

DISSERTATION

**CARCINOGENICITY BIOASSAYS WITH ARSENIC
IN CHEMICAL MIXTURES**

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER
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ABSTRACT OF DISSERTATION

CARCINOGENICITY BIOASSAYS WITH ARSENIC IN CHEMICAL MIXTURES

The carcinogenicity of arsenic has been indisputably demonstrated through diverse epidemiological data sets gathered from around the world. Evidence from environmental and occupational exposures has unequivocally established a link between exposure to arsenic and a variety of cancers, most notably in the skin, lung, liver, and urinary bladder. Yet, despite its known carcinogenic potential in humans, laboratory animal evidence for the carcinogenicity of arsenic is equivocal at best. Consequently, much remains unknown about its carcinogenic mode of action. In recent years, the scientific community invested significant resources to study the carcinogenic effects and mode of action of arsenic. This intensive research effort was driven partially by the recognition of large-scale, high-dose exposures to arsenic in drinking water in certain parts of Asia, as well as concerns in the United States regarding the protectiveness against cancer endpoints of the Environmental Protection Agency's Maximum Contaminant Level (MCL) of 50 parts per billion (ppb) in drinking water (revised in February, 2002 to 10 ppb). The need for a more complete

understanding of arsenic's carcinogenic mode action, to facilitate the development of more accurate and reliable risk assessments, had become strikingly apparent.

The preponderance of experimental evidence indicates that arsenic does not act as an initiator in the process of carcinogenesis, but rather as a promoter, progressor, or cocarcinogen. A logical experimental application of this observation, therefore, is the evaluation of arsenic in chemical mixtures composed of compounds the carcinogenic effects of which arsenic is likely to enhance. Given that humans generally encounter hazardous chemicals in the environment as mixtures rather than single compounds, the consideration of environmentally realistic chemical mixtures is equally important. Both of these issues are critical considerations in the development of a valid experimental animal model of arsenic carcinogenesis.

The overall purpose of this research is to evaluate the carcinogenic potential of a chemical mixture consisting of 1,2-dichloroethane (DCE), vinyl chloride (VC), and trichloroethylene (TCE) in addition to arsenic (As). Composition of the experimental chemical mixture was determined based on considerations of environmental relevance and the existence of a common carcinogenic endpoint. All four chemicals are frequently identified as groundwater contaminants near hazardous waste disposal sites, and three (As, DCE, and VC) have been associated with the development of hepatic angiosarcoma in humans and/or animals.

The significant results of this research were: (1) observation of hepatic sinusoidal dilatation, an early change reported in association with development of hepatic angiosarcoma, in the livers of rats treated with DCE; (2) discovery, in the framework of a

multiple organ carcinogenicity bioassay, of a dose-dependent antagonistic effect of chemical mixtures containing As, DCE, VC, and TCE on the development of a) preneoplastic lesions in the lung and liver and b) neoplastic lesions in the lung; (3) validation, in a medium-term liver foci bioassay, of the suppressive effect of the quaternary mixture on the development of preneoplastic glutathione-S-transferase π (GSTP) positive hepatic foci; (4) attribution of the suppressive effect on GSTP positive focus development to As alone; (5) development of an HPLC method for measuring S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in liver tissue; (6) evaluation of the potential involvement of altered levels of hepatic SAM and SAH in the suppressive effect of As, alone and in chemical mixtures, on the development of hepatic GSTP positive foci; and (7) *in vitro* observations of modulation of the toxicity of As and its metabolites in endothelial cells associated with altered microenvironmental pH.

In contrast to data from a variety of recent studies which support the hypotheses that As may act as a promoter, progressor, or cocarcinogen, evidence from the research presented herein indicates that under certain circumstances, As may exert an antagonistic or suppressive effect on the development of neoplasia. Indeed, the efficacy of arsenic trioxide in the treatment of a variety of hematologic malignancies is well-documented, and it is currently undergoing clinical evaluation as a chemotherapeutic agent for a variety of other cancers as well. These apparently conflicting effects of As highlight the need for a greater understanding of its paradoxical nature as both a carcinogen and a therapeutic agent. Furthermore, reconciliation of the disparity in carcinogenic effects between animals and humans represents another challenge facing researchers in the arena of As carcinogenesis.

With continued investigations, deeper knowledge of these issues will enable the development of more accurate and reliable risk assessment strategies for arsenic.

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DEDICATION

To my parents, for teaching me, by example, the valor of tenacity;

To John, for the challenge to explore unexpected depths;

To my sons, for ancient rejuvenation.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 MULTISTAGE NATURE OF CARCINOGENESIS

The process of carcinogenesis is multistep in nature and can be divided into three distinct yet continuous phases: initiation, promotion, and progression (Mastorides and Maronpot 2002; Pitot and Dragan 1996). The first stage of initiation involves the induction of an irreversible, heritable change in the genome of the cell by an initiating agent. Subsequent cell division “fixes” the genomic alteration, and the cell thus progresses on to the next stage of carcinogenesis - promotion. In this phase, the initiated cell continues to replicate and undergoes clonal expansion, resulting in an identifiable preneoplastic or neoplastic lesion. The final stage of carcinogenesis is progression. During this process, the preneoplastic or neoplastic lesions progress to states characterized by greater alteration in the genomic structure of the cell, reduced genomic stability, and greater malignancy. Malignancy is defined as the capacity for metastatic growth (secondary growth of cells separate from the primary neoplasm).

Chemical carcinogens may be classified according to their action(s) in one or more of the stages of carcinogenesis (Mastorides and Maronpot 2002; Pitot and Dragan 1996). An initiating agent (or initiator) is a chemical capable only of initiating cells, whereas the action of a promoting agent (or promoter) is limited to effecting the expansion of initiated cell clones. A progressor agent is a chemical capable of converting a cell in the stage of promotion or an initiated cell into a potentially malignant cell. Any chemical exhibiting the combined characteristics of initiating, promoting, and progressor agents - in other words, capable by itself of inducing cancer from normal cells - is known as a complete carcinogen. A cocarcinogen is defined as an agent that is not carcinogenic alone but is capable of potentiating the effect of a known carcinogen. A cocarcinogen differs from a promoter based on the timing of its critical effect; a cocarcinogen exerts its effect just prior to or together with a carcinogen, while a promoter's effect occurs after exposure to a carcinogen (Robens *et al.* 1994). Mechanisms of cocarcinogenesis include 1) increased uptake of the carcinogen, 2) increased proportion of carcinogen activated, 3) depletion of competing nucleophiles, 4) inhibition of the rate or fidelity of DNA repair, 5) enhanced conversion of DNA lesions to permanent alterations (Robens *et al.* 1994).

1.2 CHEMICAL MIXTURES: EVALUATION OF CARCINOGENICITY

Rarely are chemical pollutants encountered in the environment as single chemicals. Multiple chemical exposures are more likely to occur, as with polluted air or domestic water supplies in heavily industrialized regions or in contaminated water sources near hazardous waste disposal sites. The most realistic and accurate assessment of toxicologic effects of

environmental contaminants ought to be based not on the toxicological properties of individual chemicals but on the toxicological interaction of chemical mixtures. However, relatively few studies examining the carcinogenic effects of environmentally relevant chemical mixtures have been performed. In perhaps one of the earliest studies of chemical mixture carcinogenesis, Neish *et al.* (1967) reported the appearance of a variety of neoplasms in rats administered two noncarcinogenic aminoazo dyes in the diet over a period of one year. Subsequently, both additive and synergistic tumor development was demonstrated in studies involving the oral administration of between three and five *N*-nitrosamines at low dose levels (Berger *et al.* 1987; Lijinsky *et al.* 1983). Similarly, application of extremely low levels of benzo[*a*]pyrene in conjunction with five noncarcinogenic polycyclic aromatic hydrocarbons resulted in a significant increase in the development of neoplasms compared to control animals receiving benzo[*a*]pyrene alone (Warshawsky *et al.* 1993). In another study, rats exposed to a mixture of 40 chemical carcinogens for two years at reduced dose levels (50% of the dose required to induce neoplasia in 50% of treated animals) resulted in a significant increase in tumor incidence in the thyroid gland and liver (Takayama *et al.* 1989).

Although a two-year rodent bioassay failed to demonstrate a carcinogenic response in animals exposed to a mixture of foundry fumes, a significant increase in preneoplastic lesions was apparent compared to controls (Humfrey *et al.* 1996). Exposure of male mice to a quintary mixture of carcinogenic environmental polycyclic aromatic hydrocarbons identified greater than additive and less than additive interactions, at lower doses and higher doses, respectively, related to the development of lung adenomas (Nesnow *et al.* 1998). In contrast, rats administered a mixture of disinfection by-products (potassium bromate, 3-

chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone, chloroform, and bromodichloromethane) in the drinking water showed a less than additive effect in terms of renal adenoma and carcinoma development compared to the individual compounds (Hooth *et al.* 2002).

1.3 ARSENIC

In humans, the carcinogenicity of inorganic arsenic has been unequivocally established by a wealth of epidemiologic data from various populations. Ingestion of arsenic-contaminated drinking water has been associated with the development of skin, lung, liver, and urinary bladder cancer (Kurtio *et al.* 1999; Lewis *et al.* 1999; Rahman *et al.* 1999; Tondel *et al.* 1999; Hopenhayn-Rich *et al.* 1998; Mazumder *et al.* 1998; Subramanian and Kosnett 1998; Choprapawon and Rodcline 1997; Chowdhury *et al.* 1997; Luo *et al.* 1997; Das *et al.* 1996; Hopenhayn-Rich *et al.* 1996; Chiou *et al.* 1995; Tsuda *et al.* 1995; Cebrián *et al.* 1994; Chen and Lin 1994; Das *et al.* 1994; Chen *et al.* 1985; Chen *et al.* 1992; Chen and Wang 1990; Wu *et al.* 1989; Chen *et al.* 1988; Chakraborty and Saha 1987; Cebrián *et al.* 1983; Tseng 1977; Tseng *et al.* 1968). Remarkably, a well-established laboratory animal model for studying the carcinogenic effects of arsenic does not currently exist, in spite of many attempts to develop such models. Consequently, the carcinogenic mode of action of arsenic remains poorly understood. No evidence of mutagenic potential has been demonstrated in either bacterial or mammalian cells (Jacobson-Kram and Montalbano 1985), and it is generally accepted that arsenic does not induce point mutations (Weincke *et al.* 1997; Morton and Dunnette 1994; Li and Rossman 1989a,b; Lee *et al.* 1985). Some data indicate that arsenic may act as a promoter in cultured keratinocytes (Germolec *et al.* 1997;

Germolec *et al.* 1996) and in transgenic mouse models of skin carcinogenesis (Germolec *et al.* 1998; Germolec *et al.* 1997). In other *in vivo* studies, a metabolite of arsenic, dimethylarsinic acid (DMA), has been associated with promotional effects in the liver (Wanibuchi *et al.* 1997; Wanibuchi *et al.* 1996), lung (Yamanaka *et al.* 1996), and kidney (Yamamoto *et al.* 1997; Yamamoto *et al.* 1995). In addition, significant *in vitro* and *in vivo* evidence indicates that arsenic is capable of causing genetic effects associated with late stages of carcinogenesis (*i.e.*, progression). Arsenic is known to induce a variety of chromosomal aberrations, such as breaks and gaps, as well as sister chromatid exchanges, in human and animal cell culture systems (Hei *et al.* 1998; Moore *et al.* 1997; Rasmussen and Menzel 1997; Yamanaka *et al.* 1997; Barrett *et al.* 1989; Barrett *et al.* 1988; Lee *et al.* 1986; Jacobson-Kram and Montalbano 1985; Lee *et al.* 1985; Paton and Allison 1972). *In vivo* exposure to arsenic has also been shown to increase the frequency of chromosomal aberrations in laboratory animals and humans (Mäki-Paakkanen *et al.* 1998; Moore *et al.* 1997; Lerda 1994; Warner *et al.* 1994; Nagymajtenyi *et al.* 1985; Nordenson *et al.* 1979; Nordenson *et al.* 1978; Beckman *et al.* 1977; Burgdorf *et al.* 1977). Observations that arsenic is able to modify DNA repair processes *in vitro* suggest that it may act as a cocarcinogen (Lee-Chen *et al.* 1993, 1992).

1.4 ARSENIC AND HEPATIC ANGIOSARCOMA

Individually, As, VC, and DCE have been associated with the development of hepatic angiosarcoma (Chen and Lin 1994; Naqvi 1994; Feron *et al.* 1981; NCI 1978; Feron *et al.* 1975), a relatively rare form of cancer (Popper *et al.* 1978). While the roles of VC and

DCE in this context have been relatively well-studied, questions surrounding the action of As remain unanswered, partially because no successful animal model of As-induced carcinogenesis existed until recently. Understanding whether As acts alone or in concert with other compounds in the development of angiosarcoma could help elucidate its role in carcinogenic processes throughout the body, and investigation of the carcinogenic effects of As in chemical mixtures, particularly in environmentally relevant chemical mixtures, is highly warranted.

1.5 METHODS FOR EVALUATING CARCINOGENICITY

The traditional method for evaluating the carcinogenic potential of a chemical is the two-year chronic bioassay (Pitot and Dragan 1996; Robens *et al.* 1994). Two test species - usually the Fischer 344 (F344) rat and the B6C3F₁ strain of mouse - are chosen in order to compensate for any genetic differences in tumor susceptibility. Because the principal endpoint of the chronic bioassay is the presence or lack of tumor development resulting from exposure to the test chemical, a sufficient number of animals in each treatment group is necessary to achieve statistical significance. Fifty animals per gender per treatment group is generally considered appropriate unless the incidence of a particular tumor is of concern. Three dose levels of the test chemical are usually chosen, with the highest dose corresponding to the maximum tolerated dose (MTD). The MTD, determined in the sub-chronic toxicity study, is chosen to represent the highest dose that would not decrease the longevity of the exposed animals due to toxic effects other than tumor induction. Determination of the MTD may be based on endpoints such as body weight decrease, target

organ pathology, or adverse clinical signs. Selection of doses below the MTD depends on a variety of factors: the slope of the dose-response curve in range-finding studies, the need for a no-effect dose, and pharmacokinetic considerations. For many chronic bioassays, according to the traditional National Cancer Institute (NCI) protocol, the intermediate and low dose levels are chosen to represent one-half and one-fourth of the MTD (Robens *et al.* 1994). The National Toxicology Program (NTP) protocol frequently uses dose levels representing one-third and one-ninth of the MTD. Throughout the course of the bioassay, animals are routinely monitored for signs of toxicity and tumor development. Detailed records of food and water consumption, body weight, mortality, and pathology are a critical component of the experimental protocol. Upon death, all animals undergo a thorough necropsy examination to assess the presence of tumors or other disease and to determine the cause of death. Subsequently, a thorough histopathological examination is performed on all tissues, to include any suspect lesions and/or specific target organs.

Alternatives to the two-year chronic bioassay include the short- and medium-term rodent bioassays, such as the strain A mouse pulmonary tumor test, rat mammary neoplasm test, subcutaneous injection test, numerous variations of the medium-term rat liver carcinogenicity test, multiple organ carcinogenicity bioassays, and a variety of genetically altered mouse models (Robens *et al.* 1994; Ito *et al.* 1989c). Many of these assays rely on the multistage nature of carcinogenesis, in terms of sequential initiation and promotion events, to accelerate the development of quantifiable carcinogenic endpoints. Among these, the medium-term rat liver carcinogenicity test is particularly well-suited for studying multistage hepatocarcinogenesis. Although various models with slightly different treatment

protocols have been developed to study specific events in the hepatocarcinogenic process, most study designs incorporate the sequential administration of an initiating agent followed by test chemical administration (Ito *et al.* 1994; Ito *et al.* 1989a,b,c). A mitogenic stimulus for hepatocytes, such as two-thirds partial hepatectomy, is often incorporated into the study design (Ito *et al.* 1994; Ito *et al.* 1989a,b,c).

In the medium-term hepatocarcinogenesis models, reliance on the development and subsequent quantification of preneoplastic lesions reduces the need to wait for actual tumor development. The endpoint hepatic preneoplastic lesions of interest are usually identified as foci of altered hepatocytes which can be characterized by a variety of morphological and/or phenotypic traits, such as histological appearance (*e.g.*, altered hepatocellular foci such as basophilic, eosinophilic, or clear cell foci) or immunohistochemical staining patterns for a variety of enzymatic markers (*e.g.*, γ -glutamyltransferase, adenosine triphosphatase, glucose-6-phosphatase, and glutathione *S*-transferase π). Of these, glutathione *S*-transferase π (GST-P) has been identified as one of the most accurate markers of preneoplasia (Ito *et al.* 1989a,b).

Additionally, various *in vitro* cell transformation assays represent a short-term method for evaluating the carcinogenic potential of a chemical. In these types of assays, observation of morphologically transformed colonies or discrete cellular foci is indicative of cell transformation, which is associated with increased carcinogenic potential. Commonly used cell lines include immortalized mouse fibroblast BALB/c-3T3 or C3H 10T1/2 cells, Syrian hamster embryo cells, and virally infected rat cell lines (Pitot and Dragan 1996).

1.6 THESIS OBJECTIVES

While the carcinogenicity of arsenic in humans has been well-documented in a variety of diverse populations, its carcinogenic effects have not been duplicated in laboratory animals. As a result, relatively little is understood regarding its carcinogenic mode of action, although many researchers in the field have contributed valuable data indicating that arsenic may act as a promoter, progressor, or cocarcinogen in the multistep process of carcinogenesis. As such, evaluation of arsenic's carcinogenicity in concert with other carcinogens whose effects it may potentiate is warranted. This research need is underscored by the acknowledgment that humans encounter hazardous chemicals in the environment as mixtures rather than single agents. Thus, investigation of arsenic in environmentally relevant chemical mixtures represents a novel approach toward assessing the carcinogenic effects of arsenic.

As described in the preceding sections, the goal of the research presented herein was to evaluate the carcinogenic potential of a quaternary chemical mixture consisting of As, DCE, VC, and TCE. These compounds were selected for two reasons: 1) they are frequently identified as groundwater contaminants near hazardous waste disposal sites, and 2) exposure to As, DCE, and VC is associated with the development of angiosarcoma of the liver in humans and/or animals. The hypothesis that arsenic would demonstrate a synergistic or at least additive effect on the development of preneoplastic and neoplastic lesions in F344 rats exposed to a quaternary mixture of As, DCE, VC, and TCE was tested in a variety of bioassays. The research encompassed three primary goals: a) evaluate the effect of As in chemical mixtures on the development of preneoplastic and neoplastic lesions; b) evaluate

the effect of As alone on the development of preneoplastic and neoplastic lesions; c) examine potential mechanisms of action for As using appropriate laboratory techniques. The first step toward achieving these goals was the evaluation of hepatic angiosarcoma induction by As, alone and in combination with DCE, VC, and TCE, utilizing an animal model which had previously demonstrated that hepatic sinusoidal endothelial cells (the cell of origin of hepatic angiosarcoma), harvested from livers of diethylnitrosamine-treated rats and transplanted into weanling rats, resulted in angiosarcoma development (Chapter 4). In Chapter 5, the organ-specific carcinogenicity of quaternary mixtures (consisting of different dose levels of As, DCE, VC, and TCE) was investigated using a multiple organ carcinogenicity bioassay. This bioassay employed an initiation/promotion protocol, in which multiple initiator administration was followed by exposure to different dose levels of the quaternary chemical mixture. The hepatocarcinogenicity of the same quaternary mixture of As, DCE, VC, and TCE was subsequently evaluated using another initiation/promotion-based assay - the medium-term liver foci bioassay. Ternary (As, DCE, TCE) and binary (As, DCE) submixtures, as well as As alone, were also investigated in this study (Chapter 6). In Chapter 7, human umbilical vein endothelial cells (HUVECs) were used as surrogates for hepatic sinusoidal endothelial cells to assess the toxicity of As and its metabolites. To evaluate the effect of potential changes in microenvironmental pH in the hepatic sinusoid, *in vitro* toxicity was assessed under conditions of normal and acidotic pH.

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CHAPTER 2

BACKGROUND:

1,2-DICHLOROETHANE, VINYL CHLORIDE, AND TRICHLOROETHYLENE

2.1 1,2-DICHLOROETHANE

2.1.1 Historical and Current Human Exposures to 1,2-Dichloroethane

1,2-Dichloroethane (DCE; $C_2H_4Cl_2$) belongs to the class of chlorinated aliphatic hydrocarbons. Synonyms for DCE include 1,2-ethylene dichloride, ethylene dichloride, and dichloroethylene (ATSDR 2001). A colorless liquid at room temperature, DCE has a pleasant odor and is slightly soluble in water. It is predominantly used as a chemical intermediate in the production of vinyl chloride (VC), with 85 - 88% of its total production in the United States used for this purpose (ATSDR 2001; IARC 1999). Additionally, it is used as a chemical intermediate in the manufacture of 1,1,1-trichloroethane, tri- and tetrachloroethylene, vinylidene chloride, ethyleneamines, and aziridines (ATSDR 2001; IARC 1999). It is also used as a solvent in closed systems. In 1994, total DCE production in the US was 16.8 billion pounds (ATSDR 2003). Commercially, DCE is used as an ingredient in varnish and finish removers, textile solvents, as an extractant in organic

synthetic processes, and as a metal degreaser (ATSDR 2001; IARC 1999). It is a component of some soaps, scouring compounds, wetting and penetrating agents, paints, adhesives, and other coatings (ATSDR 2001; IARC 1999). It is also used as a lead scavenger in anti-knock fluids in gasoline, in ore flotation, and as a grain, household, and soil fumigant, although it is no longer registered for use as a fumigant on agricultural products in the United States (or in Canada, the United Kingdom, or Belize) (IARC 1999).

Occurrence of DCE in the environment is primarily due to atmospheric contamination resulting from its production and use as a chemical intermediate, solvent, and lead scavenger in gasoline (IARC 1999). However, DCE can also be released into groundwater, surface water, or soil, again resulting from its use in a wide variety of industrial processes (ATSDR 2001; IARC 1999). In the atmosphere, DCE is degraded to formyl chloride, chloroacetyl chloride, hydrogen chloride, and chloroethanol; the average lifetime of DCE in the atmosphere is estimated to be greater than five months (ATSDR 2001; USEPA 1993). In ground and surface water, DCE may be biodegraded to ethene, chloroethene, and ethane (ATSDR 2001; Hollinger *et al.* 1990). Similarly, in soil, DCE that does not volatilize into the ambient atmosphere may undergo biodegradation to ethane (ATSDR 2001; Watwood *et al.* 1991) or carbon dioxide (ATSDR 2001; Speitel and Closmann 1991; Henson *et al.* 1998).

Humans may be exposed to DCE in both environmental and occupational settings. Work-place exposures via inhalation (and potentially dermal absorption) occur predominantly in the chemical manufacturing industry, particularly in industries producing or using VC. Workers in the petroleum refining and organic chemicals industries may also be exposed. Museum and art gallery personnel may also encounter DCE in their workplaces

(ATSDR 2001; IARC 1999). Exposures in the general population to environmental sources may occur through inhalation of DCE in ambient air (especially near point sources), ingestion of contaminated water/food sources, and inhalation of DCE that may volatilize from contaminated water supplies during indoor household activities such as bathing, dishwashing, and showering. Dermal exposure to DCE is also possible (ATSDR 2001; IARC 1999).

2.1.2 Epidemiological Evidence of 1,2-Dichloroethane Carcinogenicity

2.1.2.1 Inhalation exposure

A 1979 cohort mortality study of 175 Swedish ethylene oxide production workers by Hogstedt *et al.* revealed a standardized mortality ratio (SMR) of 1.8 for all cancer deaths in workers followed from 1961 through 1967. Among the most highly exposed workers, average exposure was estimated at 100 mg/m³ from 1941-47, but exposures were expected to decrease after 1947 due to changes in production methods. SMRs for stomach cancer and leukemia were 5.0 and 11.1, respectively. Because workers were exposed to ethylene oxide, ethylene chlorohydrin, and bis(2-chloroethyl)ether in addition to DCE, it was not possible to correlate elevated SMRs with exposure to an individual chemical.

In 1980, Alexander *et al.* reported a cluster of brain tumors that occurred among workers at a petrochemical plant in the United States. A follow-up historical cohort study of 6,588 white male workers from the same population over a period of 37 years (1941-1977) revealed a greater than expected number of brain cancers compared to average national rates (SMR = 1.6, 95% confidence interval 0.8 - 2.8) (Austin and Schnatter 1983). In a subsequent

nested case-control study, Austin and Schnatter found no significant association between DCE exposure at the petrochemical plant and increased risk of workers developing brain cancer (Austin and Schnatter 1983b).

In a study of mortality in 2,510 male chemical workers in Texas followed from 1952 - 1977, Sweeney *et al.* (1986) reported excesses of laryngeal (SMR = 3.6, 90% confidence interval 0.7 - 11.5) and brain cancers (SMR = 2.1, 90% confidence interval , 0.7 - 4.9). Workers were reportedly exposed to tetraethyl lead, ethylene dibromide, inorganic lead, and VC monomer. Although levels of exposure were not reported, estimates performed by the National Institute of Occupational Safety and Health (NIOSH) in 1980 suggested DCE exposure levels were below the recommended NIOSH standard. This study was unable to link cancer incidence with exposure to any individual chemical.

Among 278 chlorohydrin production workers in the United States who had ever been employed between 1940 and 1967, excesses of pancreatic cancer (SMR = 4.9, 95% confidence interval 1.6 - 11.4) and lymphatic/hematopoietic cancers (SMR = 2.9, 95% confidence interval 1.3 - 5.8) were reported (Benson and Teta 1993). The workers were exposed to DCE, ethylene chlorohydrin, and bis(2-chloroethyl)ether (levels of exposure not reported). Although a dose-response relationship between duration of exposure and likelihood of developing these cancers was noted, correlation between cancer development and exposure to any particular chemical was not possible. In a similar study, Olsen *et al.* (1997) reported an increased risk of lymphatic/hematopoietic cancer (SMR = 1.3, 95% confidence interval 0.6 - 2.4) among 1361 male workers at two chlorohydrin production facilities in the United States. Levels of exposure were not reported and, as with most other

reported studies of occupational exposure to DCE, correlation of cancer risk with exposure level was not possible.

2.1.2.2. Oral exposure

Very little information is available regarding the development of cancer in humans following oral exposure to DCE. In a study of the relationship between cancer incidence and exposure to groundwater and surface water contaminants, an increased incidence of colon ($p = 0.009$) and rectal cancers ($p = 0.02$) was observed in males 55 years or older, although it is highly probable that multiple chemical exposures occurred in the study population (Isacson *et al.* 1985).

2.1.3 Laboratory Animal Studies of 1,2-Dichloroethane Carcinogenicity

2.1.3.1. Inhalation exposure

In a study by Maltoni *et al.* (1980) Male and female Swiss mice were exposed to 5, 10, 50, or 250 ppm DCE in air for 7 hours/day, 5 days/week for a total of 78 weeks. Due to severe toxicity, the 250 ppm exposure concentration was reduced to 150 ppm after several days of exposure. All mice were kept until spontaneous death; the total duration of the experiment was 119 weeks. Complete necropsy and histological examination performed on all animals revealed no changes in incidence of tumors normally occurring in the Swiss strain compared to controls. Additionally, no increase in incidence of any specific tumors was noted (Maltoni *et al.* 1980).

In a similar study, male and female BDF₁ mice were exposed by whole-body inhalation to 10, 30, or 90 ppm DCE for 6 hours/day, 5 days/week for 104 weeks. In males, the incidence of hepatic angiosarcoma was significantly increased in the medium and high dose groups compared to controls. An increased incidence of bronchiolar-alveolar adenomas and carcinomas, hepatocellular adenomas, mammary gland adenocarcinomas, and endometrial polyps was observed in female treatment groups compared to controls (statistics not specified) (Nagano *et al.* 1998).

Studies in male and female Sprague-Dawley rats involved exposures to 5, 10, 50, or 250 ppm DCE in air for 7 hours/day, 5 days/week for 78 weeks. The 250 ppm exposure dose was decreased after several days to 150 ppm due to severe toxic effects among exposed animals. Animals were kept from the end of the treatment period until spontaneous death; total duration of the experiment was 148 weeks. Compared to controls, female rats in the 5, 50, and 250/150 ppm treatment groups exhibited a significant increase in the incidence of mammary fibromas and fibroadenomas (Maltoni *et al.* 1980).

Neither female nor male Sprague-Dawley rats exposed to 1 or 50 ppm DCE for 7 hours/day, 5 days/week for 24 months exhibited a significant difference in tumor incidence between treatment and control groups (Cheever *et al.* 1990). In another study, male and female Fischer 344 rats were exposed by whole-body inhalation to 10, 40, or 160 ppm DCE for 6 hours/day, 5 days/week for 104 weeks. An increase in the incidence of subcutaneous fibromas, mammary gland fibroadenomas, and peritoneal mesotheliomas was observed in exposed males compared to controls (statistics not specified). Similarly, an increased incidence of subcutaneous fibromas and mammary gland adenomas, fibroadenomas, and

adenocarcinomas was noted among exposed females compared to controls (statistics not specified) (Nagano *et al.* 1998).

In a multistage inhalation study, both male and female Sprague-Dawley rats were exposed to 50 ppm DCE for 7 hours/day, 5 days/week and to 0.05% disulfiram in the diet for 24 months. All animals were then sacrificed and complete necropsy and histopathological examination were performed. A significantly increased incidence of mammary gland adenocarcinomas was observed among exposed females compared to control animals, while among exposed males, an increased incidence of interstitial cell testicular tumors was noted compared to control animals. Additionally, both genders exhibited increased intrahepatic bile duct cholangiomas and intrahepatic bile duct cysts compared to controls. Neoplastic hepatic nodules were also increased among treated males compared to controls (Cheever *et al.* 1990).

2.1.3.2 Oral exposure

In several studies performed by the National Cancer Institute (NCI 1978), male rats exposed to DCE (47 and 95 mg/kg/day) by corn oil gavage for up to 78 weeks exhibited an increased incidence of subcutaneous fibromas and angiosarcomas of the spleen, liver, pancreas, and adrenal gland compared to controls. Additionally, male rats in the high dose group (95 mg/kg/day) exhibited an increased incidence of squamous cell carcinomas of the forestomach; females in the high dose group had increased incidence of adenocarcinomas and fibroadenomas of the mammary gland. Male mice exposed to 195 mg/kg/day via corn oil gavage for up to 78 weeks exhibited an increased incidence of hepatocellular carcinomas

and pulmonary adenomas compared to controls. Increased incidences of pulmonary adenomas, adenocarcinomas of the mammary gland, and endometrial polyps/sarcomas were reported in female mice exposed to 149 and 299 mg/kg/day compared to controls. These studies were limited by dosage adjustments throughout the course of the bioassay due to the toxicity of DCE, small number of animals in control groups, poor survival rate among treated animals, imprecisions regarding the purity of DCE, and the concurrent testing of other chemicals in the same room used to house experimental animals (ATSDR 2001).

In a two-stage initiation/promotion study of DCE, B6C3F₁ mice were initiated with diethylnitrosamine (DEN) in the drinking water (10 mg/L) for 4 weeks, followed by administration of DCE in the drinking water (159 or 475 mg/kg/day) for 52 weeks (Klaunig *et al.* 1986). No increase in tumorigenicity among treatment and control groups was noted. However, this study was limited by short exposure duration, high liver tumor incidence in untreated controls, and failure to evaluate DCE purity (ATSDR 2001).

In a medium-term hepatocarcinogenesis promotion bioassay, male Osborne-Mendel rats underwent a two-thirds partial hepatectomy, followed 24 hours later with administration of a single dose of DCE (100 mg/kg body weight) via corn oil gavage. Negative controls were gavaged with the corn oil vehicle alone, positive controls with 30 mg/kg DEN. Six days post-partial hepatectomy, rats were administered phenobarbital in the diet for 7 weeks at a dose of 500 mg/kg, followed by a control diet for 7 days. Rats were then sacrificed. Evaluation of hepatic γ -glutamyltranspeptidase (GGT)-positive foci revealed no significant difference in total number of foci among treatment and negative control groups. Positive

controls exhibited a significant increase in the number of GGT- positive foci compared to negative controls (Milman *et al.* 1988).

In another initiation-promotion study, male Osborne-Mendel rats received a single intraperitoneal injection of DEN (30 mg/kg) 24 hours following a two-thirds partial hepatectomy. Beginning six days later, rats were administered 100 mg/kg DCE via corn oil gavage, 5 days/week for 7 weeks. At that time, DCE corn oil gavage was discontinued and rats were held for one week before sacrifice. No significant difference in total number of GGT-positive foci between treatment and control groups was observed (Milman *et al.* 1988).

2.1.3.3 Dermal exposure

In female Ha:ICR Swiss mice exposed to 126 mg of DCE in acetone three times weekly for life, an increased incidence of lung tumors was observed compared to controls (Van Duuren *et al.* 1979). No skin tumors were observed in this study. In a two-stage mouse skin assay in female Ha:ICR Swiss mice, a single skin application of 126 mg DCE was followed (14 days later) by application of 5 µg phorbol myristyl acetate in 0.2 mL acetone three times weekly for life. No significant differences in the occurrence of skin tumors was observed between treatment and control groups.

2.1.4 Pharmacokinetics of 1,2-Dichloroethane

1,2-Dichloroethane is readily absorbed by humans (IARC 1999; Nouchi *et al.* 1984) and experimental animals (IARC 1999; Reitz *et al.* 1982) following oral or inhalation exposures. It is also rapidly absorbed via the dermal route in rats, mice, and guinea pigs

(Tsuruta 1977, 1975). In both humans and rats, DCE is widely distributed throughout the body. In several humans who died following acute oral poisoning, concentrations of DCE ranged from 1 - 50 mg/kg in the spleen, 100 - 1000 mg/kg in the stomach, and 10 - 100 mg/kg in the liver and kidney (IARC 1999). Following inhalation by rats, DCE was detected in blood, liver, kidney, brain, and spleen, and the highest concentrations were usually noted in adipose tissue (Spreafico *et al.* 1980).

1,2-Dichloroethane is readily biotransformed in the liver. Two principal pathways are involved in the biotransformation of DCE: mixed function oxidase and glutathione S-transferase. In the mixed function oxidase pathway, DCE is oxidatively transformed to 2-chloroacetaldehyde, 2-chloroacetic acid, and 2-chloroethanol, followed by both enzymatic and non-enzymatic conjugation with glutathione (GSH) (Guengerich *et al.* 1980). Saturation of the mixed function oxidase pathway appears to occur around oral gavage doses of 25 mg/kg or higher and at inhalation concentrations of 150 ppm or higher (approximately 500 mg/kg). Both of these exposure concentrations correspond to blood levels of 5 - 10 µg/mL (D'Souza *et al.* 1988; Reitz *et al.* 1982, Spreafico *et al.* 1980). DCE may also conjugate directly with GSH to form S-(2-chloroethyl)glutathione, a sulfur half-mustard (Fouerean and Reed 1987; Schasteen and Reed 1983). Increased biotransformation via this pathway may become more important at higher doses (D'Souza *et al.* 1988; Reitz *et al.* 1982). The half-mustard may then form an episulfonium ion which can react with water to form S-(2-hydroxyethyl)glutathione, with thiols such as GSH to form ethene bis-glutathione, or with macromolecules to form adducts. Increased biotransformation by this latter pathway is likely responsible for the toxic effects noted at higher doses of DCE (D'Souza *et al.* 1988; Reitz

et al. 1982). Intermediate metabolites undergo further biotransformation to thiodiacetic acid and thiodiacetic acid sulfoxide and carbon dioxide (Cheever *et al.* 1990; Mitoma *et al.* 1985; Reitz *et al.* 1982; Spreafico *et al.* 1980). Thiodiacetic acid and thiodiacetic acid sulfoxide are excreted mostly in the urine; only a small amount of metabolites have been detected in the feces of rats (Cheever *et al.* 1990). 1,2-Dichloroethane may also be eliminated as parent compound in exhaled air (Cheever *et al.* 1990; Reitz *et al.* 1982) and in urine (Mitoma *et al.* 1985; Reitz *et al.* 1982; Spreafico *et al.* 1980).

