Add 1x vol SEVAG, and repeat steps 8 and 9.
13. Transfer top phase to tubes for the Eaton Centrifuge.
14. Add 0.1 vol 3M sodium acetate, and vortex.
15. Add 1.5 vol ice cold 100% ethanol, and invert the tube to gather the DNA.
16. Spin @ 8,300 rpm, 4°C, for 15 minutes, pour off the ethanol, and rinse 2 times with cold 70% ethanol.
17. Flip over tube and allow to dry overnight on absorbent paper.
18. Freeze DNA for future analysis.

**HPLC Analysis:**

We first tried a method outlined in Dumenco et al. (1991), using a double 10-cm SCX-5 ion-exchange column, with isocratic elution using 70 mM sodium phosphate buffer, pH 2.5, at 1.3 ml/min. We were not able to get good chromatography with this method, so we tried using the method outlined in Segerback et al. (year?), as outlined below:

Column: Spherosorb ODS reversed phase (7.8 x 250 mm)

Flow rate: 1.5 ml/min

Gradient: 25 minute linear gradient, from 0 to 50% methanol in 50 mM ammonium formate buffer, pH 4.5.

Excitation: 290

Emission: 360

HPLC chromatograms for the methylated DNA adduct standards are shown in Figure 21. We were able to achieve good separation and peak shape for the O<sup>6</sup>-m-gua (Figure 21b). However, we were not able to separate N<sup>7</sup>-m-gua from the adenine peak (compare Figures 21a and 21c).

note: x=expected levels for biologically relevant treatment concentrations.

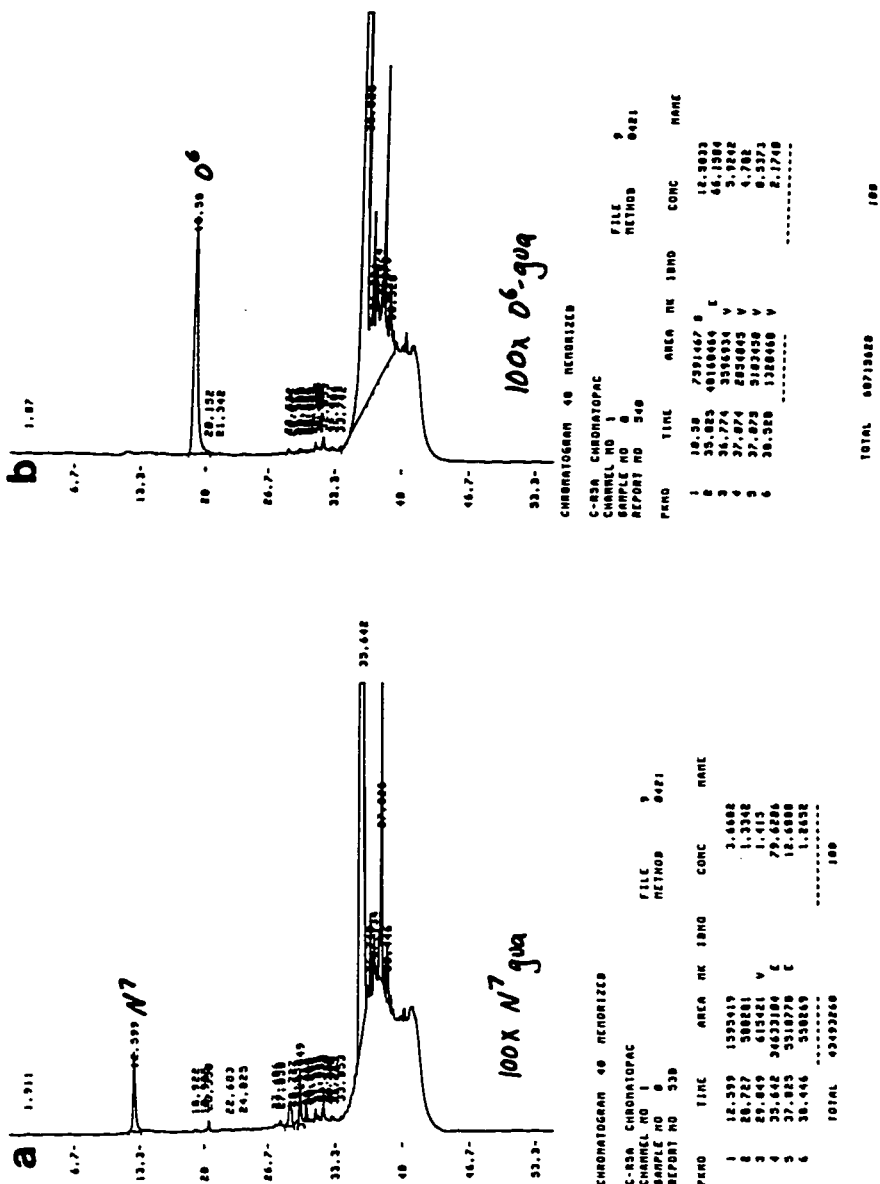


Figure 21: HPLC Chromatograms for Methylated DNA Adduct Standards

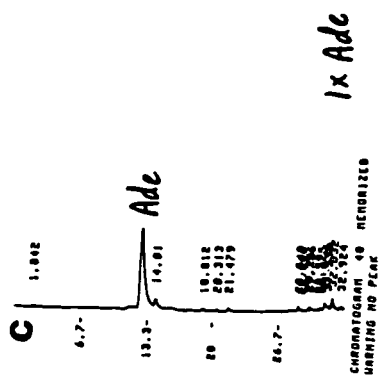
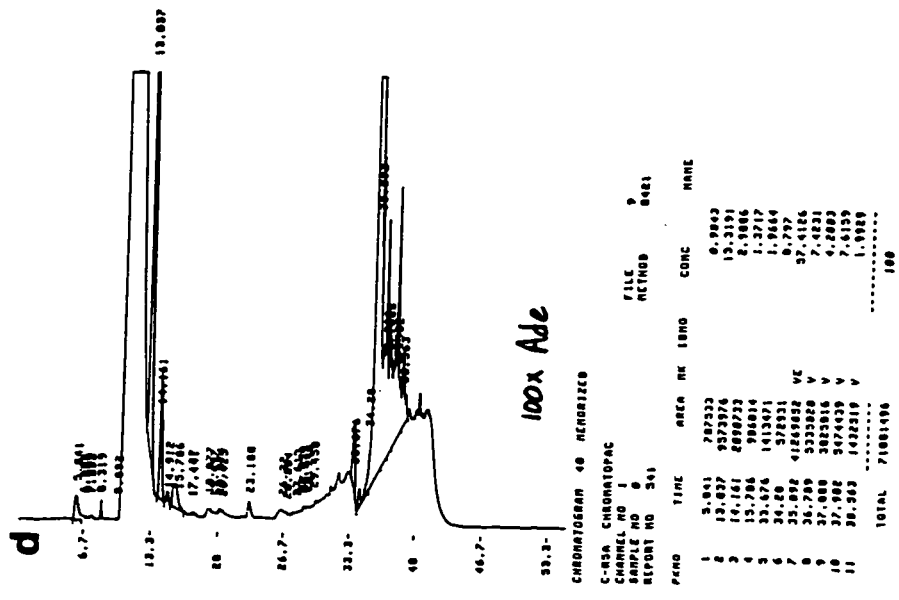


