DEPARTMENT OF THE ARMY
US ARMY PUBLIC HEALTH COMMAND (PROVISIONAL)
5158 BLACKHAWK ROAD
ABERDEEN PROVING GROUND, MD 21010-5403

MCHB-TS-THE

21 July 2010

MEMORANDUM FOR Cleanup Division (IMAE-CDP/Mr. James Daniels), US Army Environmental Command, 5179 Hoadley Road, Aberdeen Proving Ground, MD 21010-5401

SUBJECT: Toxicology Report No. 87-XE-08WR-09, Studies on Metabolism of 1,4-Dioxane, March 2010

1. Five copies of the final report are enclosed.

2. Please contact us if this report or any of our services did not meet your expectations.

3. The US Army Public Health Command (Provisional), formerly the US Army Center for Health Promotion and Preventive Medicine, point of contact is Dr. William Eck, Directorate of Toxicology, Health Effects Research Program. He may be contacted at DSN 584-7169 or commercial 410-436-7169.

FOR THE COMMANDER:

Encls

CINDY A. LANDGREN
LTC, VC
Director, Toxicology

U.S. Army Public Health Command (Provisional)

USAPHC

TOXICOLOGY REPORT NO. 87-XE-08WR-09
STUDIES ON METABOLISM OF 1,4-DIOXANE
MARCH 2010

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14. ABSTRACT

1,4-Dioxane was added as a stabilizer to the banned degreasing solvent 1,1,1-trichloroethane, and still has uses in the manufacture of small arms ammunition. Due to past and current uses, dioxane is of concern to the Army with regard to both pollution prevention and remediation of past use. The U.S. Environmental Protection Agency classifies dioxane as a probable human carcinogen based upon animal studies. One of the most important data gaps with respect to dioxane is its potential mode of action in causing cancer. Past work has identified two potential metabolites of dioxane, but definitive studies on the relationship of these possible metabolites to one another in living systems and their possible relation to carcinogenesis have remained unanswered. This report provides information supporting 2-hydroxyethoxyacetic acid (HEAA) as being the principal metabolite, and provides the first quantitative data on the chemical equilibrium between HEAA and the other potential metabolite--dioxanone. Suggestions for future research are included.

15. SUBJECT TERMS

1,4-dioxane, dioxanone, 2-hydroxyethoxyacetic acid, HEAA, dioxane metabolites, cancer

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Toxicology Report No. 87-XE-08WR-09, Oct 07-Mar 10

Sponsor

U.S. Army Environmental Command
Aberdeen Proving Ground, MD 21010

Study Title

Toxicology Study No. 87-XE-08WR-09
Studies on Metabolism of 1,4-Dioxane

Author

William S. Eck, Ph.D.

Study Completed

March 2010

Performing Laboratory

U.S. Army Public Health Command (Provisional)
(formerly U.S. Army Center for Health Promotion and Preventive Medicine)
Directorate of Toxicology
Health Effects Research Program
MCHB-TS-THE
Aberdeen Proving Ground, MD 21010-5403

Toxicology Report No. 87-XE-08WR-09, Oct 07-Mar 10

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DEPARTMENT OF THE ARMY
US ARMY PUBLIC HEALTH COMMAND (PROVISIONAL)
5158 BLACKHAWK ROAD
ABERDEEN PROVING GROUND, MD 21010-5403

MCHB-TS-THE

EXECUTIVE SUMMARY
TOXICOLOGY REPORT NO. 87-XE-08WR-09
STUDIES ON METABOLISM OF 1,4-DIOXANE
MARCH 2010

1. PURPOSE.

a. 1,4-Dioxane is a water-miscible, semi-volatile organic compound that contaminates groundwater at many military installations. Dioxane was used as a stabilizer for the degreaser 1,1,1-trichloroethane, use of which has been banned under the Montreal Protocol but whose past use has left a remediation legacy. Dioxane is currently used in the manufacture of small arms ammunition and is present in smaller quantities in many chemical formulations. The U.S. Environmental Protection Agency (USEPA) characterizes dioxane as a "probable human carcinogen" based upon animal studies.

b. The U.S. Army is facing substantial environmental restoration liability associated with remediation of dioxane-contaminated soils and groundwater. The USEPA has not established a maximum contaminant level (MCL) for dioxane but has calculated a carcinogenicity slope factor of 1.1×10^{-2} milligrams per kilograms per day (mg/kg-d) and a drinking water unit risk of 3.1×10^{-7} micrograms per liter (µg/L). Some individual states have established standards for drinking water with California's being the strictest at 3 µg dioxane/L. Inhalation standards have not been established.

c. This research had two objectives: 1) to determine the parameters of the equilibrium between the hypothesized dioxane metabolites 1,4-dioxan-2-one (dioxanone) and 2-hydroxyethoxyacetic acid (HEAA), including pH-dependence, and 2) to determine the presence and relative quantity of dioxanone and HEAA in blood and urine of rats dosed with 1,4-dioxane by gavage. Previous studies have variously reported the excreted metabolic product to be one or both of these compounds. Dioxanone has been implicated as the potential carcinogenic agent on the basis of computational modeling. Absence of dioxanone from the blood or urine will indicate that its residence time in the body is short, and the lactone is unlikely to play a role in carcinogenesis in humans. Results of this research are intended to provide information relevant to the determination of scientifically safe drinking water and groundwater remediation standards.

2. CONCLUSIONS.

a. Metabolic products. This study supports the finding that dioxanone is not the primary urinary excretion product of dioxane. It also provides the first physico-chemical of the evaluation of the equilibrium between dioxanone and HEAA, along with the relevant equilibrium constants.

b. Mode of Action. The chemical instability of dioxanone suggests that it is not likely to be involved in the mode of action for carcinogenesis of dioxane. Since HEAA is not believed to be carcinogenic, and in any event is rapidly excreted by the body, another mode of action for dioxane carcinogenesis must be evaluated. It seems likely that the true oncogenic substance in this case is dioxane itself, not a metabolic product. This line of argument is also supported by the following observations:

(1) Rodents demonstrate saturation of dioxane metabolism and develop neoplasms at concentrations above levels that saturate their metabolic capability.

(2) Humans metabolism of dioxane has not been shown to become saturated, and neoplasms have not been observed in humans. Extraordinarily large doses of dioxane are capable of causing toxicity in humans, but this chemical toxicity has not been found to result in neoplasm development.

3. RECOMMENDATIONS. It appears that future investigation of the metabolites is unlikely to provide much insight into either the mode or mechanism of action with respect to possible human carcinogenicity. At this point, a likely avenue of advance would be to investigate the impact of dioxane on the organism's metabolic pathways in the liver using commercially available microarray chips that permit investigation of the relative expression of genes after exposure of the animal. Such experiments should provide insight into how the animal (the liver in this case) is responding at a molecular level to treatment with dioxane. Such mechanistic data will provide insight into mechanisms of how dioxane exerts effects within the organism.

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TOXICOLOGY REPORT NO. 87-XE-08WR-09
STUDIES ON METABOLISM OF 1,4-DIOXANE
MARCH 2010

1. REFERENCES. See Appendix A for a listing of references used in this report.

2. PURPOSE. The objective of this research was to determine the presence and relative quantity of two projected dioxane metabolites, 1,4-dioxan-2-one (dioxanone) and 2-hydroxyethoxyacetic acid (HEAA) in blood and urine of rats dosed with 1,4-dioxane by gavage, and to determine the chemical parameters of the equilibrium between the two hypothesized metabolites. Previous studies have variously reported the excreted metabolic product to be one or both of these compounds. Dioxanone has been implicated as the potential carcinogenic agent on the basis of computational modeling. Absence of dioxanone from the blood or urine will indicate that its residence time in the body is short, and that it is unlikely to play a role in carcinogenesis in humans. Results of this research are intended to provide information relevant to the determination of scientifically safe drinking water and groundwater remediation standards.

3. AUTHORITY. This study was conducted at the request of the U.S. Army Environmental Command and funded via MIPR No. AEC MIPR8BDAT48068.

4. BACKGROUND.

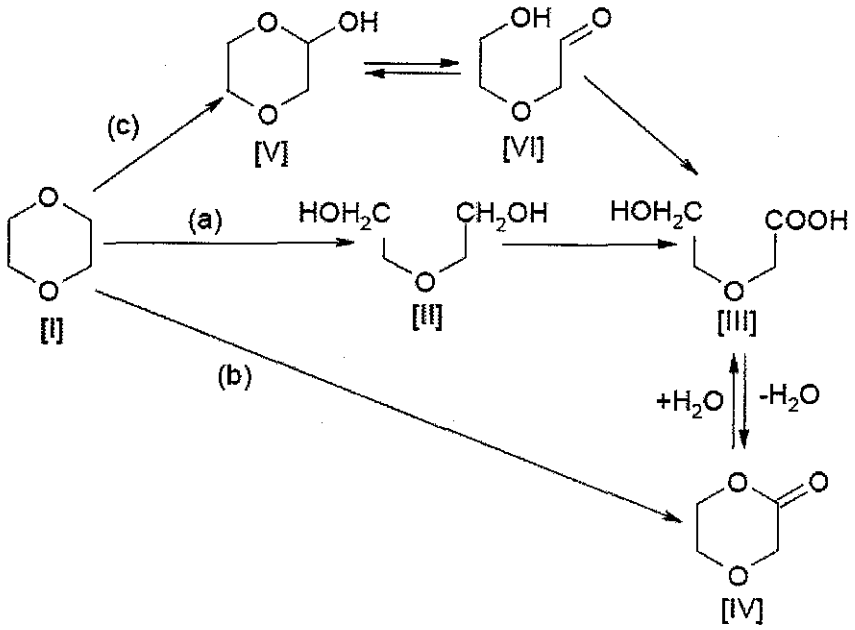
a. General. 1,4-Dioxane (Chemical Abstract Service Registry Number (CASRN): 123-91-1) is characterized by the U.S. Environmental Protection Agency (USEPA) as a "probable human carcinogen" based upon animal studies. It is a water-miscible, semi-volatile organic compound that contaminates groundwater at many military installations as a result of the use of chemical degreasers, notably 1,1,1-trichloroethane (TCA), that contained dioxane as a stabilizer. Dioxane is still used in the manufacture of small arms ammunition and is present in small quantities in many chemical formulations. A key factor in the debate surrounding carcinogenicity of dioxane relates to its metabolism. Dioxane metabolites were identified as a potential source of the carcinogenic behavior by Argus and coworkers in 1973 (Argus *et al.* 1973).

b. Metabolites.

(1) Two separate, but related, compounds have been identified as the likely products of dioxane metabolism—2-hydroxyethoxyacetic acid (HEAA or β-hydroxyethoxyacetic acid) and 1,4-dioxan-2-one (or dioxanone). Dioxanone and HEAA are related chemically as indicated in Figure 1—HEAA is simply the open-chain form of dioxanone.

Use of trademarked name(s) does not imply endorsement by the U.S. Army but is intended only to assist in identification of a specific product.

(2) Hoch-Ligeti and coworkers (Hoch-Ligeti *et al.* 1974) originally proposed that dioxane was metabolized to oxalic acid by way of diglycolic acid. This proposal was based upon chemical oxidation experiments on dioxane using nitric acid originally conducted by Fairley and coworkers (Fairley *et al.* 1936) but was later questioned by Wiley and coworkers (Wiley *et al.* 1938) who found that administration of 1,4-dioxane did not result in increased urinary excretion of oxalic acid.



I = 1,4-dioxane; II = diethylene glycol; III = β-hydroxyethoxy acetic acid (HEAA); IV = 1,4-dioxane-2-one; V = 1,4-dioxane-2-ol; VI = β-hydroxyethoxy acetaldehyde

Figure 1. Proposed Dioxane Metabolism Pathways

(3) Early reports from Woo and coworkers (Woo *et al.* 1977a; Woo *et al.* 1977b) proposed that 1,4-dioxan-2-one (dioxanone; Figure I, structure IV) was the primary metabolite. Urine samples were obtained from male Sprague-Dawley rats that received intraperitoneal injections of dioxane at doses between 50 and 400 milligrams per 100 grams (mg/100 g) body weight. Collected over a period of 2 days, the urine samples (of unspecified volume) were preserved by addition of 0.2 mL glacial acetic acid. Samples were then treated with kaolin, filtered through Whatman No. 42 filter paper, and the pH of the filtrate adjusted to between 4.0 and 4.5. Gas chromatography revealed the presence of a compound that eluted after 32 minutes on a Porapak™ Q or QS column. The presence of this proposed metabolite was pH-dependent—no metabolite was detected if the pH was above 12, but reacidification of the solution brought about the reappearance of the metabolite. Administration of diethylene glycol to rats also produced this metabolite, but it was completely excreted by the animal in less than 20 hours, suggesting diethylene glycol might represent an intermediate in the metabolism

of dioxane. Treatment of rats with diglycolic acid, ethylene glycol, and oxalic acid did not produce the metabolite, suggesting these compounds are not intermediates in the catabolism of dioxane. The proposed metabolite was isolated and analyzed by gas chromatography-mass spectrometry (GC-MS), infrared (IR), and nuclear magnetic resonance (NMR) spectrometry. A parent ion with mass/charge ratio of 102, corresponding to the molecular weight of dioxanone was observed by mass spectrometry. Attempts to confirm this molecular weight via cryoscopy and the Rast method gave inconsistent values between 147 and 565 grams per mole (g/mol). This variability in molecular weight was also noted to potentially be consistent with identification of the metabolite as dioxanone, since dioxanone will polymerize on standing. A reference standard of dioxanone procured from Aldrich Chemical Company gave IR, NMR, and GC-MS spectra identical with the metabolite. In studies where mixed function oxidases were induced by pretreatment with phenobarbital, Aroclor® 1254 or 3-methylcholanthrene, phenobarbital was reported to significantly increase the total amount of metabolite excreted and reduced the time for peak excretion. Similar results were obtained with Arochlor 1254, but 3-methylcholanthrene had no effect. Pretreatment with cobalt(II) chloride, which suppresses expression of mixed function oxidases, was reported to decrease the production of metabolite (Woo *et al.* 1977a; Woo *et al.* 1977b). (Porapak™ is a registered trademark of Waters Corporation; Aroclor® Monsanto Chemical Company.)