2.1.5 Proposed Carcinogenic Mechanisms of 1,2-Dichloroethane

Studies of the biotransformation of DCE in laboratory animals indicate that it may be biotransformed into several reactive intermediates, such as 2-chloroacetaldehyde and *S*-(2-chloroethyl)glutathione, capable of binding to cellular macromolecules such as DNA (Jean and Reed 1989; Fabricant and Chalmers 1980). As described in Section 2.1.3, DCE exhibits carcinogenic potential in laboratory animals when administered orally but not when inhaled. This apparent inconsistency may be attributable to a number of limitations associated with the experimental conditions of the studies, including poor survival rates, intermittent exposures, different strains, and doses high enough to induce acute toxicity (ATSDR 2001). Alternatively, the apparent inconsistency in cancer induction between the oral and inhalation exposure routes may be attributable to differences in biotransformation between routes of exposure (Reitz *et al.* 1982). Saturation of DCE metabolism occurred at lower doses after oral administration than after inhalation exposure (Reitz *et al.* 1982). Reitz *et al.* proposed that the carcinogenicity of DCE occurred when biotransformation processes were saturated,

thereby allowing more parent compound to circulate. When administered via inhalation, DCE may not reach high enough concentrations to saturate metabolism, thereby leading to lack of tumor development. A physiologically based pharmacokinetic (PBPK) model that predicts the amounts of DCE and its metabolites in blood and target tissues following oral and inhalation exposure has been developed (D'Souza *et al.* 1988, 1987). The model predicts that oral exposures to DCE result in more glutathione-conjugate metabolites in the lung and liver than equivalent inhalation exposures (D'Souza 1987, 1988), substantiating the observation that DCE is carcinogenic by the oral route (NCI 1978) but not by inhalation (Maltoni *et al.* 1980).

2.2 VINYL CHLORIDE

2.2.1 Historical and Current Human Exposures to Vinyl Chloride

Vinyl chloride (VC, C_2H_3Cl) belongs to the chemical class of halogenated alkenes. Synonyms for VC include chloroethene, chloroethylene, ethylene monochloride, and VC monomer. At room temperature, VC is a colorless gas with a sweet odor. The vast majority of VC produced in the United States is used in the manufacture of polyvinyl chloride (PVC); PVC is used to make a variety of plastic products, such as pipes, wire and cable coatings, packaging materials, furniture and automobile upholstery, wall coverings, housewares, and automotive parts (ATSDR 2002). Vinyl chloride is also used in copolymer products with vinyl acetate to make films and resins (ATSDR 2002). Historically, VC was used as a coolant, a spray can propellant, an extraction solvent for heat-sensitive materials, and in some cosmetics (ATSDR 2002). It is no longer used for these purposes, however, as these

practices were banned in 1974 by the United States Environmental Protection Agency (USEPA) (ATSDR 2002). In 1995, production of VC in the United States reached approximately 15 billion pounds (ATSDR 2002).

The presence of VC in the environment is largely due to releases into air and water from the plastics manufacturing industry (ATSDR 2002; Whysner *et al.* 1996). However, it can also be released at hazardous waste disposal sites (ATSDR 2002) or produced by biodegradation of other chlorinated compounds such as trichloroethylene, tetrachloroethylene, and 1,1,1-trichloroethane by bacteria in the soil (Smith and Dragun 1984). Once released into the atmosphere, VC can form a variety of compounds, such as hydrochloric acid, formaldehyde, formyl chloride, acetylene, and chloroacetaldehyde (ATSDR 2002). If released into water, most VC will volatilize (USEPA 1982a), whereas VC released into soil may either volatilize or leach into groundwater (Cowfer and Magio 1983; Verschueren 1983). Vinyl chloride is also found in tobacco smoke, and it may leach out of PVC piping or PVC plastic food wraps into drinking water or food, respectively (ATSDR 2002, Whysner *et al.* 1996).

Human populations with the highest risk for exposure to VC are those working in the PVC manufacturing industry, primarily via inhalation. Other populations with potentially high exposures include individuals located near or downwind from VC/PVC production facilities, hazardous waste disposal sites, or landfills (ATSDR 2002; Reitz *et al.* 1996; Whysner *et al.* 1996). Exposures in non-occupational settings may occur through inhalation of VC in ambient air (especially near point sources), ingestion of contaminated water/food

sources, and inhalation of VC that may volatilize from contaminated water supplies during indoor household activities such as bathing, dishwashing, and showering (ATSDR 2002).

2.2.2 Epidemiological Evidence of Vinyl Chloride Carcinogenicity

Numerous studies of workers occupationally exposed to VC have documented the association between VC exposure and development of a specific and rare form of liver cancer, hepatic angiosarcoma (Simonato *et al.* 1991; Wang *et al.* 1991; Piratsu *et al.* 1990; Teta *et al.* 1990; Wu *et al.* 1989; Jones *et al.* 1988; Rinsky *et al.* 1988; Laplanche *et al.* 1982; Theriault and Allard 1981; Weber *et al.* 1981; Fox and Collier 1977; Byren *et al.* 1976; Infante *et al.* 1976; Waxweiler *et al.* 1976; Monson *et al.* 1975; Creech and Johnson 1974). Nearly three decades after the plastics industry began using VC in the production of PVC, unusually high incidences of hepatic angiosarcoma were noted among workers exposed to high levels of VC. Because of its relatively low acute toxicity, occupational exposure standards were typically around 500 ppm (Reitz *et al.* 1996). In 1974, Creech and Johnson reported the deaths due to hepatic angiosarcoma of three workers exposed to VC at a Goodrich plant (Creech and Johnson 1974). Subsequent to this initial “sentinel health event,” numerous epidemiological studies documented increased risk of developing hepatic angiosarcoma that correlated with workers’ exposure levels and/or duration of exposure to VC (Wang *et al.* 1991; Wu *et al.* 1989; Rinsky *et al.* 1988; Jones *et al.* 1988; Weber *et al.* 1981; Fox and Collier 1977; Infante *et al.* 1976). Based on the overwhelming epidemiological evidence, VC is classified as a known human carcinogen (ATSDR 2002; USEPA 1994; IARC 1987a,b).

Vinyl chloride has been implicated as the causative agent in a variety of other cancers as well. Increased cancers of the brain and central nervous system have been reported among VC workers (Wong *et al.* 1991; Cooper *et al.* 1981; Waxweiler *et al.* 1976; Monson *et al.* 1975), but these results are balanced by other studies which indicated no association between VC exposure and increased risk of cancers of the brain or central nervous system (Simonato *et al.* 1991; Wu *et al.* 1989; Jones *et al.* 1988; Thomas *et al.* 1987; Fox and Collier 1977; Byron *et al.* 1976; Tabershaw and Gaffey 1974). In terms of cancers of the lymphatic/hematopoietic system, several epidemiological studies of VC workers indicated an increased risk associated with VC exposure (Rinsky *et al.* 1988; Weber *et al.* 1981; Monson *et al.* 1975). Conversely, other studies do not support such an association (Wong *et al.* 1991; Jones *et al.* 1988; Infante *et al.* 1976). Monson *et al.* (1975), Infante *et al.* (1976), Waxweiler *et al.* (1976), and Belli *et al.* (1987) all reported increased incidence of respiratory tract cancers among workers exposed to VC. However, such an association was not supported in studies by Simonato *et al.* (1991), Wong *et al.* (1991), Wu *et al.* (1989), Jones *et al.* (1988), Cooper *et al.* (1981), and Fox and Collier *et al.* (1977).

2.2.3 Laboratory Animal Studies of Vinyl Chloride Carcinogenicity

2.2.3.1 Inhalation exposure

The carcinogenicity of VC has been documented in multiple laboratory animals studies. Male and female Sprague-Dawley rats exposed to VC for 52 weeks at concentrations ranging from 1 to 30,000 ppm showed increased incidences of a variety of tumors compared to controls (Maltoni *et al.* 1981). In females, statistically significant increases were noted

in the development of mammary gland carcinomas at 5 ppm, in hepatic angiosarcoma at 250 ppm, in nephroblastoma at 250 ppm, and in neuroblastoma at 10,000 ppm. In males, the incidence of nephroblastoma at 100 ppm and hepatic angiosarcoma at 200 ppm was significantly elevated compared to control animals. In both males and females, the incidence of Zymbal gland carcinoma at 10,000 ppm (and above) and forestomach papillomas at 30,000 ppm was significantly increased compared to controls. The incidence of hepatic angiosarcoma and angioma was also increased among both male and female Swiss mice exposed to 50 ppm VC for 4 hours/day, 5 days/week for 30 weeks compared to control animals (Maltoni *et al.* 1981).

Rats and mice exposed to 50, 250, or 1,000 ppm VC 6 hours/day, 5 days/week for 6 months (Hong *et al.* 1981) or 12 months (Lee *et al.* 1978, 1977) exhibited a significant increase in the incidence of hepatic angiosarcoma at concentrations of 2,250 ppm compared to controls. In male rats exposed to 100 ppm VC 6 hours/day, 6 days/week for 12 months, the incidence of hepatic angiosarcoma was significantly increased (compared to control animals) when rats were sacrificed at 18 months (Bi 85). Female mice exposed to 50 ppm VC showed increased incidence of subcutaneous angiosarcoma as well as tumors of the lung and mammary gland (Drew *et al.* 1983).

2.2.3.2 Oral exposure

In a study of the effects of orally administered VC, rats were exposed to VC added to the diet (PVC powder containing a high proportion of VC monomer) for a period of 149 weeks (Til *et al.* 1991, 1983; Feron 1981). Food consumption rates and rate of VC

volatilization from food were considered in estimating total VC intake. A significantly increased incidence of hepatic angiosarcoma was observed in males at a dose of 5.6 mg/kg/day and in females at a dose of 17 mg/kg/day (Feron *et al.* 1981). Also, a significant increase in hepatic neoplastic nodules was detected in males receiving 5.6 mg/kg/day and in females receiving 1.8 mg/kg/day. Similarly, in studies performed by Til *et al.* (1991, 1983) in which rats were orally exposed to VC, the incidence of hepatocellular carcinoma was significantly increased in males (1.7 mg/kg/day) compared to controls. In females, the incidence of hepatic neoplastic nodules was significantly elevated at a dose of 1.7 mg/kg/day compared to control animals.

Sprague-Dawley rats administered VC by gavage for 52 weeks exhibited a significant increase in incidence of hepatic angiosarcomas (Maltoni *et al.* 1981). In female rats, the effect was observed at a dose of 16.65 mg/kg/day; in males, tumors developed at a dose of 50 mg/kg/day. In a similar study, hepatic angiosarcomas were observed at doses as low as 0.3 mg/kg/day (Maltoni *et al.* 1981).

2.2.4 Pharmacokinetics of Vinyl Chloride

Absorption of VC following inhalation exposure is rapid in humans and animals (Krajewski *et al.* 1980; Withey 1976). In animals, VC absorption from the gastrointestinal tract following oral exposure is also very rapid and nearly complete (Watanabe *et al.* 1976a; Withey 1976). A study of dermal absorption of VC vapor in monkeys indicated minimal absorption (Hefner *et al.* 1975a). Based on animal studies, distribution of VC appears to be rapid and widespread (Buchter *et al.* 1977; Watanabe *et al.* 1976a,b). VC metabolites have

been detected in the liver, spleen, brain, kidney, and skin, although the amounts in these tissues are relatively small (Buchter *et al.* 1977; Bolt *et al.* 1976a; Watanabe *et al.* 1976b), because storage of VC in the body is limited by rapid biotransformation and excretion.

Vinyl chloride is predominantly biotransformed in the liver by mixed function oxidases (specifically, cytochrome P450 2E1) to form 2-chloroethylene oxide, a highly reactive epoxide which nearly spontaneously rearranges to form 2-chloroacetaldehyde, a reactive α -halocarbonyl compound (Ghissassi *et al.* 1998; Gwinner *et al.* 1983; Laib *et al.* 1982; Guengerich *et al.* 1981, 1979). 2-Chloroethylene oxide can also be hydrolyzed by epoxide hydrolase (Whysner *et al.* 1996). Detoxification of 2-chloroethylene oxide and 2-chloroacetaldehyde occurs mainly through conjugation with GSH to form thiodyglycolic acid, *S*-formyl-methylcysteine, and *N*-acetyl-*S*-(2-hydroxyethyl)cysteine (Bolt *et al.* 1980; Hefner *et al.* 1975b). Metabolism of VC is a dose-dependent, saturable process (Buchter *et al.* 1980; Filser and Bolt 1979; Bolt *et al.* 1977). In rats, metabolic saturation was estimated to occur at a VC concentration of approximately 250 ppm, with a corresponding metabolic rate ($V_{\max}$) of 110 $\mu\text{mol}/\text{hour}/\text{kg}$ (Filser and Bolt 1979; Bolt *et al.* 1977). In rhesus monkeys, metabolic saturation was estimated to occur at 200 ppm VC, with a $V_{\max}$ of 50 $\mu\text{mol}/\text{hour}/\text{kg}$ (Buchter *et al.* 1980). Filser and Bolt (1979) suggested that a $V_{\max}$ of 50 $\mu\text{mol}/\text{hour}/\text{kg}$ most closely approximates the value in humans.

Within the last decade, two PBPK models for VC have been developed (Reitz *et al.* 1996; Clewell *et al.* 1995). In the model by Reitz *et al.* (1996), metabolism of VC was represented with a single saturable pathway; kinetic constants were estimated from data obtained from closed-chamber gas uptake studies in rats. The model was used to predict

total biotransformation of VC as the dose metric for the purpose of evaluating carcinogenic risk following VC exposure. In the PBPK model by Clewell *et al.* (1995), VC metabolism was represented by two saturable pathways: a CYP2E1-mediated high-affinity/low-capacity pathway and a high-capacity/low-affinity oxidative pathway mediated by other P450 isozymes. Both metabolic pathways produced the metabolite 2-chloroethylene oxide. 2-Chloroacetaldehyde was modeled as the major substrate in GSH conjugation, and GSH depletion by reaction with 2-chloroacetaldehyde was also described. The model by Clewell *et al.* (1995) successfully simulated pharmacokinetic data from a large number of studies using multiple routes of exposure and a wide range of concentrations.

Exhalation of VC does not appear to be a major pathway of excretion in humans (Krajewski *et al.* 1980). In rats, however, the importance of VC exhalation as a route of excretion is dependent upon the original exposure concentration, reflecting the saturation of metabolic pathways (Watanabe *et al.* 1978a, 1976b). Although exhalation of parent compound is a possible elimination pathway in rats, the predominant route of excretion in both humans and rats is via the urine (Krajewski *et al.* 1980; Watanabe *et al.* 1978a, 1976b). Urinary metabolites include thiodyglycolic acid, N-acetyl-S-(2-hydroxyethyl)cysteine, and S-(2-hydroxyethyl)cysteine (Watanabe *et al.* 1976a,b).

2.2.5 Carcinogenic Mechanism of Action of Vinyl Chloride

The mechanism underlying VC's carcinogenic effects involves the interaction of the reactive intermediates 2-chloroethylene oxide and 2-chloroacetaldehyde with DNA to form a variety of adducts. Most evidence indicates that 2-chloroethylene oxide is the ultimate

carcinogen. It is carcinogenic in skin and acts as an initiator in initiation/promotion assays, whereas 2-chloroacetaldehyde is negative in these assays (Zajdela *et al.* 1980). Similarly, in bacterial mutagenicity assays, 2-chloroethylene oxide exhibits mutagenic potential two orders of magnitude greater than 2-chloroacetaldehyde (Perrard *et al.* 1985). Several cyclic DNA adducts have been identified: 7-(2-oxoethyl)guanine, 1,N²-ethenoguanine, 1,N⁶-ethenoadenine, 3,N⁴-ethenocytosine, and N²-3-ethenoguanine (Ghissassi *et al.* 1998; Dosanjh *et al.* 1994, Fedtke *et al.* 1990; Laib *et al.* 1986). Of these, 7-(2-oxoethyl)guanine accounts for 98% of all DNA adducts *in vivo* (Swenberg *et al.* 1992). However, because the 7-(2-oxoethyl)guanine adduct is repaired rapidly by DNA glycosylase (Dosanjh *et al.* 1994), it is not likely to be miscoded during DNA replication and is not considered important in the carcinogenic process (Swenberg *et al.* 1992; Laib *et al.* 1986). The remaining adducts are repaired at much slower rates and, although present in significantly lower concentrations than 7-(2-oxoethyl)guanine, they are much more persistent in the genome. These adducts, therefore, are more likely to be miscoded during replication and/or transcription (Swenberg *et al.* 1992; Singer *et al.* 1987). In particular, the ethenoguanines appear to be repaired at much slower rates than the other etheno-adducts in humans (Dosanjh *et al.* 1994). Although this evidence implicates the formation of ethenoguanine adducts as a key step in the early stages of VC-induced carcinogenesis, lack of data regarding relative adduct levels in VC-exposed humans limits this interpretation to experimental systems only.

2.3 TRICHLOROETHYLENE

2.3.1 Historical and Current Human Exposures to Trichloroethylene

Like VC, trichloroethylene (TCE, C_2HCl_3) belongs to the class of compounds known as halogenated alkenes. Synonyms for TCE include trichloroethene, acetylene trichloride, and ethylene trichloride. At room temperature, TCE is a clear, colorless liquid with a sweet odor. TCE is used primarily in vapor degreasing of fabricated metal parts in the automotive and metals industries (Chemical Marketing Reporter 1986, 1983). It is also commonly used as a chemical intermediate in the production of VC (McNeil 1979), as well as in pharmaceuticals, polychlorinated aliphatics, flame retardant chemicals, and insecticides (ATSDR 2002; Mannsville Chemical Corporation 1992). TCE is an excellent solvent for extraction of greases, oils, fats, waxes, and tars, and it is used by the textile industry to scour wool, cotton, and other fabrics (also as a solvent in dyeing and finishing operations) (ATSDR 2002; Verscheuren 1983; McNeil 1979). Trichloroethylene is also found in adhesives, paints, varnishes, paint strippers, lubricants, cold metal cleaners, and pesticides (ATSDR 2002; McNeil 1979). Additionally, it is used as a refrigerant for low-temperature heat transfer (ATSDR 2002; McNeil 1979) and in the aerospace industry for flushing liquid oxygen (ATSDR 2002). The following consumer products often contain TCE: typewriter correction fluids, paint/varnish removers, spot removers, rug-cleaning fluids, and adhesives (Frankenberry *et al.* 1987). Although banned in 1977 by a United States Food and Drug Administration regulation, TCE historically was used as a general/obstetrical anesthetic, grain fumigant, pet food additive, skin/wound/surgical disinfectant, and extractant of spice oleoresins in food and of caffeine in coffee (ATSDR 2002). In 1982 (most recent data

available), total production of TCE in the United States was 200 million pounds (ATSDR 2002; US International Trade Commission 1983).

Environmental contamination with TCE is primarily due to atmospheric releases from degreasing operations (ATSDR 2002; USEPA 1985b); it can also be released into water and soil from a variety of manufacturing and processing operations (ATSDR 2002; USEPA 1985a). Releases from hazardous waste disposal sites and landfills are also common (ATSDR 2002). Several species of marine microorganisms are also known to produce TCE (Abrahamsson *et al.* 1995). In the atmosphere, TCE degrades by reacting with hydroxyl radicals with a half-life of between three and seven days (Class and Ballschmiter 1986; Singh *et al.* 1982). Degradation products include phosgene, dichloroacetyl chloride, and formyl chloride (ATSDR 2002; Atkinson 1985; Gay *et al.* 1976). Trichloroethylene in water readily volatilizes (Jensen and Rosenberg 1975) or may be biodegraded to dichloroethylene and VC (Milde *et al.* 1988; Smith and Dragun 1984). Similarly, TCE in soil will either volatilize or undergo biodegradation to VC (Vogle and McCarty 1995; Ensley 1991; Fogle *et al.* 1986).

Humans may be exposed to TCE in both occupational and environmental settings. Workers involved in degreasing operations for metals or automobile parts or those employed in the plastics manufacturing industry are likely to experience the greatest exposures (ATSDR 2002; Santodonato 1985; McNeil 1979). In the environment, the highest atmospheric levels of TCE occur in areas of concentrated industry and dense populations compared to rural, more remote areas (ATSDR 2002; Brugnone *et al.* 1994). In addition to being exposed to TCE in the ambient air in these locales, the general population can also be exposed to TCE via ingestion of contaminated drinking water (ATSDR 2002), or by contact

with consumer products containing TCE (Frankenberry *et al.* 1987). Because TCE readily volatilizes from water, another potentially major route of exposure may be inhalation of TCE in homes supplied with contaminated water, from indoor household activities such as bathing, showering, and dishwashing (Andelman 1985a,b). Indoor sources of TCE also include building products containing TCE as a solvent or other consumer products (Wallace *et al.* 1987; Hartwell *et al.* 1985).

2.3.2 Epidemiological Evidence of Trichloroethylene Carcinogenicity

2.3.2.1 Inhalation exposure

The few reported epidemiological studies of TCE inhalation are limited by small sample sizes, uncertainty in exposure data, and confounding by exposure to other chemicals (ATSDR 2002). A study of 6,929 workers exposed to TCE at an aircraft maintenance facility revealed no significant correlation between TCE exposure and excess cancer risk (Spirtas *et al.* 1991); for instance, the SMR for non-Hodgkin's lymphoma among white females was 2.86 (95% confidence interval 0.78 - 7.31). Although the work histories of female employees who died from multiple myeloma or non-Hodgkin's lymphoma indicated possible solvent exposures which may have occurred in the fabric handling departments, the authors concluded that "no significant or persuasive relationships were found between various measure of exposure to trichloroethylene and the risk of any specific malignancy" (ATSDR 2002; Spirtas *et al.* 1991).

In a cohort study of 1,424 male workers exposed to TCE, exposure levels were estimated from measured concentrations of urinary metabolites, and significant increases in

the incidence of urinary bladder cancer and lymphoma were reported (ATSDR 2002; Axelson *et al.* 1986). In an extension of this study, the cohort was expanded to include 249 female workers; no correlation between exposure level or exposure duration to TCE and development of cancer was observed (Axelson *et al.* 1994). At estimated exposure levels of approximately 20 ppm TCE, the highest standardized incidence ratio for non-Hodgkin's lymphoma was 1.56 (95% confidence interval 0.51 - 3.64) in men. However, because 80% of the cases of lymphoma occurred in the lowest exposure stratification, the authors concluded that "this study provides no evidence that trichloroethylene is a human carcinogen, *i.e.*, when the exposure is as low as for this study population" (ATSDR 2002; Axelson *et al.* 1994).

In a study of Finnish workers exposed primarily to TCE, a correlation between exposure and increased incidence of cancers of the liver, stomach, prostate, and lympho/hematopoietic system was observed (Antilla *et al.* 1995). Because workers were exposed to solvents other than TCE, however, this study was unable to link cancer incidence with exposure to TCE..

A retrospective cohort study of 169 German cardboard factory workers followed from 1956 to 1975 revealed a significant association between TCE exposure and kidney cancer (Henschler *et al.* 1995). The authors of the study reported five cases of kidney cancer among exposed workers and zero cases among the unexposed worker controls. Levels of TCE exposure were not evaluated in this study. A study of Swedish workers occupationally exposed to TCE revealed an increased risk of non-Hodgkin's lymphoma associated with TCE exposure (Hardell *et al.* 1994). However, exposures were self-reported and workers

were exposed to chemicals other than TCE. In a study of dry cleaners, a significant increase in the incidence of all malignant neoplasms was noted, in addition to increased incidences of cancer of the lung/bronchus/trachea, cervix, and skin (Blair *et al.* 1979). However, exposure to chemicals other than TCE, such as tetrachloroethylene and carbon tetrachloride, were likely in this study group.

2.3.2.2 Oral exposure

A survey performed between 1953 and 1991 of two villages in Finland with drinking water contaminated with up to 220 ppb TCE and/or up to 180 ppb tetrachloroethylene found no significant increase in standardized incidence ratios for total cancer, liver cancer, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, or leukemia (Vartiainen *et al.* 1993). Similarly, in a cohort study of 223 Michigan residents exposed to drinking water contaminated with TCE as well as other chlorinated solvents, no significant increase in cancer incidence was observed in the exposed population (Freni and Bloomer 1988). Although an ATSDR survey of 4,280 residents of Michigan, Indiana, and Illinois exposed to drinking water contaminated with TCE and other contaminants revealed an increase in respiratory tract cancers among males, the study concluded that "it would be inappropriate to relate this excess solely to trichloroethylene exposure based on the incidence of smoking in the population" (ATSDR 1994).

In an ecological study by Faglione *et al.* (1990), data collected from the New Jersey cancer registry were correlated with data regarding levels of TCE and other volatile organic hydrocarbons in contaminated drinking water. For females, the standardized incidence ratio

for leukemia was increased in towns with the highest exposure levels (volatile organic hydrocarbon levels estimated between 37 to 72 ppb). In a subsequent study, Cohn *et al.* (1994) expanded the cohort to include over one million residents in 75 towns monitored between 1979 and 1987. Females exposed to greater than 5.0 ppb TCE exhibited a significant increase in total leukemias, childhood leukemias, acute lymphatic leukemias, and non-Hodgkin's lymphoma . In males, increased incidence of non-Hodgkin's lymphoma (diffuse large cell reticulosarcoma form) was also observed. However, as with most ecological studies, major limitations of these two studies include lack of individual exposure data, uncertainties in routes of exposure, and potential exposure to multiple chemicals. In the city of Woburn, Massachusetts, some residents were exposed to municipal water contaminated with several chlorinated organic compounds, including TCE. Two studies of childhood mortality statistics between 1969 and 1979 revealed a significantly elevated rate of childhood leukemia among Woburn residents (Parker and Rosen 1981; Kotelchuck and Parker 1979). However, the extent and duration of contamination of the water in these studies was poorly defined. Subsequent studies of this population revealed increased risk of childhood leukemias, particularly acute lymphocytic leukemia, associated with ingestion of contaminated drinking water (Byers *et al.* 1988; Lagakos *et al.* 1986). However, several cases of leukemia occurred in children with no exposure to contaminated drinking water, so not all of the leukemia cases could be directly correlated with consumption of contaminated water. In addition, all of the studies of Woburn residents are limited by imprecise definition of exposure conditions. In the study by Lagakos *et al.* (1986), exposure to contaminated drinking water was classified using two approaches: categorical and continuous. The

categorical approach assigned residents into one of four categories based on level of exposure, whereas the continuous approach was based on model-derived estimates of the use and distribution of contaminated water. Leukemia risks associated with exposures based on the continuous model were less significant than risks associated with the more crude categorical approach. This comparison challenges the strength of the association between exposure to contaminated drinking water and risk of childhood leukemia. The causal relationship is also limited by small sample size, lack of evaluation of alternative/additional sources of water (*e.g.*, schools or workplaces), and multiple chemical exposures (MacMahon 1986; Prentice 1986; Rogan 1986; Swan and Robins 1986; Whittemore 1986).

A more recent study of Woburn residents performed by the Massachusetts Department of Health described an eight-fold increase in the risk of leukemia due to *in utero* TCE exposure compared to an unexposed control group (Massachusetts Department of Public Health 1996). As with previous studies of the Woburn site, these results are limited by small sample size and poorly defined exposure conditions.

2.3.3 Laboratory Animal Studies of Trichloroethylene Carcinogenicity

2.3.3.1 Inhalation exposure

Maltoni *et al.* (1988, 1986a) performed a series of experiments in which Swiss mice and B6C3F₁ mice of both genders were exposed to TCE (epoxide-free) for 78 weeks. Among male Swiss mice and B6C3F₁ mice of both genders, exposure to 600 ppm TCE was associated with a significant increase in the development of hepatomas compared to controls. Interestingly, among male Swiss mice, exposures at 100 ppm TCE were associated with a

decrease in hepatomas compared to controls. (A subsequent retest with male B6C3F₁ mice revealed no significant different difference in hepatoma incidence between exposed and control animals, although a decrease in leukemia incidence was noted). Male Swiss mice and female B6C3F₁ mice also exhibited an increase in pulmonary tumors at the 600 ppm exposure level. At the same exposure level (600 ppm), both genders of B6C3F₁ mice displayed significantly increased incidence of liver tumors compared to controls (Maltoni *et al.* 1988, 1986). In male Sprague-Dawley rats exposed to 600 ppm TCE for 104 weeks, an increased incidence of renal tubular cell adenocarcinomas was noted; a dose-related increase in testicular Leydig cell tumors was also reported (Maltoni *et al.* 1988, 1986). The results of these studies are limited by problems in study methodology, unorthodox reporting methods, lack of appropriate histopathological data regarding types of tumors observed, and lack of use of Good Laboratory Practices (ATSDR 2002).

In female ICR mice exposed to 150 or 450 ppm TCE for 5 days/week, 7 hours/day for 104 weeks, pulmonary adenocarcinoma incidence was significantly elevated compared to controls (Fukuda *et al.* 1983). However, newborn rats exposed to 2,000 ppm TCE for 10 weeks showed no evidence of preneoplastic ATPase-deficient foci (Laib *et al.* 1979). Similarly, in NMRI mice, Wistar rats, and Syrian hamsters of both genders exposed to 100 or 500 ppm TCE for 18 months, no significant differences in tumor incidence were noted between exposed and control animals (Henschler *et al.* 1980). Although an increased incidence of malignant lymphomas was noted among female NMRI mice compared to controls, the authors noted that this type of tumor is commonly reported in unexposed NMRI

mice and suggested development of this particular type of tumor may have resulted from immunosuppression.

2.3.3.2 Oral exposure

In an NCI lifetime carcinogenicity bioassay (NCI 1976), B6C3F₁ mice exposed to industrial grade TCE via corn oil gavage exhibited significant increases in hepatocellular carcinomas in both male and female mice at the low and high dose levels. However, the TCE used in this study contained small amounts of the stabilizer epichlorohydrin, which is known to form reactive radicals and may act as a tumor initiator (ATSDR 2002). Furthermore, this study was significantly limited by high rates of mortality due to toxicity in all rats (ATSDR 2002; NCI 1976). Similarly, interpretation of results of a National Toxicology Program (NTP) study of epoxide-free TCE in Fischer 344 rats, which indicated a small but statistically significant increase in renal tubular cell adenocarcinomas in males, was also limited by poor survival rates due to toxic nephrosis (NTP 1990).

In a study in mice, increased incidence of forestomach tumors was reported in ICR/Ha Swiss mice gavaged with TCE containing epoxide stabilizers (Henschler *et al.* 1984); no tumors, however, were observed in the treatment group administered TCE without stabilizers. Although male and female Sprague-Dawley rats gavaged with epoxide-free TCE in olive oil exhibited an increase in leukemia in males but not in females (Maltoni *et al.* 1986), the reliability of these results is questionable due to the absence of Good Laboratory Practices and unusual reporting methods (*e.g.*, failure to indicate the number of surviving animals) (ATSDR 2002). Mice, in contrast to rats, have exhibited tumor development

following oral exposure to TCE. For example, hepatocellular adenomas and hepatocellular carcinomas have been observed in B6C3F₁ mice exposed to epoxide-free TCE (NTP 1990).

2.3.4 Pharmacokinetics of Trichloroethylene

A variety of studies in humans and animals provide evidence that absorption of TCE occurs rapidly under inhalation, oral, or dermal exposure conditions (ATSDR 2002; Lash *et al.* 2000; Bogen *et al.* 1992; Dallas *et al.* 1991; Perbellini *et al.* 1991; Jakobson *et al.* 1982; Sato and Nakajima 1978; Tsuruta 1978; Fernandez *et al.* 1977; Astrand and Ovrum 1976; Monster *et al.* 1976; Bartonicek 1962; Defalque 1961; Kleinfeld and Tabershaw 1954). One inhalation study in rats indicated mixed uptake kinetics, with a saturable uptake process preceded by an initial slow first-order uptake process (Andersen *et al.* 1980). Oral absorption of TCE can be affected by fasting (D'Souza *et al.* 1985) and the dosing vehicle (Withey *et al.* 1983). Also, gender-based differences in the uptake of TCE have been observed in humans and animals (McCormick and Abdel-Rahman 1991). Once absorbed, TCE is widely distributed throughout the body, with highest concentrations in humans being found in the liver and fat (McConnell *et al.* 1975). In mice, TCE may be bound to proteins in the blood (Stevens *et al.* 1992); in rats, TCE has been found in liver, fat, and blood (Pfaffenberger *et al.* 1980; Savolainen *et al.* 1977).

Trichloroethylene is metabolized extensively in humans and animals (ASTDR 2002; Lash *et al.* 2000). Saturation of TCE metabolism appears to occur in rats, mice, and dogs (Templin *et al.* 1995; Maltoni *et al.* 1986b). The initial step in TCE biotransformation is either cytochrome P450 oxidation (major pathway) or conjugation with GSH (minor

pathway) (Clewell *et al.* 2000; Lash *et al.* 2000; Lipscomb *et al.* 1997). A major oxidative metabolite of TCE is chloral, which can subsequently be transformed to chloral hydrate, then oxidized to trichloroacetic acid or reduced to trichloroethanol (Clewell *et al.* 2000; Lash *et al.* 2000; Nakajima *et al.* 1992; Costa and Ivanetich 1984; Ikeda *et al.* 1980). Trichloroacetic acid is the main metabolite of TCE found in the blood (Clewell *et al.* 2000). Dichloroacetic acid, a minor metabolite of TCE in rats and mice (Dekant *et al.* 1986b; Green and Prout 1985; Hathaway 1980) which has not been detected in humans (Clewell *et al.* 2000; Curry *et al.* 1985; Lukas *et al.* 1980), is produced from trichloroacetic acid (Clewell *et al.* 2000; NTP 1990).

Although the liver is the main site of TCE biotransformation, biotransformation in the kidney and lungs is known to occur. A minor pathway of TCE biotransformation is conjugation with GSH (in the liver) followed by formation of the cysteine conjugate dichlorovinylcysteine (in the kidney) (Clewell *et al.* 2000; Lash *et al.* 2000; Elfarra *et al.* 1986). Dichlorovinylcysteine may be bioactivated to form a reactive thioacylating intermediate (Dekant *et al.* 1988), or it may be acetylated and excreted in the urine (Dekant *et al.* 1990, Vamvakis *et al.* 1987). Additionally, cytochrome P-450 in Type II alveolar and Clara cells in the lung may biotransform TCE (Nichols *et al.* 1992; Forkert *et al.* 1985; Miller and Guengerich 1983).

In humans exposed to TCE, elimination may occur via exhalation of parent compound or trichloroethanol (following inhalation exposure) (Monster *et al.* 1979, 1976), or via urinary excretion of trichloroethanol, trichloroacetic acid, or trichloroethanol glucuronide (following inhalation or oral exposure) (Vartiainen *et al.* 1993; Monster *et al.*

1979; Sato *et al.* 1977). Gender differences in the excretion of urinary metabolites of TCE have been reported (ATSDR 2002; Inoue *et al.* 1989; Nomiyama and Nomiyama 1971). Excretion pathways and metabolites are similar in rats, with the exception of the additional minor urinary metabolites oxalic acid, dichloroacetic acid, and N-(hydroxyacetyl)-aminoethanol (Dekant *et al.* 1986b, 1984).

Numerous PBPK models have been developed for TCE (Clewell *et al.* 1995; Cronin *et al.* 1995; Allen and Fisher 1993; Fisher *et al.* 1991; Sato *et al.* 1991; Droz *et al.* 1989a,b; Bogen *et al.* 1988; Fernandez *et al.* 1977; Sato *et al.* 1977). Many of these have been employed to help study the rate of excretion of inhaled TCE and its major metabolites. A PBPK model developed by Bogen *et al.* (1988) assumed continuous pulmonary uptake to accurately model metabolism and urinary excretion data from humans occupationally exposed to TCE (ATSDR 2002; Bogen *et al.* 1988). An earlier model by Sato *et al.* (1977) described TCE metabolism using first order kinetics. Subsequent refinement and expansion of this early model expanded its application to include prediction of the effects of differences in body weight, body fat content, and gender on TCE biotransformation and excretion (Sato *et al.* 1991). Other PBPK models have been developed for use in evaluating cancer risk associated with TCE exposure (Clewell *et al.* 2000, Fisher 2000; Clewell *et al.* 1995, Cronin *et al.* 1995; Allen and Fisher 1993; Fisher *et al.* 1991).

2.3.5 Proposed Carcinogenic Mechanisms for Trichloroethylene

A variety of studies in laboratory animals indicate that several metabolites of TCE may act either as tumor promoters or as complete carcinogens. In studies in mice and rats,

14-day exposure (via gavage) to TCE resulted in significantly increased hepatocyte DNA synthesis in males, but not in females (Klaunig *et al.* 1991). No changes in renal DNA proliferation were observed in either species or in either gender. Exposure to the TCE metabolites chloral hydrate, dichloroacetic acid, or trichloroacetic acid has been shown to directly induce liver tumors in mice (Templin *et al.* 1993; Daniel *et al.* 1992; DeAngelo *et al.* 1991). Similarly, exposure to dichloroacetic acid and trichloroacetic acid, with and without prior exposure to the initiating agent ethylnitrosourea, resulted in a significantly increased incidence of liver tumors in mice (Herren-Freund *et al.* 1987).

