THE URINARY EXCRETION OF MERCAPTURIC ACIDS IN FREE-LIVING ADULT MALES

by

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Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Human Nutrition and Foods

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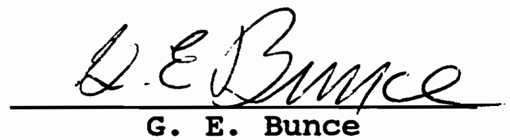

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The Urinary Excretion of Mercapturic Acids in Free-Living Adult Males

ABSTRACT

In order to establish a profile of detoxification via glutathione conjugation, the level of urinary mercapturic acid excreted by a free-living male population and the effect of external environmental and genetic factors, such as consumption of vegetables, fruits, and meat, charbroiled food intake, tobacco, alcohol, caffeinated coffee, and marijuana use, exposure to chemicals and familial cancer incidence, were investigated. A subgroup of 30 subjects was randomly selected from 117 subjects who complied with the collection protocol. Three consecutive 24 hr urine samples of this subgroup were analyzed. The modified method of Seutter-Berlage et al(Chemical Porphyria in Man. Elsevier/North-Holland Biomedical Press, N.Y. 1979:233-236) was used for the quantitation of urinary mercapturic acid.

The mean excretion of mercapturic acid was 0.27 mmole mercapturate(-SH)/24 hr and 18.1 umole -SH/mmole creatinine. An analysis of variance showed a large degree of inter- and intraindividual variability. The interindividual coefficients of variation in mmole -SH/24 hr and umole -SH/mmole creatinine were 37.7% and 31.2%, respectively. The intraindividual coefficients of variation in mmole -SH/24 hr and umole -SH/mmole creatinine were 32.4% and 30.1%, respectively.

A higher($p \leq 0.05$) excretion of mercapturic acid was observed among subjects with a high frequency of exposure to chemicals. The lack of significance of the other dietary, non-dietary, and genetic factors on the observed mercapturic acid excretion may be due to the large inter- and intra-variability, the use of food consumption frequency in food intake analysis and unequal sample sizes of subgroups.

ACKNOWLEDGEMENTS

I sincerely express my gratitude to my committee members, Doctors Ryland E. Webb, Forrest W. Thye and George E. Bunce for their patience and persistence regarding my thesis work.

In addition, I would like to thank Jeff Morgan for the statistical analysis of the data and his tireless patience. Special thanks are due my colleagues Rita Lugogo and Yuni Kim for their technical advice, assistance and moral support.

Loving thanks to my friends and family, especially to my parents, who gave me the opportunity and encouragement to pursue my dreams.

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CHAPTER I: INTRODUCTION

Humans live in a chemical environment which consists of nutrients(macronutrients and micronutrients) and non-nutrients or xenobiotics. Xenobiotics are exogenous compounds of no known functional value to individuals. They can not yield energy or contribute to structures(1). They are encountered by individuals (a) deliberately, due to their use as drugs, cosmetics, etc., (b) coincidentally, in the food or contaminants in the diet; or (c) accidentally, from industrial, agricultural or waste sources(2).

The predominant routes by which xenobiotics enter the human body are gastrointestinal, respiratory, and epidermal(3). Absorption at these portals of entry is primarily attributed to the lipophilic nature of the xenobiotic. This property also facilitates the diffusion of the xenobiotic through the lipid membranes of many cells and the interaction with various blood lipoproteins necessary for its distribution throughout the body(3,4). Xenobiotics, which are absorbed through the gastrointestinal tract, travel directly to the liver, mainly via the portal vein, while those absorbed through the skin and lungs are distributed to various body tissues and organs by more indirect routes(3). Xenobiotic metabolism occurs to a variable degree in the portals of entry and in the kidneys; however, both quantitatively and qualitatively, the liver is the predominant

site of metabolism(3,5).

The fate of xenobiotics varies: (a) the xenobiotic may be excreted unchanged, if the compound is already very polar and unreactive to other cellular constituents; (b) some may undergo spontaneous reactions(non-enzymatic) to form new products that may be more or less reactive than the parent compound; and (c) the majority of xenobiotics undergo enzymatic metabolism, biotransformation(6).

Most biotransformations can be divided into phase I and phase II reactions. Phase I reactions, including oxidation, reduction, and hydrolysis, expose functional groups or introduce a polar functional group(-OH,-NH2,-SH, or -COOH) into parent compounds. The functional group serves as the site for the phase II reactions(6,7). A complete discussion of phase I metabolism may be found in Hodgson and Dauterman(4), and in Alvares(5).

During phase II metabolism, the parent xenobiotic and/ or its phase I metabolite(s) conjugate with an endogenous moiety, such as glucuronic acid, glutathione, amino acid(s), etc.(6,7). Although numerous classes of conjugation reactions are recognized in mammals, the six major reactions are glucuronidation, sulfation, methylation, acetylation, glutathione conjugation, and amino acid conjugation(2). The products of the principal conjugation reactions are mainly acidic, water-soluble compounds and are primarily excreted in

the urine. In general, phase II conjugation reactions serve to end the biological activity of the compound and are thus termed detoxifications(6,7). However, it has become increasingly clear that the process of converting a lipid soluble xenobiotic into a water soluble metabolite also can result in the formation of a more biologically reactive compound. A review of bioactivation is presented by Monks and Lau(8). Nevertheless, for the majority of xenobiotics, the ultimate effect of biotransformation is inactivation and enhanced excretion(7). More recently, the term biotransformation has been adopted to collectively describe all of the phase I and phase II reactions involved in xenobiotic metabolism, without reference to the consequences of bioactivation(9).

Xenobiotics and/or their metabolites are excreted from the body predominantly in urine and bile, but also via expired air, perspiration, vomitus, hair, and mammary secretions(9). The excretory mechanisms are similar to those involved in the elimination of endogenous waste products(10,11). Hence, elimination via hepatic and renal pathways predominate. Furthermore, urinary and biliary pathways of excretion are known to be complementary; however, the mechanisms which dictate the principal routes remain unclear(10-12).

Given this overview of toxicology from exposure to excretion, it should be evident that the biological response

of an organism to a xenobiotic is most critically dependent on the highly complex realm of the phase I and phase II biotransformations. In the past, most toxicological investigations have rigorously focused attention on the phase I biotransformation, leaving the phase II conjugation reactions relatively neglected(7). From a pharmacological viewpoint(specifically product development, therapeutic effectiveness, and safety) such an emphasis is understandable because metabolic activation occurs primarily during the phase I biotransformations(5). Consequently, knowledge concerning the multiple variables potentially influencing the conjugation reactions as a whole is lacking.

In 1979, Caldwell(7) pointed out the importance of conjugation reactions in drug metabolism and toxicity and suggested that the two phases of biotransformations should not be considered separately. The significance of conjugation as a detoxication mechanism becomes obvious only when the reaction is impaired due to saturation or a metabolic defect. In such cases, the protective effect is voided and the toxicity is manifested in some manner(6,7). For example, calf kidneys do not acetylate cysteine conjugates to form mercapturic acids. The absence of N-acetyltransferase may be the reason why calves are susceptible to the toxic reactions of dichlorovinylcysteine(DCVC)(13).

From a toxicological viewpoint, one of the most important

aspects of six conjugation reactions is the low capacity of the glutathione conjugation(7). Many of the highly reactive electrophilic compounds which enter the body or are generated during the enzymatic oxidation bind covalently to the macromolecular constituents such as proteins, RNA and/or DNA by a reaction with nucleophilic -SH, -NH2, or -OH groups(14). The subsequent structural modification of vital cellular macromolecules can result in allergy, necrosis, teratogenesis, mutagenesis, or carcinogenesis(7). And, the most versatile of the ways in which the body protects itself from these electrophiles is by their conjugation with the highly reactive nucleophilic tripeptide, glutathione, via the influence of a family of enzymes.

The fate of glutathione conjugates varies from biliary excretion to further metabolism of the tripeptide. However, intestinal microfloral hydrolysis and subsequent enterohepatic circulation suggests that biliary elimination is relatively insignificant(10). On the other hand, subsequent catabolism of glutathione conjugates results in the formation of S-substituted, N-acetylcysteines (mercapturic acids), which are excreted in the urine. Of biliary and urinary excretion pathways, the formation of urinary mercapturic acid is quantitatively the more important(2).

The purpose of this research was to quantitatively assess the urinary excretion of mercapturic acids in a free-living

population of young male adults, in order to establish a profile of detoxication via glutathione conjugation.

CHAPTER II: REVIEW OF LITERATURE

Mercapturic acid biosynthesis is considered to be a detoxication pathway. First, the endogenous nucleophilic glutathione(GSH) conjugates with an exogenous electrophilic compound. Second, subsequent metabolism of the glutathione conjugate generates an organic acid, a mercapturic acid. The acquired water-solubility facilitates the excretion of the xenobiotic(2,15). The following review will discuss this pathway in more detail. The review begins with the introduction of the whole pathway followed by a discussion of the nature of substrates and the properties and functions of enzymes. Finally, the factors affecting urinary excretion of mercapturates and previous studies determining urinary mercapturates are discussed.

I. The Mercapturic Acid Pathway

A century ago, N-acetylcysteine conjugates(mercapturic acids) were first discovered by Bauman and Preusse after administration of bromobenzene to dogs(15). However, the mechanism was not conclusively established until Barnes et al(16) and Bray et al(17) in 1959 found that the cysteinyl moiety was mainly derived from GSH and that GSH conjugation was catalyzed by GSH S-transferase(14). The formation of mercapturic acid (Figure 1)(15,18) begins with the elimination of a leaving group and the formation of a thioether conjugate

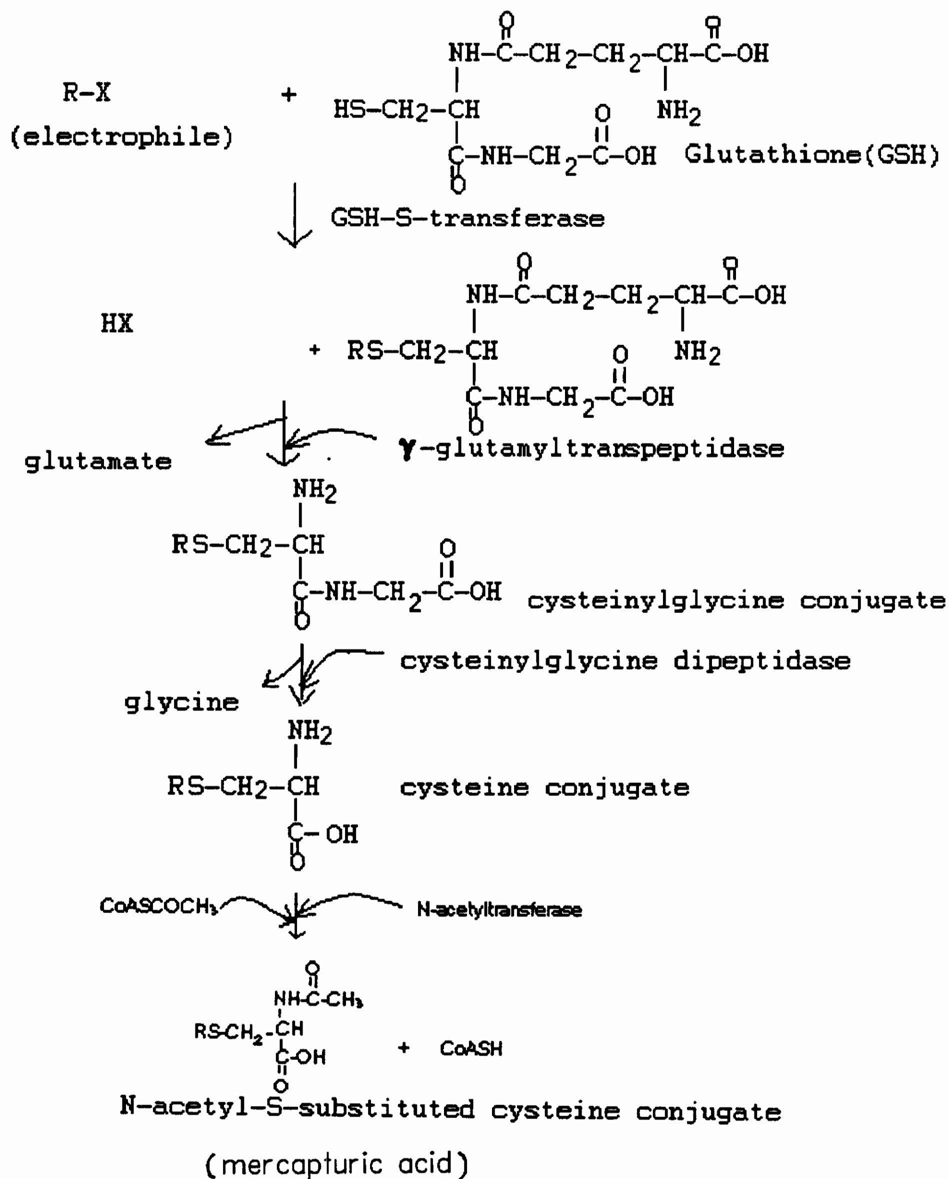

Figure 1. The Pathway of Mercapturic Acid Formation. Modification from Stevens and Jones(15) and Jakoby et al(18).

of GSH. Conjugation is followed by the removal of the γ-glutamyl moiety of the GSH conjugate by the action of γ-glutamyltranspeptidase. In turn, a glycine residue is released from S-substituted cysteinylglycine dipeptide by cysteinylglycine dipeptidase hydrolysis. Mercapturic acid biosynthesis is completed by N-acetylation to give an N-acetyl-S-cysteine conjugate(18).