(4) In direct contradiction to the position taken by Woo and coworkers, research by Braun and Young (Braun and Young 1977) asserted that β-hydroxyethoxyacetic acid (HEAA, Figure I, structure II) was the primary metabolite. This group conducted an experiment where rats were administered ^{14}C-labelled dioxane and the urinary metabolites analyzed by thin-layer chromatography (TLC). The initial TLC work indicated the presence of one major and two minor radioactively-labeled compounds. The two minor components were identified as dioxane and diethylene glycol; the major component was identified as HEAA. In an alternative development system, HEAA separated upon chromatography into two separate spots, reportedly corresponding to HEAA and dioxanone.

(5) Braun (Braun 1977) published a method for simultaneous determination of dioxane and HEAA in plasma and urine. In this method, HEAA was converted to the methyl ester and analyzed by gas chromatography. The method was reported to have a detection limit of 0.07 micrograms per gram (µg/g) for 1,4-dioxane in both urine and plasma and 0.1 and 0.5 µg/g of HEAA in urine and plasma, respectively. Plant workers exposed to dioxane vapors at an average concentration of 1.6 parts per million (ppm) dioxane for a period of 7.5 hours were found to excrete both HEAA and dioxanone in urine samples collected at the end of the work day. Conversion to HEAA was extensive, with the urinary ratio being 118:1 for HEAA:dioxane (Young *et al.* 1975).

c. Carcinogenicity.

(1) Correct identification of the metabolite is of some consequence, since in addition to the assertions made by Argus' group, quantitative structure-activity relationship (QSAR) modeling by Blake (Blake 1995) and Gombar (Gombar 1995) suggested that dioxanone is carcinogenic, but HEAA is not. Because of this uncertainty over the identification of the true dioxane metabolite and the potential significance of the answer to determining the mode of action for dioxane, understanding the relationship between these two metabolites remains one of the primary unresolved issues in dioxane carcinogenesis (DeRosa *et al.* 1996).

(2) Dioxane was first reported to be carcinogenic by Argus and coworkers (Argus *et al.* 1965). Rats were orally dosed with 1 percent dioxane in drinking water for a period of 63 weeks, euthanized, and evaluated histopathologically. These animals were estimated to have consumed 1.32×10^5 milligrams (mg) of dioxane over the course of 63 weeks; the estimated dose rate was 600 milligrams per kilograms per day (mg/kg-d). At the end of the experiment, six rats were found to have hepatocarcinomas, while one had additional tumors of the kidney and leukemia, the latter two cancers judged to not be treatment-related. The paper went on to hypothesize that the mode of action for tumor induction by dioxane involved denaturation of cellular macromolecules with resulting changes in morphology of endoplasmic membranes causing changes in the conformation, and thus, activity of individual enzymes.

(3) In a follow-on study (Hoch-Ligeti *et al.* 1970), rats received dioxane orally in their drinking water for a period of 13 months at concentrations of 0.75, 1.00, 1.40, or 1.80 percent. Tumors of the nasal cavity, determined to be squamous cell carcinomas, developed in six rats; liver carcinomas were also present in four animals.

(4) Hoch-Ligeti and Argus (Hoch-Ligeti and Argus 1970) administered dioxane in drinking water to 22 male guinea pigs over a 23-month period. The concentration of dioxane was regulated to between 0.5 percent and 2.0 percent, so that the normal growth of the guinea pigs was maintained. The total amount of dioxane received by the animals ranged from 588 to 635 grams over the experimental period. The investigators found hyperplasia and nodular mononuclear infiltration in the lungs of 9 animals. Two animals were found to have carcinoma of the gall bladder, three animals had early hepatomas, and one had adenoma of the kidney. No tumors of the nasal cavity were observed.

(5) Involvement of a dioxane metabolite in the carcinogenicity of dioxane was first suggested by Argus and coworkers (Argus *et al.* 1973) in 1973. This conclusion was based on the observation that the lethal dose (LD_{50}) for dioxane in rats decreased from 5.60 ± 0.06 grams per kilogram (g/kg) in animals receiving oral dioxane alone to

5.18 ± 0.06 g/kg in rats treated with 10 mg of 3-methylcholanthrene, an inducer of liver degradative enzymes. This decrease was statistically significant at the $p<0.001$ level.

(6) King and coworkers (King *et al.* 1973) found evidence that dioxane was a promotor of tumor activity in mice. Groups of 30 male and 30 female Swiss-Webster mice received a dermal application of dioxane in acetone. Mice in the promotion study were treated with 50 micrograms (µg) dimethylbenzanthracene 1 week prior to administration of dioxane. Tumors were observed in skin, lung, and kidney. In a companion oral study, Osborne-Mendel rats (35/sex/group) and B6C3F1 mice (50/sex/group) were exposed to either 0.5 percent or 1.0 percent dioxane in drinking water for a period of about 42 weeks. While no mice were observed to have either lung or liver tumors from the oral dosing experiment at the end of the experimental period, significant numbers of both lung and liver neoplasms, as well as significant mortality, were observed in the rats.

(7) A seminal study in the research on dioxane was conducted by Kociba and coworkers (Kociba *et al.* 1974). Groups of Sherman rats were dosed with 0, 0.01, 0.1, or 1.0 percent dioxane in their drinking water for up to 716 days. Rats at the highest doses had significantly greater numbers of hepatocellular carcinomas but showed no significant increase in renal carcinoma and only three nasal carcinomas. Rats receiving 1 percent dioxane exhibited greatly increased mortality beginning in the 4th month of the study, until only 3 animals survived at the end of the study. This high mortality rate indicates the maximum tolerated dose was exceeded, which under current guidelines, is inappropriate for a long-term cancer study (EPA 2005). Animals at lower dose rates showed survivability comparable to the controls. Rats receiving dioxane at 0.1 or 1.0 percent showed histopathological evidence of renal tubule regeneration, but there were no such effects at the 0.01 percent dose level. A companion study (Torkelson *et al.* 1974) of inhalation exposure of rats at a single concentration of 111 ppm for a period of 2 years found no increase in hepatic or other carcinomas compared to control animals.

(8) Some studies have suggested there is a threshold, at least in rats, for the development of carcinogenicity and the possibility that detoxification mechanisms can be saturated. Young and Gehring (Young and Gehring 1975) treated rats with ^{14}C-labeled dioxane at dosages of 10, 100, or 1000 mg dioxane/kg. They found that as the dosage increased, the blood concentration of dioxane and the concentration of unchanged dioxane in expired air increased disproportionately with the increase in dose. In a follow-on study reported the next year, this group (Young *et al.* 1975) found that as the dosage of dioxane increased, proportionally less dioxane was metabolized to the product HEAA and more was excreted as un-metabolized dioxane. This result suggested both that the metabolism of dioxane had a threshold and could, therefore, become saturated, and that toxicity of dioxane occurred only when the pathway became saturated.

(9) Stott and coworkers evaluated dioxane for impact on rat DNA synthesis, hepatocyte DNA repair, and Ames mutagenicity. Treatment of rats with 1 g/kg-d of dioxane in drinking water for 11 weeks resulted in significant increases in both hepatic and renal DNA synthesis. Treatment-related histopathological changes were observed in treated animals. Negative results were obtained in *in vitro* Ames mutagenicity and hepatocyte DNA repair tests. These results indicate dioxane's mode of action for cancer production in the rat does not involve a genetic mode of action (Stott *et al.* 1981).

(10) An early study of dioxane carcinogenicity administered in drinking water to rats had noted the presence of squamous cell carcinomas (Hoch-Ligetti *et al.*, 1970b). Because of the absence of this type of tumor in other species tested, it was suspected to be a unique response of the rat to this form of dose administration. Goldsworthy and coworkers examined chemically-induced DNA repair in nasal turbinate epithelial cells and hepatocytes of male Fischer-344 rats. No DNA repair activity was observed in either hepatocytes or nasal turbinate cells of rats exposed to up to 2 percent dioxane in drinking water or a single oral dose of up to 1000 mg/kg for a period of 1 week; DNA repair was only observed after 2 weeks of treatment. Reexamination of pathology slides from the National Toxicology Program (NTP) bioassay revealed that the primary site of nasal tumor formation was the anterior third of the dorsal meatus, supporting the hypothesis that these nasal tumors result from deposition of inhaled dioxane-contaminated drinking water. No increase in cell proliferation was noted in cells obtained from the nasal turbinates isolated from rats exposed to 1 percent dioxane in drinking water for a period of 1 week (Goldsworthy *et al.* 1991).

(11) Japanese researchers have recently repeated the oral and inhalation studies conducted by the group at Dow Chemical in the late 1970s. Kano and coworkers (Kano *et al.* 2009; Yamazaki *et al.* 1994) administered dioxane in drinking water to rats and mice at concentrations of 0, 200, 1000, and 5000 ppm in rats and 0, 500, 2000, and 8000 ppm in mice. The highest dose levels were claimed to not exceed the maximum tolerated dose. Over the course of the 2-year study, rats were found to develop nasal squamous cell carcinomas in females and both hepatocellular adenomas and carcinomas in males and females. In mice, there was a significant induction of hepatocellular tumors in both males and females, and two rare nasal tumors in the 8000 ppm dose group were attributed to dioxane exposure.

(12) Kasai and coworkers (Kasai *et al.* 2009) conducted a chronic inhalation study by exposing 50 rats to dioxane vapor at 0, 50, 250, or 1250 ppm (v/v) for 6 hours/day, 5 days/week for 104 weeks. Survival rates at the 250 and 1250 ppm exposures were decreased near the end of the study period attributed to malignant tumors. A slight decrease in body weight was observed in the 1250 ppm-exposed group. Changes in plasma levels of the enzymes aspartate aminotransferase (AST),

alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GTP) and relative liver weight were also observed in the 1250 ppm exposure group. A lowest-observed adverse effect level (LOAEL) of 50 ppm was reported based upon the nasal endpoint for general chronic toxicity.

(13) Evidence for carcinogenicity in humans is weak. Barber (Barber 1934) reported the deaths of five silk industry workers whose necrosis of the liver and kidney was attributed to exposure to dioxane vapors, but cancer was not implicated in the deaths. Buffler and coworkers (Buffler *et al.* 1978) conducted a mortality study on 165 employees occupationally exposed to dioxane for a period of up to 24 years, finding the observed deaths from cancer in this cohort were not significantly different from the expected number of deaths.

d. Other data.

(1) In evaluating clearance rate constants resulting from a human study, Young and coworkers found the metabolic clearance of dioxane to be 75 mL/min. The renal clearance for un-metabolized dioxane was only 0.34 Ml/min, while the renal clearance of HEAA was found to be 121 Ml/min. Hence, conversion of dioxane to HEAA greatly increases the rate at which it is eliminated from the body (Young *et al.* 1977). These data can be used to demonstrate that at the inhalation exposure limit of 50 ppm mandated by the Occupational Safety and Health Administration (OSHA), any dioxane absorbed by the body during an 8-hour work shift will have been completely cleared from the body by the beginning of the next work shift, resulting in no potential for accumulation of dioxane within the human body over time.

(2) Inhalation toxicity. An acute inhalation toxicity study in humans was conducted by Young and coworkers (Young *et al.* 1977). Four healthy male volunteers were exposed to 50 ppm dioxane vapor for 6 hours. The half-life for elimination of dioxane was found to be 59 ± 7 minutes, with 99.3 percent of the eliminated product being HEAA, and the remaining 0.7 percent being un-metabolized dioxane. A physiologically based pharmacokinetic (PBPK) simulation of repeated daily exposures for 8 hours/day indicated that dioxane would never accumulate to concentrations above those attained after a single 8-hour exposure so long as the exposure concentration was 50 ppm or less. [Note: the OSHA time-weighted average (TWA) permissible exposure limit (PEL) is 100 ppm, although some states still enforce the previous PEL of 25 ppm-Hazard Substance Database (HSDB®).] (HSDB® is a registered trademark of the U.S. National Library of Medicine.)

(3) Impact of induction of cytochrome P450 in rats following treatment with dioxane was studied by Nannelli and coworkers (Nannelli *et al.* 2005). Rats received dioxane either as an oral gavage dose of 2 g/kg for a period of 2 days or as a 1.5

percent solution in drinking water for a period of 10 days. Both of these treatment regimes were found to induce cytochrome P450 2B1/2 and 2E1-dependent microsomal monooxygenase activities in the liver, but only the 2E1 marker was enhanced in the kidney and nasal mucosa. An induction of 2α-testosterone hydroxylase (associated with the constitutive and hormone-dependent P450 2C11) was also noted in the liver. Collectively, the results of these investigations failed to support the hypothesis of reactive or toxic intermediates being formed during biotransformation of the solvent, even when metabolism was enhanced by P450 inducers. Induction of palmitoyl CoA-reductase, a marker of peroxisome proliferation, was also not induced after the 10-day oral administration of dioxane. The authors hypothesized that induction of cytochrome P450 2E1 might result in an increase of reactive oxygen species (ROS) leading to organ-specific toxicity.