Hepatic peroxisome proliferation has also been proposed as a basis for the carcinogenic effects of TCE. In male mice and rats exposed to TCE, elevated levels of cyanide-insensitive palmitoyl CoA oxidase, an indicator of peroxisome proliferation, were reported (Goldsworthy and Popp 1987). Exposure of mice and rats to TCE has also been shown to result in increased peroxisome proliferation in mice but not rats (Elcombe *et al.* 1985). Although it is unclear how peroxisome proliferation may induce cancer, it has been speculated that indirect DNA damage may be the result of increased levels of reactive oxygen species in peroxisomes (Bentley *et al.* 1993). Also, the chronic cellular injury, necrosis, and regeneration associated with peroxisome proliferation may contribute to a promotional effect (Bentley *et al.* 1993; Steinberg and Desesso 1993). Notably, peroxisome proliferation has not been reported in humans at TCE exposure levels that are known to elicit a response in rodents (Bentley *et al.* 1993).

Carcinogenic mechanisms for renal tubular cancer development in male rats may be attributed to the formation of hyalin protein droplets associated with alpha-2- μ -globulin

accumulation in lysosomes (Goldsworthy *et al.* 1988). The increase in renal cellular proliferation resulting from alpha-2- μ -globulin deposition may give rise to a promotional effect in the development of renal cancer (Mirsalis *et al.* 1989; Doolittle *et al.* 1987). However, because alpha-2- μ -globulin is a rat (male)-specific protein, this proposed carcinogenic mechanism of action is not likely to be relevant in humans. Another proposed mechanism for the development of renal cancer associated with TCE exposure in laboratory animals and humans involves the production of the cysteine conjugate dichlorovinylcysteine following conjugation with GSH. Cleavage of this metabolite by an enzyme present in the renal tubule, β -lyase, may lead to the formation of potentially carcinogenic metabolites (Dekant *et al.* 1986a). Dichlorovinylcysteine has been found in the urine of TCE-exposed rats (Dekant *et al.* 1986a) and humans (Birner *et al.* 1993).

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CHAPTER 3

THE PHARMACOKINETICS, METABOLISM, AND CARCINOGENICITY OF ARSENIC

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3.1 HISTORICAL AND CURRENT HUMAN EXPOSURES TO ARSENIC

In civilizations throughout history, arsenic has enjoyed a reputation of power and mystery, a means of effecting outcomes both virtuous and sinister in intent. Egyptian and Arabic alchemists were perhaps among the earliest to value its properties, believing that it could mediate the transmutation of less valuable metals and ores into silver and gold (Azcue and Nriagu 1994). Its notoriety as a powerful poison has also contributed to its rich historical legacy. In 55 A.D., in order to secure his right to the Roman throne, Nero used arsenic to poison Britannicus (Gorby 1994; Joliffe 1993). Aristotle and Socrates were aware of its toxic characteristics as well (Gorby 1994). From the Middle Ages through the mid-1800s, arsenic trioxide was such a popular homicidal agent among professional poisoners (the equivalent of modern day assassins) that it became known as “inheritance powder” (Azcue

and Nriagu 1994; Gorby 1994; Joliffe 1993). Toward the end of the 19th century, arsenic's popularity as a poison began to fade, likely due, at least in part, to the development of sensitive and dependable assays for its detection in body tissues and fluids (Gorby 1994; Joliffe 1993; Hindmarsh and McCurdy 1986).

In addition to its use as a poison, arsenic has been a major constituent of the pharmacologic repertoire of healers throughout time, ingested as a liquid or solid, inhaled as a vapor, injected intravenously, intramuscularly, or intrathecally, even administered as an enema (Gorby 1994; Joliffe 1993). Aristotle, along with the Greek physician Hippocrates and the Roman naturalist Pliny the Elder, reportedly used various sulfur-containing arsenicals in the treatment of a wide variety of medical conditions (Gorby 1994; Joliffe 1993). During the 1800s and early 1900s, arsenic appeared in many medicinal preparations, such as Fowler's solution, a 1% solution of potassium arsenite, and arsphenamine, also known as salvarsan (diamino-dihydroxy-arsenobenzene hydrochloride) (Gorby 1994; Joliffe 1993). These medications were used in the treatment of diseases ranging from asthma, psoriasis, rheumatism, and blood dyscrasias to tuberculosis and syphilis (Gorby 1994; Joliffe 1993). By the mid-1900s, the use of arsenic in medicine had declined significantly, probably due to an increasing awareness of its carcinogenic potential as well as the emergence of the more effective antibiotics (Gorby 1994; Joliffe 1993). Today, arsenicals may be used to treat parasitic infections in animals and trypanosomiasis and amebiasis in humans (Gorby 1994; Joliffe 1993). Additionally, in recent years arsenic trioxide has reappeared as a chemotherapeutic agent in the treatment of acute promyelocytic leukemia (Coco *et al.* 1998; Huang *et al.* 1998; Kizaki *et al.* 1998; Soignet *et al.* 1998; Chen *et al.* 1996). Interestingly,

the traditional Chinese medications pi shuang and xiong huang, known to contain arsenic, have been used for over 1000 years as remedies for a variety of medical conditions, including cancer (Li and Broome 1999).

At present, humans are exposed to arsenic in environmental and occupational settings. Naturally-occurring high levels of arsenic in groundwater have been reported in some areas of the world rich in geologic deposits of arsenic, such as the southwest coast of Taiwan (Chiou *et al.* 1995; Chen and Lin 1994; Chen and Wang 1990; Wu *et al.* 1989; Chen *et al.* 1988; Chen *et al.* 1986; Chen *et al.* 1985; Tseng 1977; Tseng *et al.* 1968) and areas of Mexico (Cebrián *et al.* 1994; Cebrián *et al.* 1983), Argentina (Hopenhayn-Rich *et al.* 1998; Hopenhayn-Rich *et al.* 1996a), Chile (Smith *et al.* 1998; Borgoño *et al.* 1977; Rivara *et al.* 1997), India (Mazumder *et al.* 1998; Subramanian *et al.* 1998; Chowdhury *et al.* 1997; Das *et al.* 1996; Das *et al.* 1994; Chakraborty and Saha 1987), Thailand (Choprapawon and Rodcline 1997), China (Ma *et al.* 1999; Sun *et al.* 1999; Luo *et al.* 1997), and Bangladesh (Chowdhury *et al.* 1999; Rahman *et al.* 1999; Tondel *et al.* 1999). Geologic deposits of arsenic can be disturbed by mining activities, resulting in its liberation into the environment where weathering processes may result in further release (Hindmarsh and McCurdy 1986). The burning of fossil fuels can also contribute to elevated levels of environmental arsenic (Naqvi *et al.* 1994; Hindmarsh and McCurdy 1986). Industries in which arsenic-containing compounds are used include glassblowing and the manufacture of semiconductors and metal alloys (Azcue and Nriagu 1994; Naqvi *et al.* 1994; Vahter 1994). During the smelting of many metals, arsenic may be released into the air or remain as a by-product of the smelting process (Naqvi *et al.* 1994; Vahter 1994; Hindmarsh and McCurdy 1986). Agriculturally,

various arsenic preparations have been used as pesticides, wood preservatives, and cotton dessicants (Naqvi *et al.* 1994; Vahter 1994; Hindmarsh and McCurdy 1986). Arsenic has been identified as one of the most common groundwater contaminants near hazardous waste disposal sites in the United States (Faroon *et al.* 1994), likely the result of its widespread production and use in industry and agriculture.

Arsenic exposure via the diet originates mainly from marine sources (National Research Council 1999; Vahter 1999; Buchet *et al.* 1996; Yamauchi and Fowler 1994). Approximately 0.5-1% of arsenic detected in seafood is inorganic (Vahter 1999; Edmonds and Francesconi 1993); the rest occurs in the forms of various organic compounds, the most common of which is arsenobetaine (National Research Council 1999; Vahter 1999; Vahter 1994; Yamauchi and Fowler 1994). *In vivo*, arsenobetaine has demonstrated low toxicity and is rapidly excreted in the urine unchanged (Vahter 1999; Buchet *et al.* 1996; Le *et al.* 1994; Vahter *et al.* 1983). In addition to arsenobetaine, other organic arsenic compounds detected in fish and crustaceans include arsenocholine, trimethylarsine oxide, trimethylarsine, and tetramethylarsonium salts, though at much lower levels than arsenobetaine (National Research Council 1999; Vahter 1994 National Research Council 1999). In seaweed and mussels, most arsenic occurs in the form of arsenosugars, which, compared to arsenobetaine, are metabolized *in vivo* and exhibit a longer retention time in the human body (National Research Council 1999; Vahter *et al.* 1999; Buchet *et al.* 1996; Le *et al.* 1994). Aside from seafood, most other human foods contain low amounts of arsenic - generally less than 0.2 mg/kg (Vahter 1999; Schoof *et al.* 1998; Yost *et al.* 1998). Thus, historical and current human exposure to arsenic occurs mainly in occupational and environmental milieu; by far

the most common form of arsenic encountered in these settings is inorganic. In addition to its organic and inorganic forms, arsenic may exist in several valence states: 0 (metalloid), ± 3 , or $+5$. The toxicity of the various forms of arsenic, in descending order from most to least toxic, is as follows: arsines > inorganic, trivalent compounds (arsenites) > organic, trivalent compounds > inorganic, pentavalent compounds (arsenates) > organic pentavalent compounds > arsonium compounds > metalloid (Gorby 1994; Morton and Dunnette 1994; Hindmarsh and McCurdy 1986). For the remainder of this discussion, inorganic arsenic will be referred to simply as arsenic; when necessary, its valence state and/or specific organic forms of arsenic will be distinguished.

3.2 EPIDEMIOLOGIC EVIDENCE OF ARSENIC CARCINOGENICITY

The carcinogenic effects of arsenic in humans have long been recognized; earliest case reports may date back to the time of Paracelsus, who, in the 1500s, described a fatal lung disease among central European miners, subsequently identified as lung cancer (Hindmarsh and McCurdy 1986). While exposure to radium was later identified as the probable primary cause of these cancers, inhalation of airborne arsenic has been proposed as a contributing factor (Hindmarsh and McCurdy 1986). During the late 1800s and early 1900s, several clinical studies conducted in Great Britain suggested an association between ingestion of arsenical medicinal preparations, prescribed mainly for the treatment of psoriasis, and the development of skin cancer (Hindmarsh and McCurdy 1986). Various epidemiologic studies have since substantiated the association between exposure to arsenic

and the development of skin and lung cancers. Many of these studies have also implicated arsenic in the etiology of cancers of the urinary tract, liver, and other organs.

3.2.1 Drinking Water Exposures

3.2.1.1. Taiwan and other parts of Asia

The largest epidemiologic database of a population environmentally exposed to arsenic consists of residents in villages located along the southwest coast of Taiwan. In the early 1900s, in response to high salinity of shallow well water sources (6-8 meters in depth), villagers switched to deeper artesian wells (100-200 meters in depth) as an alternative water source (Tsai *et al.* 1998; Abernathy *et al.* 1996; Hsueh *et al.* 1995; Tseng *et al.* 1968). Water in the artesian wells contained very high levels of arsenic, ranging in concentration from 0.01 to 1.82 mg/L (Bates *et al.* 1992; Tseng 1977; Tseng *et al.* 1968). This area of Taiwan, sometimes referred to as the “Blackfoot disease endemic region” has been recognized for its high prevalence of Blackfoot disease (BFD), a chronic peripheral vascular disorder which has been attributed to high levels of arsenic in the drinking water (Abernathy *et al.* 1996; Bates *et al.* 1992; Wu *et al.* 1989; Chen *et al.* 1988; Tseng 1977) as well as ergot (Tsai *et al.* 1998; Chen *et al.* 1988; Carlson-Lynch *et al.* 1994; Lu 1990), which has also been identified in the artesian well water. BFD is characterized by numbness, gangrene, ulceration, blackening, and spontaneous amputation of the extremities (Chiou *et al.* 1995; Bates *et al.* 1992; Chen *et al.* 1988; Tseng 1977).

In a landmark ecologic study, Tseng *et al.* stratified 40,421 residents of the BFD endemic region according to concentration of arsenic in village wells: <0.3 mg/L, 0.3-0.6

mg/L, >0.6 mg/L, and a fourth group consisting of villages either using arsenic-free water or those which demonstrated such high variability in arsenic concentrations as to render classification into the other three groups impossible (Tseng 1977; Tseng *et al.* 1968). An increased prevalence of skin cancer was associated with exposure to the low, mid, and high levels of arsenic in the drinking water, with prevalence rates (per 1,000) in the low, mid, and high exposure categories ranging from 1.3 - 27.1, 2.2 - 106.2, and 11.5 - 192.0, respectively (Tseng 1977; Tseng *et al.* 1968). In subsequent studies of this population, a clear dose-response relationship between well water arsenic concentration and increased standardized mortality ratios (SMRs) due to skin cancer has also been reported (Wu *et al.* 1989; Chen *et al.* 1988; Chen *et al.* 1985;), with risks in the range of 1.87 - 3.24 in the highest exposure category (Wu *et al.* 1989). Additionally, increased incidence and SMRs of urinary bladder, lung, kidney, liver, colon, and prostate cancers have been observed in this population in association with consumption of drinking water containing high levels of arsenic (Chiou *et al.* 1995; Chen and Lin 1994; Chen *et al.* 1992; Chen and Wang 1990; Wu *et al.* 1989; Chen *et al.* 1988; Chen *et al.* 1986; Chen *et al.* 1985). Chiou *et al.* (1995) have also reported a higher risk of arsenic-induced cancers among smokers than non-smokers in this population. Beginning in the late 1950s, tap water supplies began to replace arsenic-contaminated artesian well water sources, becoming widely available during the 1970s and early 1980s (Hsueh *et al.* 1995). Notably, in a recent epidemiologic study, termination of exposure to arsenic-contaminated drinking water was associated with a decline in SMRs from skin and internal cancers (Tsai *et al.* 1998).

The carcinogenicity of arsenic in drinking water has also been observed in parts of Asia other than Taiwan. Its effects on the development of skin lesions and skin cancer have been reported in India (Subramanian and Kosnett 1998; Mazumder *et al.* 1998; Chowdhury *et al.* 1997; Das *et al.* 1996; Das *et al.* 1994; Chakraborty and Saha 1987), Thailand (Choprapawon and Rodcline 1997), China (Luo *et al.* 1997), and Bangladesh (Rahman *et al.* 1999; Tondel *et al.* 1999). In a Japanese cohort study, 454 individuals residing in Niigata Prefecture were exposed for approximately five years to industrially contaminated well water containing arsenic (Tsuda *et al.* 1995). Exposure was classified as high (≥ 1 mg/L), intermediate (0.05-0.99 mg/L), or low (< 0.05 mg/L). Increased SMRs associated with lung (15.69) and urinary tract cancers (31.18) were observed in the high exposure group. This study also provided evidence for a synergistic effect between arsenic exposure and smoking.

3.2.1.2 Central and South America

Epidemiologic studies conducted in areas of Central and South America geologically rich in arsenic have further documented its carcinogenic effects. Among residents of Region Lagunera in the central part of northern Mexico, elevated levels of arsenic in the drinking water (0.41 mg/L) have been associated with an increase in the incidence of ulcerative lesions classified as skin cancer compared to a nearby control population consuming drinking water containing 0.007 mg/L arsenic (Cebrián *et al.* 1994; Cebrián *et al.* 1983). In Argentina, high levels of arsenic in the drinking water have been reported in eastern regions of the province of Cordoba (Hopenhayn-Rich *et al.* 1998, 1996a). In a series of two studies, residents of the 26 counties of Cordoba were categorized according to level of arsenic

exposure (low, medium, or high) based on historical data; a crude average arsenic concentration of 178 µg/L was estimated in the high exposure group (Hopenhayn-Rich *et al.* 1998, 1996a). Increased SMRs for cancers of the urinary bladder (men - 2.14; women - 1.82) (Hopenhayn-Rich *et al.* 1996a), lung (men - 1.77; women - 2.16), and kidney (men - 1.57; women - 1.81) (Hopenhayn-Rich *et al.* 1998) were observed in the highest exposure category. In another study conducted in northern Chile, residents of Region II exposed to high levels of arsenic via the drinking water (population weighted average of up to 570 µg/L) also exhibited elevated SMRs from urinary bladder cancer (men - 6.0; women - 8.2) and lung cancer (men - 3.8; women - 3.1) (Smith *et al.* 1998).

3.2.1.3 Europe and the United States

In a study conducted on a large group of residents of southeast Hungary, concentrations of arsenic in the drinking water were reported ranging from 170-330 µg/L (Borzsonyi *et al.* 1992). Despite chronic consumption of the contaminated drinking water, no significant increase in cancer incidence was observed among residents compared to a control population living in a nearby rural area. In Belgium, Buchet and Lison studied residents of communities located near nonferrous metal smelters; levels of arsenic exposure ranged from 20-50 µg/L in the drinking water and reached a concentration of 0.3 µg/m³ in air (Buchet and Lison 1998). Chronic exposure to these levels of arsenic did not cause an observable increase in cancer incidence among exposed residents. A recent study in Finland evaluated residents exposed to arsenic in drinking water obtained from drilled wells; concentrations of arsenic in the water ranged from <0.05-64 µg/L (Kurtio *et al.* 1999).

Consumption of well water containing $>0.5 \mu\text{g/L}$ arsenic was associated with an increased risk of urinary bladder cancer (risk ratio of 2.44). In addition, a synergistic effect between arsenic exposure and smoking was noted.

Epidemiologic studies of US populations exposed to arsenic-contaminated drinking water have also yielded equivocal results. In Millard County, Utah, a cohort of 2,203 residents exposed to median drinking water arsenic concentrations ranging from 14-166 $\mu\text{g/L}$ was stratified according to an “arsenic exposure index”: low ($<1,000 \mu\text{g/L}\cdot\text{years}$), medium (1,000 - 4,999 $\mu\text{g/L}\cdot\text{years}$), or high ($\geq 5,000 \mu\text{g/L}\cdot\text{years}$) (Lewis *et al.* 1999). Irrespective of exposure level, an increased SMR for prostate cancer (1.45) was observed among male members of the cohort; moreover, comparison between low, medium, and high groups suggested a dose-response relationship. In another study of Utah residents, responses to a National Bladder Cancer Study were evaluated for an association between low level arsenic exposure and urinary bladder cancer (Bates *et al.* 1995). Arsenic concentrations ranged from 0.5-160 $\mu\text{g/L}$ (mean concentration of 5.0 $\mu\text{g/L}$); exposure was interpreted in terms of total cumulative exposure (mg) and intake concentration (mg/L $\cdot\text{years}$). No correlation between arsenic exposure and urinary bladder cancer risk was observed. Among smokers, however, exposure to arsenic was associated with an increased risk of urinary bladder cancer. Among 3,237 residents of Lane County, Oregon exposed to arsenic-contaminated drinking water at concentrations of up to 2,150 $\mu\text{g/L}$ (mean of 8.6 $\mu\text{g/L}$), no association between arsenic concentration and the incidence of skin cancer was detected (Morton *et al.* 1976).

Significantly, several European and US studies have documented the carcinogenic effects of arsenic at very low environmental exposure levels (Kurttio *et al.* 1999; Lewis *et*

al. 1999). These studies provide valuable data regarding the nature of the dose-response curve at very low exposure levels for risk assessment purposes. Both positive and negative studies, however, may be limited by small study size, relatively short duration of exposure, and/or low arsenic concentrations. Many researchers recognize the need for confirmatory studies (Kurttio *et al.* 1999; Bates *et al.* 1995).

3.2.2 Occupational and Medicinal Exposures

3.2.2.1 Copper smelting

The weight of evidence from epidemiologic studies of populations environmentally exposed to arsenic confirms the carcinogenic effects of arsenic via ingestion of contaminated drinking water. In addition, studies of occupationally exposed populations substantiate the carcinogenicity of arsenic via a different route of exposure - inhalation. Copper smelter workers in the US and Sweden exhibit a strong correlation between inhalation of arsenic-containing dusts and the development of lung cancer. In a study of 8,047 white male workers at a copper smelter in Anaconda, Montana, exposure was classified as light, medium, or heavy based on the relative amount of arsenic trioxide in the atmosphere of different work areas throughout the smelter (Lee and Fraumeni 1969). A dose-response relationship between the level of arsenic exposure and SMR was apparent, with a SMR of 6.67 for the heavy exposure category. A correlation between duration of exposure and SMR was also demonstrated. Subsequent analyses of this cohort redefined exposure categories as low ($<100 \mu\text{g}/\text{m}^3$), medium ($100\text{--}499 \mu\text{g}/\text{m}^3$), high ($500\text{--}4,999 \mu\text{g}/\text{m}^3$), and very high ($\geq 5,000 \mu\text{g}/\text{m}^3$) (Welch *et al.* 1982), or light ($0.38 \text{ mg}/\text{m}^3$), medium ($7.03 \text{ mg}/\text{m}^3$), and heavy (61.99

mg/m³) (Lee-Feldstein 1986). Both studies revealed a strong dose-response relationship between cumulative arsenic exposure (mg/m³·years) and SMR for respiratory tract cancer (Lee-Feldstein 1986; Welch *et al.* 1982). Among 2,802 workers at a copper smelter in Tacoma, Washington, a clear dose-response relationship between inhalation of arsenic (using levels of urinary arsenic as a surrogate of exposure level) and SMRs for respiratory tract, kidney, and bone cancer was evident (Enterline *et al.* 1995; Enterline *et al.* 1987; Enterline and Marsh 1982). Evaluation of 3,916 smelter workers in Rönnskär, Sweden with airborne arsenic exposures ranging from less than 0.25 mg/m³ to over 100 mg/m³ demonstrated elevated SMRs associated with lung cancer (Jarup and Pershagen 1991; Jarup *et al.* 1989). In one study, a SMR of 11.37 was observed for the highest exposure category (Jarup *et al.* 1989). Increased mortality from other cancers, such as liver (Tokudome and Kuratsune 1976), gastrointestinal tract (Enterline *et al.* 1995; Enterline and Marsh 1982; Wall 1980; Tokudome and Kuratsune 1976), bone (Enterline *et al.* 1995), hemolymphatic (Enterline and Marsh 1982; Axelson 1978), kidney (Enterline *et al.* 1995), urinary bladder (Tsuda *et al.* 1990; Welch *et al.* 1982), and brain/nervous system (Wall 1980) has also been reported among copper smelter workers exposed to airborne arsenic.

3.2.2.2 *Manufacture and application of pesticides*

Workers in other occupations exposed to high levels of arsenic have also demonstrated elevated risks for a variety of cancers. Vintners from Moselle, Germany were exposed to pesticides containing arsenic trioxide through direct pesticide application and indirectly, through consumption of contaminated haustrunk (“house drink”), a wine

substitute containing 2-90 mg/L arsenic trioxide made from the skins of previously pressed grapes (Bates *et al.* 1992; Luchtrath 1983). Among these workers, an increased incidence of cancers of the skin and respiratory tract (IARC 1987; Luchtrath 1983), gastrointestinal tract (Luchtrath 1983), and liver, including both hepatocellular carcinoma (Luchtrath 1983; Popper *et al.* 1978) and hepatic angiosarcoma, a very rare form of liver cancer (Bates *et al.* 1992; IARC 1987; Kasper *et al.* 1984; Falk *et al.* 1981; Popper *et al.* 1978), was noted. Increased mortality rates from lung cancer (Mabuchi *et al.* 1979; Ott *et al.* 1974) and hemolymphatic cancer (Ott *et al.* 1974) have also been observed among workers exposed to arsenic at pesticide manufacturing and packaging plants.

3.2.2.3 *Treatment with medicinal preparations*

Epidemiologic studies which utilize case reports of cancers associated with consumption of medicinal preparations containing arsenic may be considered less powerful than large-scale drinking water and occupational studies due to many factors, including limited study size and the potential for selection bias (Bates *et al.* 1992). Nonetheless, they provide valuable evidence for the carcinogenic properties of arsenic. In a study of 262 patients treated with Fowler's solution (1% potassium arsenite) for periods ranging from weeks to several years (cumulative dose of 10-2600 mL), the risk of skin cancer was shown to increase in accord with total ingested dose of arsenic (Fierz 1965). Similarly, in another analysis of 478 patients from Lancashire, England treated with Fowler's solution for a duration of two weeks to twelve years, in which cumulative arsenic exposure ranged from <250 mg to over 10,000 mg, an increased risk of skin cancer was observed (Cuzick *et al.*

1982). In a follow-up study, treatment with Fowler's solution was associated with an increased SMR for urinary bladder cancer with evidence of a weak dose-response relationship (Cuzick *et al.* 1992). An increased incidence of hepatic angiosarcoma has been observed in patients receiving Fowler's solution (Bates *et al.* 1992; IARC 1987; Kasper *et al.* 1984; Falk *et al.* 1981; Popper *et al.* 1978), and consumption of arsenic-containing medicinal preparations has been correlated with the development of lung cancer as well (Robson and Jelliffe 1963).

3.3 LABORATORY ANIMAL STUDIES OF ARSENIC CARCINOGENICITY

3.3.1 Oral Exposure

Despite overwhelming evidence of the carcinogenicity of arsenic in humans, its carcinogenic effects have not been definitively demonstrated in laboratory animal models. In chronic carcinogenicity bioassays, dietary administration of arsenate to Wistar rats (100 or 400 ppm) (Kroes *et al.* 1974) or Osborne-Mendel rats (up to 400 ppm) (Byron *et al.* 1967) did not result in an observable increase in tumor development. Similarly, beagle dogs exposed for two years to as much as 125 ppm arsenate in the diet did not exhibit increased tumor incidences (Byron *et al.* 1967). Cynomolgus monkeys (*Macaca fascicularis*) orally administered arsenate, 0.1 mg/kg/day, 5 days/week for 15 years did not demonstrate an increased probability of development of malignant tumors (Thorgeirsson *et al.* 1994). In the dog and monkey, however, exposure periods of two and fifteen years, respectively, may not be considered representative of a chronic exposure sufficient for tumor induction. In addition, Wistar rats chronically administered a combination of 100 ppm arsenate in the diet

and 5 µg diethylnitrosamine (DEN) via daily intubation, 5 days/week, did not exhibit an increase in tumor incidence (Kroes *et al.* 1974). However, in C57B1/6J mice and metallothionein knock-out transgenic mice administered 500 ppb arsenate in the drinking water for up to 26 months, the incidence of tumors of the gastrointestinal tract, lungs, liver, spleen, bone, skin, reproductive system, and eye was increased (Ng *et al.* 1999). Notably, in this study no tumors were observed among mice in control groups.

Chronic studies with arsenite have yielded similar results. Lifetime exposure of Osborne-Mendel rats to as much as 250 ppm arsenite in the diet did not result in enhanced tumor development (Byron *et al.* 1967). Likewise, beagle dogs administered up to 125 ppm arsenite in the diet for two years did not exhibit an increase in tumor incidence (Byron *et al.* 1967). Chronic administration of arsenite in the drinking water (5 ppm) of Long-Evans rats was not associated with any increase in tumor incidence in either species (Falk *et al.* 1981; Kanisawa and Schroeder 1967).

Interestingly, several studies in mice suggest that arsenic may actually decrease the formation of a variety of tumors. In Swiss mice, lifetime administration of 5 ppm arsenite via the drinking water led to a significant decrease in the development of spontaneous tumors of the lung (Kanisawa and Schroeder 1967). Similarly, administration of arsenate (80 ppm) for 12 weeks yielded a decrease in the incidence of urethane-induced pulmonary tumors (Blakley 1987). In C3H mice, exposure to 10 ppm arsenite in the drinking water for 15 months resulted in a decrease in the incidence of spontaneous mammary tumors, although an increase in tumor size was also noted (Schrauzer and Ishmael 1974). Arsenite (0.01%) given in drinking water to C x C3H mice during methylcholanthrene initiation and promotion

by syngeneic skin grafting resulted in a decrease in the development of skin papillomas (Milner 1968). Although C3H/St mice chronically exposed to drinking water containing 2 ppm arsenite demonstrated no significant difference in tumor incidence, an increase in tumor onset age (latency) in treated rats was observed (Schrauzer *et al.* 1978).

Data from multistep carcinogenicity bioassays designed to investigate the promoting and/or cocarcinogenic potential of test compounds indicate that arsenic may act as a tumor promoter, progressor, and/or cocarcinogen. In male Wistar-King rats intratracheally administered a mixture of 0.26 mg arsenic trioxide and 0.4 mg benzo[a]pyrene, a higher incidence of pulmonary carcinoma was observed compared to controls receiving only 0.4 mg benzo[a]pyrene (Ishinishi *et al.* 1977). Male Wistar rats administered 160 ppm sodium arsenite in the drinking water following intraperitoneal (*i.p.*) injection of DEN (30 mg/kg) developed significantly more renal adenocarcinomas compared to control rats treated with either sodium arsenite alone or DEN alone (Shirachi *et al.* 1983).

Investigation of dimethylarsinic acid (DMA), a metabolite of arsenic, in multistep carcinogenesis bioassays has also yielded positive results. In one study utilizing the Ito Medium-Term Liver Foci Bioassay model of rat hepatocarcinogenesis (Ito *et al.* 1996; Hasegawa *et al.* 1994; Ito *et al.* 1989; Ito *et al.* 1988), male Fischer 344 (F344) rats were injected with DEN (200 mg/kg *i.p.*) as an initiator at the onset of the study (Wanibuchi *et al.* 1997). Two weeks later, rats were administered DMA (25, 50, or 100 ppm) via the drinking water for six weeks then euthanatized; partial hepatectomy was performed at week three. A dose-dependent increase in area and number of hepatic glutathione S-transferase π (GST-P) positive foci, a reliable marker of hepatic preneoplasia in the rat (Hasegawa and Ito 1994; Ito

et al. 1988), was reported among DMA-treated groups compared to DEN controls (Wanibuchi *et al.* 1997). Wanibuchi *et al.* (1996) have also reported the promotional effect of DMA in a rat urinary bladder carcinogenesis model. Male F344 rats were treated with 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) for four weeks followed by administration of DMA (2,10, 25, 50, or 100 ppm) in the drinking water for 32 weeks. A dose-dependent increase in the incidence of preneoplastic lesions, papillomas, and carcinomas in the urinary bladder was observed. In another study based on a short-term pulmonary carcinogenesis model (Inayama 1986), male ddY mice were subcutaneously (*s.c.*) injected with 4-nitroquinoline 1-oxide (4NQO; 10 mg/kg) followed by administration of DMA in the drinking water (200 or 400 ppm) for 25 weeks (Yamanaka *et al.* 1996). An increased incidence of pulmonary nodules was reported among treatment groups compared to two control groups: one initiated with 4NQO only and another initiated with 4NQO followed by administration of a 5% glycerol solution (an established promoter of 4NQO-initiated cells). Further histopathological analysis of pulmonary nodules revealed a higher degree of cellular atypism suggestive of tumor progression (*e.g.*, nuclear hyperchromasia, increased nuclear:cytoplasmic ratio, irregular cell arrangement) among DMA treatment groups compared to those controls treated with 4NQO and glycerol.

Similar “multiple organ” initiation/promotion studies in which rodents were administered several organospecific initiating agents followed by administration of DMA have also revealed enhanced tumor development in various organs. In one 30-week study, male F344/DuCrj rats were administered a series of five initiators during the first 30 days: DEN (100 mg/kg *i.p.* on day 1); N-methyl-N-nitrosourea (MNU, 20 mg/kg *i.p.* on days 5, 8,

11, and 14); 1,2-dimethylhydrazine (DMH, 40 mg/kg *s.c.* on days 18, 22, 26, and 30); BBN (0.05% in the drinking water during weeks 1 and 2); and N-bis(2-hydroxypropyl)nitrosamine (DHPN, 0.1% in the drinking water during weeks 3 and 4) (Yamamoto *et al.* 1997; Yamamoto *et al.* 1995). Two weeks after the conclusion of the initiation period, rats were administered DMA (50, 200, or 400 ppm) in the drinking water until euthanasia at the end of week 30. Treatment with DMA was associated with an increased incidence of tumors in the liver, kidney, urinary bladder, and thyroid gland, as well as an increased induction of preneoplastic lesions in the liver and kidney (GST-P positive foci and atypical tubules, respectively).

Most laboratory animal evidence suggests that arsenic does not act as an initiator in the process of multistage carcinogenesis, but rather as a promoter, progressor, or cocarcinogen. Demonstration and analysis of the carcinogenic potential of arsenic in laboratory animals, therefore, demands experimental models designed with these characteristics in mind. Since humans encounter hazardous chemicals in the environment as mixtures rather than single agents, it is important to investigate the carcinogenic effect(s) of arsenic in environmentally relevant chemical mixtures comprised of other carcinogens, in addition to arsenic, with actions and/or effects that might be potentiated by arsenic.

In our laboratory, we have conducted a series of studies designed to investigate the carcinogenic effects of a chemical mixture containing arsenic. Components of the chemical mixture were chosen based on their environmental relevance and similarity in carcinogenic effects. In addition to arsenic, some of the most commonly identified groundwater contaminants near hazardous waste disposal sites include 1,2-dichloroethane (DCE), vinyl

chloride (VC), and trichloroethylene (TCE) (Faroon *et al.* 1994; ATSDR 1993). Exposure to arsenic has been associated with the development of hepatic angiosarcoma in humans (Bates *et al.* 1992; IARC 1987; Kasper *et al.* 1984; Falk *et al.* 1981; Popper *et al.* 1978). Both DCE (National Cancer Institute 1978) and VC (Maltoni *et al.* 1984; Popper *et al.* 1981; Feron *et al.* 1981; Popper *et al.* 1978; Waxweiler *et al.* 1976) have also been associated with the development of hepatic angiosarcoma in laboratory animals and/or humans. In our earliest study, using a protocol described by Luquette *et al.* (1985), we attempted to induce hepatic angiosarcomas in F344 rats by exposing them to a four-chemical mixture consisting of arsenite, DCE, VC, and TCE for 16 weeks (Pott *et al.* 1997). Exposures to each individual chemical were also carried out. Although we were unable to induce tumors in any of the treatment groups, rats exposed to arsenite (75 ppm) or DCE (300 ppm) in drinking water exhibited hepatic sinusoidal dilatation (Pott *et al.* 1997), an early change reported in association with angiosarcoma development (Popper *et al.* 1981, 1978).

In a second study, we utilized a multistep multi-organ carcinogenesis bioassay (Ito *et al.* 1996; Hasegawa and Ito 1994; Ito *et al.* 1991; Ito *et al.* 1989) to investigate whether arsenic would potentiate the carcinogenic effects of DCE, VC, and TCE (Pott *et al.* 1998). In this 20-week study, male F344 rats were administered a series of three broad spectrum (multiple organ) initiators during the first 28 days (“DMD” initiation protocol, representing the first letter of each initiator): DEN (100 mg/kg *i.p.* on day 1); MNU (20 mg/kg *i.p.* on days 2, 5, 8, and 11); and DHPN (0.1% in the drinking water during weeks 3 and 4) (Pott *et al.* 1998). Control groups were administered vehicle controls (saline, citrate buffered saline, and deionized drinking water, respectively) during this period. For 16 weeks following the four-

week initiation period, rats were administered one of three dose levels of the four-chemical mixture: low (7.5 ppm arsenite, 3 ppm DCE, 1.5 ppm VC, 19 ppm TCE); medium (25 ppm arsenite, 30 ppm DCE, 5 ppm VC, 190 ppm TCE); or high (75 ppm arsenite, 300 ppm DCE, 15 ppm VC, 1900 ppm TCE). Arsenic and DCE were administered daily via the drinking water; VC and TCE via once daily corn oil gavage, five times per week. Control groups were administered deionized drinking water and corn oil. All rats were euthanatized at the end of week 20 (Pott *et al.* 1998).

