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Characterization of Chemical and Radiation-Induced DNA Damage in the MDBK Cell Culture System

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CHARACTERIZATION OF CHEMICAL AND RADIATION-INDUCED DNA DAMAGE IN THE MDBK CELL CULTURE SYSTEM

by

Jeffrey R. Hincks

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

of

DOCTOR OF PHILOSOPHY

in

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Major Professor	Committee Member
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DEDICATION AND ACKNOWLEDGEMENTS

This dissertation is dedicated to my wife Karla for her love, understanding and support of my work. I am also very grateful to my mother and father for their love, encouragement and support.

I wish to express my gratitude to Dr. Roger Coulombe, my major advisor, who gave his time, guidance and support throughout this study and I am proud to be his first graduate student.

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ABSTRACT

Characterization of Chemical and Radiation-Induced DNA Damage in the MDBK Cell Culture System

by

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A mechanism common to many carcinogens and mutagens is the interaction with cellular DNA. Alkaline elution is a sensitive and commonly used technique to detect cellular DNA damage in the form of DNA single-strand breaks (SSB), DNA-protein cross-links (DPC), and DNA interstrand cross-links (ISC). In the present study, gravity-flow alkaline elution was developed and validated to rapidly detect DNA damage in the MDBK cell culture system by genotoxic agents.

The system was first validated by detecting a dose dependent induction of DNA SSB in cells exposed to 25-1500 rad of X-ray. The assay reliably detected the DNA damage of seven genotoxic carcinogens and showed no detectable DNA damage in six non-genotoxic compounds.

Gravity-flow alkaline elution was then used to detect the induction of DNA SSB and subsequent repair in cells exposed to ultraviolet (UV) radiation from sunlight, suntan booth, or germicidal lamp (254 nm). The induction of DNA SSB was dose-dependent for all

three exposure systems. Repair of sunlight and suntan induced DNA SSB were similar, whereas cells exposed to 254 nm UV did not show any repair. Repair studies using the DNA repair inhibitors 1-B-D-arabinofuranosylcytosine and hydroxyurea indicated DNA excision repair was involved. The mechanisms underlying UV induced DNA damage and repair appear similar for both sunlight and suntan booth, but different than 254 nm UV.

The gravity-flow system was modified to rapidly detect DNA cross-links in MDBK cells exposed the cross-linking agents, nitrogen mustard, mitomycin C, and 254 nm UV radiation. Both drugs induced DPC and ISC whereas UV radiation induced protein-associated DNA strand breaks.

The gravity-flow system was used to characterize the DNA damage in cells exposed to seven pyrrolizidine alkaloids (PAs). Five of the PAs induced both DPC and ISC, while heliosupine induced only DPC, retronecine induced no cross-links, and none of the PAs induced DNA SSB. Since these PAs differ only in the extent of substitution of the diester side chain, these results indicate that these substituents are an important determinant in the induction of DNA damage by pyrrolizidine alkaloids.

(100 pages)

CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

INTRODUCTION

Many environmental agents are capable of disrupting the integrity of cellular DNA. The genotoxic results could have a profound effect on human health if the exposure is substantial enough to become permanently expressed in the cellular genome. Adverse changes in the genome may evolve into mutational events, or may develop further into the initiation steps of cancer. As a result, there is great value in studying the specific interactions of agents with cellular DNA.

The alkaline elution technique, developed by Kohn and Grimek-Ewig [1973], has been proven to be one of the more sensitive and reliable methods to detect specific DNA lesions [Kohn et al., 1976]. This technique has been employed in determining the genetic damage induced by UV light, X-irradiation and chemical agents in vitro [Bradley and Erickson, 1981; Fornace,1982; Johnston et al., 1983] and in vivo [Eastman and Bresnick, 1978; Petzold and Swenberg, 1978; Bolognesi et al., 1981]. Several studies have shown a high correlation between the carcinogenic potential of compounds and the ability to induce DNA strand breaks as measured by alkaline elution [Swenberg et al., 1976; Parodi et al.,1982; Sina et al., 1983].

The alkaline elution procedure utilizes filtration to discriminate various lengths of single-stranded DNA in an alkaline solution. The technique depends on an agent to either induce direct DNA strand

breaks and/or alkali-liable lesions, or for DNA repair endonucleases to excise the damaged sites leaving temporary repair nicks in the DNA. The DNA damage is detected by an increase in DNA single-strand breaks (SSB) as measured by the increase in DNA eluting through the filter in the alkaline (pH 12.2) solution.

This technique has also been modified to detect total DNA cross-links as well as specific interactions, eg. DNA-protein cross-links and DNA interstrand cross-links [Fornace and Kohn, 1976; Kohn et al., 1981]. Determination of cross-links by alkaline elution is based on the reduction of DNA eluting through the filter. The occurrence of interstrand cross-links (ISC) can be differentiated from DNA-protein cross-links (DPC) based on the resistance of ISC to proteinase digestion during the alkaline elution assay.

Major drawbacks of conventional alkaline elution include the excessive time required to pump the eluting solution (ca. 15 hr) and the pumping system limits the number of samples processed per day. In addition, the collection of several fractions per sample makes the system very cumbersome. As a result, a modification of the alkaline elution method, whereby the alkaline solution is eluted by gravity and collected as one fraction, would greatly enhance this procedure. This new system would appear especially attractive as a screening method in the detection of potential genotoxic agents, by maintaining the sensitivity and reliability yet reducing the time and expense compared to conventional alkaline elution.

In the following study, one of two major objectives was to validate the new gravity-flow system in the detection of chemical and

radiation-induced DNA damage by measuring DNA SSB and DNA cross-links. The gravity-flow system was validated for the detection of DNA SSB by exposing MDBK epithelial cells to various doses of X-irradiation or seven genotoxic carcinogens, as well as six non-genotoxic agents. The system was also validated for the detection of DNA cross-links by exposing MDBK cells to the known cross-linking agents nitrogen mustard (HN2), mitomycin C (MMC) and ultraviolet (UV) radiation.

The second major objective of this project was to apply the gravity-flow system in assessing potential environmental genotoxic hazards. One such hazard involves the environmental exposure of humans to UV radiation. Many researchers have investigated the biological effects of UV radiation using 254 nm UV germicidal lamps, which primarily cause the induction of DNA pyrimidine dimers [Jagger, 1976]. Although 254 nm lamps are a useful laboratory tool, they are environmentally irrelevant. Many studies have demonstrated that nondimer DNA lesions have a significant role in the mechanisms of sunlight induced DNA damage [Fornace et al., 1976; Elkind and Han, 1978; Parsons and Goss, 1980; Parsons and Musk, 1982; Francis and Regan, 1986]. As a result, inferences about sunlight genotoxicity should not be made based on results from 254 nm exposure studies.

Due to the increased human exposures to UV radiation from sunlight and suntan booths, additional information is needed regarding UV-induced genotoxicity. Thus, the following study compared the induction of DNA SSB and subsequent repair in epithelial cells exposed to UV radiation from sunlight, a commercial suntan booth and a

germicidal lamp (254 nm) using gravity-flow alkaline elution. This part of the study was designed with the intention of better understanding the mechanisms underlying the inherent carcinogenic risks to humans exposed to these UV sources.

Another environmental problem associated with genotoxicity is the poisoning of animals and humans by plant alkaloids. Pyrrolizidine alkaloids (PAs) are naturally-occurring plant alkaloids which are primarily hepatic and pulmonary toxicants [McLean, 1970]. Data from a limited number of studies indicate that some PAs are carcinogenic [Svoboda and Reddy, 1972; Newberne and Rogers, 1973; Kuhara et al., 1980]. The reactive metabolite(s) is thought to be a pyrrole derivative with bifunctional alkylating capabilities.

Several PAs have tested positive for mutagenicity by the Salmonella/mammalian microsomal (Ames) assay [Yamanaka et al., 1979] or for genotoxicity by various unscheduled DNA synthesis assays [Green et al., 1981; Mori et al., 1985; Griffin and Segall, 1986]. Petry et al. [1984; 1986] characterized the DNA damage of two PAs, monocrotaline and jacobine, as inducing DNA cross-links in primary liver hepatocytes. Thus, the following project also characterized the mechanism by which seven structurally related PAs interact with DNA in MDBK cells by gravity-flow alkaline elution. The results indicated possible structure-activity relationships of PA induced DNA damage.

REVIEW OF THE LITERATURE

Genetic Toxicology

Genetic toxicology is a subdiscipline of toxicology which investigates the adverse effects of agents on the hereditary components (nucleic acids) in living systems. Many toxic agents damage the genetic material of cells to such a severe extent that it produces cell death. The primary interest of genetic toxicology is to study the effects of sublethal doses of agents on the cellular genome as well as the possible genetic consequences in subsequent daughter cells. Adverse changes in the cellular genome may result in mutation which may also develop into a carcinogenic event.

Chemical carcinogenesis is a multistage process in which the initiation stage results in an irreversible mutagenic event [Weinstein et al, 1984]. These mutations result from the reactive chemical or metabolite interacting with the cellular DNA [Miller and Miller, 1981; Farber, 1981]. There are supporters of a different theory of carcinogenesis involving epigenetic mechanisms, also known as developmental switching. This theory contends that epigenetic interactions, which are defined as changes in gene expression without changes in DNA base sequences, are the causative mechanisms in chemical carcinogenesis. Straus [1981] cited evidence of the similarities of cellular differentiation and cancer-involving mutational mechanisms, and showed that mutational and epigenetic events are not mutually exclusive. Nevertheless, there is a great need to study chemical interactions with DNA to better understand the

mechanisms of chemical carcinogenesis and, as a result, better predict the carcinogenic potential of chemicals.

Genotoxic Assays

Mutagenesis

There is considerable evidence supporting the fact that most carcinogens are also mutagens [McCann and Ames, 1975; Poirer and Weisburger, 1979; Rinkus and Legator, 1979]. This has lead to the development of a variety of mutagenic assays to determine the carcinogenic potential of chemicals. The most commonly used *in vitro* mutagenicity assay is the *Salmonella*/mammalian microsomal (Ames) test which was first validated by McCann and Ames [1975] and described in detail by Maron and Ames [1983]. Briefly, the assay detects a chemically-induced back mutation at the histidine locus in various strains of *Salmonella typhimurium*. Some strains are more sensitive to some compounds than others and certain strains are more specific for the type of mutations induced by the chemical, e.g. tester strain TA 100 detects base-pair substitutions and TA 98 detects various frameshift mutations [Maron and Ames, 1983]. So that mutagens which require bioactivation may be detected, the *Salmonella* test can be coupled with a mammalian liver S9 preparation which contains cytochrome P-450 monooxygenases. The Ames assay is commonly used because it is simple and relatively inexpensive, relative to *in vivo* carcinogenesis assays. However, there are several drawbacks to this assay. The assay only measures a biological end-point, mutation, as the genotoxic insult; other interactions, with DNA not resulting in a

mutational event, go undetected. Futhermore, the use of a bacterial cell as a target is considered by many to be a poor model for mammalian systems.

Cytogenetics

A common genotoxic technique used to detect chromosome aberrations is cytogenetics, in which metaphase chromosomes are morphologically examined. Many staining techniques have been developed, e.g. quinacrine and giemsa, that reveal individual chromosomes with a distinctive banding pattern [Sanchez and Yunis, 1977]. Chromosomal abnormalities induced by excessive translocations, aneuploidy and clastogenic events can be detected by changes in the banding pattern. The detection of abnormal banding patterns usually indicate severe DNA damage while minor changes in DNA are not detected. The sister chromatid exchange is a commonly used cytogenetic assay and will be described in detail under the topic of primary DNA damage.

Primary DNA damage

Another major class of genotoxic assays is those which detect primary DNA damage, which can be measured by the stimulation of DNA repair, recombination between chromatids, or the detection of DNA strand breaks and DNA cross-links.

Unscheduled DNA synthesis. The most common assay used in the detection of DNA repair is the unscheduled DNA synthesis (UDS) assay. In this technique, cells are exposed to the test compound and [^{3}H]-thymidine with the premise that the incorporation of labeled thymidine is due to repair (UDS) of the DNA damage induced by the test compound.

The detection of DNA repair and subsequent incorporation of [^{3}H]-thymidine may be performed by isolation of cellular DNA and liquid scintillation counting or by autoradiography. DNA replication synthesis may increase the incorporation of labeled thymidine, thus interfering with the detections of UDS. A modification of this method, using primary hepatocytes [Williams, 1980], eliminated problems inherent in earlier methods caused by the slow replicating capabilities of hepatocytes within the first 48 hours after isolation. Thus, there is less uptake of labeled thymidine in these cells, due to replication. Furthermore, primary hepatocytes contain higher levels of cytochrome P-450 monooxygenase activity than most cell culture systems.

Problems with the UDS assay involve the simultaneous incubation of cells with the test agent and [^{3}H]-thymidine. Some agents may induce changes in cell membrane permeability which may increase the uptake of the labeled thymidine, or the test agent may stimulate replicative DNA synthesis. Such events may result in false positives in the UDS assay. Snyder and Van Houten [1986] showed that human fibroblasts exposed to formaldehyde exhibited different UDS responses depending on whether labeled thymidine or labeled deoxycytidine was used in the assay. This suggested that formaldehyde changed the cell membrane permeability, which selectively altered the uptake of the labeled deoxynucleoside precursors.

Another drawback with the UDS assay includes the large number of genotoxic carcinogens testing negative or weakly positive [Williams et al, 1982; Mitchell et al, 1983]. In addition, the assay does not

determine the molecular interaction between a potential genotoxic compound and DNA. These problems taken together indicate the potential of missed detection by UDS of agents that induce low levels of DNA damage.

Sister chromatid exchange. Detection of genotoxicity by measuring sister chromatid exchanges (SCE) was first established by Taylor [1958]. Better staining techniques have improved the performance of this assay. The method depends upon the incorporation into DNA of the nucleotide analog 5-bromodeoxyuridine (BrdU). After two cell divisions, the sister chromatids in cells exposed to BrdU are asymmetrically labeled with BrdU, and as a result stain differently with Hoechst 33258 dye. SCEs are visualized by light and darkly stained areas within the same chromatid. The formation of SCEs is not necessarily a genotoxic event, yet SCE correlates with potential genotoxicity. Problems associated with SCE in the detection of genotoxicity include: 1) the mechanisms of chemical-induced SCE are not known; 2) SCEs are not genotoxic events; and 3) similar to UDS, SCE does not reveal the molecular interactions of potential genotoxic agents with DNA.

Alkaline elution. Another technique used in the detection of primary DNA damage and one which may be used to characterize the molecular interactions with DNA, is alkaline elution. This method was developed by Kohn and Grimek-Ewig [1973] and has proven to be one of the more sensitive and reliable methods to detect specific DNA damage [Kohn et al., 1974; Kohn et al., 1976]. The principles of alkaline elution have been explained in detail previously [Kohn et al., 1981]

and will be mentioned here briefly. The technique employs a filter to discriminate various lengths of single-strand DNA in an alkaline solution, whereby short strands of DNA elute at a faster rate than longer strands. Variations of the method allow for detailed characterization of the types of DNA damage induced by a genotoxic agent such as DNA single-strand breaks (SSB), alkali-labile sites, DNA-protein cross-links (DPC), DNA-DNA interstrand cross-links (ISC), and protein associated DNA SSB. Alkaline elution has the sensitivity to detect one DNA lesion per 10^7 nucleotides [Kohn et al., 1981], which is more sensitive than alkaline sucrose sedimentation [Kohn et al., 1974].

To detect DNA SSB, protein adsorption to the filter is minimized by using polycarbonate filters, lysing cells with a solution containing sodium dodecyl sulfate (SDS) with proteinase K, and eluting solution (pH 12.2-12.4) containing SDS. When cells are lysed, most of the cellular protein and RNA are washed through the filter leaving the double-stranded DNA on the filter.

The DNA is eluted by slowly pumping the alkaline solution through the DNA, which is in single-stranded form, and collecting the DNA elution fractions. Elution is generally complete in 12-16 hours and DNA SSB are detected by an increased elution rate of DNA. Alkali-labile sites are DNA lesions that become DNA SSB in high alkaline solutions. These lesions are detected by comparing the eluting rates of two different samples using eluting solutions of different pH (e.g., pH 12.1 and pH 12.6). Chemically-induced alkali-labile sites are detected as an increase in DNA SSB measured with the higher pH

eluting solution compared to the results utilizing the lower pH solution.

The extent of chemically-induced DNA cross-links is determined by first exposing the cells to the test agent, then producing a background level of DNA SSB in the cell (usually by x-irradiation or gamma-irradiation), followed by DNA elution. A reduction in DNA eluted in cells treated with x-ray and the test agent, compared to x-ray alone, indicates agent-induced DNA cross-links. The formation of DNA ISC may link short DNA strands together thus reducing the amount of DNA which elutes through the filter. In the case of DPC, the test agent may covalently bind proteins to DNA, which reduces the amount of DNA that elutes through the filter.

DNA cross-links are assessed under conditions which promote adsorption of possible cross-linking proteins to the membrane filter. Such conditions include the use of polyvinyl chloride filters and lysing and eluting solutions without SDS. Proteinase K is included in the lysing solution to hydrolyze possible DPC. Resistance to proteinase K digestion indicates the presence of DNA ISC. A variation in the cross-link assay was developed to specifically detect DPC [Kohn and Ewig, 1979]. This procedure utilizes a higher dose of x-ray (from 2000 to 10,000 rad) to induce very short fragments of single-stranded DNA. These fragments will elute rapidly unless they are bound to a protein, in which case the DNA-protein fragments may be retained on the filter reducing the amount of DNA eluted.

Characterization of DNA damage by alkaline elution was first studied by detecting DNA SSB in cells exposed to X-rays [Kohn and

Grimek-Ewig, 1973]. A dose-dependent increase in elution rate was seen with X-ray exposure suggesting a dose-dependent increase DNA SSB damage. Cells exposed to X-ray followed by a post-incubation in culture medium showed a decrease in elution rates, which showed that cells are capable of repairing the X-ray induced DNA SSB. DNA from cells exposed to a dose of 1000 rad was completely repaired within 30 minutes [Kohn and Grimek-Ewig, 1973].

Fornace et al. [1976] evaluated UV-induced DNA SSB and repair in both normal and repair-deficient fibroblasts from xeroderma pigmentosum (XP) patients. Detection of DNA SSB in the normal cells required a short post-incubation period after UV exposure. These DNA SSB which were produced by endonuclease repair enzymes were slowly resealed. In XP cells, no DNA SSB were detected because these cells are endonuclease repair deficient. In a variant form of XP cells which are partially deficient in DNA repair, the formation of DNA SSB was similar to normal cells, but resealing of the SSB was much slower. Thus, the alkaline elution technique can be used to characterize DNA damage repair capabilities of cells using the described post-incubation techniques.

DNA repair inhibitors may be used with alkaline elution to detect repair of DNA damage. The combination of 1-$\underline{B}$-D-arabinofuranosylcytosine (Ara C) and hydroxyurea (HU) has been shown to inhibit the polymerase step of excision repair in cells with damaged DNA [Dunn and Regan, 1979]. Fornace [1982] used Ara C and HU to inhibit DNA excision repair in cells exposed to the DNA cross-linkers trans-platinum or formaldehyde. Inhibition of the polymerase

step of DNA excision repair creates unsealed DNA repair fragments which are detected by an increase in DNA elution. Fornace [1982] showed that an increase in DNA elution rates occurred in cells exposed to either cross-linking agent and subsequent post-incubation with Ara C and HU. This finding suggested that DNA excision repair is important in the repair of DNA damage induced by trans-platinum or formaldehyde. Other specific DNA repair inhibitors have been used with alkaline elution to investigate the enzymes involved in DNA repair after exposure to various genotoxic agents. These techniques have been recently reviewed [Collins et al, 1984].

The induction of DNA SSB has been correlated with the carcinogenic potential of various compounds. Swenberg et al. [1976] used alkaline elution to detect DNA SSB in Chinese hamster V79 cells exposed to various pro- and complete carcinogens. The responses were generally dose-dependent and none of the noncarcinogens induced DNA damage. Parodi et al. [1982] showed a good correlation between the carcinogenic potential of 21 compounds and the induction of DNA SSB. These results imply that the detection of DNA SSB by alkaline elution is an indicator of chemical carcinogenic potential. Rat hepatocytes have also been used to predict the carcinogenic potential of 91 compounds by the induction of DNA SSB as assessed by alkaline elution [Sina et al., 1983].

DNA cross-links as assessed by alkaline elution were first characterized using UV radiation in normal and repair deficient XP cells [Fornace and Kohn, 1976]. A dose-dependent reduction in the rate of DNA elution was observed in cells exposed to UV radiation and

x-ray compared to cells exposed to x-ray alone, indicating an induction of DNA cross-links. DNA cross-links were induced in both normal and XP cells, but damage was partially repaired after 12 hours post-UV incubation, whereas no repair occurred in the XP cells.

The use of this system to detect chemically-induced DNA cross-links was validated using the bi-alkylating agents nitrogen mustard (HN2) and mitomycin C (MMC) [Fornace and Little, 1977]. These compounds produced a dose-dependent induction of DNA cross-links (both DPC and ISC) in human fibroblasts. Alkaline elution has been used in the detection and characterization of DNA cross-links induced by the chemical carcinogens N-acetoxy-2-acetylaminofluorene and 7,12-dimethylbenz[a]-anthrene [Fornace and Little, 1979], as well as formaldehyde [Bedford and Fox, 1981; Craft et al., 1987] and acrolein [Crook et al., 1986].