Figure 21 (continued): HPLC Chromatograms for Methylated DNA Adduct Standards

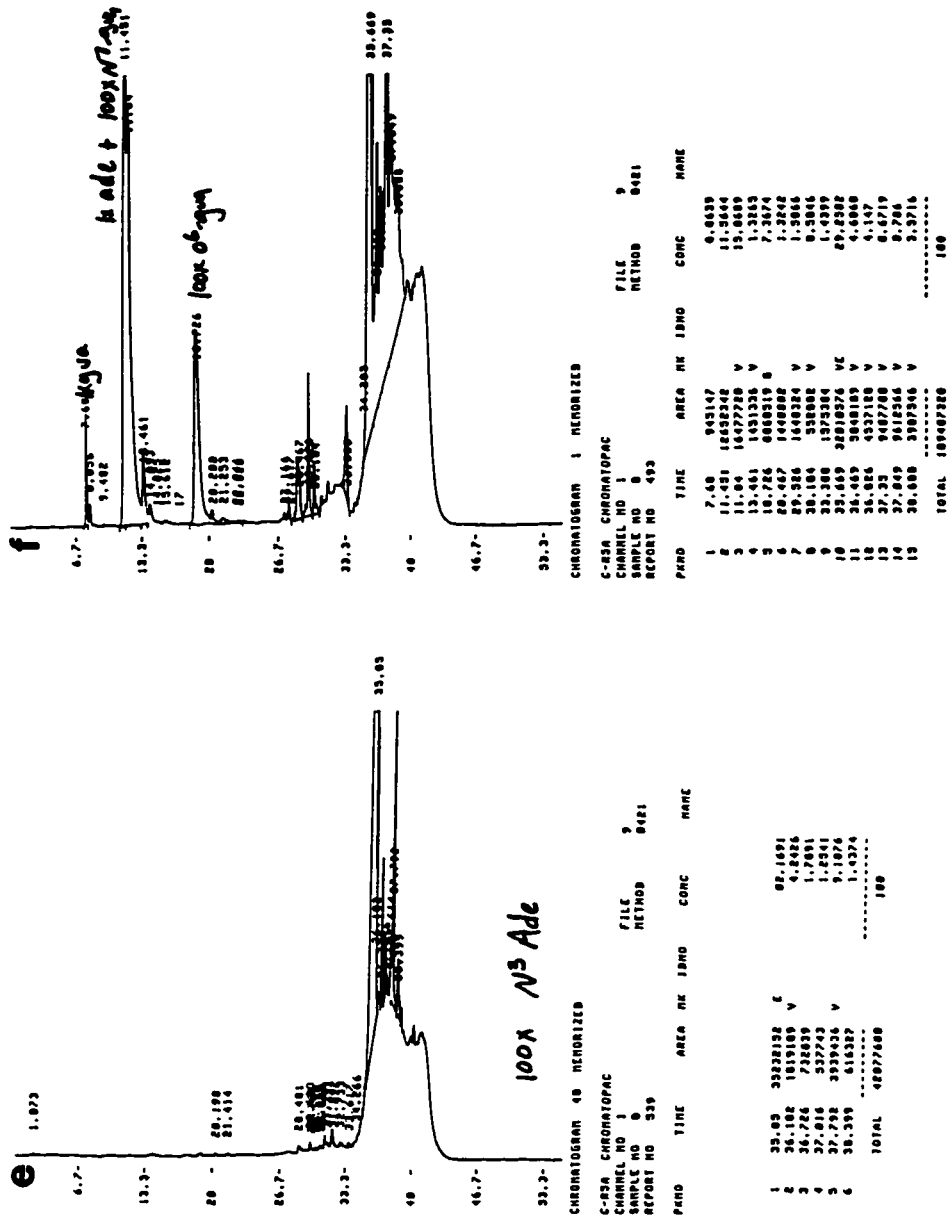


Figure 21 (continued): HPLC Chromatograms for Methylated DNA Adduct Standards

Therefore, we tried a method outlined in Dumenco et al. (1991) which separates out the N7 adducts from the guanine, adenine and O<sup>6</sup> adducts. This method is outlined below.

Neutral and Acid Hydrolysis of DNA:

1. Dissolve samples in 1ml of 10 mM sodium cacodylate, pH 7
2. Heat samples at 100°C for 30 minutes (neutral hydrolysis)  
*Labilizes the N7-m-gua adducts, which are released into solution*
3. Add 100 µl of 1N HCl at 4°C  
*Precipitates DNA, including O<sup>6</sup>-m-gua, guanine and adenine.*
4. Centrifuge at 13,000 rpm in microcentrifuge at 4°C for 10 minutes
5. Remove supernatant which contains N7-methylguanine adducts
6. Resuspend the pellet in 1ml of 0.1 N HCl (pH 1.2)
7. Heat sample at 80°C for 45 minutes (acid hydrolysis)  
*Releases guanine, adenine, and O<sup>6</sup>-m-gua into solution*
8. Centrifuge at 13,000 rpm in microcentrifuge at 4°C for 10 minutes
9. Remove supernatant containing guanine, adenine and O<sup>6</sup>-m-gua
10. Take OD readings of both the N7-m-gua fraction and the guanine/O<sup>6</sup>-m-gua fraction, to determine proper loading for HPLC

Figure 22 shows chromatograms for DNA hydrolyzed using neutral and acid hydrolysis, for cells treated either with 30 µM MMS, for low levels of O<sup>6</sup>-m-gua adducts; or with 2646 µM MNU, for high levels of O<sup>6</sup>-m-gua adducts. Figures 22a and b show elution times for guanine, adenine, N7-m-gua and O<sup>6</sup>-m-gua. The chromatogram for the guanine/O<sup>6</sup>-m-gua fraction for cells treated with 30 µM MMS show peaks for guanine and adenine, and an additional peak eluting at 16.9 minutes. This peak is probably not the O<sup>6</sup>-m-gua peak, since that peak eluted at 18.5 minutes for the standards (Figure 22b) and levels of O<sup>6</sup>-m-gua adducts should be low in this sample. Figure 22d shows that there was not a sizable peak for the N7-m-gua adduct in cells treated with 30 µM MMS. In cells treated with 2646 µM MNU, there was a sizable peak eluting at 12.4 minutes, which could represent N7-m-gua. Unfortunately, we loaded too much DNA for the guanine/O<sup>6</sup>-m-gua fraction, and the guanine/adenine peak ran past the elution point for O<sup>6</sup>-m-gua.