Contrary to our expectations, the four-chemical mixture consisting of known carcinogens demonstrated an antagonistic effect on the development of hepatic and pulmonary preneoplastic and neoplastic lesions. Compared to initiated controls, treatment with the four-chemical mixture significantly decreased the area and number of all altered hepatocellular foci (AHF), as well as the area of GST-P positive foci in a dose-dependent manner (Table 3.1) (Pott *et al.* 1998). Additionally, exposure to the four-chemical mixture was associated with a decrease in the frequency of pulmonary bronchioalveolar hyperplasia and adenoma formation (Table 3.2) (Pott *et al.* 1998).

Table 3.1
Altered hepatocellular foci (AHF) and hepatic glutathione S-transferase π positive (GST-P⁺) foci in a multi-organ carcinogenesis bioassay (Pott *et al.* 1998).

Treatment Group	AHF Total Area**	AHF Total No.**	GST-P ⁺ Foci Total Area	GST-P ⁺ Foci Total No.	Large [†] GST-P ⁺ Foci Area	Large GST-P ⁺ Foci No.
DMD*/Control	0.652 (0.396) [§]	12.0 (5.3)	0.450 (0.064)	77.2 (14.4)	0.081 (0.063)	1.7 (1.2)
DMD/Low Mix	0.232 (0.153) ^a	7.5 (3.4) ^a	0.377 (0.108) ^b	69.2 (13.9)	0.043 (0.051) ^c	0.7 (0.7) ^c
DMD/Medium Mix	0.116 (0.080) ^a	5.6 (2.7) ^a	0.293 (0.062) ^b	69.0 (10.6)	0.036 (0.034) ^c	0.7 (0.6) ^c
DMD/High Mix	0.032 (0.023) ^{a,d}	1.9 (1.0) ^a	0.214 (0.083) ^b	63.0 (12.5)	0.015 (0.021) ^c	0.3 (0.4) ^c
Vehicle/Control	0.001 (0.001)	0.1 (0.1)	0.010 (0.008)	2.8 (1.6)	0.002 (0.006)	0.1(0.1)
Vehicle/High Mix	0.001 (0.001)	0.1 (0.1)	0.005 (0.004)	2.0 (1.4)	0	0

* DMD: DEN (100 mg/kg *i.p.*); MNU (20 mg/kg *i.p.*); DHPN (0.1% in drinking water).

** Foci area (mm²) per unit liver area (cm²); foci number per unit liver area (cm²).

† Diameter > 0.2 mm.

§ Mean (standard deviation).

^a Significantly different from DMD/Control ($p < 0.001$) according to a one-way ANOVA.

^b Significantly different from DMD/Control ($p < 0.01$) according to a one-way ANOVA.

^c Significantly different from DMD/Control ($p < 0.05$) according to a one-way ANOVA.

^d Significantly different from DMD/Low Mix ($p < 0.05$) according to Tukey's method for pairwise comparisons.

Table 3.2
Bronchioalveolar hyperplasia and pulmonary adenoma formation
in a multi-organ carcinogenesis bioassay (Pott *et al.* 1998).

Treatment Group	Bronchioalveolar Hyperplasia **	Pulmonary Adenomas **
DMD*/Control	12.3 (3.3) [§]	0.251 (0.446)
DMD/Low Mix	7.2 (2.9) ^a	0 ^a
DMD/Medium Mix	7.2 (2.5) ^a	0 ^a
DMD/High Mix	5.8 (2.4) ^a	0 ^a
Vehicle/Control	0	0
Vehicle/High Mix	0	0

* DMD: DEN (100 mg/kg *i.p.*); MNU (20 mg/kg *i.p.*); DHPN (0.1% in drinking water).

** Number of lesions per unit lung area (cm²).

[§] Mean (standard deviation).

^a Significantly different from DMD/Control ($p < 0.001$) according to a one-way ANOVA.

In our third study, to assess whether the antagonistic effect on the development of GST-P positive hepatic foci was a function of the four-chemical mixture, a submixture thereof, or simply arsenite alone, we utilized the Ito Medium-Term Liver Foci Bioassay model (Ito *et al.* 1996; Hasegawa and Ito 1994; Ito *et al.* 1989; Ito *et al.* 1988). On day one of this eight-week study, male F344 rats were administered DEN (200 mg/kg *i.p.*). During weeks three through eight, a four-chemical (75 ppm arsenite, 300 ppm DCE, 1900 ppm TCE, 15 ppm VC), 3- chemical (arsenite, DCE, TCE) or 2-chemical (arsenite, DCE) mixture, or arsenite alone was administered orally to rats. An additional treatment group received the four-chemical mixture with a higher dose level of arsenite (300 ppm weeks three and four, 150 ppm weeks five through eight). Partial hepatectomy was performed at week three, and rats were euthanatized at the end of week eight (Pott *et al.* 2000). Preliminary morphometric analysis revealed that exposure to either arsenite alone (75 ppm) or the four-chemical mixture containing the high dose of arsenite (300/150 ppm) significantly decreased the total area of GST-P positive foci compared to DEN-treated positive controls (Pott *et al.* 2000). These results suggest that arsenite is responsible for the observed antagonistic effect.

Laboratory animal evidence for the carcinogenicity of arsenic is equivocal at best. Early studies document its impotency as a complete carcinogen in chronic carcinogenicity bioassays. Data from more recent studies support the hypothesis that arsenic or its metabolite DMA may act as a promoter, progressor, or cocarcinogen. These data, however, are contradicted by evidence from our laboratory and others that suggest arsenic may exert an antagonistic or protective effect on the development of neoplasia. Yet the carcinogenic effect of arsenic in humans remains undeniable. Reconciliation of the disparities among

animal studies and between human and animal data is characteristic of the many challenges facing arsenic researchers.

The inability to demonstrate the carcinogenicity of arsenic in laboratory animal models may reflect a unique sensitivity of humans to its carcinogenic effects. If so, differences in the carcinogenicity of arsenic between humans and laboratory animals may be interpreted from two perspectives. First, species differences in arsenic pharmacokinetics may result in different target tissue concentrations of arsenic and its metabolites; such differences in target tissue doses may be responsible for the differential carcinogenicity between humans and laboratory animals (Clewell *et al.* 1998). Secondly, species differences in the cellular and molecular interactions of arsenic and/or its metabolites in target tissues may ultimately be responsible for the species differences in its carcinogenic effects (Clewell *et al.* 1998). Alternatively, apparent differences in the carcinogenic effects of arsenic between humans and laboratory animals may be a function of our choice of appropriate experimental models. If arsenic exerts its carcinogenic effect via pathways or mechanisms incongruous with established experimental models of carcinogenicity, it should not be surprising that we are unable to duplicate its carcinogenic effects in the laboratory.

3.4 ARSENIC AS A CHEMOTHERAPEUTIC AGENT

Over the last decade, arsenic trioxide has emerged as a chemotherapeutic agent for the treatment of acute promyelocytic leukemia (APL). APL patients are generally treated with a standard therapy of all-*trans*-retinoic acid (ATRA) (Coco *et al.* 1998, Kizaki *et al.* 1998). ATRA acts by inducing differentiation of leukemia cells, but this remission can be

short-lived and APL patients can become resistant to the effects of ATRA (Kizaki *et al.* 1998, Soignet *et al.* 1998; Warrell RP 1993). Recent studies in China and the United States have indicated that arsenic trioxide is capable of inducing complete remission in cases of relapsed or refractory APL (Huang *et al.* 1998; Soignet *et al.* 1998; Shen *et al.* 1997; Chen *et al.* 1996). The efficacy of arsenic trioxide has also been demonstrated in ATRA-sensitive APL patients (Coco *et al.* 1998). Although the mechanism of arsenic trioxide's clinical effects remains unclear, it has been shown to induce apoptosis in leukemic (Ishitsuka *et al.* 1998; Soignet *et al.* 1998; Chen *et al.* 1996) and lymphoid cell lines (Bazarbachi *et al.* 1999; Zhang *et al.* 1998) *in vitro*. Inhibition of microtubule formation by arsenic trioxide has been demonstrated (Li and Broome 1999). Furthermore, *in vitro* evidence suggests that arsenic trioxide might be clinically useful in the treatment of malignant lymphoma and chronic lymphocytic leukemia (Zhang *et al.* 1998), adult T-cell leukemia (Bazarbachi *et al.* 1999; Ishitsuka *et al.* 1998), and lymphoma (Dai *et al.* 1999).

3.5 PHARMACOKINETICS OF ARSENIC

3.5.1 Absorption

Gastrointestinal and pulmonary absorption of arsenic correlates closely with the compound's aqueous solubility: in general, the more water-soluble the compound, the greater its absorption (Vahter 1983). Studies in the hamster have shown that absorption of the poorly soluble lead arsenate administered orally or intratracheally is very low compared to that of the highly soluble sodium arsenate and sodium arsenite (Marafante and Vahter 1987). Likewise, gallium arsenide, a compound with low water solubility, has been shown to be

poorly absorbed when administered orally to hamsters (Yamauchi *et al.* 1986). Studies in rats and cats have revealed that enteral absorption of arsenic occurs mainly in the small intestine (Vahter 1983). Among species, the Syrian golden hamster demonstrates low rates of enteral arsenic absorption, with approximately 30% of the administered dose being absorbed (Charbonneau *et al.* 1980), compared to more than 90% in humans and most other laboratory animal species (Vahter 1983; Charbonneau *et al.* 1980; Vahter and Norin 1980; Bettley and O'Shea 1975). In the rat, intestinal perfusion with phosphate has been shown to decrease absorption of arsenate, presumably due to a shared transport mechanism (Gonzalez *et al.* 1995). Dietary composition may also affect enteral absorption (Vahter 1983). In the case of airborne exposures, absorption of arsenic depends not only on its solubility but also on the size of the inhaled particle (Vahter 1983). Terminal lung cancer patients exposed to either cigarette smoke containing ^{74}As -labeled arsenite or an aerosol of ^{74}As -arsenite demonstrated a cumulative absorption after four days ranging from 70-85% of the total amount deposited in lungs which rose to 85-90% after 14 days (Vahter 1983; Holland *et al.* 1959).

3.5.2 Distribution

In the bloodstream, arsenic is distributed between the erythrocytes and the plasma, and the relative amounts in each compartment depend on a variety of factors, including the valence and dose of arsenic administered, as well as the species of animal. In mice administered a single oral dose of ^{74}As -labeled arsenate (0.4 or 4.0 mg/kg), arsenic was approximately equally distributed between the erythrocyte and plasma compartments (Vahter

1983; Vahter and Norin 1980). If mice were instead administered ^{74}As -labeled arsenite using the same dosing regime, the erythrocyte:plasma ratio of arsenic shifted to approximately 2:1 (Vahter 1983; Vahter and Norin 1980). In the rat, most arsenic, regardless of dose, is bound to the erythrocytes as DMA (Vahter 1994; Lerman and Clarkson 1983; Rowland and Davies 1982; Odanaka *et al.* 1980). DMA forms a stable complex with rat hemoglobin (Lerman and Clarkson 1983); a mixed structure of the hemoglobin protein, glutathione, and arsenite has been proposed (Winski and Carter 1995). In humans, arsenic has been shown to accumulate in erythrocytes as doses increase. In unexposed subjects, the distribution of arsenic between erythrocytes and plasma was approximately equal (2.7 and 2.4 $\mu\text{g/L}$, respectively) (Heydorn 1970). Among non-Black Foot Disease residents of the Black Foot Disease-endemic region in Taiwan, the erythrocyte:plasma ratio shifted to approximately 2 (32.7:15.4 $\mu\text{g/L}$); those residents with Black Foot Disease exhibited a ratio of over 3 (106:32.3 $\mu\text{g/L}$) (Heydorn 1970).

In humans and most laboratory animals, clearance of arsenic from the blood conforms to a three exponential curve (Lerman and Clarkson 1983; Vahter and Norin 1980). The half-time of the first phase is one to two hours, during which greater than 90% of blood arsenic is cleared (Vahter 1983). The half-times of the second and third phases have been estimated at 30 and 200 hours, respectively (Vahter 1983). In the rat, due to binding of DMA to erythrocytes, the half-time of either arsenate or arsenite is approximately 60-90 days (Vahter 1983; Ariyoshi and Ikeda).

The binding of arsenic and its metabolites in different organs is complex and incompletely characterized in terms of tissue kinetics. In general, keratin-rich tissues with

high sulfhydryl group content, such as the hair, nails, and skin, exhibit the highest concentrations and longest retention of arsenic (Vahter 1999; Hindmarsh and McCurdy 1986; Liebscher and Smith 1968). Specifically, trivalent arsenic exhibits a high affinity for vicinal thiols (Delnomdedieu 1993; Wiencke and Yager 1992; Squibb and Fowler 1983). In rabbits administered a single dose of ^{74}As -labeled arsenite (50 $\mu\text{g/kg}$ or 100 $\mu\text{g/rabbit i.p.}$), in the plasma and lung, most of the ^{74}As was bound to low molecular weight fractions of the cytosol (Marafante *et al.* 1981). In the liver and kidney, however, most ^{74}As was associated with high molecular weight components of the cytosol (Marafante *et al.* 1981). In mice orally administered a single dose of ^{74}As -labeled arsenite or arsenate (0.4 or 4.0 mg/kg), greater retention of arsenic was observed with arsenite compared to arsenate 24 hours after dosing (Vahter and Norin 1980). At the high dose (4.0 mg/kg arsenate or arsenite), differences in arsenic accumulation were noted in the liver (0.39 $\mu\text{g/g}$ when dosed with arsenite compared to 0.04 $\mu\text{g/g}$ with arsenate), kidney (0.78 $\mu\text{g/g}$ with arsenite versus 0.06 $\mu\text{g/g}$ with arsenate), and skin (0.76 $\mu\text{g/g}$ with arsenite versus 0.04 $\mu\text{g/g}$ with arsenate) (Vahter and Norin 1980). In experiments of mice orally administered ^{74}As -labeled arsenite or arsenate three times a week for 12 weeks, the highest concentrations of arsenic were found in the hair (17 mg/kg three days after the first dose of arsenite and 1,110 mg/kg three days after the last dose) and skin (13 mg/kg after the first dose and 104 mg/kg after the last dose), followed by the liver, kidneys, lungs, and intestinal mucosa, which exhibited two- to three-fold increases in arsenic content over time (Vahter 1983). Tissue accumulation of arsenic in these mice was higher after administration of arsenite compared to arsenate (Vahter 1983). Investigation of the accumulation of arsenic in tissues of hairless mice exposed to airborne

arsenite (average concentration of $179.4 \mu\text{g}/\text{m}^3$) six hours/day, five days/week for six weeks revealed an unexpected decrease in liver and kidney arsenic accumulation over time (Bencko and Symon 1970). After two weeks of exposure, concentrations of arsenic in the liver and kidney ranged from 63-85 mg/kg and 173-208 mg/kg (wet tissue weight), respectively (Bencko and Symon 1970). After four weeks of exposure, however, liver and kidney arsenic concentrations dropped to about 5 and 12 mg/kg (wet tissue weight), respectively (Bencko and Symon 1970). In another experiment, strain H mice were administered a single dose of ^{74}As -arsenate intramuscularly after having been previously exposed to arsenite (50 ppm) in the drinking water for 4, 16, or 64 days (Bencko *et al.* 1973). Mice exposed to arsenite in the drinking water for four days exhibited higher levels of ^{74}As in the liver and kidneys than those receiving the same dose of arsenite in the drinking water for 64 days, suggesting enhanced excretory function with prolonged arsenic exposure (Bencko *et al.* 1973).

In marmoset monkeys administered a single dose (0.4 mg/kg) of radiolabeled arsenate intraperitoneally (Vahter *et al.* 1982) or intravenously (Vahter *et al.* 1985), a high degree of arsenic accumulation in the liver was noted one to four days after dosing, which was attributed to binding to the rough microsomal membranes (Vahter *et al.* 1985; Vahter *et al.* 1982). Arsenic accumulation was also noted to a lesser extent in the oral cavity, esophagus, skin, kidneys, and gall bladder (Vahter and Marafante 1985). In mice and rats orally administered ^{74}As -labeled DMA (0.4 mg/kg), DMA accumulated in the kidneys, lungs, intestinal mucosa, stomach, and testes within six hours after dosing (Vahter *et al.* 1984). While the concentration of DMA decreased rapidly in most tissues, the tissues with the

longest retention times were the lungs, thyroid, intestinal mucosa, and the ocular lens (Vahter *et al.* 1984).

In the average human with no known occupational or environmental exposure to arsenic, the highest concentrations are found in the hair (0.46 mg/kg dry weight) and nails (0.2 mg/kg), with fairly high accumulation also occurring in the skin (0.08 mg/kg) and lungs (0.08 mg/kg) (Liebscher and Smith 1968). In Swedish smelter workers occupationally exposed to high levels of airborne arsenic, the highest concentrations appeared in the lung (35 µg/kg wet weight) (Gerhardsson *et al.* 1988). Residents of West Bengal, India, exposed to concentrations of arsenic in the drinking water ranging from 0.05-3.7 mg/L demonstrated elevated levels of arsenic in the hair (1.18-31.05 mg/kg), nails (1.47-52.03 mg/kg), skin (3.05-4.26 mg/kg), and liver (1.41-2.20 mg/kg) (Das *et al.* 1994). In five samples of arsenic-related skin tumors from residents of arsenic endemic regions in Taiwan, Thailand, and Japan, arsenic levels were measured as 0, 2.72, 5.71, 17.1 and 61.8 mg/kg, while in samples of arsenic-unrelated skin tumors, no arsenic was detectable (Matsui *et al.* 1999).

3.5.3 Metabolism

The extent of biotransformation of arsenic varies considerably among species. In most mammals, the metabolic pathway of arsenic involves a series of methylation steps, resulting in the formation of MMA and DMA (Vahter 1999; Aposhian 1997; Vahter 1994; Marafante *et al.* 1985; Vahter 1983). The chimpanzee (Vahter *et al.* 1995b), marmoset (Zakharyan *et al.* 1996; Vahter and Marafante 1985; Vahter *et al.* 1982), tamarin (Zakharyan *et al.* 1996), squirrel monkey (Aposhian 1997), guinea pig (Healy *et al.* 1997), gorilla

(Goering *et al.* 1999), and orangutan (Goering *et al.* 1999), however, do not methylate arsenic, as they are either deficient in or lack the arsenic methyltransferase enzyme(s) responsible for methylation (Goering *et al.* 1999; Vahter 1999; Healy *et al.* 1997; Aposhian 1997; Zakharyan *et al.* 1996). While many details about the methylation process remain unknown, the general pattern involves sequential donation of a methyl group from S-adenosylmethionine (SAM) to a trivalent form of arsenic (Zakharyan and Aposhian 1999; Aposhian 1997; Vahter 1994; Buchet and Lauwerys 1985; Marafante and Vahter 1984; Vahter 1983). Cyanocobalamin (Vitamin B12), coenzyme B12, and methylcobalamin have also been identified as potential methyl donors (Vahter 1999; Buchet and Lauwerys 1985). The methylation pathway is illustrated in Figure 3.1 below.

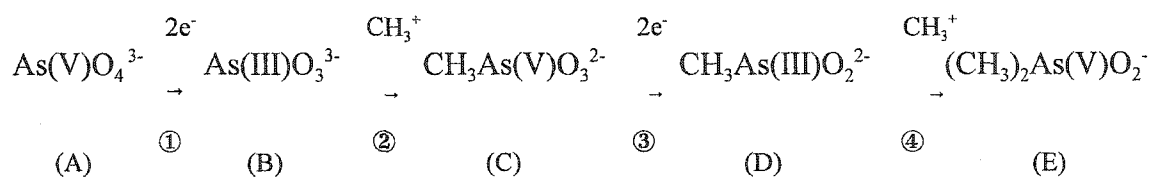


Figure 3.1: Biotransformation of arsenic. (A) arsenate; (B) arsenite; (C) monomethylarsonic acid (MMA^V); (D) monomethylarsonous acid (MMA^{III}); (E) dimethylarsinic acid (DMA); ① arsenate reductase; ② arsenite methyltransferase; ③ MMA^V reductase; ④ MMA^{III} methyltransferase.

The usual, though not exclusive, reducing agent appears to be glutathione (GSH) (Vahter 1999; Zakharyan *et al.* 1995; Styblo *et al.* 1995; Vahter 1994; Buchet and Lauwerys 1987; Buchet and Lauwerys 1985). Thus, absorbed arsenate is first reduced to arsenite, which may occur mainly in the plasma (Vahter 1999; Winski and Carter 1995; Vahter 1994; Bertolero *et al.* 1987; Marafante *et al.* 1985; Vahter and Marafante 1985). An arsenate reductase has been partially purified from human liver (Radabaugh and Aposhian 2000). Arsenite then undergoes oxidative methylation in the liver by addition of a carbonium ion (from SAM), resulting in the formation of MMA^V (monomethylarsonic acid) (Vahter 1999; Vahter 1994; Buchet and Lauwerys 1985; Vahter 1983). Arsenite methyltransferases have been identified in livers of the rabbit (Zakharyan *et al.* 1995), hamster (Wildfang *et al.* 1998), and rhesus monkey (Zakharyan *et al.* 1996). The pentavalent arsenic in MMA^V is then reduced to the trivalent form in MMA^{III} (monomethylarsonous acid); rabbit liver MMA^V reductase has been isolated and characterized as the rate limiting step in the biotransformation of arsenic in the rabbit (Zakharyan and Aposhian 1999). MMA^V reductase has also been identified in human liver (Zakharyan and Aposhian 1999). Formation of MMA^{III} facilitates the addition of a second carbonium ion via oxidative methylation to yield DMA (Vahter 1999; Vahter *et al.* 1994; Vahter 1984; Vahter 1983). MMA^{III} methyltransferase has been isolated from rabbit liver and Chang human hepatocytes (Zakharyan *et al.* 1999; Zakharyan *et al.* 1995). In rabbit liver, arsenite methyltransferase and MMA^{III} methyltransferase have been putatively identified as the same enzyme, perhaps with two different active centers (Zakharyan *et al.* 1999; Aposhian 1997; Zakharyan *et al.*

1996; Zakharyan *et al.* 1995). DMA is generally considered the endpoint of arsenic biotransformation (Vahter 1999; Vahter 1994; Buchet and Lauwerys 1985).

Experimentally-induced chemical inhibition of methyl donation from SAM has been associated with increased tissue concentrations of arsenic, particularly in the skin (Marafante *et al.* 1985; Marafante and Vahter 1984). As arsenic becomes sequentially methylated in its biotransformation, depletion of the methyl donor SAM and concurrent accumulation of its demethylated by-product, S-adenosylhomocysteine (SAH) could potentially occur. A decreased SAM/SAH ratio has been shown to inhibit a variety of methyltransferases (Mato *et al.* 1994; Pascale *et al.* 1991; Garcea *et al.* 1989) and could potentially inhibit methyltransferases involved in the biotransformation of arsenic. If so, the resulting accumulation of arsenic in critical tissues may be involved in the modulation of its carcinogenicity *in vivo*.

Because the methylated metabolites of arsenic are less reactive with tissue macromolecules and are eliminated more rapidly than arsenic itself, methylation has classically been considered a detoxification pathway (Goering *et al.* 1999; Vahter 1999; Vahter 1994; Yamauchi and Fowler 1994; Squibb and Fowler 1983; Buchet *et al.* 1981). Some recent data, however, indicate that the methylated metabolites of arsenic may be at least partially responsible for its carcinogenic effects. Recent investigations using Chang human hepatocytes have demonstrated that monomethylarsonous acid (MMA^{III}) is more toxic than arsenite, arsenate, monomethylarsonic acid (MMA^{V}), and DMA (Petrick *et al.* 2000). *In vitro* studies in human alveolar epithelial type II (L-132) cells have also demonstrated the genotoxic potential of DMA at concentrations ranging from less than 100 μM to 10 mM in

terms of its ability to produce DNA single strand breaks, DNA-protein crosslinks, and induce DNA repair synthesis (Yamanaka *et al.* 1997a,b; Yamanaka *et al.* 1995; Kato *et al.* 1994; Tezuka *et al.* 1993; Yamanaka *et al.* 1993). *In vivo*, DMA orally administered to rats and mice at concentrations of up to 400 ppm has also been shown to induce pulmonary DNA damage (Brown *et al.* 1997; Okada *et al.* 1994; Yamanaka *et al.* 1994; Yamanaka *et al.* 1989a,b). In addition, studies evaluating the promotional effects of DMA, in which rodents were administered DMA following single- or multiple-agent initiation, revealed increased incidence of cancers of the liver, kidney, thyroid gland (Yamamoto *et al.* 1997; Yamamoto *et al.* 1995), lung (Yamanaka *et al.* 1996), and urinary bladder (Yamamoto *et al.* 1997; Wanibuchi *et al.* 1997; Yamamoto *et al.* 1995).

Conversely, other data indicate that arsenate and/or arsenite, not MMA or DMA, are the active chemical species. *In vitro* studies using the mouse lymphoma cell L5178Y/TK^{+/−} assay revealed that arsenate (10-14 µg/mL) and arsenite (1-2 µg/mL) induced chromosomal damage while much higher concentrations of MMA (2500-5000 µg/mL) and DMA (almost 10,000 µg/mL) were required to induce DNA damage (Moore *et al.* 1997). Data from *in vivo* initiation/promotion carcinogenicity bioassays also suggest that arsenite acts as a promoter in the rat lung and kidney. Wistar-King rats intratracheally administered arsenic trioxide (0.26 mg) together with benzo[*a*]pyrene (0.4 mg/kg) exhibited an increased incidence of squamous cell carcinomas the lung compared to controls receiving benzo(a)pyrene only (Ishinishi *et al.* 1977). Wistar rats receiving 160 ppm arsenite via the drinking water following partial hepatectomy and DEN injection (30 mg/kg *i.p.*) demonstrated an increased incidence of renal tumors compared to partially hepatectomized,

DEN-treated control rats (Shirachi *et al.* 1983). Thus, it remains unclear whether the critical chemical species in the carcinogenicity of arsenic is arsenate/arsenite or one of the methylated metabolites. Demonstration of the carcinogenic effects of arsenic in laboratory animals models, therefore, may be a function of identification and investigation of the appropriate active chemical species.

3.5.4 Excretion

While the general biotransformation pathway of arsenic is qualitatively similar among species (with the exceptions noted above), the urinary excretion patterns of arsenic and its methylated metabolites are highly variable among species. The differences in urinary excretion profiles among species reflect significant variations in methylation rates (Vahter 1994). Humans are the only species to excrete MMA in significant amounts, with normal urinary excretion profiles ranging from 10-20% arsenic, 10-20% MMA, and 60-80% DMA (Vahter 1999; Vahter 1994; Vahter 1986). While rats, mice, and dogs are all very efficient at methylating arsenic, the binding of DMA to rat erythrocytes results in a low urinary excretion of DMA in rats (approximately 4%) compared to mice and dogs (70%) (Vahter 1994). The urinary excretion profiles most similar to humans are found in the hamster and rabbit, although both species excrete less MMA and more DMA than humans (Vahter 1999; Vahter 1994).

3.6 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS OF ARSENIC

Several physiologically based pharmacokinetic (PBPK) models reflecting the metabolism of arsenic have been developed (Yu 1999; Menzel 1997; Mann *et al.* 1996a,b). The most recent of these is a flow-limited model based on short-term oral exposures to arsenate via the drinking water in humans (Yu 1999). The model consists of the following compartments: stomach, small intestine, large intestine, liver, kidney/richly perfused tissues, muscle, fat, skin, lung, urine, and bile/feces (Figure 3.2). Submodels for absorption (stomach, small intestine, and large intestine) and excretion (urine and bile/feces) are included; both processes are modeled as first-order.

The model accounts for the major steps in the biotransformation of arsenic and includes the species arsenate, arsenite, MMA, and DMA in the bloodstream and tissue compartments. The reduction of arsenate to arsenite via glutathione is assumed to occur in every perfused tissue compartment according to a second-order process. Production of MMA and DMA in sequential steps of methylation is represented using saturable Michaelis-Menten kinetics in the liver and kidney. Tissue/blood partition coefficients and methylation rate constants were estimated based on data from a report of a fatal poisoning of a child (Saady *et al.* 1989). Allometric scale-up calculations were employed to convert values for a child to those appropriate for an adult. Absorption and excretion rate constants were based on experimental values for blood concentrations and urinary/fecal excretion data in rats and humans (Odanaka *et al.* 1980; Pomroy *et al.* 1980). Tissue specific glutathione values were based on literature values for rodents (Yu 1999).

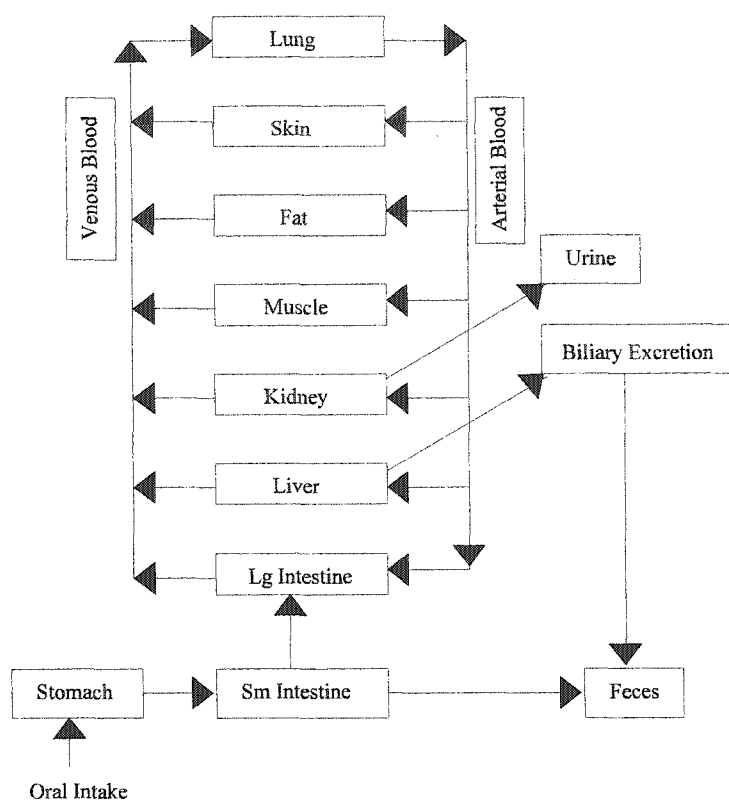


Figure 3.2: Structure of a PBPK model for short-term oral exposure to arsenic (redrawn after Yu 1999).

The model is able to account for scenarios of single and multiple oral exposures at varying concentrations. Model predictions for tissue and urinary concentrations of arsenate, arsenite, MMA, and DMA are consistent with experimental observations following short-term exposures in laboratory animals. Compared to published human data on urinary excretion of arsenite, arsenate, MMA, and DMA over a period of four days following a single oral exposure, the model underpredicts urinary excretion values at time points prior

to 24 hours and overpredicts these values after 24 hours. With respect to repeated dosing, the model is consistent with urinary excretion values in humans. The limitations of this model are associated with the validity of assumptions regarding tissue/blood partition coefficients, urinary excretion constants, tissue glutathione concentrations, and reduction and metabolism constants ($V_{\max}$, K_m). Accurate estimation of these parameters is limited by lack of experimental data.

PBPK models initially developed for rabbits and hamsters (Mann *et al.* 1996a) have subsequently been extended to humans (Mann *et al.* 1996b). The original model in rabbits and hamsters is diffusion-limited and accounts for oral, intravenous, and intratracheal routes of exposure (Figure 3.3). All four species of arsenic (arsenate, arsenite, MMA, and DMA) are accounted for in the model, which is based on five tissue compartments: liver, skin, lung, kidney, and other (*e.g.*, bone and muscle). Gastrointestinal absorption is considered in a transit (nontissue) compartment. A submodel for intratracheal exposure includes nasopharyngeal, tracheobronchial, and pulmonary transit compartments. Absorption is described according to first-order kinetics; absorption constants were derived from experimental data. Intravenous administration is modeled as a single bolus into the plasma. Diffusion of nonionized compounds from the plasma into tissues is described as free diffusion through capillary membranes. Ionized compounds are considered to diffuse only through capillary pores. Tissue affinity constants for the different metabolites of arsenic were determined from experimental data and were assumed to be linear and independent of animal species. The model also accounts for the biotransformation of arsenic. Reduction of arsenate to arsenite is assumed to occur in the plasma according to a first-order process.

The sequential methylation of arsenite to MMA and then to DMA is described according to Michaelis-Menten kinetics in the liver compartment. Species-specific metabolic rate constants were determined by fitting the model to experimental data. Elimination of arsenic is accounted for via the urine, feces (including biliary excretion), and desquamation of the skin.

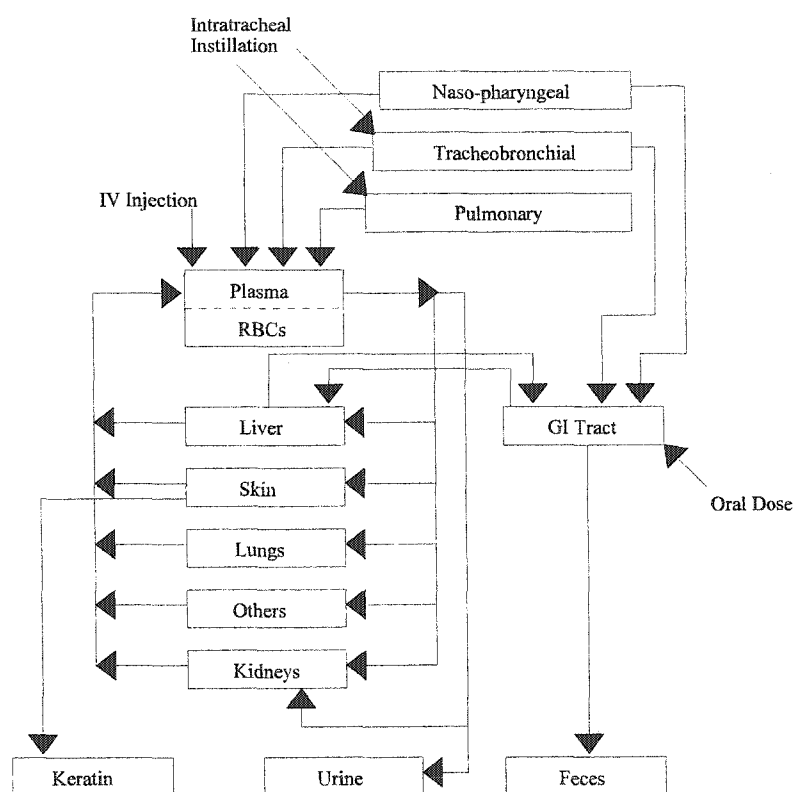


Figure 3.3: Structure of a PBPK model for oral, intravenous, and intratracheal exposure to arsenic (redrawn after Mann *et al.* 1996 a,b).

A PBPK model for human exposure to arsenic (oral and inhalation) also has been developed (Mann *et al.* 1996b), based on the rabbit and hamster models described above (Mann *et al.* 1996a). The model consists of six tissue compartments (blood, liver, kidney, lung, skin, and other), four absorption compartments (gastrointestinal, nasopharyngeal, tracheobronchial, and pulmonary), and three excretion compartments (urine, feces, and skin). Absorption from all compartments is described according to first-order processes. The tissue distribution and metabolism of arsenic are based on the diffusion-limited model developed for rabbits and hamsters (Mann *et al.* 1996a).

Both the rabbit/hamster model and the human model were able to adequately predict urinary concentrations of arsenate, arsenite, MMA, and DMA for each species. In rabbits and hamsters, however, some discrepancies between tissue concentrations and model predictions were noted. In rabbits, the model overpredicted the total amount of arsenic in the skin, possibly due to overprediction of reduction of arsenate to arsenite. In hamsters, two sets of data were used for validation. In the first, the model overpredicted urinary and fecal excretion, although the ratio of metabolites in the urine and feces were accurately described. Model predictions for liver and kidney concentrations of arsenic and its metabolites were generally accurate throughout the first 24 hours, but not after. Use of a different set of experimental data improved the model predictions in hamsters. The model predictions for arsenate were generally more accurate than those for arsenite.

A PBPK model of oral exposure to arsenate in mice has also been developed (Menzel 1997). The model assumes efficient absorption of arsenate from the gastrointestinal tract and primarily hepatic metabolism. Due to limitations of the model, all organs were assumed to

metabolize arsenate to some extent. The model was able to accurately predict tissue binding of arsenite based on experimental data from animals.