A variety of antitumor drugs have been shown to exert their cytotoxic action on tumor cells by cross-linking DNA. Examples of these include nitrosoureas [Ewig and Kohn, 1977], the platinum coordination compounds trans-platinum [Kohn and Ewig, 1979] and cis-platinum [Zwelling et al., 1979], cyclophosphamides [Erickson et al., 1980] and diaziquone [Szmigiero et al., 1984].

Alkaline elution has also been used to detected protein-associated DNA SSB in cells exposed to DNA intercalating agents [Ross et al., 1979; Zwelling et al., 1981]. These agents induced both DPC and DNA SSB, but the SSB were only detected upon proteinase K digestion. These researchers suggested the intercalating agents induced distortion in the DNA helical structure, thus stimulating DNA repair

enzymes to relieve the torsional stress by breaking, unwinding and resealing the DNA strands. As a result, the proteins bound to the strand breaks were thought to be repair nucleases, such as topoisomerase. Incubation with proteinase K acts to remove the nucleases and allow the detection of DNA SSB induced by repair enzymes using the alkaline elution technique.

Problems inherent with the conventional alkaline elution method revolve around the pumping process and collection of the eluted DNA fractions. In order to process several samples, expensive pumps and fraction collectors are needed. In addition, excessive time is required to pump the eluting solution (usually 15 hours) and the processing of several fractions per sample makes the system very cumbersome. A modification to reduce time and expense would make the alkaline elution technique more feasible as a screening method in the detection of potential genotoxic agents.

Ultraviolet Radiation and Genotoxicity

Ultraviolet (UV) radiation represents the range of wavelengths between the violet region of visible light and x-rays. The ultraviolet wavelengths are generally subdivided into three regions, UV-C (200-290 mm), UV-B (290-320 mm) and UV-A (320-400 mm). Solar UV radiation consists of all but UV-C since these wavelengths are absorbed by ozone in the stratosphere [Parrish et al., 1978].

There is a considerable amount of evidence supporting the view that UV radiation in sunlight has a role in the induction of human basal cell and squamous cell carcinoma [for review see Parrish et al.,

1978; Van der Leun, 1984; Daynes et al., 1985]. These carcinomas are found primarily over the head and neck regions and are more common in people who work outdoors. Additionally, both basal cell and squamous cell carcinomas are more prevalent in geographical locations of high sun exposure [Urbach et al., 1972]. It has been shown that UV-B radiation has greater carcinogenic potential than radiation from UV-C or UV-A in experimental animals [Urbach et al., 1974]. It has been postulated that UV-C radiation does not penetrate skin efficiently to cause the necessary genetic damage to the underlying cells. But UV-C, specifically 254 nm radiation, has been termed "germicidal radiation" because of its ability to readily kill single-cell organisms [Parrish et al., 1978]. Willis et al. [1981] showed evidence of synergism between UV-A and UV-B radiations in the formation of cancer, but other studies have not supported this [for review see Van der Leun, 1984].

The connection between UV radiation and the formation of tumors involves the ability of UV radiation to induce DNA damage. Many researchers have investigated UV-induced DNA damage and repair using 254 mm UV germicidal lamps which primarily produce DNA pyrimidine dimers in exposed cells [Jagger, 1976]. Pyrimidine dimers are repaired from DNA in mammalian cells by DNA excision repair [Lindahl, 1979]. DNA excision repair begins by an incision step close to the dimer, followed by DNA chain breakage at the 5' side of the dimer and removal of the dimer by an exonuclease, which releases a small oligonucleotide [Lindahl, 1979]. The repair is completed by a DNA polymerase which replicates the missing segments of DNA then subsequent ligation by DNA ligase.

Cleaver [1969] showed that xeroderma pigmentosum (XP) cells were deficient in the rate of excision repair of UV-induced DNA damage. Thus, the formation of DNA dimers by 254 nm UV radiation and the inability to properly repair the damage may result in mutagenesis and/or carcinogenesis. But studies utilizing 254 nm or UV-C radiation to induce DNA damage may not accurately depict the mechanisms involved with sunlight-induced carcinogenesis.

Mammalian cells exposed to sunlight showed a much higher ratio of nondimer (e.g. DNA strand breaks or DNA protein cross-links) to dimer DNA lesions, compared to that of 254 nm UV irradiated cells [Elkind and Han, 1978]. Also, many studies have demonstrated the importance of nondimer lesions in sunlight-induced DNA damage [Fornace et al., 1976; Parsons and Gross, 1980; Parsons and Musk, 1982; Rosenstein and Ducore, 1983; Francis and Regan, 1986].

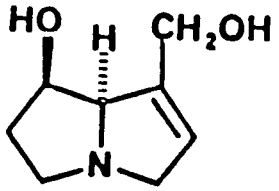
Enninga et al. [1986] investigated the dependence of UV wavelength as it related to pyrimidine dimer formation, cell killing and mutation in human fibroblast cells. They demonstrated that UV-induced pyrimidine dimers (especially at the longer wavelengths) did not correlate with the induction of mutation or cell killing. Thus, nondimer DNA lesions may be important in solar UV radiation-induced mutagenesis. Recently, DNA excision repair inhibitors were used to inhibit the repair of UV induced DNA dimer and nondimer lesions [Rosenstein et al., 1986]. The results indicated that different repair pathways are involved in the repair of DNA dimer lesions compared to nondimer lesions in DNA damage induced by UV wavelengths greater than 310 nm.

Taken together, the above studies indicate that there is a difference in the type of DNA damage and repair that is induced by 254 nm UV radiation than is induced by sunlight. As a result, risk estimates of sunlight-induced carcinogenesis should be based on studies utilizing sunlight as a source of UV radiation (UV-A and UV-B) and not 254 nm UV radiation.

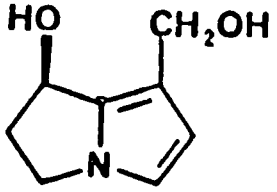
Pyrrolizdine Alkaloids and Genotoxicity

Pyrrolizidine alkaloids (PAs) are constituents found in a variety of plants in the genera Senecio, Crotalaria, Heliotropium, and Symphytum [Bull et al., 1968; Smith and Culvenor, 1981]. Many of the PAs are toxic and have been involved in livestock and human poisonings. The toxicities include veno-occlusive disease of the liver, gastrointestinal lesions, pulmonary hypertension, right ventricular hypertrophy and central nervous disorders [McLean, 1970]. Human poisonings have occurred from exposure to contaminated herbal teas or medicinals [Stillman et al., 1977; Huxtable, 1980].

The structure of the PA retronecine is shown in Figure II-1. McLean [1970] noted that essential structures for PA toxicity include a double bond in the pyrrolizidine ring at C1,2, esterified hydroxyl groups on the pyrrolizidine ring, and at least one branched carbon on the ester side chains. The conversion of PAs to a toxic metabolite is mediated by P-450 monooxygenases [Mattocks, 1968]. The major toxic metabolite of PAs is thought to be a reactive pyrrole formed by allylic oxidation in the pyrrolizidine ring followed by dehydration [Mattocks and White, 1971]. Figure I-1 shows the structure of



RETRONECINE



DEHYDRORETRONECINE

Figure I-1. Structure of retronecine and the reactive pyrrole metabolite dehydroretronecine.

dehydroretronecine an example of a pyrrole metabolite. These pyrrolic metabolites can form alkylating groups at the site of either or both ester linkages, thus producing bi-alkylating agents. Another toxic metabolite is formed from N-oxides which are less toxic than the free bases but may also form reactive pyrrole metabolites [Mattocks, 1971].

Other reactive metabolites may be involved in the toxicity of PAs. The toxic properties of PAs may involve the epoxidation of a ring double bond, as is the case of aflatoxin B_1 [Schoental, 1970]. Segall et al. [1985] identified trans-4-hydroxy-2-hexanol, a possible fragmentation product of the pyrrolizidine ring, as a reactive metabolite of the PA senecionine. Futhermore, LaFranconi et al., [1985] isolated a biliary metabolite of the PA monocrotaline which contained the pyrrole ring structure with one of the esterified side chain groups partially degraded. Thus, several metabolites may be involved in the toxic activities of PAs.

The DNA-alkylating capabilities of the reactive pyrrole metabolites would suggest PAs have mutagenic and carcinogenic activities. In fact, various PAs were shown to be mutagenic in the Salmonella/mammalian microsomal (Ames) assay [Yamanaka et al., 1979] and carcinogenic in animal studies [Bull et al., 1968; Hirono, 1981]. Furthermore, the PA indicine N-oxide has been used in clinical trials as an antitumor agent [King et al., 1987].

The genotoxic potential of several PAs has been investigated. Senecephylline and senecionine were shown to covalently bind DNA in rat liver, lung, and kidney tissue [Candrian et al., 1985]. A dehydro-metabolite of monocrotaline has been shown to form adducts at

the N^2 of deoxyguanosine [Robertson, 1982]. Mori et al. [1985] showed that 13 out 15 pyrrolizidine alkaloids tested positive for genotoxicity using the hepatocyte primary culture-DNA repair test. Monocrotaline [Petry et al., 1984] and jacobine [Petry et al., 1986] induced DPC and ISC, but not DNA SSB, as detected by alkaline elution. Further studies are needed to better understand structure-activity relationships and possible mechanisms involved in PA-induced genotoxicity.

CHAPTER II

MATERIALS AND METHODS

CHEMICAL AGENTS

The following compounds were dissolved in spectral grade dimethyl sulfoxide (DMSO; J.T. Baker): methyl methanesulfonate (MMS), methylnitrosourea (MNU), 2-acetylaminofluorene (AAF), N-diethylnitrosamine (DEN), and 12-O-tetradecanoyl phorbol-13-acetate (TPA; Sigma Chemical Co.); N-amyl-N'-nitro-N-nitrosoguanidine (ANNG), ethanol (ETOH), and acetaldehyde (AA; Aldrich Chemical Co.); aflatoxin B_1 (AFB_1; Calbiochem); 2-hydroxy-benzo[a]pyrene (BP; Chemical Carcinogen Reference Repository of the National Cancer Institute); saccharin (SAC; Matheson Coleman and Bell); phenobarbital (PB; Merck and Co., Inc.).

The cross-linking agents bis(2-chloroethyl)methylamine (nitrogen mustard; HN2) and mitomycin C (MMC) were purchased from Sigma Chemical Co. (St. Louis, MO). These agents were dissolved in phosphate buffered saline (PBS; 0.12 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, and 0.1 mM KH_2PO_4, pH 7.4) and the stock solutions were added directly to the cell culture media in a volume that did not exceed 1.0 percent.

The pyrrolizidine alkaloids (PAs) seneciphylline, senecionine, retrorsine, and retronecine were extracted and isolated from Senecio vulgaris (common groundsel) as outlined by Segall [1979 a,b]. Riddelline was isolated from Senecio sp. and monocrotaline from Crotalaria sp. by the method of Molyneux et al. [1979]. Heliosupine was isolated from Cynoglossum officianale (hounds-tongue) as described

by Knight et al. [1984]. The PAs were dissolved in DMSO and the stock solutions were added to the cell culture media in a volume that did not exceed 1.0 percent. Proteinase K was obtained from Sigma Chemical Co.

IRRADIATION

Cells were X-irradiated at 0°C with an X-ray generator (Rich-Seifert and Co.) operating at 150 kV and 12 mA supplying a dose rate of 200 rad/min for doses of 600 rad or less, 400 rad/min for 800 and 1200 rad doses, and 1000 rad/min for the dose of 3000 rad. Other cells were exposed to gamma rays using a ^{137}Cs-irradiator at a dose rate of 171 rad/min. Both sources were calibrated using the Fricke's ferrous sulfate dosimetry method [ICRU, 1969].

Spectral irradiance scans were performed on all three UV light sources using a spectral radiometer (Optronics, Model 742). Cells were exposed to the different sources of UV radiation while attached to 60 mm culture dishes with the plastic covers removed and containing 2 ml of PBS. The dishes were placed on ice during UV exposures to prevent a rise in temperature and reduce enzymatic repair of the damaged DNA.

In the sunlight eposure studies, cells were exposed for various time periods between 1200 and 1400 hours on clear days in September and October, 1985 in Logan, Utah (elevation ca. 4,500 above sea level). For cells exposed to a suntan booth, cells were placed 10 cm from the source (Bellarium S, SA1-10-100W lamps) at a local suntanning parlor. Cells exposed to the germicidal lamp were irradiated with a

UV 254 nm lamp in a Chromatic-Vue cabinet (Ultra-Violet Products, Inc., San Gabriel, CA), at a distance of 16 cm, producing a dose rate of 0.861 J/m^2-sec.

CELL CULTURE AND TREATMENT CONDITIONS

The Madin-Darby bovine kidney (MDBK) epithelial cell line, strain CCL 22, was originally obtained from American Type Culture Collection (Rockville, MD) and has been maintained in our laboratory for several years. Cells were grown in Eagle's minimum essential medium with nonessential amino acids and L-glutamine (Gibco Labs., Grand Island, NY), supplemented with 1 mM sodium pyruvate and 5% fetal bovine serum (Hyclone Labs, Logan, UT), and maintained at 37°C in an atmosphere of 97% air with 3% CO_2.

Cells were seeded in 60 mm culture dishes for 22 hr, then cellular DNA was labeled for 22 hr with 0.1 uCi/ml of methyl [^{3}H]-thymidine (2 Ci/mmol, New England Nuclear, Boston, MA). Prior to UV- or X-irradiation, the medium was removed from the dishes and replaced with ice cold PBS (pH 7.4). Cells were kept at 0°C throughout the X-ray and UV exposures to minimize DNA repair activity.

Carcinogen-Induced DNA SSB Study

After the labeling period, cells were exposed for 4 hr in fresh media to various concentrations of the test compounds. The final solvent concentration did not exceed 1 percent.

UV Radiation Study

In the DNA repair studies, the appropriate dose of UV from each light source was used to produce similar amounts of DNA SSB (ca. 50 percent DNA eluted). Following UV exposures the ice-cold PBS was removed from the dishes and replaced with fresh medium. The dishes were incubated at 37°C for specified time periods to allow for rejoining (repair) of the UV-induced DNA SSB. Repair of DNA SSB was determined by a decrease in percent DNA eluted over time, as measured by alkaline elution. During this post-incubation repair period, some of the cultures were treated with 20 uM 1-B-D-arabinofuranosylcytosine (Ara C) and 2 mM hydroxyurea (HU; Sigma Chemical Co., St. Louis, MO) to inhibit DNA repair.

DNA Cross-Linking Study

After the labeling period, the medium was decanted and replaced with fresh medium containing HN2 or MMC. After 1 hr, the medium was removed and cells were washed twice with ice-cold PBS and kept at 0°C in PBS. UV irradiation was performed after the medium had been removed and replaced with ice-cold PBS. After UV exposure, the PBS was removed, replaced with medium and cells were incubated at 37°C for 30 min to allow for cross-linking.

Pyrrolizidine Alkaloid Study

Following the labeling period, the medium was decanted and replaced with fresh medium containing a PA and incubated for the appropriate time at 37°C. The PAs, which were dissolved in

dimethylsulfoxide (DMSO), were added directly to the culture media in a volume not to exceed 1.0 percent.

Some of the treatments required the use of an external metabolizing system to increase metabolic activation. The external metabolizing system consisted of a NADPH generating system and rat liver S9 fraction as described by Madle et al. [1986]. The S9 fraction was prepared from Aroclor 1254-induced male Sprague-Dawley rats according to Maron and Ames [1983]. These fractions (protein content = 46.0 mg/ml) were pooled, stored at -80^{o}C and the same batch was used in all experiments . The co-factors in the S9 mix were 0.67 mM NADP, 0.83 mM glucose-6-phosphate, 1.33 mM $MgCl_2$, 10mM phosphate buffer, with 2 percent rat S9 fraction (all as final concentrations in the culture medium).

ALKALINE ELUTION

The alkaline elution procedure was based on the methods and principles outlined by Kohn et al. [1981] and Fornace et al. [1982] with several modifications. The filtration assemblies consisted of a 50 ml syringe barrel mounted on a 25 mm Swinex filter holder (Millipore Corp.) attached to a 16 gauge blunt needle. Plastic tubing (Tygon; 3/32 I.D., 5/32 O.D.) was fitted to the needle. These filter assemblies were mounted on a 20-syringe rack.

After chemical and/or radiation exposures, cell suspensions (containing 1 x 10^6 cells) were gently loaded into the filter assemblies. The filter holders contain 2 um pore size polycarbonate filters (Nucleopore, Pleasanton, CA) for the DNA SSB assay or 2 um

pore size polyvinylchloride filters (Millipore Corp., San Francisco, CA) for the DNA cross-link assay. The cells were washed with ice cold PBS and lysed with 5 ml of lysing solution, followed by an additional 2 ml of lysing solution either with or without 0.5 mg/ml of proteinase K (Sigma Chemical Co., St. Louis, MO). The DNA was washed with 4 ml wash solution and eluted by gravity with 40 ml eluting solution.

For the detection of DNA SSB, the cells were lysed with 2% SDS, 0.02 M Na_2EDTA, and 0.1 M glycine (pH 9.6); 2 ml of lysing solution (containing proteinase K) was then added, the filters were washed with 0.02 M Na_2EDTA and 0.1 M glycine (pH 9.6), and DNA was eluted with 0.1% SDS, 0.02 M EDTA (acid form) adjusted to pH 12.4 with tetraethylammonium hydroxide. For the detection of DNA cross-links, cells were lysed with 2.0 M NaCl, 0.02 M Na_2EDTA, and 2.0% N-lauroyl-sarcosine (pH 10.0) followed by a 1 h incubation with the same lysing solution in the presence or absence of proteinase K, washed with 0.02 M Na_2EDTA (pH 10.0) and then the DNA was eluted with 0.02 M EDTA (acid form) containing enough tetraethylammonium hydroxide for a pH 12.4. All steps, from cell lysing through DNA elution, were performed in the dark.

After DNA elution, which was usually completed within 1 h, the filters were removed, placed in vials with 0.8 ml of 1 N HCl and heated for 1 hr at 60°C. One ml of 2 N NaOH was added to the vials, vortexed, cooled to 25°C, and 8.2 ml of 0.4 N NaOH was added. The filter assemblies were washed with 10 ml of 0.4 N NaOH. The DNA content of the eluate, filter, and filter assembly wash was determined by taking 0.5 ml aliquots of each fraction and measuring radioactivity

by scintillation counting (Beckman Model LS-3801) using 4 ml of scintillation cocktail. The cocktail contained 3L toluene, 11.7 g PPO, 0.24 g bis-MSB, 850 ml surfactant and 0.7% acetic acid (all from Fisher Scientific Co., Farlawn, NJ).

Results were presented as percent DNA eluted which were determined by the following formula:

$$\text{percent DNA eluted} = \frac{\text{total DPM in eluate}}{\text{total DNA activity}} \times 100$$

where total DNA activity = total DPM in eluate + DPM on filters + DPM in filter wash fraction. An increase in percent DNA eluted over control cells represented an induction of DNA SSB. Total DNA cross-links (TDC) were assessed by the reduction in the percent DNA eluted from cells exposed to the test agent plus irradiation compared to cells exposed to irradiation alone. DNA interstrand cross-links (ISC) were differentiated from DNA-protein cross-links (DPC) by the resistance of ISC to proteinase digestion during the lysing step of the alkaline elution assay [Kohn et al., 1981].

In the DNA cross-linking study, the formation of DPC was also detected using a variation of the above procedure, which involved exposing cells to a large dose of X-ray (3000 rad). This amount of X-ray produces a large number of very short fragments of DNA which pass easily through the membrane filter unless DNA is covalently bound to proteins, in which case the protein-bound DNA is retained on the filter [Kohn and Ewig, 1979].

In the PA study, DNA cross-link factors were calculated in order to better estimate the actual numbers of cross-links induced based on the alkaline elution data [Ewig and Kohn, 1978]. The DNA cross-link factors were calculated by the following equation:

$$\text{Cross-link Factor} = (E_c/E_t)^{0.5} - 1$$

where E_c is the percent DNA eluted in control cells (cells exposed to irradiation only) and E_t is the mean percent DNA eluted in treated cells plus irradiation exposure. For the calculation of TDC factors, E_t is the mean percent DNA eluted from cells treated with a PA-plus irradiation. To calculate DNA ISC factors, E_t is the mean percent DNA eluted from cells exposed to a PA plus irradiation and incubated with proteinase K. The DPC factor is determined by the difference between the TDC factor and DNA ISC factor for the same PA treatment. For further explaination and derivation of the formula see Ewig and Kohn [1978].

CELL VIABILITY AND COLONY FORMING EFFICIENCY

Cell viability was determined by trypan blue dye exclusion assay. Only cells which were attached to the culture dishes were used in the alkaline elution procedures.

Cell mitotic indices were determined by colony-forming efficiency. The latter was determined by seeding 500-600 cells in 10 mm culture dishes 24-48 hr prior to treatment with PAs plus S9 mix for 2 hr at 37^{o}C. After the 2 hr exposure, the medium was replaced with fresh medium (containing 40 ug/ml gentamycin and 2.5 ug/ml amphotericin B), and cells were incubated for 7-8 days with changes in medium every

second day. After the incubation period, cultures were fixed with methanol and stained with 0.2% methylene blue. The number of surviving colonies were counted in the treated groups, divided by the number of colonies in the control groups and designated as the surviving fraction. Experiments were performed in duplicate and repeated at least twice.