A. Glutathione(GSH)

GSH, the tripeptide γ-glutamylcysteinylglycine, is found in bacteria, plant, insect and animal tissues. It is one of the most important sulfhydryl compound in living cells at relatively high intracellular concentrations(3mM seems average)(19). The intracellular concentration of GSH represents a balance between the catabolic and biosynthetic processes in response to a variety of biochemical and physiological influences(20,21). For example, after administration of dimethylazoamino benzene and other azo dyes, the amount of glutathione conjugates excreted is two to three times greater than the normal level of liver GSH(22). The implication is that the liver can rapidly synthesize large quantities of GSH to cope with the depletion of GSH. Hence, an adequate resynthesis of GSH is essential for the conjugation-detoxification system.

GSH carries on a variety of physiological and metabolic

functions. One such function is the detoxification of hydrogen peroxide, organic peroxides, free radicals, and foreign compounds(23). The importance of GSH in xenobiotic metabolism is due to the reactive thiol group and the combination of polarity and size associated with its tripeptide structure(19). Thiol groups are strong nucleophiles, which can inactivate electrophiles and form glutathione conjugates. As GSH reacts with lipophilic xenobiotics, it bestows polarity on the lipophilic compounds. The increased polarity reduces affinity for cell membranes and facilitates the removal of xenobiotics from the cells and the body(19).

B. Foreign Compounds Forming Mercapturic Acids

Based on in vitro studies of conjugations with GSH and of glutathione S-transferase, Chasseaud(24) suggested that compounds undergoing mercapturic acid formation usually contain an electrophilic center such as a carbon, sulfur, nitrogen or oxygen atom. These electrophilic centers may already be present in the molecule or they may arise as a result of an earlier biotransformation(2). As previously discussed, GSH is a highly reactive compound and is present in a relatively high concentration. Therefore, nonenzymatic conjugation reactions also can occur at a measurable rate(19). The rate of spontaneous reaction between GSH and an

electrophile depends on the nature of the electrophile. In general, GSH will react more rapidly with an electrophile having a low positive charge density than with one having a high positive charge density. In the case of a reactive electrophile with a high positive charge density, enzymatic conjugation is essential for any success in GSH-dependent detoxification(25).

According to Wood(26), the mercapturic acid precursors may be classified into four types. Each type is catalyzed by a specific enzyme.

Type I. Aromatic hydrocarbons or their halogen derivatives which require oxidation to an intermediate, presumably an epoxide.

Type II. Aromatic hydrocarbons with a displaceable halide or nitro group which is activated by nucleophilic substitution.

Type III. Alkyl esters such as alkyl halides, sulfonates, and nitrates.

Type IV. Alkenes activated by a conjugated group such as an aromatic ring, e.g., styrene.

C. Enzymes of Mercapturic Acid Formation

1. GSH-S-transferase

The initial reaction in the mercapturic acid pathway is catalyzed by a family of isoenzymes, glutathione-S-

transferases(GST). These enzymes are homo- and hetero-dimers with a molecular weight of approximately 46,000 daltons(19,27). Based on their similar structure and enzymatic properties, the subunits from rats, man and mice are grouped into three gene families: alpha, mu and pi(28). Each subunit seems to be present in almost every organ; however, the level of expression differs significantly from tissue to tissue as well as from cell to cell. In rats, the liver has the highest amount of total GST protein. In general, most epithelial cells express high levels of several subunits, and in hepatocytes, kidney tubular epithelia and epithelia of the gastrointestinal tract, the GST protein consists of a large fraction of the total cytosolic protein(27). GST activity is clearly in the cytosol. However, many of the xenobiotics are water-insoluble and would be expected to partition into cellular membranes. Thus, Boyer et al(29) in studying whether the soluble GST could interact directly with membrane-bound molecules or not observed that soluble transferases did not bind membrane-bound substrates. In addition, the rate of catalysis depended on the partition coefficient of substrates between lipid phase and water.

Recently, membrane-bound enzymes in the endoplasmic reticulum and the nuclei of rats also have been reported(27,30). There are no significant sequence similaries between cytosolic and microsomal enzymes(27,30). In addition,

nuclear GSTs may play a role in the detoxification of peroxided DNA(27).

The function of the GSTs as catalysts is twofold: (1) nonspecific binding proteins for hydrophobic xenobiotics, and (2) catalyzing a glutathione conjugation if the hydrophobic ligand possesses an electrophilic center(27). Therefore, these enzymes express broad and overlapping substrate specificities towards a variety of mutagenic, carcinogenic and pharmacologically or metabolically active substances(27). Adang et al(31) postulated a model of the interactions of GSH in the G-site in GSTs. The γ-glutamyl moiety of GSH, especially the carboxylate group, is the major binding determinant that allows the thiol group to align properly at the reaction site. The GSTs appear to be very critical with respect to the correct position of the thiol group for conjugation. The glycyl subsite is the least restrictive domain in the GSTs. Additionally, kinetics analysis revealed that alpha class and the mu class of GST isoenzymes have different acceptance of modified GSH analogues(31). Therefore, Adang et al(31) explained that different classes of isoenzymes probably have different G-sites. Five types of glutathione reactions have been classified by Ketterer (Table 1)(19).

In order to reach the active site of extracellular degradative enzymes, the GSH conjugate has to be extruded from

Table 1. Classification of Glutathione Conjugations(19)

Classification	Examples
nucleophilic Displacement at a Saturated Carbon	$O_2N-C_6H_4-CH_2Cl \xrightarrow{GSH} O_2N-C_6H_4-CH_2SG$
Nucleophilic Displacement at an Aromatic Carbon	$O_2N-C_6H_3(NO_2)-Cl \xrightarrow{GSH} O_2N-C_6H_3(NO_2)-SG$
Nucleophilic Attack Involving the Opening of a Strained Ring	$O_2N-C_6H_4-OCH_2CH(O)CH_2 \xrightarrow{GSH} O_2N-C_6H_4-OCH_2CH(OH)CH_2SG$
Michael Additions	$C_6H_5(H)C=C(H)COCH_3 \xrightarrow{GSH} C_6H_5-CH(SG)CH_2COCH_3$
Reactions with Electrophilic Cations	$C_6H_5-N=N-C_6H_4^{+}=NCH_3 \xrightarrow{GSH} C_6H_5-N=N-C_6H_3(SG)-NHCH_3$

the hepatic and renal cells. It is assumed that GSH conjugates as well as GSH are secreted into lumen via the same carrier transport system(32).

2. Degradative Enzymes of GSH-Conjugates

(a). Gamma-Glutamyl Transpeptidase

The degradation of glutathione conjugates is initiated by the loss of the γ-glutamyl residue. So far, γ-glutamyltranspeptidase(γ-GT), a membrane bound protein, is the only enzyme known to cleave the γ-glutamyl peptide bond(15,18). It is located exclusively on the extracellular surface of the plasma membrane. The location of the enzyme implies that this degradation is an extracellular process(15). It is generally accepted that the kidney is the major site of the catabolism of GSH and GSH conjugates because the most commonly used experimental animal, the rat, has a lower level of activity of hepatic γ-glutamyltransferase and a higher renal γ-GT activity level(33). In a recent study, Hinchman and Ballatori(34) examined the relative importance of hepatobiliary catabolism of GSH and GSH conjugates by comparing γ-GT in the liver, the gallbladder, and the kidneys of six mammalian species and found that in species such as the guinea pig and perhaps humans, the liver and biliary system also play a large role in GSH and GSH conjugates catabolism.

As a general rule, cells with a secretory or absorptive function have higher enzyme activity levels. Included among these cells are pancreatic acinar and ductile epithelial cells, the epithelial cells of renal proximal tubule, and small intestinal epithelium(33).

γ-GT is a hetero-dimer with heavy and light subunits. The heavy subunit probably provides binding domains for glutathione and other γ-glutamyl peptides, thereby imposing reaction specificity in the dimeric enzyme toward γ-glutamyl compounds; the light subunit would provide the catalytic center for cleavage of the peptide bond in the substrate(33). Although γ-glutamyl transpeptidase can catalyze two types of reactions, hydrolysis and transpeptidation, to yield cysteinylglycine thioether, hydrolysis is the major reaction catalyzed in vivo(15).

(b) Cysteinylglycine Dipeptidase or Aminopeptidase

Following γ-GT-catalyzed hydrolysis, microsomal aminopeptidase or the more specific cysteinylglycine dipeptidase hydrolyzes the second peptide bond of glutathione conjugates(15). Hughey et al(35) demonstrated that this particular peptidase is located with γ-GT in the brush border membranes. The enzyme exhibits greater activity with S-derivatized substrates than with simple cysteinylglycine. In addition, the membrane-bound renal aminopeptidase prefers

substrates containing a N-terminal amino acid residue with a hydrophobic side chain. Heat-inactivation studies show that all of these activities are attributable to a single enzyme(35). Therefore, characteristics of this dipeptidase indicate that it, as well as γ-GT, has a broad substrate specificity(33,35). In general, the highest activity of dipeptidase is present in the kidney, the liver, and the intestinal mucosa(33).

As cysteine conjugates are formed, they are reabsorbed by the renal tubule cells, especially the renal proximal tubule, and hepatocytes for N-acetylation(32).

(c). Cysteine S-conjugate N-Acetyltransferase

Following the two extracellular hydrolytic steps, mercapturate biosynthesis is completed by N-acetylation of the remaining thioether of cysteine. Cysteine S-conjugate N-acetyltransferase is presumably responsible for the reaction(36,37). Topological studies show that the active site of N-acetyltransferase is located on the outer surface of the endoplasmic reticulum in rat kidney and livers(35,37). Moreover, the proximal straight tubule plays an important role in the N-acetylation of a cysteine conjugate after it is absorbed(32). The efficiency of acetylation in rat kidney increases with the lipophilicity of the specific substitute on the sulfur(36). A hypothesis has been proposed that the

kidney should be mainly responsible for acetylation of hydrophobic substrates such as S-benzylcysteine, whereas hepatocytes would primarily be responsible for acetylation of hydrophilic substrates such as S-carbamidomethyl-cys(32). More studies are needed to verify this hypothesis.

Mercapturic acids formed in the kidneys will be resecreted in the proximal straight tubule to a great extent. On the other hand, mercapturic acids formed in the liver finally reach the kidney no matter whether they are released into the systemic circulation or the bile. Then, part of the mercapturic acids(25-35%) are filtered and stay in the tubular lumen(32). The others, escaping from filtration, are preferentially taken up by renal tubule cells from the basolateral side of plasma membranes via the probenecid-sensitive transtubular transport system and excreted rapidly into the lumenal space(39).

D. Factors Affecting Mercapturic Acid Formation

Xenobiotic metabolism, including GSH conjugation and mercapturic acid formation, is highly influenced by an extremely large number of variable or "host" factors(40-43). These factors can be broadly classified as environmental, physiological, and genetic. However, in spite of such classification, there is a complex and dynamic

interrelationship between all host factors(40,43). This complex and dynamic interrelationship makes it virtually impossible to examine the impact of an isolated variable on the rate and pattern of metabolism, let alone on the overall toxicity of xenobiotic compounds(40,43). Ultimately, the impact of such a complex and dynamic process is manifested as interindividual and intraindividual variations in xenobiotic metabolism(40-43).

The major factors responsible for interindividual variations are genetics and environmental conditions of the individual(44,45). On the other hand, the major determinants of intraindividual variations are the environmental and physiological conditions of the individual. Yet, it is well demonstrated that a slight alteration in any one critical host factor can have a profound effect on the metabolism of xenobiotics(43). The influence of these factors on xenobiotic metabolism will be discussed in the following review.

1. Nutritional Parameters Altering Xenobiotic Metabolism

Dietary nutrients can alter the metabolic pathways that are responsible for activation and elimination of xenobiotics. It has been reported that protein deficiency limits the availability of cysteine needed for synthesis of GSH. Indeed, GSH levels were determined to be reduced during protein deficiency(46). The potential for detoxification of many

xenobiotic compounds can be directly related to the hepatic GSH concentration. For example, depletion of GSH with diethylmaleate increases the toxicity of many xenobiotics(4,5). In 1988, Bauman et al(47) determined the effect of a dietary supplement of a cysteine derivative on hepatic GSH concentration. The results indicated that in addition to cysteine availability, previous dietary protein status plays a key role in the regulation of the feeding-induced diurnal rhythm of hepatic GSH concentration in rats. Additionally, Hagen et al(48) demonstrated that dietary GSH can be absorbed intact and results in a substantial increase in blood plasma GSH. This finding provides the basis for the therapeutic or prophylactic use of GSH against a wide variety of pathophysiological conditions.