Availability of information regarding the tissue kinetics of arsenic and its metabolites in laboratory animals and humans is poor. Of the data that is available, reliability is restricted by inconsistency. The major limitations of the models discussed above are associated with this lack of reliable information. While these models are able to predict the urinary excretion of arsenic and its metabolites under different exposure conditions, they are not able to accurately represent the kinetic behavior of arsenic and its metabolites in tissues throughout the body, in particular those tissues which are targets for the carcinogenic effects of arsenic. Once the experimental data base regarding the kinetic behavior of arsenic is expanded, these models will be able to provide valuable information regarding the target tissue doses of relevant chemical species and enhance our understanding of the role of pharmacokinetics in the carcinogenicity of arsenic.

3.7 FACTORS AFFECTING THE METHYLATION OF ARSENIC

3.7.1 Does a threshold for the methylation of arsenic exist?

Some researchers have observed increased urinary ratios of MMA:DMA in humans chronically exposed to arsenic in the drinking water (Goering *et al.* 1999; Del Razo *et al.* 1997; Cebrián *et al.* 1994; Del Razo *et al.* 1994). Based on these alterations in urinary excretion profiles of MMA and DMA, the existence of a methylation threshold for arsenic biotransformation in humans has been proposed (Goering *et al.* 1999; Vahter 1999; Abernathy *et al.* 1996; Carlson-Lynch *et al.* 1994; Petit and Beck 1990; Buchet *et al.* 1982;

Buchet *et al.* 1981). According to this hypothesis, when arsenic exposure reaches a certain level (threshold), the methylation pathway becomes saturated and its efficiency begins to decline (Vahter 1999; Hopenhayn-Rich *et al.* 1993). As a result, arsenic and/or MMA may accumulate in tissues. If either of these chemical species represents a critical component in the carcinogenic mechanism, a methylation threshold could be crucially involved in the carcinogenic response of humans to arsenic. To date, however, no evidence has been presented implicating MMA as somehow involved in the carcinogenicity of arsenic, and for now, at least, the significance of alterations in urinary MMA and DMA content remains unknown.

3.7.2 Methylation polymorphisms

Human polymorphisms which may alter the body's ability to metabolize and excrete arsenic have been proposed or identified (Vahter 1999; Chiou *et al.* 1997; Vahter *et al.* 1995). Genetic polymorphisms of glutathione S-transferase (GST) M1 and T1, identified in a Taiwanese population exposed to arsenic, were shown to affect the extent of arsenic methylation (Chiou *et al.* 1997). Additionally, urinary excretion profiles from an arsenic-exposed Argentinian population suggest a genetic polymorphism controlling methyltransferase activity responsible for the biotransformation of arsenic (Vahter *et al.* 1995). Depending on the chemical species of arsenic critically involved in the carcinogenic mechanism (arsenate/arsenite versus MMA versus DMA), these polymorphisms may prove highly relevant in understanding the carcinogenicity of arsenic in humans.

3.7.3 Nutritional considerations

Nutritional status may also affect the degree of methylation and potentially the carcinogenicity of arsenic. Methionine- and/or cysteine-deficient diets in humans could lead to decreased biotransformation of arsenic, resulting in accumulation of arsenate/arsenite, as well as a decreased formation and excretion of the more soluble (and presumably less toxic) MMA and DMA (National Research Council 1999; Peraza *et al.* 1998; Petito and Beck 1990). Lack of cyanocobalamin (vitamin B12) and/or its cofactors may also be involved (National Research Council 1999; Buchet and Lauwerys 1985). In the rabbit, deficiencies of methionine, choline, or protein have been shown to decrease the methylation of arsenic (Vahter and Marafante 1987). It has been suggested that methyl deficient diets may have enhanced the carcinogenicity of arsenic in Taiwanese populations (Carlson-Lynch *et al.* 1994; Petito and Beck 1990). However, populations in Argentina, known to have sufficient levels of methyl-containing amino acids in the diet, also showed increased risks of cancer associated with exposure to arsenic-contaminated well water (Hopenhayn-Rich *et al.* 1995). Dietary deficiencies in selenium and zinc have also been proposed as modulators of the toxic and carcinogenic effects of arsenic (National Research Council 1999).

3.8 PROPOSED CARCINOGENIC MECHANISMS FOR ARSENIC

Because of the inability to reproduce the carcinogenic effects of arsenic in laboratory animals, the cellular and molecular mechanisms responsible for its carcinogenicity are currently the focus of intense research activities. In light of the available data, a variety of

hypotheses regarding arsenic's carcinogenic mode of action have been generated; no single hypothesis, however, enjoys unanimous support.

3.8.1 Mutagenicity

Despite the lack of a single, unifying theory accounting for the carcinogenicity of arsenic in humans, it is generally believed that it does not induce point mutations (Wiencke *et al.* 1997; Morton and Dunnette 1994; Li and Rossman 1989a,b; Lee *et al.* 1985). No evidence of mutagenic potential has been demonstrated in either bacterial or mammalian cells (Jacobson-Kram and Montalbano 1985). Evaluation of sodium arsenite in five different strains of *E. coli*, each carrying different mutations for DNA repair capacities, using three different protocols for the quantification of mutant cells, did not reveal any mutagenic activity (Rossman *et al.* 1980). Interestingly, a dose-dependent inhibition of mutation in one of the strains was observed, possibly due to suppression of error prone DNA repair (Jacobson-Kram and Montalbano 1985; Rossman *et al.* 1980). In the yeast *Saccharomyces cerevisiae*, arsenite demonstrated weak mutagenic activity and no potential for inducing gene conversion (Singh 1983). Both sodium arsenate and arsenite (in the presence and absence of rat microsomal S-9 mix) were tested in mouse L5178Y cells for mutagenic activity at the thymidine kinase locus; mutation frequencies in arsenic-exposed cells did not exceed the range of mutation frequencies in control cells (Jacobson-Kram and Montalbano 1985; Oberly *et al.* 1982). In V79 cells exposed to sodium arsenite, no mutations were observed at either the hypoxanthine guanine phosphoribosyl transpose (HGPRT) or membrane-bound sodium-potassium ATPase loci (Rossman *et al.* 1980).

Human-hamster A_L cells have also been used to investigate the mutagenic potential of arsenic (Hei *et al.* 1998). A_L cells are a hybrid cell line consisting of a single human chromosome 11, which encodes a series of human cell surface antigens, and a standard set of hamster chromosomes. The presence or loss of a particular cell surface antigen within the human chromosome 11, due to either an intragenic or multilocus mutation, can be detected using a complement-mediated cytotoxicity assay. Only a small part of human chromosome 11 is necessary for A_L cells to survive; therefore, relatively large mutations in chromosome 11 can still be detected. In A_L cells exposed to low micromolar concentrations of sodium arsenite, large multilocus deletions were detected (Hei *et al.* 1998). Further experimentation has suggested that the mutagenic action of arsenite in this assay may involve the production of reactive oxygen species (see below).

3.8.2 Promotion

The promotional effects of arsenic have been demonstrated in a variety of *in vitro* and *in vivo* models. In cultured human keratinocytes, low micromolar concentrations of arsenite have been shown to induce overexpression of the growth factors granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor- α (TGF- α) (Germolec *et al.* 1997; Germolec *et al.* 1996). Proliferation of keratinocytes, assessed by increase in cell numbers, expression of *c-myc* oncogene, and DNA incorporation of (³H)thymidine was also observed (Germolec *et al.* 1997; Germolec *et al.* 1996). These data support a promotional role for arsenic in a known human target tissue. Using Tg.AC transgenic mice which carry the v-Ha-*ras* oncogene, Germolec *et al.* subsequently demonstrated the co-promotional effect

of arsenic on the development of skin papillomas (Germolec *et al.* 1998; Germolec *et al.* 1997). Mice administered 0.02% arsenite in the drinking water and treated with a low-dose application of tetradecanoyl phorbol acetate (TPA) exhibited a significant increase in skin papillomas (Germolec *et al.* 1998). Additionally, increased mRNA transcripts of GM-CSF and TGF- α were observed in the epidermis of arsenite-treated mice. Similar alterations in growth factor expression were noted in samples of skin lesions obtained from residents of the BFD-endemic region in Taiwan (Germolec *et al.* 1998). *In vivo* experimental evidence also indicates that arsenic may act as a promoter in a variety of organs. In an investigation of the effect of arsenic on hepatocellular proliferation, Sprague-Dawley rats were orally administered sodium arsenite (1.6, 8.2, or 24.6 mg/kg) 21 and 4 hours prior to sacrifice (Brown and Kitchin 1996). An increase in activity of hepatic ornithine decarboxylase, a marker of cell proliferation (Matsui-Yuasa *et al.* 1992; Babaya *et al.* 1983; O'Brien *et al.* 1975), was observed (Brown and Kitchin 1996). In two case reports of humans acutely exposed to high levels of arsenic, an unusually high level of hepatocellular mitotic activity was noted on histological examination of biopsied liver tissue (Brenard *et al.* 1996). Increased hepatocellular proliferation was confirmed by nuclear expression of Ki67 and PC 10. As reported earlier, in our laboratory, a four-chemical mixture consisting of arsenic, DCE, VC, and TCE actually demonstrated an anti-promotional antagonistic effect on the development of hepatic GST-P positive foci in F344 rats (Pott *et al.* 1998).

Promotional effects have also been observed *in vivo* with DMA. F344 rats administered DMA in the drinking water (10, 25, or 100 mg/L) for eight weeks demonstrated a dose-dependent increase in the 5-bromo-2'-deoxyuridine labeling index in urinary bladder

epithelial cells, indicative of a proliferative response to DMA exposure (Wanibuchi *et al.* 1996). Furthermore, in F344 rats treated with DMA in the drinking water (2, 10, 25, 50, or 100 mg/L) for 32 weeks following exposure to 0.05% BBN for four weeks, a dose-dependent increase in the incidence of preneoplastic lesions, papillomas, and carcinomas in the urinary bladder was observed.

In another study, two weeks after injection with DEN (200 mg/kg *i.p.*), male F344 rats were administered drinking water containing DMA (10 or 50 mg/L) for two weeks (Wanibuchi *et al.* 1997). Rats underwent partial hepatectomy at week three and were euthanatized at week four. In the liver, activity of ornithine decarboxylase was increased in DMA-treated rats. In a parallel study, male F344 rats were injected with DEN (200 mg/kg *i.p.*); two weeks later, they were administered DMA via the drinking water (25, 50, or 100 mg/L) for six weeks (Wanibuchi *et al.* 1997). Rats underwent partial hepatectomy at week three and were euthanatized at the end of week eight. A dose-dependent increase in area and number of hepatic GST-P positive foci was observed among treatment groups compared to DEN-treated controls.

In a study of the effect of DMA on pulmonary carcinogenesis, male ddY mice were administered drinking water containing DMA (200 or 400 mg/L) for 25 weeks following injection 4NQO (10 mg/kg *s.c.*). Mice exposed to DMA displayed an increased incidence of pulmonary nodules compared to controls (Yamanaka *et al.* 1996). In a multiple organ bioassay, male F344/DuCrj rats were administered DMA (50, 200, or 400 mg/L) for 24 weeks following exposure to five organ specific initiating agents over a period of 30 days: DEN (100 mg/kg *i.p.*); MNU (20 mg/kg *i.p.*); DMH (40 mg/kg *s.c.*); BBN (0.05% in the

drinking water); and DHPN (0.1% in the drinking water) (Yamamoto *et al.* 1997; Yamamoto *et al.* 1995). Ornithine decarboxylase activity was elevated in kidneys of rats treated with 100 mg/L DMA, suggestive of a proliferative effect. Additionally, an increased incidence of tumors of the liver, kidney, urinary bladder, and thyroid gland was noted among DMA-exposed rats. Increased induction of hepatic GST-P positive foci and atypical renal tubules was also observed.

3.8.3 Progression

Significant *in vitro* and *in vivo* evidence indicates that arsenic is capable of causing genetic effects associated with late stages of carcinogenesis (*i.e.*, progression). The stage of progression is characterized by karyotypic instability (Pitot and Dragan 1996; Ruddon 1995; Harris 1991; Maronpot 1991). Large-scale genetic abnormalities such as aneuploidy, chromosomal aberrations (*e.g.*, translocations and rearrangements), and gene amplification (Ottaggio *et al.* 1993), as well as inhibition of DNA repair (Fornace *et al.* 1980) may be manifestations of such instability (Pitot and Dragan 1996; Ruddon 1995; Cheng and Loeb 1993; Ottaggio *et al.* 1993; Maronpot 1991). Arsenic has been shown to induce a variety of chromosomal aberrations, such as breaks and gaps, as well as sister chromatid exchanges, in human and animal cell culture systems. Syrian hamster embryo (SHE) cells exposed to low micromolar concentrations of sodium arsenite and arsenate exhibited a dose-dependent increase in chromosomal aberrations, with arsenite being more active than arsenate (Barrett *et al.* 1989; Lee *et al.* 1985). Exposure to both the trivalent and pentavalent forms of arsenic was also associated with an increased frequency of sister chromatid exchanges (Barrett *et al.*

1989; Lee *et al.* 1985). In Chinese hamster ovary (CHO) cells, exposure to low micromolar concentrations of sodium arsenite during the G2 phase of the cell cycle enhanced chromatid breaks and exchanges induced by ultraviolet light or 4NQO (Lee *et al.* 1986). Chinese hamster V79 cells exposed to 4mM MNU followed by incubation with 10 μ M sodium arsenite demonstrated an increase in DNA strand breakage compared to MNU-treated controls (Li and Rossman 1989b). The authors attributed these observations to a possible interference with DNA repair by arsenite. In another study using the L5178/TK^{+/+} mouse lymphoma assay, micromolar concentrations of sodium arsenite and sodium arsenate were associated with an increased frequency of chromosomal breaks and rearrangements compared to controls (Moore *et al.* 1997). Low micromolar concentrations of sodium arsenite and arsenate have also been shown to induce amplification of the dihydrofolate reductase gene in mouse 3T6 cells (Barrett *et al.* 1988; Lee *et al.* 1988).

Similar effects of arsenic have been demonstrated in human cells *in vitro*. Human peripheral lymphocytes and diploid fibroblast cell lines WI.38 and MRC₅ exposed to arsenite in the micro- and nanomolar dose ranges for 24-48 hours demonstrated an increased incidence of chromosomal breaks. Exposure to arsenate in the same dose ranges was not associated with increased chromosomal damage (Jacobson-Kram and Montalbano 1985; Paton and Allison 1972). In human lymphocytes and lymphoblastoid cell lines treated with low micromolar concentrations of sodium arsenite, a dose-dependent increase in sister-chromatid exchanges has been reported (Rasmussen and Menzel 1997). Like arsenate and arsenite, DMA has also demonstrated clastogenic effects *in vitro*. In L-132 human pulmonary epithelial cells exposed to DMA (micro- and millimolar dose ranges), an increase

in DNA single strand breaks has been associated with inhibition of repair polymerization (Yamanaka *et al.* 1997).

In vivo exposure to arsenic has also been shown to increase the frequency of chromosomal aberrations in laboratory animals and humans. In pregnant mice exposed to airborne arsenic (28.5 mg/m³) for 4 hours on days 9, 10, 11, and 12 of gestation, an increase in hepatocellular chromosomal damage (chromosomal breaks and chromatid exchanges) was observed in fetuses examined on day 18 of gestation (Nagymajtenyi *et al.* 1985). Lymphocytes harvested from Swedish smelter workers exposed to arsenic demonstrated an increase in chromosomal gaps and aberrations, as well as chromatid aberrations, compared to controls (Beckman *et al.* 1977). Interpretation of this study, however, is limited by inadequate quantification of arsenic exposure and potential exposure to other metals such as lead and selenium. In a follow-up study in which workers were characterized according to level of arsenic exposure, a dose-dependent increase in frequency of chromosomal breaks and aberrations in lymphocytes was demonstrated (Nordenson *et al.* 1978). Further analysis also suggested an interaction between arsenic exposure and smoking. In cultured lymphocytes from psoriasis patients treated with medicinal preparations containing arsenic, the frequency of chromosomal aberrations (including chromosomal gaps and chromatid aberrations) was elevated in comparison to lymphocytes from psoriasis patients not exposed to arsenic (Nordenson *et al.* 1979). An elevated rate of sister chromatid exchange has also been reported in lymphocytes of patients treated with Fowler's solution (Burgdorf *et al.* 1977).

Among residents of the Province of Córdoba in Argentina chronically exposed to arsenic in the drinking water (mean concentration of 0.13 mg/L), the frequency of sister chromatid exchanges in lymphocytes was increased compared to a control population from a neighboring province (mean drinking water arsenic concentration of 0.02 mg/L) (Lerda 1994). Studies of populations exposed to arsenic-contaminated drinking water in Nevada (1,312 µg/L average concentration) (Warner *et al.* 1994) and Chile (600 µg/L average concentration) (Moore *et al.* 1997) have demonstrated an increased level of micronucleated cells (indicative of genetic damage) in exfoliated bladder cells associated with elevated levels of arsenic in the drinking water. In a group of residents of southwestern Finland, exposure to arsenic via contaminated well water (median concentration 410 µg/L) was associated with an increase in the frequency of chromosomal aberrations in peripheral lymphocytes (including gaps, breaks, and rearrangements) (Mäki-Paakkanen *et al.* 1998). Notably, the correlation between arsenic exposure and frequency of chromosomal aberrations was stronger among residents currently exposed to arsenic-contaminated well water than among residents who had stopped using contaminated water two to four months prior to sampling.

Data from epidemiological studies of cancer risk among individuals occupationally exposed to high levels of arsenic have been analyzed in mathematical models based on the multistage theory of carcinogenesis. According to this theory, patterns of excess cancer risk may be attributed to age at initial exposure, duration of exposure, and follow-up time since termination of exposure (Brown and Chu 1983a,b). Results of multistage analysis of lung cancer mortality patterns among copper smelter workers suggest that the carcinogenic effect of arsenic occurs during the later stages of carcinogenesis (Brown and Chu 1983a,b). These

results, in combination with experimental evidence of chromosomal aberrations and gene amplification, have fostered the development of the hypothesis that arsenic acts during the stage of progression (Barrett 1992). It is therefore not surprising that arsenic does not test positive in medium-term or chronic rodent carcinogenicity bioassays which were designed to evaluate the initiating and/or promoting activity of a test compound, but not events typically associated with later stages of the carcinogenic process. Failure to demonstrate the carcinogenic effects of arsenic in laboratory animals, then, may be due to the lack of an appropriate animal model for late stage carcinogenesis.

3.8.4 Cocarcinogenicity

Observations that arsenic is able to modify DNA repair processes *in vitro* suggest that it may act as a cocarcinogen. In normal human and xeroderma pigmentosum (XP) fibroblasts, pyrimidine dimers were induced by ultraviolet (UV) irradiation; fibroblasts were then incubated with micromolar concentrations of arsenite and arsenate for 24 hours (Okui and Fujiwara 1986). Treatment with both forms of arsenic was associated with inhibition of pyrimidine dimer excision; arsenite exerted a greater effect on inhibition than arsenate (Okui and Fujiwara 1986). Repair of DNA damage induced with UV irradiation in CHO cells has also been shown to be inhibited by micromolar concentrations of arsenite by inhibition of DNA ligation and post-replication repair (Lee-Chen *et al.* 1993, 1992). Inhibition of repair of methylmethane sulfonate (MMS)-induced DNA damage in CHO cells has also been demonstrated (Lee-Chen *et al.* 1993). Low doses of MMS and arsenite (0.2 mM and 40 μ M, respectively) were associated with increased frequency of alkali-labile sites

in CHO cells. Alkali-labile sites are transient apurinic/apyrimidinic sites due to enzymatic or chemical hydrolysis of alkylated bases which are converted to strand breaks in a pH-dependent reaction. Higher doses of MMS and arsenite (0.8 mM and 80 μ M, respectively), were required to enhance frequency of chromosomal breaks in these cells (Lee-Chen *et al.* 1993).

In a series of studies designed to investigate the effects of arsenic on DNA repair, Chinese hamster V79 cells were exposed to 4 mM MNU for 15 minutes to induce DNA strand breaks, followed by treatment with 10 μ M sodium arsenite for three hours (Li and Rossman 1989b). Cells treated with MNU and arsenite exhibited a higher frequency of DNA strand breaks compared to cells treated with MNU alone. Exposure to arsenite alone was not associated with an increase in DNA single strand breakage. The effect of arsenite on MNU-induced DNA strand breaks was attributed to inhibition of DNA excision repair or ligation (Li and Rossman 1989b). In further investigations using the same treatment protocol, arsenite was shown to inhibit the ligation step of MNU-induced DNA repair (Li and Rossman 1989a). More specifically, inhibition of DNA ligase II, and to a lesser extent DNA ligase I, was demonstrated. Evidence also indicates that arsenite does not directly inhibit DNA repair, but rather acts indirectly (Hu *et al.* 1998; Li and Rossman 1989a;), perhaps by interfering with signal transduction pathways regulating DNA repair (Hu *et al.* 1998). Indirect inhibition of DNA repair could also be accounted for by a decrease in induction of accessory or other key proteins involved in cellular control of DNA repair pathways (Clewell *et al.* 1998).

Investigation of cDNA expression libraries isolated from arsenite-resistant sublines of Chinese hamster V79 cells led to the identification of a gene sequence capable of conferring arsenite resistance to arsenite-hypersensitive V79 sublines (Rossman and Wang 1999). The sequence was nearly completely homologous with the rat *fau* tumor suppressor gene, which contains a ubiquitin-like region. Because arsenite has previously been shown to inhibit ubiquitin-mediated proteolysis (Klemperer and Pickart 1989), it is possible that the *fau* tumor suppressor gene is somehow disrupted by arsenic (Rossman and Wang 1999). Involvement of the ubiquitin system in the process of growth regulation and, potentially, in carcinogenesis has been established. The protein products of the protooncogenes *c-jun* and *v-mos* are known to be degraded by ubiquitin-dependent proteolytic processes (Rossman and Wang 1999; Hochstrasser 1996). Mutant forms of these proteins cannot be ubiquitinated, resulting in resistance to degradation and prolonged cellular half-life (Rossman and Wang 1999; Hochstrasser 1996). The ubiquitin system also appears to play a role in DNA repair via activation of enzymes critically involved in DNA repair (Smith *et al.* 1996; Bailly *et al.* 1994; Jentsch *et al.* 1987).

Based on the experimental evidence for the cocarcinogenicity of arsenic, an hypothesis has been developed for a mechanism of arsenic comutagenicity in a cell which has theoretically lost cell cycle control at the G1-S checkpoint (Rossman and Wang 1999; Clewell *et al.* 1998). In the absence of G1-S checkpoint control, an arsenic-induced delay in the completion of DNA repair would enhance the chances of cell division despite unrepaired strand breaks. This, in turn, would enhance chromosomal aberrations and genomic instability, leading to the development of more aggressive, malignant phenotypes

(Clewell *et al.* 1998). In the presence of functional cell cycle check points, the co-mutagenic effect of arsenic is absent; therefore, when tested in inappropriate carcinogenicity bioassays (*e.g.*, models in which cell cycle control remains functional), arsenic would demonstrate no carcinogenic potential.

3.8.5 Alterations in DNA methylation

Another hypothesis regarding the mechanism of carcinogenicity of arsenic proposes that, in its consumption of methyl groups from SAM during biotransformation, arsenic alters genomic methylation patterns and therefore gene expression (Goering *et al.* 1999; Zhao *et al.* 1997; Mass and Wang 1997). In human lung adenocarcinoma A549 cells, arsenite (0.08-2 μ M) and arsenate (30-300 μ M) induced hypermethylation of the *p53* promoter region in a dose-responsive manner (Mass and Wang 1997). Hypermethylation of DNA can be associated with gene silencing (Bird 1996; Counts and Goodman 1995) which, in the case of a tumor suppressor gene, could enhance the carcinogenic process. Conversely, in a rat liver epithelial cell line (TRL 1215) malignantly transformed by chronic exposure to sodium arsenite (0.5 μ M), global hypomethylation of DNA occurred in conjunction with decreased levels of SAM (Zhao *et al.* 1997). Hypomethylation of DNA can be associated with enhanced gene expression which, in the case of an oncogene, may be critically involved in the carcinogenic process (Counts and Goodman 1995). In transformed TRL 1215 cells, expression of the metallothionein gene, which is known to be regulated by DNA methylation (Waalkes *et al.* 1988; Compere and Palmiter 1981), was increased (Zhao *et al.* 1997),

suggesting that gene expression in the transformed TRL 1215 cells could be regulated by the methylation status of DNA.

3.8.6 Production of reactive oxygen species

Several *in vivo* and *in vitro* studies have suggested that arsenic produces reactive oxygen species (ROS) such as the dimethylarsinic peroxy radical, via dimethylarsine, a metabolite of DMA (Yamanaka 1997; Brown *et al.* 1997; Yamanaka and Okada 1994; Yamanaka *et al.* 1989). *In vitro* exposure of mouse lung DNA to dimethylarsine, but not to DMA, resulted in the production of DNA single-strand breaks (Yamanaka *et al.* 1989). Dimethylarsine-induced single strand breaks were attenuated by addition of superoxide dismutase and catalase, indicating that ROS produced by dimethylarsine were somehow involved in the production of DNA single strand breaks.

Human-hamster A_L cells have also been used to investigate the ROS-generating potential of arsenic (Hei *et al.* 1998). Large multilocus deletions were detected in A_L cells exposed to low micromolar concentrations of sodium arsenite. A_L cells coexposed to arsenite and dimethyl sulfoxide, an oxygen radical scavenger, exhibited reduced mutagenic activity, suggesting the involvement of ROS in the mutagenic action of arsenite in this assay. In cultured human lymphocytes, diminution of arsenite-induced sister chromatid exchanges by superoxide dismutase and catalase has also been demonstrated (Nordenson and Beckman 1991).

The role of ROS in the carcinogenicity of arsenic has also been investigated *in vivo*. In ICR mice orally administered a single dose of DMA (1500 mg/kg), increased pulmonary

DNA single strand breaks, associated with production of ROS, were reported (Yamanaka and Okada 1994; Yamanaka *et al.* 1989). Single strand breaks were also observed in the liver, kidney, and spleen, although to a much lesser extent than in the lung (Yamanaka *et al.* 1989). In F344 rats administered DMA (100 mg/L in the drinking water) as a promoter following initiation with DEN (200 mg/kg *i.p.*), increased levels of 8-hydroxydeoxyguanosine, an indicator of oxygen-radical mediated DNA damage, were observed (Wanibuchi *et al.* 1997). If the primary carcinogenic mechanism of action of arsenic involves ROS production, one might expect that high levels of ROS would be produced in rodents exposed to arsenic, since rodents are very efficient at methylating arsenic to DMA. One would therefore expect to observe increased tumor formation in rodents compared to humans, an effect which has not been demonstrated *in vivo*. Thus generation of ROS is not likely the primary mechanism of arsenic carcinogenicity, although ROS may somehow be secondarily involved (Clewell *et al.* 1998).

3.8.7 Inducible tolerance to arsenic

Observations that human cells are more sensitive to arsenite toxicity than Chinese hamster cells have raised questions about differences in arsenic tolerance among species. In cultured human fibroblasts and Chinese hamster ovary cells, the concentrations of sodium arsenite associated with 50% lethality were ten-fold higher for Chinese hamster ovary cells compared to human fibroblasts (Lee and Ho 1994; Lee *et al.* 1989). Treatment with sodium arsenite induced cell cycle disturbances in Chinese hamster ovary cells, but not human fibroblasts, suggesting that cell cycle arrest may allow time for recovery in Chinese hamster

ovary cells but not in human fibroblasts (Lee *et al.* 1989). Furthermore, treatment with vitamin E decreased arsenite-induced lethality in human fibroblasts, but not Chinese hamster ovary cells, suggesting that human fibroblast sensitivity to arsenite may be due, at least in part, to oxidative damage (Lee and Ho 1994). While some variants of the Chinese hamster V79 cells exhibited an inducible tolerance mechanism to arsenite (arsenite-resistant V79 variants), human cells from a variety of origins (several normal human fibroblast lines, fibroblasts from an individual with Gardner's syndrome, HeLa cells, meduloblastoma cells, and keratinocytes) showed no evidence of inducible tolerance to arsenite (Rossman *et al.* 1997). Notably, compared to V79 cells, all human cell lines were more sensitive with respect to arsenite-induced toxicity (Rossman *et al.* 1997). It has been postulated that these differences may be attributed to alterations in intracellular levels of glutathione (GSH), induction of heat shock proteins, or intracellular accumulation of arsenic (Rossman *et al.* 1997). In the arsenite-resistant Chinese hamster V79 variants, the presence of an energy-dependent arsenic efflux pump has been demonstrated (Wang *et al.* 1996). Further research is necessary to evaluate the presence or absence of these mechanisms in human cells. These studies, however, provide evidence that fundamental differences in the presence of inducible arsenic tolerance mechanisms among species may underlie species differences in the carcinogenic effects of arsenic.

3.9 CONCLUSION

The carcinogenicity of arsenic in humans has been unambiguously demonstrated in a variety of epidemiologic studies encompassing geographically diverse study populations

and multiple exposure scenarios. Despite the abundance of human data, our knowledge of the mechanism(s) responsible for the carcinogenic effects of arsenic remains incomplete. A deeper understanding of these mechanisms is highly dependent on the development of appropriate experimental models, both *in vitro* and *in vivo*, for future mechanistic investigations.

Suitable *in vitro* models would facilitate further investigation of the critical chemical species (arsenate/arsenite/MMA/DMA) involved in the carcinogenic process, as well as the evaluation of the generation and role of ROS. Mechanisms underlying the clastogenic effects of arsenic, its role in modulating DNA methylation, and the phenomenon of inducible tolerance could all be more completely investigated using *in vitro* models. The mechanisms involved in arsenic's inhibition of ubiquitin-mediated proteolysis demand further attention, particularly with respect to its effects on cell proliferation and DNA repair. Exploration of the mechanisms responsible for the protective or anticarcinogenic effects of arsenic could also enhance our understanding of the cellular and molecular interactions which influence its carcinogenicity. In addition, appropriate *in vivo* models must be developed which consider the action of arsenic as a promoter and/or progressor. *In vivo* models that allow further investigation of the co-mutagenic effects of arsenic are also especially necessary. Such models may employ initiation-promotion-progression bioassays or transgenic animals.

Both *in vitro* and *in vivo* models have the potential to greatly enhance our current understanding of the cellular and molecular interactions of arsenic and its metabolites in target tissues. However, refinement of our knowledge of the mechanistic aspects of arsenic carcinogenicity is not alone sufficient; an understanding of the pharmacokinetics and target

tissue doses of the critical chemical species is essential. Additionally, a more thorough characterization of species differences in the tissue kinetics of arsenic and its methylated metabolites would facilitate the development of more accurate and relevant PBPK models. Improved models could be used to further investigate the existence of a methylation threshold for arsenic and its relevance to arsenic carcinogenicity in humans. The significance of alterations in relative tissue concentrations of SAM and SAH deserves further attention, particularly with respect to their role in modulating methyltransferases involved in arsenic metabolism and DNA methylation. The importance of genetic polymorphisms and nutrition in influencing methyltransferase activities must not be overlooked. *In vivo* models are necessary to evaluate these factors; transgenic or knockout models would be particularly useful in the investigation of methylation polymorphisms. Further evaluation of methylation polymorphisms in human populations is also warranted. Other *in vivo* models incorporating dietary manipulation could provide valuable insight into the role of nutrition in the carcinogenicity of arsenic.

With more complete knowledge of the pharmacokinetics of arsenic metabolism and the mechanisms associated with its carcinogenic effects, development of more reliable risk assessment strategies will be possible. Integration of data, both pharmacokinetic and mechanistic in nature, will lead to more accurate descriptions of the interactions that occur between the active chemical species and cellular constituents which lead to the development of cancer. This, in turn, will facilitate the development of more accurate and reliable risk assessment strategies for arsenic.

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CHAPTER 4

STUDIES ON ANGIOSARCOMA DEVELOPMENT POTENTIAL OF A MIXTURE OF ARSENIC, 1,2-DICHLOROETHANE, VINYL CHLORIDE, AND TRICHLOROETHYLENE IN FISCHER 344 RATS USING A DONOR-RECIPIENT PROTOCOL

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ABSTRACT

Arsenic (As), 1,2-dichloroethane (DCE), vinyl chloride (VC), and trichloroethylene (TCE) are commonly identified groundwater contaminants near hazardous waste disposal sites, and As, DCE, and VC are known to induce hepatic angiosarcoma in humans and/or animals. To investigate the hypothesis that a quaternary mixture consisting of As, DCE, VC, and TCE would synergistically induce hepatic angiosarcoma compared to each individual constituent, we utilized a donor-recipient animal protocol that had previously demonstrated angiosarcoma development in weanling rats following transplantation of hepatic sinusoidal endothelial cells (SECs), harvested from livers of diethylnitrosamine (DEN)-treated donor rats

(Luquette *et al.* 1985). In our studies, we used DEN as a positive control to repeat the Luquette *et al.* (1985) studies. In parallel, we evaluated the angiosarcoma development potential of As, DCE, VC, and TCE, singly and in combination as a quaternary mixture. Two negative control groups (deionized water and corn oil gavage) were also included. Donor rats were treated with one of three dose levels of the quaternary mixture, or one of three dose levels of each individual chemical, or DEN. Following a 16-week intermittent treatment period, which consisted of 6 weeks of treatment, 2 weeks of no treatment, 3 weeks of treatment, and 5 weeks of no treatment, a SEC suspension was prepared from donor rat livers of each group, then injected into the respective transplant recipients. In DEN-treated donor rats, hepatocellular adenomas and adenocarcinomas were observed; however, we were not able to reproduce the results of Luquette *et al.* (1985) in which angiosarcomas were formed in the recipient rats following subcutaneous injection of SEC suspensions prepared from the livers of DEN-treated donor rats. Treatment with As or DCE was associated with focal hepatic sinusoidal dilatation (an early change reported in the development of hepatic angiosarcoma). Sinusoidal dilatation was distributed asymmetrically around central veins. Morphometric analysis revealed statistically significant dilatation in the DCE treatment group only. Angiosarcomas did not develop in any transplant recipient. Using sinusoidal dilatation as an index, our original hypothesis of synergistic toxicological interaction by the quaternary mixture was not substantiated.

INTRODUCTION

Arsenic (As) is a well-known human carcinogen, yet its mode of action remains unclear. Most evidence indicates that it does not act as an initiator in the process of multistage carcinogenesis, but rather as a promoter, progressor, co-mutagen, or co-carcinogen (Kitchin 2001; National Research Council 1999; Germolec *et al.* 1998). Thus, evaluation of its carcinogenic action in chemical mixtures is relevant. Furthermore, because humans encounter hazardous chemicals in the environment as mixtures rather than single agents, investigation of the carcinogenic activity of As in environmentally relevant chemical mixtures is highly warranted. Thus, the carcinogenic effects of a chemical mixture containing As were investigated in our laboratory. Components of the chemical mixture were chosen based on their environmental relevance and similarity of carcinogenic endpoint.

Some of the most commonly identified groundwater contaminants near hazardous waste disposal sites include, in addition to As, DCE, VC, and TCE (Faroon *et al.* 1994; Agency for Toxic Substances and Disease Registry 1993). Exposure to As and VC has been associated with the development of hepatic angiosarcoma in humans (Bates *et al.* 1992; IARC 1987; Kasper *et al.* 1984; Falk *et al.* 1981; Popper *et al.* 1978). In addition, both DCE (National Cancer Institute, 1978) and VC (Maltoni *et al.* 1984; Feron *et al.* 1981; Popper *et al.*, 1981; Popper *et al.* 1978; Waxweiler *et al.* 1976) have been associated with the development of angiosarcoma in laboratory animals. Thus, we hypothesized that As would demonstrate a synergistic or at least additive effect on the development of hepatic angiosarcoma in rats exposed to a chemical mixture consisting of As, DCE, VC, and TCE. To evaluate hepatic angiosarcoma induction by As alone and in combination with DCE, VC,

and TCE, we utilized an animal donor-recipient protocol which had previously demonstrated that hepatic SECs (the cell of origin of hepatic angiosarcoma), harvested from livers of DEN-treated Fischer 344 (F344) donor rats and transplanted into weanling F344 rats, resulted in angiosarcoma development (Luquette *et al.* 1985).

MATERIALS AND METHODS

All procedures describe in the subsequent sections were identical to those reported by Luquette *et al.* (1985).

Chemicals

DEN, sodium arsenite, DCE, Joklik's modification of Minimum Essential Medium (JTCM), and Pronase were purchased from Sigma Chemical Company (St. Louis, MO). VC gas (certified 2010 ppm in nitrogen carrier gas) was purchased from Scott Specialty Gases (Pasadena, TX). TCE was purchased from Aldrich Chemical Company (Milwaukee, WI). All other reagents were of analytical grade.