STATISTICAL ANALYSIS

Data were analyzed with ANOVA [Dowdy and Weardon, 1983]. The least significant difference was used for the multiple means comparison tests; $p \leq 0.05$ was considered significant. Correlation coefficients (r) were determined by linear regression.

CHAPTER III

RESULTS OF CARCINOGEN-INDUCED DNA STRAND BREAKS DETECTED BY GRAVITY-FLOW ALKALINE ELUTION

The gravity-flow alkaline elution system was first validated by examining the effects of X-irradiation on MDBK cells. X-ray exposures of 25-1500 rad showed dose-dependent responses ($p<0.05$; Fig. III-1).

The effect of the alkaline eluting solution (pH 12.2) on DNA hydrolysis was tested. After 3-hr preincubation in the eluting solution, the X-ray exposed cells showed an increase in precent of DNA eluted (95 percent) and remained there for the later time periods. In the control cells, preincubation significantly increased DNA fragmentation (Fig. III-2). Ninety-five percent of the total DNA was eluted from control cells after 24 hr of preincubation in the eluting solution.

The results of gravity-flow alkaline elution from MDBK cells exposed to three concentrations of seven genotoxic carcinogens for 4 hr are shown in Table III-I. Rad-equivalents (rad-eq) were extrapolated from the line in Figure III-1 using the elution results obtained for each compound and dose. The direct-acting carcinogens (MNU, ANNG and MMS) all significantly damaged DNA at 0.3 and 3.0 mM, as shown by the high percent of DNA eluted (Table III-I). The rad-eq values indicate that the DNA damage induced at the highest doses of MNU, ANNG and MMS were equivalent to X-ray exposures of 1550, 1600 and 1270 rad, respectively. At 0.3 mM, BP-induced extensive DNA

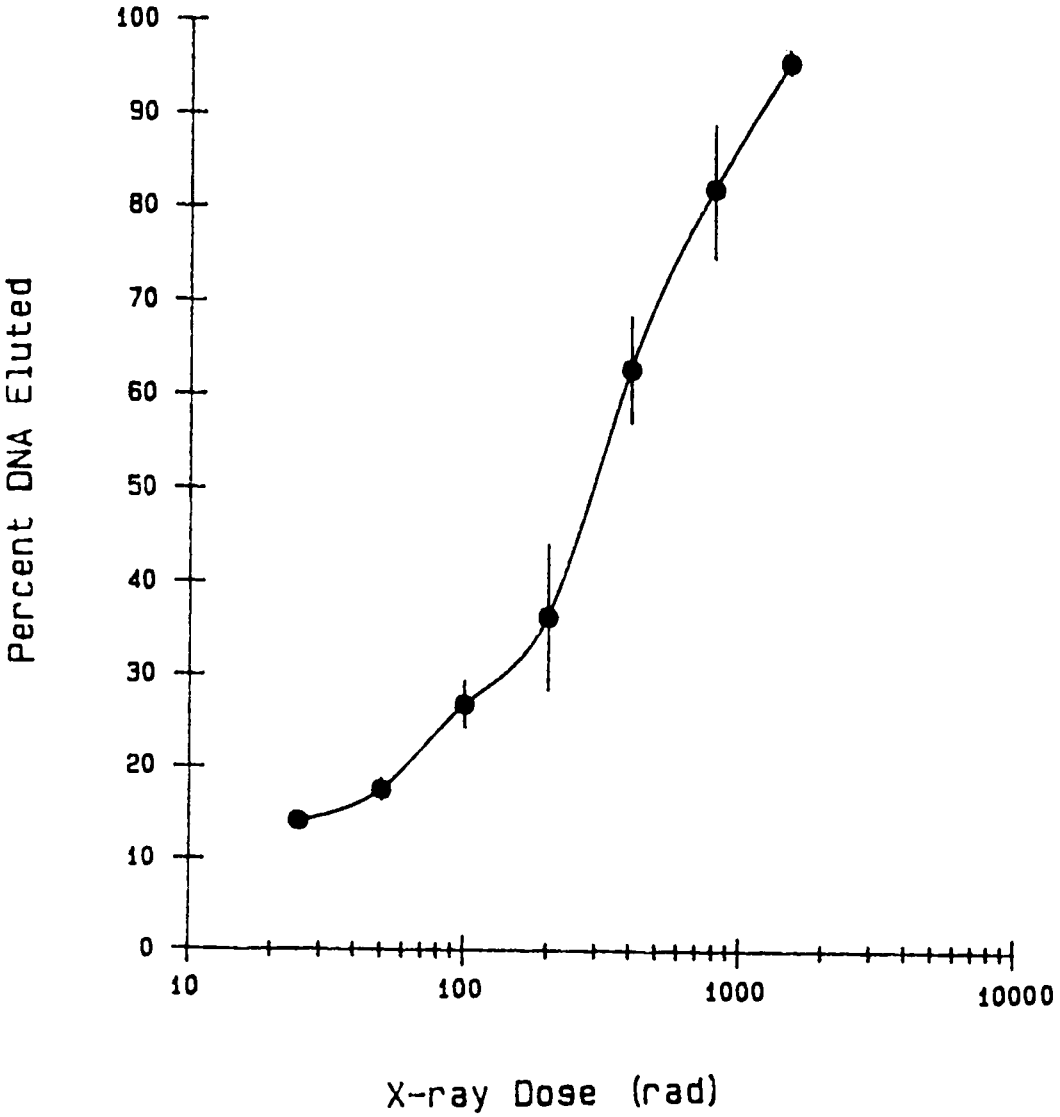


Figure III-1. Effects of 25, 50, 100, 200, 400, 800 or 1500 rad x-ray irradiation on the percent of DNA eluted in MDBK cells. Each data point represents the mean of experiments performed on different days ± SD, n=3-8.

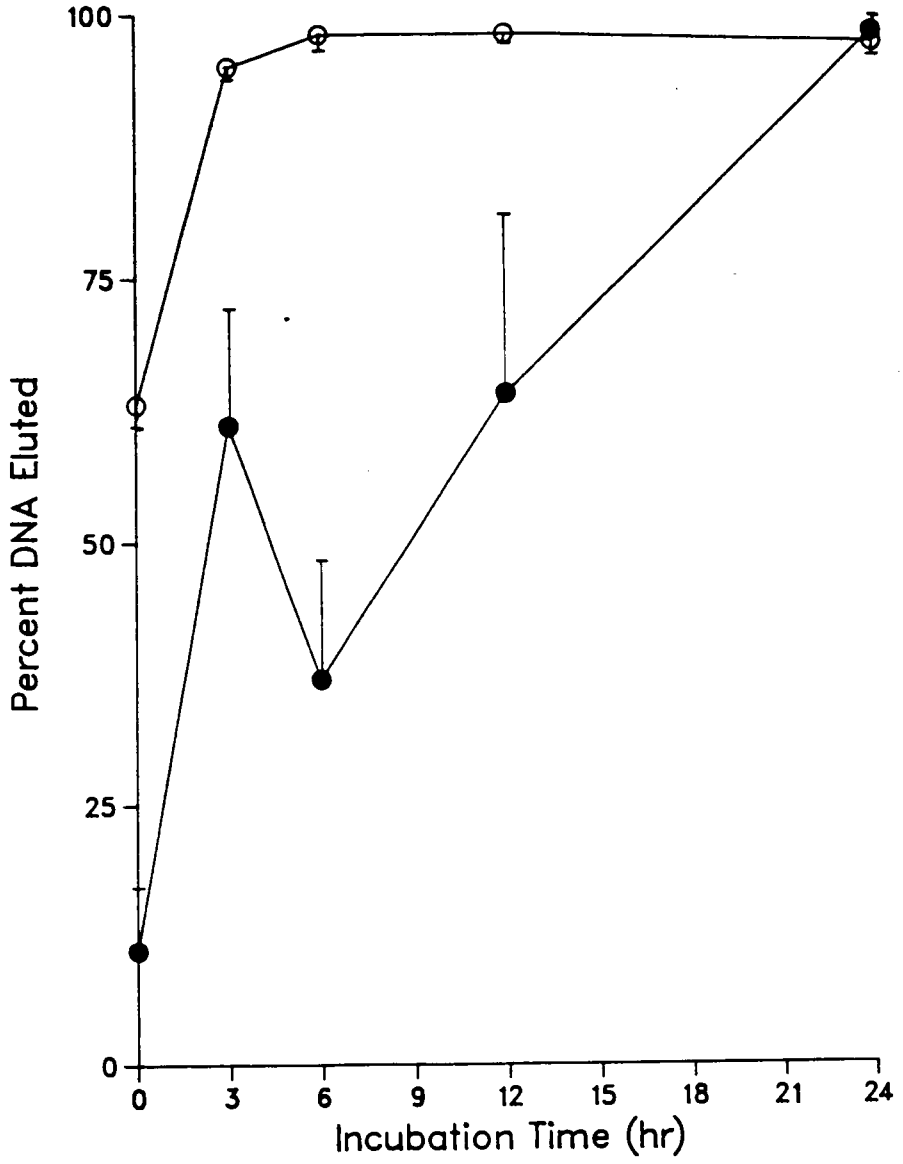


Figure III-2. Effects of incubation time in eluting solution (pH 12.2) prior to gravity-flow elution on the percent DNA eluted in MDBK cells. Cells exposed to 400 R x-ray irradiation (O-O) and control cells with no exposure (●-●). Each point is the mean of triplicate samples ± SD.

TABLE III-I. Effect of *In Vitro* Treatment with Genotoxic Agents on Alkaline Elution from MDBK Cell DNA.

Compound-Dose (mM)	n[a]	% Viability[b]	% DNA Eluted[c] (mean±S.D.)	X-ray R-equivalent[e]
MNU 0.03	3	90	31.2±14.6[d]	95
0.3	4	90	69.0± 9.6	560
3.0	4	92	96.7± 1.6	>1500
ANNG 0.03	3	93	47.0± 9.8	190
0.3	4	86	66.5± 9.8	500
3.0	4	37	97.8± 1.0	>1500
BP 0.03	3	90	33.6±11.6	110
0.3	4	79	74.2± 8.6	640
3.0	2	30	94.5± 3.0	>1500
MMS 0.03	3	88	32.1± 1.0	100
0.3	4	97	90.0± 5.6	1500
3.0	4	90	91.9± 7.8	>1500
AAF 0.03	3	95	46.8± 9.9	190
0.3	4	90	44.2± 8.5	160
3.0	4	87	59.4± 7.1	320
AFB_1 0.003	3	94	52.0± 9.2	260
0.03	4	90	43.2±11.7	150
0.3	4	93	54.4± 3.0	280
DEN 0.03	3	95	24.1± 6.8[d]	62
0.3	4	96	29.5± 7.0	82
3.0	4	95	44.5± 3.9	165
CONTROL[f]	5	96	17.0± 6.9	---

[a]Total number of experiments each run on different days.

[b]As determined by trypan blue dye exclusion.

[c]Significantly different ($p<0.005$) relative to controls, unless otherwise indicated.

[d]Significantly different ($p<0.025$) relative to controls.

[e]Calculated from the regression line shown in Figure 2.

[f]Cells exposed to solvent only (1% DMSO).

strand breaks but had little effect on cell viability. Other procarcinogens, such as AAF, AFB_1 and DEN, produced extensive DNA strand breakage but less breakage than BP at the medium and high doses. The maximum rad-eqs for AAF, AFB_1 and DEN were less than produced by the medium dose of BP. Table III-I demonstrates the effects of genotoxic carcinogen concentrations on DNA elution. With the exception of AFB_1 and AAF_1, the effects of the carcinogens on DNA strand breaks were dose-related. In general, the genotoxic carcinogens caused minimal cytotoxicity, except for 3.0 mM ANNG and 3.0 mM BP which reduced cell viability to 37 percent and 30 percent, respectively (Table III-I).

The effects of 4-hr exposures to five non-genotoxic agents on MDBK cell DNA are shown in Table III-II. Results clearly show that tested levels of AA, ETOH, DMSO, PB, SAC and TPA did not significantly increase cellular DNA damage compared to the control of no compound ($p>0.10$). Cell viability exceeded 90 percent (Table III-II).

TABLE III-II. Effect of *In Vitro* Treatment with Non-genotoxic Agents on Alkaline Elution from MDBK Cell DNA.

Compound-Dose (mM)	n	% Viability[a]	% DNA Eluted[b] (mean±S.D.)
AA 0.3	3	95	14.4±2.0
3.0	3	94	14.4±5.5
PB 0.3	4	98	17.4±3.1
3.0	3	93	13.2±6.0
SAC 0.3	4	93	16.3±2.8
3.0	3	95	14.3±3.4
TPA 0.003	4	97	18.8±5.8
0.03	4	94	16.6±4.5
1% ETOH	5	92	14.7±5.0
1% DMSO	5	96	17.0±6.9
CONTROL[c]	7	97	14.3±5.94

[a] As determined by trypan blue dye exclusion.

[b] Each treatment resulted in no significant difference ($p>0.10$) relative to untreated controls.

[c] No compound.

CHAPTER IV

INDUCTION OF DNA SINGLE-STRAND BREAKS AND SUBSEQUENT REPAIR BY THREE SOURCES OF ULTRAVIOLET RADIATION

Figure IV-1 shows the spectral irradiance scans of the three sources of UV radiation used in this study. The germicidal lamp had a peak irradiance at 254 nm of 861 mW/m^2, which dropped sharply on either side so that irradiance at 251 nm and at 257 nm was only 4.0 mW/m^2. The wavelength scans of sunlight were similar to the suntan booth, but the radiant energy was higher in the tanning booth. In the UV-B region for example, sunlight produced 0.1 mW/m^2 at 295 nm which increased to 224 mW/m^2 at 320 nm; whereas the tanning rays, at 10 cm from the source produced 0.3 mW/m^2 at 290 nm and increased to an intensity of 618 mW/m^2 at 320 nm. Thus, the relative intensity of the tanning booth was 2.7 times sunlight at these wavelengths. The intensities continued to rise rapidly in the UV-A regions, with radiant energy as high as 2,048 and 2,213 mW/m^2 at 340 and 350 nm, respectively, which were 5 times the intensity that sunlight produced at those wavelengths.

The effects of sunlight-exposure time on the induction of DNA SSB are presented in Figure IV-2. The production of DNA SSB was dose-dependent on exposure time in sunlight ($p \leq 0.05$) with a good correlation, $r=0.99$. Cells exposed to 45 min of sunlight produced a mean response of 55.2 percent DNA eluted, and this exposure time was used in subsequent repair studies. There was a dose-dependent

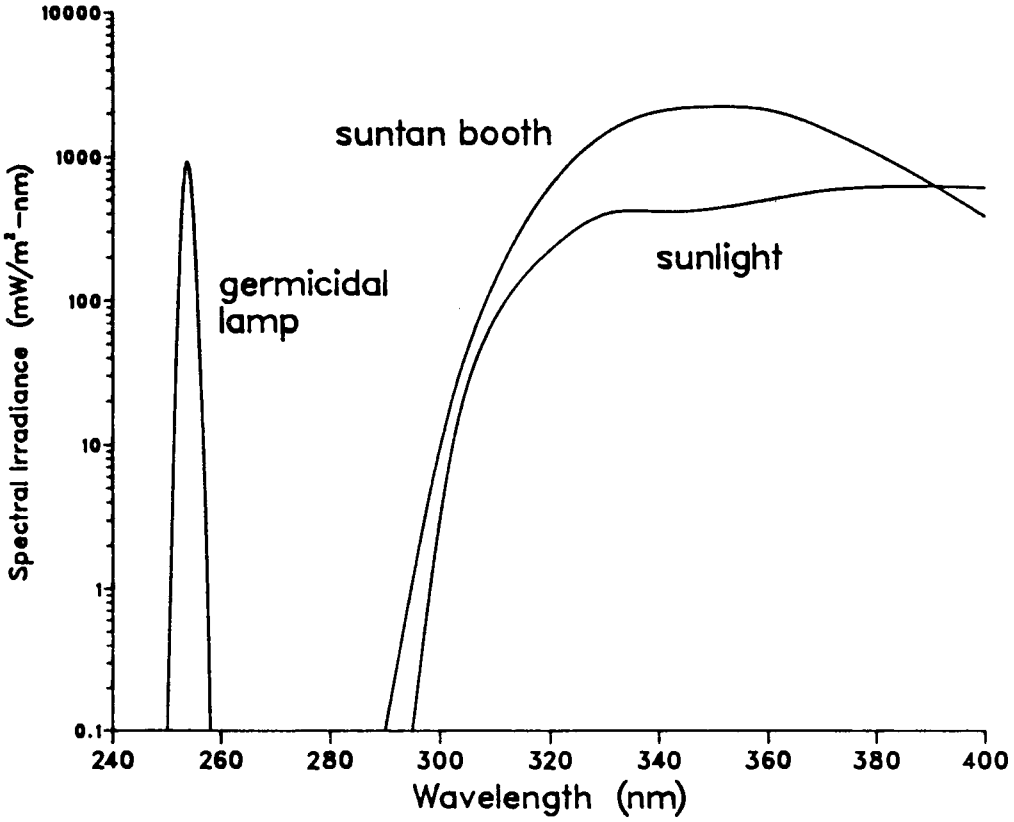


Figure IV-1. Spectral irradiance scans of the three UV light sources used in this study.

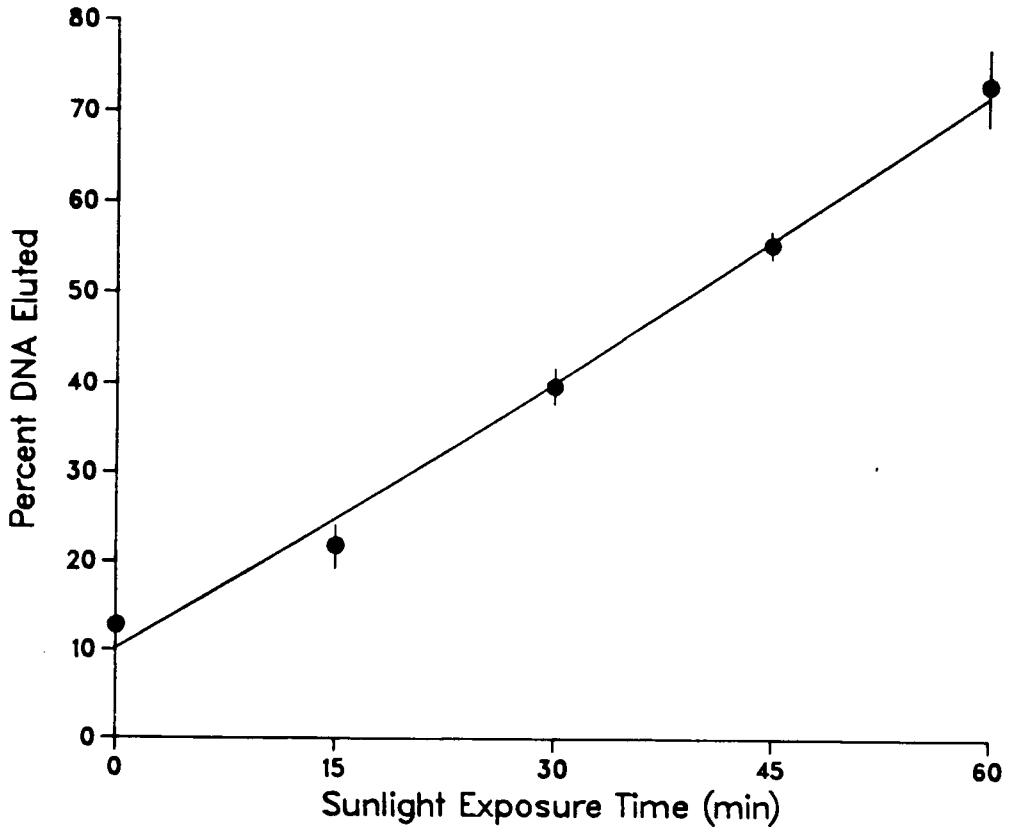


Figure IV-2. Effects of sunlight exposure time on the induction of DNA SSB. Each point represents the mean ± SE, n=3-7. Sunlight produced a significant dose-dependent response at each time point tested ($p \leq 0.05$; $r=0.99$).

induction of DNA SSB in cells exposed to UV radiation from the suntan booths at exposure times between 2.5 min and 30 min (Fig. IV-3; $p \leq 0.05$; $r=0.99$). The 20 min exposure produced a mean response of 53.7 percent DNA eluted; this exposure time was used in the repair studies. Likewise, cells exposed to 254 nm UV radiation showed a dose-dependent induction of DNA SSB for all intervals (Fig. IV-4; $p \leq 0.05$; $r=0.99$). The doses of 40 J/m^2 and 80 J/m^2 produced 53.7 percent and 29.8 percent DNA eluted, respectively. Both doses were used in the repair study, because of the lack of repair seen in the higher dose of 80 J/m^2.

Strand breaks in DNA from cells exposed to 45 min of sunlight were rapidly repaired during the first 15 min of post-incubation, and approached control values (i.e., complete repair) by 2 hr (Fig. IV-5). The inhibition of DNA repair was shown by an increase in DNA SSB in cells post-incubated with Ara C and HU for 1 and 4 hr. The rejoining of DNA SSB in cells exposed in the suntan booth for 20 min was rapid over the first hour, and 50 percent of the DNA SSB was removed between 30 min and 1 hr (Fig. IV-6). Repair was complete by 1 hr when the percent DNA eluted was not significantly different from control levels ($p>0.05$). Cells post-incubated with Ara C and HU showed an increase in DNA SSB between 1 and 4 hr.