One factor involving the sensitivity towards alkylating agents is the modulation of GSH S-transferases(28). The activity of isoenzymes may be changed by the presence of specific inhibitors or may be increased by the presence of inducing agents, such as xenobiotics, antioxidants, nutrients, etc.. Siddik et al(49) demonstrated that vitamin A-deficiency increases glutathione S-transferase activities in rat liver and kidney but not lung tissue. The authors suggested that the greater susceptibility of vitamin A-deficient respiratory tracts to chemical carcinogens is due to mechanisms other than the decreased GSH S-transferase activity(49). On the other

hand, in liver, the increased transferase activity may protect vitamin A-deficient rats against hepatocarcinogens metabolized there.

Recently, many researchers have focused on the inductive effect of cruciferous vegetables on glutathione S-transferases. An induction pattern study indicated that in brussels sprouts at least two compounds, probably allyl isothiocyanate and goitrin, are responsible for the induction of glutathione S-transferase(50). In addition, allyl isothiocyanate results in a much stronger enhancement of subunit 2(50). As a result, this enhancement of GSH S-transferase may protect cells from attack by electrophiles.

2. Environmental Factors

Chemical pollutants as well as dietary factors can alter the mercapturic acid pathway. Cigarettes contain hundreds of compounds, many of which are biologically highly reactive. As compared with nonsmokers, smokers tend to increase urinary thioethers(51). Van Doorn et al(52) and Lafuente and Mallol(53) further demonstrated a positive relationship between the number of cigarettes smoked each day and the urinary excretion of thioethers.

Besides the smoking factor, occupational chemical exposure also affects urinary excretion of mercapturic acids. Workers in chemical plants, exposured to complex mixtures of

chemicals, excreted more thioether compounds than workers in the metal industry(54). Similarly, an increase of mercpaturic acid excretion was also seen in rubber plants(55), retail petroleum sales(56), and dry cleaning workshops(57). In 1983, Buffoni et al(58) determined the concentration of mercapturic acids in the urine of smoking and non-smoking chemical employees and controls. Smoking did not have a significant effect on mercapturic acids excretion. Also, chemical workers, smokers and non-smokers, excreted significantly lower level of mercapturic acids than did the control group. It was suggested that the chemical employees may have impaired glutathione metabolism or depletion of glutathione stores. However, dietary factors, use of medications, mode of exposure, and a variety of other critical host factors could also be responsible for these unexpected results.

3.Physiological Factors and Sex

After the claim that the urinary excretion of mercapturic acid could be an indicator of exposure to alkylating agents(52,54), Pentz(59) investigated the influence of certain diseases in man and rats on the urinary excretion of mercapturic acid. The results showed patients with cirrhotic diseases and diabetics had higher mercapturate excretion. Correspondingly, rats with diabetes, hyperthyroidism, and partialy hepatectomies have higher mercapturic acid excretion.

Pentz(59) also reported that there was no significant difference in urinary mercapturic acid between males and females. However, Vainio et al(51) demonstrated that female clerks in chemical plants tended to excrete more mercapturic acid in urine than did male clerks. Furthermore, the rat and mouse studies have shown that males and females have different isoenzyme patterns of GSH S-transferases in liver(28).

In summary, only the study by Vainio et al(51) attempted to systematically account for a number of variable factors which could confound effects. But, the consumption of caffeine, alcohol, diet ,and other confounding factors were not examined. In addition, most of the control subjects were recruited from administrative departments in plants. As compared with a normal healthy population, they still have a greater chance to be contaminated by chemicals. Therefore, additional research considering the effect of confounding factors on urinary mercapturic acid excretion is needed.

II. Determination of Mercapturic Acid

In the past, the yield of mercapturic acids after exposure of animals to electrophilic compounds was measured gravimetrically after isolation from urine. However, the quantification of urinary mercapturic acid is often influenced by losses during the crystallization isolation(26). Shortly thereafter, acidification and solvent extraction replaced

crystallization isolation to enhance quantification of the small amounts of urinary mercapturic acids. Although the acidification procedure and solvent extraction is commonly used as the first step in the determination of urinary mercapturic acids, solvent extraction yields intractable gum and acidification makes the acid-labile components unidentifiable(26).

Since 1936, the liberation of thiol(-SH) group from mercapturic acid by alkali has been used as the basis of an iodometric, or colorimetric procedure(26). The colorimetric method is more specific than the iodometric method, but the iodometric method is not suitable for the small amounts of mercapturic acids present in urine. The iodometric method for the determination of mercapturic acid is influenced by hydroxyquinol because hydroxyquinol can react with I_2. This reaction may give high results (60). Parke and Williams(60) developed a turbidimetric method to measure phenylmercuric mercaptide after alkaline hydrolysis of phenylmercapturic acid. The results showed that mercapturic acids were completely hydrolyzed by less than 1N NaOH in half an hour, whereas 5N NaOH was needed for complete hydrolysis of arylcysteines. In other words, N-acetylphenylcysteines were more sensitive to alkaline hydrolysis than phenyl-cysteines. Besides, the unsubstituted phenylmercapturic acids were more stable to alkali hydrolysis than the substituted. The

researchers also suggested that 40 minutes was required to completely hydrolyze rabbit urine by 5N NaOH(60).

Recently, the method of Ellman has become a basic method in indirect spectrophotometric analysis for the estimation of sulfhydryl groups released during alkaline hydrolysis. In 1977, Seutter-Berlage et al(54) proposed the use of urinary mercapturic acids and thioethers as an indicator of exposure to alkylating agents. The developed method of thioether analysis consisted of initial deproteinization and centrifugation of the crude urine, followed by NaOH hydrolysis of the supernatant under nitrogen to prevent oxidation of the SH groups. After neutralization, Ellman's reagent, DTNB, 5,5'-dithiobis(2-nitrobenzoic acid), was used for colorimetric analysis, and the values were corrected by the non-hydrolyzed supernatant as blanks(54). Moreover, Vainio et al(51) systematically determined the content of mercapturic acids and thioethers in morning urine from various divisions of a chemical plant. The basic method of analysis was similar to that of Seuter-Berlage et al(54), except that ascorbic acid was added and standard thioglycolic acid was used. But, the whole alkali hydrolysis procedure was not carried out under nitrogen(51). However, vitamin C has a much higher redox-potential than GSH. In other words, in the presence of ascorbate, metal-EDTA complex can catalyze the conversion of ascorbate to dehydroascorbate. Then, this process enhanced

the oxidation of simple thiols to disulfides. Thus, Seutter-Berlage et al(61) suggested that instead of adding antioxidants, it was better to keep the pH of the final reaction mixture between 6.8-7.1.

Van Doorn et al(52) in 1979 further modified the analytical procedure in order to reduce the relatively high background values obtained in previous studies(54). This improved procedure consisted of the extraction of acidified urine samples with ethyl acetate and the reduction of all disulfide compounds with $NaBH_4$ before the alkaline hydrolysis of the thioethers. This method eliminated the interference of urinary disulfides normally coming from cystine(49umole SH/mmole creatinine). In addition, the values obtained here represented more specific compounds, ethyl acetate-soluble acidic thioethers such as mercapturic acids(52).

In order to decrease the large intraindividual variations in estimated thioether excretion by humans(52,54), Buffoni et al(62) used a cation-exchange resin to partially remove free cysteine and yellow urinary pigments. $NaBH_4$ reduction and affinity chromatography were employed to remove free -SH and -SS- groups in the urine before hydrolysis. Although the recovery(88%) and accuracy were good, the preparation of columns and the whole procedure was laborious. Clearly, this would be impractical for application to a large population. Therefore, the development of a simple practical procedure to

detect urinary mercapturate is needed.

III.Justification

Humans are continually exposed to an imposing number of xenobiotics through combinations of environmental, occupational, dietary, social and medicinal sources. Although xenobiotic metabolism is highly complex, phase II conjugation reactions are most often successful in terms of detoxification(7).

Like other conjugation reactions, mercapturic acid biosynthesis has been considered a detoxification pathway. The majority of substrates involving mercapturic acid formation are highly reactive and potentially alkylating electrophilic xenobiotics(63). In addition, most of the unconjugated xenobiotics are not soluble in water, but via the mercapturic acid pathway they become organic acids, mercapturates. The increased water solubility and the active secretion of organic acids facilitate the excretion of xenobiotics and, hence, detoxification(15).

Monitoring the air concentration of toxic chemicals has been widely used to estimate external exposure. However, determination of internal exposure is more related to health risks related to the mode of exposure, the extent of absorption, the mode of interaction, i.e., competition,

inhibition, induction, etc., and the metabolism of xenobiotics. The internal approach includes the measurement of the metabolite concentration in blood, urine, or expired air(14). Although GSH conjugates are extensively excreted into bile, they usually undergo enterohepatic circulation and are consequently excreted in the urine as mercapturic acids. Thus, the biliary and fecal excretion become less significant(10). In 1977, Seutter-Berlage et al(54) first proposed the use of urinary mercapturic acid excretion as a biological parameter of xenobiotic exposure. Van Doorn et al(52) found a dose-response relationship between urinary mercapturic acid excretion and the number of cigarettes consumed. Furthermore, an increase in urinary mercapturic acid excretion was found in chemical and rubber workers(53-57). Since the developed method for determination of urinary mercapturic acid involves simple techniques and inexpensive equipment, the use of urinary mercapturate excretion as a biological parameter of xenobiotic exposure would appear to be feasible.

Considering previous studies which used urinary excretion of mercapturic acid as a parameter of xenobiotic exposure, it would seem apparent that establishing the usual pattern of urinary mercapturic acid excretion on a population basis is needed. In order to propose a biological threshold limit of exposure and to meaningfully interpret the health significance

of such a threshold, it is essential to first determine the range of the excretion of urinary mercapturic acids in a normal population and to take into consideration the overall effect of a number of critical host factors on this range. Hence, the purpose of this research was to quantitatively assess the urinary excretion of mercapturic acids in a free-living population of young male adults, in order to establish a profile of detoxification via GSH conjugation.

The primary objectives of this research were

1. to develop and refine the methodology for quantification of urinary mercapturic acid;
2. to determine the range and interindividual variation of mercapturic acid excretion on a twenty-four hour basis;
3. to determine the intraindividual variation of mercapturic acid excretion over three consecutive days for a subgroup of the sample population; and
4. to examine the interindividual population profile(objective 2) for correlations between the amount of mercapturic acids excreted and the exposure to certain variables which could affect such a profile; for example, dietary patterns, caffeine, tobacco, alcohol, and other social drugs.

CHAPTER III: METHODOLOGY

A. Subject Recruitment

A pre-experimental survey, food frequency questionnaire and urine collection were completed by all subjects. The detailed methods are described in " The Urinary Excretion of Sulfoconjugates in an Adult Male Population" by Robichaud (64). The following is a summary of the procedure.

One hundred and thirty-five healthy adult males in Blacksburg, Virginia, who were not currently taking medications and who were not under a physician's care were recruited. Prior to urine collection, each subject was required to complete the Pre-Experimental Survey (Appendix 1). In an attempt to describe the subject population, this survey quantified the frequency of exposure to caffeine, alcohol, tobacco, social drugs, medications, and environmental xenobiotics. During the collection period, each subject was required to complete the Food Frequency Questionnaire (Appendix 2). The questionnaire provided descriptive information on the dietary patterns of the population and presumptive evidence of dietary adequacy.

Urine samples were collected over three consecutive 24-hour periods from each subject. A 24-hour period was defined as beginning with the collection of the second voiding on day one through the first voiding on day two. Also, a handout, "Instructions for Collection and Handling of Urine," was

provided to each subject. Upon the delivery of the urine specimen, total volume and pH were measured. Several aliquots from each specimen were immediately frozen (-20^{0}C) for future analysis.

B. Analysis of Urinary Creatinine

The determination of urinary creatinine had been completed previously. The procedure was based on the Jaffe reaction, which consists of the alkaline formation of a red 1:1 addition product between creatinine and sodium picrate. The intraindividual and interindividual variability in creatinine excretion/kg body wt/24 hour period was determined for the population. Each subject whose creatinine value was beyond two standard deviations from the mean was excluded from the study.

C. Selection of a Subgroup for Analysis of Urinary Mercapturic Acids

A previous study of the urinary excretion of conjugated sulfates and glucurondies showed similar interindividual variation between all the subjects in this study and a randomly selected subgroup of 40 subjects(64). In the current study, a subgroup of 30 subjects derived from the original subgroup of 40 subjects was used for determining both inter-

and intra-individual variability of mercapturic acid excretion.