5. MATERIALS AND METHODS.

a. Dioxanone and HEAA were synthesized by Dr. James Fishbein, University of Maryland, Baltimore County, in partial fulfillment of contract number W91ZLK-08-P-1038. Analytical data relating to the chemical composition and purity of these compounds is included at Appendix B.

b. Test animals were male and female Sprague-Dawley strain rats obtained from Charles River Laboratories, Wilmington, Massachusetts. Actual weights of test animals upon receipt was 250–300 g. Each test animal was pre-implanted with a femoral artery catheter to facilitate obtaining blood samples. On the week of the experiment, surgery to implant the catheters was performed by the supplier on Monday and the animals were shipped on Tuesday to arrive at U.S. Army Public Health Command (Provisional) (USAPHC) (Prov) on Wednesday. The test animals were inspected by the USAPHC (Prov) animal care staff upon arrival and their health status confirmed prior to being signed over to the Study Director. Upon receipt, animals were placed in solid-bottom cages until dose administration. Each animal received a Nylabone® in its cage for environmental enrichment. Experiments were performed on either Thursday or Friday. If the experiment was to be conducted on Friday, catheters were flushed and tested for patency on Thursday. Flushing involved removing the lock solution from the catheter using a syringe to ensure the patency of the catheter, followed by injection of 0.2 Ml of normal saline and 0.1 Ml heparin solution, generally in accordance with the blood sampling procedure outlined below in subparagraph d. (Nylabone® is a registered trademark of Central Garden and Pet Company, Inc.)

c. All animal manipulations were performed according to USAPHC(Prov) Animal Use Protocol 08WR-54-08-06-01, with modifications (Appendix C). This protocol and the test procedure were approved by the USAPHC (Prov) Institutional Animal Care and Use Committee (IACUC). Animals were administered single, oral gavage doses of

dioxane in water, equivalent to 0, 10, or 1000 mg dioxane/kg body weight. Treatment groups contained a single male and a single female animal, the pair being selected so that the average weights for each of the treatment groups were approximately equal. After dosing, each animal was transferred to a metabolism cage where feces-free urine was collected. Water, but not food, was provided in the metabolism cages over the 8-hour duration of the experiment.

d. To draw a blood sample from the catheter, one person gently restrains the animal while a second person performs the procedure. The catheter plug, together with the catheter, is pulled 1–2 inches caudally out of the skin pocket. While holding the junction of the plug and the polyurethane tubing with forceps, a hemostat is used to remove the plug. A 1 mL Luer-Lok® syringe and blunted 23 gauge needle are inserted into the catheter tubing and the lumen lock solution (heparinized saline) is removed. The catheter is then crimped with a cushioned hemostat. A clean 1 mL syringe and blunted 23 gauge needle are inserted into the catheter and the cushioned hemostat is removed to draw the blood sample. Volume of the blood sample drawn is 0.10 mL from each rat per sample time. The catheter is again crimped with a hemostat and a sterile physiological saline-filled syringe is inserted into the catheter. The catheter is then flushed with 0.20 mL of saline. A syringe filled with heparinized saline is then inserted into the catheter and the dead volume is filled to prevent clotting. The catheter is then wiped with an alcohol swab, and fed back into the skin flap up to the wound clip. (Luer-Lok® is a registered trademark of Becton, Dickinson and Company.)

e. pH measurements were performed on urine samples at the time of collection using a Corning® semi-micro combination Ph electrode, Catalog No. 476156. The pH meter employed was a Corning model 220. Standardization of the electrode was carried out using pH 4.0 and pH 7.0 standard reference buffers. (Corning® is a registered trademark of Corning, Inc.)

f. Blood samples are delivered directly into a vial containing 1.0 mL of acidified acetone, and urine samples are delivered into vials containing 1.0 mL of methylene chloride. Methylene chloride (1.0 mL) is added to the blood samples, and then both blood and urine samples are agitated on a flat-bed agitator for 1 hour to ensure disruption of cells and extraction of the analytes. Samples are then centrifuged to settle particulates and the methylene chloride layer is drawn off for analysis. Methylene chloride extracts are stored in the freezer at -20 degrees Celsius (°C) until analyzed.

g. Samples were analyzed via gas chromatography on a Hewlett Packard HP6890 instrument fitted with an HP7673A autosampler and flame ionization detector (FID). Separations were performed on a 30.00 meter Stabilwax (primary analytical) or DB-1 (confirmatory) capillary column. Initial column temperature was 50 °C for 1.0 minute, with a programmed temperature increase of 10 °C/minute until a final temperature of

130 °C that is held for 10.0 minutes. Quantitation was performed automatically by the instrument based on calibration run data using peak height or peak area as the basis of measure.

h. In order to investigate the stability of potentially excreted dioxanone in urine, a test tube experiment was conducted where equal quantities of HEAA and dioxanone were incubated for a period of 4 hours at room temperature in rat urine. Aliquots were extracted at times 0, 2 and 4 hours and analyzed for dioxanone using the method described above. Since both potential metabolites are present, a conversion of one to the other should be readily apparent by the relative increase or decrease in dioxanone. Urinary pH was between 6.5 and 7.0 prior to extraction, as determined via pH test strip. The results of this experiment are given below.

i. A contract was awarded to Dr. James Fishbein, University of Maryland, Baltimore County, to perform studies designed to evaluate the equilibrium constant and pH-dependence of the equilibrium between dioxanone and HEAA. This study could not be undertaken at USAPHC (Prov) because instrumentation required to determine these data were not available in-house. Specifically, Dr. Fishbein was asked to determine:

(1) The pH profile for the interconversion of dioxanone and HEAA and its pH-dependence over pH range 1.0 to 9.0 using nuclear magnetic resonance techniques, so as to not induce changes in the position of the equilibrium that might occur as a result of derivatization and extraction of the subject compounds.

(2) Determine the equilibrium constant (K_{eq}) for the equilibrium at Ph 7.2, presumed physiological pH, and the pK for the reaction in buffered aqueous media.

(3) Determine the reaction order and rate constant for both directions of the equilibrium reaction at both physiological pH (7.2) and the pK for the reaction.

(4) Synthesize in pure form (99.5+percent) a minimum of 10.0 grams of both dioxanone and HEAA, to be submitted to USAPHC (Prov) for use in quality control evaluation and metabolism experiments.

6. RESULTS.

a. Dioxanone as a Metabolic Product.

(1) No dioxanone was detected in any of the samples analyzed, either blood or urine (see Tables 1 through 3). This suggests that previous literature reporting of dioxanone as the urinary product of dioxane metabolism is incorrect. This absence of dioxanone occurred even under conditions where large quantities of un-metabolized dioxane were detected in both blood and urine. Although not measured directly, it is assumed that any dioxane metabolized was excreted as HEAA. We had hoped to be able to demonstrate the presence of HEAA by acidification and reanalysis of metabolic samples, but the chemical and physical properties of the excreted product made this impossible.

(2) Results obtained in the metabolism studies are displayed in Tables 1 through 3 and Figures 2 and 3. No dioxanone was detected in blood or urine samples at any time point. Dioxane was found to be absorbed somewhat slowly from the gut, with a blood maximum being detected at approximately 4 hours. Lack of urine samples from the study animals impacted analysis of dioxane and dioxanone in urine, but un-metabolized dioxane was found in the blood within 1.0 hour of dosing in the higher dose animals of either sex, indicating that a substantial amount of dioxane was directly and rapidly excreted in the urine. Within the timeframe of this study, metabolism of dioxane to dioxanone was not demonstrated.

Table 1. Dioxane Metabolism Experiment Results, Run 1 (24 April 2009)

Role	Control Male	High Male	Low Male	Low Female	Control Female	High Female	Control Male	High Male	Low Male	Low Female	Control Female	High Female
Rat No.	694	696	697	699	700	701	694	696	697	699	700	701
Time (hours)	Blood dioxanone concentration (µg/mL)						Blood dioxane concentration (µg/mL)					
0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.5	nd	nd	nd	nd	nd	nd	nd	28.8	nd	nd	nd	79.2
1.0	nd	nd	nd	nd	nd	nd	nd	31.5	0.8	nd	nd	80.3
2.0	nd	nd	nd	nd	nd	nd	nd	51.6	nd	nd	nd	107.8
4.0	nd	nd	nd	nd	nd	nd	nd	87.3	nd	nd	nd	111.1
8.0	nd	nd	nd	nd	nd	nd	nd	95.8	nd	nd	nd	91.3
Rat No.	694	696	697	699	700	701	694	696	697	699	700	701
Time (hours)	Urine dioxanone concentration (µg/mL)						Urine dioxane concentration (µg/mL)					
0	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
0.5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
1.0	ns	nd	nd	ns	nd	ns	ns	175	4.5	ns	nd	ns
2.0	nd	nd	ns	nd	nd	ns	nd	234	ns	nd	nd	ns
4.0	nd	ns	nd	nd	ns	ns	nd	ns	nd	nd	ns	ns
8.0	nd	ns	nd	nd	ns	ns	nd	ns	nd	nd	ns	ns

Legend:
nd = nothing detected; below Method Reporting Limit (MRL)
ns = no sample obtained

Table 2. Dioxane Metabolism Experiment Results, Run 2. (13 August 2009)

Role	Low Male	Control Female	High Female	High Male	Control Male	Low Female	Low Male	Control Female	High Female	High Male	Control Male	Low Female
Rat No.	798	801	804	800	797	802	798	801	804	800	797	802
Time (hours)	Blood dioxanone concentration (µg/mL)						Blood dioxane concentration (µg/mL)					
0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.5	nd	nd	nd	nd	nd	nd	nd	nd	72.6	53.9	nd	4.2
1.0	nd	nd	nd	nd	nd	nd	6.2	nd	70.4	60.5	nd	nd
2.0	nd	nd	nd	nd	nd	nd	3.8	nd	57.2	75.9	nd	5.3
4.0	nd	nd	nd	nd	nd	nd	nd	5.6	82.5	94.6	nd	nd
8.0	nd	nd	nd	nd	nd	nd	nd	nd	114.4	100.1	nd	4.6
Rat No.	798	801	804	800	797	802	798	801	804	800	797	802
Time (hours)	Urine dioxanone concentration (µg/mL)						Urine dioxane concentration (µg/mL)					
0 hr	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
0.5	nd	ns	ns	ns	nd	ns	nd	ns	ns	ns	nd	ns
1.0	nd	nd	ns	nd	ns	nd	1.0	nd	ns	233	ns	1.6
2.0	ns	ns	nd	nd	ns	ns	nd	ns	ns	276	ns	ns
4.0	nd	ns	nd	nd	nd	nd	nd	ns	367	299	nd	0.6
8.0	nd	nd	nd	ns	nd	nd	nd	nd	656	ns	nd	nd

Key:
nd = nothing detected; below Method Reporting Limit (MRL)
ns = no sample

Table 3. Dioxane Metabolism Experiment, Run 3 (28 August 2009)

Role	Low Male	Control Female	High Female	High Male	Control Male	Low Female	Low Male	Control Female	High Female	High Male	Control Male	Low Female
Rat No.	807	812	810	806	805	809	807	812	810	806	805	809
Time (hours)	Blood dioxanone concentration (µg/mL)						Blood dioxane concentration (µg/mL)					
0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.8	nd
1.0	nd	nd	nd	nd	nd	nd	nd	nd	91.3	147.4	4.1	nd
2.0	nd	nd	nd	nd	nd	nd	nd	nd	95.7	221.1	3.3	nd
4.0	nd	nd	nd	nd	nd	nd	nd	nd	130.9	174.9	1.8	nd
6.0	nd	nd	nd	nd	nd	nd	nd	nd	144.1	251.9	10.4	nd
8.0	nd	nd	nd	nd	nd	nd	nd	nd	211.2	148.5	nd	nd
Rat No.	807	812	810	806	805	809	807	812	810	806	805	809
Time (hours)	Urine dioxanone concentration (µg/mL)						Urine dioxane concentration (µg/mL)					
0	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
1.0	nd	nd	ns	nd	nd	ns	0.5	nd	ns	306	12	ns
2.0	ns	nd	nd	ns	nd	ns	ns	nd	466	ns	15	ns
4.0	nd	nd	nd	nd	ns	nd	nd	nd	340	520	9.4	ns
6.0	ns	nd	nd	nd	nd	ns	ns	nd	361	462	1.6	ns
8.0	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Legend:
nd = nothing detected; below Method Reporting Limit (MRL)
ns = no sample obtained