In contrast to DNA from cells exposed to either sunlight or in a suntan booth, SSB from cells exposed to either 40 or 80 J/m^2 of 254 nm UV radiation were not repaired during lengthy post-incubation periods (Fig IV-7). Instead, there was an increase in DNA SSB after

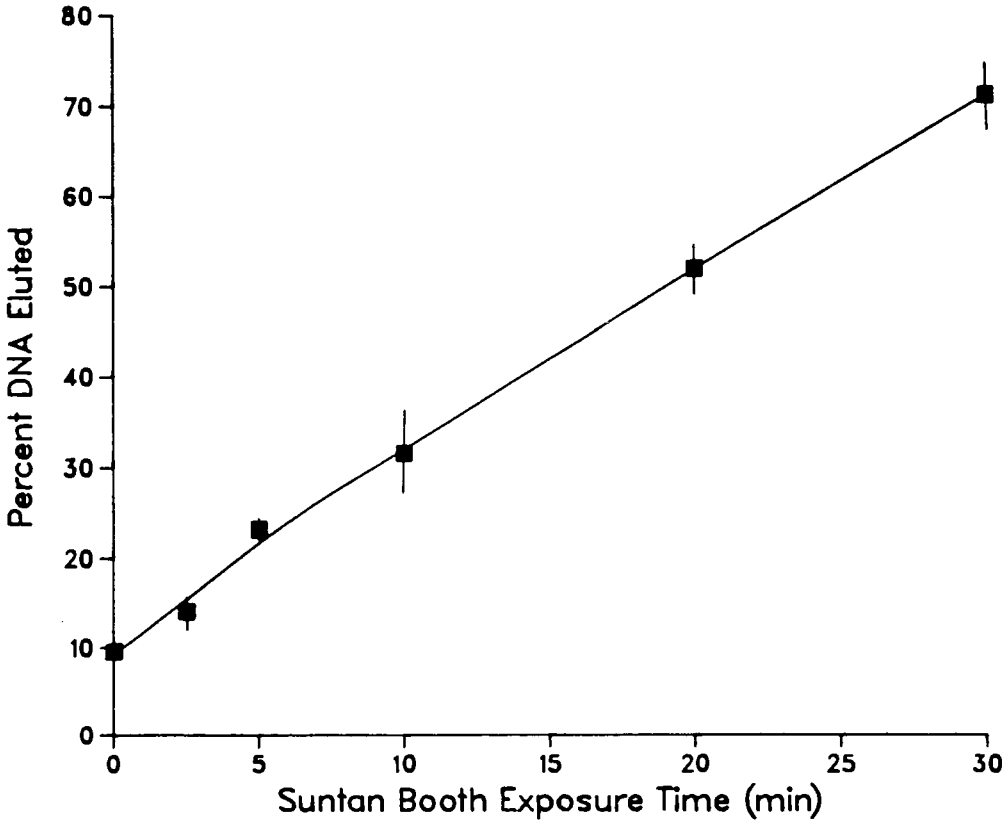


Figure IV-3. Effects of suntan booth exposure time on the induction of DNA SSB. Each point represents the mean ± SE, n=3-8. Suntan booth exposures (>2.5 min) produced a significant dose-dependent response ($p \leq 0.05$; $r=0.99$).

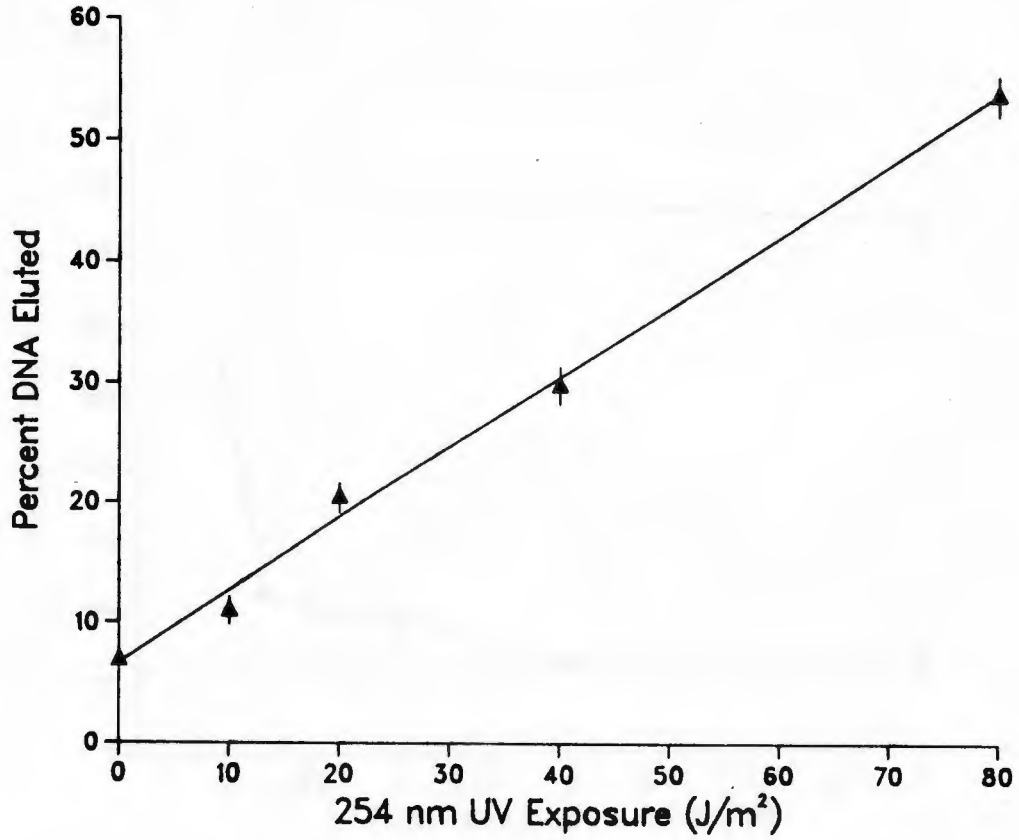


Figure IV-4. Effects of 254 nm UV exposure on the induction of DNA SSB. Each point represents the mean ± SE, n=3-7. 254 nm UV light produced a significant dose-dependent response at all doses tested ($p \leq 0.05$; $r=0.99$). Data points at 10, 20, 40, and 80 J/m^2 correspond to 11, 23, 46, and 93 sec of exposure respectively.

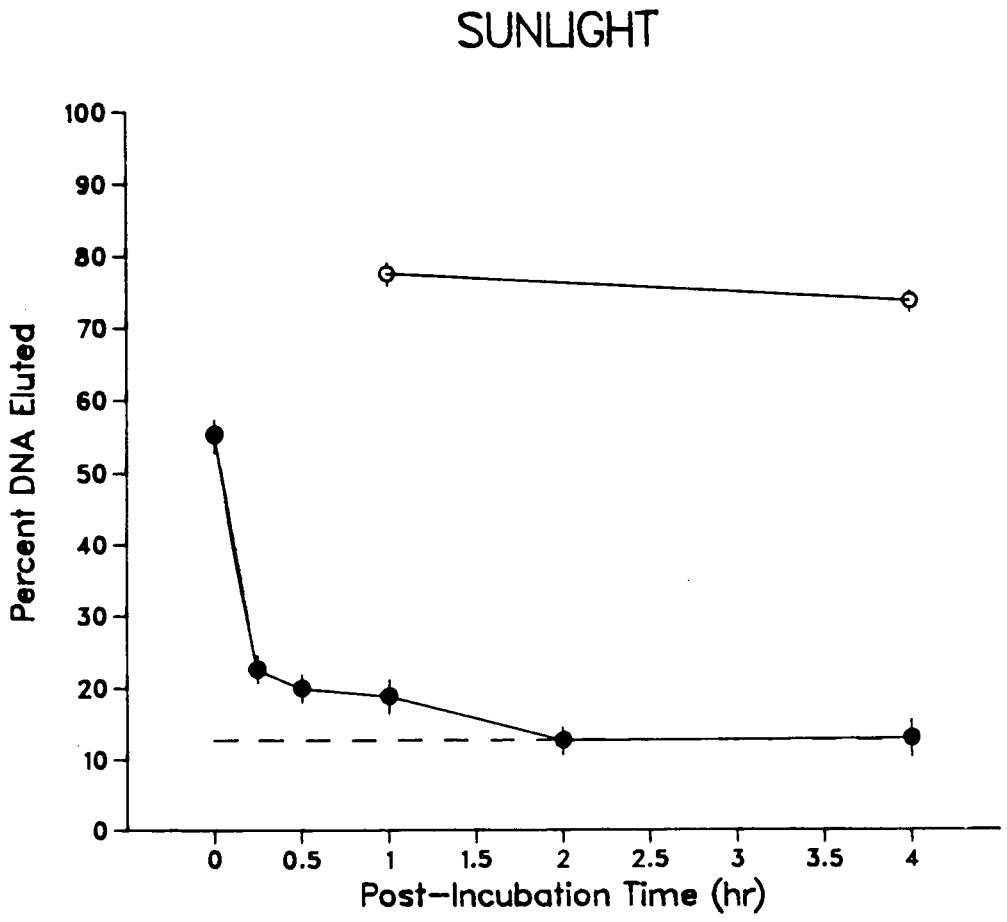


Figure IV-5. The induction and rejoining of DNA SSB in cells exposed to 45 min of sunlight. Data represents the mean ± SE, n=3-6. Sunlight exposure + post-incubation with media at 37°C (●). Sunlight exposure + post-incubation at 37°C with media containing Ara C and HU (○). The dashed line indicates % DNA eluted from cells with no treatment (---).

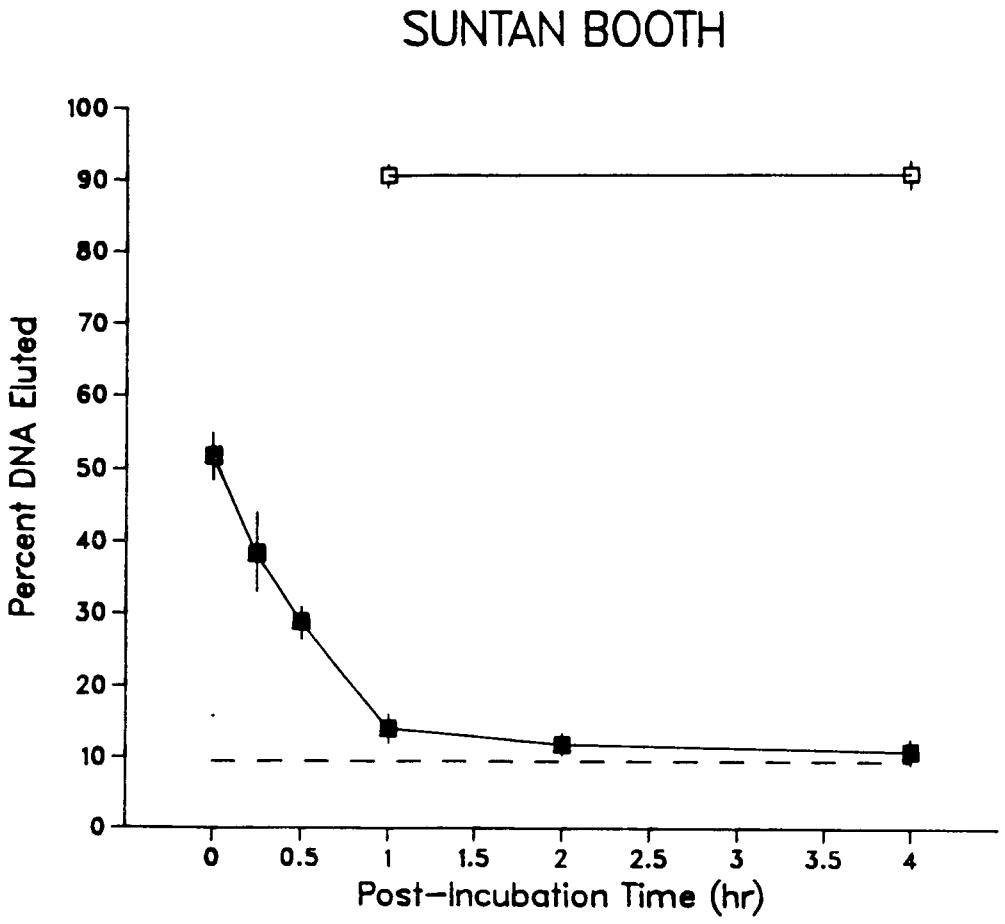


Figure IV-6. The induction and rejoining of DNA SSB in cells exposed to the suntan booth for 20 min. Data represents the mean ± SE, n=3. Suntan booth exposure + post-incubation with media at 37°C (■), suntan booth + post-incubation at 37°C with media containing Ara C + HU (□). The dashed line indicates % DNA eluted from cells with no treatment (---).

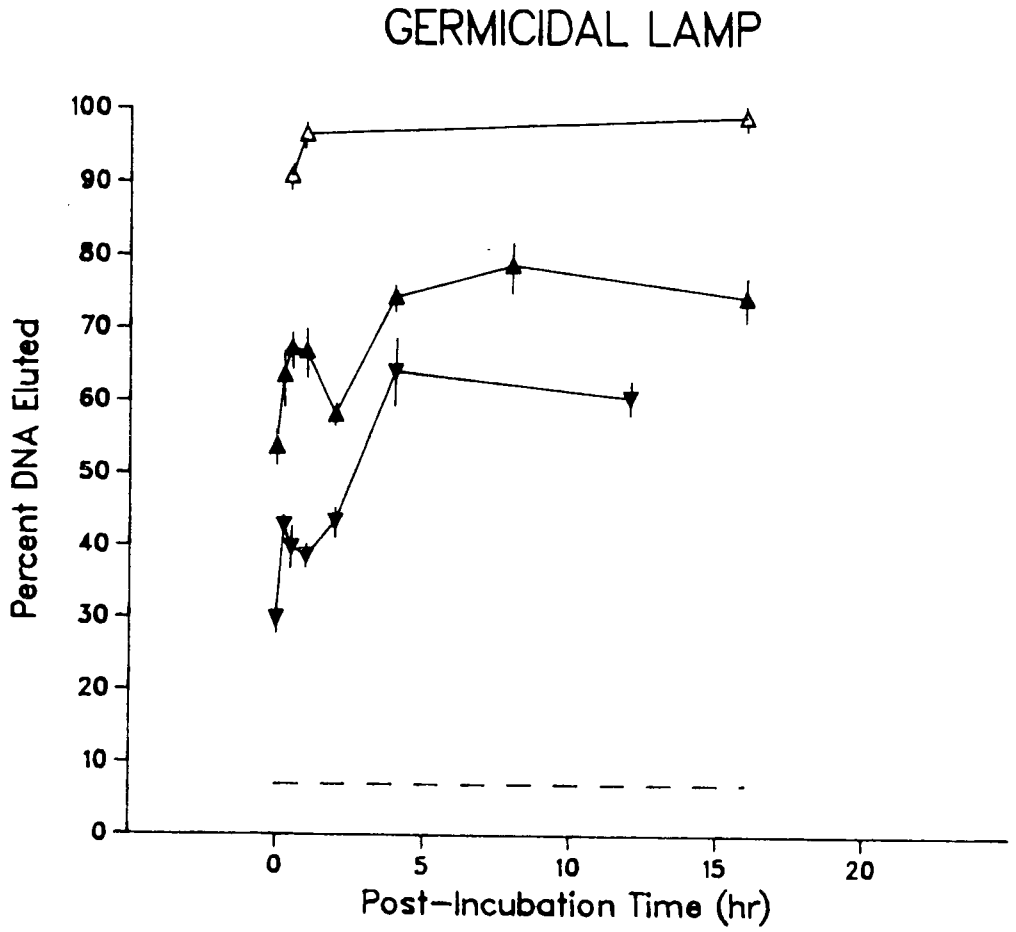


Figure IV-7. The effect of post-incubation time with media at 37°C after exposure to ultraviolet radiation from a germicidal lamp (254 nm), 40 J/m^2 (▼) or 80 J/m^2 (▲), 80 J/m^2 + post-incubation with Ara C + HU (Δ). The dashed line indicates % DNA eluted from cells with no treatment (---). Data represents mean ± SE, n=3.

Treatments of 40 and 80 J/m^2 correspond to exposure times of 46 and 93 sec respectively.

2 hr, which remained until the end of post-incubation time periods. Cells exposed to 80 J/m^2 and Ara C and HU during post-incubation produced a still higher amount of DNA eluted (Fig. IV-7).

CHAPTER V

RESULTS OF CHEMICAL AND ULTRAVIOLET RADIATION-INDUCED DNA CROSS-LINKS DETECTED BY GRAVITY-FLOW ALKALINE ELUTION

The viability of MDBK cells after 1 hr exposure to various doses of HN2 or MMC, or after exposure to UV radiation, was >95 percent as determined by trypan blue dye exclusion (data not shown).

Agents which induce DNA strand breaks cause an increase in the amount of DNA eluted under alkaline conditions of this assay, whereas agents which induce DPC or DNA ISC act to increase the retention of cellular DNA on the filter and appear as a decrease in DNA eluted. In order to detect DNA cross-linking, a controlled amount of DNA strand breaks were introduced in a controlled manner by X-irradiation of the cells. Figure V-1 shows the amount of DNA eluted from cells exposed to X-irradiation (100-1200 rad). Between 200-800 rad, the increase in percent DNA eluted was dose-dependent ($p \leq 0.01$) with good correlation by linear regression ($r=0.985$). A dose of X-ray (either 600 or 800 rad) that was in the linear range of DNA strand break induction, was used in the subsequent DNA cross-linking assays.

The induction of total DNA cross-links (TDC) by HN2, MMC and UV radiation are presented in Figures V-2 - V-4. HN2 produced a dose-dependent decrease ($p \leq 0.01$) in the amount of DNA eluted with increasing doses of HN2 (Fig. V-2). The percent DNA eluted was 26.8, 20.2 and 13.8 in cells exposed to 600 rad X-ray with 0.25, 1.0, or 4.0 uM HN2 respectively, while 600 rad alone produced 31.9 percent DNA

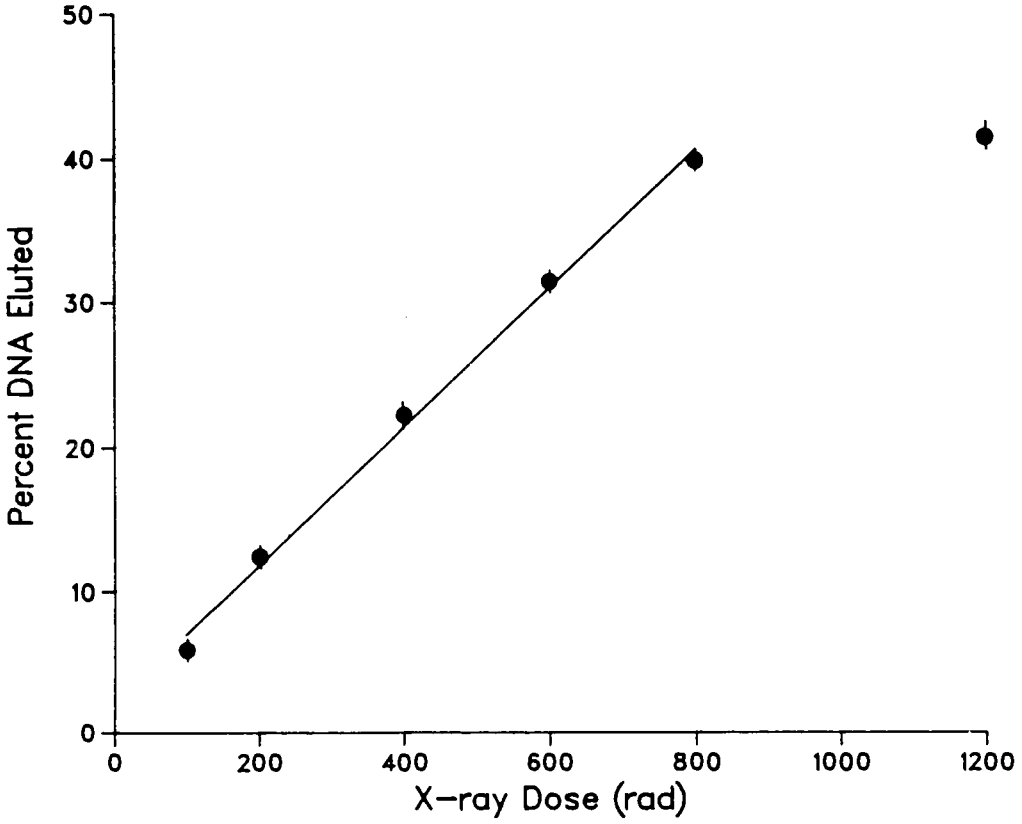


Figure V-1. Effects of X-ray on the induction of DNA strand breaks in MDBK cells. Each point represents the mean ± S.E., n=3-7. Significant dose-dependent increase in % DNA eluted ($p \leq 0.01$) at all doses except 1200 rad. Linear regression analysis on doses between 100-800 rad has a good correlation, r=0.985.