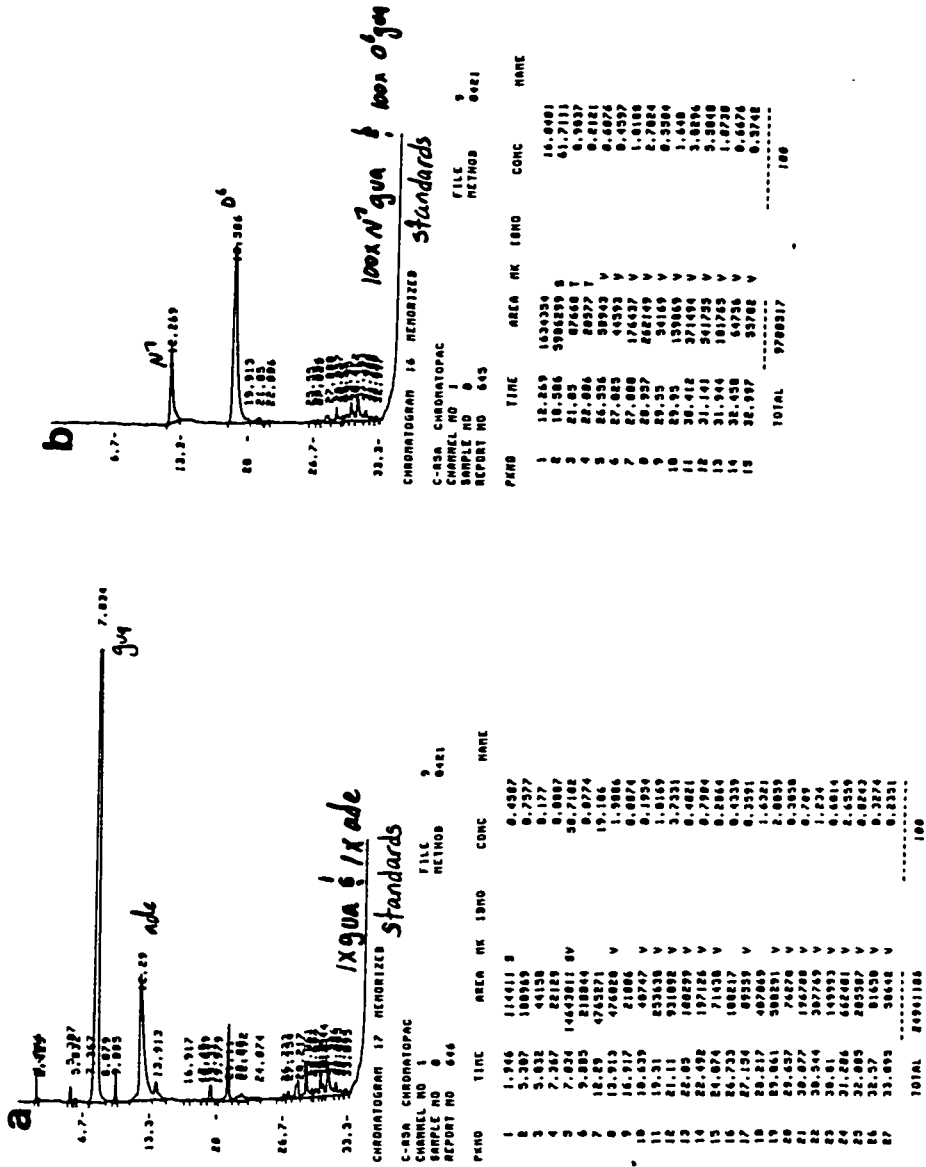
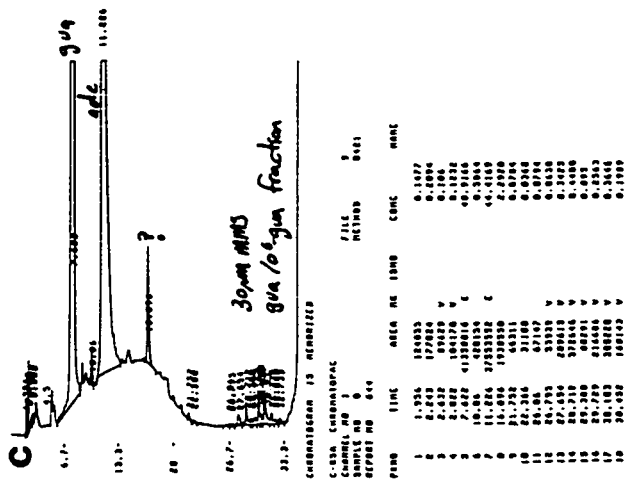
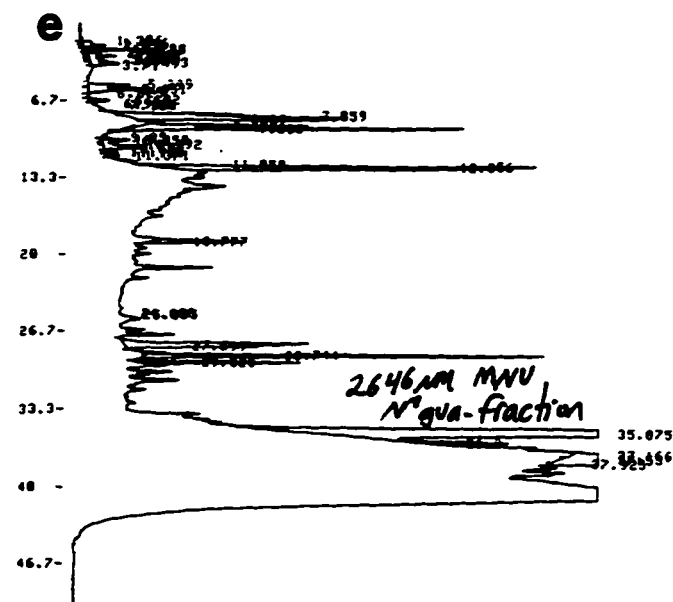


Figure 22: HPLC Chromatograms with Neutral and Acid Hydrolysis: Low versus High O<sub>6</sub>-Methylguanine Alkylation





CHROMATOGRAM 11 MEMORIZED

C-RSA CHROMATOPAC  
CHANNEL NO 1

FILE 9

SAMPLE NO	0	METHOD	0421			
REPORT NO	648					
PKNO	TIME	AREA	HK	IBNO	CONC	NAME
1	1.646	38968	V		0.122	
2	1.95	181428	V		0.2899	
3	2.288	167238	V		0.3461	
4	2.67	57385	V		0.1186	
5	2.843	182788	V		0.2127	
6	3.055	62238	V		0.1288	
7	3.219	63889	V		0.1386	
8	3.493	168985	V		0.333	
9	5.299	277617			0.5745	
10	5.651	391854	SV		0.8892	
11	6.602	86278			0.1785	
12	7.859	55283			1.1441	
13	8.89	1899836	V		3.9314	
14	8.773	595288			1.2318	
15	9.888	1387851	V		2.8783	
16	10.859	139627			0.2889	
17	10.392	56964	V		0.1179	
18	10.651	291926	V		0.6841	
19	11.185	41373	V		0.8856	
20	11.371	57996			0.12	
21	11.958	38631			0.1048	
22	12.356	2616759			5.415	
23	18.777	474989			0.9827	
24	27.699	1689887			3.4951	
25	28.744	1719867			3.5573	
26	29.323	555727			1.15	
27	35.075	31828968	E		64.2885	
28	36.2	414472	V		0.8577	
29	37.166	1368458			2.8152	
30	37.52	1448327	V		2.9971	
31	37.925	414951	V		0.8587	
TOTAL		48324684			188	

Figure 22 (continued): HPLC Chromatograms with Neutral and Acid Hydrolysis:Low versus High O<sup>6</sup>-Methylguanine Alkylation

Next, we decided to compare chromatography for treated (2646  $\mu$ M MNU) versus untreated DNA, as shown in Figure 23. As shown in Figure 23c, the two main peaks for the N7-m-gua fraction of MNU-treated DNA eluted at 14.3 and 18.8 minutes, neither of which correspond with the N7-m-gua elution time of 12.9 (Figure 23a). To confirm the location of the N7-m-gua peak, we spiked the sample with 100x expected biological levels, obtaining a new peak at 12.2 minutes. We concluded that we were not able to detect N7-m-gua adducts with this method. The peak at 18.8 does correspond with the elution time for O<sup>6</sup>-m-gua, which is interesting since we should not have O<sup>6</sup>-m-gua if the procedure is working properly. Interestingly, this same peak was present in the N7-m-gua fraction of the untreated DNA (Figure 23e) and was probably not due to residual DNA present on the column from the previous run since the peak size was reduced by 50% when the sample was diluted 1:2. This peak could represent an artifact of either the DNA isolation or hydrolysis, which co-elutes with the O<sup>6</sup>-m-gua peak. The chromatogram for the guanine/O<sup>6</sup>-m-gua fraction of treated cells (Figure 23g) shows a peak eluting at 18.7 minutes, which could be the O<sup>6</sup>-m-gua peak since peak size increases when spiked with 100x O<sup>6</sup>-m-gua. The guanine/O<sup>6</sup>-m-gua fraction for untreated DNA shows a mystery peak eluting at 17.1 minutes (Figure 23i). We also decided to try to run the O<sup>6</sup>-m-gua standard, and were only able to detect a very small peak even at 10x O<sup>6</sup>-m-gua (Figure 23j).

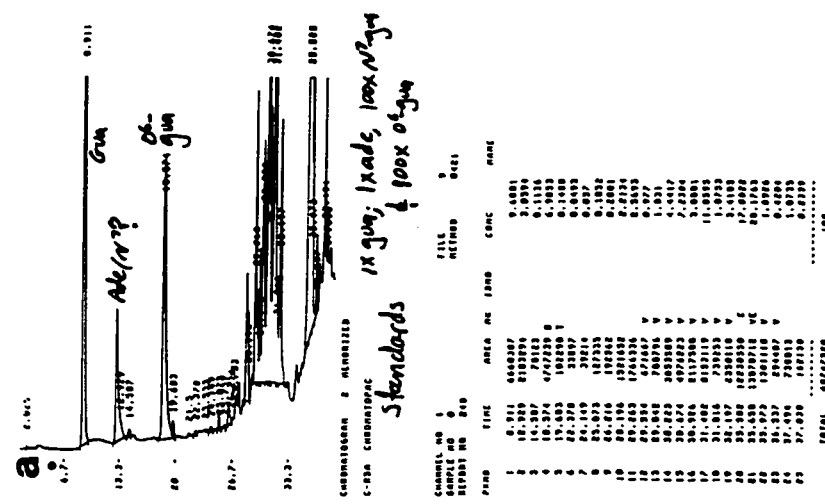
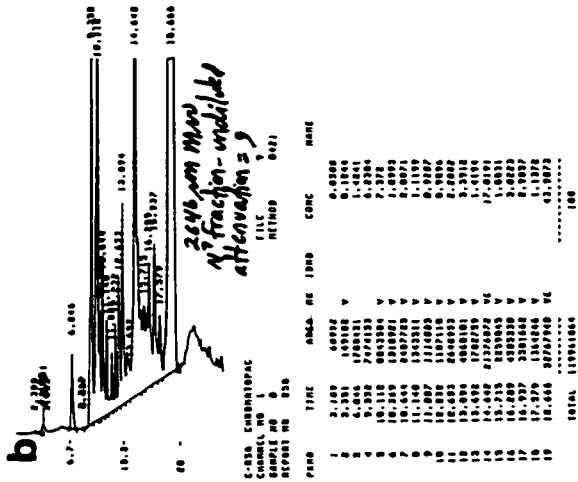


Figure 23: HPLC Chromatograms with Neutral and Acid Hydrolysis: Treated versus Untreated DNA



Figure 23 (continued): HPLC Chromatograms with Neutral and Acid Hydrolysis: Treated versus Untreated DNA

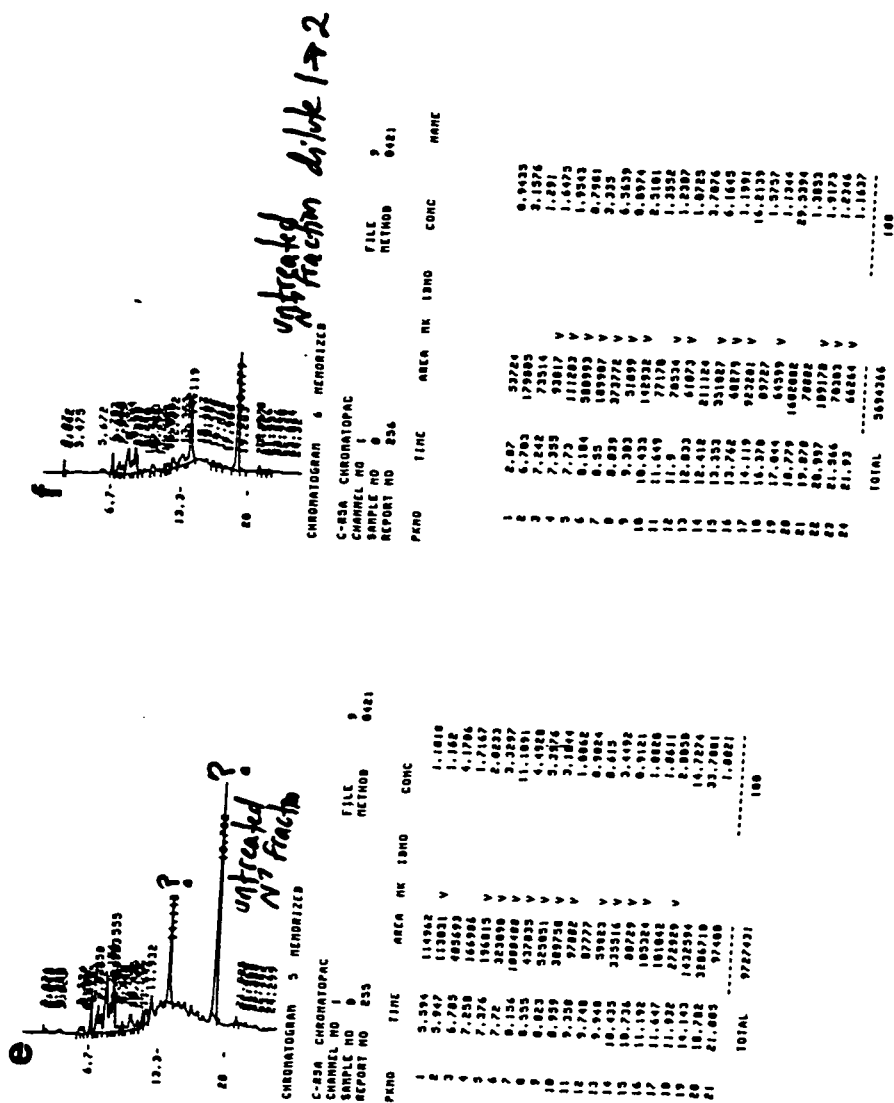


Figure 23 (continued): HPLC Chromatograms with Neutral and Acid Hydrolysis: Treated versus Untreated DNA

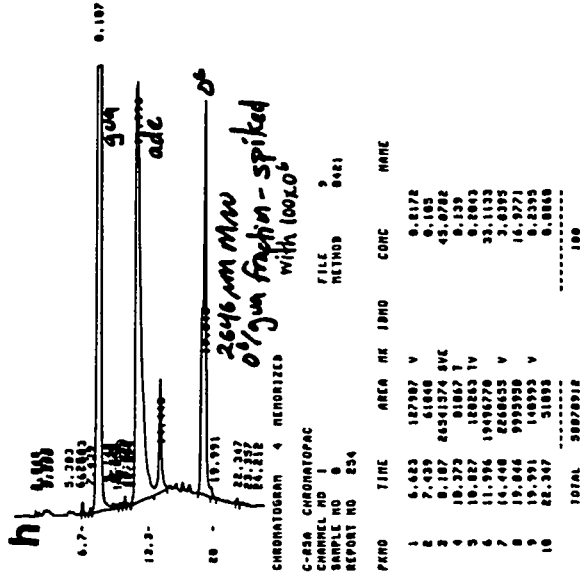
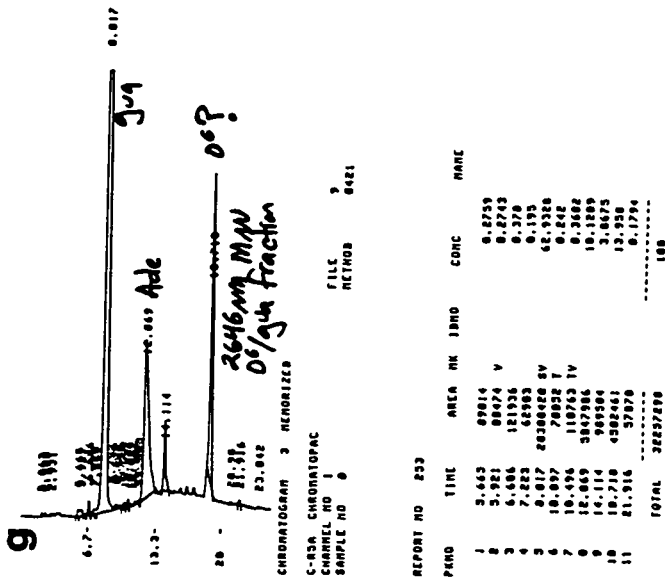