Dosing Solutions

Drinking water solutions were prepared twice weekly by dissolving DEN, sodium arsenite, or DCE in deionized water. Due to their low solubility in water, VC and TCE were administered via oral corn oil gavage. VC gavage solutions were prepared by bubbling VC gas through corn oil, and the amount of VC in the corn oil was analyzed by gas chromatography (Feron *et al.* 1975). TCE solutions were prepared by dissolving neat TCE

directly in corn oil. Time-course volatilization studies dictated weekly VC and TCE solution preparation. To prepare VC/TCE solutions, VC corn oil preparations were spiked with TCE. All dosing solutions were stored at -80°C in daily aliquots prior to administration.

Donor Animals and Treatment

Four-week-old male Fischer 344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed three to a cage and located in a fully accredited American Association for Accreditation of Laboratory Animal Care (AAALAC) facility. Lighting was set on a 12-hour light/dark cycle. All animals received food (Harlan Teklad NIH-07 diet; Madison, WI) and water *ad libitum*. Following a two-week acclimation period, rats were randomized by weight into treatment groups (6 - 12 rats/treatment group). The following eighteen treatment groups were included (as shown in Table 4.1): four single chemical groups (As, DCE, VC, and TCE) at three different dose levels, one quaternary mixture group (As + DCE + VC + TCE) at three different dose levels, DEN (positive control), deionized water (negative control), and oral corn oil gavage (negative control). Rats (6 rats/dose) were exposed to each of the following single chemical drinking water solutions: 75, 25, or 7.5 ppm As; 300, 30, or 3 ppm DCE; or deionized water (negative control; 12 rats). Additional treatment groups (6 rats/dose) were gavaged once daily (5 days/week) with the following single-chemical corn oil solutions: 15, 5, or 1.5 ppm VC; 1900, 190, or 19 ppm TCE; or pure corn oil (negative control; 12 rats). Quaternary mixture exposures consisted of the following three treatment groups (6 rats/dose): high (75 ppm As, 300 ppm DCE, 15 ppm VC, 1900 ppm TCE); medium (25 ppm As, 30 ppm DCE, 5 ppm VC, 190 ppm TCE)

or low (7.5 ppm As, 3 ppm DCE, 1.5 ppm VC, 19 ppm TCE). An additional treatment group (12 rats) received 90 ppm DEN in the drinking water as a positive control. As illustrated in Figure 4.1, rats were intermittently exposed to the test chemical(s) according to the following schedule: 6 weeks with treatment, 2 weeks without treatment, 3 weeks with treatment, 5 weeks without treatment. At the end of the 16-week intermittent treatment period, rats were sacrificed and their livers harvested using sterile technique. A section from each liver was reserved for histopathological examination and the remainder used for preparation of SEC suspensions.

Sinusoidal Endothelial Cell (SEC) Suspensions

The following experimental procedures were reported by Luquette *et al.* (1985). After harvest, livers were sectioned, cooled in JTCM at 4°C for 5 minutes, then minced in JTCM. After weighing, the minced tissue was digested with 3.5 to 4.5 mL of 0.1% Pronase solution per gram of liver tissue at room temperature for 20 minutes. The supernatant was decanted into tubes containing 10 to 20 mL of 10% horse serum in JTCM. The suspension was cooled at 4°C for 10 minutes, then centrifuged at 97g for 10 minutes at 4°C. The pellet was resuspended in 2 to 3 mL of JTCM with 10% horse serum and stored in an ice bath until 10 successive digestions were completed. The first 2 digestions were discarded, and digestions 3 - 10 were pooled then filtered through 250 µm mesh. Cell viability was assessed periodically throughout the digestion process by the Trypan Blue exclusion assay. The resulting cell suspensions, containing an enriched population of SECs, were quantified then injected subcutaneously into 12- to 15-day-old (weanling) male F344 rats (Harlan Sprague

Dawley; Indianapolis, IN). Twenty-five million cells per each of two subcutaneous injections sites were deposited in the dorsal interscapular region. Five to eight weanling transplant recipient rats were injected with SEC suspensions per each dosing group. For a subset of SEC suspensions (including those harvested from DEN-treated positive controls), irradiated nude mice (4-6 mice per group) were also used as transplant recipients. Tumor development at the site of injection was monitored for six months in transplant recipients.

Histopathology and Morphometric Analysis

Tissue sections from donor rat livers were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (H & E) for histopathologic evaluation. Under light microscopy, a point-count-intersect method was used to quantify sinusoidal area. Each intercept was categorized as sinusoid or non-sinusoid, and the area density (relative ratio/unit area) of sinusoids was then calculated (Weibel *et al.*, 1969). Twenty random fields/section were analyzed.

Statistical Analysis

Results were analyzed using one-way analysis of variance. All statistical tests were performed using the Minitab statistical software package, release 11.21 (Minitab, Inc., State College, PA).

RESULTS

While our SEC viability for all groups was consistently above 95%, we were not able to reproduce the findings of Luquette *et al.* (1985) in that angiosarcomas did not develop in perinatal transplant recipient rats or irradiated nude mice injected with SECs harvested from any of the treatment groups, including the putative positive control group treated with DEN. On histologic examination of donor rat livers, sinusoidal dilatation, an early change reported in association with angiosarcoma development (Popper *et al.* 1981, 1978) was evident in treatment groups exposed to 75 ppm As or 300 ppm DCE (Figure 4.2). As illustrated in Table 4.2, morphometric analysis revealed a significant increase in hepatic sinusoidal area in rats exposed to 300 ppm DCE (sinusoidal area density = 0.1502 ± 0.003 per cm^2) compared to corn oil-treated controls (sinusoidal area density = 0.1113 ± 0.017 per cm^2), but in livers of rats exposed to 75 ppm As, sinusoidal dilatation was not statistically significantly different (sinusoidal area density = 0.1358 ± 0.024 per cm^2) compared to deionized water controls (sinusoidal area density = 0.1098 ± 0.027 per cm^2). Hepatic sinusoidal dilatation was not present in the medium or low dose As or DCE treatment groups or in any of the quaternary mixture treatment groups (data not shown). In the livers of rats treated with DEN (putative positive angiosarcoma controls), multiple adenomas and adenocarcinomas were observed which frequently exhibited varying degrees of sinusoidal dilatation. Hepatic adenomas were identified in 6/12 rats and hepatic adenocarcinomas in 4/12 rats; 2/12 rats exhibited both adenomas and adenocarcinomas of the liver. Consequently, hepatic sinusoidal dilatation was not evaluated morphometrically in the DEN positive control group.

DISCUSSION

Our principal hypothesis of a synergistic toxicological interaction regarding angiosarcoma development by the quaternary mixture was not substantiated in this study. We were unable to reproduce the Luquette *et al.* (1985) results of inducing angiosarcoma with a rat donor-recipient system using DEN or a chemical agent. The development of hepatic angiosarcoma in humans has been linked with exposure to VC, Thorotrast, and As. Hepatic angiosarcomas associated with VC exposure have been reported in occupationally exposed workers (IARC 1987; Popper *et al.* 1981, 1978). Development of radiation-induced hepatic angiosarcoma has been linked with exposure to Thorotrast (thorium dioxide), an imaging agent historically administered for diagnostic radiological procedures (Popper *et al.* 1978). Work-related, environmental, and medicinal exposures to As have also been associated with hepatic angiosarcoma (Bates *et al.* 1992; Kasper *et al.* 1984; Falk *et al.* 1981; Popper *et al.* 1978; Waxweiler *et al.* 1976). In a review of over 100 hepatic angiosarcoma case studies, Popper *et al.* (1978) reported a similar disease pattern and evolution in nearly all instances, regardless of etiology. A “precursor stage” was characterized by hepatocellular proliferation associated with focal sinusoidal dilatation and proliferation of SECs. Following the “precursor stage,” five distinct processes were identified, each contributing in varying degrees to the transition to hepatic angiosarcoma: 1) proliferation of intralobular endothelial cells with increasing anaplasia; 2) atrophy and disappearance of hepatocytes following earlier hyperplasia; 3) increasing perisinusoidal fibrosis; 4) sinusoidal dilatation progressing to peliosis; 5) transformation of SECs and portal capillary endothelial cells to sarcomas (Popper *et al.* 1978). A similar pattern was observed in VC-induced hepatic lesions in rats (Popper

et al. 1981). In our studies, morphometric analysis revealed a statistically significant increase in focal sinusoidal dilatation in livers of rats exposed to 300 ppm DCE (compared to corn oil vehicle controls). Sinusoidal dilatation was observed on histological examination of livers of rats exposed to 75 ppm As, and, while morphometric analysis revealed increased sinusoidal dilatation, it was not statistically significant compared to deionized water controls. It is possible that the highly focal distribution of sinusoidal dilatation in the livers of rats exposed to 75 ppm As contributed to the observed non-significant increase in sinusoidal volume. Using sinusoidal dilatation as an index, we did not observe any toxicological interaction in the quaternary mixture group. No proliferation of SECs or hepatocytes was observed on histologic examination of livers of rats from any treatment group.

Notably, angiosarcomas did not develop in perinatal transplant recipient rats injected with SECs harvested from any of the treatment groups, including the putative positive control group treated with DEN. Lack of tumor development in transplant recipients was not likely due to technical problems in the preparation of SEC suspensions, as SEC viability at numerous stages throughout liver digestion procedure and preparation of SEC suspensions consistently remained above 95%. Nor could host-mediated transplant rejection account for lack of tumor formation, since angiosarcomas did not develop in irradiated nude mice. Based on these observations, we concluded that lack of tumor development in perinatal transplant recipients and nude mice was likely due to delivery of inadequate numbers of transformed SECs (insufficient numbers of transformed SECs produced in livers of donor rats).

In the model on which we based our study (Luquette *et al.* 1985), the investigators originally utilized F344 rats from a colony maintained in their own laboratory. When F344 rats from a commercial breeding laboratory were used instead of those from a private colony, the induction of angiosarcomas in transplant recipients was not repeatable (personal communication, TP Pretlow, 1997). Unfortunately, this information was not available to us at the inception of our studies. The inability to reproduce the results of the original study in F344 rats from different colonies suggests a predisposition for angiosarcoma development in the private breeding colony, perhaps due to genetic drift.

While our experiments did not prove the hypothesis that the quaternary mixture would act in an additive or synergistic fashion in angiosarcoma induction, there was some evidence that sinusoidal dilatation, an early event in angiosarcoma formation, was present in DCE-treated and to a lesser degree in As-treated rats. That such an effect was not observed in the mixture-treated rats suggests that there was neither an additive nor synergistic response for that finding. It is of interest that previous studies of hepatocarcinogenesis in our laboratory actually showed inhibition of preneoplastic hepatocellular foci development in rats treated with As alone or with a quaternary mixture consisting of As, DCE, VC, and TCE (Pott *et al.* 1998). The lack of an additive or synergistic response in this study is consistent with our previous findings with the same chemical mixture.

ACKNOWLEDGMENTS

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Table 4.1. Experimental Treatment Groups

Treatment Group	Treatment (ppm)						
	DI*	CO**	DEN***	As†	DCE‡	TCE§	VC*
Deionized Water (negative control)	+	-	-	-	-	-	-
Corn Oil (negative control)	-	+	-	-	-	-	-
DEN (positive control)	-	-	+	-	-	-	-
As High	-	-	-	75	-	-	-
As Medium	-	-	-	25	-	-	-
As Low	-	-	-	7.5	-	-	-
DCE High	-	-	-	-	300	-	-
DCE Medium	-	-	-	-	30	-	-
DCE Low	-	-	-	-	3	-	-
TCE High	-	-	-	-	-	1900	-
TCE Medium	-	-	-	-	-	190	-
TCE Low	-	-	-	-	-	19	-
VC High	-	-	-	-	-	-	15
VC Medium	-	-	-	-	-	-	5
VC Low	-	-	-	-	-	-	1.5
Quaternary Mixture High	-	-	-	75	300	1900	15
Quaternary Mixture Medium	-	-	-	25	30	190	5
Quaternary Mixture Low	-	-	-	7.5	3	19	1.5

* DI, deionized drinking water (12 rats).

** CO, corn oil gavage, once daily, 5 days/week (12 rats).

*** DEN, diethylnitrosamine, 90 ppm, administered in the drinking water (12 rats).

† As, sodium arsenite, administered in the drinking water (6 rats/dose).

‡ DCE, 1,2-dichloroethane, administered in the drinking water (6 rats/dose).

§ TCE, trichloroethylene, administered via once daily corn oil gavage, 5 days/week (6 rats/dose).

* VC, vinyl chloride, administered via once daily corn oil gavage, 5 days/week (6 rats/dose).

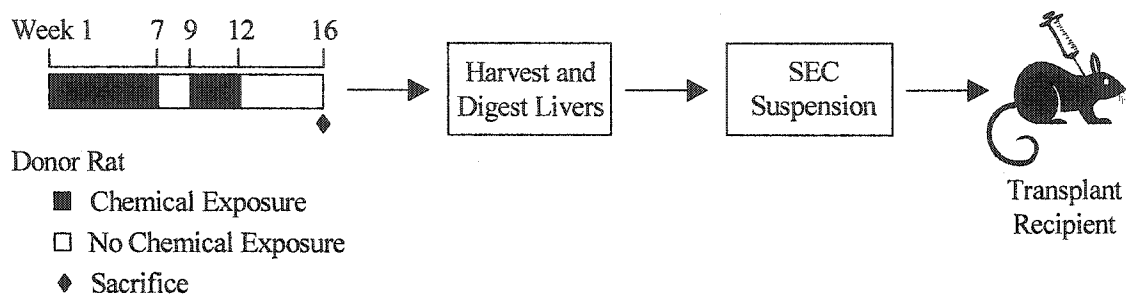


Figure 4.1: Experimental design entailed an intermittent 16-week exposure period followed by the harvest and digestion of donor rat livers (Luquette *et al.*, 1985). The resulting sinusoidal endothelial cell suspensions were injected subcutaneously into transplant recipients.

Table 4.2. Hepatic Sinusoidal Dilatation in Donor Rats
Receiving Highest Chemical Doses

Treatment Group ^a	Sinusoidal Area Density(/cm ²) ^b
Deionized Water (negative control)	0.1098 ± 0.027
Corn Oil (negative control)	0.1113 ± 0.017
As High	0.1358 ± 0.024
DCE High	0.1502 ± 0.003 ^c
TCE High	0.1018 ± 0.006
VC High	0.0987 ± 0.027
Quaternary Mixture High	0.1037 ± 0.013
DEN (positive control)	— ^d

^a As, arsenite; DCE, 1,2-dichloroethane; TCE, trichloroethylene; VC, vinyl chloride; DEN, diethylnitrosamine.

^b Values represent mean ± SD.

^c Significantly different ($P < 0.05$) from negative controls according to one-way ANOVA.

^d Morphometric analysis not performed due to the presence of multiple hepatic adenomas/adenocarcinomas exhibiting sinusoidal dilatation.

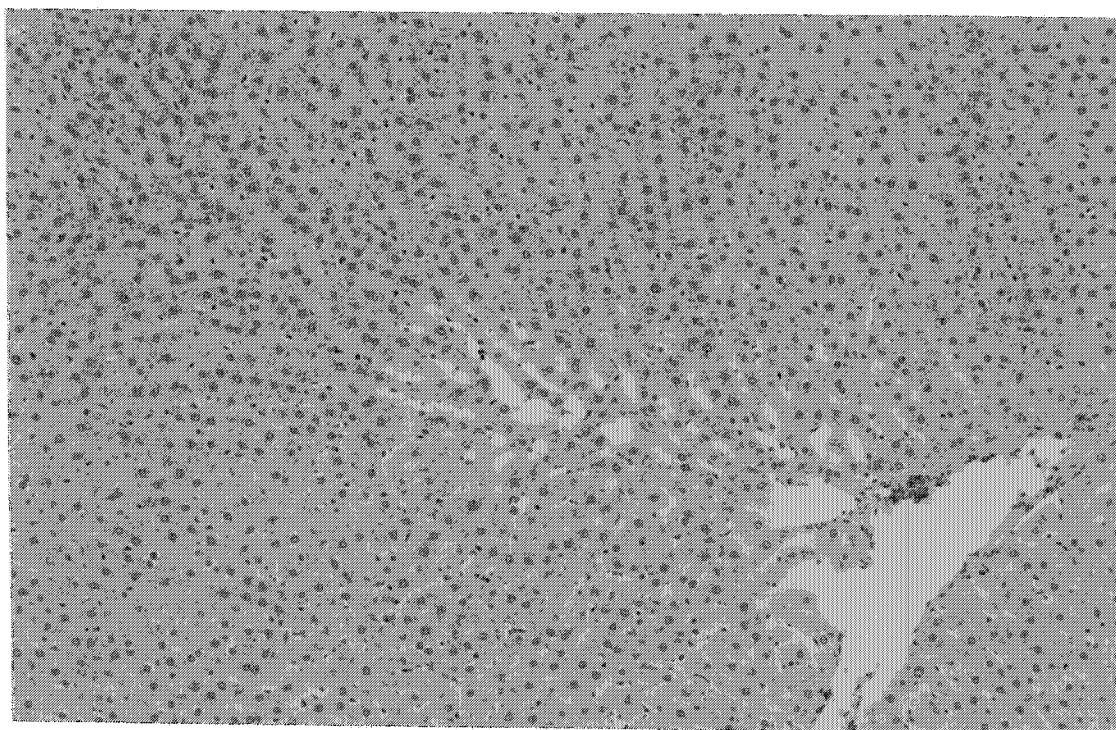


Figure 4.2: Photomicrograph of liver from rat treated with 300 ppm DCE depicting marked focal sinusoidal dilatation near a central vein.

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CHAPTER 5

ANTAGONISTIC INTERACTIONS OF AN ARSENIC-CONTAINING MIXTURE IN A MULTIPLE ORGAN CARCINOGENICITY BIOASSAY

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ABSTRACT

Inorganic arsenic (As), 1,2-dichloroethane (DCE), vinyl chloride (VC), and trichloroethylene (TCE) are frequently identified as groundwater contaminants near hazardous waste disposal sites. While the carcinogenicity of each of these chemicals has been extensively studied individually, little information exists regarding their carcinogenic potential in combination. We therefore investigated the carcinogenic promoting potential of chemical mixtures containing arsenic, DCE, VC, and TCE following multiple initiator administration in a multiple organ carcinogenicity bioassay. Our results reveal a dose-responsive antagonistic effect of this four-chemical mixture on the development of preneoplastic hepatic lesions (altered hepatocellular foci and

glutathione s-transferase π positive foci) as well as bronchioalveolar hyperplasia and adenoma formation.

INTRODUCTION

Arsenic (As), 1,2-dichloroethane (DCE), vinyl chloride (VC), and trichloroethylene (TCE) are often identified as groundwater contaminants near hazardous waste disposal sites (Naqvi *et al.* 1994; ATSDR 1993). Chronic ingestion of inorganic arsenic has been linked with the development of a variety of human cancers, including squamous cell and basal cell carcinomas of the skin, hepatic angiosarcoma, and cancers of the respiratory tract, kidney, and urinary bladder (Naqvi *et al.* 1994; Bates *et al.* 1992). Strikingly, most carcinogenicity assays of inorganic arsenic in laboratory animals have yielded negative results (Naqvi *et al.* 1994). Although its mechanism of action remains unclear, arsenic may act as a comutagen or cocarcinogen, inhibiting DNA repair (Morton *et al.* 1994).

DCE exposure has been linked with the development of pancreatic cancer in humans (Benson and Teta 1993), and in chronic carcinogenicity assays in laboratory animals, DCE has induced angiosarcoma, hepatocellular carcinoma, squamous cell carcinoma of the stomach, and subcutaneous fibrosarcoma (NCI 1978). Exposure to VC has been associated with the development of hepatic angiosarcoma and cancers of the brain, respiratory tract, and hematopoietic/lymphopoietic systems in humans (Popper *et al.* 1978; Waxweiler *et al.* 1976). In laboratory animals, VC has induced a similar array of neoplastic lesions (Maltoni *et al.* 1984; Feron *et al.* 1981). TCE has been linked with the development of lymphoma, urinary tract tumors, and childhood leukemia in humans (Davidson *et al.* 1991). TCE has also been

shown to cause hepatocellular carcinoma and respiratory tract cancer in laboratory animals (Davidson *et al.* 1991). Notably, three (As, DCE, and VC) of the four chemicals studied in this project are known to cause angiosarcoma in animals and/or humans.

Environmental exposures to hazardous chemicals generally involve long-term, low-level exposures to chemical mixtures. Interactions among chemical mixture components may potentially alter the effects associated with each individual component, such as the development of cancer. Because arsenic is suspected to act as a cocarcinogen, investigation of its carcinogenic effects in environmentally relevant chemical mixtures is especially warranted.

To study the organ-specific carcinogenicity of 4-chemical mixtures consisting of arsenic, DCE, VC, and TCE, we used a Multiple Organ Carcinogenicity Bioassay developed by Ito and colleagues (Ito *et al.* 1991, Ito *et al.* 1989). This assay employs an initiation/promotion protocol, in which exposure to the test chemical(s) follows multiple initiator administration. The carcinogenic effects of the test chemical(s) are assessed by comparative analysis of neoplastic lesions and preneoplastic lesions, such as altered hepatic foci expressing glutathione s-transferase π (GST-P) (Ito *et al.* 1991, Ito *et al.* 1989a,b).

MATERIALS AND METHODS

Chemicals

Diethylnitrosamine (DEN), N-methyl-N-nitrosourea (MNU), sodium arsenite, and DCE were purchased from Sigma Chemical Company (St. Louis, MO). VC gas (certified 2010 ppm in nitrogen carrier gas) was purchased from Scott Specialty Gases (Pasadena, TX).

TCE was purchased from Aldrich Chemical Company (Milwaukee, WI). Dihydroxy-di-N-propylnitrosamine [N-bis (2-hydroxypropyl)nitrosamine] (DHPN) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents were of analytical grade.

Dosing Solutions

Drinking water solutions were prepared twice weekly by dissolving sodium arsenite and DCE in deionized water. VC and TCE were administered via corn oil gavage due to their low water solubilities. To prepare gavage solutions of VC and TCE, VC gas was bubbled through corn oil, and the amount of VC in the corn oil was analyzed by gas chromatography based on methods described by Feron *et al.* (1975). Time-course volatilization studies dictated weekly VC solution preparation. VC/corn oil solutions were spiked with TCE and stored at -80°C in daily aliquots prior to administration.

Animals and Treatment

Four-week-old male Fischer 344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed three to a cage in Thoren units (self-contained laboratory animal housing racks designed for continuous external ventilation of individual cages) located in a fully accredited American Association for Accreditation of Laboratory Animal Care (AAALAC) facility. All animals received food (Harlan Teklad NIH-07 diet; Madison, WI) and water *ad libitum*, and lighting was set on a 12-hour light/dark cycle.

Following a two-week acclimation period, rats were randomized by weight into six treatment groups (15 rats/treatment group) as shown in Figure 1. During the first four weeks

of the bioassay, Groups I, II, III, and IV received a series of three broad spectrum (multiple organ) initiators (“DMD” initiation protocol, representing the first letter of each initiator). Groups V and VI received vehicle controls during this period. Briefly, on day 1 of the “DMD” protocol, Groups I, II, III, and IV were administered DEN (100 mg/kg) dissolved in 0.9% saline via intraperitoneal (*i.p.*) injection. Groups V and VI received an injection of 0.9% saline. On days 2, 5, 8, and 11, Groups I, II, III, and IV were injected (*i.p.*) with MNU (20 mg/kg) in citrate buffer solution. Groups V and VI received injections of citrate buffer solution. During days 15–28, Groups I, II, III, and IV received 0.1% DHPN in the drinking water; Groups V and VI received deionized drinking water. For 16 weeks following the four-week initiation period, Groups II, III, IV, and VI were administered one of three dose levels of the 4-chemical mixture. Groups I and V received deionized drinking water and corn oil vehicle gavage. For treatment groups exposed to the 4-chemical mixture, arsenic and DCE were administered daily via the drinking water; VC and TCE via once daily corn oil gavage, five times per week. Group II received the low dose mixture (7.5 ppm As, 3 ppm DCE, 1.5 ppm VC, 19 ppm TCE); Group III the medium dose mixture (25 ppm As, 30 ppm DCE, 5 ppm VC, 190 ppm TCE); and Groups IV and VI the high dose mixture (75 ppm As, 300 ppm DCE, 15 ppm VC, 1900 ppm TCE).

At the end of the 20-week assay period, rats were anesthetized with CO₂/ether and euthanatized by aortic exsanguination. Tissue specimens from thyroid gland, lung, esophagus, forestomach, glandular stomach, liver, kidney, and urinary bladder, as well as any grossly visible lesions, were fixed in 10% neutral buffered formalin, embedded in paraffin,

serially sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) for histopathological examination.

Immunohistochemical Staining

For analysis of focal hepatic GST-P expression, additional formalin-fixed liver sections (unstained) were deparaffinized in xylene and rehydrated by passage through an alcohol series. Following a 10-minute bath in 3% hydrogen peroxide, the slides were rinsed with deionized water and placed in phosphate buffered saline. Using an immunoperoxidase kit specific for the GST-P primary antibody (Vector Labs, Burlingame, CA), a standard avidin/biotin (ABC) protocol was employed to identify GST-P positive foci in liver sections. Following incubation with anti-GST-P primary antibody (Binding Site, San Diego, CA), slides were incubated with 3-amino-9-ethyl carbazole (AEC; Biomeda, Foster City, CA). Slides were then counterstained with hematoxylin for histologic evaluation.

Analysis of Hepatic Foci

All liver sections were prepared from the same lobes from each animal. Areas of altered hepatocellular foci (clear cell, acidophilic, or basophilic foci) were evaluated on H&E stained sections. GST-P positive foci were measured on immunohistochemically stained liver sections. On each liver section, foci outlines were manually traced using the Bioquant image analysis system (version IV; R&M Biometrics, Inc., Nashville, TN), and the system software computed the area within the tracing. A Dage CCD72 MTI camera (Dage Corporation, Michigan City, IN) coupled with a Bioquant system (Version 2.60) was used

to measure the total areas of liver sections. Results for treatment and control groups were expressed as areas of altered hepatocellular foci or GST-P positive foci more than 0.2 mm in diameter per unit area of liver (Ito *et al.* 1989a).

Quantification of Bronchioalveolar Hyperplasia and Adenomas

Bronchioalveolar hyperplastic lesions and adenomas were expressed as total number of lesions per unit area of lung. Total areas of lung sections were determined as described above for liver sections. All lung sections were prepared from the same lobes from each animal.

Statistical Analysis

Results were analyzed using one-way analysis of variance. To evaluate the dose-response effect of the 4-chemical mixture, between group post-tests were performed using Tukey's method for pair-wise comparisons (Kleinbaum *et al.* 1998). All statistical tests were performed using the Minitab statistical software package, release 11.21 (Minitab, Inc., State College, PA).

RESULTS

Histopathological examination of H&E stained tissue sections revealed altered hepatocellular (primarily clear cell) foci, proliferative pulmonary lesions (ranging from bronchioalveolar hyperplasia to adenomas), and thyroid follicular cell hyperplasia and adenomas in all initiated treatment groups. One case of renal myxosarcoma was present in

Group I, and one case of undifferentiated hepatic sarcoma was noted in Group IV. Occurrence of thyroid follicular cell hyperplasia and adenomas was not significantly different between treatment and control groups (data not shown).

As shown in Table 5.1, exposure to the 4-chemical mixture significantly decreased the total area of altered hepatocellular foci compared to controls ($p < 0.001$). Treatment with the 4-chemical mixture also significantly decreased the number of altered hepatocellular foci compared to controls ($p < 0.001$). Pairwise comparisons between treatment groups revealed total number of altered hepatocellular foci was significantly different between Groups II and IV ($p < 0.05$).

Results of focal hepatic GST-P expression are summarized in Table 5.2. Exposure to the 4-chemical mixture significantly decreased the total area of GST-P foci compared to controls ($p < 0.01$). Pairwise comparisons between treatment groups showed total area of GST-P foci was significantly different between Groups II and IV ($p < 0.05$). Additionally, exposure to the 4-chemical mixture significantly decreased the area of large foci (> 0.2 mm diameter) ($p < 0.05$) and the number of large foci ($p < 0.01$) compared to controls.

Finally, as illustrated in Table 5.3, the 4-chemical mixture significantly decreased the frequency of pulmonary bronchioalveolar hyperplasia and adenoma formation compared to controls ($p < 0.001$). A total of four pulmonary adenomas occurred in Group I (DMD/Control), yet none of the treatment groups exposed to the 4-chemical mixture developed pulmonary adenomas. These results suggest that under the given experimental conditions, the 4-chemical mixture suppressed adenoma formation.

DISCUSSION

A variety of *in vivo* medium-term models, such as the Multiple Organ Carcinogenicity Bioassay used in this study, have been established to examine the promoting ability of test chemicals by measuring the development of preneoplastic and neoplastic lesions in single (*e.g.*, liver) or multiple organs (Ito *et al.* 1991, Ito *et al.* 1989a). Numerous investigators have used these and other similar bioassays to demonstrate the inhibitory effects of antioxidants and other chemopreventive compounds on the development of preneoplastic and neoplastic lesions in various organs (Ito *et al.* 1996; Kojima *et al.* 1993; Pascale *et al.* 1993). Data from these studies suggest that the mechanisms responsible for suppression of the development of preneoplastic and neoplastic lesions may involve decreased cell proliferation and/or enhanced apoptosis (Kojima *et al.* 1993; Pascale *et al.* 1993).

Additionally, preneoplastic lesion development during promotion may be modulated by a phenomenon known as remodeling, also described as redifferentiation or phenotypic maturation (Tatematsu *et al.* 1983). Preneoplastic cells undergoing remodeling revert back to their normal phenotypic appearance and may no longer express characteristic biochemical markers (Pascale *et al.* 1993). Administration of the chemopreventive agent S-adenosyl-L-methionine has been shown to enhance remodeling of preneoplastic liver lesions, resulting in decreased expression of biochemical markers associated with enzyme-altered hepatocellular foci (Pascale *et al.* 1993).

In the data presented here, we report a dose-responsive inhibitory effect of a 4-chemical mixture containing arsenic, DCE, VC, and TCE on the promotion of preneoplastic and neoplastic lesions in rat lung and liver. Paradoxically, each of these chemicals alone

exhibits carcinogenic potential in humans and/or laboratory animals. Mechanisms which could potentially account for this antagonistic effect include decreased cell proliferation, increased apoptosis, and/or enhanced remodeling in preneoplastic lesions. Further investigations to determine the effects of the 4-chemical mixture on hepatocyte proliferation and apoptosis as well as hepatic levels of S-adenosylmethionine are currently underway.

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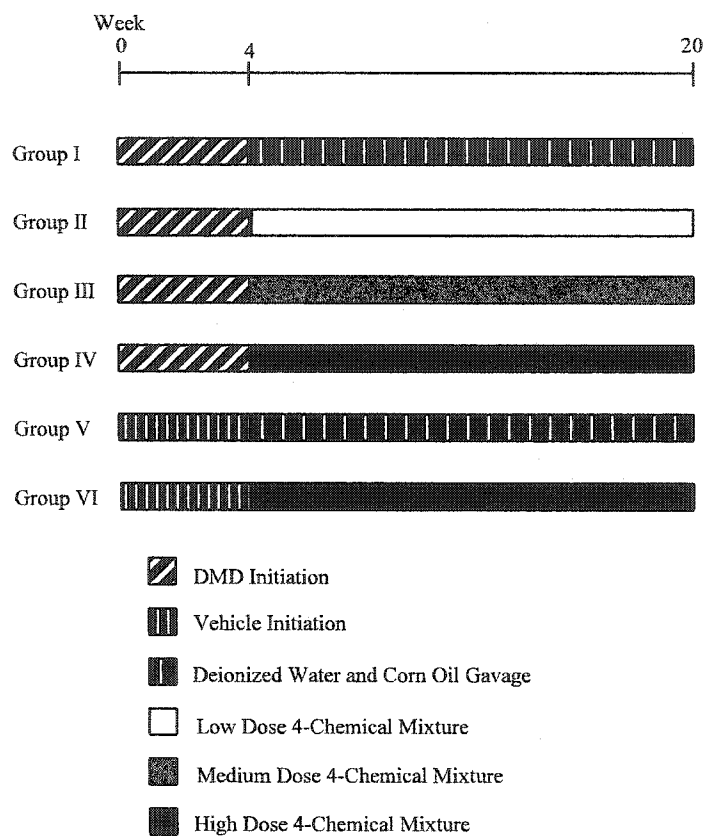


Figure 1

Figure 5.1: Experimental treatment protocol for evaluation of As, 1,2-DCE, TCE, and VC in Multiple Organ Carcinogenicity Bioassay.

**Table 5.1. Altered Hepatocellular Foci in Four-Chemical Mixture
Multiple Organ Carcinogenicity Bioassay**

Treatment Group	Total Foci Area ^a	No. of Foci ^b
I (DMD/Control) (15)	0.652 ± 0.396*	12.0 ± 5.3*
II (DMD/Low) (15)	0.232 ± 0.153 [†]	7.5 ± 3.4 [†]
III (DMD/Medium) (15)	0.116 ± 0.080 [†]	5.6 ± 2.7 [†]
IV (DMD/High) (11)	0.032 ± 0.023 [†]	1.9 ± 1.0 ^{†‡}
V (Vehicle/Control) (15)	0.001 ± 0.001	0.1 ± 0.1
VI (Vehicle/High) (13)	0.001 ± 0.001	0.1 ± 0.1

Number of animals in each group is in parentheses; values represent the mean ± standard deviation.

^a Total foci area is expressed as foci area (mm²) per unit liver area (cm²).

^b No. of foci is expressed per unit liver area (cm²).

* Significantly different ($p < 0.001$) from Group V (Vehicle/Control) according to a Student's t-test.

[†] Significantly different ($p < 0.001$) from Group I (DMD/Control) according to a one-way ANOVA.

[‡] Significantly different ($p < 0.05$) from Group II (DMD/Low) according to Tukey's method for pairwise comparisons.

Table 5.2. Hepatic Glutathione S-Transferase π Positive Foci in Four-Chemical Mixture Multiple Organ Carcinogenicity Bioassay

Treatment Group	Total Foci Area ^a	No. of Foci ^b	Large Foci Area ^c	No. of Large Foci ^d
I (DMD/Control) (15)	0.450 $\pm$ 0.064*	77.2 $\pm$ 14.4*	0.081 $\pm$ 0.063 [†]	1.7 $\pm$ 1.2 [†]
II (DMD/Low) (15)	0.377 $\pm$ 0.108 [‡]	69.2 $\pm$ 13.9	0.043 $\pm$ 0.051 [§]	0.7 $\pm$ 0.7 [‡]
III (DMD/Medium) (15)	0.293 $\pm$ 0.062 [‡]	69.0 $\pm$ 10.6	0.036 $\pm$ 0.034 [§]	0.7 $\pm$ 0.6 [‡]
IV (DMD/High) (11)	0.214 $\pm$ 0.083 ^{‡**}	63.0 $\pm$ 12.5	0.015 $\pm$ 0.021 [§]	0.3 $\pm$ 0.4 [‡]
V (Vehicle/Control) (15)	0.010 $\pm$ 0.008	2.8 $\pm$ 1.6	0.002 $\pm$ 0.006	0.1 $\pm$ 0.1
VI (Vehicle/High) (13)	0.005 $\pm$ 0.004	2.0 $\pm$ 1.4	0	0

Number of animals in each group is in parentheses; values represent the mean $\pm$ standard deviation.

^a Total foci area is expressed as foci area (mm²) per unit liver area (cm²).

^b No. of foci is expressed per unit liver area (cm²).

^c Large foci area is expressed as the area of foci with diameters > 0.2 mm (mm²) per unit liver area (cm²).

^d No. of large foci is expressed as the number of foci with diameters > 0.2 mm per unit liver area (cm²).

* Significantly different ($p < 0.001$) from Group V (Vehicle/Control) according to a Student's t-test.

[†] Significantly different ($p < 0.02$) from Group V (Vehicle/Control) according to a Student's t-test.

[‡] Significantly different ($p < 0.01$) from Group I (DMD/Control) according to a one-way ANOVA.

[§] Significantly different ($p < 0.05$) from Group I (DMD/Control) according to a one-way ANOVA.