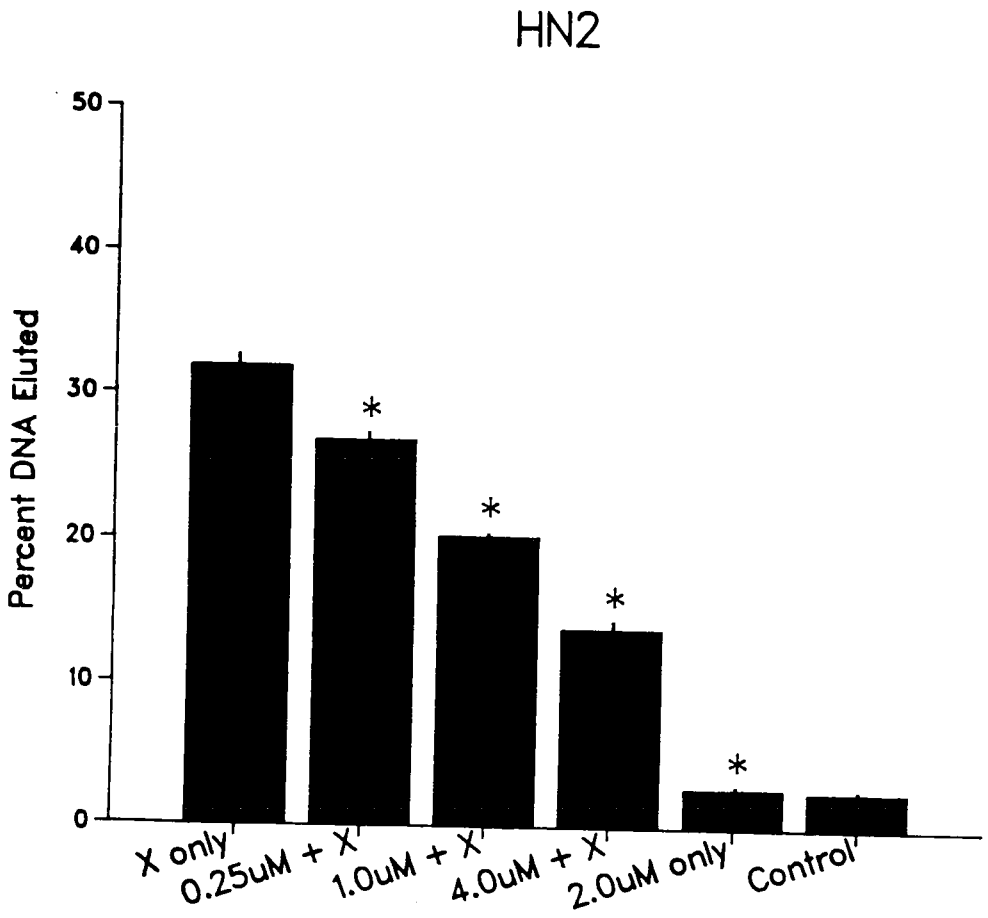


Figure V-2. Induction of DNA cross-links by HN2 exposures in MDBK cells. X-ray dose was 600 rad (X) and control represents cells treated with saline only. Data is shown as mean $\pm$ S.E., n=3-4.
*Results significantly lower ($p \leq 0.01$) than the previous treatment.

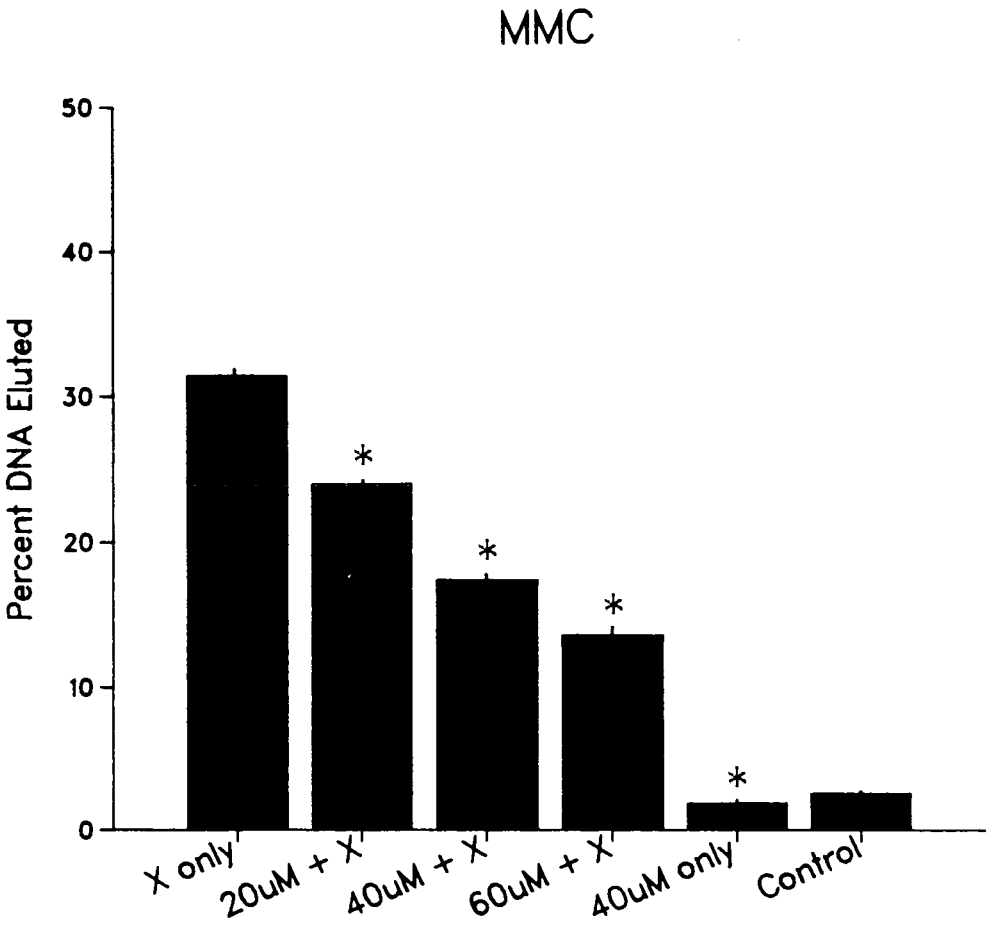


Figure V-3. Induction of DNA cross-links by MMC exposures in MDBK cells. X-ray dose was 600 rad (X) and control represents cells treated with saline only. Data is shown as mean ± S.E., n=3-6.
*Result is significantly lower ($p \leq 0.01$) than previous treatment.

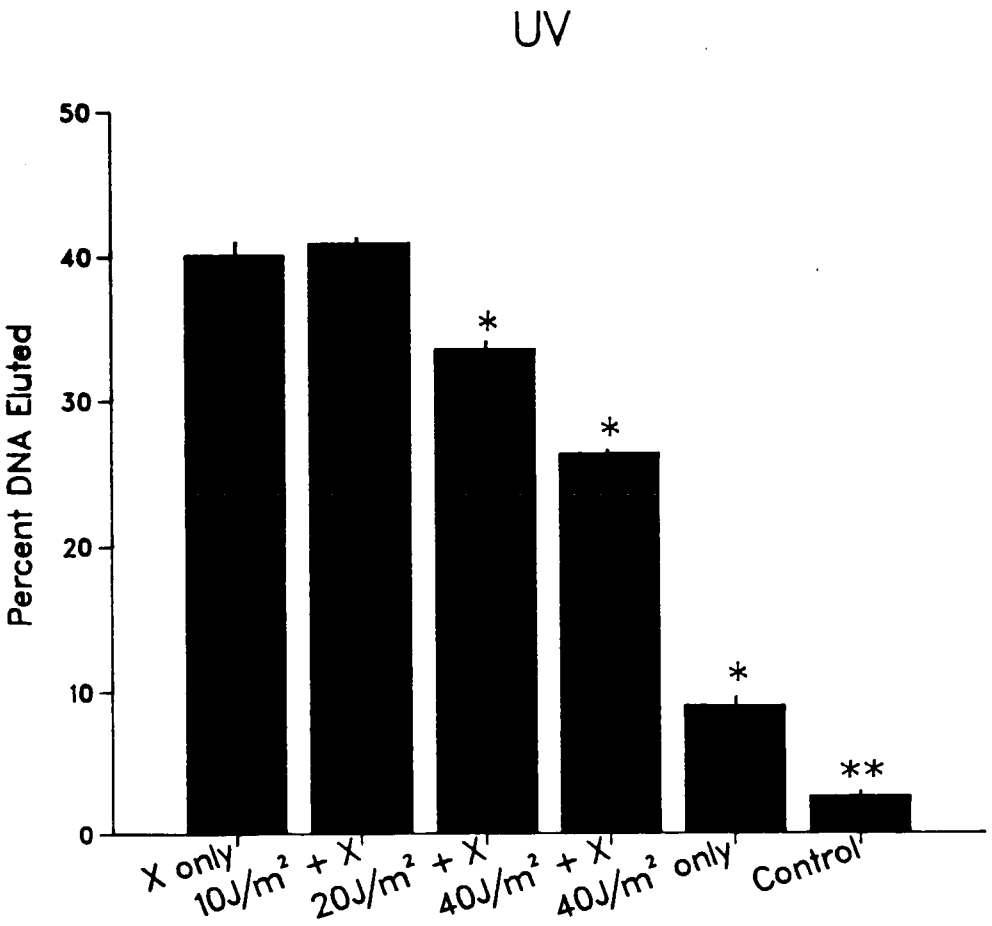


Figure V-4. Induction of DNA cross-links by UV exposures in MDBK cells. X-ray dose was 800 rad (X) and control represents cells with no treatment. Data shown as mean ± S.E., n=3-6.

*Result is significantly lower ($p \leq 0.01$) or **($p \leq 0.05$) than previous treatment.

eluted. A reduction in the amount of DNA eluted represents an induction of TDC into the cells exposed to HN2. There was no difference in the amount of DNA eluted between cells exposed to only 2.0 uM HN2 (2.8 percent) or in control cells (2.6 percent), indicating that HN2 did not induce DNA strand breaks in these cells. A similar dose-dependent induction of TDC ($p \leq 0.01$) was seen in cells exposed to MMC at exposure levels of 20, 40 and 60 uM (Fig. V-3). There was no indication of DNA strand breaks, since the amount of DNA eluted in cultures exposed to 40 uM MMC alone (1.9 percent) was similar to that of the control (2.6 percent).

Figure V-4 shows the induction of TDC in cells exposed to UV irradiation. There was a significant dose-dependent decrease ($p \leq 0.01$) in the amount of DNA eluted from cells exposed to 20 or 40 J/m^2 of UV irradiation plus 800 rad of X-irradiation (33.5 and 26.2 percent, respectively). A dose of 10 J/m^2 did not elicit enough TDC to be detected in this assay, since the combination of 10 J/m^2 + X-ray resulted in a similar amount of DNA eluted (40.9 percent) as with X-ray alone (40.1 percent). Cells exposed to 40 J/m^2 of UV irradiation also contained DNA strand breaks (9.0 percent), as determined by the significant increase ($p \leq 0.05$) in the percent DNA eluted for 40 J/m^2 alone, compared to the control of no treatment (2.6 percent).

The type of DNA cross-linking that was induced by HN2, MMC, and UV was then characterized. The induction of DPC by exposure of cells to 3000 rad X-ray along with the exposure to cross-linking agents, is shown in Table V-I. Both HN2 (2.0 uM) and MMC (40.0 uM) produced DPC

TABLE V-I. Detection of DNA-protein Cross-links in Cells Exposed to HN2, MMC, and UV.

Treatment	n	%DNA Eluted (Mean ± S.E.)
X-ray only (3000 rad)	6	73.3±1.1
2.0 uM HN2 + X-ray	3	57.0±1.3*
40.0 uM MMC + X-ray	5	65.0±1.2*
40 J/m^2 UV + X-ray	4	70.8±1.4

Cells were either untreated or treated with HN2, MMC, or UV radiation, cooled to 0^oC, and X-irradiated with 3000 rad.

as demonstrated by reduction in the amount of DNA eluted compared to X-ray alone. In this assay, DPC were not detected in cells exposed to 40 J/m^2 UV irradiation, since there was no difference in amount of DNA eluted in cells exposed to X-ray alone, compared to cells exposed to UV + X-ray.

To determine the presence of DNA ISC after exposure to the three cross-linking agents, the cell lysates were incubated with proteinase K (Prot) prior to the elution step, which disassociates proteins from DNA. An increased retention of DNA on the filters relative to samples not treated with proteinase indicates the formation of DNA ISC (Kohn et al., 1981). Figure V-5 shows that HN2 induced both DPCs and DNA ISCs. DNA from cells exposed to HN2 + X-ray + Prot showed an increase in the amount of DNA eluted ($p \leq 0.01$) over HN2 + X-ray without Prot, which indicated a removal of DPC, yet this effect was significantly lower than X-ray alone + Prot ($p \leq 0.01$). The latter response

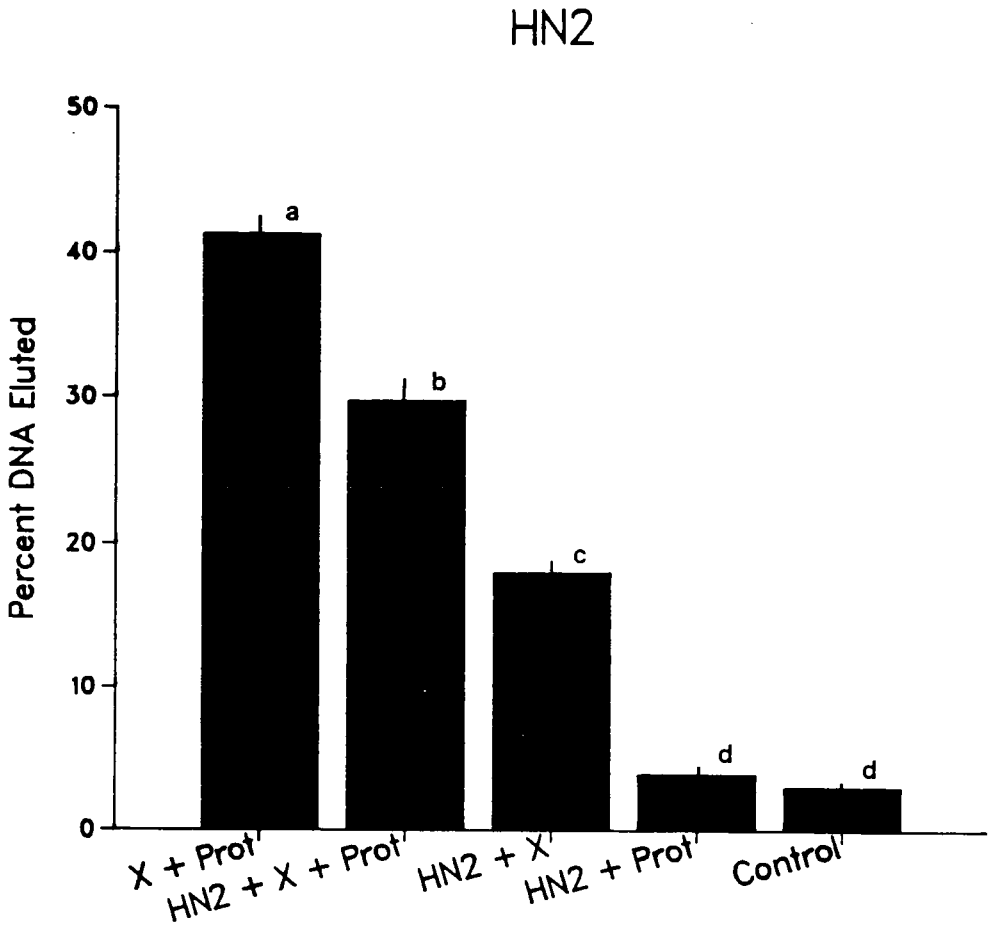


Figure V-5. Detection of DNA ISC by 2.0 uM HN2 exposure in MDBK cells. X-ray dose was 600 rad (X) and control represents cells treated with saline only. The increase in % DNA eluted in cells treated with drug + X + Proteinase (Prot) over drug + X only, represent removal of DPC. The lower % DNA eluted in cells treated with drug + X + Prot compared to X + Prot only, represents the detection of DNA ISC. Data shown as mean $\pm$ S.E., n=3-8. Results with different letters, represent significant difference ($p \leq 0.01$).

represented the presence of DNA interstrand cross-links. The same effect was also seen with 40 uM MMC (Fig. V-6). There was no change in the amount of DNA eluted in cells treated with only HN2 or MMC followed by Prot compared to controls, indicating that neither HN2 nor MMC induced DNA strand breaks.

A high amount of DNA was eluted from cells exposed to 40 J/m^2 of UV + X-ray and treated with Prot (63.1 percent; Fig. V-7). This was significantly greater ($p \leq 0.01$) than the response seen in cells exposed to UV + X-ray (20.5 percent) or X-ray + Prot (41.0 percent). Furthermore, there was a significant increase ($p \leq 0.01$) in DNA eluted (37.0 percent) in cells exposed to UV + Prot compared to UV alone (9.0 percent; Fig. V-7). These results indicated that UV irradiation induced the formation of DNA-associated proteins which were removed after proteinase digestion allowing the detection of DNA strand breaks.

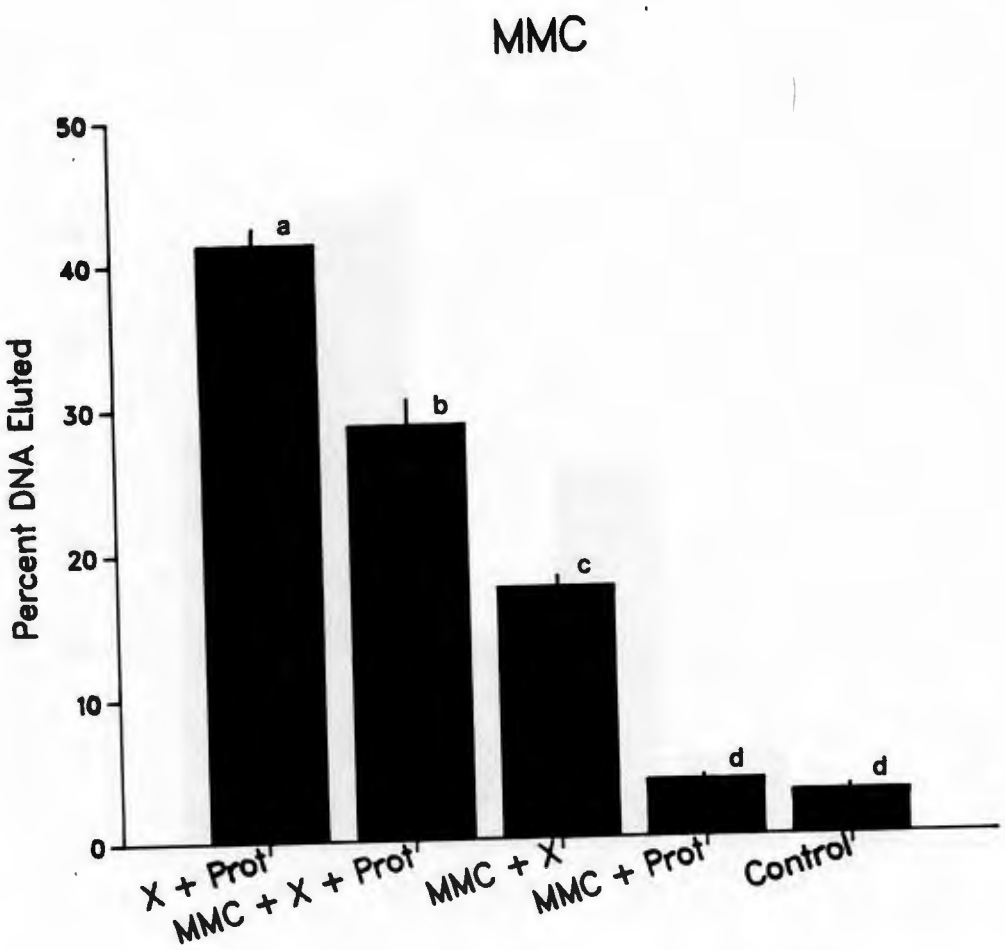


Figure V-6. Detection of DNA ISC by 40 uM MMC exposure in MDBK cells. See Figure V-5 for details.