Figure 23 (continued): HPLC Chromatograms with Neutral and Acid Hydrolysis: Treated versus Untreated DNA

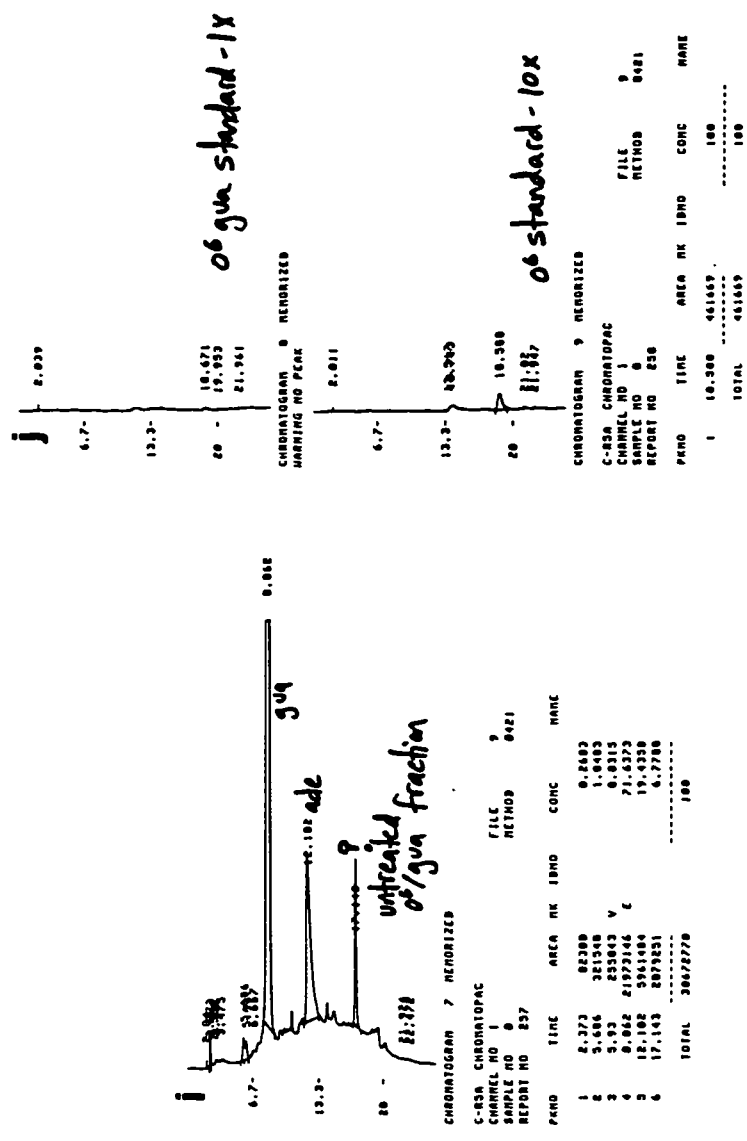


Figure 23 (continued): HPLC Chromatograms with Neutral and Acid Hydrolysis: Treated versus Untreated DNA

From the previous HPLC run, we decided that trying to detect the N7-m-gua adduct at biologically relevant treatment concentrations would probably be very difficult with the current system, and decided to concentrate on detection of the O6-m-gua adducts. We first tried a standard acid hydrolysis of DNA, as described by Herron and Shank (1979).

Acid Hydrolysis of DNA:

1. Dissolve DNA in 1 ml of 0.1 N HCl
2. Heat samples at 70°C for 30 minutes  
*Releases all bases into solution*
3. Filter through 0.65  $\mu$ M filter

We also changed the running conditions by lengthening the gradient from 25 to 30 minutes, to try to separate the peak which was co-eluting with the O<sup>6</sup>-m-gua peak.

Figure 24 shows chromatograms for cells treated with 2646  $\mu$ M MNU, using acid hydrolysis and the new longer gradient. Figure 24b shows that lengthening the gradient results in two separate peaks eluting close to the O<sup>6</sup>-m-gua retention time. This chromatogram shows a very high baseline, which did not go down even after the sample was centrifuged (Figure 24c).

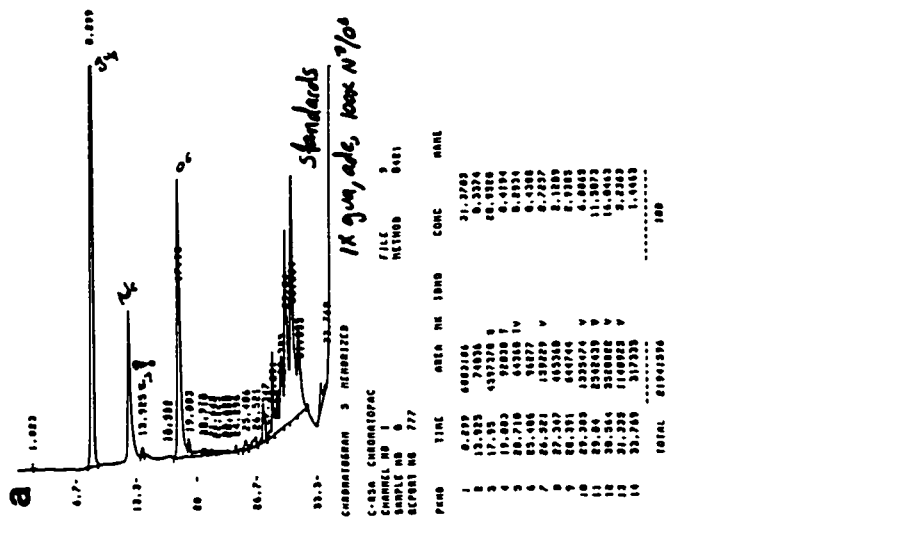
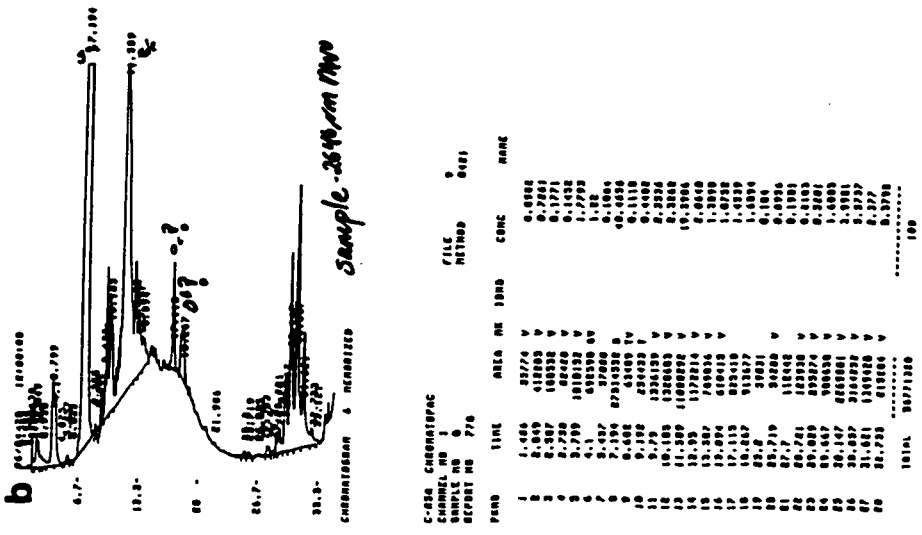
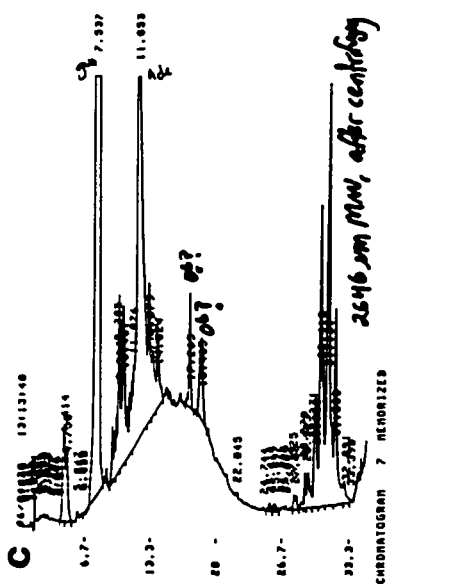
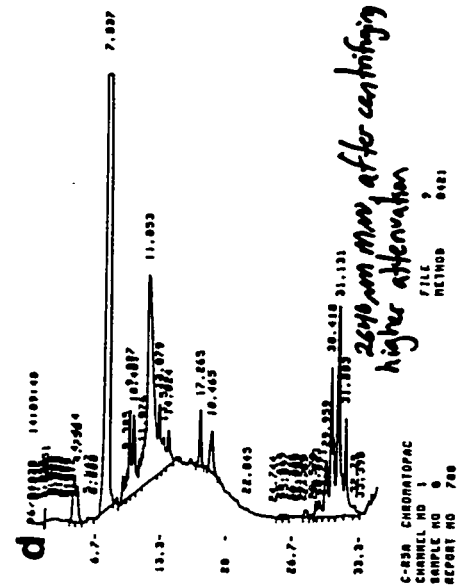


Figure 24: HPLC Chromatograms with Acid Hydrolysis



C-830 CHROMATOGRAPH  
CHANNEL NO 1  
SAMPLE NO 0  
REPORT NO 740

FILE 0481  
METHOD 7

PERO	TIME	AREA	HE	ISNO	CONC	NAME
1	1.031	31764	V		0.0841	
2	4.414	971956	V		1.3798	
3	4.728	630138	V		1.8963	
4	7.337	2799498	E		45.4078	
5	9.385	385407	V		0.9038	
6	9.387	1488234	V		2.2582	
7	16.491	1650822	V		2.2925	
8	11.053	10927427	V		17.6833	
9	13.079	1294853	V		1.8055	
10	13.079	1209458	V		1.7168	
11	13.317	740911	V		1.0400	
12	14.024	725019	V		1.0091	
13	17.265	748778	V		1.0491	
14	18.465	972044	V		1.361	
15	25.097	72974	V		0.1194	
16	25.097	72974	V		0.1194	
17	27.85	172865	V		0.2384	
18	28.379	42298	V		0.5812	
19	29.257	49098	V		0.7145	
20	29.257	49098	V		0.7145	
21	29.255	1238453	V		17.654	
22	30.418	3115408	V		43.657	
23	31.131	4459132	V		7.2612	
24	31.083	1007682	V		2.0659	
25	32.051	210738	V		0.3054	
TOTAL						6130786
						100

C-850 CHROMATOGRAPH  
CHANNEL NO 8  
SAMPLE NO 0  
REPORT NO 779

FILE 0481  
METHOD 7

PERO	TIME	AREA	HE	ISNO	CONC	NAME
1	1.031	31764	V		0.0829	
2	4.414	971956	V		1.3748	
3	4.728	630138	V		1.8314	
4	7.337	2799498	E		45.2622	
5	9.385	385407	V		0.9003	
6	9.387	1488234	V		2.0917	
7	16.491	1650822	V		2.0748	
8	11.053	10927427	V		17.7489	
9	13.079	1294853	V		1.8122	
10	13.079	1209458	V		1.722	
11	13.317	725019	V		1.0174	
12	14.024	725019	V		1.0174	
13	17.265	748778	V		1.0456	
14	18.465	972044	V		1.3766	
15	25.097	72974	V		0.119	
16	25.097	72974	V		0.1192	
17	27.85	172865	V		0.2384	
18	28.379	42298	V		0.1009	
19	29.257	49098	V		0.2145	
20	29.257	49098	V		0.2145	
21	29.255	1238453	V		17.657	
22	30.418	3115408	V		43.657	
23	31.131	4459132	V		7.2612	
24	31.083	1007682	V		2.0659	
25	32.051	210738	V		0.3054	
TOTAL						6171064
						100

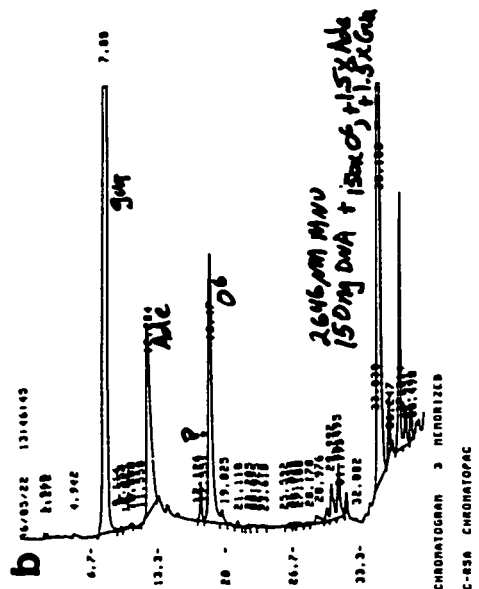
Figure 24 (continued): HPLC Chromatograms with Acid Hydrolysis

In order to try to lower our baseline, we decided to go back to the neutral and acid hydrolysis, with a few modifications (indicated in bold).

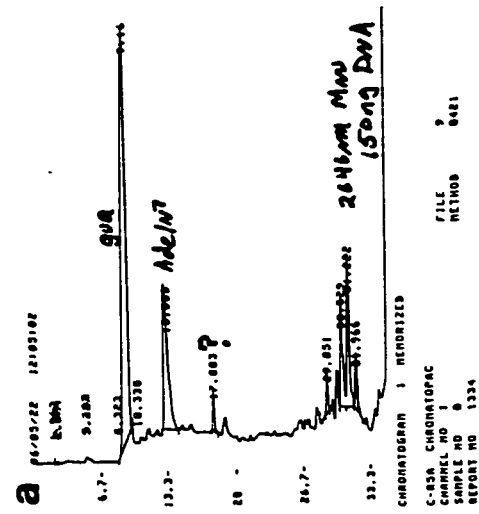
Neutral and Acid Hydrolysis of DNA:

1. Dissolve samples in 1ml of **deionized, distilled water, pH 6.2**
2. Heat samples at **80°C** for **5** minutes (neutral hydrolysis)  
*Labilizes the N7-m-gua adducts, which are released into solution?*
3. Add 100 µl of 1N HCl at 4°C  
*Precipitates DNA, including O<sup>6</sup>-m-gua, guanine and adenine.*
4. Centrifuge at 13,000 rpm in microcentrifuge at 4°C for 10 minutes
5. Remove supernatant, **to make sure DNA adducts are not lost in this step**
6. Resuspend the pellet in 1ml of 0.1 N HCl (pH 1.2)
7. Heat sample at 80°C for 45 minutes (acid hydrolysis)  
*Releases guanine, adenine, and O<sup>6</sup>-m-gua into solution*
8. Centrifuge at 13,000 rpm in microcentrifuge at 4°C for 10 minutes
9. Remove supernatant containing guanine, adenine and O<sup>6</sup>-m-gua
- 10. Filter through 0.65 µM filter**
11. Take OD readings of both the first supernatant (supernatant 1) and the guanine/O<sup>6</sup>-m-gua fraction, to determine proper loading for HPLC

Chromatograms for treated and untreated DNA, using the modified neutral and acid hydrolysis are shown in Figure 25. When we loaded 150 ng of DNA, there was a very small O<sup>6</sup>-m-gua peak (Figures 25a and 25b), which was significantly larger when we loaded 2.25 µg DNA. We also saw the mystery peak in the treated DNA, as well as the untreated DNA (Figure 25d).