D. Quantification of Urinary Mercapturic Acids

In the present study, the method of Seutter-Berlage et al(65) was used except that standard curves were developed to determine urinary mercapturic acid levels instead of using a molar extinction coefficient. Additionally, the volume of reaction mixture used for Ellman's method was increased from 0.25 ml to 0.75 ml. Comparable results were found in the current study and the study of Seutter-Berlage et al(65).

Generally, the basic procedures included reduction of disulfides with $NaBH_4$, followed by NaOH hydrolysis of the thioether bond. A thiol-disulfide exchange reaction between the free sulfhydryl compound and Ellman's reagent, DTNB, generated the highly colored dianion TNB^{2-} for indirect spectrophotometric analysis. Corrections were made for the contribution to the absorbance by the color of the urine pigments and the DTNB reagent. The differences in concentration of free -SH groups between hydrolyzed and non-hydrolyzed samples represented the concentration of urinary mercapturic acid.

To determining the urinary mercapturic acid for the subjects, standard curves for prehydrolysis (Figure 2) and posthydrolysis (Figure 3) were developed separately. Five

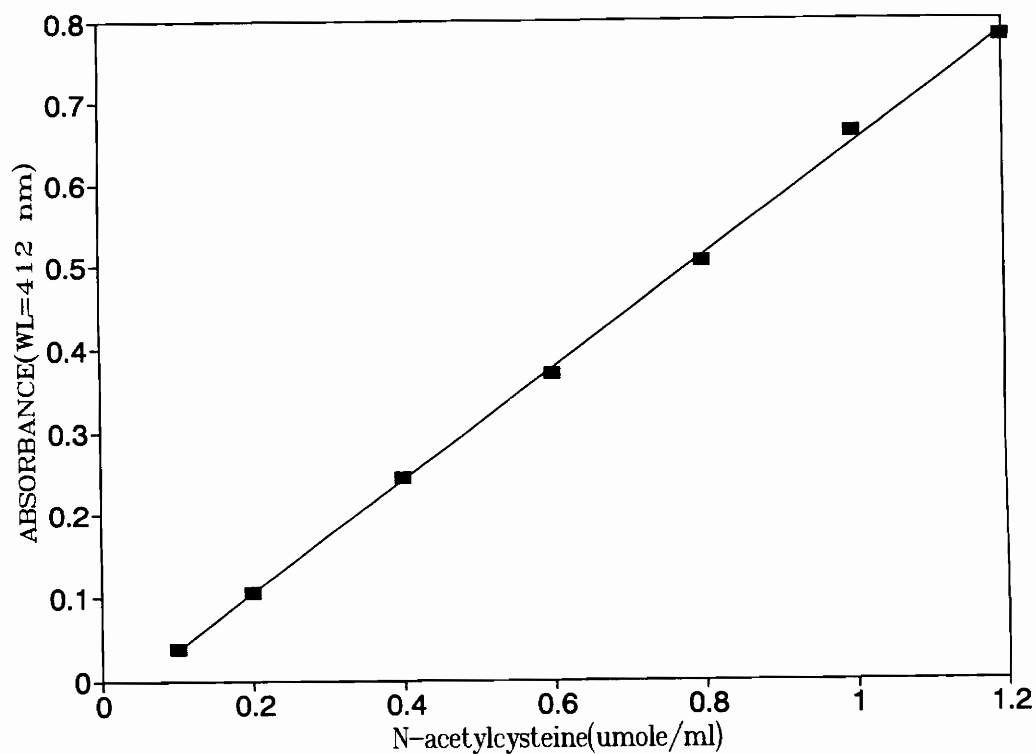

Figure 2. Prehydrolysis Standard Curve.
The concentration range of N-acetylcysteine was from 0.1 to 1.2 umole. The reaction mixture was subjected to the procedure for determination of free SH groups after reduction. Each point represents the mean of five determinations (r=0.999).

different urine samples were spiked with a known concentration of N-acetylcysteine (NAC). Simultaneously, these five urine samples were run without spiking. The difference in absorbance between a spiked urine sample and a urine sample alone represented the absorbance of NAC at a given concentration. Each point of a standard curve represents the mean of these five determinations. The spiked concentrations for the standard curves of prehydrolysis and posthydrolysis were 0.1-1.2 and 0.1-1.6 umole/ml, respectively. There was a linear relationship between absorbance and NAC concentration. The mean recoveries of 0.5 umole NAC added to the urine assay system for prehydrolysis and posthydrolysis were 85.4 and 86.1%, respectively. The interassay variation for three determinations was less than 5.5%.

A flow chart for this procedure is given in Figure 4. The detailed procedure of the mercapturic acid test was as follows:

(I) Free SH-groups after reduction of disulphides with $NaBH_4$: Two ml of urine were centrifuged for 5 min at 2500 rpm. One ml of 5% $NaBH_4$ was added to 1 ml of the centrifuged urine. The reaction mixture was vortexed and heated at 60^0C for 15 min and cooled in ice-water bath for 10 min. In order to decompose excess $NaBH_4$, 1 ml of acetone was added and the mixture was vortexed. After 5 min, 1 ml of 2.5 M HCl was added to totally destroy excess $NaBH_4$. Ten minutes later, 0.2

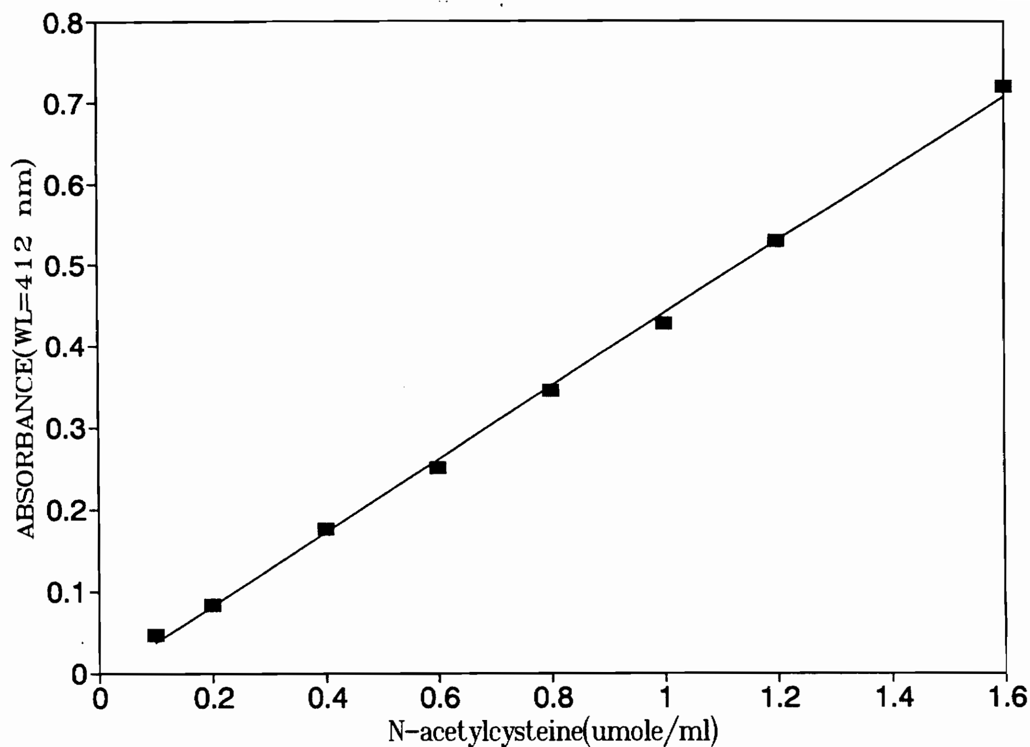

Figure 3. Posthydrolysis Standard Curve.
The concentration range of N-acetyl cysteine was from 0.1 to 1.6 umole. The reaction mixture was subjected to the entire procedure for determination of mercapturic acids. Each point represents the mean of five determinations (r=0.998).

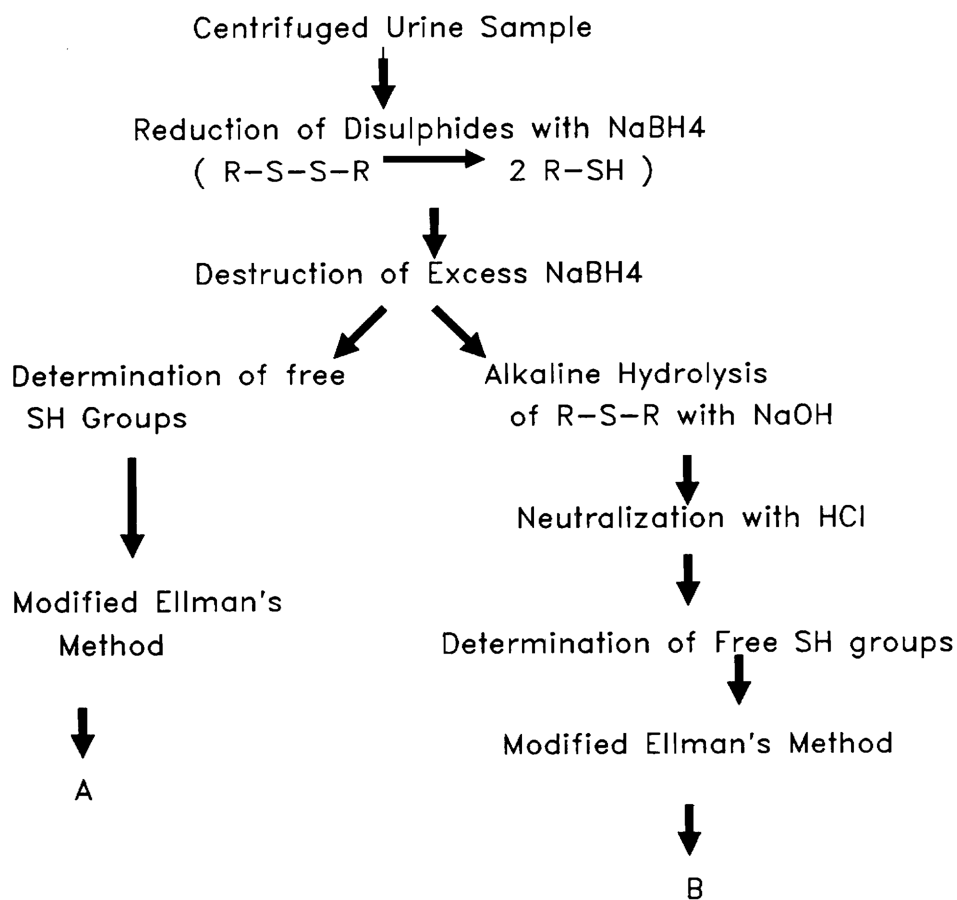

Figure 4. **Flow Chart for the Determination of Mercapturic Acids. A= urinary concentration of free -SH groups before hydrolysis. B= urinary concentration of free -SH groups after alkaline hydrolysis. B-A= urinary concentration of mercapturic acids**

ml of metaphosphoric acid was added. The solution was mixed and after 2 min, free SH groups were assayed by the method of Ellman: 0.75 ml of the reaction mixture was mixed with 2 ml, 0.25 M phosphate buffer (pH=7.1) and 0.3 ml of DTNB. Eight to ten minutes after mixing, the absorbance was read against a blank at 412 nm in a Spectronic 401, visible, single-beam spectrophotometer. The contribution of urine color was measured by substitution of 0.3 ml deionized water for the DTNB. The actual absorbance of the urine reaction mixture was the difference in absorbance between the reaction mixture with DTNB and the reaction mixture withdeionized water.

After pipetting out 1.5 ml of the urine mixture for Ellman's reaction, the remaining mixture continued to be used for alkaline hydrolysis of R-S-R.

(II) SH-groups after alkaline hydrolysis of R-S-R:

One ml 5M NaOH was mixed with the mixture left from the prehydrolysis. Nitrogen gas was released into each test tube for 15 seconds. The test tubes were capped immediately, heated at 97-100 ^{0}C for 40 min, and placed in an ice-water bath for 10 min. Then, the hydrolysate was neutralized with 1 ml 5N HCl. Free SH groups were assayed as mentioned in the prehydrolysis : 0.75 ml of reaction mixture was mixed with 2 ml of 0.25M phosphate buffer(pH= 7.1) and 0.3 ml of DTNB. Eight to 10 minutes after mixing, the absorbance was read

against a blank at 412 nm. The contribution of urine color was measured by substitution of 0.3 ml deionized water for the DTNB. The calculation of actual absorbance of the urine reaction mixture after hydrolysis was similar to prehydrolysis.

The concentrations of urinary free SH groups in prehydrolysis and posthydrolysis were determined from the standard curves. The differences in the concentrations of the free SH groups between hydrolyzed and nonhydrolyzed samples represented the concentration of urinary mercapturic acid. Eventually, the concentrations of the urinary mercapturic acids were expressed as mmole -SH/ 24 hr and umole -SH/ mmole creatinine/ 24 hr.

E. Variable Assessment:

(a) Dietary Factors- Vegetable, Fruit, and Meat Consumption

Protein deficiency has been shown to increase the toxicity of many xenobiotics due to the limited availability of cysteine needed for GSH synthesis(4,5,46). Since meat is a good source of protein, meat was chosen as a variable affecting mercapturic acid excretion. According to the consumption frequency of meat, subjects were categorized as either low or high consumers as follows:

Low(L)= ≤ 2 times/d

High(H)= > 3 times/d

Vegetables were chosen as a variable affecting mercapturic acid excretion because cruciferous vegetables has been shown to induce glutathione s-transferase which may protect cells from attack by electrophiles(50). The subjects were classified as follows:

Low(L)= < 2 times/day

High(H)= ≥ 3 times/day

Polycyclic, aromatic hydrocarbons, such as benzo(a) pyrene, have been found in certain smoked and cooked foods. These compounds are known to induce hydroxylase activity and increase the metabolism of several drugs(67). Therefore, charbroiled meat intake was chosen as a variable affecting mercapturic acid excretion. The subjects were classified as follows:

Low(L)= ≤ 3 times/month

High(H)= > 3 times/month

(b) Non-Dietary Factors:

The pre-experimental survey was used to ascertain each subject's exposure to various substances. Tobacco and marijuana were chosen as variables affecting mercapturic acid excretion because tobacco and marijuana smoke contain high levels of polycyclic hydrocarbons which may induce hydroxylase activity and increase metabolism of certain drugs(68,69). The subjects were classified as follows:

Low(L)= < 1 cigarette/day

marijuana < 1 time/week

High(H)= ≥ 20 cigarette/day

marijuana ≥ 1 time/week

Alcohol and caffeinated coffee intakes were chosen as variables affecting mercapturic acid excretion since some studies have shown that alcohol and caffeine affect drug metabolism by inhibiting and inducing drug clearance, respectively(70,71). The users of alcohol and caffeinated coffee were categorized as follows:

Low(L)= alcohol ≤ 3 times /month (including non-users)

caffeinated coffee ≤ 2 cups/week

High(H)= alcohol > 24 times / month

caffeinated coffee ≥ 1 cup/day

Since an increase of mercapturic acid excretion has been found in chemical and rubber workers, and in workers in the paper industry, chemical exposure was chosen as a variable affecting mercapturic acid excretion(54-58). The chemical factors included exposure to organic solvents such as xylene, benzene, gasoline, carbon tetrachloride, acetone, insecticides and herbicides. Subjects were classified as follows:

Low(L)= exposure to chemical factors < 1 time/wk

High(H)= exposure to chemical factors ≥ 1 time/wk

(c) Genetic Factor:

Genetic factor was chosen as a variable affecting mercapturic acid excretion since genetics contributes to intervariability of xenobiotic metabolism(41). In this study, the incidence of cancer among family members was assumed to be reflective of a genetic factor. Subjects were classified as follows (Appendix 1):

Yes(Y)= a family member has or had cancer

No(N)= no known cancer among their family members

F. Data Analysis

In this study, only the frequency of intake or exposure of the previous identified variables(except genetics) was recorded. No quantification of variables was made.

A nonparametric Kruskal-Wallis test was used to test the difference between the subgroups in the respective variable groups. The Kruskal-Wallis test was used because the sample sizes of subgroups in each variable group were not equal. Additionally, variances of subgroups in respective variable groups were assumed to be constant and measurements were continuous. A probability of 0.05 or less was considered as statistically significant.

CHAPTER IV: RESULTS

Subjects

Subjects used in this study were derived from a group of 135 males with a mean age 20.7 years. The frequency of food consumption indicated the subjects' food intake to be nutritionally adequate(64).

As mentioned in Chapter III, Section B, three-day urinary creatinine excretions were previously measured and analyzed for intra-(within) and inter-(between) subject variability(64). Creatinine excretion for an individual normally remains relatively constant from day to day. For this study, subjects whose daily creatinine excretions were beyond two standard deviations from the mean(24.55 mg/kg of body weight/ 24 hr) were excluded from further analysis. A subgroup of 30 subjects was randomly selected from the 117 subjects whose creatinine excretions were within two standard deviation from the mean.

The mean, standard deviation, and range for the urinary excretion of mercapturic acid for the three-day samples in mmole -SH/24 hr and in umole -SH/mmole creatinine/24 hr are shown in Table 2. The inter- and intra-variations of subjects relative to mercapturic acid excretion are presented in Table 3.

Table 2. Urinary excretion of mercapturic acids in free-living adult males. (N=30)

Mercapturic Acid	Daily Excretion for 3 Days Mean ± SD	Range
mmole -SH/24 hr	0.27 ± 0.08	0.13-0.42
umole -SH/mmole creatinine/24hr	18.05 ± 4.07	9.92-28.23

Table 3. Inter- and intra- subject variation in urinary excretion of mercapturic acids in free-living adult males. (N=30)

Mercapturic Acid	Variation	
	inter CV*	intra CV*
mmole -SH/24 hr	37.7	32.4
umole -SH/mmole creatinine/24hr	31.2	30.1

* CV= coefficient of variation(%)

Dietary Factors:

Subjects were classified as being low or high consumers according to the consumption frequency of vegetable, fruit, meat and charbroiled meat. The mean and standard deviation of the urinary excretion of mercapturic acids for each category of dietary variable in mmole -SH/24 hr and umole -SH/mmole creatinine/24 hr are shown in Table 4.

No statistical difference in mercapturic acid excretion between high and low consumers for each dietary factor was observed. In addition, no significant correlation between mercapturic acid excretion and protein, fat, carbohydrate, or energy intake was found.

Non-Dietary Factors

According to the use of alcohol, marijuana, caffeinated coffee and exposure to chemicals, subjects were classified into low or high consumers. The mean and standard deviation of mercapturic acid excretion for different categories of non-dietary factors are listed in Table 5. Again, no statistical difference in mercapturic acid excretion between high and low groups for each variable was noted except for the chemicals factors.

Table 4. Urinary excretion of mercapturic acids according to dietary factors in free-living adult males.

Dietary Factor		N	Mercapturic Acids mmole -SH/24hr	Mercapturic Acids umole -SH/mmole creatinine/ 24hr
Vegetable				
< 2 times/d	L	18	0.27 ± 0.09*	17.06 ± 3.77
≥ 3 times/d	H	12	0.28 ± 0.07	19.53 ± 4.21
Fruit				
≤ 1 times/d	L	23	0.28 ± 0.09	17.63 ± 3.80
≥ 3 times/d	H	7	0.26 ± 0.04	19.42 ± 4.94
Meat				
≤ 2 times/d	L	21	0.26 ± 0.09	18.60 ± 4.57
> 3 times/d	H	9	0.31 ± 0.06	16.76 ± 2.28
Charbroil				
≤ 3 times/mo	L	24	0.26 ± 0.08	17.88 ± 3.84
> 3 times/mo	H	6	0.32 ± 0.09	18.72 ± 5.26

* Mean ± SD

Table 5. Urinary excretion of mercapturic acids according to non-dietary factors in free-living adult males

Non-Dietary Factor & Frequency	Rank[b]	N	Mercapturic Acids: mmole -SH/24hr	Mercapturic Acids: umole -SH/mmole creatinine /24hr
Alcohol				
≤ 3 times/mo	L	22	0.28 ± 0.09[a]	18.24 ± 3.92
> 24 times/mo	H	8	0.25 ± 0.06	17.53 ± 4.72
Marijuana				
< 1 time/wk	L	26	0.28 ± 0.08	18.05 ± 4.26
≥ 1 time/wk	H	4	0.26 ± 0.07	18.05 ± 3.02
Caffeine				
< 2 cups/wk	L	13	0.25 ± 0.08	18.76 ± 4.02
≥ 1 cup/day	H	13	0.28 ± 0.07	17.06 ± 4.27
Chemical				
< 1 time/wk	L	13	0.26 ± 0.09	16.02 ± 3.11**
≥ 1 time/wk	H	17	0.29 ± 0.07	19.60 ± 4.11
Cancer Incidence				
	Yes	12	0.26 ± 0.08	18.01 ± 4.98
	No	18	0.29 ± 0.08	18.08 ± 3.50

a Mean ± SD
b L=Low; H=high
** Significantly different(P≤0.05)

In terms of umole -SH/ mmole creatinine/24 hr, subjects with high frequency of exposure to chemicals excreted significantly ($p \leq 0.05$) higher levels of mercapturic acids than did subjects with a low frequency of exposure.

Cigarette-smoking factor was not tested in this study because of insufficient sample size of the subgroup with a high frequency of cigarette-smoking.

Genetics:

Subjects were classified according to the incidence of cancer among relatives (Appendix 1). The mean and standard deviation for the urinary excretion of mercapturic acids in mmole -SH/24 hr and umole -SH/mmole creatinine/24 hr are listed in Table 5. There was no significant difference in mercapturic acid excretion between Y and N groups.

CHAPTER V: DISCUSSION

The primary purpose of this study was to determine the amount of mercapturic acids excreted in a free-living male population. The mean value of urinary mercapturic acids was found to be 18.05 umoles -SH/mmole creatinine/24 hr. In comparison with the urinary mercapturate levels in the literature (Table 6), the present study shows much lower values than those found in the studies of Seutter-Berlage et al(54) and Vainio et al(51). This might be explained, in part, by the different analytical procedures used. The previous studies(51,54) did not reduce urinary disulfides to sulfhydryl groups with $NaBH_4$. In other words, the contribution of urinary disulfides, especially cystine, to the amount of sulfhydryl groups finally measured was not taken into account. The normal value of urinary cystine is 49 umole -SH/mmole creatinine/24 hr(52) and would contribute to the higher values noted(51,54). However, the values reported by Van Doorn et al(52,72) were much lower than the mean value obtained in this study. A possible reason may be due to the fact that ethyl acetate was used to selectively extract acidic, sulfur-containing compounds in the previous studies(52,57). In other words, only thioethers which were extracted into ethyl acetate were measured. Therefore, this extraction procedure caused the loss of part of thioethers and resulted in the lower values observed by

Table 6. A comparison of urinary mercapturate acid levels in the literature and the present study

Source	N	Mean umole -SH/mmole creatinine/24hr
Seutter-Berlage et al(54)	50	54
Vainio et al(51)	63	37
Seutter-Berlage et al(65)	5	19.8
Van Doorn et al (52)	20	1.6
Van Doorn et al (72)	196	3.8
Present Study	30	18.05

Van Doorn et al(52,72).

A secondary purpose of this study was to determine if the dietary patterns and nondietary habits of subjects affect the excretion of urinary mercapturic acids. The results of the present study showed no significant differences in mercapturate excretion(mmole -SH/24 hr and umole -SH/mmole creatinine/24 hr) for dietary factors, such as the frequency of vegetable, fruit, meat, and charbroiled beef consumption. Furthermore, no significant correlations between mercapturate excretion and protein, fat, carbohydrate, and calories intake was observed. Several factors might account for these observations.

Firstly, the dietary information used in this study was obtained from the food frequency questionnaire instead of food diaries. A food frequency questionnaire is useful for qualitative assessment rather than quantitative measurement. Secondly, in some cases, differences in sample sizes among subgroups of the respective variable group were extreme. Thirdly, the overall effect of dietary and environmental factors on mercapturic acids excretion was considered and each variable was not controlled for separately and strictly. This lack of control may have masked the effect of each dietary factor on mercapturic acids excretion.

As mentioned in the literature review, an increase of mercapturic acid excretion has been found in chemical and

rubber workers and in workers in the paper industry(54-58). This phenomenon was supported by our present finding that subjects with high frequency of exposure to chemicals, such as acetone, xylene, benzene, insecticides, and herbicides, excreted significantly more mercapturic acids than did subjects with a low frequency of exposure to chemicals. This finding suggests the usefulness of urinary mercapturic acids as a biological parameter of chemical exposure.

Large inter-individual variations have been found in the metabolism of drugs in man(73). Some studies have shown that genetic factors are the major causes of the inter-individual difference when the subjects are under environmentally controlled conditions. On the other hand, Alvares et al(42) suggested that external environmental factors, such as diet, smoking, medications, alcohol, and exposure to environmental chemicals are the major sources of inter-individual differences. Thus, the relative importance of genetic factors and the external environment in determining the capacity of xenobiotic metabolism depends on whether or not the external environmental factors are controlled.

In this study, no attempt was made to control for the lifestyles and diets of subjects. The differences observed are probably due to both external environment and genetic factors. Inter-variation in the current study was 37.7% in mmole -SH/24 hr. This coefficient of variation is lower than

the variation found in other detoxification pathways, i.e., glucuronide conjugation, 48.2%(74), sulfation, 69.8%(74), amino acid conjugation, 50.8-53.2%(75).

In order to minimize the large inter-individual variation, which likely masks any effects of diet and environment on urinary mercapturic acid excretion, the means of the 3-day samples were used to determine the 32.4% intra-variation in mmole -SH/24 hr. As compared with other detoxification pathways, this coefficient of variation is close to the variations found in glucuronide conjugation, 29.2%(74), and amino acid conjugation, 31.4-32.4%(75), but, is lower than the variation found in the sulfation, 57.4%(74). Similarly, Alvares et al(42) reported that high intra-variation, 42.5%, in plasma half-lifes was found for a drug, phenacetin.

The 95th percentile of urinary excretion of mercpaturic acids was 25.85 umoles -SH/ mmole creatinine/24 hr. In other words, merely 5% of a free-living male population have urinary mercapturate values above 25.85 umoles -SH/ mmole creatinine/24 hr. On the other hand, the 5th percentile was 12.54 umoles -SH/mmole creatinine/24 hr. That is, only 5% of a free-living male population have urinary mercapturate values below 12.54 umoles -SH/mmole creatinine/24 hr. In order to propose a biological threshold limit of exposure, these values could be suggested as warning limits. Therefore, if a person's urinary mercapturate level is higher than 25.85

umoles -SH/mmole creatinine/24 hr, the environment to which he or she is exposed probably needs to be monitored. Thus, if a employee has a value below 12.54 umoles -SH/mmole creatinine/24 hr, the employee might not need to be as concerned about exposure.

The complexity of the subject matter represented by this study with its many interactive factors is recognized. Therefore, further research to clarify the mercapturic acid excretion pattern of a free-living population is recommended. In addition, research with more control of the subjects' diet and lifestyle to identify factors which affect mercapturic acid excretion is needed.

CHAPTER VI: SUMMARY

This study was designed to investigate the amount of mercapturic acid excreted by a free-living male population and the effect of external environment and physiological factors, such as vegetable, fruit, meat, charbroiled food intake, tobacco, alcohol, caffeinated coffee, and marijuana use, exposure to chemicals and familial cancer incidence.

The mean excretion of mercapturic acid was 0.27 mmole -SH/24 hr or 18.05 umole -SH/mmole creatinine/24 hr. An analysis of variance revealed a large degree of inter-(between subjects) and intra-(within subjects) variability. The inter-individual coefficients of variation in mmole -SH/ 24 hr and umole -SH/mmole creatinine/24 hr were 37.7% and 31.2%, respectively. The intra-individual coefficients of variation in mmole -SH/24 hr and umole -SH/mmole creatinine/ 24 hr were 32.4 % and 30.1%, respectively.

The large variability may mask the effects of diet, environment, or genetics on the observed mercapturate excretion. In spite of the observed high variability, a higher excretion of mercapturic acid was observed among subjects with high frequency of exposure to chemicals. Therefore, further research is needed to identify the relationship between mercapturic acid excretion and genetic and environmental factors in a free-living male population.

REFERENCE

1. Dutton, G.J. Introduction- the biological function of glucuronidation. In: Dutton, G.J.,eds. Glucuronidation of Drugs and Other Compounds. CRC Press, Inc., Baco Raton, Florida. 1980:3-12.

2. Caldwell, J. Conjugation mechanisms of xenobiotic metabolism: mammalian aspects. In: Paulson, G.D., Caldwell, J., Hutson, D.H., and Menn, J.J., eds. Xenobiotic Conjugation Chemistry. Am. Chem. Soc., Washington, D.C. 1986:2-28.

3. Hodgson, E., and Guthrie, F.E. Biochemical toxicology: definition and scope. In: Hodgson, E., and Guthrie, F.E., eds. Introduction to Biochemical Toxicology. Elsevier, New York. 1980:1-9.

4. Hodgson, E., and Dauterman, W.C. Metabolism of toxicants: phase I reactions. In: Hodgson, E., and Guthrie, F.E., eds. Introduction to Biochemical Toxicology. Elsevier, New York. 1980:67-91.

5. Alvares, A.P. Oxidative biotransformation of drugs. In: Arias,I., Popper, H., Schachter, D., and Shafritz, D.A., ed. The Liver: Biology and Pathobiology. Raven Press, New York. 1982:265-280.

6. Bidlack, W.R. Toxicant metabolism and the role of nutrients. Food Technology. 1982, 10:106-112.

7. Caldwell, J. The significance of phase II(conjugation)

reactions in drug diposition and toxicity. Life Science. 1979, 24:571-578.

8. Monks, T.J., Lau, S.S. Reactive intermediates and their toxicological significance. Toxicology. 1988, 52:1-53.

9. Neal, R.A. Metabolism of toxic substances. In: Doull, J., Klaassen, C.D., and Amdur, M.O., eds. Casarett and Doull's Toxicology, 2nd Ed. Macmillan Publ. Co., Inc. New York. 1980:56-69.

10. Klaassen, C.D. Absorption, distribution, and excretion of toxicants. In: Casarett, L.J., and Doull, J., eds. Casarett and Doull's Toxicology. 2nd Ed. Macmillan Publ. Co. Inc., New York. 1980:28-51.

11. Matthews, H.B. Elimination of toxicants and their metabolites. In: Hodgson, E., and Guthrie, F.E., eds. Introduction to Biochemical Toxicology. Elsevier, New York. 1980:162-179.

12. Hirom, P.C., Millburn, P., and Smith, R.L. Bile and urine as complementary pathways for the excretion of foreign organic compounds. Xenobiotica. 1976, 6:55-64.

13. Bakke, J.E., and Davison, K.L. Renal processing of glutathione conjugates by rat, chicken, pig and calf. Toxicol. Letters. 1990, 53:127-129.

14. Van Doorn, R., Leijdekkers, Ch.M., Bos, R.P., Brouns, R.M.E., and Henderson, P.Th. Detection of human exposure to electrophilic compound by assay of thioether

detoxication products in urine. Ann. Occup. Hyg. 1981, 24:77-92.

15. Stevens, J.L., and Jones, D.P. The mercapturic acid pathway: biosynthesis, intermediary metabolism, and physiological disposition. In: Dolphin, D., Poulson, R., and Avramovic, O., eds. Glutathione: Chemical, Biochemical, and Medical Aspects. Part B. John Wiley & Sons, Inc. New York. 1989:45-75.

16. Barnes, M.M., James, S.P., and Wood, P.B. The formation of mercapturic acids. 1. Formation of mercapturic acid and the levels of glutathione in tussues. Biochem. J. 1959, 71(4):680-690.

17. Bray, H.G., Franklin, T.J., and James, S.P. The formation of mercapturic acids. 2. The possible role of glutathionase. Biochem. J. 1959, 71(4):690-696.

18. Jakoby, W.B., Stevens, J., Duffel, M.W., and Weisiger, R.A. The terminal enzymes of mercapturate formation and the thiomethyl shunt. In: Hodgson, E., Bend, J.R., and Philpot, R.M., eds. Reviews in Biochemical Toxicology. Elsevier Science Publ. Co., Inc. New York. 1984:97-115.

19. Ketterer, B. The role of nonenzymatic reactions of glutathione in xenobiotic metabolism. Drug Metab. Rev. 1982, 13(1):161-187.

20. Kosower, N.S., and Kosower, E.M. The glutathione status of cells. In: Bourne, G.H., and Danielli, J.F., eds.

International Review of Cytology. Academic Press, New York. 1978, 65:109-160.

21. Reed, D.J., and Beatty, P.W. Biosynthesis and regulation of glutathione: toxicological implications. Rev. Biochem. Toxicol. 1980, 2:213-241.

22. Jefcoate, C.R. Integration of xenobiotic metabolism in carcinogen activation and detocication. In: Caldwell, J., and Jakoby, W.B., ed. Biological Basis of Detoxification. Academic Press, New York. 1983:31-76.

23. Meister, A. Glutathione. In: Arias, I., Popper, H., Schachter, D., and Shafritz, D.A., eds. The Liver: Biology and Pathology. Raven Press, New York. 1982:297-308.

24. Chasseaud, L.F. Conjugation with glutathione and mercapturic acid excretion. In: Arias, I.M., and Jakoby, W.B., eds. Glutathione: Metabolism and Function. Raven Press, Pub. New York. 1975:77-114.

25. Ketterer, B. Detoxication reactions of glutathione and glutathione transferase. Xenobiotica. 1986, 16:957-973.

26. Wood, J.L. Biochemistry of mercapturic acid formation. In: Fishman, W.H.,eds. Metabolic Conjugation and Metabolic Hydrolysis. Vol. II. Academic Press, New York. 1970:261-299.

27. Mantle, E.J., McCusker, F.M., Phillips, M., and Boyce, S. Glutathione S-transferases. Biochem. Soc. Trans. 1990,

18:175-177.

28. Vos, R.M.E., and Van Bladeren, P.J. Glutathione S-transferases in relation to their role in the biotransfromation of xenobiotics. Chem. Biol. Interactions. 1990, 75:241-265.

29. Boyer, T.D., Zakim, D., and Vessey, D.A. Do the soluble glutathione S-transferases have direct access to membrane-bound substrates? Biochem. Pharmacol. 1983, 32(1):29-35.

30. Depierre, J.W. Comparison of the distribution of microsomal and cytosolic gultathione S-transferase activities in different organs of the rat. Biochem. Pharmacol. 1983, 32(4):721-723.

31. Adang, A.E.P., Brussee, J., Gen, A.V.D., and Mulder, G.J. The glutathione-binding site in glutathione S-transferases: investigation of the cysteinyl, glycyl and r-glutamyl domains. Biochem. J. 1990, 269:47-54.

32. Silbernagl, S., and Heuner, A. Renal transport and metabolism of mercapturic acids and their precursors. Toxicol. Letters. 1990, 53:45-51.

33. Tate, S.S. Enzymes of mercapturic acid formation. In: Jakoby, W.B. Enzymatic basis of detoxication. Vol. I. Academic Press, New York. 1980:95-120.

34. Hinchman, C.A., and Ballatori, N. Glutathione-degrading capacities of liver and kidney in different species.

Biochem. Pharmacol. 1990, 40:1131-1135.

35. Hughey, R.P., Rankin, B.B., Elce, J.S., and Curthoys, N.P. Specificity of a particulate rat renal peptidase and its localization along with other enzymes of mercapturic acid synthesis. Arch. Biochem. Biophys. 1978, 186(2):211-217.

36. Duffel, M.W., and Jakoby, W.B. Cysteine S-conjugate N-acetyltransferase from rat kidney microsomes, Molecular Pharmacology. 1981, 21:444-448.

37. Bakke, J.E., Davison, K.L., and Larsen, G.L. Evidence for the absence of cysteine S-conjugate N-acetyltransferase activity in the metabolism of porpachlor, naphthalene, and dichlobanil in calves. Xenobiotica. 1990, 20(8):801-807.

38. Okajima, K., Inoue, M., Morino, Y., and Itoh, K. Topological aspects of microsomal N-acetyltransferase, an enzyme responsible for the acetylation of cysteine S-conjugates of xenobiotics. Eur. J. Biochem. 1984, 142:281-286.

39. Inoue, M., Okajima, K., and Morino, Y. Renal transtubular transport of mercapturic acid in vivo. Biochimica et Biophysica Acta. 1981, 641:122-128.

40. Dauterman, W.C. Physiological factors affecting metabolism of xenobiotics. In: Hodgson, E., and Guthrie, F.E., eds. Introduction to Biochemical Toxicology.

Elsevier, New York. 1980:133-142.

41. Vesell, E.S., and Penno, M.B. Intraindividual and interindividual variations. In: Caldwell, J., and Jakoby, W.B., eds. Biological Basis of Detoxication. Academic Press, New York. 1983:369-410.

42. Alvares, A.P., Kappas, A., Eiseman, J.L., Anderson, K.E., Pantuck, C.B., Pantuck, E.J., Hsiao, K.C., Garland, W.A. and Conney, A.H. Intraindividual variation in drug disposition. Clin. Pharmacol. Therap. 1979, 26(4):407-419.

43. Vesell, E.S. On the significance of host factors that affect drug disposition. Clin. Pharmacol. Therap. 1982, 31(1):1-7.

44. Weinshilboum, R.M. Human pharmacogenetics. Federation proceedings. 1984, 43(8):2295-2297.

45. Hodgson, E. Chemical and environmental factors affecting metabolism of xenobiotics. In: Hodgson, e., and Guthrie, F.E., eds. Introduction to Biochemical Toxicology. Elsevier, New York. 1980:143-161.

46 Bidlack, W.R., Brown, R.C., and Mohan, C. Nutritional parameters that alter hepatic drug metabolism, conjugation, and toxicity. Federation Proc. 1986, 45:142-148.

47. Bauman, P.F., Smith, T.K., and Bray, T.M. Effect of dietary protein deficiency and L-2-oxothiazolidine-4-

carboxylate on the diurnal rhythm of hepatic glutathione in the rat. J. Nutr. 1988, 118:1048-1054.

48. Hagen, T.M., Wierzbicka, G.T., Sillau, A.H., Bowman, B.B., and Jones, D.P. Bioavailability of dietary glutathione: effect on plasma concentration. Am. Physiol. 1990, 259:G530-535.

49. Siddik, Z.H., Mimnaugh, E.G., Trush, M.A., and Gram, T.E. The effect of vitamin A deficiency on hepatic, renal and pulmonary glutathione S-transferase activities in the rat. Biochem. J. 1980, 188:889-893.

50. Bogaards, J.J.P., Van Ommen, B., Falke, H.E., Willems, M.I., and Van Bladeren, P.J. Glutathione S-transferase subunit induction patterns of brussels sprouts, allyl isothiocyanate and goitrin in rat liver and small intestinal mucosa: a new approach for the identification of inducing xenobiotics. Fd. Chem. Toxic. 1990, 28(2):81-88.

51. Vainio, H., Savolainen, H., and Kilpikari, I. Urinary thioether of employees of a chemical plant. Br. J. Indust. Med. 1978, 35:232-234.

52. Van Doorn, R., Bos, R.P., Leijdekkers, Ch.M., Wagenaas-Zegers, M.A.P., Theuws, J.L.G., and Henderson, P.Th. Thioether concentration and mutagenicity of urine from cigarette smokers. Int. Arch. Occup. Environ. Hlth. 1979, 43:159-166.

53. Lafuente, A., and Mallol, J. Urinary thioethers in low tar cigarette smokers. Public Health. 1986, 100:392-398.

54. Seutter-Berlage, F., Van Dorp, H.L., Kosse, H.G.J., and Henderson, P.Th. Urinary mercapturic acid excretion as a biological parameter of exposure to alkylating agents. Int. Arch. Occup. Environ. Hlth. 1977, 39:45-51.

55. Kilpikari, I. Correlation of urinary thioethers with chemical exposure in a rubber plant. Br. J. Indust. Med. 1981, 38:98-100.

56. Stock, J.K., and Priestly, B.G. Urinary thioether output as an index of occupational chemical exposure in petroleum retailers. Br. J. Indust. Med. 1986, 43:718-720.

57. Lafuente, A., and Mallol, J. Thioethers in urine during occupational exposure to tetrachloroethylene. Bri. J. Indust. Med. 1986, 43:68-69.

58. Buffoni, F., Santoni, G., Albanes, V., and Dolara, P. Urinary mercapturic acid in chemical workers and in control subjects. J. Appl. Toxicol. 1983, 3(2):63-65.

59. Pentz, R. Urinary excretion of bound thio groups in healthy and diseased man and rats. Naunyn-Schmiedebergs Arch. Pharmacol. 1979, 308: R51.

60. Parke, D.V., and Williams, R.T. The metabolism of benzene. (a) The determination of phenylmercapturic acid in urine. (b) Mercapturic acid excretion by rabbits

receiving benzene. Biochem. J. 1951, 48:524-629.

61. Seutter-Berlage, F., Van Doorp, H.L., Kosse, H.J.J., Hoog Antink, J.M.T., and Wagenaars-Zegers, M.A.P. The estimation of mercapturic acids and other thioethers in urine. In: Strike, J.J. T.W.A., and Koeman, J.H., eds. Chemical Porphyria in Man. Elsevier/North-Holland Biomedical Press, New York. 1979:225-232.

62. Buffoni, F., Coppi, C., and Santoni, G. Determination of thioethers in urine. Clin. Chem. 1982, 28(1):248-250.

63. Jocelyn, P.C. The function of thiols and disulphides in enzyme catalysed reactions. In: Jocelyn, P.C. Biochemistry of the SH group. Academic Press, New York. 1972.

64. Robichaud, V. The urinary excretion of sulfoconjugates in an adult male population. Masters Thesis, Department of Human Nutrition and Food, VPI & SU, Blacksburg, Virginia.

65. Seutter-Berlage, F., Selten, G.C.M., Oostendorp, S.G.M.L. and Hoog Antink, J.M.T. The modified thioether test. In: Strike, J.J.T.W.A., and Koeman, J.H., eds. Chemical Porphyria in Man. Elsevier/North- Holland Biomedical Press, New York. 1979:233-236.

66. Kappas, A., Anderson, K.E., Conney, A.H., and Alvares, A.P. Influence of dietary protein and carbohydrate on antipyrine and theophylline metabolism in man. Clin. Pharmacol. Ther. 1976, 20:643-653.

67. Pantuck, E.J., Hsiao, K.C., Conney, A.H., Garland, W., Kappas, A., and Anderson, K. Effect of charcoal-broiled beef on phenacetin metabolism in man. Science. 1976, 194:1055-1057.

68. Jusko, W.J., Schentag, J.J., Clark, J.H., Gardner, M., and Yrchak, A.M. Enhanced biotransformation of theophylline in marijuana and tobacco smokers. Clin. Pharmacol. Ther. 1978, 14:406-410.

69. Pantuck, E.J., Hsiao, K.C., Maggio, A., Nakamura, K., Kuntzman, R., and Conney, A.H. Effect of cigarette smoking on phenacetin metabolism. Clin. Pharmacol. Ther. 1974, 15:9-17.

70. Graham, D.M. Caffeine- its identity, dietary source, intake, and biological effects. Inter. Rev. 1978, 36:97-102.

71. Gibson, G.G., and Skett, P. Introduction to Drug Metabolism. Chapman and Hall, London. 1986:158.

72. Van Doorn, R., Leijdekkers, CH-M., Bos, R.P., Brouns, R.M.E., and Henderson P. TH. Enhanced excretion of thioethers in urine of operators of chemical waste incinerators. Br. J. Indust. Med. 1981, 38:187-190.

73. Alvan, G. Individual differences in the disposition of drugs metabolized in the body. Clin. Pharmacokinet. 1978, 3:155-175.

74. Murano, P.S., Robichaud, V., and Webb, R.E. Urinary

excretion of sulfate and glucuronate conjugates in a free living population of adult males. Bull. Environ. Contam. Toxicol. 1989, 43:13-16.

75. Webb, R.E., and Kim, Y. The urinary excretion of amino acid conjugates in free living adult males. Toxicol. Indust. Hlth. 1991, 7(3):119-124.

APPENDIX 1.

DATE ______________

NUMBER ______________

DETOXIFICATION PROFILE STUDY
1984
PRE-EXPERIMENTAL SURVEY

In order to assess your relative ability to detoxify foreign substances (xenobiotics), we must learn about the frequency of your exposure to them. Please answer all of the following questions as accurately and as honestly as possible, remembering that any information disclosed, will remain confidential. To ensure anonymity, please notice the number in the space provided above. This number will be used as a code for purposes of data analyses.

A. GENETICS

1. Has anyone, genetically related to you, ever had any form of cancer? (Please circle the appropriate letter.)

   a. Yes   b. No

2. If yes, please indicate which relative by circling the appropriate letter. If more than one relative in each category had cancer, please indicate how many in the space provided.

   a. Mother
   b. Father
   c. Brother(s) _____
   d. Sister(s) _____
   e. Son(s) _____
   f. Daughter(s) _____
   g. Maternal grandparent(s) _____
   h. Paternal grandparent(s) _____
   i. Aunt(s) _____
   j. Uncle(s) _____
   k. First cousin(s) _____

B. DIETARY

1. Are you a Vegetarian? (Please circle the appropriate letter.)

   a. No.
   b. Yes, I avoid all meats, eggs, and dairy products.
   c. Yes, I avoid all meats, but consume eggs and/or dairy products.

2. During the past month, on the average, how many 6 oz. cups of hot/iced coffee did you consume? (Circle the appropriate letter.)

   a. None
   b. 1-2 cup(s)/week
   c. 3-4 cups/week
   d. 5-6 cups/week
   e. 1-2 cup(s)/day
   f. 3-5 cups/day
   g. 6-10 cups/day
   h. more than 10 cups/day

3. If coffee was consumed, describe the kind most frequently used.

   a. caffeinated, instant.
   b. caffeinated, brewed.
   c. decaffeinated, brewed.
   d. decaffeinated, instant.

4. During the past month, on the average, how many 5 oz. cups of hot/iced tea did you consume?

a. None
b. 1-2 cup(s)/week
c. 3-4 cups/week
d. 5-6 cups/week
e. 1-2 cup(s)/day
f. 3-5 cups/day
g. 6-10 cups/day
h. more than 10 cups/day

5. If tea was consumed, describe the kind most frequently used. (Please circle the appropriate letter and specify the brand name in the space provided.)

a. Regular or Black Teas ______________________
b. Oriental or Green Teas ______________________
c. Herbal Teas ______________________

6. Is the tea described in question #5, decaffeinated? (Circle the appropriate letter.)

a. Yes   b. No   c. Uncertain

7. During the past month, how frequently did you consume EACH of the following kinds of alcoholic beverages? Using the code below, circle the number following each product that most closely corresponds to your intake.

0. None the entire Month
1. 1-3 times/month
2. 1-2 times/week
3. 3-4 times/week
4. 5-6 times/week
5. once/day
6. 2-3 times/day
7. more than 3 times/day

a. Beer	0	1	2	3	4	5	6	7
b. Wine	0	1	2	3	4	5	6	7
c. Mixed Drinks	0	1	2	3	4	5	6	7
d. Cordials	0	1	2	3	4	5	6	7

C. TOBACCO PRODUCTS

1. Do you smoke tobacco products? (Circle the appropriate letter.)

a. Yes   b. No

2. If yes, which product(s) do you use? (Circle EACH appropriate letter, and specify the Brand Name most frequently used.)

a. Cigarettes ______________(brand)
b. Cigars ______________(brand)
c. Pipe Tobacco ______________(brand)
d. Other ______________(describe)

3. If you smoke, how much of each product do you use? (Circle the appropriate letter under each applicable category.)

Cigarettes

a. less than 1/day
b. 1-5/day
c. 6-10/day
d. 11-15/day
e. 16-20/day
f. 21-25/day
g. 26-30/day
h. 30+/day

Cigar

a. less than 1/day
b. 1/day
c. 2/day
d. 3/day
e. 4/day
f. 5+/day

Pipefills

a. less than 1/day
b. 1/day
c. 2/day
d. 3/day
e. 4/day
f. 5+/day

4. If you smoke, do you regularly inhale the smoke into your lungs? (Circle the appropriate letter.)

a. Yes, most of the time.
b. Occasionally, some of the time.
c. No, I try not to.

5. If you use other tobacco products, please indicate each form used, and the frequency with which each is used. (Please check (✓) the appropriate box.)

	Monthly	Weekly	Daily
a. Snuff	[]	[]	[]
b. Chewing Tobacco	[]	[]	[]
c. Other ______________	[]	[]	[]

D. OTHER SOCIAL DRUGS

Please check (✓) the appropriate box which indicates the relative frequency with which you have used the following types of drugs within the last month.

	Never Tried	USED, But Not in last month	USED 1-3 times in last month	USED at least 1 time/week	USED at least 4 time/week	USED DAILY
1. Cocaine						
2. Hallucinogens (LSD, Mescaline)						
3. Inhalents (glue)						
4. Heroin						
5. Marijuana or Hash						
6. Stimulants (Amphetamines, diet pills, speed)						
7. Tranquilizers (Valium, quaaludes, phenabarbitol)						
8. Other Narcotics (Codeine, Opium)						

E. MEDICATIONS

1. During the past month, did you take any prescription medication for any reason? (i.e. hypertension, diabetes, seizure, pain, colds or flu, insomnia, etc.)

   a. Yes   b. No

2. If yes, please name the medication and estimate the amount taken.

Name of Drug	Number of Days Taken Last Month	Number of Doses or Pills Taken Each Day Used
EXAMPLE: AMPICILLIN	10 DAYS	3 TSP/DAY

3. During the past month, did you take any non-prescription medication for any reason? (i.e. aspirin, cough medicine, vitamin/mineral/herb supplements, laxatives, antacids, etc.)

   a. Yes   b. No

4. If Yes, please name the medication and estimate the amount taken.

Name of Drug	Number of Days Taken Last Month	Number of Doses or Pills Taken Each Day Used
EXAMPLE: EXCEDRIN	20 DAYS	4 PILLS/DAY

F. ENVIRONMENTAL

We are interested in knowing if you are exposed to any chemicals during work-hours or while pursuing a hobby which might effect your health. Such chemicals may be organic solvents such as xylene, benzene, gasoline, carbon tetrachloride, acetone, or agents used in farming such as insecticides and herbicides. Paints, glues, and binding agents could also be included. Please answer the following questions so that we can assess your level of exposure.

1. Please list the names of substances which you know you have handled or been exposed to in the last month. If you do not know the name, write a description. We are chiefly interested in organic (carbon-based) compounds. Such compounds often have distinctive odors. If you are doubtful about whether a substance qualifies, list it and let us decide.

EXAMPLE: ACETONE

a. ________ f. ________
b. ________ g. ________
c. ________ h. ________
d. ________ i. ________
e. ________ j. ________

2. Place a star (*) by each substance listed above, which you have been exposed to in the last two weeks.

3. Please RANK your level of exposure to each substance (listed in question 1) according to the following categories:

	DAILY OR ALMOST DAILY EXPOSURE. LOW PRECAUTIONS TAKEN TO PREVENT EXPOSURE (VERY HIGH)	DAILY OR ALMOST DAILY EXPOSURE. GREAT PRECAUTION TAKEN TO PREVENT EXPOSURE (HIGH)	WEEKLY EXPOSURE OR LESS. LOW PRECAUTION TAKEN TO PREVENT EXPOSURE (MEDIUM)	WEEKLY EXPOSURE OR LESS. GREAT PRECAUTION TAKEN TO PREVENT EXPOSURE (LOW)
a				
b				
c				
d				
e				
f				
g				
h				
i				
j				

4. To the best of your recollection, have you ever been exposed to organic substances such as pesticides, oil-based paints, etc., for periods of two weeks or more? (Circle the appropriate letter.)

   a. Yes   b. No.

5. To the best of your recollection, have you ever sprayed a cropfield with an herbicide or been downwind of a farm which does?

   a. Yes   b. No

6. If you answered yes to either question #4 or #5, how long ago did this exposure occur? (Circle the appropriate letter.)

   a. less than 6 months ago   c. 1-5 years ago
   b. 6 months - 1 year ago   d. more than 5 years ago

7. If you answered yes to either questions #4 or #5, what was the extent of your exposure at that time? (Use the levels of very high, high, medium, and low, as defined in question #3 of this section.)

   a. Very high   c. Medium
   b. High   d. Low

8. If you answered either a or b in question #7, and if you can still remember the name of the substance(s) to which you were exposed, please list them below.

   ____________   ____________   ____________
   ____________   ____________   ____________

9. Do you have a wood-burning stove or fireplace in your home?

   a. Yes   b. No

10. If yes, how many days did you use it last month? (Circle the appropriate letter.)

   a. None   d. 5-6 times/week
   b. 1-2 times/week   e. daily
   c. 3-4 times/week

G. EXERCISE HABITS

1. How would you classify your daily exercise regime? (Circle the appropriate letter.)

   a. light (sedentary)
   b. mild (1 HR structured activity, i.e. sports, dance, etc.)
   c. moderate (1-2 HR/Day, structured activity, i.e. sports, dancing)
   d. vigorous (construction work, etc.)

2. If you answered Vigorous above, please describe:

3. Do you participate in any type of marathon races such as running, cycling, etc?

   a. Yes   b. No

4. If yes, did this participation occur within the past month?

   a. Yes   b. No.

APPENDIX 2.

DATE ____________
NUMBER ____________

DETOXIFICATION PROFILE STUDY
1984
FOOD FREQUENCY QUESTIONNAIRE

I. A variety of common food items are listed below according to major food categories. Please use the following coding system to indicate how frequently you consumed each of the foods listed below over the past month. (Please circle the appropriate number.)

CODE	RESPONSE
0	Never
1	Once a month
2	2-3 times/month
3	Once a week
4	2-4 times/week
5	5-7 times/week
6	2-3 times/day
7	4-6 times/day
8	over 6 times/day

	FOODS	CODE								
A.	MILK GROUP									
	Whole milk and ice cream	0	1	2	3	4	5	6	7	8
	Skim or low fat milk	0	1	2	3	4	5	6	7	8
	Buttermilk	0	1	2	3	4	5	6	7	8
	Canned, evaporated milk	0	1	2	3	4	5	6	7	8
	Reconstituted powdered milk	0	1	2	3	4	5	6	7	8
	Yogurt, fruit flavored	0	1	2	3	4	5	6	7	8
	Yogurt, plain	0	1	2	3	4	5	6	7	8
B.	VEGETABLES									
	Alfalfa sprouts	0	1	2	3	4	5	6	7	8
	Artichoke	0	1	2	3	4	5	6	7	8
	Asparagus	0	1	2	3	4	5	6	7	8
	Bean sprouts	0	1	2	3	4	5	6	7	8
	Beets	0	1	2	3	4	5	6	7	8
	Broccoli	0	1	2	3	4	5	6	7	8
	Brussel sprouts	0	1	2	3	4	5	6	7	8
	Cabbage	0	1	2	3	4	5	6	7	8
	Carrots	0	1	2	3	4	5	6	7	8
	Cauliflower	0	1	2	3	4	5	6	7	8
	Celery	0	1	2	3	4	5	6	7	8
	Chicory	0	1	2	3	4	5	6	7	8
	Cucumbers	0	1	2	3	4	5	6	7	8
	Eggplant	0	1	2	3	4	5	6	7	8
	Green peppers	0	1	2	3	4	5	6	7	8
	Beet greens	0	1	2	3	4	5	6	7	8
	Chard greens	0	1	2	3	4	5	6	7	8
	Collard greens	0	1	2	3	4	5	6	7	8
	Dandelion greens	0	1	2	3	4	5	6	7	8
	Endive or Escarole	0	1	2	3	4	5	6	7	8
	Kale greens	0	1	2	3	4	5	6	7	8
	Lettuce	0	1	2	3	4	5	6	7	8

	FOODS	CODE								
B.	VEGETABLES (cont.)									
	Mustard greens or seeds	0	1	2	3	4	5	6	7	8
	Spinach greens	0	1	2	3	4	5	6	7	8
	Turnip greens	0	1	2	3	4	5	6	7	8
	Mushrooms	0	1	2	3	4	5	6	7	8
	Okra	0	1	2	3	4	5	6	7	8
	Onions	0	1	2	3	4	5	6	7	8
	Radishes	0	1	2	3	4	5	6	7	8
	Parsley	0	1	2	3	4	5	6	7	8
	Parsnips	0	1	2	3	4	5	6	7	8
	Rhubarb	0	1	2	3	4	5	6	7	8
	Rutabaga	0	1	2	3	4	5	6	7	8
	Sauerkraut	0	1	2	3	4	5	6	7	8
	String beans, green or yellow	0	1	2	3	4	5	6	7	8
	Summer squash	0	1	2	3	4	5	6	7	8
	Tomatoes	0	1	2	3	4	5	6	7	8
	Turnips	0	1	2	3	4	5	6	7	8
	Vegetable juice	0	1	2	3	4	5	6	7	8
	Zucchini	0	1	2	3	4	5	6	7	8
C.	FRUITS (fresh, dried or juice included)									
	Apple	0	1	2	3	4	5	6	7	8
	Applesauce	0	1	2	3	4	5	6	7	8
	Apricots	0	1	2	3	4	5	6	7	8
	Banana	0	1	2	3	4	5	6	7	8
	Berries	0	1	2	3	4	5	6	7	8
	Cherries	0	1	2	3	4	5	6	7	8
	Cider	0	1	2	3	4	5	6	7	8
	Dates	0	1	2	3	4	5	6	7	8
	Figs	0	1	2	3	4	5	6	7	8
	Grapefruit	0	1	2	3	4	5	6	7	8
	Grapes	0	1	2	3	4	5	6	7	8
	Honey	0	1	2	3	4	5	6	7	8
	Mango	0	1	2	3	4	5	6	7	8
	Melons	0	1	2	3	4	5	6	7	8
	Nectarine	0	1	2	3	4	5	6	7	8
	Orange	0	1	2	3	4	5	6	7	8
	Papaya	0	1	2	3	4	5	6	7	8
	Peach	0	1	2	3	4	5	6	7	8
	Pear	0	1	2	3	4	5	6	7	8
	Persimmon	0	1	2	3	4	5	6	7	8
	Pineapple	0	1	2	3	4	5	6	7	8
	Plums	0	1	2	3	4	5	6	7	8
	Prunes	0	1	2	3	4	5	6	7	8
	Raisins	0	1	2	3	4	5	6	7	8
	Tangerine	0	1	2	3	4	5	6	7	8
D.	BREADS/CEREALS									
	1. Breads									
	White, French, Italian	0	1	2	3	4	5	6	7	8
	Wheat	0	1	2	3	4	5	6	7	8
	Rye or pumpernickel	0	1	2	3	4	5	6	7	8
	Raisin	0	1	2	3	4	5	6	7	8

	FOODS	CODE								
D.	BREADS/CEREALS (cont.)									
	1. Breads (cont.)									
	Bagel	0	1	2	3	4	5	6	7	8
	Muffins	0	1	2	3	4	5	6	7	8
	Rolls	0	1	2	3	4	5	6	7	8
	Buns	0	1	2	3	4	5	6	7	8
	2. Cereals									
	Ready-to-eat cereals	0	1	2	3	4	5	6	7	8
	Cooked cereals	0	1	2	3	4	5	6	7	8
	Grits, rice or barley	0	1	2	3	4	5	6	7	8
	Pasta noodles	0	1	2	3	4	5	6	7	8
	Bran flakes	0	1	2	3	4	5	6	7	8
	Wheat germ	0	1	2	3	4	5	6	7	8
	Popcorn	0	1	2	3	4	5	6	7	8
	3. Crackers									
	Saltines or soda	0	1	2	3	4	5	6	7	8
	Graham	0	1	2	3	4	5	6	7	8
	Butter-type crackers	0	1	2	3	4	5	6	7	8
	Wheat or rye wafers	0	1	2	3	4	5	6	7	8
	Matzoth or Oyster	0	1	2	3	4	5	6	7	8
	4. Legumes									
	Beans (except lima)	0	1	2	3	4	5	6	7	8
	Peas or lentils	0	1	2	3	4	5	6	7	8
	5. Starchy Vegetables									
	Corn	0	1	2	3	4	5	6	7	8
	Lima beans	0	1	2	3	4	5	6	7	8
	Potato, white (except fried)	0	1	2	3	4	5	6	7	8
	Pumpkin	0	1	2	3	4	5	6	7	8
	Winter squash, acorn, etc.	0	1	2	3	4	5	6	7	8
	Sweet potato or yam	0	1	2	3	4	5	6	7	8
	6. Other breads									
	French fried potatoes	0	1	2	3	4	5	6	7	8
	Potato or corn chips	0	1	2	3	4	5	6	7	8
	Other fried snacks	0	1	2	3	4	5	6	7	8
	Pancakes or waffles	0	1	2	3	4	5	6	7	8
E.	MEATS									
	Beef or veal	0	1	2	3	4	5	6	7	8
	Lamb	0	1	2	3	4	5	6	7	8
	Poultry	0	1	2	3	4	5	6	7	8
	Pork, ham, or sausage	0	1	2	3	4	5	6	7	8
	Shellfish	0	1	2	3	4	5	6	7	8
	Fish	0	1	2	3	4	5	6	7	8
	Liver, kidney or tongue	0	1	2	3	4	5	6	7	8
	Cold cuts	0	1	2	3	4	5	6	7	8
	Hotdogs	0	1	2	3	4	5	6	7	8
	Eggs	0	1	2	3	4	5	6	7	8
	Peanutbutter	0	1	2	3	4	5	6	7	8
	Cottage cheese	0	1	2	3	4	5	6	7	8
	Hard cheeses	0	1	2	3	4	5	6	7	8
	Soft, spreadable cheese	0	1	2	3	4	5	6	7	8

	FOODS	CODE								
F.	FATS									
	Butter	0	1	2	3	4	5	6	7	8
	Margarine	0	1	2	3	4	5	6	7	8
	Bacon	0	1	2	3	4	5	6	7	8
	Cream	0	1	2	3	4	5	6	7	8
	Cottonseed oil	0	1	2	3	4	5	6	7	8
	Other oils	0	1	2	3	4	5	6	7	8
	Nuts	0	1	2	3	4	5	6	7	8
	Mayonnaise	0	1	2	3	4	5	6	7	8
	Lard	0	1	2	3	4	5	6	7	8
G.	MISCELLANEOUS									
	Black pepper	0	1	2	3	4	5	6	7	8
	Chocolate or cocoa	0	1	2	3	4	5	6	7	8
	Horseradish	0	1	2	3	4	5	6	7	8

II. If there are any foods which are not included in Part I and which you regularly consume, please list below and indicate the frequency.

FOODS	CODE								
Caffeinated Sodas	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8
	0	1	2	3	4	5	6	7	8

III. During the past month, how frequently did you consume char-broiled or burnt foods? (Circle the appropriate letter.)

a. None
b. 1-3 times/month
c. 1-3 times/week
d. 4-6 times/week
e. 1-2 times/day
f. over 2 times/day

VITA

Hui-Chuen Chen was born in Nantou, Taiwan on April 30, 1964. She was awarded a Bachelor of Sciences in Nutrition and Foods from the Providence University in June, 1986. She worked as a research assistant in the Food Science Technology Department of Tunghai University before she enrolled in the Master of Science degree in Human Nutrition and Foods at Virginia Polytechnic Institute and State University on August, 1988. She intends to apply for a 1992 dietetic internship.