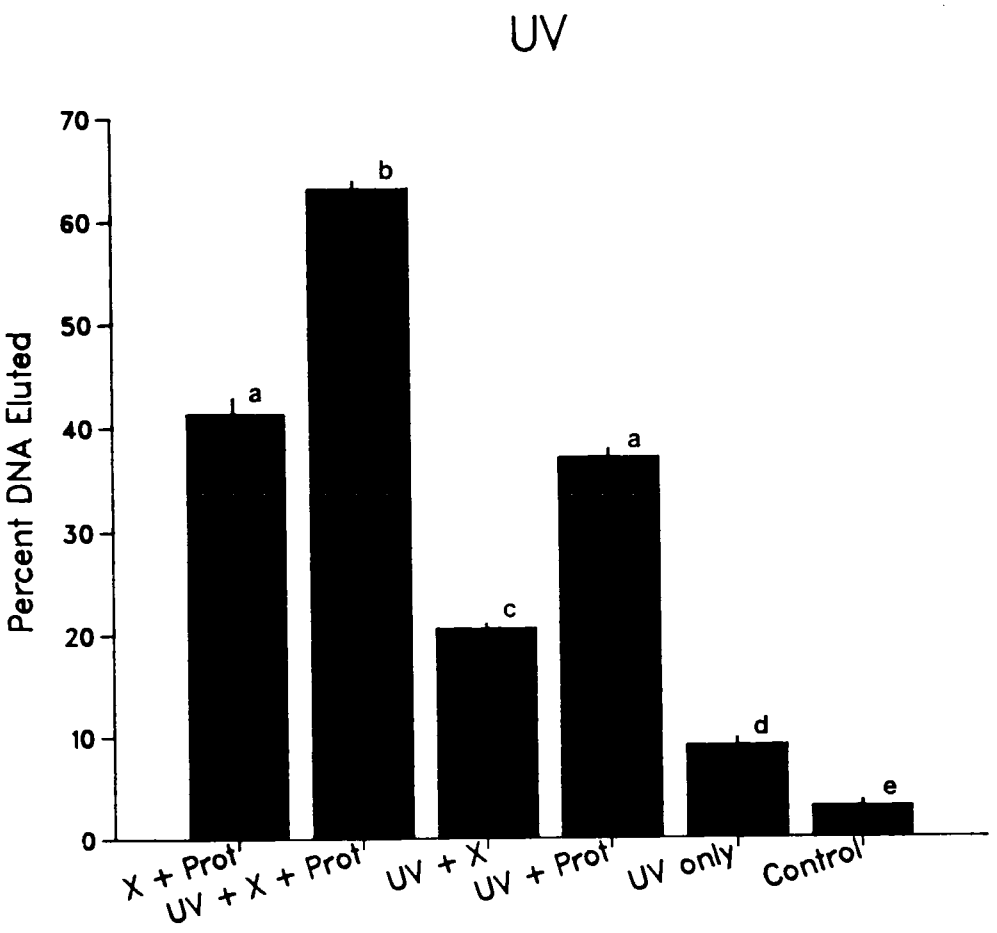


Figure V-7. Induction of DPC or protein associated DNA strand breaks by 40 J/m^2 UV exposure in MDBK cells. X-ray dose was 600 rad (X) and control represents cells with no treatment. Cells treated with UV + X + Proteinase (Prot) showed an increase in % DNA eluted over both UV + X and X + Prot and cells treated with UV + Prot showed an increase in DNA eluted over UV alone. These results indicated UV induction of protein associated DNA strand breaks. Data shown as mean $\pm$ S.E., n=3-8. Bars with different letters, are significant different ($p \leq 0.01$; for d-e comparison $p \leq 0.05$).

CHAPTER VI

CHARACTERIZATION OF DNA DAMAGE BY PYRROLIZIDINE ALKALOIDS

Cell viabilities for all treatment groups were > 90 percent as determined by trypan blue dye exclusion assay (data not shown), and only cells which adhered to the culture dishes after treatment exposures were used in the alkaline elution procedures.

In preliminary experiments using 4 hr incubations, cells treated with high concentrations of PAs had minimal or no detectable DNA single-strand breaks (Table VI-I). Cells exposed to PAs for an additional 8 hr also did not induce detectable amounts of DNA SSB (data not shown). DNA single-strand breaks (SSB) are detected as a significant increase in the amount of DNA eluted compared to controls. Methylnitrosourea was used as a positive control for the induction of DNA SSB. This treatment produced a significant increase in the amount of DNA eluted compared to the control values (Table VI-I).

For the detection of DNA cross-links, cells were exposed to the PAs for 1 hr, after which time the medium was removed, fresh medium was added, and cells were incubated for an additional 8 hr. This post-incubation time was performed in case additional time was needed for the formation of the PA bialkylating species. DNA cross-links were indicated by a significant decrease in amount of DNA eluted in cells exposed to PA + X-ray compared to X-ray exposure alone (Table VI-II). The exposure to X-ray induced a background level of DNA strand breaks,

Table VI-I. Results of Experiments to Detect DNA Single-strand Breaks in Cells Exposed to Various PAs for 4 hr.

Chemical	Dose (mM)	%DNA Eluted (Mean ± S.E.;n≥3)
Senecionine	1.0	8.9±1.3
	5.0	9.6±0.6*
Seneciphylline	1.0	11.4±0.8*
	5.0	9.9±2.5
Riddelline	1.0	7.8±2.6
	5.0	13.0±0.5*
Monocrotaline	1.0	7.6±1.1
	5.0	8.4±1.4
Heliosupine	0.1	8.4±1.9
	1.0	7.5±1.0
Methylnitrosourea	0.3	69.0±4.8*
	3.0	96.7±0.8*
DMSO 1%		5.2±0.3
Control, no treatment		5.4±1.8

*Significantly higher ($p \leq 0.05$) than controls.

as shown by a significant increase in the amount of DNA eluted compared to control cells. If the PAs induced DNA cross-links, more DNA would be retained and therefore less DNA would be eluted through the filter.

Since only minimal amounts of DNA cross-links were detected at the previous time exposures, cells were then exposed to a representative PA, senecionine, for 20 hr (Table VI-III). This PA induced DNA cross-links as indicated by the significant reduction in amount of DNA eluted in cells exposed to senecionine + X-ray compared to X-ray

Table VI-II. The Induction of DNA Cross-links in Cells Exposed to Various PAs.

Treatment[1]	Dose (mM)	%DNA Eluted (Mean ± S.E.; n≥3)
X-ray only (800 rad)		47.2±1.3
Senecionine + X-ray	1.0	31.9±1.2*
Seneciphylline + X-ray	1.0	33.9±0.4*
Riddelline + X-ray	1.0	35.4±2.0*
Monocrotaline + X-ray	1.0	32.4±1.8*
Heliosupine + X-ray	1.0	39.4±1.5*
Control, no treatment		1.9±0.2*

[1]Cells were exposed to compound for 1 hr, compound was removed and cells incubated for 8 hr in fresh media.

*Significantly lower ($p \leq 0.05$) than X-ray alone.

Table VI-III. The Induction of DNA Cross-links in Cells Exposed to Senecionine for 20 hr.

Treatment	Dose (mM)	%DNA Eluted (Mean ± S.E.;n≥3)
X-ray only (600R)		34.4±1.7
Senecionine + X-ray	0.01	22.7±1.6*
	0.1	24.4±1.3*
	1.0	23.1±1.9*
Control, no treatment		2.6±0.4*

*Significantly lower ($p \leq 0.05$) than X-ray alone.

alone. The fact that large doses of PAs and long exposure times were needed to detect minimal changes in the amount DNA eluted lead us to believe that MDBK cells may not have adequate metabolic capabilities to bioactivate the PA compounds. Thus, in subsequent experiments, these cells were co-cultured with an external metabolizing system consisting of a NADPH generating system and rat liver S9 fraction. Preliminary studies showed there was no difference on the induction of DNA cross-links between 2 and 4 hr incubations containing 100 uM senecionine and S9 mix (data not shown). Post-incubations in fresh medium for 4, 8 or 12 hr after 2 hr exposures to 100 uM senecionine + S9 mix, did not show any increase in the detection of DNA cross-links (data not shown). Thus, subsequent experiments were performed using S9 mix during the PA exposures without post-incubations.

Figures VI-1 - VI-7 show the relative cross-linking activities of the PAs when cells were exposed for 2 hr to concentrations of 100, 300, or 500 uM of each PA. An induction of DNA SSB would show a higher amount of DNA eluted in cells exposed to 500 uM of the PAs compared to cells exposed to DMSO alone (vehicle control). In all seven PA treatments, cells exposed to 500 uM did not show a difference in the amount of DNA eluted compared to the DMSO treatment (Fig. VI-1 - VI-7, the last 2 bars in each graph), which indicated DNA SSB were not detected after any of the PA exposures at 500 uM concentration, even with the addition of S9 mix.

In experiments to characterize the possible induction of DNA cross-links induced by the PAs (as shown in Fig. VI-1 - VI-7), a controlled amount of DNA SSB was introduced into the cells by 1000 rad

gamma-irradiation (G). Gamma-irradiation alone produced roughly 50 percent DNA eluted, which is shown by the first bar on the left-hand side of each graph in Figures VI-1 - VI-7. A reduction in the amount of DNA eluted from DMSO + G, compared to PA exposures of 100, 300 or 500 uM + G, was an indication of DNA cross-linking, which was designated as total DNA cross-links (TDC). To characterize the type of DNA cross-linking, cells were exposed to an intermediate concentration of PA (300 uM) followed by irradiation and subsequent proteinase K digestion (Prot). An increase in the amount of DNA eluted from cells exposed to 300 uM PA + G + Prot, compared to cells exposed to 300 uM PA + G, indicated the removal of DNA protein cross-links (DPC). If the amount of DNA eluted from cells exposed to 300 uM PA + G + Prot was less than the cells exposed to DMSO + G alone, then the DNA cross-links represented DNA interstrand cross-links (ISC).

Figure VI-1 shows the DNA cross-linking capabilities of seneciphylline. The decrease in amount of DNA eluted from control cells exposed to DMSO + G, compared to the treatment of 100 uM seneciphylline + G, indicates a significant induction of TDC. An increase in cross-links was seen with increasing doses of seneciphylline. An increase in the amount of DNA eluted was seen in cells exposed to 300 uM seneciphylline + G + Prot, relative to cells exposed to 300 uM + G with no proteinase K digestion. This result indicates the removal of DPC and the detection of DNA ISC.

The response of cells exposed to riddelline (Fig. VI-2) or retrorsine (Fig. VI-3) was similar to that of senecephylline; i.e., there was a large induction of TDC at 100, 300, and 500 uM

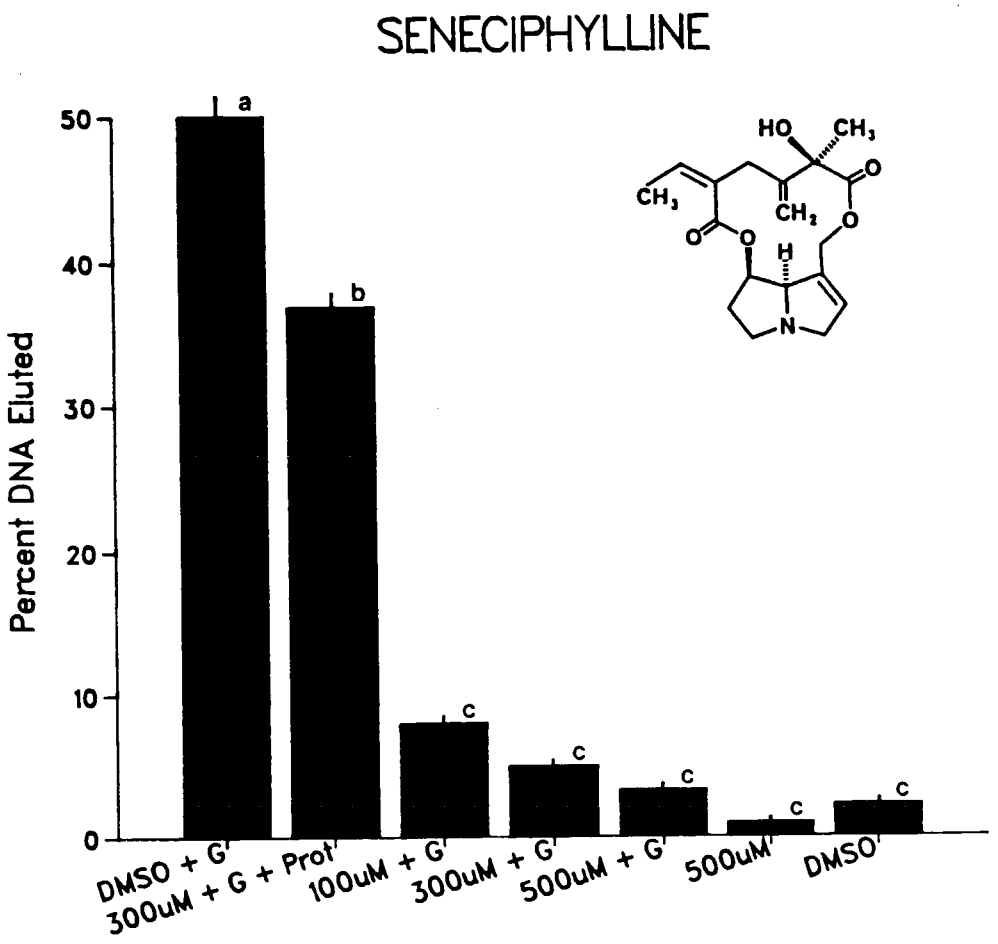


Figure VI-1. The structure of seneciphylline and the characterization of the induced DNA damage. Cells were co-cultured with seneciphylline and S9 mix for 2 hr, some of the treatments were also exposed to 1000 rad gamma-irradiation (G), and in some of the groups the cells were incubated with proteinase K (Prot). Bars with the different letters were significantly different ($p \leq 0.05$).

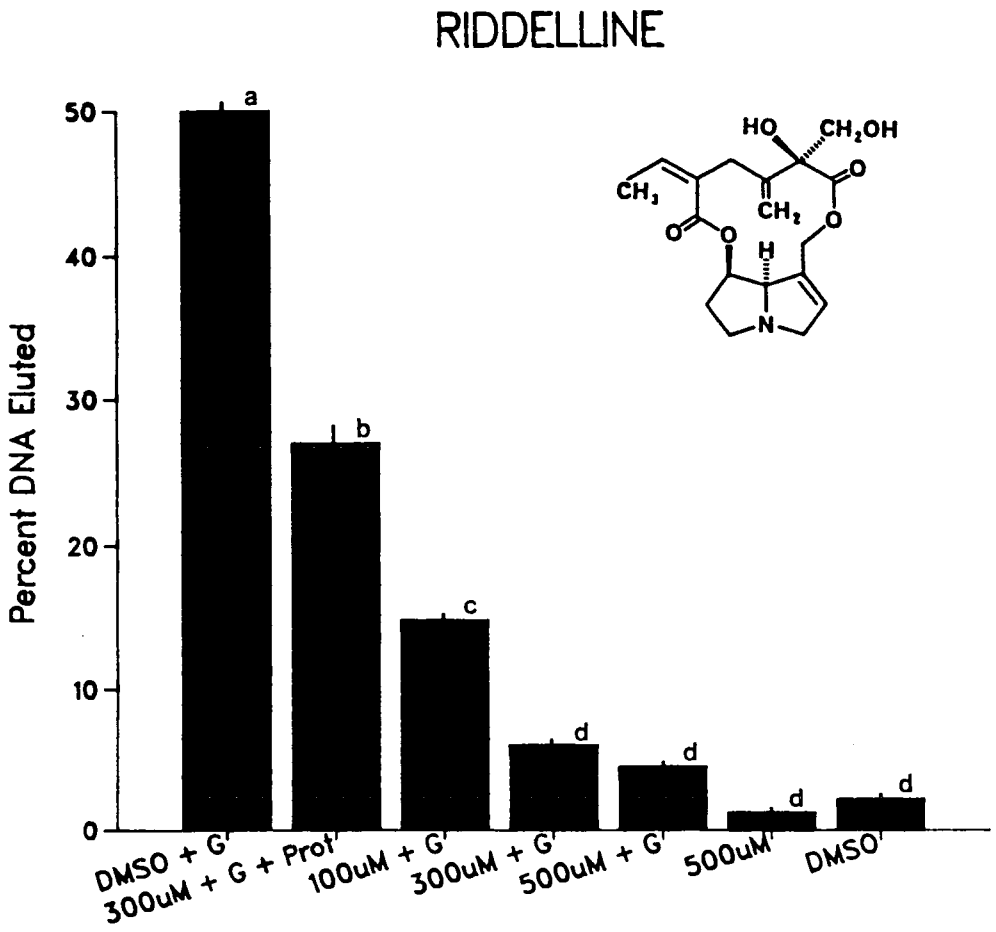


Figure VI-2. The structure of riddelline and the characterization of the induced DNA damage. See Figure VI-1 for details.

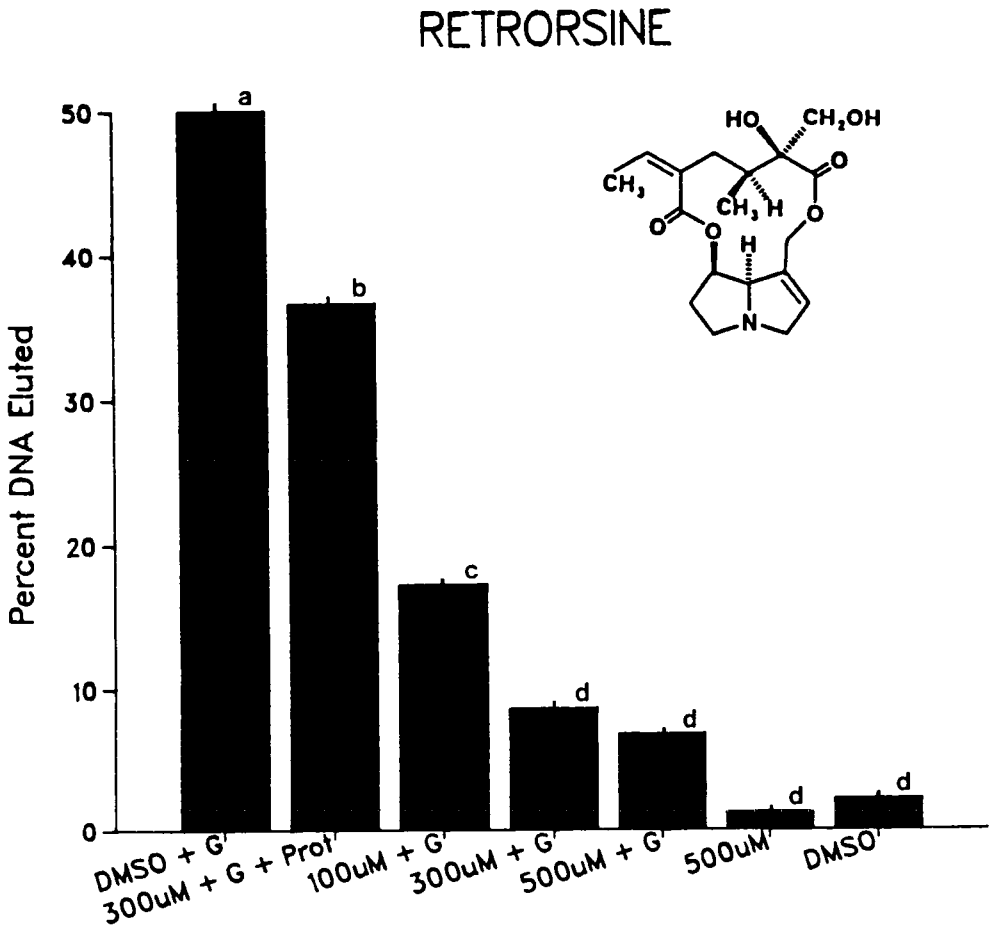


Figure VI-3. The structure of retrorsine and the characterization of the induced DNA damage. See Figure VI-1 for details.

concentrations. The resistance to proteinase K digestion at 300 uM indicates these alkaloids induced both DPC and DNA ISC.

Senecionine (Fig. VI-4) and monocrotaline (Fig. VI-5) showed less TDC at the same dose levels tha seneciphylline, riddelline, or retrorsine, but DPC and DNA ISC were still detected.

Figure VI-6 shows the detection of TDC in cells exposed to 300 and 500 uM heliosupine, but only DPC were detected since proteinase K digestion eliminated any cross-linking effect. Retronecine did not induce any DNA cross-links at 100, 300 or 500 uM concentrations (Fig. VI-7).

The DNA cross-linking capabilities of the seven PAs studied at 300 uM concentration were determined by calculating the DNA cross-linking factors as shown in Table VI-IV. The PAs, ranked from highest to lowest along with the type of cross-link produced, were: seneciphylline (DPC>ISC), riddelline (DPC>ISC), retrorsine (DPC>ISC), senecionine (DPC>ISC), heliosupine (DPC only), monocrotaline (ISC>DPC), and retronecine (neither DPC nor ISC). The differences expressed in the cross-link factors, in comparison to the alkaline elution data, represent the manipulation of the data by the cross-linking formula. The formula takes into account that the rate that DNA elutes is nearly by first order kinetics [Ewig and Kohn, 1978]. Thus, the calculated cross-link factors gives an indication of the actual number of PA-induced DNA cross-links.