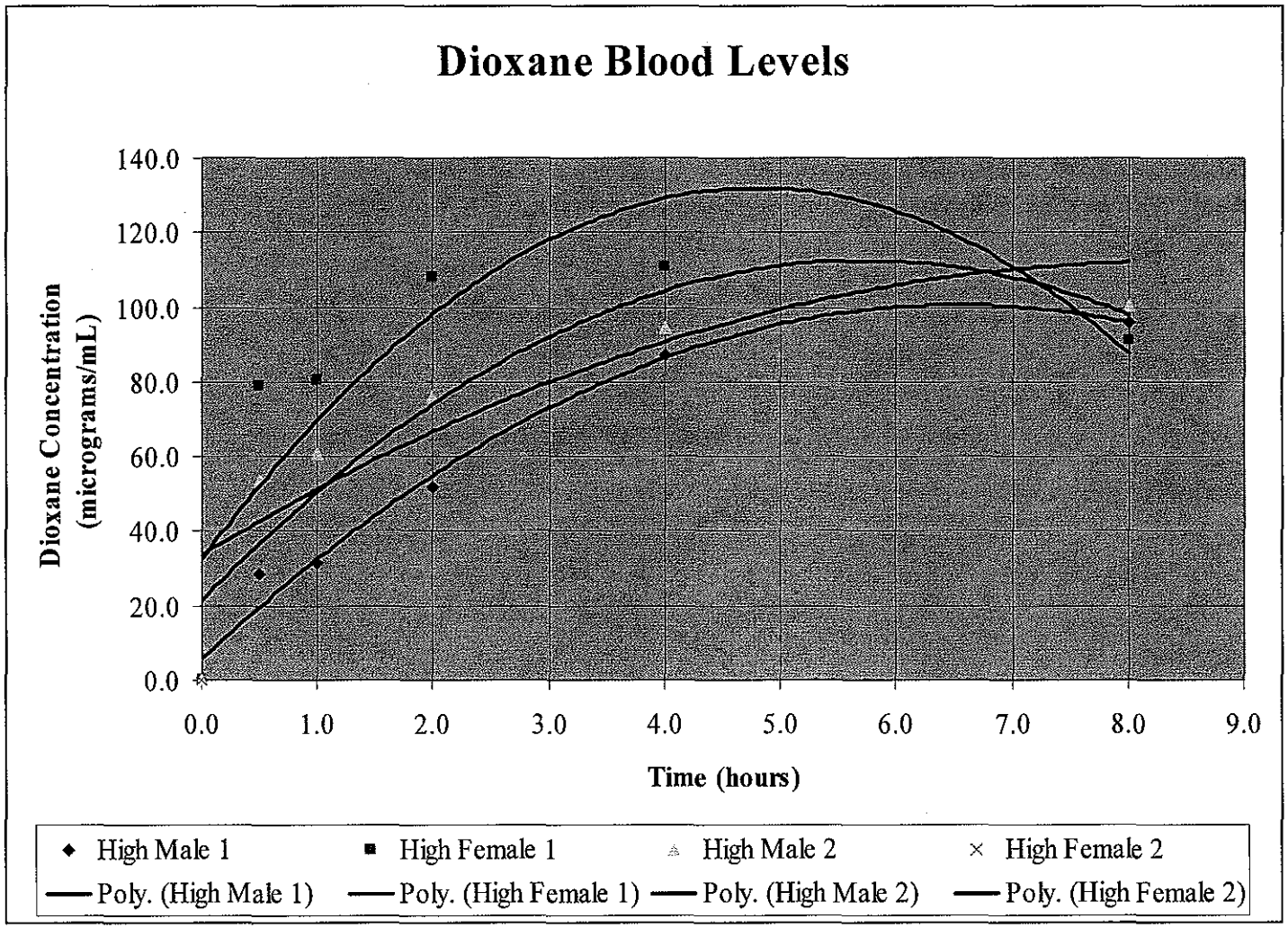


Figure 2. Time Course for Blood Dioxane Concentrations, Runs 1 and 2

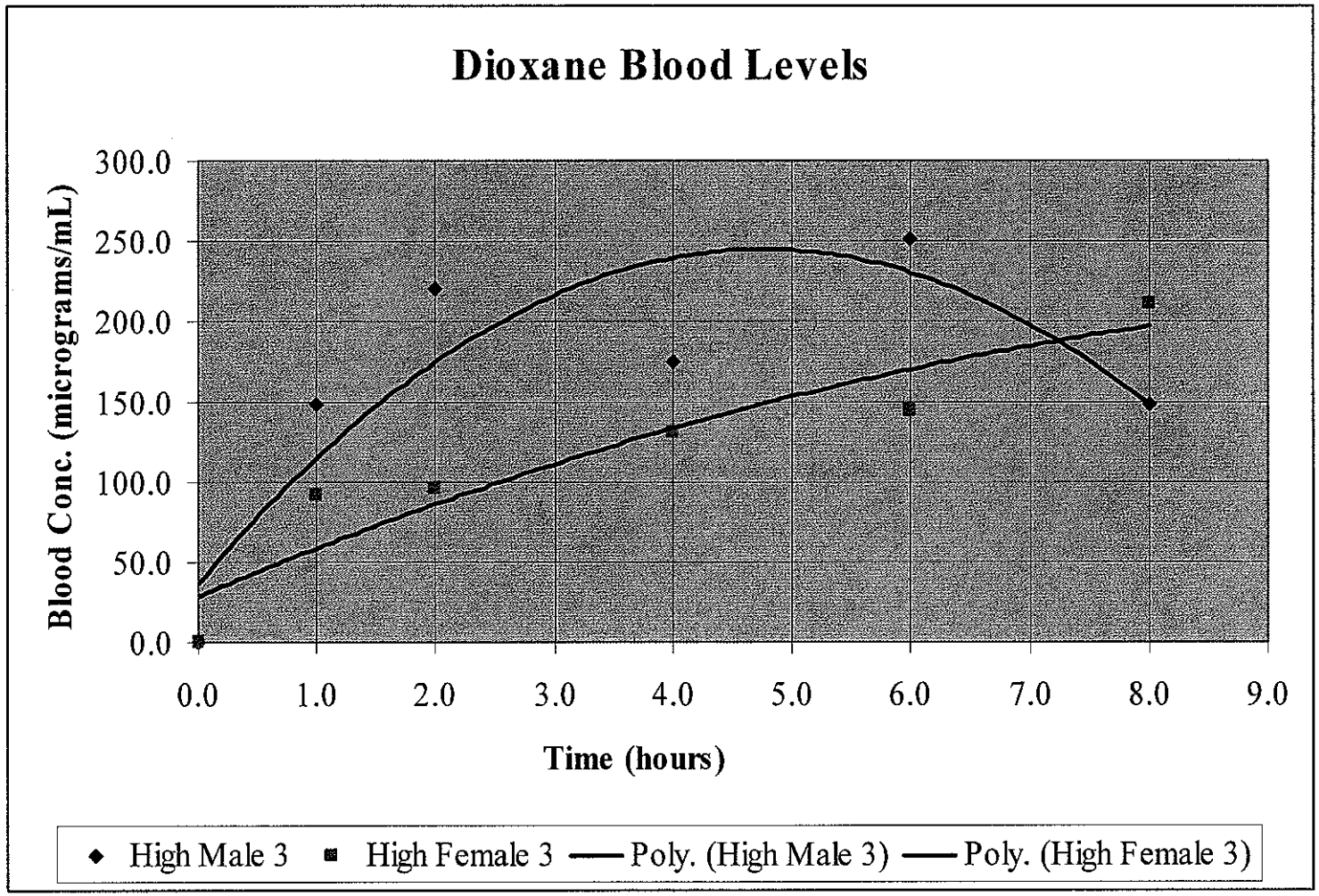


Figure 3. Time Course for Blood Dioxane Concentrations, Run 3

b. Dioxanone Stability in Urine.

(1) As noted above in the results, no dioxanone was detected in any of the urine samples analyzed. As an empirical evaluation of the stability of dioxanone in urine, a test to determine its stability in urine was conducted over a 4-hour period, the longest time that a urine sample would be unrefrigerated in the metabolism cage tube. A test sample was prepared in rat urine by adding equivalent amounts of dioxanone and HEAA. With both potential metabolites present, the stability of metabolites and the direction of the equilibrium could be readily determined. The experiment was run induplicate; results are displayed in Table 4.

Table 4. Results of Dioxanone Stability Test

Exposure Time (hours)	[Dioxanone] µg/mL	
	Sample 1	Sample 2
Blank	2.0448	---
0	23.2476	23.7056
2	0.6519	0.3450
4	0.2455	0.2505

(2) These data indicate that any dioxanone that might be excreted as a result of metabolism is rapidly and completely broken down with a half-life of approximately 0.4 hours, presumably to HEAA, when exposed to an aqueous environment, and there is no conversion of HEAA to dioxanone. This not only clarifies why dioxanone is not observed in any of the aqueous samples, but also corroborates the findings of the chemical equilibrium study, outlined below, and reinforces the position that dioxanone is too unstable in aqueous environments to be implicated in the hypothesized mechanism of dioxane carcinogenicity.

c. Chemical Equilibrium Studies.

(1) Dr. Fishbein was asked to determine the chemical equilibrium parameters for the dioxanone-HEAA equilibrium in an attempt to elucidate the non-biologically-mediated aspects of this equilibrium. These studies were carried out without derivatization of the two compounds so that the equilibrium would not be disturbed by the analytical process. The results are depicted in Figure 4.

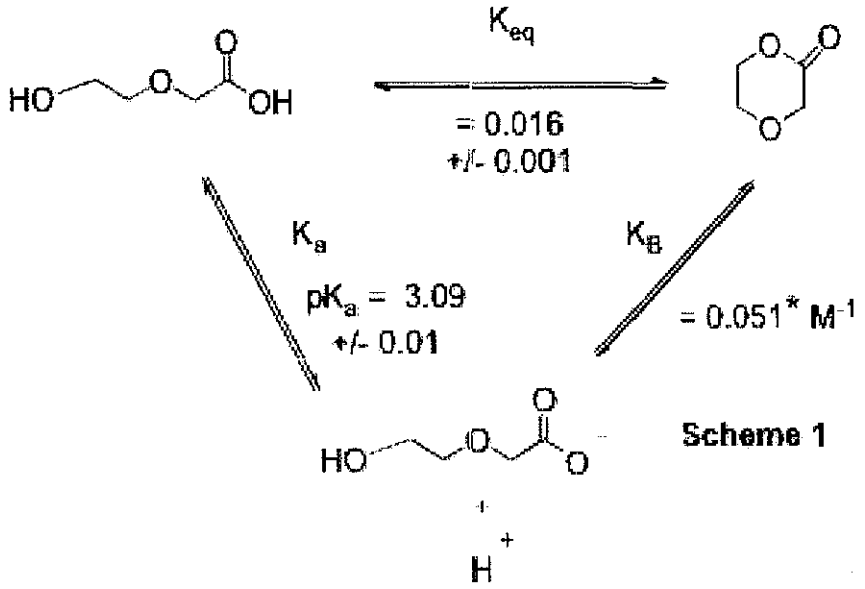


Figure 4. Thermodynamic Cycle for the equilibrium between HEAA and dioxanone

(2) The primary equilibrium between HEAA and dioxanone was found to have an equilibrium constant of 0.016±0.001, indicating that thermodynamically, HEAA is very strongly favored in the equilibrium. Any dioxanone that might be formed as a result of biological oxidation is expected to rapidly decompose in the presence of water to the open-chain form of HEAA purely on the basis of its chemical properties.

(3) The second question addressed by Dr. Fishbein was the pH-dependence of this ring-opening reaction. This was of some concern because previous researchers had acidified their biological extracts to an unknown pH, and acidification was presumed to result in a situation favoring ring closure to dioxanone. The rate constant for the hydrolysis of the lactone (the reverse of the ring closure reaction) was found to have a minimum value of approximately 0.001 at pH ≈ 5. The rate constant increased in a symmetrical fashion at pHs either above or below 5, as indicated in Figure 5.

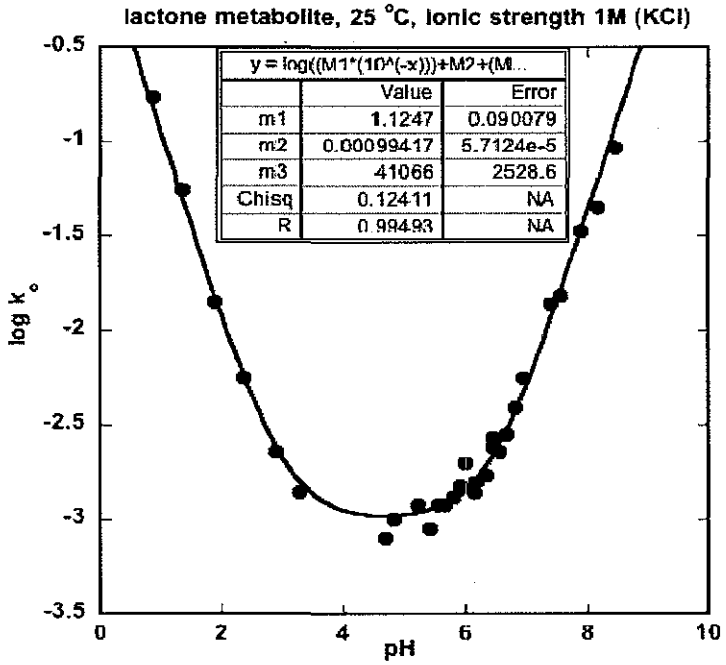


Figure 5. pH-Dependence of lactone hydrolysis rate constant

7. DISCUSSION.

a. Characterization of the Dioxanone-HEAA Equilibrium.

(1) Previous attempts to identify the metabolic product of dioxane metabolism have resulted in the assertion that either dioxanone (Woo *et al.* 1977; Woo *et al.* 1977) or HEAA (Braun and Young 1977; Young and Gehring 1975) is the true metabolite. Clarifying the identity of the metabolites has been complicated by apparently contradictory validation against reference materials and a tendency to under-report details of laboratory manipulations that could result in chemical change in the products. For these reasons, it was decided to undertake a study to experimentally determine the chemical constants for the equilibrium reaction in an environment that would not involve extraction, separation, or derivatization of the separate compounds. The only way in which such a study could be reasonably undertaken would involve the use of advanced nuclear magnetic resonance techniques and instrumentation that was not available at USAPHC (Prov).

(2) Determination of the chemical parameters of the dioxanone-HEAA equilibrium clearly indicates that it is essentially impossible for dioxanone to exist more than a few minutes in an aqueous environment. The value of the equilibrium constant so greatly favors HEAA that any dioxanone that might be formed as a result of dioxane

metabolism will be immediately hydrolyzed to HEAA. This result provides experimental validation that the true excretion product in animal systems is HEAA and not dioxanone. This finding advances understanding of the carcinogenic mode of action for dioxane by arguing against the possibility that dioxanone, a substance that was predicted by QSAR modeling to be carcinogenic, is the true carcinogen, and suggests the true focus attention on the parent compound, dioxane, and its impacts on target organs.

b. *In vivo* Study of Dioxane Metabolism. Results from the *in vivo* study reinforce the findings of the nuclear magnetic resonance (or NMR) studies of the equilibrium. Although the presence of dioxane could clearly be followed over the time course of the experiment, no dioxanone was detected in either the blood or urine samples. This again demonstrates that in the living, physiological system, dioxanone is unlikely to be relevant to the development of cancers observed in the long-term carcinogenicity studies. These results are also consistent with the findings of Goldsworthy et al. (1991), who found no evidence for dioxanone initiating DNA repair activity in the rat hepatocyte DNA repair assay.

c. Implications of the Results to the Question of Carcinogenicity. What do these studies tell us about the possible mode of action for carcinogenicity of dioxane? *If* dioxanone is formed by the action of cytochromes P450, *and* is not being eliminated by the subject animal, then there would seem to be only two possibilities for its metabolic fate.

(1) The first possibility would be that as soon as it is formed, dioxanone reacts with compounds in its immediate vicinity in such a way that the reaction product would be concentrated in the tissue. If such were the case, one would expect to see foci of tissue modification in the liver as rapidly as dioxanone were formed, and tissue damage would be quickly apparent. This level of histopathology has only been observed when extraordinarily high levels of dioxane were administered (more than 7000 mg/kg-d; Stott et al., 1981).

(2) The second possibility is that dioxanone is rapidly hydrolyzed to HEAA, does not chemically react with tissue macromolecules, and is unlikely to be the proximate cause of cancer observed in the rat. Under these circumstances, it would be more productive to look at other aspects of dioxane's interaction with target tissues, especially at concentrations above which it is known the metabolic pathways, at least in rodents, become saturated.

8. CONCLUSIONS

a. Metabolic products. This study supports the finding that dioxanone is not the primary urinary excretion product of dioxane. It also provides the first physico-chemical evaluation of the equilibrium between dioxanone and HEAA, along with the relevant equilibrium constants.

b. Mode of Action. The chemical instability of dioxanone suggests that it cannot be involved in the mode of action for carcinogenesis of dioxane. Since HEAA is not believed to be carcinogenic and is rapidly excreted by the body, another mode of action for dioxane carcinogenesis must be evaluated. It seems likely that the true oncogenic substance in this case is dioxane itself, not a metabolic product. This line of argument is also supported by the following observations:

(1) Rodents demonstrate saturation of dioxane metabolism and develop neoplasms at concentrations above levels that saturate their metabolic capability.

(2) Human metabolism of dioxane has not been shown to become saturated, and neoplasms have not been observed in humans. Extraordinarily large doses of dioxane are capable of causing toxicity in humans, but this chemical toxicity has not been found to result in neoplasm development.

c. Suggestion for Future Work. It appears that future investigation of the metabolites is unlikely to provide much insight into either the mode or mechanism of action with respect to possible human carcinogenicity. At this point, a likely avenue of advance would be to investigate the impact of dioxane on the liver's metabolic pathways using commercially available microarray chips that permit investigation of the relative expression of genes after exposure of the animal. Such experiments should provide insight into how the animal (the liver in this case) is responding at a molecular level to treatment with dioxane. Such mechanistic data will provide insight into exactly how dioxane exerts effects within the organism.

APPENDIX A

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APPENDIX B

ANALYTICAL CHARACTERIZATION OF TEST COMPOUNDS

ATLANTIC MICROLAB, INC.

Sample No. KN-1

P.O. Box 2288
Norcross, Georgia 30091
(770) 242-0082
www.atlanticmicrolab.com

PROFESSOR/SUPERVISOR: Dr. Fishbein
P.O. # CH 81039

SUBMITTER
Company / School: UMBC
Address: 1000 Hilltop Circle
Dept of Chem / Biochemistry
Baltimore, MD 21250
NAME: Kiss Nagy DATE: 3/14/08

Element	Theory	Found	
C	33.8	33.82	33.86
H	4.97	5.00	5.01

Single ☐ Duplicate ☒
Elements Present: $C_4H_7O_4Na$
Analyze for: CH
Hygroscopic ☐ Explosive ☐
M.P. _____ B.P. _____
To be dried: Yes ☐ No ☒
Temp. _____ Vac. _____ Time _____
FAX Service ☒
FAX Phone # 410-455-2608
Rush Service ☐ (SEE CURRENT PRICE LIST)
Phone Service ☐
Phone No. _____

Date Received MAR 17 2008 Date Completed MAR 18 2008
Remarks:
PO CH 81039. Charge to VSM on file.

Figure B-1: Elemental analysis of 2-hydroxyethoxyacetic acid (HEAA)

ATLANTIC MICROLAB, INC.

Sample No. KN- LAC2

P.O. Box 2288
Norcross, Georgia 30091
(770) 242-0082
Dr. FISHBEIN

SUBMITTER

Name UMBC /KOISSI NIANGORAN
Address 1000 HILLTOP CIRCLE
Dept CHEM/BIOCHEM
BALTIMORE, MD 21250
Date 09/08/08

Element	Theory	Found	
C	47.06	46.94	46.90
H	5.92	6.08	5.94

Single ☐ Duplicate ☒
Elements Present: $C_4H_6O_3$
Analyze for:
Hygroscopic ☒ Explosive ☐
M.P. ____ B.P. 204-206°C
To be dried: Yes ☒ No ☐
Temp. 0°C Vac. high Time 1h
FAX Service ☒
FAX Phone 410 455 2608
Rush Service ☐ ($5/sample)
Phone Service ☐ ($5/phone call)
Phone No.

Date Received SEP 09 2008 Date Completed SEP 10 2008
Remarks:

Figure B-2: Elemental analysis of dioxanone

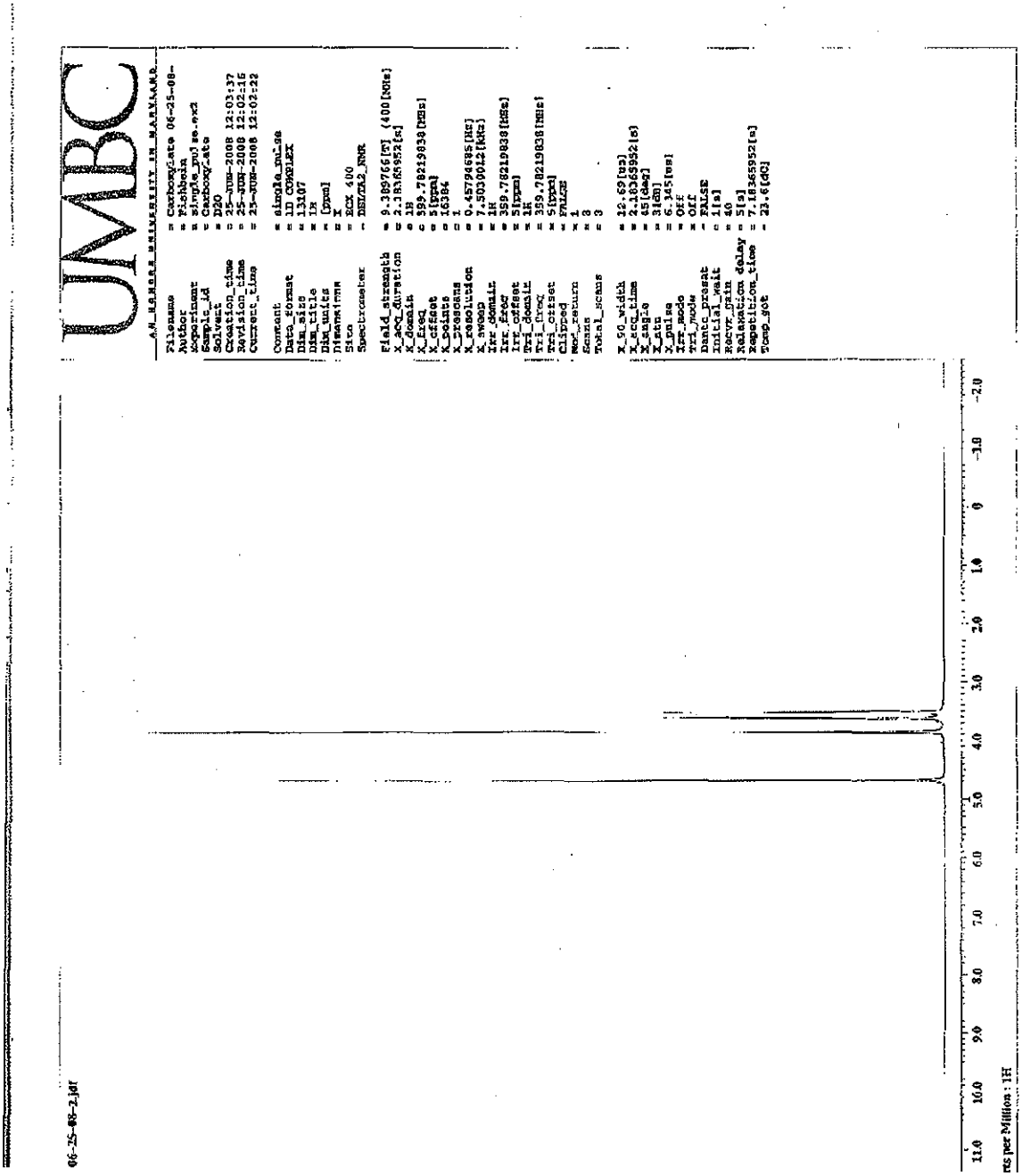

Figure B-3: Proton NMR spectrum of HEAA

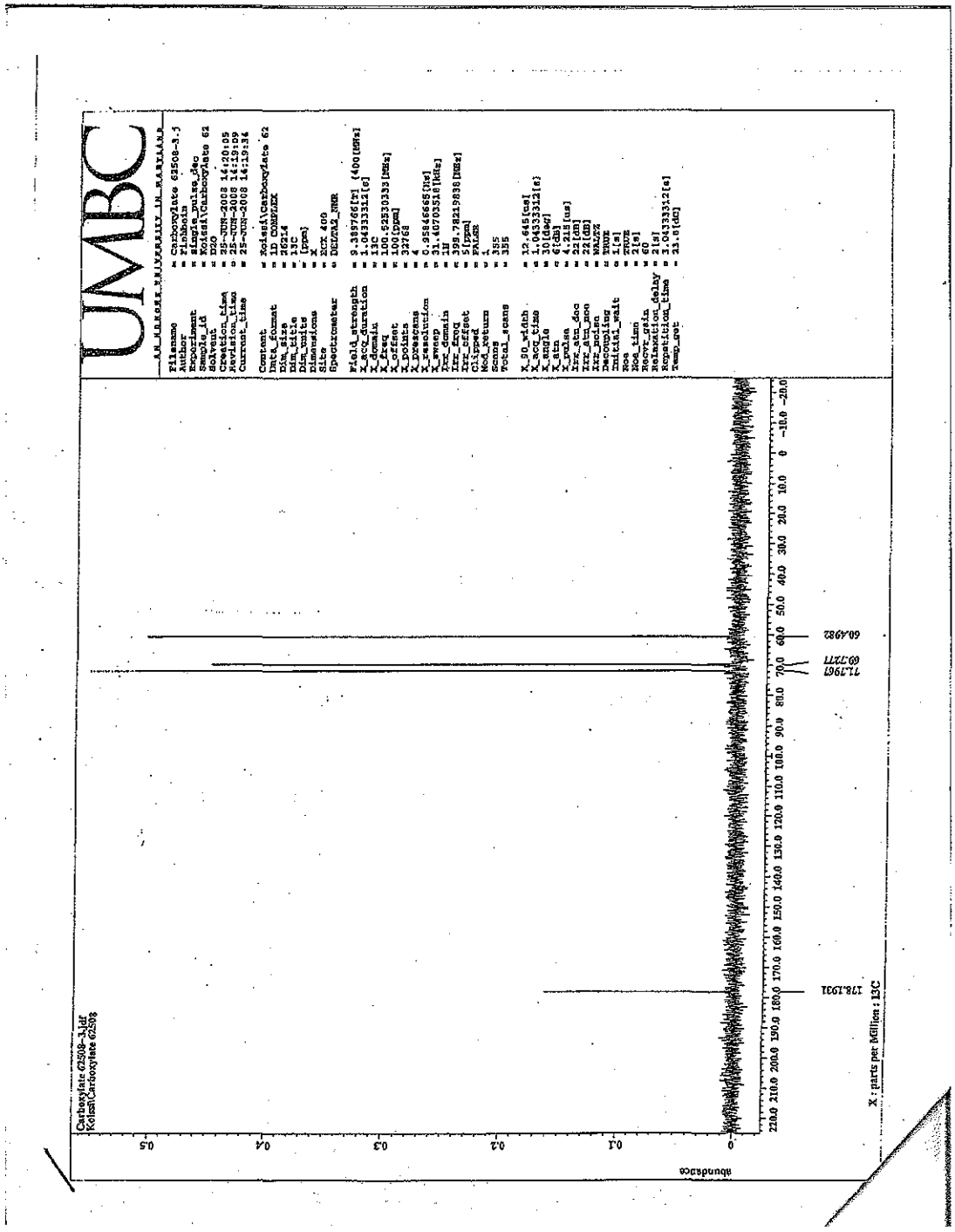

Figure B-4: ^{13}C- NMR spectrum of HEAA

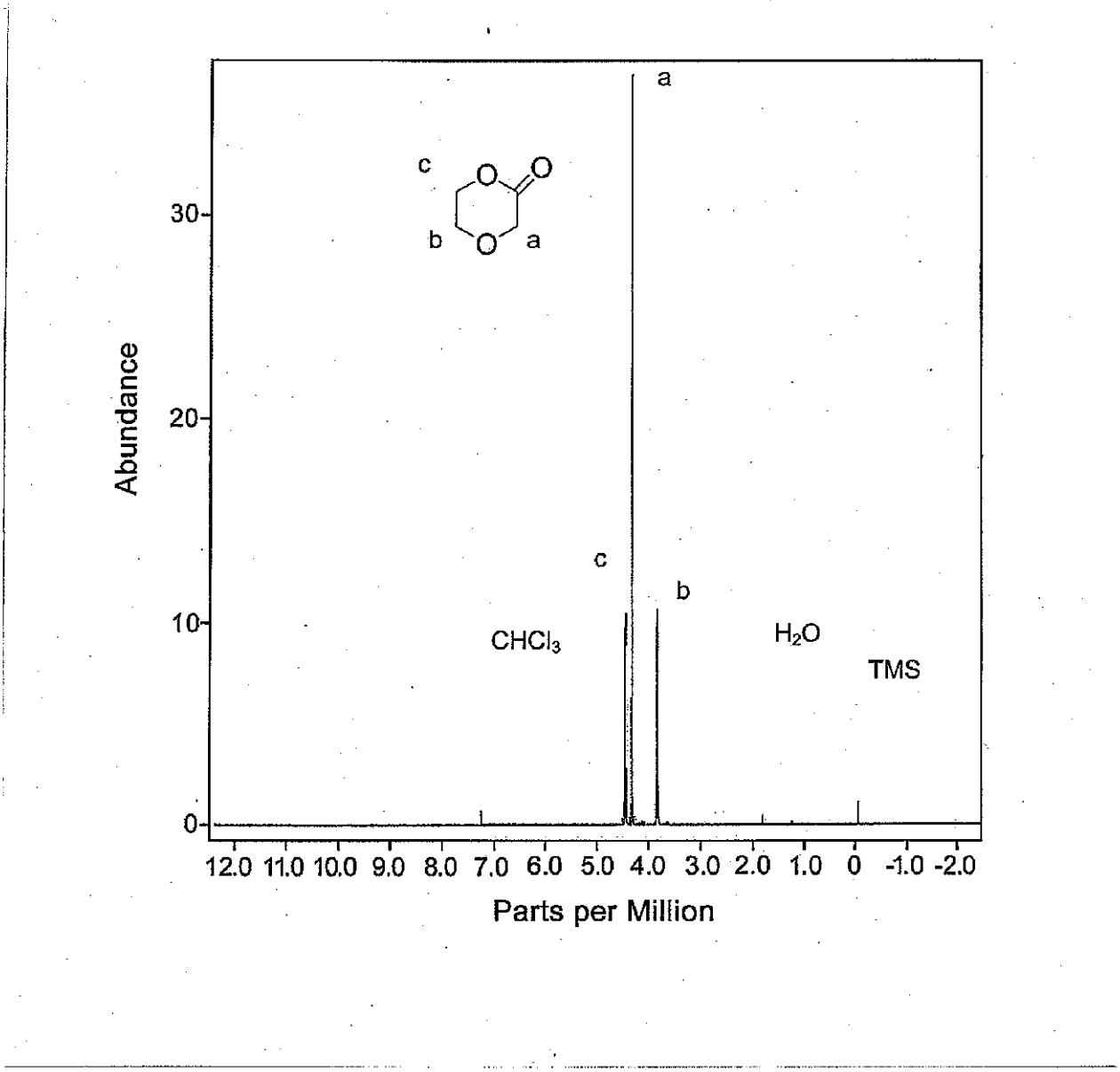

Figure B-5: Proton Nuclear Magnetic Resonance spectrum of dioxanone.

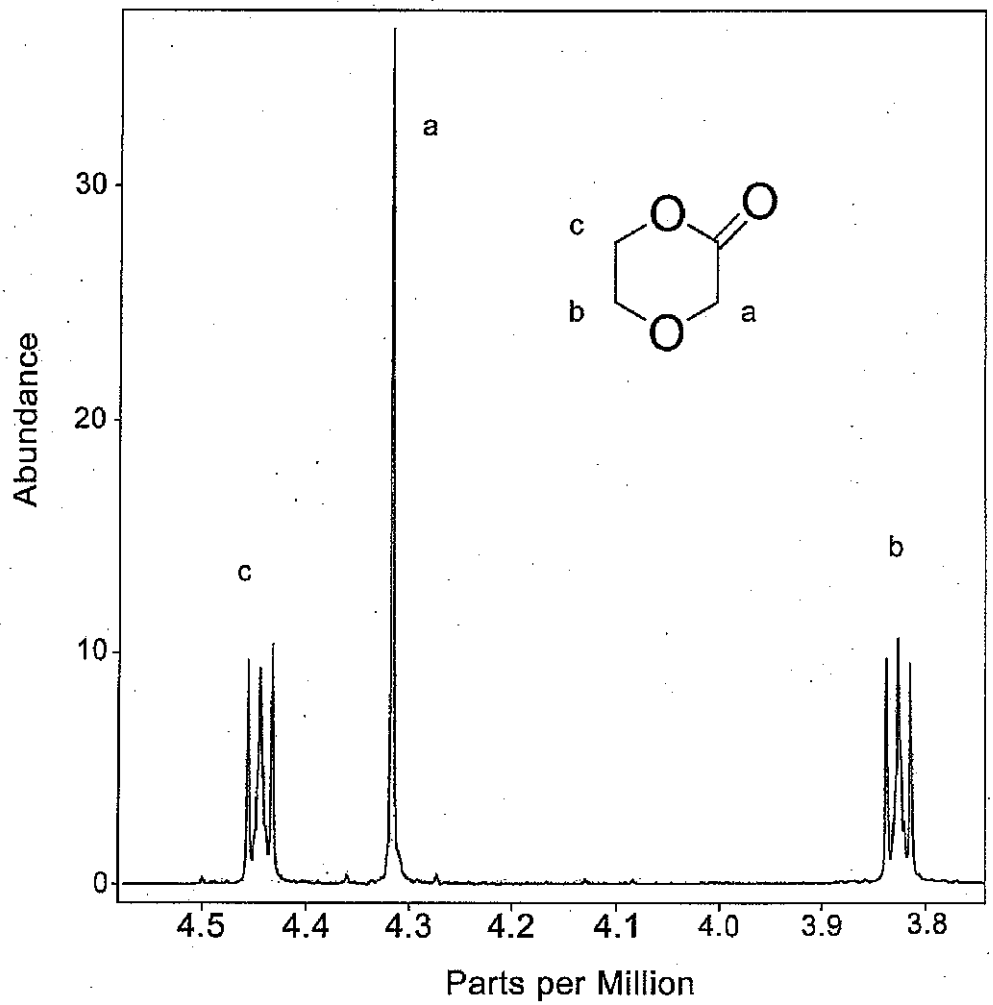

Figure B-6: Detail of proton NMR spectrum for dioxanone.

APPENDIX C

ANIMAL USE PROTOCOL
TOXICOLOGY DIRECTORATE
U.S. ARMY CENTER FOR HEALTH PROMOTION
AND PREVENTIVE MEDICINE
ABERDEEN PROVING GROUND, MD 21010-5403

PROTOCOL TITLE: Metabolism of 1,4-Dioxane in Rats

PROTOCOL NUMBER: 08WR-54-08-06-01

PRINCIPAL INVESTIGATORS/STUDY DIRECTOR:

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Toxicologist
Health Effects Research Program
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USACHPPM

SPONSOR:

U.S. Army Environmental Command
ATTN: Cleanup Division
5179 Hoadley Road
Aberdeen Proving Ground, MD 21010-5401

I. NON-TECHNICAL SYNOPSIS:

The USEPA classifies 1,4-dioxane (hereafter "dioxane") as a probable human carcinogen on the basis of drinking water studies in rats conducted during the late 1960's and early 1970's. Reports in the scientific literature suggest that the carcinogenicity of dioxane is overstated (especially with regard to low-level exposures), and current exposure standards are based upon inappropriate experiments and model application.

A key factor in the debate surrounding carcinogenicity of dioxane relates to its metabolism. Disagreement exists in the literature regarding which of two identified complementary metabolites, 2-hydroxyethoxy acetic acid (HEAA) and 1,4-dioxan-2-one (the "lactone"), is actually produced during metabolism in mammals. This is an important question because only one of the two metabolites is suspected of having carcinogenic properties.

In this investigation, rats with pre-implanted vascular catheters will be administered 1,4-dioxane in water by oral gavage. Each rat will receive a dose equivalent to the amount they would consume in a single day by drinking water containing either 0.01% or 1% dioxane. Blood and urine samples will be collected 0, 0.5, 1, 2, 4, and 8 hours after dosing. The blood and urine samples will be analyzed for the presence of the two metabolites.

II. BACKGROUND:

II.1 Background:

1,4-Dioxane (CASRN: 123-91-1; "dioxane," not to be confused with dioxin, or TCDD) is characterized by the EPA as a "probable human carcinogen" based upon animal studies. It is a water-miscible, semi-volatile organic compound that contaminates groundwater at many military installations as a result of the use of chemical degreasers that contained dioxane as a stabilizer, notably 1,1,1-trichloroethane (TCA). Dioxane is still used in the manufacture of small arms ammunition, and is present in small quantities in many chemical formulations. Environmental releases of dioxane have decreased significantly since the Montreal Protocol banned use of TCA.

In a study published in 1965, dioxane was found to cause liver, kidney, and nasal cancers in laboratory rats (1). By 1973, investigators hypothesized that a metabolite, rather than dioxane itself, was probably responsible for the carcinogenic activity (2). It has been established that the two identified potential metabolites, 2-hydroxyethoxyacetic acid (HEAA, Fig. 1, structure III) and 1,4-dioxan-2-one (the "lactone", Fig. 1, structure IV) are in equilibrium with one another (34), and the

distribution of the compounds in the equilibrium is dependent upon the pH of the solution. Published work on the metabolites is discussed in the following section.

Quantitative Structure-Activity Relationship (QSAR)-modeling has been carried out on both metabolite structures. Blake (3) and Gombar (4) wrote the EPA indicating that the lactone is a potential carcinogen, while HEAA, the open-chain form is not. At face value, this appears to support the position of those favoring the lactone being the true metabolite. However, since dioxane is only a weak hepatocarcinogen (2), HEAA-supporters indicate this argues in favor of their position, and the relative amount of lactone must be small. Goldsworthy, et al. (6) found the lactone metabolite failed to exhibit any activity in the *in vitro* primary rat hepatocyte repair assay.

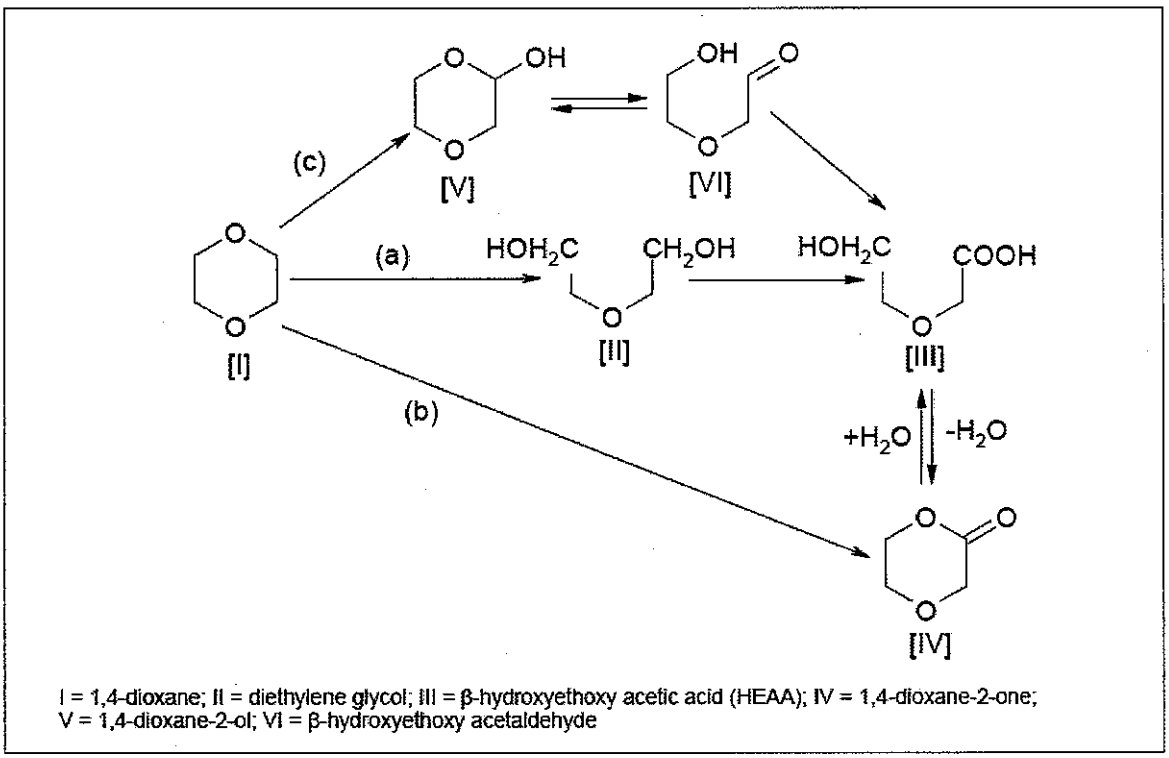

Figure 1. Proposed Metabolic Pathways for Dioxane Catabolism

EPA's cancer slope factor assessment was performed using a linear multi-stage model (LMSM) and data from rats, mice, and guinea pigs receiving dioxane in drinking water (23). While a LMSM is appropriate for carcinogens that are genotoxic (i.e. interact directly with the DNA in genes), it is currently considered inappropriate for compounds such as dioxane that exhibit threshold behavior (5). There is general agreement in the scientific literature that dioxane does not function via a genotoxic mechanism (6, 22, 35). The EPA is presently reviewing the scientific data on dioxane's mode of action,

providing an opportunity to create a science-based standard for drinking water exposures.