PKNO	TIME	AREA	PK	IDNO	CONC	NAME
1	9.16	15056088			56.0688	
2	12.060	5241517			19.1116	
3	17.003	474181			1.4811	
4	20.051	2034098			7.4481	
5	21.311	6817488	V		18.2328	
7	31.966	767389			2.7871	
TOTAL						87310346
						100



PKNO	TIME	AREA	PK	IDNO	CONC	NAME
1	7.08	2689124	0		32.0529	
2	9.323	59811	0		0.0826	
3	12.284	627063	V		7.1483	
4	16.321	7581271	V		18.7481	
5	17.023	242121	V		0.3268	
7	21.118	59812	.		0.1082	
8	22.003	56531	.		0.0756	
9	23.618	39888	V		0.0838	
10	24.234	184539	V		0.1535	
11	26.714	60544	V		0.6964	
12	27.108	64416	V		0.0933	
13	28.178	36696	V		0.0914	
14	28.974	233798	V		0.3071	
15	29.981	244384	V		0.3082	
16	30.495	102984	V		1.4919	
17	31.130	102984	V		1.4919	
18	31.130	2382182	E		34.2578	
19	34.847	634688	E		0.9188	
20	37.214	3681823	V		3.2031	
21	37.931	658399	V		0.9469	
22	38.488	482339	V		0.6688	
TOTAL						69678618
						100

Figure 25: HPLC Chromatograms with Modified Neutral and Acid Hydrolysis: Treated versus Untreated DNA

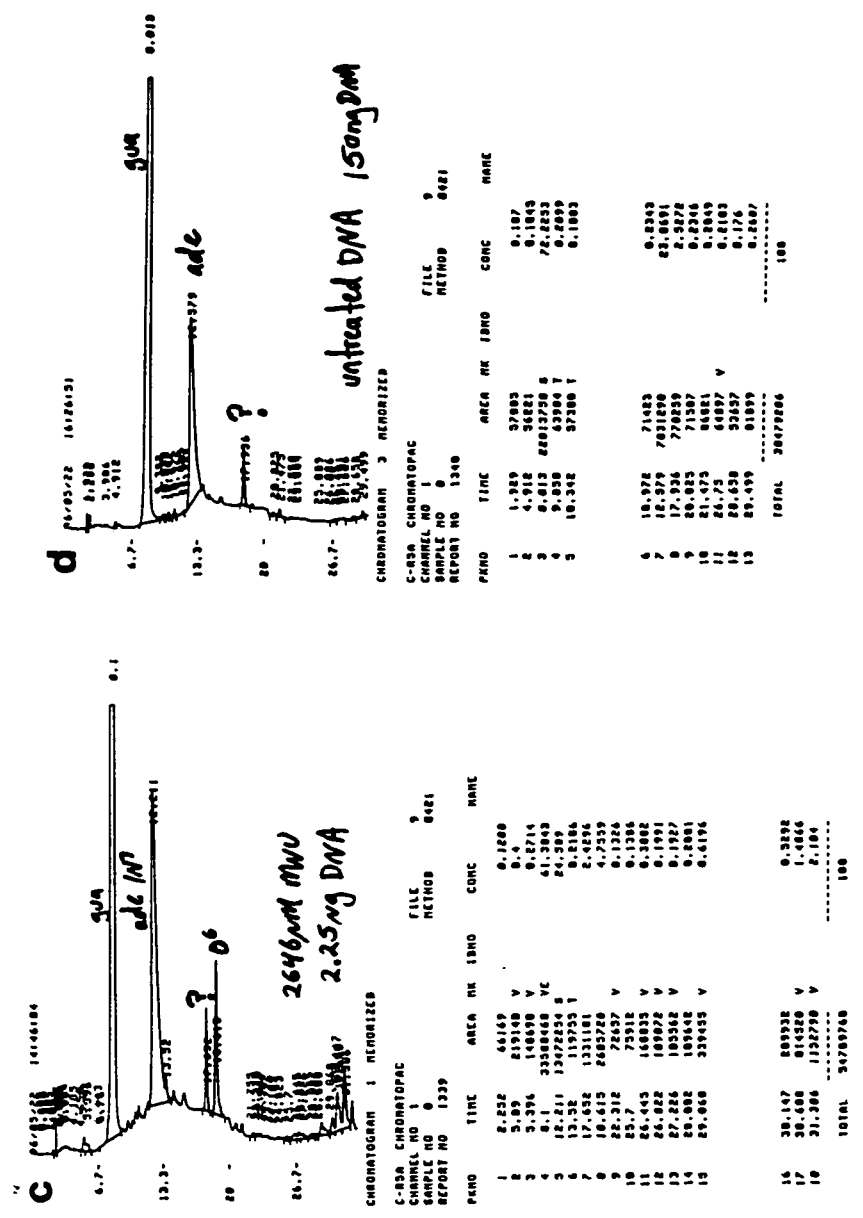


Figure 25 (continued): HPLC Chromatograms with Modified Neutral and Acid Hydrolysis: Treated versus Untreated DNA

We decided that we had optimized the method as much as possible, and that to maximize the number of adducts we could detect, we pooled three experiments for each treatment. Figures 26 shows chromatograms for the MMS samples. We were not able to detect the O<sup>6</sup>-m-gua adduct in these samples.

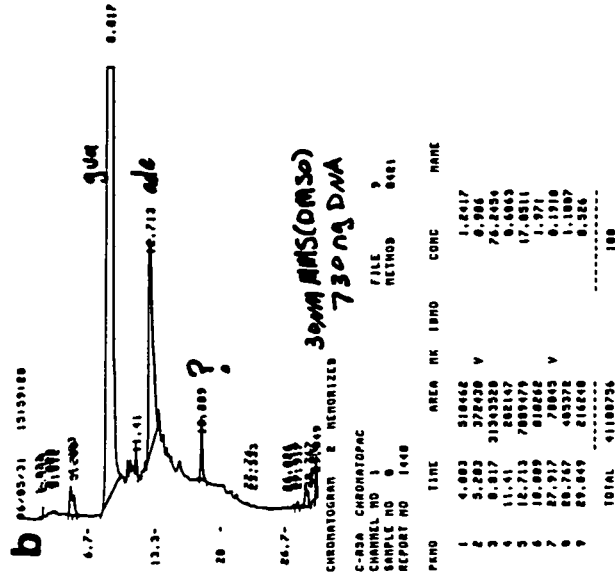
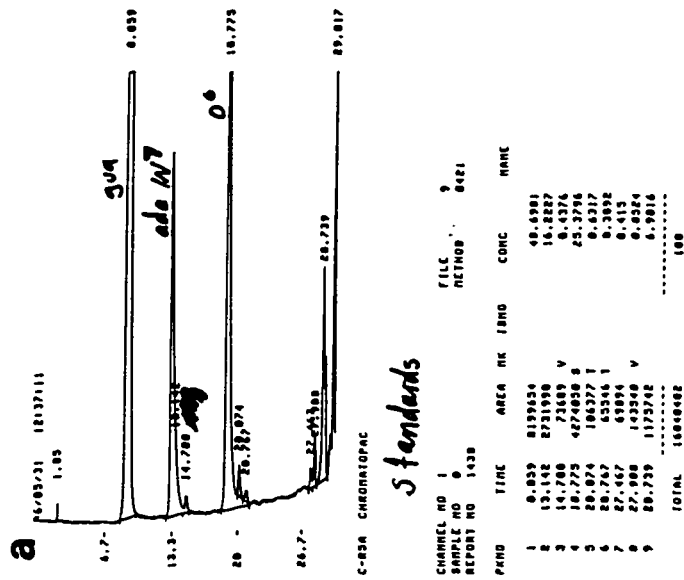


Figure 26: HPLC Chromatograms for MMS Samples

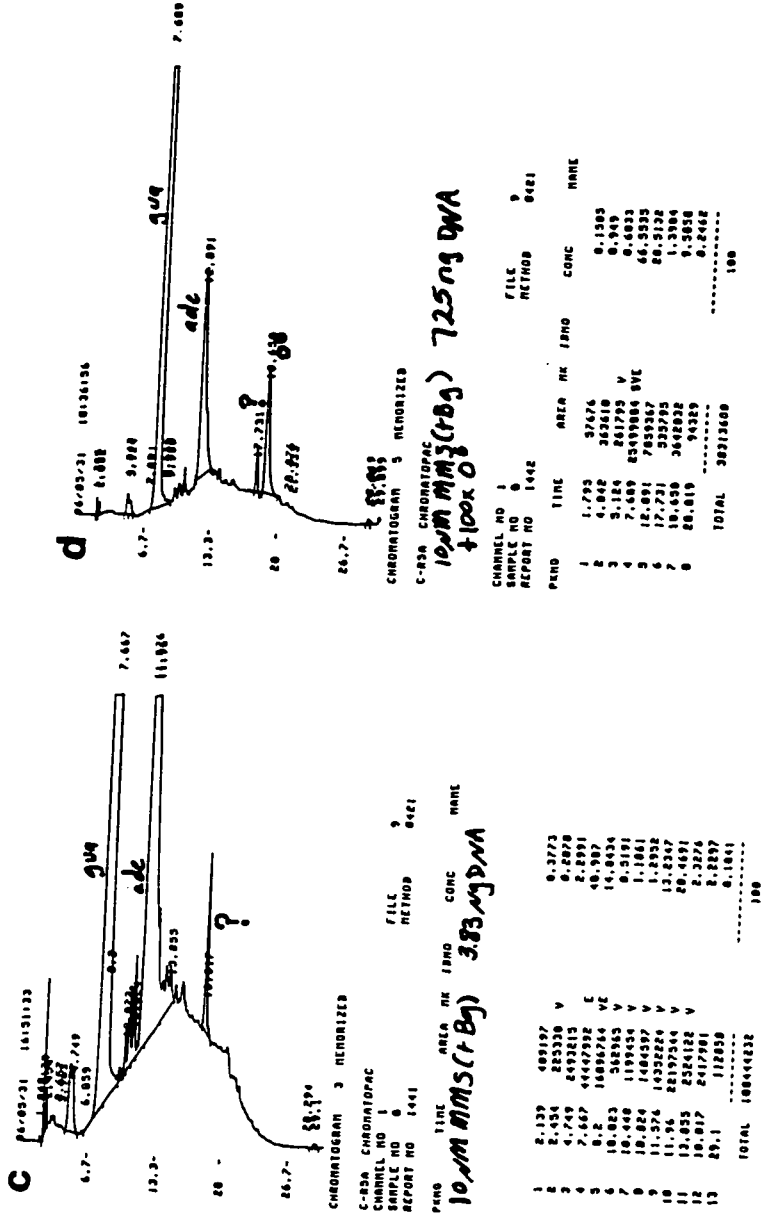


Figure 26 (continued): HPLC Chromatograms for MMS Samples

Next we ran the MNU treated samples (Figure 27). Again, we were not able to detect O<sup>6</sup>-m-gua, possibly due to a very high baseline where the adduct is eluting from the column. We also checked the first supernatant, to make sure that we were not losing the O<sup>6</sup>-m-gua adduct in the first neutral hydrolysis. As indicated in Figure 27 e-g, this did not seem to be the case.



Figure 27: HPLC Chromatograms for MNU Samples

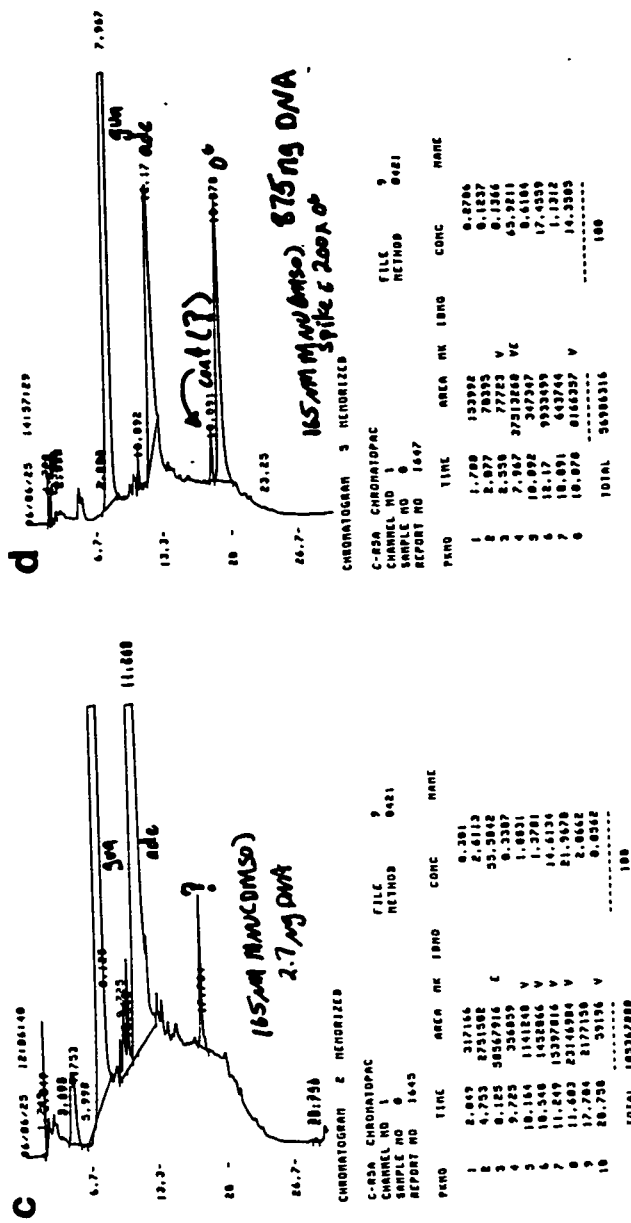


Figure 27 (continued): HPLC Chromatograms for MNU Samples



Figure 27 (continued): HPLC Chromatograms for MNU Samples

So it appears that the HPLC assay with fluorescence detection may not be sensitive enough to detect adducts at the low levels of alkylation present in our samples. Unfortunately, we were limited in the amount of DNA we could load onto the column because of high background, and size of the guanine peak. When there is too much DNA, the guanine and adenine peaks are too big and extend past the point where O6 elutes. Additionally, the baseline gets so high that low levels of alkylation which we would expect in our samples would be below the baseline. One possible reason for the high baseline is that there are many of organic molecules which excite and emit at the wavelengths which we used, and also because these wavelengths are so close together

If there is future interest in measuring DNA adducts, it may be necessary to either use radiolabeled alkylating agents, or adduct-specific antibodies.

## VITA

### EDUCATION

- Ph.D.**, Toxicology - University of Washington (August, 1996)  
Dissertation topic: Developmental toxicity of alkylating agents. DNA repair and modes of cell death  
Thesis: *Effects of Alkylating Agents on Differentiating Cell Cultures*
- M.S.**, Environmental Engineering and Science - University of Washington 1986  
Research focus: Stream productivity  
Thesis: *Use of Artificial Channels to Study How Nutrients, Velocity and Turbidity Affect Periphyton.*
- B.A.**, Biology, *cum laude* - Wellesley College 1983

### PUBLICATIONS

- Faustman, E.M., R.A. Ponce, **M.R. Seeley** and S.G. Whittaker (1996) Mechanisms of Developmental Toxicity. In *Handbook of Teratology*, Ronald Hood, editor. Boca Raton: CRC Press.
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