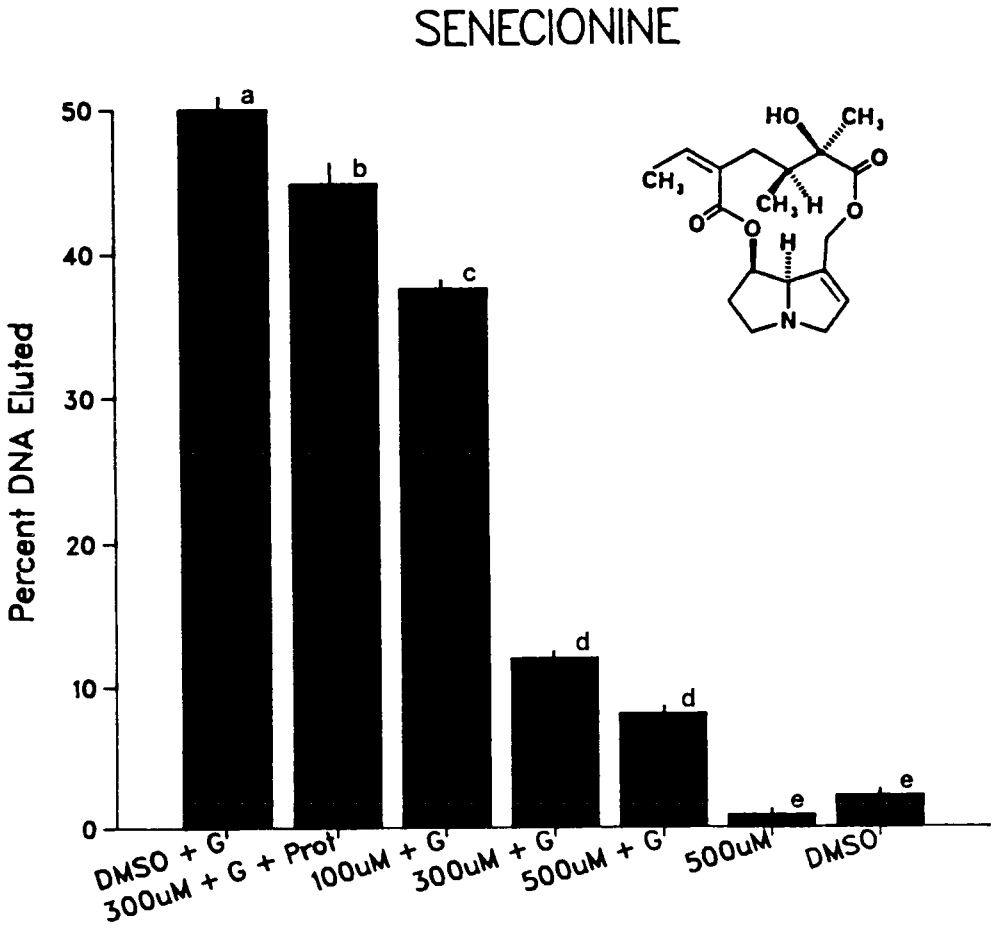


Figure VI-4. The structure of senecionine and the characterization of the induced DNA damage. See Figure VI-1 for details.

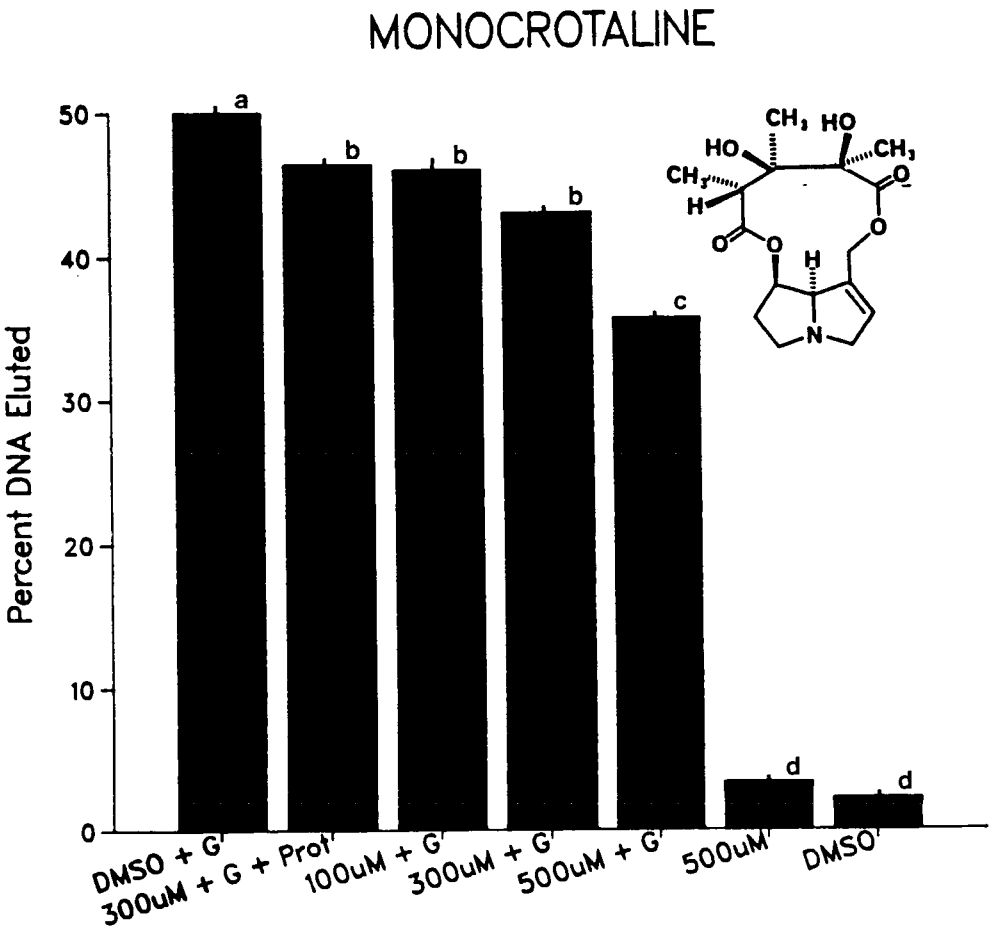


Figure VI-5. The structure of monocrotaline and the characterization of the induced DNA damage. See Figure VI-1 for details.

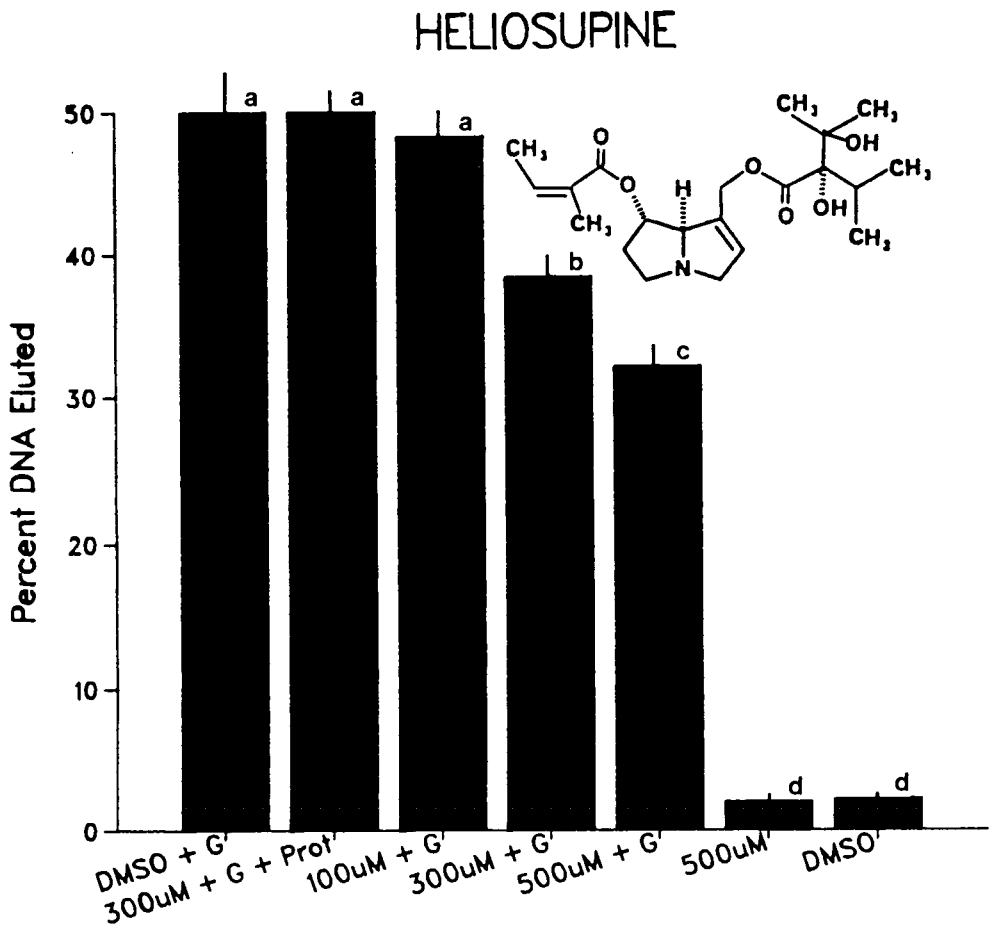


Figure VI-6. The structure of heliosupine and the characterization of the induced DNA damage. See Figure VI-1 for details.

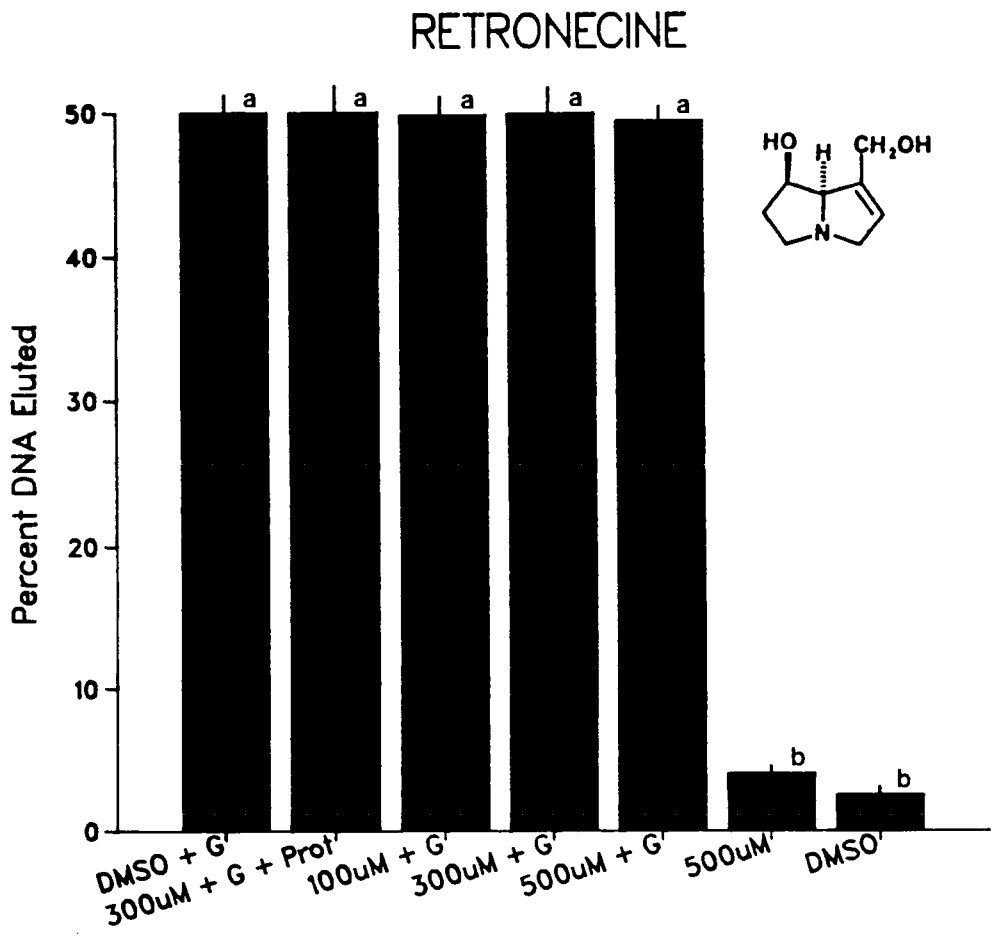


Figure VI-7. The structure of retronecine and the characterization of the induced DNA damage. See Figure VI-1 for details.

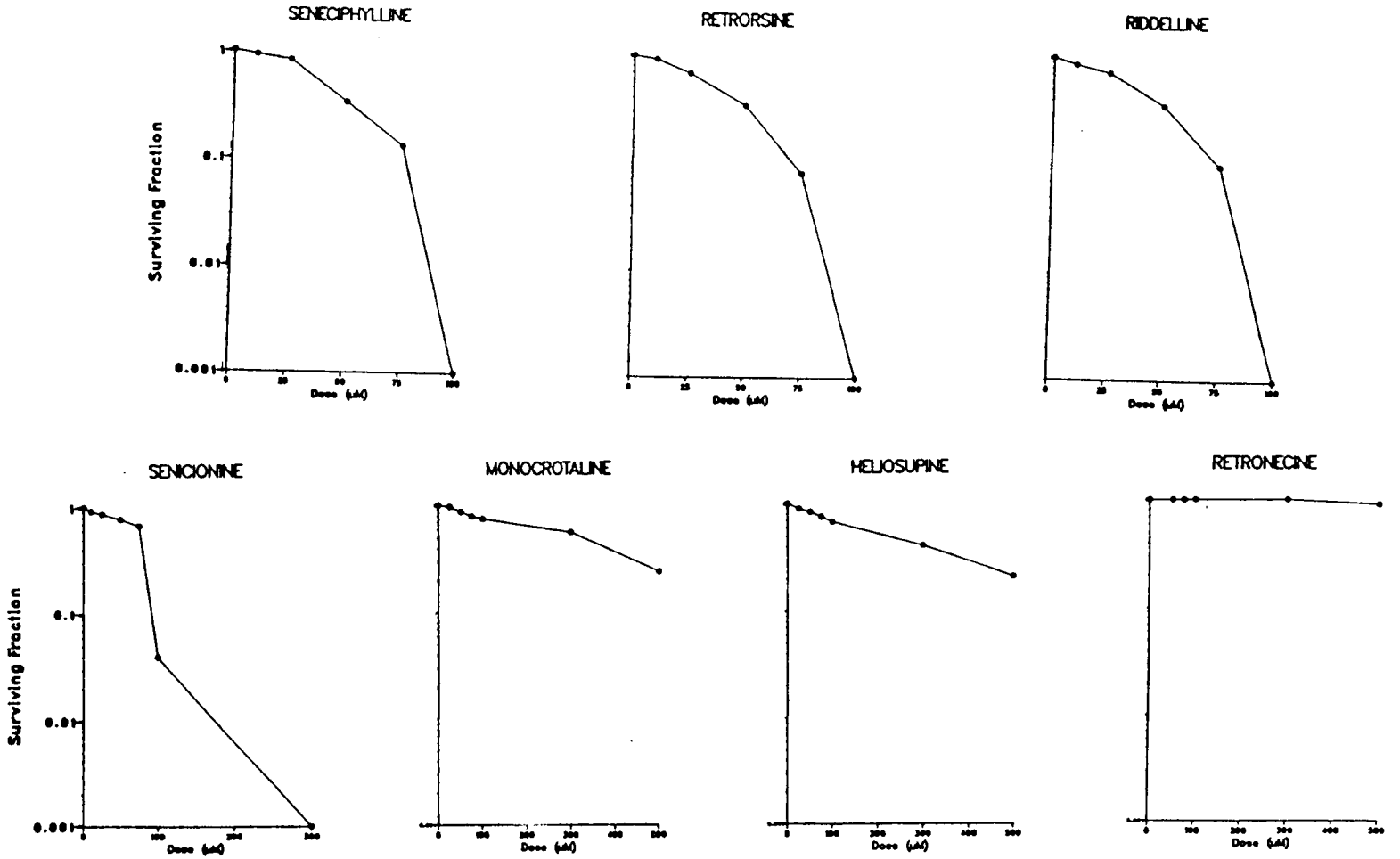
Table VI-IV. The Characterization of DNA Cross-links Induced in Cells Exposed to Various PAs.

COMPOUND (300 uM)	DNA CROSS-LINK[1] FACTOR	
Seneciphylline	TDC	2.198
	DPC	2.031
	ISC	0.167
Riddelline	TDC	1.887
	DPC	1.526
	ISC	0.361
Retrorsine	TDC	1.440
	DPC	1.041
	ISC	0.399
Senecionine	TDC	1.080
	DPC	1.008
	ISC	0.072
Heliosupine	TDC	0.156
	DPC	0.156
	ISC	0.000
Monocrotaline	TDC	0.089
	DPC	0.041
	ISC	0.048
Retronecine	TDC	---
	DPC	---
	ISC	---

[1]Calculated from the formula shown in Methods; DPC factors were determined by the difference between the calculated values of TDC and ISC factors.

Figure VI-8 shows the colony-forming efficiencies of cells exposed to various concentrations of the PAs studied. The results demonstrate that the PAs that induced greater amounts of DNA cross-links were more active in suppressing colony-forming efficiencies. Cells exposed to the highest concentrations of some of the PAs showed no colony formation, yet they were still viable, as determined by trypan blue after eight days post-incubation (data not shown). This implies that high concentrations of the most potent PAs (seneciphylline, retrorsine, riddelline, and senecionine) inhibited cell division indicating cytotoxicity but not lethality.

Figure VI-8. Colony forming efficiencies of cells exposed to the test PA at various doses. Cells were co-cultured with the PA and S9 mix for 2 hr then washed, replaced with fresh medium and cultured for 7-8 days. Results are presented as surviving fraction vs. dose (uM), where 1.0 represents no inhibition of colonies and 0.001 represents 99.9% inhibition of colony formation.


SENECIPHYLLINE
RETRORSINE
RIDDELLINE
SENICIONINE
MONOCROTALINE
HELIOSUPINE
RETRONECINE
Surviving Fraction
1
0.1
0.01
0.001
Dose (μM)

CHAPTER VII

DISCUSSION

DETECTION OF CARCINOGEN-INDUCED DNA STRAND BREAKS BY GRAVITY-FLOW ALKALINE ELUTION

The present modification of the alkaline elution assay was developed to simplify sample analysis when screening single-stranded DNA breaks induced by potential genotoxic compounds. The gravity-flow alkaline elution technique sensitively and reproducibly detected DNA strand breaks following exposure to X-irradiation and chemical carcinogens. The present method detected statistically significant increases in DNA damage over controls at very low x-ray doses (as low as 25 rad), similar to the results of Kohn et al. [1976].

No significant increases in sensitivity were seen when DNA from MDBK cells were preincubated for various periods in eluting solution. In fact, for the control cells, after a 3 h preincubation, results showed an unusually high level of DNA breaks. In the X-ray treated cells, preincubation also resulted in increased DNA breakage. This implies that DNA breakage, induced by preincubation in the eluting solution for 3 hr or longer, was probably due to alkaline hydrolysis, which has been reported to be unrelated to damage induced by genotoxic agents [Kohn et al., 1976]. As a result, alkaline hydrolysis is reduced to a minimum in the gravity-flow method since elution time is less than 2 hr.

To further validate the gravity-flow system, MDBK cells were exposed to seven genotoxic carcinogens. All carcinogens tested

significantly increased single-strand DNA breakage. The test compounds and doses used in these experiments were patterned after a recent study [Sina et al., 1983], in which the alkaline elution method employed the conventional pumping technique to detect DNA damage in rat hepatocytes. The gravity-flow method accurately detected the carcinogenic potential of the test compounds, and sensitivities were similar to those presented in the study by Sina et al. [1983].

Six non-genotoxic agents produced no significant increases in DNA eluted, relative to controls. These results were expected because ETOH, DMSO, TPA and PB have been reported to be negative in the Ames mutagenicity assay [McCann and Ames, 1975], as were AA [Rosenkranz,1977] and SAC [Kroes, 1981]. Also, these compounds elicited no more DNA damage than in controls using the conventional alkaline elution method [Sina et al., 1983].

Conventional alkaline elution procedures [Bradley and Erickson, 1981; Fornace, 1982; Johnston et al., 1983; Eastman and Bresnick, 1978; Kohn et al., 1981] utilize a pumping system to draw the eluting solution through a DNA sample, which may require up to 15 hr. Initially, Kohn et al. [1974] used a pumping rate of 0.5 ml/min (similar to my flow rate) to accurately measure single-stranded DNA breaks induced by X-irradiation. In a later study [Kohn et al., 1976], the pumping rate was slowed to 0.03-0.06 ml/min to study DNA elution kinetics. In that study, two DNA elution phases were resolved: an initial phase, which is related to single-stranded DNA breaks induced by the genotoxic agents, and a second phase which represented strand breaks due to alkaline hydrolysis of the DNA. The

authors stated that the initial phase is of primary importance in determining DNA damage produced by exposure to X-ray or other genotoxic agents. The present technique was designed primarily to detect the initial phase of elution by decreasing the time the DNA is exposed to the alkaline solution.

The chief advantages of the presented assay are that no pump or fraction collectors are required, only three fractions are collected per sample, as many as 20 samples may be completed in 8 hr, statistical analyses can be easily applied to the results, and the sensitivity is similar to that found in conventional alkaline elution procedures. Since the gravity-flow method measures the total amount rather than the rate of single-stranded DNA eluted the assay is best suited for rapid screening of potential genotoxic agents.

INDUCTION OF DNA SINGLE-STRAND BREAKS AND REPAIR BY THREE SOURCES OF UV RADIATION

The purpose of this study was to characterize the DNA strand break potential and subsequent repair of UV radiation from sunlight, suntan booth and germicidal lamp (254 nm). Previous studies have determined that UV-B radiation has a much greater carcinogenic potential in experimental animals than wavelengths within the UV-C or UV-A regions [Urbach et al., 1974]. It has been postulated that radiation from the UV-C region does not penetrate skin efficiently to cause the necessary genetic damage to the underlying cells. It is also important to note that the ozone layer in the earth's upper atmosphere absorbs all the UV-C radiation from sunlight so that only UV-B and UV-A are environmentally relevant. The irradiance scans (Fig. IV-1) show the

energy intensities for the sources of radiation used in the present study. One can see that the suntan booth produced wavelengths similar to those of sunlight, but with roughly 2.7 times the intensity in the UV-B (280-320 nm) region and as high as 5 times that in the UV-A (320-400) region.

Exposures to UV-A alone have the potential of inducing tumors but are much less carcinogenic than exposures to UV-B [Van der Luen, 1984; Daynes et al., 1985]. Willis et al. [1981] showed evidence of synergism between UV-B and UV-A in the induction of cancer, although other studies [Van der Leun, 1984] have not substantiated this. Thus, the contention that the cancer risk posed by a significant UV-A intensity in the suntan booth is greater than that posed by sunlight is left open for debate.