II.2 Literature Search for Duplication:

Studies have previously been carried out on products of dioxane metabolism, but this previous work is the source of the present controversy and the issue remains unresolved. Determining which metabolite is actually present is important because QSAR modeling (3,4) predicts that the lactone structure is carcinogenic, but HEAA is not. The experiment that is outlined in this protocol has not been reported in the literature, and no comparable study has been identified. Previously-reported work relevant to the current study is outlined below.

The lactone was identified as the primary metabolite of dioxane in work reported by Woo, et al. (7). They used glacial acetic acid to preserve the urine specimens, reducing the pH to an unknown value, then readjusted it to between 4.0 and 4.5. Reduced pH favors the formation of the lactone structure over the open-chain form, HEAA. If the pH is reduced below the pK of the equilibrium, the lactone will form spontaneously, and its presence in urine is simply an artifact of the analytical procedure. The pK of the equilibrium is presently unknown.

Studies in rats and human volunteers by a group at Dow Chemical (9-14) assert that the true metabolic product is 2-hydroxyethoxyacetic acid (HEAA). This is based upon their identification of HEAA by thin-layer chromatography; a procedure not believed to cause ring closure to the lactone.

Studies conducted in bacteria have supported the argument that HEAA is the metabolic product (15,16), but such findings are not definitive for higher organisms. In contrast, the presence of HEAA was not detected in studies employing the fungus *Cordyseps sinensis*, where dioxane degradation appears to occur via ethylene glycol, glycolic acid, and oxalic acid (17).

II.2.1 Literature Source(s) Searched:

a. Dialog results
MEDLINE (R) 1950-2008
Adverse Reaction Database 2007/Q3
ToxFile 1965-2008/Mar W4
Inside Conferences 1993-2008/ Apr 01
Biol. & Agric. Index 1983-2008/Feb
AGRICOLA 70-2008/Feb
Int. Pharm. Abs 1970-2008/Feb B2

Pharmline (R) 1978-2002/Dec W3
Pascal 1973-2008/Mar W4
BIOSIS Toxline (R) 1969-2008/Mar W4
The Merck Index Online(SM) 2005/S2
FEDRIP 2008/Feb
Global Health 1983-2008/Feb
CAB Abstracts 1972-2008/Feb
EMBASE 1974-2008/Apr 01
NTIS 1964-2008/Apr W1
Biosis Previews (R) 1926-2008/Mar W4
RTECS 2008/Q1
Science 1996-1999/Jul W3
SciSearch(R) Cited Ref Sci 1990-2008/Mar W5

b. DTIC Private STINET 1965-2008/Apr 01

c. TOXNET

d. CBRN START

e. PubMed 1965-2008

f. DOD Biomedical Research Database

II.2.2 Date of Search: 1 April 2008. Performed by MRICD research librarian.

II.2.3 Period of Search: 1950-2008

II.2.4 Key Words of Search: dioxane, 1,4-dioxane, metabolism, metabolite*, pharmacokinetic, cancer, carcinogenesis, carcinoma, tumor, rat, rats, mouse, mice, guinea pig*, rodent. (The asterisks in search terms are a standard "wild card" symbol meaning that any text character(s) may be included.)

II.2.5 Results of Search:

The proposed experiment has not been duplicated by any previous work. Woo, et al. (7,8) employed gas chromatography of acidified urine samples for initial analytical work. A mass spectrum and NMR spectrum were obtained for the "metabolite" from a lyophilized sample purified by preparative GC. The parent ion in the mass spectrum was found to have a mass/charge ratio of 102, corresponding to the molar mass of the lactone.

In identifying HEAA as the primary metabolite of dioxane, Braun and Young (13) initially employed thin-layer chromatography (TLC; 2 development systems). One major and two minor products were detected in urine. The two minor products had R_f (relative migration of a compound with respect to the solvent front in a chromatographic system, considered a physical property of the compound that can contribute to structure identification) values corresponding to 1,4-dioxane and diethylene glycol. The major product was found to have an R_f value corresponding to HEAA. Analysis was confirmed using ion exchange chromatography, gas chromatography, NMR and mass spectroscopy.

HEAA is not volatile, but can be analyzed as its methyl ester by gas chromatography. Braun prepared samples for GC/MS analysis by evaporating an ion exchange fraction containing the "major metabolite" to dryness and redissolving the resulting solid in 0.1M methanolic HCl. Residual water was removed by addition of acetic anhydride (18). The impact of this treatment on the lactone is unknown.

III. OBJECTIVE/HYPOTHESIS:

The objective of this research is to determine the presence and relative quantity of two compounds (2-hydroxyethoxyacetic acid (HEAA) and 1,4-dioxan-2-one, the lactone) in blood and urine of rats dosed with 1,4-dioxane via gavage. Absence of lactone in the blood and urine will eliminate this compound from involvement in carcinogenesis by 1,4-dioxane in the rat. Such a finding would be relevant to the derived human health assessment. Presence of HEAA will be inferred by analysis of split samples; details of the analytical scheme are presented in section V.4.4.2, below. The null hypothesis in this experiment is that the lactone is the physiological metabolite of dioxane in the rat, and by implication, other mammals.

IV. MILITARY RELEVANCE:

1,4-Dioxane is a water-miscible, semi-volatile organic compound that contaminates groundwater at many military installations. Dioxane was used as a stabilizer for the degreaser 1,1,1-tricholoroethane, use of which is banned under the Montreal Protocol. Dioxane is currently used in the manufacture of small arms ammunition, and is present in smaller quantities in many chemical formulations. The EPA characterizes dioxane as a "probable human carcinogen" based upon animal studies.

The Army is presently facing substantial environmental restoration liability associated with remediation of dioxane-contaminated soils and groundwater. The EPA has established neither inhalation nor drinking water standards for this substance, but has made assessments on the carcinogenicity of dioxane (23), with an oral slope factor

of 1.1 x 10^{-2} mg/kg-day, and a drinking water unit risk of 3.1 x 10^{-7} µg/L. In the absence of Federal standards, the individual states have established their own standards for water contamination, the most stringent being California, with a drinking water limit of 3 µg dioxane/L.

Determination that the presumed carcinogenic lactone metabolite is not present in rat urine will support the establishment of less-stringent cleanup standards for groundwater contamination at Army and other DoD installations. Less-stringent standards could potentially save the Army and taxpayers millions of dollars in unnecessary remediation cost.

V. MATERIALS AND METHODS:

V.1 Experimental Design and General Procedures:

V.1.1. Experimental Design:

a. Dosing and sample collection: A total of 6 Sprague-Dawley rats are required for each repetition of this experiment. Four animals, two of each sex, will receive gavage doses comparable to a daily ingestion of 0.01% or 1.0% 1,4-dioxane in water, and one male and one female will receive only water and serve as controls. Provision is made in this protocol for 3 repetitions of the experiment, however repetitions will occur only if data analysis indicates they are necessary. Animals used in this experiment will be outfitted with vascular catheters (*syn.* cannulated rats) to enable time-dependent determination of blood levels of metabolites. These dosage levels have been chosen because it is known that the rat's metabolism of dioxane is subject to saturation, and the Maximum Tolerated Dose (MTD; derived from drinking water studies) that does not appear to cause toxicity from causes other than cancer is believed to be between 0.01% and 0.02% (19). Long-term studies in rats receiving dioxane in drinking water at the 1.0% level result in hepatic, renal, and nasal cancers (20). One male and one female rat will be employed at each dosage level, the minimum number of animals necessary to cover possible gender factors. Each experimental run can be completed in one day, with 100 µL blood samples being obtained at 0, 0.5, 1, 2, 4, and 8 hours.

Each rat will be received from Charles River Laboratories with a subcutaneous femoral artery catheter already in place. To draw a blood sample from the catheter, one person will gently restrain the animal while a second person performs the procedure. The catheter plug, together with the catheter, is pulled 1-2 inches caudally out of the skin pocket. While holding the junction of the plug and the polyurethane tubing with forceps, a hemostat is used to remove the plug. A 1 cc Luer lock syringe and blunted 23 gauge needle are inserted into the catheter tubing and the lumen lock solution (heparinized saline) is removed. The catheter is then crimped with a cushioned hemostat. A clean 1 cc syringe and blunted 23 gauge needle are inserted into the

catheter and the hemostat is removed to draw the blood sample. 100 µL of blood will be drawn from each rat per sample time. The catheter is again crimped with a hemostat and a sterile physiological saline-filled syringe is inserted into the catheter. The catheter is then flushed with 200 µL of saline. A syringe filled with heparinized saline is inserted into the catheter and the dead volume is filled to prevent clotting. The catheter is then wiped with an alcohol swab, inserted back into the skin flap, and locked into place with the wound clip (24).

The animals will be housed in metabolism cages after the initial dosing and through the completion of the experiment 8 hours later. Urine samples will be collected from the metabolism cages, via a receptacle attached to the bottom of the metabolism cage. The receptacle will be cooled to near 0 °C using an external ice bath, and collected at the same interval as the blood samples. The pH of each urine sample will be determined upon collection by use of a calibrated pH meter and a combination electrode. The integrity of individual urine samples will be maintained. If no urine is produced during a given time interval, the receptacle will remain in position and be collected at the next interval.

Both blood and urine samples will be split, with one portion being analyzed directly for the presence of the lactone, and the other being acidified using 6.0 M hydrochloric acid (to cause formation of the lactone) and analyzed for the lactone. The general scheme of biosample analysis is laid out in section V.4.4.2, below. Directorate of Laboratory Sciences, USACHPPM, will conduct all analysis of the biosamples.

Data collected during the course of this experiment will be treated as described in SOP 052, Handling and Storage of Test Records, Data, and Specimens (33).

b. Assignment of animals: Each animal will be identified by cage card only. All animals will be assigned a unique identification number as per SOP 003 (28).

c. Housing: The animals will be individually housed in solid bottom (8 in x 9 in x 8 in) shoebox cages from the time of receipt and given water *ad libitum*. Animals will be given certified rodent feed *ad libitum*. Animal rooms will be maintained at a constant temperature range (64 -79°F) and humidity range (30 - 70%) with a 12-hour light/dark cycle (31).

d. Administration of test substance. Oral dosing will be performed using a stainless steel 16 ga x 2 inch gavage needle. Per EPA Health Effects Test Guidelines, the volume given will not exceed 10 mL/kilogram of body weight (27). Chromatographic Analysis Division (CAD), Directorate of Laboratory Sciences (DLS), USACHPPM will perform all analyses for purity, concentration, and stability of the dosing material.

e. Observation of animals. Observations made of the animals upon receipt and during the course of the experiment will be detailed and carefully recorded in the laboratory notebook. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards).

f. Study conduct. The study described will be conducted in compliance with the Good Laboratory Practice (GLP) regulations in the Toxic Substances Control Act (TSCA): 40 CFR (Code of Federal Regulations) 792, plus amendments (24). The investigators and technicians will adhere to The Guide for Care and Use of Laboratory Animals (U.S. Department of Health, Education, and Welfare Publication No. NIH 86-23, 1996) (26).

V.2 Data Analysis:

No formal statistical tests to determine population characteristics are planned on the experimental data. The data analysis concept in this experiment is very simple; the desired endpoint being simply to distinguish between the two possible metabolites. By collecting blood and urine samples at several different time points, we expect to observe a rise and fall of detectable compounds in both biological media. Because multiple samples are being obtained from each animal, the presence of a curve over time effectively provides a larger n-value, and each animal is, to a degree, self-validating. The baseline assumption is that the animals will excrete one or the other of the metabolites, and any appearance of the other metabolite is a result of spontaneous chemistry (we can control for this by knowing the pH of the samples, especially urine). Absence of the lactone in the directly-analyzed samples, but its presence in the acidified samples, will indicate the presence of HEAA rather than the lactone, and disprove the null hypothesis. Should multiple replications of this experiment occur, data from each time period for each gender of test animal will be averaged and standard deviations computed, with further data analysis performed as the situation requires.

V.3 Laboratory Animals Required and Justification:

V.3.1 Non-animal Alternatives Considered: No *in vitro* or non-animal alternatives are known. The animal's absorption, digestion, metabolism, and excretion (ADME) processes are essential to carry out metabolic changes that occur in the substrate.

V.3.2 Animal Model and Species Justification: Most previous studies in this area have been carried out in the rat. The rat is also the only species that has demonstrated

susceptibility to the nasal carcinoma from dioxane in drinking water that is the foundation of the EPA drinking water risk assessment.

V.3.3 Laboratory Animals:

V.3.3.1 Genus and Species: *Rattus norvegicus*

V.3.3.2 Strain/Stock: Sprague-Dawley

V.3.3.3 Source Vendor: Charles River Laboratories, Wilmington, MA (USDA 14-R-0144)

V.3.3.4 Age: Rats aged 4-6 weeks will be employed. Rats to be outfitted with vascular catheters typically have a mass between 125 and 350 g each, dictated by their age and the desired number of catheters (21). Animals furnished with single catheters tend toward the bottom of this weight range. Animals aged 4-6 weeks typically have a body mass of 125-150 grams.

V.3.3.5 Sex: Male and female. Both sexes are required to eliminate the impact of any gender differences in metabolism of the compound.

V.3.3.7 Special Considerations: Animals will be obtained from Charles River Labs. This lab performs routine health testing and can provide written reports of this testing to the Attending Veterinarian. Animals will be tested for and certified free from common pathogens of rats. In addition, rats will be received from Charles River Labs implanted with femoral artery catheters. The standard acclimation period will be waived and the study will begin the day after receipt of the animals to avoid complications with the femoral catheters.

V.3.4 Number of Animals Required (By Species): 6 animals are required for each run of this experiment (3 male; 3 female); plus one of each sex will be ordered for weight matching and unexpected complications. Animals will be procured only in groups of 8. A second and third run of this experiment would require an additional 8-16 animals, but will be undertaken only if at least 3 acceptable data points are not obtained from each experimental animal during the first trial. Additional animals will not be ordered if their use is unnecessary based on the results of the first experimental trial.

V.3.5.1 Refinement:

Procedures employed in this experiment are not anticipated to cause pain or undue distress in the animals. Vascular catheters avoid repeated vein puncture for blood sampling. Animals will be single-housed to minimize potential dislocations of the

catheters. Animals will be used the day after receipt to minimize the possibility of malfunctioning catheters and animal discomfort resulting from the presence of the catheter.

V.3.5.2 Reduction:

Each run of this experiment will use only 6 animals, the minimum necessary to detect potential gender differences in response and accommodate 2 treatment groups and a control. Two additional animals will be procured in the event of catheter failure or other experimental exigencies. Group sizes are based only upon the number of experimental treatments, and are not based upon statistical considerations.

V.3.5.3 Replacement:

There are no practical replacement alternatives, since the entire ADME cycle is necessary to determine the outcome. *In vitro* methods were considered, but cannot sufficiently replicate the physiology and metabolism of the intact animal.

V.4 Technical Methods:

V.4.1 Pain/Distress Assessment

The gavage procedure is not considered painful or distressful when conducted by trained individuals.

Monitoring: The short time-frame of the experiment and the continuous attention required during its execution will ensure that the animals will be continuously monitored over the first four hours of the experiment, ensuring rapid response to any exigencies that develop. During the period between the 4 and 8 hour samplings, animals may be briefly unattended. Should any animal distress behavior, such as seizures, ruffled fur, self-mutilation, or extraordinary emotional withdrawal be observed during the first 4-hour period, monitoring will be continuous over the 8-hour duration of the experiment.

V.4.1.1 APHIS Form 7023 Information:

V.4.1.1.1 Number of Animals:

V.4.1.1.1.1 Column B: 0

V.4.1.1.1.2 Column C: 24

V.4.1.1.1.3 Column D: 0

V.4.1.1.1.4 Column E: 0

V.4.1.2 Pain Relief/Prevention: Since catheters will be pre-implanted and gavage is not considered a painful procedure when performed by trained individuals, it is not anticipated that the animals will experience pain during the experiment. Should evidence of pain appear, the attending veterinarian will be consulted, and the animals euthanized upon his/her recommendation.

V.4.1.2.1 Anesthesia/Analgesia/Tranquilization: N/A

V.4.1.2.2 Pre- and Post-Procedural Provisions: Animals will be observed for signs of pain or discomfort, to include seizures, ruffled fur, or extraordinary emotional withdrawal, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards).

V.4.1.2.3 Paralytics: N/A

V.4.1.3 Literature Search for Alternatives to Painful or Distressful Procedures: N/A

V.4.1.3.1 Source(s) Searched: N/A

V.4.1.3.2 Date of Search: N/A

V.4.1.3.3 Period of Search: N/A

V.4.1.3.4 Key Words of Search: N/A

V.4.1.3.5 Results of Search: N/A

V.4.1.4 Unalleviated Painful/Distressful Procedure Justification: N/A

V.4.2 Prolonged Restraint: N/A

V.4.3 Surgery:

V.4.3.1 Pre-Surgical Provisions: N/A

V.4.3.2 Procedure: N/A

V.4.3.3 Post-Surgical Provisions: N/A

V.4.3.4 Location: N/A

V.4.3.5 Surgeon: N/A

V.4.3.6 Multiple Major Survival Operative Procedures: N/A

V.4.3.6.1 Procedures: N/A

V.4.3.6.2 Scientific Justification: N/A

V.4.4 Animal Manipulations:

V.4.4.1 Injections: N/A

V.4.4.2 Biosamples: 100 µL blood samples will be drawn through pre-implanted vascular catheters on the schedule previously indicated, resulting in 6 samples being taken from each animal over an 8-hour period. Urine samples will be collected in the trap of the metabolism cage and will involve no discomfort to the animal.

Biological fluid samples will be split into two aliquots. Aliquot 1 will be analyzed directly for the presence of the lactone by extraction using methylene chloride, and analyzed via gas chromatography utilizing a flame-ionization detector (FID). Aliquot 2 will be acidified by addition of a single drop of 6.0 M HCl to promote formation of the lactone from HEAA, the pH determined, and extraction performed in the same manner as aliquot 1.

Absence of detectable lactone in aliquot 1 combined with its presence in aliquot 2 will indicate HEAA is the physiological metabolite and demonstrate that detection of the lactone in previous studies was simply an artifact of analytical procedures.

V.4.4.3 Adjuvants: N/A

V.4.4.4 Monoclonal Antibody Production: N/A

V.4.4.5 Animal Identification: Cage cards will be used in accordance with SOP 003 (28).

V.4.4.6 Behavioral Studies: N/A

V.4.4 7 Other Procedures: Oral gavage is performed using a 16 ga x 2 inch gavage needle. The animal is grasped such that the index and middle finger of the handler's

hand restrain movement of the head while the thumb and remaining fingers support and restrain the animal's thorax. The restraining hand is rotated so that the animal is oriented nearly vertically, but inclined slightly dorsally. The gavage syringe is then inserted in the animal's mouth and gently inserted down the esophagous. The gavage dose is then administered by depressing the plunger of the syringe. After the dose has been administered, the gavage needle is removed by reversing the procedure used during insertion, and the animal is restored to its feet.

V.4.4.8 Tissue Sharing: N/A

V.4.5 Study Endpoint: The study will be completed within 8 hours of initiation. Test and control animals will be euthanized immediately following the completion of the final blood and urine samples at 8 hours after gavage.

V.4.6 Euthanasia: Rats will be injected with a lethal dose of a pentobarbital-based euthanasia solution via the existing vascular catheter, in accordance with SOP 066 (29).

V.5 Veterinary Care:

V.5.1 Husbandry conditions: Rats will be confined to metabolism cages during the execution of the experiment; at other times, they will be singly-housed in solid bottom cages. Animals will be housed and fed according to existing standards (30, 31). Feed will consist of certified rat chow; water will be provided *ad libitum*. Animal rooms will be maintained at a constant temperature (64-79°F) and humidity (30-70%) with a 12-hour light/dark cycle.

V.5.1.1 Study Room: Studies will be conducted at the USACHPPM Directorate of Toxicology facilities, Building E-2100/E-2101, Aberdeen Proving Ground, MD 21010-5403. The animal care facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

V.5.1.2 Special Husbandry Provisions: There will be no acclimation period because the rats must be used the day after receipt to avoid failure of the vascular catheters.

V.5.1.3 Exceptions: N/A

V.5.2 Veterinary Medical Care:

V.5.2.1 Routine Veterinary Medical Care: All animals will be observed and handled on a daily basis by the animal caretakers and a member of the study staff to assess their health and welfare. Appropriate methods of animal care shall be maintained to prevent,

control, diagnose, and treat diseases and injuries. Animal users have been trained in the handling, immobilization, anesthesia, and euthanasia of the laboratory animals.

V.5.2.2 Emergency Veterinary Medical Care: During the course of this study it is not expected that the animals will become sick due to dioxane ingestion. However, if an animal becomes moribund, is unable to feed, is injured, or vascular access complications arise, the observer will report to the attending veterinarian. Moribund animals may be humanely euthanized according to SOP 066 (29). Emergency veterinary care will be available at all times including weekends and holidays.

V.5.3 Environmental Enrichment:

V.5.3.1 Enrichment Strategy: Animal caretakers and principle investigators will handle rats upon receipt and prior to the dosing regimen. All rats will be evaluated and handled as necessary to maintain health, and will be individually housed. Rats will be provided a certified Nylabone on day of receipt, and receive food and water *ad libitum* while in the solid bottom cages.

V.5.3.2 Enrichment Restrictions: Presence of the vascular catheters prevents employment of other environmental enrichment strategies. Neither food nor Nylabones will be provided to the rats while they are housed in the metabolism cages, however water will be available.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Dr. William Eck has a Ph.D. in Biochemistry. He has previously conducted studies employing mice and rats, with experience in techniques including oral gavage, cardiac puncture, and organ recovery and processing.

Dr. Craig McFarland has graduate training and experience in Wildlife Biology (MS) and Environmental Toxicology (Ph.D). He has animal care and use training while at Texas Tech University with a small mammal focus (GLP conditions). He has 12 years field and laboratory experience in studies of small mammal toxicology entailing extensive trapping and handling, assessment, blood and tissue collection, humane euthanasia, and necropsy. As a veterinarian, he practiced small animal medicine and surgery for 20 years.

All investigators named in this study have demonstrated an understanding of the humane care and use of research animals through successful completion of courses offered by the American Association for Laboratory Animal Science (AALAS). They have taken part in discussions of pertinent laws and regulations concerning the use of animals in biomedical research in the Department of Defense as required by Public

Laws 89-544, 91-579, and 99-198 (The Animal Welfare Act and Amendments, DOD Directives, and Army Regulations). They are familiar with the concepts for the reduction or elimination of the use of animals and have concluded that there is a need for the use of animals in this study. They have been familiarized in the proper methods for minimizing and/or alleviating pain in the animal species selected for study. They will either have an animal technician assigned to assist them who is an expert in the animal techniques required for the study, or have exhibited sufficient proficiency themselves to justify allowing them to work unassisted or without direct guidance from the laboratory veterinary staff. If their training in required procedures has not been of a formal, documented nature, they will demonstrate proficiency in these procedures prior to performing them on study animals. The Attending Veterinarian will observe them and determine proficiency or additional training needs. They have been advised on the animal care and use policy at this institution and are aware of the established reporting mechanisms for the observed deficiencies in animal care and treatment. Appendix A contains a list of personnel supporting this protocol and Table 1 lists the personnel that will be performing procedures during this study.

Table 1. Study Personnel Qualifications

Staff Member	Procedure	Training	Experience	Qualifications
William Eck	Oral gavage, necropsy	Rat handling & gavage training (CHPPM, 7/2007); Rat necropsy training (CHPPM, 12/2007 & 2/2008); AALAS Training online (CHPPM Animal Users' Track)	5 years animal research	Ph.D. Biochemistry
Lee Crouse	Oral gavage, blood sampling, urine collection, euthanasia	Humane Care and Use of Laboratory Animals (May 2000); Rodent Handling Techniques, (WRAIR. 1996); Rat handling & gavage training (CHPPM, 7/2007)	15 years animal research	M.S. Environmental Science
Craig McFarland	Oral gavage, euthanasia	Rodent & Small Animal Handling Workshop (MRICD 10/2006); Rat handling & gavage training (CHPPM, 7/2007); Rat euthanasia (CHPPM, 10/2007)	12+ years animal and toxicological investigations	DVM, Ph.D. Environmental Toxicology

VII. BIOHAZARDS/SAFETY: Adherence to standard chemical and animal handling procedures will be required during the performance of this study. Dioxane is considered

a potentially hazardous, carcinogenic material, and will be handled in accordance with SOP 083(32). In addition, all personnel participating in this study will conform to USACHPPM Occupational Health and Safety Regulations 385-1(36) and 385-5(37).

VIII. ENCLOSURES

a. References

IX. STUDY TIME FRAME

IX.1 Estimated Experimental Start Date: June 2008

IX.2 Estimated Experimental End Date: June 2008

X. ASSURANCES: As the Principle Investigator on this protocol, I provide the following assurances:

A. Animal Use: The animals for use in this protocol will be used only in the activities and in the manner described herein, unless a deviation is specifically approved by the IACUC.

B. Duplication of Effort: I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal.

D. Biohazard/Safety: I have taken into consideration, and have made the proper coordination regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, etc., in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" which the DOD has embraced, namely,

"Responsibility" for implementing animal use alternatives where feasible, and conducting humane and lawful research.

G. Scientific Review: The proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful procedures: I am conducting biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals that WILL NOT be relieved with the use of anesthetics, analgesics and/or tranquilizers.

William S. Eck, Ph.D.

First name, MI, Last name of Study Director/Principal Investigator

(S.D. Signature) (Date)

ANNEX 1 TO APPENDIX C

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