** Significantly different ($p < 0.05$) from Group II (DMD/Low) according to Tukey's method for pairwise comparisons.

Table 5.3. Bronchioalveolar Hyperplasia and Pulmonary Adenoma Formation in Four-Chemical Mixture Multiple Organ Carcinogenicity Bioassay

Treatment Group	No. of Hyperplastic Lesions ^a	No. of Adenomas ^b
I (DMD/Control) (15)	12.3 ± 3.3 [*]	0.251 ± 0.446 [†]
II (DMD/Low) (15)	7.2 ± 2.9 [‡]	0 [‡]
III (DMD/Medium) (15)	7.2 ± 2.5 [‡]	0 [‡]
IV (DMD/High) (11)	5.8 ± 2.4 [‡]	0 [‡]
V (Vehicle/Control) (15)	0	0
VI (Vehicle/High) (13)	0	0

Number of animals in each group is in parentheses; values represent the mean ± standard deviation.

^a Mean no. of lesions is expressed per unit lung area (cm²).

^b Mean no. of adenomas is expressed per unit lung area (cm²).

^{*} Significantly different ($p < 0.001$) from Group V (Vehicle/Control) according to a Student's t-test.

[†] Significantly different ($p < 0.01$) from Group V (Vehicle/Control) according to a Student's t-test.

[‡] Significantly different ($p < 0.001$) from Group I (DMD/Control) according to a one-way ANOVA.

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CHAPTER 6

ARSENIC SUPPRESSES PRENEOPLASTIC FOCUS DEVELOPMENT IN A MEDIUM-TERM LIVER FOCI BIOASSAY

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ABSTRACT

Despite significant data linking inorganic arsenic (As) exposure with the development of a variety of human cancers, relatively little is known about its carcinogenic mode of action. Because it may function in concert with other carcinogens as a promoter, progressor, co-mutagen, or co-carcinogen, investigation of its effects as a component of environmentally relevant chemical mixtures is appropriate. Our previous studies using a multiple organ carcinogenesis bioassay indicated that a quaternary mixture consisting of As, 1,2-dichloroethane (DCE), trichloroethylene (TCE), and vinyl chloride (VC) suppressed the development of hepatic preneoplastic glutathione S-transferase π (GST-P) positive foci in the rat in a dose dependent manner. To test the hypothesis that As, whether alone or in chemical mixtures, was

responsible for the suppressive effect on the development of GST-P positive foci, we utilized a medium-term liver foci bioassay to evaluate GST-P positive foci development associated with exposure not only to the quaternary mixture, but also to a ternary (As, DCE, TCE) and binary (As, DCE) submixture as well as to As alone. Additionally, to evaluate the effect of As dose level on the development of GST-P positive hepatic foci, we included treatment groups with higher doses of As in the quaternary mixture. Consistent with our earlier data, exposure to As, whether alone or in the quaternary, ternary, or binary chemical mixture, was associated with a significant decrease in the total area and number of GST-P positive foci compared to controls. We also sought to determine if alterations in hepatic S-adenosylmethionine (SAM) and/or S-adenosylhomocysteine (SAH) content could be associated with suppression of GST-P positive foci growth. No significant difference in SAM or SAH levels was observed between treatment and control groups.

INTRODUCTION

The carcinogenicity of As in humans has been unequivocally established by epidemiologic data gathered from diverse populations throughout the world. Exposure via ingestion of contaminated drinking water has been linked with the development of cancers of the skin, lung, liver, urinary bladder, and possibly other organs (Chiang *et al.* 1998; Hopenhayn-Rich *et al.* 1998; Smith *et al.* 1998; Guo *et al.* 1997; Hopenhayn-Rich *et al.* 1996; Bates *et al.* 1995; Chiou *et al.* 1995; Tsuda *et al.* 1995; Bates *et al.* 1992; Chen *et al.* 1992; Smith *et al.* 1992; Wu *et al.* 1989; Tseng *et al.* 1968). Remarkably, despite many

attempts to duplicate the carcinogenic effects of As in laboratory animals, a well-established model does not currently exist. Recently, however, a transplacental carcinogenicity study in which mice were administered varying doses of As in the drinking water revealed an increased incidence of hepatocellular carcinomas and adrenal tumors among male offspring and an increased incidence of ovarian tumors and lung carcinomas in female offspring (Waalkes *et al.* 2003). Although these results represent highly significant progress toward establishing a reliable *in vivo* model of As carcinogenesis, the carcinogenic mode of action of As remains poorly understood. It has been proposed to act in concert with other carcinogens as a promoter, progressor, co-mutagen, or co-carcinogen (Chen *et al.* 2001a,b; Kitchin 2001; Vogt and Rossman 2001; Trouba *et al.* 2000; Clewell *et al.* 1999; Germolec *et al.* 1998, 1997, 1996; Li and Rossman 1989a,b). Therefore, investigation of the carcinogenic effects of As in chemical mixtures, particularly in environmentally relevant chemical mixtures, is highly warranted.

In previous studies utilizing a multiple organ carcinogenicity bioassay, we investigated the carcinogenic potential of a chemical mixture containing, in addition to As, DCE, TCE, and VC (Pott *et al.* 1998). As commonly identified groundwater contaminants near hazardous waste disposal sites (Faroon *et al.* 1994; ATSDR 1993), these four chemicals represent an environmentally relevant chemical mixture. Additionally, while each of these compounds on its own is known to induce a wide array of tumors in humans and/or laboratory animals, As, DCE, and VC have all demonstrated the capacity to induce angiosarcomas in animals and/or humans (Maltoni *et al.* 1984; Feron *et al.* 1981; NCI 1978; Popper *et al.* 1978; Waxweiler *et al.* 1976). Thus, we hypothesized that a quaternary mixture

consisting of As, DCE, TCE, and VC would induce tumors, particularly angiosarcomas, in a synergistic or at least additive fashion compared to each chemical individually. Contrary to our expectations, our studies demonstrated that, in the rat, the quaternary mixture actually suppressed the development of preneoplastic GST-P positive hepatic foci, a reliable marker of hepatocellular neoplastic potential, in a dose-dependent manner (Pott *et al.* 1998).

Enhanced remodeling of preneoplastic foci could account for the observed suppressive effect of the quaternary mixture on the development of preneoplastic GST-P positive foci. Remodeling of preneoplastic foci has been reported in association with alterations in normal levels of hepatic SAM and its by-product, SAH (Garcea *et al.* 1989). Because the biotransformation of As occurs via sequential addition of methyl groups supplied by SAM (Thomas *et al.* 2001; NRC 1999; Vahter 1999; Aposhian 1997; Vahter 1983), chronic exposure to As would consume cellular methyl groups and could be associated with aberrations in hepatocellular pools of SAM/SAH. Perturbation of hepatic SAM/SAH levels might thus alter the cellular environment to favor the remodeling of preneoplastic lesions.

To further investigate the role of As in the quaternary mixture (As, DCE, TCE, and VC) on the development of GST-P positive hepatic foci, as well as the possible role of SAM/SAH in mediating the suppression of foci development, we utilized the Ito medium-term liver foci bioassay (Ito *et al.* 1994; Ito *et al.* 1989). In this initiation/promotion assay, exposure to the test chemical(s) follows administration of diethylnitrosamine (DEN) as an initiating agent. One week into the 6-week course of treatment with test chemical(s), a two-thirds partial hepatectomy is performed to enhance clonal expansion of initiated hepatocytes.

The carcinogenic effects of the test chemical(s) are then assessed by comparative morphometric analysis of preneoplastic GST-P positive hepatic foci. Our studies were designed to test the hypothesis that As, whether alone or in chemical mixtures, is responsible for the suppressive effect on the development of GST-P positive foci. We assessed the role of As in the quaternary mixture by evaluating GST-P positive foci development associated with exposure to not only the quaternary mixture, but also a ternary (As, DCE, TCE) and binary (As, DCE) submixture, as well as to As alone. Additionally, to evaluate the effect of As dose level on the development of GST-P positive hepatic foci, we included treatment groups with higher doses of As in the quaternary mixture. Finally, we sought to determine if alterations in hepatic SAM/SAH content could be associated with suppression of GST-P positive foci growth.

MATERIALS AND METHODS

Chemicals

DEN, As (as sodium arsenite), and DCE were purchased from Sigma Chemicals (St. Louis, MO). TCE was purchased from Aldrich Chemicals (Milwaukee, WI). VC gas, certified 2010 ppm in nitrogen carrier gas, was purchased from Scott Specialty Gases (Pasadena, TX). SAM, SAH, and 1-heptanesulfonic acid were purchased from Sigma Chemicals (St. Louis, MO). All other reagents were of analytical grade.

Dosing Solutions

As and DCE drinking water solutions were prepared twice weekly in deionized water. Due to their low aqueous solubilities, TCE and VC were administered in corn oil via once daily oral gavage, 5 days/week. VC corn oil solutions were prepared by bubbling VC gas through corn oil for 4 hours; VC content was then determined by gas chromatography (Pott *et al.* 1998; Feron *et al.* 1975). Based on time-course volatilization studies, gavage solutions were prepared on a weekly basis. VC/TCE and TCE corn oil solutions were prepared by spiking VC solutions or corn oil, respectively, with TCE. VC/TCE and TCE corn oil solutions were prepared weekly, aliquoted into daily doses, and stored at -80°C.

Animals and Treatment

Four-week old male Fischer 344 (F344) rats (Harlan Sprague Dawley, Indianapolis, IN) were housed three to a cage in Thoren units (self-contained laboratory animal housing racks designed for continuous external ventilation of individual cages) located in a temperature (25°C) and humidity (55%) controlled environment in a fully accredited American Association for Accreditation of Laboratory Animal Care (AAALAC) facility. Animals were fed Harlan Teklad NIH-07 diet (Madison, WI) and deionized water *ad libitum* and housed on corn cob bedding; lighting was set on a 12 hour light/dark cycle.

Following a four-week acclimatization period, rats were randomized by weight into eleven treatment groups (18 rats per treatment group) as shown in Table 6.1. On day 1, groups III, IV, V, VI, VII, VIII, IX and XI were administered DEN (200 mg/kg in saline intraperitoneally) while groups I, II, and X were injected with saline as controls (Figure 6.1).

During weeks 3-8, groups II, IV, X, and XI were orally administered a standard quaternary mixture (75 ppm As, 300 ppm DCE, 1900 ppm TCE, 15 ppm VC; abbreviated as “std 4^o”); groups V and VI received a ternary (As, DCE, TCE; abbreviated as “3^o”) or binary (As, DCE; abbreviated as “2^o”) submixture, respectively; group VII was administered As alone; and group III received deionized drinking water and corn oil gavage. To assess the dose-response effect of As in the 4^o mixture, two additional treatment groups received the 4^o mixture with higher dose levels of As. Group VIII (designated as “DEN, high 4^o”) was administered 300 ppm As during weeks 3-4 and 150 ppm during weeks 5-8, and group IX (designated as “DEN, medium 4^o”) received 150 ppm As during weeks 3-8. The reduction of As concentration from 300 ppm to 150 ppm in the DEN, high 4^o group (Group VIII) was necessitated by clinical evidence of toxicity (decreased activity, postural changes, decreased food/water consumption). These treatment groups (VIII and IX) also received DCE, TCE, and VC at the same dose levels as in the standard 4^o mixture. In all treatment groups, As and DCE were administered in the drinking water, TCE and VC via once daily oral corn oil gavage, 5 days/week. On day 22 of the bioassay, groups I-IX underwent two-thirds partial hepatectomy (PH) while groups X and XI did not. At the end of the 8-week assay period, all rats were anesthetized with isoflurane and euthanatized by abdominal aortic exsanguination. Tissue specimens from the lung, liver, duodenum, spleen, and kidney were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μ m and stained with hematoxylin and eosin (H&E) for histopathologic evaluation.

Immunohistochemical Staining

Focal expression of hepatic GST-P was analyzed on unstained formalin-fixed liver sections that were deparaffinized in xylene and rehydrated by passage through a series of ethanol baths. Following a 10 minute bath in hydrogen peroxide (0.3%) to quench endogenous peroxide, the slides were rinsed with deionized water and placed in phosphate-buffered saline. Using an immunoperoxidase kit specific for the GST-P primary antibody (Vector Laboratories, Burlingame, CA), a standard avidin/biotin complex (ABC) protocol was employed to identify GST-P positive foci in liver sections. Following incubation with anti-GST-P primary antibody (Binding Site, San Diego, CA), slides were incubated with 2-amino-9-ethyl carbazole (AEC, Biomed, Foster City, CA). Slides were then counterstained with hematoxylin for histomorphometric evaluation.

Histomorphometric Analysis

All liver sections were prepared from the same lobes from each animal. GST-P positive foci were quantified on immunohistochemically stained liver sections. Using a Bioquant image analysis system (Version IV, R&M Biometrics, Nashville, TN), foci outlines were manually traced on each liver section, then area within the tracing was computed by the system software. A Dage CCD72 MTI camera (Dage Corporation, Michigan City, IN) coupled with a Bioquant system (Version 2.60, R&M Biometrics, Nashville, TN) was utilized to measure the total area of each liver section. Results were expressed as area of GST-P positive foci per unit area of liver.

Hepatic SAM and SAH Analysis

Levels of hepatic SAM and SAH were analyzed using reverse-phase HPLC (Molloy *et al.* 1990). The system consisted of a Beckman Gold Nouveau 126 solvent module connected to a 508 autosampler and a 166 variable UV detector module (Beckman Coulter, Fullerton, CA). Liver tissue was homogenized in 40% trichloroacetic acid, then centrifuged at 10,000g for 20 minutes. The supernatant was collected and washed 3 times with 4 volumes of peroxide-free diethyl ether, then filtered through a 0.2 μ m PVDF syringe top filter (Fisher Scientific, Pittsburgh, PA). Aliquots were stored at -20°C. Analyses were carried out using a linear gradient elution method (Molloy *et al.* 1990) on an Alltech Altima C₁₈ column (5 μ m, 250 mm) equipped with a C₁₈ guard column (Alltech, Deerfield, IL). Buffer A consisted of 25 mM sodium phosphate and 10 mM heptanesulfonic acid (pH adjusted to 3.2 with orthophosphoric acid), Buffer B of 10 mM heptanesulfonic acid in methanol. Initial conditions were 85% Buffer A and 15% Buffer B. Over the first 10 minutes of the analysis, the percentage of Buffer B was increased from 15% to 25%, then held at 25% for 3 minutes before returning to initial conditions over a period of 5 minutes. All analyses were performed at room temperature with a flow rate of 1.0 mL/minute and a sample volume of 50 μ L. Retention times for SAH and SAM were 11.4 and 14.8 minutes, respectively. Data were analyzed with Beckman System Gold Nouveau software (Beckman Coulter, Fullerton, CA). Results were expressed as nmol SAM or SAH per gram of liver tissue.

Statistical Analysis

Results were analyzed using Student's *t*-test and one-way analysis of variance. To evaluate the dose-response effect of As in the quaternary mixtures, between group post-tests were performed according to Tukey's method for pairwise comparisons (Kleimbaum *et al.* 1988). All statistical tests were performed using the Minitab statistical software package, release 11.21 (Minitab, State College, PA).

RESULTS

Histomorphometric Analysis

As shown in Table 6.2, treatment with DEN plus partial hepatectomy (group III) resulted in a significant increase in total area and number of GST-P positive foci compared with saline controls (groups I and II) or the groups with no PH (groups X and XI). Treatment groups initiated with DEN then exposed to the 4° (standard, high, or medium), 3°, or 2° mixture, or As alone (groups IV - IX) exhibited a significant decrease in the total area of all GST-P positive foci compared to DEN, DI/CO controls (group III) ($P < 0.001$). Two groups showed a difference in number of foci. Group IV, DEN 4° was lowered compared with DEN, DI/CO controls (group III). Exposure to the medium 4° mixture following DEN initiation (group IX) was associated with a very significant increase, almost 3-fold, in the total number of all GST-P positive foci compared to DEN, DI/CO controls (group III; $P < 0.001$). The area and number of large GST-P positive foci (> 0.2 mm diameter) were decreased in DEN-initiated treatment groups exposed to the 4° (standard, high, or medium), 3°, or 2° mixtures, or As alone (groups IV - IX; $P < 0.001$) when compared to DEN, DI/CO

controls. Multiple scattered individual and small clusters of GST-P positive hepatocytes were observed in the livers of DEN-initiated rats exposed to the medium 4° mixture (group IX), but not in any other treatment group.

SAM/SAH Analysis

Results of hepatic SAM and SAH analysis are summarized in Table 6.3. We observed no significant differences in SAM or SAH levels among DEN-initiated treatment groups compared to DEN, DI/CO controls (group IV). However, in rats that did not undergo partial hepatectomy, those initiated with DEN and subsequently exposed to the standard quaternary mixture (group XI) exhibited a significantly lower level of hepatic SAH compared to partially hepatectomized rats receiving the same treatment (group IV). Accordingly, the SAM/SAH ratio was significantly increased in group XI compared to group IV. Additionally, the SAM/SAH ratio was increased in group XI compared to unhepatectomized rats that received a saline injection (rather than DEN) and were then exposed to the standard quaternary mixture (group X).

DISCUSSION

In confirmation of our earlier studies in a multiple organ carcinogenicity bioassay (Pott *et al.* 1998), As, alone and in 4° (As, DCE, TCE, VC), 3° (As, DCE, TCE), and 2° (As, DCE) chemical mixtures, demonstrated a suppressive effect on the development of preneoplastic GST-P positive hepatic foci in Ito's medium-term liver foci bioassay in the rat. Both number and area of foci were decreased in association with As exposure compared to

controls. Among treatment groups receiving the standard 4°, 3°, or 2° mixture, or As alone, comparison of area of large foci revealed a non-significant trend toward decreased area associated with decreasing number of chemical mixture components. Elevated dose levels of As in the quaternary mixture (groups VIII and IX) did not affect its potential to inhibit the development of GST-P positive hepatic foci. An increase in the number of all GST-P positive foci associated with exposure to the medium 4° mixture (group IX) was likely due to the presence of multiple individual and small clusters of GST-P positive hepatocytes in this treatment group. The cause of this GST-P expression pattern in this specific treatment group is unknown.

Remodeling of hepatic preneoplastic foci has been reported in association with altered levels of SAM and SAH (Garcea *et al.* 1989). We hypothesized that the suppressive effect of As on the development of GST-P positive foci might be associated with perturbations of hepatocellular pools of SAM/SAH, as As is biotransformed by sequential methylation, with methyl groups supplied by SAM (Thomas *et al.* 2001; NRC 1999; Vahter 1999; Aposhian 1997; Vahter 1983). Chronic exposure to As could potentially alter hepatocellular pools of SAM and SAH to favor the remodeling of preneoplastic GST-P positive foci. In our studies, however, no perturbation in hepatic SAM or SAH content was observed in association with As exposure, whether alone or in 2°, 3°, or 4° chemical mixtures compared to controls. Interestingly, lack of partial hepatectomy was associated with alterations in hepatic SAH content and consequently in SAM/SAH ratios compared to hepatectomized rats receiving comparable treatments (see Table 6.3). Indeed, partial hepatectomy has been shown to significantly alter hepatic SAM and SAH levels and

SAM/SAH ratios within 24 - 72 hours following partial hepatectomy surgery (Huang *et al.* 1998; Garcea *et al.* 1989a,b). Importantly, interpretation of hepatic SAM and SAH levels with respect to remodeling of GST-P positive foci in our studies may be limited by availability of data at only one time point - the termination of the study. It is possible that compensatory or other mechanisms predominating by the end of the study (8 weeks) may not be representative of earlier changes in SAM and/or SAH levels, particularly in the hours or days immediately following surgery.

As illustrated in 6. 2, the biotransformation of As involves its stepwise reduction and oxidative methylation, resulting in the formation of a series of mono- and dimethylated metabolites as tri- or pentavalent moieties (Kitchin 2001; Thomas *et al.* 2001; NRC 1999; Zakharian *et al.* 1999). At the present time, the role of these metabolites in the carcinogenic process remain unknown, but substantial data exist to suggest that the methylated metabolites are capable of significant biological activity and as such may somehow be involved in the carcinogenic process (Kitchin 2001; Thomas *et al.* 2001). It has also been proposed that, in the process of biotransformation, As may perturb various cellular methylation processes which may modulate its carcinogenic effects (Chen *et al.* 2001a,b; Goering *et al.* 1999; Mass *et al.* 1997; Mass 1992). DNA methylation status, whether hypomethylated or hypermethylated, may contribute to control of the expression of a variety of genes (Counts and Goodman 1995). Continuous methyl depletion due to chronic As exposure may deplete cellular pools of SAM, an essential cofactor for a variety of methyltransferases, including the DNA methyltransferases that are responsible for methylation of DNA (Goering *et al.* 1999; Cheng 1995). Overexpression of hypomethylated oncogenes could lead to a succession of

increased proliferation, cell cycle disruption, and malignant transformation (Chen *et al.* 2001a,b; Goering *et al.* 1999; Mass and Wang 1997; Zhao *et al.* 1997; Henderson *et al.* 1982). Conversely, prolonged exposure to As may lead to hypermethylation of DNA, perhaps via selective inhibition of SAM-dependent methyltransferases. Decreased utilization and subsequent accumulation of SAM would follow, potentially driving uninhibited methyltransferase enzymes to overmethylate their substrates (Goering *et al.* 1999; Mass and Wang 1997). In the case of a tumor suppressor gene, genomic hypermethylation could lead to decreased expression, which could also contribute to a sequence of hyperproliferation, disruption of the cell cycle, and subsequent malignant transformation (Mass and Wang 1997).

During periods of active cell division and growth, however, such as occurs in the liver following partial hepatectomy, methylation of As may be decreased due to 1) diversion of methionine and cysteine to protein synthesis instead of to the methyltransferases responsible for the biotransformation of As and 2) increased requirements for spermine and spermidine generation at the expense of SAM regeneration (Donohue and Abernathy 2001).

It is possible that decreased methylation of As may be associated with the suppression of GST-P positive foci development we observed in our studies. Clearly, in order to understand the role of As in mediating the suppression of the development of preneoplastic hepatic foci, it is essential to understand not only how its methylation may modulate the carcinogenic process, but also the carcinogenic potential of the different methylated metabolites.

Importantly, inorganic As displays, in addition to its carcinogenic activity, anticarcinogenic properties as well. In concert with research centers across the United States,

the National Cancer Institute (NCI) is currently evaluating the clinical efficacy of As trioxide in a variety of hematologic malignancies, including acute promyelocytic leukemia, acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, chronic lymphocytic leukemia, myelodysplastic syndrome, and multiple myeloma (Murgo 2001). The NCI also supports research to evaluate As trioxide for treatment of solid tumors such as advanced hormone-refractory prostate cancer and refractory transitional cell carcinoma of the bladder, renal cell cancer, and cervical cancer (Murgo 2001). Although the mechanism underlying As trioxide's clinical action remains unclear, it is known to cause cytotoxicity in neoplastic cells by inhibiting proliferation, inducing apoptosis, and promoting cellular differentiation (Murgo 2001; Roboz *et al.* 2000; Bazarbachi *et al.* 1999; Ishitsuka *et al.* 1998; Zhang *et al.* 1998; Chen *et al.* 1996). Notably, in considering the dynamics of preneoplastic foci growth in our studies, decreased proliferation and/or increased apoptosis of hepatocytes in GST-P positive foci could be responsible for the decrease in foci area and number that we observed.

Full understanding of the role of As in suppressing the development of preneoplastic hepatic GST-P positive foci in our studies depends on a deeper knowledge of the dual nature of As as a carcinogen and anti-cancer therapeutic agent. Time-course experiments designed to evaluate the development of GST-P positive foci in conjunction with rates of hepatocellular proliferation/apoptosis (within foci and in the surrounding parenchyma) would yield important information on the dynamics of foci growth. Kinetic data on hepatic concentrations of SAM and SAH and each methylated metabolite of As are crucial to determine critically active metabolites and more completely understand the role of

methylation in modulating the carcinogenic effects of As. The effect of partial hepatectomy on these parameters should also be evaluated. In summary, we have clearly demonstrated that As, as sodium arsenite, has a suppressive effect on the induction of hepatocellular preneoplastic foci in a rat initiation/promotion protocol. These findings are of significance for further evaluation of the carcinogenic and antineoplastic effects of this common environmental contaminant.

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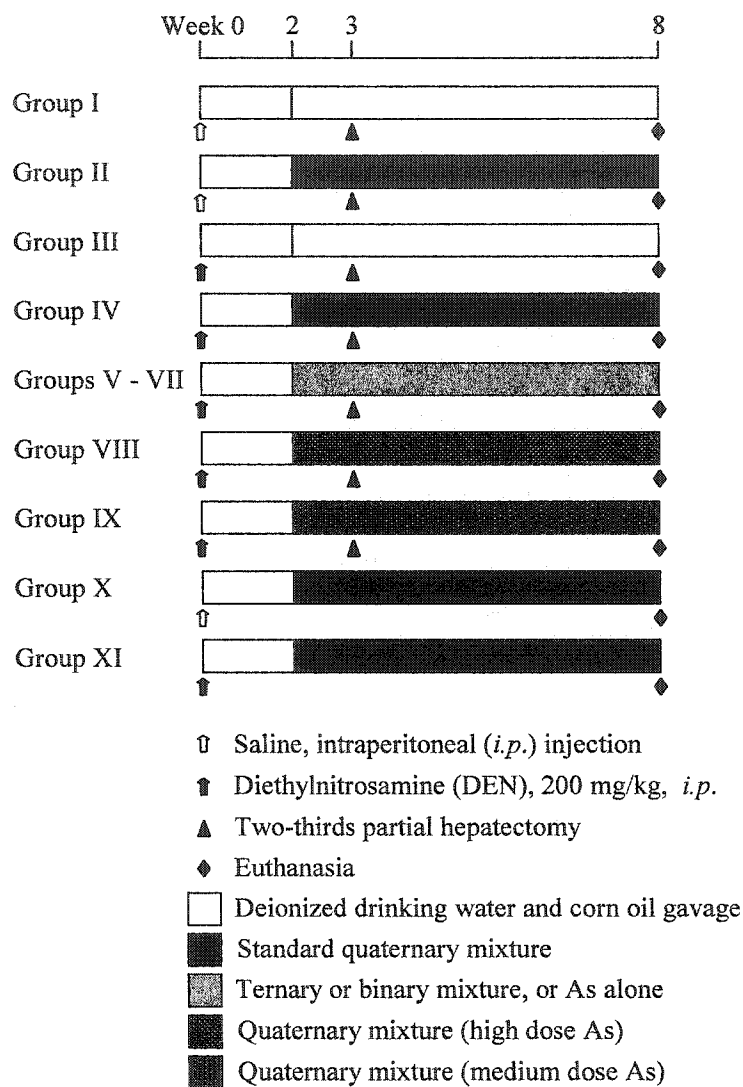


Figure 6.1: Experimental design for the Ito medium-term liver foci bioassay. Rats were initiated with DEN (200 mg/kg, *i.p.*) on day 1 and a quaternary, ternary, or binary chemical mixture or arsenite alone was orally administered during weeks 2-8. Two-thirds partial hepatectomy was performed on day 22.

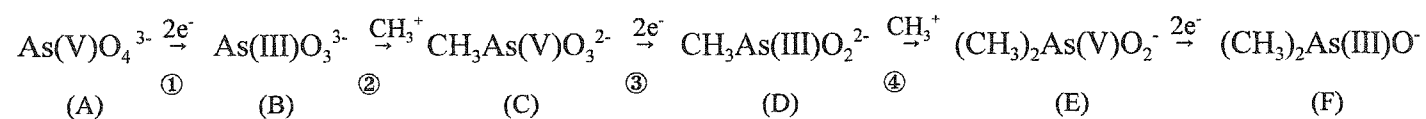


Figure 6.2: Biotransformation of arsenic. (A) arsenate; (B) arsenite; (C) monomethylarsonic acid (MMA^V); (D) monomethylarsonous acid (MMA^{III}); (E) dimethylarsinic acid (DMA^V); (F) dimethylarsinous acid (DMA^{III}). ① arsenate reductase; ② arsenite methyltransferase; ③ MMA^V reductase; ④ MMA^{III} methyltransferase. (Redrawn after Aposhian 1997).

Table 6.1. Experimental treatment groups for the Ito Medium-Term Liver Foci Bioassay								
Treatment	Initiator*	PH**	Treatment (ppm)					
Group			DI***	CO****	As†	DCE‡	TCE§	VC*
I (saline, DI/CO)	saline	+	+	+	—	—	—	—
II (saline, std 4°)	saline	+	—	—	75	300	1900	15
III (DEN, DI/CO)	DEN	+	+	+	—	—	—	—
IV (DEN, std 4°)	DEN	+	—	—	75	300	1900	15
V (DEN, 3°)	DEN	+	—	—	75	300	1900	—
VI (DEN, 2°)	DEN	+	—	+	75	300	—	—
VII (DEN, As)	DEN	+	—	+	75	—	—	—
VIII (DEN, high 4°)	DEN	+	—	—	300/150 [‡]	300	1900	15
IX (DEN, medium 4°)	DEN	+	—	—	150	300	1900	15
X (saline, std 4°, no PH)	saline	—	—	—	75	300	1900	15
XI (DEN, std 4°, no PH)	DEN	—	—	—	75	300	1900	15

* DEN, diethylnitrosamine, 200 mg/kg *i.p.*

** PH, two-thirds partial hepatectomy.

*** DI, deionized drinking water.

**** CO, corn oil gavage, once daily, 5 days/week.

† As, sodium arsenite, administered in the drinking water.

‡ DCE, 1,2-dichloroethane, administered in the drinking water.

§ TCE, trichloroethylene, administered via once daily corn oil gavage, 5 days/week.

* VC, vinyl chloride, administered via once daily corn oil gavage, 5 days/week.

[‡] quaternary mixture with 300 ppm As for the first two weeks, 150 ppm As for the remaining 4 weeks.

Table 6.2. Hepatic Glutathione S-Transferase π Positive Foci in Ito Medium-Term Liver Foci Bioassay				
Treatment group	Total foci area (mean $\pm$ SD) [*]	No. foci (mean $\pm$ SD) [†]	Large foci area (mean $\pm$ SD) [‡]	No. large foci (mean $\pm$ SD) [§]
I (saline, DI/CO)	0.001 $\pm$ 0.001	0.212 $\pm$ 0.813	0	0
II (saline, std 4°)	0.001 $\pm$ 0.001	0.259 $\pm$ 0.726	0	0
III (DEN, DI/CO)	0.307 $\pm$ 0.084 ^a	37.5 $\pm$ 8.9 ^a	0.117 $\pm$ 0.070 ^a	2.0 $\pm$ 1.1 ^a
IV (DEN, std 4°)	0.186 $\pm$ 0.089 ^{b, c}	33.9 $\pm$ 13.6 ^b	0.064 $\pm$ 0.054 ^{b, c}	0.9 $\pm$ 0.8 ^{b, c}
V (DEN, 3°)	0.199 $\pm$ 0.084 ^c	37.6 $\pm$ 11.4	0.054 $\pm$ 0.055 ^c	1.0 $\pm$ 0.9 ^c
VI (DEN, 2°)	0.177 $\pm$ 0.074 ^c	33.6 $\pm$ 12.1	0.045 $\pm$ 0.038 ^c	0.8 $\pm$ 0.6 ^c
VII (DEN, As)	0.185 $\pm$ 0.058 ^c	35.2 $\pm$ 9.9	0.040 $\pm$ 0.030 ^c	0.8 $\pm$ 0.5 ^c
VIII (DEN, high 4°)	0.186 $\pm$ 0.102 ^c	27.2 $\pm$ 12.7	0.032 $\pm$ 0.053 ^c	0.4 $\pm$ 0.4 ^c
IX (DEN, medium 4°)	0.110 $\pm$ 0.072 ^c	111.6 $\pm$ 79.5 ^d	0.023 $\pm$ 0.026 ^c	0.5 $\pm$ 0.5 ^c
X (saline, std 4°, no PH)	0.001 $\pm$ 0.001	0.03 $\pm$ 0.11	0	0
XI (DEN, std 4°, no PH)	0.072 $\pm$ 0.032 ^{e, f}	37.2 $\pm$ 13.1 ^f	0.006 $\pm$ 0.008 ^{e, g}	0.1 $\pm$ 0.2 ^{e, g}

^{*} Total foci area is expressed as foci area (mm²) per unit liver area (cm²).

[†] Number of foci is expressed per unit liver area (cm²).

[‡] Large foci area is expressed as the area of foci with diameters > 0.2 mm (mm²) per unit liver area (cm²).

[§] Number of large foci is expressed as the number of foci with diameters > 0.2 mm per unit liver area (cm²).

^a Significantly different ($P < 0.001$) from group I according to a Student's t-test.

^b Significantly different ($P < 0.001$) from group II according to a Student's t-test.

^c Significantly different ($P < 0.001$) from group III according to one-way ANOVA.

^d Significantly different ($P < 0.001$) from groups III, IV, V, VI, VII, & VIII according to one-way ANOVA.

^e Significantly different ($P < 0.001$) from group IV according to a Student's t-test.

^f Significantly different ($P < 0.001$) from group X according to a Student's t-test.

^g Significantly different ($P < 0.05$) from group X according to a Student's t-test.

Table 6.3. Hepatic S-adenosylmethionine and S-adenosylhomocysteine levels (nmol/gram liver tissue)			
Treatment group	SAM (mean $\pm$ SD)	SAH (mean $\pm$ SD)	SAM/SAH
I (saline, DI/CO)	66.7 $\pm$ 8.7	60.1 $\pm$ 4.8	1.1 $\pm$ 0.10
II (saline, std 4°)	55.4 $\pm$ 11.9	50.8 $\pm$ 9.3	1.1 $\pm$ 0.09
III (DEN, DI/CO)	64.0 $\pm$ 3.8	54.7 $\pm$ 5.9	1.2 $\pm$ 0.1
IV (DEN, std 4°)	63.5 $\pm$ 14.4	57.0 $\pm$ 4.9	1.1 $\pm$ 0.2
V (DEN, 3°)	60.2 $\pm$ 8.6	57.9 $\pm$ 10.4	1.1 $\pm$ 0.2
VI (DEN, 2°)	60.2 $\pm$ 11.0	54.4 $\pm$ 5.9	1.1 $\pm$ 0.1
VII (DEN, As)	62.3 $\pm$ 8.3	60.8 $\pm$ 7.8	1.0 $\pm$ 0.1
VIII (DEN, high 4°)	61.7 $\pm$ 14.1	52.0 $\pm$ 4.8	1.2 $\pm$ 0.2
IX (DEN, medium 4°)	64.2 $\pm$ 9.3	56.1 $\pm$ 9.3	1.2 $\pm$ 0.2
X (saline, std 4°, no PH)	61.8 $\pm$ 7.5	55.5 $\pm$ 8.7	1.1 $\pm$ 0.1
XI (DEN, std 4°, no PH)	67.4 $\pm$ 8.4	49.9 $\pm$ 4.0 ^a	1.3 $\pm$ 0.1 ^{b, c}

^a Significantly different ($P < 0.03$) from group IV according to a Student's t-test.

^b Significantly different ($P < 0.06$) from group IV according to a Student's t-test.

^c Significantly different ($P < 0.02$) from group X according to a Student's t-test.

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CHAPTER 7

ENDOTHELIAL CELL INVESTIGATIONS

7.1 HEPATIC SINUSOIDAL ENDOTHELIAL CELL FUNCTION

The liver is composed of both parenchymal and non-parenchymal cells. Hepatic parenchymal cells (hepatocytes) account for approximately 70% of all the cells in the liver; the remaining 30% consists of Kupffer cells, sinusoidal endothelial cells (SECs), Ito cells, and pit cells (Laskin 1990). Of the non-parenchymal cells, hepatic SECs make up approximately 48% of the total (Laskin 1990). Hepatic SECs line the walls of the hepatic sinusoids, with long, extended processes and fenestrae (pores) arranged in clusters known as “sieve plates”. Hepatic SECs differ from their vascular counterparts in that they do not have basement membranes (Hooper *et al.* 1981). As evidence of their well-developed endocytic capacity, hepatic SECs possess “bristle coated” invaginations in their plasma membrane (Hooper *et al.* 1981). They are capable of actively participating in the clearance of a variety of macromolecules and particles via pinocytic vesicles, lysosomes, and receptor-mediated endocytosis (Smedsrod *et al.* 1990, 1985). In the presence of impaired Kupffer cell function, hepatic SECs may increase their phagocytic capacity (Steffan *et al.* 1986).

7.2 ARSENIC AND ENDOTHELIAL CELL DISEASE

Notably, prolonged exposure to arsenic (As) has been correlated with the development of hepatic angiosarcoma (a malignant cancer originating from hepatic sinusoidal endothelial cells) (Falk *et al.* 1981). Chronic exposure to As also has been associated with a variety of other endothelial cell diseases, including arteriosclerosis and other peripheral vascular disorders, cardiovascular manifestations, and Black Foot Disease (Rahman *et al.* 1999; Hsueh *et al.* 1998; Engel *et al.* 1994). Under normal physiological conditions, such as during physical exercise, endothelial cells may be exposed to local acidotic conditions (D'Arcangelo *et al.* 2000). Endothelial cells may also encounter an acidotic microenvironment under a variety of pathological conditions, including ischemia and subsequent neovascularization, diabetic ketoacidosis, wound healing, respiratory failure, uremia, and tumorigenesis (D'Arcangelo *et al.* 2000). It is therefore plausible that under conditions of microenvironmental acidosis, modulation of As-induced endothelial cell disease, including cancer, may occur. Indeed, the potential for an acidotic microenvironment to somehow modulate the carcinogenic effects of As in hepatic SECs remains unknown and deserves further attention, particularly in light of the lack of understanding surrounding the carcinogenic mode of action of As.

7.3 ROLE OF BIOTRANSFORMATION IN ARSENIC CARCINOGENICITY

As described in Chapter 3, the biotransformation of As entails the serial donation of a methyl group from S-adenosylmethionine (SAM) to a trivalent form of As (Zakharyan and Aposhian 1999; Aposhian 1997; Vahter 1994; Buchet and Lauwerys 1985; Marafante and

Vahter 1984; Vahter 1983). The first step of As biotransformation is reduction of arsenate (As^{V}) to arsenite (As^{III}) (Vahter 1999; Winski and Carter 1995; Vahter 1994; Bertolero *et al.* 1987; Marafante *et al.* 1985; Vahter and Marafante 1985). Via oxidative methylation, As^{III} gains a carbonium ion to form MMA^{V} (monomethylarsonic acid) (Vahter 1999; Vahter 1994; Buchet and Lauwerys 1985; Vahter 1983). The pentavalent As in MMA^{V} is then reduced to the trivalent form in MMA^{III} (monomethylarsonous acid), and formation of MMA^{III} facilitates the addition of a second carbonium ion via oxidative methylation to yield DMA^{V} (dimethylarsinic acid) (Vahter 1999; Vahter *et al.* 1994; Vahter 1983). DMA^{V} is generally considered the endpoint of As biotransformation (Vahter 1999; Vahter 1994; Buchet and Lauwerys 1985).

The methylated metabolites of As may be at least partially responsible for its carcinogenic effects. MMA^{III} is more toxic to Chang human hepatocytes (an hepatic cell line established by HeLa contamination of human liver tissue, tumorigenic in Syrian hamsters) than As^{III} , As^{V} , MMA^{V} , or DMA^{V} (Petrick *et al.* 2000). At concentrations ranging from less than 100 μM to 10 mM, DMA^{V} induced single strand breaks in DNA and DNA-protein crosslinks and induced DNA repair synthesis in human alveolar epithelial type II (L-132) cells (Yamanaka *et al.* 1997a,b; Yamanaka *et al.* 1995; Kato *et al.* 1994; Tezuka *et al.* 1993; Yamanaka *et al.* 1993). DMA^{V} orally administered to rats and mice at concentrations of up to 400 ppm has also been shown to induce pulmonary DNA damage (Brown *et al.* 1997; Okada *et al.* 1994; Yamanaka *et al.* 1994; Yamanaka *et al.* 1989a,b). In addition, studies evaluating the promotional effects of DMA^{V} have revealed increased incidence of cancers of the liver, kidney, thyroid gland (Yamamoto *et al.* 1997; Yamamoto *et al.* 1995), lung

(Yamanaka *et al.* 1996), and urinary bladder (Yamamoto *et al.* 1997; Wanibuchi *et al.* 1997; Yamamoto *et al.* 1995).

Other investigations, however, indicate that As^{III} and/or As^V are the chemical species responsible for the carcinogenic effects of As. In mouse lymphoma cells, both As^{III} and As^V induced chromosomal damage at much lower concentrations (10-14 µg/mL and 1-2 µg/mL, respectively) than MMA^V or DMA^V (2500-5000 µg/mL and almost 10,000 µg/mL, respectively) (Moore *et al.* 1997). As^{III} has also been shown to act as a promoter in the rat lung and kidney. (Shirachi *et al.* 1983; Ishinishi *et al.* 1977). Thus, it remains unclear whether the critical chemical species in the carcinogenicity of As is As^V, As^{III}, or one of the methylated metabolites .

7.4 HYPOTHESES

We hypothesized that 1) As^V, As^{III}, MMA^V, and DMA^V would exhibit differential carcinogenic potencies in rat hepatic SECs, and 2) an acidotic microenvironment would enhance the carcinogenic potential of each compound. To evaluate the carcinogenic potential of each compound in hepatic sinusoidal endothelial cells, *in vitro* cell transformation assays represent a valid short-term approach. In such studies, observation of morphologically transformed colonies or discrete cellular foci, indicative of cell transformation, is correlated with increased carcinogenic potential (Pitot and Dragan 1996). To evaluate the capability of As^V, As^{III}, MMA^V, and DMA^V to induce transformation of cultured endothelial cells under normal physiological pH (7.4) and acidotic pH (7.0), we originally chose rat primary hepatic SECs as our preferred model. Because no hepatic SEC

lines are commercially available (regardless of species), techniques were developed in our laboratory for the isolation and culture of hepatic sinusoidal endothelial cells derived from Fischer 344 rats (described below in Section 7.5.1). As with all primary cell isolates, culture and maintenance of this cell type proved to be particularly challenging, requiring a highly supplemented culture medium with maximum survival times ranging from 24 to 48 hours. Due to the significant decrease in rat hepatic sinusoidal endothelial cell viability at the 24-48 hour time-point, we sought an alternative *in vitro* endothelial cell model. Commercially available human endothelial cell lines include both arterial and venous endothelial cells derived from pulmonary, iliac, and femoral vessels (both arterial and venous), as well as the aorta, vena cava, and umbilical vein. Based on literature reports, human umbilical vein endothelial cells (HUVECs) represent the best available model for *in vitro* studies of endothelial cell transformation (Seki *et al.* 2000; Burger *et al.* 1998; Rhim *et al.* 1998; Cockerill *et al.* 1994; Ide *et al.* 1988) and thus were chosen for our investigations. Basic procedures for culture and maintenance of HUVECs are detailed in Section 7.5.3 below. A first step toward developing an *in vitro* HUVEC transformation model to evaluate the transforming capabilities of As^V, As^{III}, MMA^V, and DMA^V (under both normal and acidotic conditions) required establishing toxicity curves for each compound (under both normal and acidotic conditions). These methods are described in Sections 7.5.4 and 7.5.5 below. Preliminary results and discussion are provided in Sections 7.6 and 7.7, respectively.

7.5 MATERIALS AND METHODS

7.5.1 Hepatic SEC isolation and culture

The following procedure was developed based on a protocol described by the European Collection of Animal Cell Cultures, Centre for Applied Microbiology and Research (1994). A male Fischer 344 rat, 8 to 16 weeks old, was injected intraperitoneally with pentobarbital (65 mg/mL, 60 - 80 mg/kg body weight), and heparin (0.1 mL + 0.1 mL/100 g body weight). Upon reaching an appropriate level of anesthesia, the abdomen was shaved and scrubbed with 70% ethanol. The abdominal cavity was then opened with a U-shaped incision, and the liver reflected cranially to reveal the hepatic portal vein and the caudal vena cava. Two ligatures were placed around the portal vein and one around the caudal vena cava proximal to the right renal vein. The portal vein was catheterized using an 18 gauge 1.16 inch (1.3 x 30 mm) catheter (Insite, San Diego, CA) which was subsequently secured with the preplaced sutures. The caudal vena cava was immediately severed distal to the right renal vein ligature. Following opening of the thoracic cavity, the thoracic portion of the inferior vena cava was severed and the right renal vein ligated with the preplaced suture.

The liver was then perfused with Buffer A (see below) at 37°C (35-40 mL/min; minimum 4-5 mL/g liver) for 7-10 minutes or until the perfusate buffer was free of blood, at which time the perfusate was switched to Buffer B (collagenase buffer; see below) with the same flow rate for perfusion over 5 to 12 minutes at 37°C. The liver was then excised and transferred to a plastic Petri dish. Five mL of 1x phosphate-buffered saline (PBS) were added, Glisson's capsule removed, and the liver gently shaken to release cells. The cell

suspension was filtered through a 100 mesh screen (Sigma, St. Louis, MO) to remove cellular aggregates and tissue debris. The filtrate was washed with approximately 100 mL PBS. Cells were then transferred to 50 mL polycarbonate centrifuge tubes and PBS added to a total volume of 50 mL. After centrifugation of the cell suspension at 50 g (500 rpm) in a swing-out rotor at 4°C for 2 minutes, the cell supernate (enriched with non-parenchymal cells) was transferred to a 50 mL polycarbonate tube (repeated twice).

The supernatant was centrifuged in a swing-out rotor at 800 g (2000 rpm) at 4°C for 10 minutes. Pellets were then collected and pooled into 50 mL polystyrene tubes to which PBS was added to achieve a final volume of 10 mL. Twenty mL of 25% Percoll was gently added to each 50 mL polystyrene tube, followed by 15 mL of 50% Percoll (at the bottom of each tube). Tubes, containing the Percoll layers and non-parenchymal cells, were then centrifuged at 800 g (2000 rpm) in a swing-out rotor at 4°C for 10 minutes. An extremely well-balanced centrifuge was necessary for this step. Upon centrifugation, the non-parenchymal cells were distributed into 5 distinct zones, discernable with careful visual inspection. The second and third zones from the bottom of the centrifuge tube were enriched with Kupffer cells and SECs (total volume of these zones was between 24 and 27 mL). These fractions were collected and transferred to 50 mL polycarbonate tubes. The total volume of each tube was adjusted to 50 mL by addition of PBS; tubes were then gently mixed.

After centrifugation at 800 g (2000 rpm) in a swing-out rotor at 4°C for 10 minutes, the pelleted cells were then resuspended with RPMI cell culture medium (supplemented with glutamine but not fetal bovine serum [FBS]) to a final concentration of $7-9 \times 10^6$ cells/mL.

The cells were seeded into 24-well culture plates at a concentration of 0.8 to 1.0×10^6 cells (SECs + Kupffer cells). In the absence of FBS, SECs will attach poorly to cell culture dishes, allowing for selective attachment of KC. Following incubation at 37°C , $5\% \text{CO}_2$ for 30 minutes to allow selective attachment of Kupffer cells, the non-attached SECs were collected by gentle washing of plates and transfer of wash fluid to centrifuge tubes. Following collection, the SECs were diluted with RPMI to achieve a final concentration of $3\text{-}4 \times 10^6$ cells/mL and seeded at a density of 0.5×10^6 cells on fibronectin-coated 24-well plates. After incubation at 37°C , $5\% \text{CO}_2$ for 45 minutes, the non-attached cells were removed and the remaining monolayers of SECs were washed extensively with RPMI. Finally, RPMI supplemented with 2 mM glutamine, 10% FBS, 5 $\mu\text{g}/\text{mL}$ hydrocortisone, 1 $\mu\text{g}/\text{mL}$ insulin, 10 ng/mL endothelial cell growth factor (ECGF), 100 U/mL Penicillin, 100 $\mu\text{g}/\text{mL}$ Streptomycin, and 100 $\mu\text{g}/\text{mL}$ Gentamicin was added to the wells. Poor survival rates of cultured SECs were attributed to cell detachment, which occurred at approximately 48 hours (maximum confluency of 50 - 60%).

7.5.2 Reagents Used in SEC Isolation and Culture

Buffer A consisted of 145 mM NaCl, 7 mM KCl, and 10 mM HEPES in sterile water (pH 7.5 at 37°C). Buffer B contained 70 mM NaCl, 7 mM KCl, 5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 165 mM HEPES, 1.5% and bovine serum albumin (BSA) (pH adjusted to 7.6 at 37°C). Phosphate-buffered saline (10x; without Ca^{2+} and Mg^{2+}) was prepared with 80.0 g/L NaCl, 2.0 g/L KCl, 29.0 g/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 2.0 g/L KH_2PO_4 . A 1:10 dilution of 10x PBS was performed to achieve 1x PBS. One hundred percent solution of Percoll consisted of 10

mL 10x PBS and 90 mL Percoll (Sigma, St. Louis, MO); pH was adjusted to 7.4. Other Percoll solutions used in the density centrifugation steps of sinusoidal endothelial cell isolation were prepared by adding 1x PBS to achieve the desired concentration.

Fibronectin-coated plates were prepared by coating cell culture plates with a 100 µg/mL solution of bovine fibronectin (Sigma; St. Louis, MO). RPMI used in isolation of sinusoidal endothelial cells contained 2mM L-glutamine. RPMI used for culturing sinusoidal endothelial cells contained 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 100 µg/mL Gentamicin, 1% v:v endothelial cell growth factor, 1 µg/ml insulin, 5 µg/ml hydrocortisone, and 10 mg/mL insulin (all reagents obtained from Sigma, St. Louis, MO).

7.5.3 Human Umbilical Vein Endothelial Cell Culture

Pooled human umbilical vein endothelial cells (HUVECs) and the recommended EGM-2 culture medium (EGM-2) were purchased from Clonetics/BioWhittaker (Charlotte, NC). The EGM-2 culture medium contained human recombinant epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, hydrocortisone, human recombinant insulin-like growth factor, heparin, gentamicin, and amphotericin-B (concentrations proprietary), and 2% FBS. Cells were seeded at a density of 10,000 cells/cm² in cell culture plates and incubated at 37°C, 5% CO₂. Cells reached confluency of 75 - 80% in approximately 48 to 72 hours.

7.5.4 Experimental Exposure Conditions

For assessing the toxicity of arsenical compounds at normal physiological and acidotic pHs, HUVECs (75-85% confluent) were placed in 37°C incubators dedicated to the following conditions: 95% air:5% CO₂ (media pH = 7.00 ± 0.05), or 80% air:20% CO₂ (media pH = 7.40 ± 0.03). Cells were incubated for 24 hours with serum-free EGM-2 medium containing incremental concentrations of As^V (sodium arsenate; Sigma Chemicals, St. Louis, MO), As^{III} (sodium arsenite; Sigma Chemicals, St. Louis, MO), MMA^V (monomethylarsonic acid; Chem Service, Inc., West Chester, PA), or DMA^V (dimethylarsinic acid; Strem Chemicals, Inc., Newburyport, MA). Cell culture medium was collected periodically to confirm pH.

7.5.5 Assessment of Cytotoxicity

HUVECs were rinsed with PBS following removal of serum-free EGM-2 medium containing arsenical test compounds. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (Sigma, St. Louis, MO) was added at a concentration of 0.5 mg/mL in serum-free EGM-2 medium. HUVECs were then incubated at 5% or 20% CO₂, 37°C, for 4 hours, after which time the MTT/EGM-2 medium was removed and the HUVECs gently rinsed with PBS. Following addition of dimethylsulfoxide (DMSO), absorbance of the dissolved formazan crystals was measured at 550 nm. Cytotoxicity in the treatment groups exposed to the arsenical test compounds was expressed as a percentage of cytotoxicity in the untreated control groups.

7.5.6 Statistical Analysis

Results were analyzed using Student's *t*-test; all statistical tests were performed using the Minitab statistical software package, release 11.21 (Minitab, State College, PA).

7.6 RESULTS

Under conditions of 95% air:5% CO₂, simulating a normal physiological pH of 7.4, the mean LC₅₀s of As^V, MMA^V, and DMA^V in HUVECs were 7.1 ± 0.4, 25.1 ± 0.4, and 48.7 ± 3.1 mM, respectively (Table 7.1). At 95% air:5% CO₂, the mean LC₅₀ of As^{III} was 33.0 ± 6.4 μM. Under acidotic conditions (80% air:20% CO₂, pH=7.0), the mean LC₅₀s of As^V, MMA^V, and DMA^V in HUVECs were 10.2 ± 0.9, 15.6 ± 1.4, and 76.6 ± 5.3 mM, respectively. At 20% CO₂, the mean LC₅₀ of As^{III} was 127 ± 24.7 μM, approximately 4 times higher than at 5% CO₂. According to a Student's *t*-test, in HUVECs exposed to As^V, As^{III}, or DMA^V, the LC₅₀s at 20% CO₂ were significantly higher compared to the LC₅₀s for the same compounds at 5% CO₂ (*p* < 0.01). The LC₅₀ of MMA^V at 20% CO₂ was significantly lower than at 5% CO₂ (*p* < 0.01).

7.7 DISCUSSION

In HUVECs, toxicities of the pentavalent As compounds As^V, MMA^V, and DMA^V the trivalent As^{III}, were significantly different at 80% air:20% CO₂ (pH = 7.0) compared to 95% air:5% CO₂ (pH = 7.4). As^{III} was more toxic to HUVECs than any of the pentavalent As compounds, whether under conditions of acidosis (20% CO₂) or normal physiological pH (5% CO₂). As^V, As^{III}, and DMA^V were less toxic to HUVECs under acidotic conditions

(20% CO₂) than under normal physiological pH (5% CO₂). MMA^V was more toxic to HUVECs under acidotic conditions (20% CO₂) than under normal physiological pH (5% CO₂). These data indicate that, consistent with toxicity studies in a variety of other cell lines (Goering *et al.* 1999; Aposhian 1997; Gorby 1994; Morton and Dunnette 1994; Aposhian 1989; Bertolero *et al.* 1987; Hindmarsh and McCurdy 1986), As^{III} is more toxic in HUVECs, by several orders of magnitude, than As^V, MMA^V, or DMA^V. Further experiments are necessary to evaluate the toxicity of the trivalent methylated metabolite MMA^{III} in HUVECs. The data from our studies also indicate that an acidotic microenvironment may modulate the toxicity of As compounds in HUVECs. The mechanism for this effect may involve alterations in endothelial cell apoptotic pathways (D'Arcangelo *et al.* 2000), and additional evaluation of this effect is warranted. Finally, further investigations are necessary to determine whether HUVECs represent a useful *in vitro* cell transformation model for studying the carcinogenic effects of As and its metabolites, under conditions of normal physiological pH and acidosis.

Table 7.1: Toxicity of Arsenic (As) Compounds in HUVECs under Normal and Acidotic Conditions *In Vitro*

As Compound	LC ₅₀ (μM)	
	5% CO ₂	20% CO ₂
As ^V	7,064 ± 397 (5)	10,200 ± 901 * (4)
As ^{III}	33 ± 6.4 (5)	127 ± 24.7 * (4)
MMA ^V	25,086 ± 435 (4)	15,639 ± 1,377 * (5)
DMA ^V	48,740 ± 3,056 (4)	76,597 ± 5,264 * (4)

Values represent the mean ± SD of values for multiple experiments (n).

As^V: arsenate.

As^{III}: arsenite.

MMA^V: monomethylarsonic acid.

DMA^V: dimethylarsinic acid.

* According to a Student's t-test, significantly different from LC₅₀ value for the same compound at 5% CO₂ ($p < 0.01$).

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CHAPTER 8

SUMMARIZING DISCUSSION

Inorganic arsenic (As) ranks among the oldest known human carcinogens, with cancers of the skin, lung, liver, and urinary bladder well-documented in a variety of epidemiological studies of diverse populations around the world. Yet, despite the wealth of evidence indicating its ability to induce cancer in humans, its carcinogenic mode of action remains poorly understood. Until recently, no robust laboratory animal model of its carcinogenicity existed. Some progress has been made in understanding the role of As in dermal carcinogenesis: two mouse skin models have recently been described (Rossman *et al.* 2001; Germolec *et al.* 1998, 1997) which support a cocarcinogenic or promotional role of As in the development of skin cancer. Notably, a recent transplacental carcinogenicity study in mice demonstrated that inorganic As, administered orally via the drinking water, induced tumor formation in multiple organs (Waalkes *et al.* 2003), providing evidence that As is capable of acting as a complete transplacental carcinogen in mice. In their study, Waalkes *et al.* administered sodium arsenite to pregnant C3H mice via the drinking water (*ad libitum*) at concentrations of 0, 42.5, and 85 ppm from day 8 to 18 of gestation. Offspring were born, weaned, and sacrificed at 74 weeks (males) and 90 weeks (females) and

received no additional As treatment beyond that administered during gestation. Among male offspring, dose-related increases in the incidence of hepatocellular carcinomas and adrenal tumors were noted, as well as increased multiplicity of both liver and adrenal tumors. In female offspring, dose-related increases in the incidence of ovarian tumors and lung carcinoma were observed. Inorganic As exposure was also associated with an increase in the incidence of proliferative lesions of the uterus and oviduct. Results from this study are highly significant in that they demonstrate that exposure to inorganic As in the drinking water induced transplacental tumor formation in C3H mice, thus establishing inorganic As as a complete transplacental carcinogen in mice. As such, these results carry important implications regarding its carcinogenic mode of action.

Generally, it has been accepted that inorganic As does not cause point mutations (Weincke *et al.* 1997; Morton and Dunnette 1994; Li and Rossman 1989a,b; Lee *et al.* 1985). Admittedly, some experimental data do suggest that it may act by inducing point mutations, perhaps through the generation of reactive oxygen species (Hei *et al.* 1998; Nordenson and Beckman 1991). However, until now, most experimental data have indicated that inorganic As (or one or more of its metabolites) acts as a promoter, progressor, or cocarcinogen in the multistep process of carcinogenesis. Recall that an initiator is capable of inducing an irreversible, heritable change in a cell's genome under conditions of limited exposure (Mastorides and Maronpot 2002; Pitot and Dragan 1996). A promoter effects the clonal expansion of initiated cells, an outcome which is considered to be both operationally reversible and dependent on continued exposure to the promoting agent (Mastorides and Maronpot 2002; Pitot and Dragan 1996). Progressors are chemical agents capable of

converting a cell in the stage of promotion or an initiated cell into a potentially malignant cell, characterized by greater alteration in the genomic structure of the cell, reduced genomic stability, and greater malignancy. The action of progressors is considered irreversible (Mastorides and Maronpot 2002; Pitot and Dragan 1996). A cocarcinogen does not demonstrate carcinogenic ability alone but is capable of potentiating the effect of a known carcinogen (Robens *et al.* 1994).

In the Waalkes *et al.* (2003) study, the relatively short period of exposure (*i.e.*, an 11-day exposure ending prior to birth) and the very early stage of exposure (*i.e.*, prenatal) provide evidence of a mode of action more suggestive of an initiator rather than a promoter, progressor, or cocarcinogen. Carcinogenic outcomes were associated with a very short period of exposure and displayed neither reversibility over time following cessation of exposure, nor the need for prolonged and continued exposure for effect. Thus, in this transplacental model of carcinogenicity, inorganic As does not appear to act in ways characteristic of a promoting agent. In contrast, other recent studies provide significant evidence that inorganic As may act as a tumor promoter (Rossman *et al.* 2001; Germolec *et al.* 1998, 1997; Brown and Kitchin 1996). Promotional effects have also been observed with dimethylarsinic acid (DMA), a metabolite of inorganic As (Wanibuchi *et al.* 1997; Yamamoto *et al.* 1997; Wanibuchi *et al.* 1996; Yamanaka *et al.* 1996; Yamamoto *et al.* 1995). Additionally, results from the Waalkes *et al.* (2003) study do not support the role of inorganic As as a progressor, as progression is generally a late stage event in carcinogenesis. Some experimental and epidemiological data, however, do indicate that As may exert its carcinogenic effects as a progressor (Mäki-Paakkanen *et al.* 1998; Moore *et al.* 1997;

Rasmussen and Menzel 1997; Yamanaka *et al.* 1997; Lerda 1994; Warner *et al.* 1994; Barrett *et al.* 1989; Lee *et al.* 1986, 1985; Jacobson-Kram and Montalbano 1985; Nagymajtenyi *et al.* 1985; Nordenson *et al.* 1979, 1978; Beckman *et al.* 1977; Paton and Allison 1972). Some data also indicate that inorganic As may act as a cocarcinogen (Hu *et al.* 1998; Lee-Chen *et al.* 1993, 1992; Li and Rossman 1989a,b; Okui and Fujiwara 1986), but characterization of it as such based on the results of the Waalkes *et al.* (2003) study is not possible due to lack of coadministration of another known carcinogen in addition to inorganic As. Clearly, the specific effects of inorganic As in the carcinogenic process remain an enigma, particularly with respect to its carcinogenic mode(s) of action. Recent animal models, however, provide valuable information. Results from the Waalkes *et al.* (2003) study offer particularly compelling evidence for inorganic As as a transplacental carcinogen.

In humans, *in utero* exposure to certain agents has been associated with an increased risk of cancer in childhood and early adulthood (Anderson *et al.* 2000). Among these, ionizing radiation (Wakeford 1995; Mole 1990) and diethylstilbesterol (Herbst *et al.* 1971) are the most well-established. In laboratory animals, a variety of research has demonstrated that gestation is often a period of unique susceptibility to chemical carcinogens. Transplacental carcinogenic events have been associated with a variety of agents in laboratory animals including ionizing radiation (Anderson *et al.* 2000; Benjamin *et al.* 1998, 1991; Sasaki 1991; Morris *et al.* 1983; Sasaki *et al.* 1978), dimethylbenzanthracene (Anderson *et al.* 2000; Morris *et al.* 1983; Goerttler *et al.* 1980), ethylnitrosourea (Anderson *et al.* 2000; Branstetter *et al.* 1987; Diwan and Meier 1974; Vesselinovitch 1973), 3-methylcholanthrene

(Miller *et al.* 2000), diethylnitrosamine (Anderson *et al.* 2000; Mohr *et al.* 1995), lead (Waalkes *et al.* 1995), cisplatin (Diwan *et al.* 1995, 1993), and nickel (Diwan *et al.* 1992).

Regardless of the species, the susceptibility of the embryo or fetus to transplacental carcinogenic effects may be determined by a variety of factors. Fundamentally, the success of a transplacental carcinogenic agent may be dependent on the number of target cells at risk (Anderson *et al.* 2000), suggesting that the later stages of gestation may be at highest risk, because it is then when the population of susceptible cells is largest. In other words, the greater the number of potential target cells, the greater the probability that a mutational carcinogenic event (initiating event) will occur. If, as suggested by the results of the Waalkes *et al.* (2003) study, inorganic As acts as an initiator, the number of target cells at risk would likely be important, implicating the later stages of gestation as particularly vulnerable. The average murine gestation period ranges from 18 - 21 days, and exposures in the Waalkes *et al.* study spanned gestation days 8-18. Further experimentation, particularly during the later stages of gestation, is necessary to determine the critical time windows for the transplacental carcinogenic effects of inorganic As.

In addition to the number of cells at risk, the rate of cell division may also influence transplacental carcinogenic processes. The greater the rate of mitosis, the more likely a genomic mutation will become “fixed” by replication before it can be repaired. During periods of high cell division, a genomic mutation is more likely to result in cellular initiation. Thus, if inorganic As acts as an initiator, the time windows of highest cell division rates and the corresponding tissues/organs would be expected to be most susceptible to its transplacental carcinogenic effects. Furthermore, because many of the genes expressed

during gestation are important for regulating cell proliferation and differentiation, (*e.g.*, protooncogenes), mutation in these genes could further enhance clonal expansion of initiated cells.

Susceptibility to transplacental carcinogens during development may also be affected by embryonic or fetal capacity for DNA repair (Anderson *et al.* 2000). Some DNA repair enzymes, such as O⁶-alkylguanine-DNA alkyltransferase, which is responsible for repairing promutagenic adducts caused by DNA alkylating agents, may be less active in fetal compared to adult tissue (Coccia *et al.* 1988; Chen *et al.* 1983). Other DNA repair enzymes, however, such as 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (active in the repair of oxidative lesions of guanosine triphosphate), occur at higher levels in fetal tissue than in maternal (Bialkowski *et al.* 1999). If inorganic As acts as a cocarcinogen by inhibiting DNA repair, its cocarcinogenic effects could be augmented by decreased expression of critical DNA repair enzymes in embryonic or fetal tissues. Clearly, more mechanistic data are required regarding the specific DNA repair enzymes and pathways potentially affected by inorganic As.

Another important factor affecting susceptibility of the embryo or fetus to transplacental carcinogenic agents is the development of differentiated characteristics, such as the ability to biotransform xenobiotics (Anderson *et al.* 2000), significant in terms of both metabolic activation as well as detoxification. In the case of inorganic As, the relative rates of methylation in fetal versus adult tissue may influence transplacental carcinogenic outcomes. Substantial experimental data implicate the process of inorganic As biotransformation as playing an important role in its carcinogenicity, whether by production

of key carcinogenic metabolites or simply as a by-product of the biotransformation process itself. A variety of studies have provided evidence that the methylated metabolites of inorganic As may be involved in the carcinogenic process (Thomas *et al.* 2001; Petrick *et al.* 2000; Goering *et al.* 1999; Wanibuchi *et al.* 1997; Yamamoto *et al.* 1997; Wanibuchi *et al.* 1996; Yamanaka *et al.* 1996; Yamamoto *et al.* 1995), although the precise nature of their role is not completely understood. Different production rates of methylated metabolites of inorganic As due to differences in fetal/embryonic biotransformation processes could result in accumulation of potentially carcinogenic metabolites. Furthermore, the biotransformation process itself has also been implicated in modulating the carcinogenic effects of inorganic As, perhaps via perturbation of various cellular and genomic methylation processes (Chen *et al.* 2001a,b; Goering *et al.* 1999; Mass and Wang 1997; Mass 1992). Recent data indicate that dietary methyl supplementation in pregnant mice increases genomic methylation and subsequently alters phenotypic expression of hair coat color in offspring (Waterland and Jirtle 2003). Similarly, perturbation of genomic methylation patterns during gestation due to differential inorganic As biotransformation capabilities between embryo/fetus versus adult could potentially modulate carcinogenic outcomes in offspring. Interpretation of the role of inorganic As in transplacental carcinogenesis is difficult due to lack of mechanistic information as well as lack of definitive data indicating the biotransformation product(s) responsible for its carcinogenic effects.

In our laboratory, we hypothesized that inorganic As would act in concert with other known carcinogens in a synergistic or at least additive fashion in the carcinogenic process. An initial study using an environmentally relevant quaternary mixture consisting of inorganic

As, 1,2-dichloroethane (DCE), vinyl chloride (VC), and trichloroethylene (TCE) attempted to induce hepatic angiosarcomas in male Fischer 344 rats, based on a protocol described by Luquette *et al.* (1985), by exposing them to the quaternary mixture for 16 weeks. Individual chemical exposures were also performed. Although no tumors were induced in any of the treatment groups, the results presented in Chapter 4 demonstrate dilatation of hepatic sinusoids, an early change reported in association with angiosarcoma development (Popper *et al.* 1981, 1978), in rats exposed to drinking water containing 75 ppm As or 300 ppm DCE. These results provide important histological evidence of early changes in the liver which previously have been correlated with eventual development of hepatic angiosarcoma.

In a second study (Pott *et al.* 1998), the potential for inorganic As to enhance the carcinogenic effects of DCE, VC, and TCE was investigated utilizing a multistep multi-organ carcinogenesis bioassay. Surprisingly, the quaternary mixture (consisting of known carcinogens) demonstrated a dose-dependent antagonistic effect on the development of hepatic preneoplastic lesions and pulmonary preneoplastic and neoplastic lesions. Compared to livers of initiated treatment control rats, livers of rats exposed to the quaternary mixture exhibited a significant decrease in the area and number of all altered hepatocellular foci (AHF), as well as the area of glutathione-S-transferase π (GST-P) positive foci in a dose-dependent manner (Chapter 5). Additionally, exposure to the quaternary mixture was associated with a decrease in the frequency of pulmonary bronchioalveolar hyperplasia and adenoma/adenocarcinoma formation.

To assess whether the antagonistic effect on the development of GST-P positive hepatic foci was a function of the quaternary mixture, a submixture thereof, or simply

inorganic As alone, a third study utilizing a medium-term liver foci bioassay model was performed. As described in Chapter 6, exposure to the quaternary (As, DCE, TCE, VC), ternary (As, DCE, TCE), or binary (As, DCE) mixture, or to As alone significantly decreased the total number and area of hepatic GST-P positive foci compared to controls. These results indicate that inorganic As alone is responsible for the suppressive effect on hepatic preneoplastic lesion development. Clearly, in order to understand the role of inorganic As in suppressing the development of preneoplastic hepatic foci, it is essential to understand the kinetics of its methylation as well as the carcinogenic potential of the different methylated metabolites.

As a preliminary step toward evaluating the differential effects of inorganic As and its metabolites, the toxicities of arsenate (As^{V}), arsenite (As^{III}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}) were investigated in cultured human umbilical vein endothelial cells (HUVECs). As reported in Chapter 7, As^{III} is more toxic in HUVECs than As^{V} , MMA^{V} , or DMA^{V} , consistent with findings in toxicity studies using other cell lines (Goering *et al.* 1999; Aposhian 1997; Gorby 1994; Morton and Dunnette 1994; Aposhian 1989; Bertolero *et al.* 1987; Hindmarsh and McCurdy 1986). Under the influence of microenvironmental acidosis (such as occurs during tumorigenesis), As^{V} , As^{III} , and DMA^{V} were less toxic to HUVECs than under normal physiological pH. MMA^{V} , however, was more toxic to HUVECs under acidotic conditions. Additional experiments to evaluate the toxicity of the trivalent methylated As metabolite monomethylarsonous acid (MMA^{III}) in HUVECs are necessary for a complete understanding of the differential effects of inorganic As and its metabolites in HUVECs.

In considering the carcinogenic mode of action of inorganic As, its anticarcinogenic properties must also be acknowledged. Over the past decade, arsenic trioxide has emerged as a chemotherapeutic agent for the treatment of acute promyelocytic leukemia. In fact, the National Cancer Institute is involved in a variety of clinical studies to assess the efficacy of arsenic trioxide in a variety of hematologic malignancies as well as other cancers (Murgo 2001). The mechanism underlying arsenic trioxide's chemotherapeutic effects appears to involve inhibition of cellular proliferation, induction of apoptosis, and promotion of cellular differentiation in neoplastic cells (Murgo 2001; Roboz *et al.* 2000; Bazarbachi *et al.* 1999). Notably, in terms of the growth dynamics of preneoplastic foci, the suppressive effect of inorganic As on the development of GST-P positive hepatic foci reported herein may be tied to decreased proliferation and/or increased apoptosis of hepatocytes. Inorganic As has also been reported to induce cellular senescence *in vitro* (Liao *et al.* 2001), but the mechanism underlying this effect is unknown. The suppressive effects noted in our *in vivo* studies may be related to the effects observed *in vitro*.

In conclusion, reconciliation of the apparently paradoxical nature of inorganic As rests in an improved understanding of its pharmacokinetics as well as its mechanistic effects at the cellular and molecular levels. The pharmacokinetics of inorganic As may differ to varying degrees among species and even among the different life stages of a given species. Such differences could result in variable target tissue concentrations of inorganic As and its metabolites. Additionally, the kinetics of inorganic As biotransformation may be affected by a variety of experimental (*e.g.*, partial hepatectomy) and perhaps some naturally-occurring (*e.g.*, nutritional status or stage of gestation) conditions. Such changes could alter target

tissue concentration profiles of inorganic As and its metabolites and, depending on the mechanism of action of the metabolite present, could ultimately result in differential carcinogenic or anticarcinogenic effects. Clearly, more complete knowledge of the pharmacokinetic behavior of inorganic As will improve our understanding of its concentration (and that of its metabolites) in various target tissues. In turn, this information may illuminate our interpretation of its differential effects among animal species and life stages, and in carcinogenic and anticarcinogenic pathways. Enhanced pharmacokinetic knowledge alone, however, is not sufficient; equally important is a more refined understanding of how inorganic As and its metabolites interact with cellular and molecular targets. Such information may elucidate factors underlying different carcinogenic effects among species or life stages. It will most certainly improve our understanding of its carcinogenic and anticarcinogenic effects. With continued investigations, improved knowledge in both pharmacokinetic and mechanistic arenas will enable the development of more accurate and reliable risk assessment strategies for arsenic.

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