The greater energy emitted from the suntan booth appears to induce a faster rate of cellular DNA damage than from sunlight, as sunlight produced a significant amount of DNA SSB (ca. 50 percent DNA eluted) at 45 min of exposure, and the suntan booth produced similar amounts of DNA SSB in less than half the exposure time (20 min). In comparison, 254 nm UV radiation produced approximately 50 percent DNA eluted at a dose of 80 J/m^2, which corresponded to an exposure time of 93 sec. This amount of strand breakage is equivalent to that produced by 300 rad of X-irradiaton, as shown in the carcinogen-induced DNA SSB study.

The rejoining of DNA SSB was rapid and displayed similar kinetics for both sunlight and the suntan booth. Post-incubation with the polymerase inhibitor Ara C and an inhibitor of ribonucleotide

reductase, HU, has the combined effect of inhibiting the polymerase step of excision repair of damaged DNA [Fornace, 1982; Snyder et al., 1984]. The accumulation of DNA SSB, when the post-incubation media contains Ara C and HU, indicates that excision repair is important in the rejoining of DNA SSB induced by all three UV radiation sources.

The accumulation of DNA SSB during a post-incubation period in cells exposed to 254 nm UV exhibited biphasic kinetics, in that there was an immediate increase in DNA SSB followed by a second increase at 4 hr post-incubation. The amount of DNA SSB was unchanged after 16 hr of post-incubation. The accumulation of DNA SSB was probably due to additional DNA breaks produced during excision repair of pyrimidine dimers [Elkind and Han, 1978], as well as to the inability to repair all the damage induced by 254 nm UV. This response was not seen in the sunlight and suntan booth exposures, since there is a very low formation of dimers in UV-A and UV-B radiation exposures [Elkind and Han, 1978]. Thus, it would appear that mechanisms involved with DNA dimer repair differ from mechanisms of nondimer repair, which is in agreement with the conclusions reached by Rosenstein et al. [1986].

Nondimer DNA photolesions play a significant role in the mechanism of sunlight-induced DNA damage. Enninga et al. [1986] showed that in the UV-A and UV-B regions, cell killing and mutation induction did not correlate with DNA dimer formation. Overwhelming evidence indicates damage to cellular DNA represents the primary target for UV radiation-induced carcinogenesis [for reviews see
Parrish et al, 1978; Daynes et al., 1985]. Therefore, the induction

of DNA SSB and repair induced by sunlight and the suntan booth further illustrates the carcinogenic potential of these UV radiation sources.

DETECTION OF CHEMICAL AND ULTRAVIOLET RADIATION-INDUCED DNA DAMAGE BY GRAVITY-FLOW ALKALINE ELUTION

As mentioned previously, the binding of chemical carcinogens with cellular DNA is thought to be a necessary first step in the initiation of carcinogenesis. Some chemical carcinogens cross-link DNA, which may be an important factor underlying their carcinogenic potential. Alkaline elution has been used to detect DNA cross-linking by various chemical carcinogens [Fornace and Little, 1979] and other alkylating agents such as HN2 and MMC [Fornace and Little, 1977]. In addition, the method can be modified to differentiate between DNA ISC and DPC, since only ISC are resistant to proteinase digestion [Kohn et al., 1981].

Another variation of the alkaline elution method used to detect DPC is the use of a high X-ray dose (2000-15000 rad), which severely fragments DNA, producing a large amount of background SSB. Subsequent retention of DNA from cells exposed to a compound plus X-ray, compared to X-ray alone, is believed to be due to an increase in proteins covalently bound to DNA [Kohn and Ewig, 1979].

Inherent drawbacks associated with the conventional alkaline elution method, which were mentioned previously, were overcome by utilizing the gravity-flow alkaline method. In this part of the project, gravity-flow alkaline elution was modified to rapidly detect

potential cross-linking in DNA from cells exposed to either HN2, MMC, or UV radiation.

Nitrogen mustard is a bifunctional alkylating agent, shown to produce cross-links by various methods [Lawley and Brookes, 1967; Klatt et al. 1969]. Conventional alkaline elution techniques detected the formation of DNA-protein and DNA interstrand cross-links following exposure of this compound at concentrations of 0.25-50 uM [Ewig and Kohn, 1977; Fornace and Little, 1977]. The antitumor antibiotic MMC has also been shown to produce DNA cross-links [Iyer and Szybalski, 1963; Lown, 1979]. More recently Dorr et al. [1985] showed that MMC induced both DPC and DNA ISC. Neither compound has been shown to induce DNA strand breaks by conventional alkaline elution methodology.

In the present study, which employed concentrations similar to previous studies, gravity-flow alkaline elution accurately detected HN2- and MMC-induced DPC and ISC . Also, using a high dose of X-ray (3000 rad) to produce very small DNA fragments, both HN2 and MMC reduced the amount of DNA eluted as measured by gravity-flow alkaline elution. This reduction represents increased proteins covalently bound to DNA, due to the bialkylating capabilities of HN2 and MMC, and is in agreement with the results of Kohn and Ewig [1979].

Ultraviolet radiation (254 nm) has been shown to induce both DNA single-strand breaks [Fornace et al., 1976; Elkind and Han, 1978; Rosenstein and Ducore, 1983] and DNA-protein cross-links [Fornace and Kohn, 1976; Peak et al., 1985] by conventional elution techniques. The results presented here using similar UV doses are in agreement with these studies, but the alternate procedure, utilizing high dose

(3000 rad) X-ray exposures without proteinase, detected little if any DPC. In fact, cells exposed to 40 J/m^2 UV + 600 rad + Prot had a higher amount of DNA eluted than 600 rad + Prot alone. This implies that proteinase digestion removed DNA associated proteins and allowed for the detection of the DNA strand breaks.

Protein-associated DNA strand breaks have been detected with certain intercalating agents [Zwelling et al., 1981]. Such proteins are postulated to be nucleases (such as topoisomerase), which become bound to one of the terminal ends of the strand break it produces. Proteinase treatment removes these repair enzymes revealing DNA SSB as measured by alkaline elution [Zwelling et al., 1981]. Ultraviolet radiation (254 nm) induces excessive DNA damage by the formation of pyrimidine dimers [Jagger, 1976]; removal of pyrimidine dimers occurs by excision repair [Snyder et al., 1984]. Thus, the protein-associated DNA strand breaks detected after UV irradiation may also be due to nucleases bound to the damaged DNA, and the increase in detection of DNA strand breaks after proteinase digestion may be due to the removal of DNA-associated repair enzymes.

CHARACTERIZATION OF DNA DAMAGE BY PYRROLIZIDINE ALKALOIDS

Chemical carcinogenesis is a multistage process involving an initiation step in which a compound or a reactive metabolite interacts with cellular DNA [Miller and Miller, 1981; Farber, 1981]. Several classes of antitumor drugs are thought to induce their cytotoxic effects on tumor cells by the interaction with cellular DNA. Thus, investigating possible DNA interactions of various compounds may allow

for the assessment of carcinogenic potential as well as the discovery of new antitumor drugs.

Several studies have shown a high correlation between the carcinogenic potential of compounds and their ability to induce DNA SSB [Swenberg et al., 1976; Parodi et al., 1982] or DNA cross-links [Fornace and Little, 1979] as measured by alkaline elution. This technique has also been used to assess the formation of DNA cross-links in relation to cell killing for various antitumor drugs. These include nitrogen mustard [Murnane and Byfield, 1981], Mitomycin C [Dorr et al., 1985] and the platinum coordination compound cisplatin [Zwelling et al., 1979].

In the present study, none of the seven PAs induced appreciable amounts of DNA SSB, even when exogenous S9 mix was present to increase metabolic activation. However, since many of the PAs were active in producing DNA cross-links, small amounts of DNA SSB, if present, may go undetected. Our results did show seneciphylline, retrorsine, riddelline, senecionine, and monocrotaline induced significant amounts of DPC and DNA ISC, while heliosupine induced only DPC. Retronecine did not induce any DNA cross-links. Mori et al. [1985] showed a similar relationship with respect to induction of unscheduled DNA repair in isolated hepatocytes. In addition, monocrotaline [Petry et al., 1984] and Jacobine [Petry et al., 1986] did not induce detectable levels of DNA SSB in isolated hepatocytes, but did induce both DPC and DNA ISC.

A differential induction of DNA cross-links was observed for the seven PAs in the present study, indicating possible structure-

activity relationships. Seneciphylline, riddelline, and retrorsine induced the highest levels of DNA cross-linking and were the most cytotoxic, as measured by the reduction in colony formation efficiency. This implies that PA-induced total DNA cross-links were closely related to the inhibition of cell division.

McLean [1970] stated that PAs are most toxic if the pyrrolizidine ring contains a C1,2 double bond and the hydroxyl groups are esterified. Many PAs form highly reactive pyrroles by allylic oxidation of the pyrrolizidine ring followed by dehydration [Mattocks and White, 1971], which is mediated by cytochrome P-450 monooxygenases [Mattocks, 1968]. These pyrrolic metabolites can form alkylating groups at either or both ester linkages [McLean, 1970], producing bi-alkylating agents similar to those of nitrogen mustard and mitomycin. These bi-alkylating products may be the reactive metabolites inducing DNA cross-links in MDBK cells.

Other reactive metabolites may be involved in the toxicity of PAs. As is the case for aflatoxin B_1, the carcinogenic and toxic activity of PAs may involve epoxidation of a ring double bond [Schoental, 1970]. Segall et al. [1985] identified trans-4-hydroxy-2-hexanol as a possible fragmentation product of the pyrrolizidine ring as a reactive metabolite of senecionine.

A structure common to the seven PAs that were studied is the pyrrolizidine ring with a C1,2 double bond with the only structural differences involving the diester substituents. Our results indicate that structural differences in the diester substituents are involved in the differential activities of DNA cross-linking. Seneciphylline

and riddelline, the PAs most active in inducing cross-links, have six carbons in the cyclic diester ring and two unsaturated carbons in the diester side chain. Retrorsine and senecionine contain a similar C-6 cyclic diester ring, but only have one unsaturated carbon bond in the diester group. These two compounds were less active in the induction of DNA cross-links than seneciphylline and riddelline. Monocrotaline has a C-5 cyclic diester ring and no unsaturated carbons in the diester side chain, while heliosupine contains an open chain diester configuration. Both were lower in the induction of DNA cross-links and cytotoxicity than the PAs containing the six carbon cyclic diester ring. Retronecine has no ester substituents and produced no DNA crosslinking.

Schoental [1970] suggested that PAs with bulky, branched chain diester groups are more effective toxicants, due to the rigidity of the molecule allowing for easier epoxidation at the C1,2 double bond in the pyrrolizidine ring. The results of the present study would fit this hypothesis, but recently a metabolite of monocrotaline containing a partially metabolized side chain was found to be a reactive metabolite involved in pneumotoxicity [LaFranconi et al., 1985]. This study supports the contention for the possible involvement of the diester side chain in the bifunctional alkylating metabolite which is responsible for the induction of DNA cross-links.

CHAPTER VIII

CONCLUSIONS

VALIDATION OF GRAVITY-FLOW ALKALINE ELUTION

The interaction of chemical carcinogens with cellular DNA is thought to be a necessary step in the initiation of carcinogenesis. Techniques utilized to detect the molecular interactions of various agents with cellular DNA are useful in determining the genotoxic and possible carcinogenic potential of these agents. The technique of DNA alkaline elution is a sensitive and commonly used short-term method to detect DNA damage. This technique is quite cumbersome and expensive, since excessive time is required to pump eluting solution, and several pumps and fraction collectors are needed to process samples. As a result, a rapid modification of this technique (the gravity-flow alkaline elution method) was developed and validated in the present study.

The gravity-flow technique was first validated by evaluating DNA SSB in epithelial cells exposed to X-irradiation and seven known genotoxic carcinogens. The results show that this modification accurately detected a dose-dependent induction of DNA SSB in cells exposed to 25-1500 rad X-irradiation. In addition, DNA damage was generally dose-dependent and significantly different from control values for all genotoxic carcinogens tested. Of six non-genotoxic compounds tested, none showed any detectable DNA damage. Thus, the assay proved to be a rapid, sensitive and reliable method to detect chemical and X-irradiation-induced DNA damage in the form of DNA SSB.

Since a mechanism common to many carcinogens is the induction of DPC or ISC, a rapid method may be useful to fully study molecular interactions of these agents with cellular DNA. Further experiments were to needed to adapt the gravity-flow system to enable detection of DNA damage in the form of DNA cross-links. The experiments included three known cross-linking agents, nitrogen mustard (HN2), mitomycin C (MMC), and UV radiation (254 nm).

One hr exposures of HN2 at 0.25, 1.0 and 4.0 uM or MMC at 20, 40 and 60 uM produced a dose-dependent increase in DNA cross-links. Subsequent digestion with proteinase K revealed that HN2 and MMC induced both DNA-protein cross-links and DNA interstrand cross-links. Ultraviolet radiation induced DNA cross-links at 20 and 40 J/m^2. However, upon incubation with proteinase, these effects were eliminated, indicating UV radiation produced protein-associated DNA strand breaks.

Thus, gravity-flow alkaline elution is a sensitive and accurate method of detecting and characterizing the molecular events of both DNA strand breaks as well as DNA cross-linking. Due to the reduction in time and cost of performing gravity-flow alkaline elution, this method may be useful as a screening assay for genotoxicity and/or a predictive assay for potential mutagenic or carcinogenic agents.

DNA DAMAGE AND REPAIR BY THREE SOURCES OF UV RADIATION

The induction of DNA SSB in cultured epithelial cells was dose-dependent for sunlight, suntan booth, and 254 nm UV. The suntan booth however, required half the time to produce an equivalent amount of DNA

SSB compared to sunlight. Repair of DNA SSB was very rapid and involved DNA excision repair for both sunlight and the suntan booth, yet the induction of DNA damage and the repair of cells exposed to 254 nm UV radiation appeared different. The 254 nm UV radiation may have induced larger amounts of DNA dimers than produced by sunlight or the suntan booth, creating the differences seen in the induction of DNA SSB and rate of repair. The similarities of DNA damage and repair in cells exposed to sunlight and the suntan booth indicate human exposure to either source may pose similar carcinogenic risks.

CHARACTERIZATION OF DNA DAMAGE BY PYRROLIZIDINE ALKALOIDS

In this study, the mechanism by which seven pyrrolizidine alkaloids (PAs) interact with DNA in cultured MDBK cells were characterized using gravity-flow alkaline elution. Sensitivity in the detection of DNA damage was increased in cells co-cultured with an external metabolizing system consisting of a NADPH generating system and rat liver S9 fraction. Because the fact that PAs require activation is known a priori, these results indicated that MDBK cells were deficient in metabolizing PAs to the reactive genotoxic metabolite.

Six of the seven PAs studied were found to induce DNA cross-linking; five of these produced both DPC and DNA ISC while one produced only DPC. None of the PAs induced appreciable amounts of DNA SSB, even when co-cultured with exogenous S9 mix. The induction of DNA cross-links by the PAs was inversely related to the colony

formation efficiency, implying that DNA cross-links play a role in the inhibition of cell division.

Previous studies have indicated that pyrrole formation is involved in the ultimate PA metabolite. However, since the PAs in the present study differ only in the extent of substitution of the diester side chain, the results indicate that these substituents are an important determinant in the induction of DNA damage by pyrrolizidine alkaloids. The diester substituents may be directly involved in the genotoxic metabolite or they may direct enzymatic activation at other sites in the pyrrolizidine molecule.

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EDUCATION:

Ph.D. in Toxicology, expected Jan. 1988; Utah State University, Logan, UT.

M.S. in Toxicology, 1983; Utah State University, Logan, UT.

B.S. in Wildlife Biology/Veterinary Science, 1979; Michigan State University, East Lansing, MI.

EXPERIENCE:

3/86-Present — Graduate School Fellowship, Toxicology Program, Utah State University, Logan, UT.

9/82-3/86 — NIEHS Predoctoral Trainee in Toxicology, Toxicology Program, Utah State University.

Dissertation: "Characterization of Chemical and Radiation-Induced DNA Damage and Repair in the MDBK Cell Culture System."

Techniques utilized: tissue culture, monitor genotoxicity by alkaline elution, DNA adduct formations and repair, Ames assay, HPLC analysis, gel electrophoresis, and radioisotopic tracers. Familiar with x-ray, gamma, UV light, and chemical interactions with cellular DNA.

9/80-9/82 — Graduate Teaching and Research Assistant, Department of Biology, Toxicology Program, Utah State University, Logan,UT

Thesis: "Alterations in Serum Enzyme Levels,Activity of Hepatic Monooxygenases, and Hepatocyte Morphology Due to Liver Enzyme Inducers in Microtus Montanus."

Techniques utilized: Hepatic microsomal enzyme preparation, spectrophotometric measurement of monooxygenase activity, gas, column and thin layer chromatography, pathological analysis by histological examinations.

3/80-8/80 Lawn Care Specialist, Chem Lawn Corporations, Lansing, MI

Techniques utilized: handling and application of lawn care pesticides.

6/79-12/79 Wildlife Biologist, Bureau of Land Management, Department of Interior United States Government, Worland, Wyoming.

Techniques utilized: small mammal trapping, wildlife habitat utilization and analysis.

PUBLICATIONS:

Hincks, J.R. R.A. Coulombe, Jr., Hea-Young Kim, H.J. Segall R.J. Molyneux and F.R. Stermitz. 1987. Characterization of DNA damage in mammalian cells by pyrrolizidine akaloids. Manuscript submitted.

Hincks, J.R. and R.A. Coulombe. 1987. Detection of chemical and ultraviolet radiation-induced DNA cross-links by gravity-flow alkaline elution. Manuscript submitted.

Hincks, J.R. and R.A. Coulombe. 1987. Induction of DNA single-strand breaks and subsequent repair by three sources of ultraviolet radiation in cultured epithelial cells. Manuscript submitted.

Margaretten, N.C., J.R. Hincks, R.P. Warren and R.A. Coulombe. 1987. Effects of phenytoin and carbamazepine on human natural killer cell activity and genotoxicity in vitro. Toxicol. Appl. Pharmacol. 87:10-17.

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Hincks, J.R. and R.A. Coulombe. 1986. Gravity-Flow alkaline elution: a modified method to rapidly detect carcinogen induced DNA strand breaks. Biochem. Biophys. Res. Commun. 137:1006-1014.

Hincks, J.R. and W.A. Brindley. 1986. Effects of varying inducer type and dose on hepatic microsomal activities in the mountain vole Microtus montanus. Comp. Biochem. Physiol. 85C:385-389.

ABSTRACTS AND PRESENTATIONS:

J.R. Hincks, H.Y. Kim, and R.A. Coulombe, Jr. Further characterization of the genotoxicity of a variety of pyrrolizidine alkaloids. Presented at the 1987 Mt. West Chapter, SOT, Meeting, Boulder, CO.

Hincks, J.R. and R.A. Coulombe. 1987. Detection of DNA cross-linking by gravity-flow alkaline elution. Presented at 1987 Society of Toxicology Meeting, Washington, D.C.

Hincks, J.R. and R.A. Coulombe. 1987. Genotoxicity of pyrrolizidine and larkspur alkaloids detected by alkaline elution. Presented at 1987 Society of Toxicology Meeting, Washington, D.C.

Hincks, J.R. and R.A. Coulombe. 1986. Comparisons of UV induced DNA damage and repair by sunlight, suntan booth, and germicidal lamp. Presented at 1986 Society of Toxicology Meeting, New Orleans, LA.

Margaretten, N.C., J.R. Hincks, R.P. Warren and R.A. Coulombe. 1986. Phenytoin suppresses immune function and causes damage to DNA in vitro and in vivo. Presented at 1986 Society of Toxicology Meeting, New Orleans, LA.

Margaretten, N.C., J.R. Hincks, R.P. Warren and R.A. Coulombe. 1985. Phenytoin suppresses killer cell activities and causes damage to DNA in vitro and in vivo. Presented at Mountain-West Chapter, SOT, Albuquerque, NM.

Hincks, J.R. and R.A. Coulombe. 1985. A rapid method to detect carcinogen-induced single-stranded DNA breaks. Presented at the 1985 Society of Toxicology Meeting, San Diego, CA.

Hincks, J.R. and R.A. Coulombe. 1984. The feasibility of gravity-flow alkaline elution to detect carcinogen-induced single-stranded DNA breaks. Presented and chaired session at the Mountain-West Chapter, SOT, Logan, UT.

Hincks, J.R. and W.A. Brindley. 1983. The relationship of hepatic monooxygenase activity to histological changes observed in Microtus montanus due to the inducers phenobarbital, B-naphthoflavone, and Aroclor 1254. Presented and chaired session at the 1983 Third International Congress on Toxicology, San Diego, CA.

Hincks, J.R. and W.A. Brindley. 1983. Hepatic monooxygenases in relation to type and dose of inducer in the mountain vole. Presented and chaired session at Utah Academy of Sciences, Logan, UT.

Hincks, J.R. and W.A. Brindley. 1983. Comparison of dose response relationships between the inducers phenobarbital, B-naphthoflavone, and Aroclor 1254 in Microtus montanus. Presented at the 1983 Society of Toxicology Meeting, Las Vegas, NV.

PROFESSIONAL ORGANIZATIONS:

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PERSONAL:

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ACTIVITIES:

Participated in the 1987 Molecular Biology and DNA Sequencing Workshop sponsored by Promega Corp. held in Logan, UT.

Participated in 1986 Inhalation Workshop sponsored by the Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM.